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Joshua R. Shak Date

Bacterial population dynamics of pneumococcal colonization of the nasopharynx

By

Joshua R. Shak

Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Program in Population Biology, Ecology and Evolution

_____	_____
Keith P. Klugman Advisor	Jorge E. Vidal Advisor
_____	_____
Bruce R. Levin Committee Member	Timothy D. Read Committee Member
_____	_____
William M. Shafer Committee Member	David S. Stephens Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.

Dean of the James T. Laney School of Graduate Studies

_____

Date

Bacterial population dynamics of pneumococcal colonization of the nasopharynx

By

Joshua R. Shak

B.A., Amherst College, 2006

Advisors: Keith P. Klugman, MD, PhD
Jorge E. Vidal, PhD

An abstract of
A dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies
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Abstract

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By Joshua R. Shak

Streptococcus pneumoniae (the pneumococcus) is a common commensal inhabitant of the nasopharynx and a frequent etiologic agent in diseases responsible for approximately 1.6 million deaths annually. Colonization of the nasopharynx by *S. pneumoniae* (Sp) is a necessary precursor to pneumonia, otitis media, bacteremia, and meningitis. Intraspecies and interspecies interactions influence pneumococcal carriage in important ways. Biofilms – multicellular, surface-associated communities – are an intraspecies interaction that is particularly critical for Sp nasopharyngeal colonization and pathogenesis. We found that pneumolysin (Ply) – the primary toxin of Sp and an important vaccine target – is vital to the assembly of pneumococcal biofilms. Ply was expressed during biofilm development and localized to cellular aggregates. Knockout mutants deficient in Ply produced significantly less biofilm mass than wild-type strains, both on polystyrene and on human respiratory epithelial cells. Immunogold transmission electron microscopy of biofilms demonstrated that Ply was present both on pneumococcal cell surfaces and in the extracellular biofilm matrix. Altogether, we demonstrate a novel role for pneumolysin in the assembly of biofilms that is likely important during both carriage and disease. To examine interspecies interactions we examined nasopharyngeal densities of *Staphylococcus aureus* (Sa), *Haemophilus influenzae* (Hi) and *Moraxella catarrhalis* (Mc) following controlled challenge of healthy human adults with Sp. Of the 52 subjects challenged with Sp, 33 (63%) carried Sp at 2, 7, or 14 days post-inoculation. While baseline presence and density of Sa, Hi, and Mc were not associated with likelihood of successful Sp colonization at a statistically significant level, trends in the data suggest a protective effect of baseline Sa colonization and a facilitative effect of Hi colonization. At 14 days post-challenge, the proportion carrying Sa was significantly lower among those colonized with Sp as compared to those not colonized with Sp ($p = 0.008$). These are the first data reported of bacterial densities in relation to experimental human pneumococcal colonization and provide estimated effect sizes that will be useful in designing future studies. Through investigation of both intraspecies and interspecies interactions, this dissertation highlights the importance of the nasopharyngeal bacterial ecosystem for understanding pneumococcal carriage.

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Chapter 1: Introduction

Streptococcus pneumoniae (also known as the pneumococcus), is a Gram-positive bacterium that is a common asymptomatic resident of the human nasopharynx as well as an etiologic agent in diseases that cause significant morbidity and mortality worldwide. Pneumococcal diseases – including pneumonia, otitis media, sinusitis, meningitis, and bacteremia – lead to expensive healthcare utilization, lifelong disability, and death in both developing and developed countries [1,2]. Research has rightly focused on the aspects of the pneumococcus that enable invasion, tissue damage, antibiotic resistance, and mortality. However, the vast majority of the time, this bacterium exists as a commensal inhabitant of the human upper respiratory tract. This introduction will provide background information necessary to understand why the commensal phase of the pneumococcal lifecycle is an important topic of study, and outline the investigations of bacterial population dynamics conducted for this dissertation.

A brief history of the pneumococcus

The pneumococcus has been a cornerstone of microbiology since its discovery in 1881 by George Sternberg in the United States and Louis Pasteur in France. Both microbiologists isolated the microbe from rabbit blood after injecting the laboratory animals with human saliva [3,4]. Five years later, German scientist Albert Fraenkel was the first to refer to the new organism as The Pneumococcus because of its association with pulmonary disease

[4]. The 1880s also saw the discovery of the etiologic role of the pneumococcus in otitis media and meningitis [3,4] and the development of Christian Gram's famous stain using pathology samples from lobar pneumonia [5].

As the subject of Frederick Griffith's famous 1928 experiment showing the transference of virulence determinants, the pneumococcus was key to the development of the field of genetics. Griffith showed that while heat-killed virulent bacteria and live avirulent bacteria left mice unharmed when administered independently, concurrent administration had the ability to kill [6]. Sixteen years later, Avery, MacLeod, and McCarty demonstrated that it was DNA that allowed for the transmission of pneumococcal virulence [7], establishing DNA – and not protein – as the encoding material for genetic information.

Interestingly, both Sternberg and Pasteur first isolated the pneumococcus from human saliva rather than pathology samples [3]. Therefore, from the beginning it was known that the pneumococcus could be carried in the upper respiratory tract. By 1905, physicians in New York City had begun careful observational studies of the pneumococci isolated from mouths of healthy adults and pneumococci isolated from pathology specimens [8-10]. The main findings of these studies were that pneumococci isolated from carriers and pneumococci isolated from fatalities were impossible to distinguish based on morphology and that there were other organisms isolated from the mouth that were distinguishable from the pneumococcus only by metabolic assays and agglutination reactions [8-10]. Interestingly, the rate of colonization among healthy adults living in New York City at

the turn of the 20th century was 40-50% – a rate very similar to that observed in the developing world today.

Epidemiology of pneumococcal colonization

Pneumococcal colonization of the nasopharynx begins at birth [11,12], with rates peaking around 50-70% at 2-3 years of age [13,14] and stabilizing after 10 years of age at 5-10% in developed countries [13,15-17] and at 25-60% in developing countries [18,19]. The predominance of social determinants – ethnicity, crowding, family size, and smoking – as risk factors for pneumococcal colonization highlights the importance of person-to-person transmission in carriage prevalence [20]. High population density and poor sanitation can lead to high rates of nasopharyngeal carriage; for example, a prevalence of 82% was observed among infants living in a French orphanage [21] and a prevalence of 97% was documented in children in a Gambian village [22].

The median duration of carriage is estimated to be 31 days in adults and 60.5 days in children, with length of carriage dependent on capsular serotype, previous immunologic exposure, as well as host age and immunocompetence [22,23]. A recent study using intensive sampling of Kenyan children found a shorter duration of carriage and predicted that each child undergoes eight colonization events per year [24]. Repeated carriage events during childhood expose the host immune system to serotype-specific and serotype-independent antigens. Although repeated exposure to capsular antigen was previously thought to be responsible for the age-dependent decrease in pneumococcal

colonization, recent evidence has indicated that CD4 and IL-17 mediated mechanisms may be responsible for the age-dependent increase in ability to prevent and clear pneumococcal colonization [25,26].

While natural protection against carriage may not be dependent on serotype-specific antibodies, polyvalent vaccines using toxoid-conjugated capsular antigens have proven very effective in altering the epidemiology of pediatric carriage. Currently only 7-13 of the more than 90 serotypes can feasibly be included in the pneumococcal conjugate vaccine (PCV) for children. The serotypes included in the vaccines were those most likely to cause invasive disease in young children [27]. After the introduction of PCV, the replacement of vaccine serotypes with non-vaccine serotypes has resulted in overall rates of carriage remaining relatively constant [28,29]. However, this shift in serotype carriage has lead to a dramatic decrease in invasive pneumococcal disease; in the United States alone, pediatric pneumococcal pneumonia hospitalizations declined 65% with the introduction of heptavalent PCV and the incidence of invasive pneumococcal diseases caused by vaccine serotypes has decreased by as much as 95% [30,31]. In addition, through herd immunity, the use of PCV in children has also reduced the incidence of invasive pneumococcal disease in adults [32]. The reduction in invasive pneumococcal disease resulting from alteration of pneumococcal serotypes carriage frequencies represents a substantial accomplishment for public health and is projected to prevent 1.5 million deaths in developing countries between 2011 and 2020 [33].

Molecular mechanisms of nasopharyngeal colonization

On entering the nasal passage, the pneumococcus must bind and proliferate on the host epithelium. Attachment to the human respiratory endothelium is dependent on the pneumococcal cell binding to carbohydrates on the human cell surface, such as N-acetyl-glycosamine [34]. Exoglycosidases such as neuraminidase (NanA) facilitate colonization by removing terminal sugars on human glycoconjugates and exposing N-acetyl-glycosamine while simultaneously providing a source of nutrition to the bacteria [35]. The surface of the pneumococcus displays a family of choline binding proteins (CBPs) that facilitate adhesion to human endothelial and immune cells. In particular, it appears that the CBPs PsaA and CbpA (a.k.a. PspC; Figure 1) are critical to successful adhesion to epithelial cells [36,37]. In addition, the CBPs as a whole increase pneumococcal hydrophobicity and electrostatic charge, which may independently facilitate attachment to host cells [38].

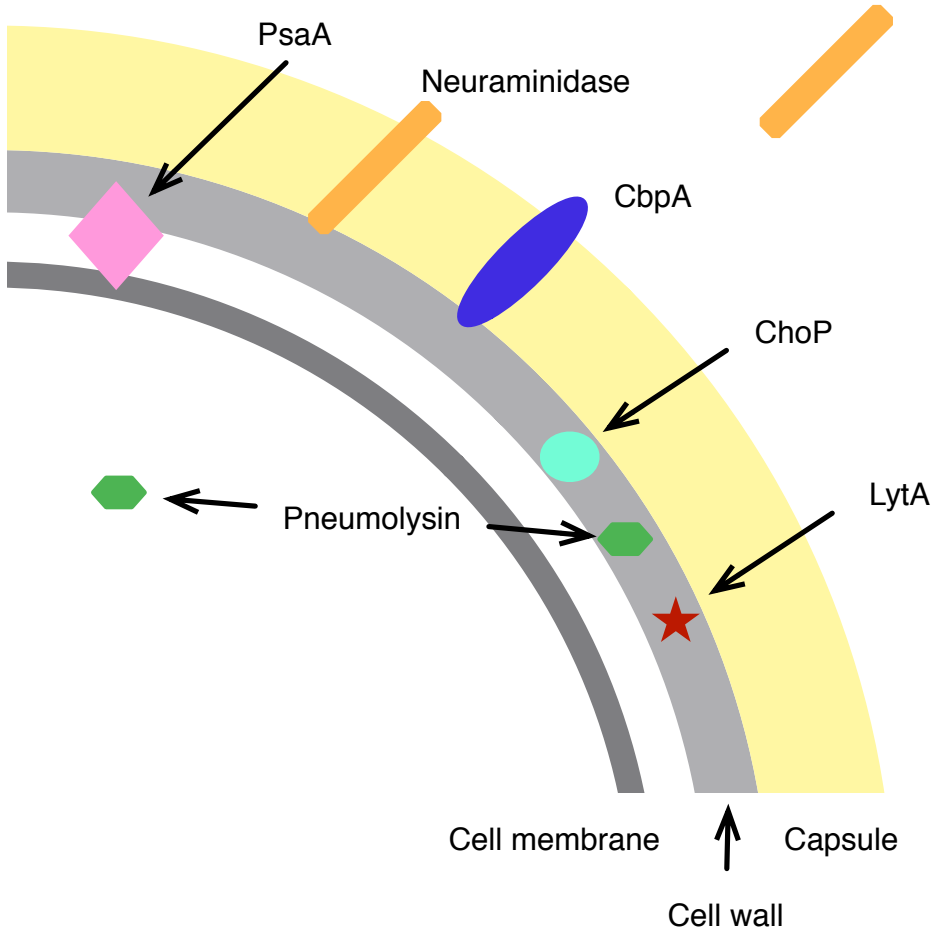

Figure 1. Important adhesins and virulence factors associated with the pneumococcal cell wall and membrane. Figure inspired by Jedrzejas, 2001 [39] and Harrison's, 18e.

The polysaccharide capsule is perhaps the single most studied virulence determinant and has an important role in immune evasion. With more than 90 immunologically distinct variants [40,41], the capsule limits the ability of the host immune system to fix complement and bind opsonins that facilitate phagocytosis and clearance. In addition, the capsule reduces entrapment in mucus, allowing the bacteria access to the nasal epithelium for long-term colonization [42]. The polysaccharide capsule is essential for virulence: almost all invasive isolates display a capsule and acapsular mutants have a greatly attenuated ability to cause invasive disease [43]. Phase variation of the capsule allows the

bacteria to adjust to the differing demands of colonization and invasion: the “transparent” variant displays increased capacity for binding to host tissue and the “opaque” variant is more resistant to phagocytosis during invasion [44,45]. Thus, the regulatory transition from the transparent to the opaque variant, possibly mediated by the decrease in oxygen associated with invasion, is an important step in the transition from commensalist to pathogen [46].

Pneumolysin is the primary toxin of the pneumococcus, but it is also expressed during colonization. During carriage, release of pneumolysin causes rhinorrhea, facilitating transmission from person to person and perhaps from the nasopharynx to other organ sites [47]. Once the pneumococcus has invaded the blood stream or lung tissue, pneumolysin is the primary mediator of the disease process. Injection of pneumolysin alone into rat lungs recreates many of the key pathologic features of pneumonia [48]. Pneumolysin may also assist bacteria in evading human dendritic cell surveillance by impairing dendritic cell maturation and the induction of inflammatory cytokines [49]. Although pneumolysin activity can occur at the bacterial cell wall independently of lysis, the toxin is most rapidly released when cells undergo autolysis (the seemingly spontaneous degradation of the cell wall) [50]. Gram-positive bacteria require cell-wall hydrolases, or lytic enzymes, to undergo cellular restructuring. In the pneumococcus, the autolysin encoded by the *lytA* gene, a lysozyme encoded by the *lytC* gene, and a murein hydrolase encoded by the *cbpD* gene are required for cellular lysis [51-53].

Role of biofilms during carriage

Biofilms – surface-associated aggregates of microorganisms in an extracellular matrix – are a current area of intensive research in microbiology because they are key to understanding problems ranging from catheter-associated nosocomial infections to "biofouling" of industrial equipment. Biofilms are also the most likely natural state of pneumococcal carriage in the nasopharynx. While biofilms of *Haemophilus* and *Bordatella* have been documented in the human nasopharynx [54-56], pneumococcal biofilms have yet to be directly observed in the human nasopharynx. However, pneumococcal biofilms have been found in nasopharyngeal lavage samples of mice [57] and pneumococcal mutants lacking the ability to form biofilms have an impaired ability to colonize mice [58]. Pneumococcal biofilms have been directly observed in the middle ear mucosa of human subjects with chronic otitis media [59]. These findings, and the semi-dry mucosal environment of the nasopharynx, make biofilms the most likely form of pneumococcal carriage.

Mechanisms of pneumococcal biofilm formation are an ongoing area of research. All biofilms go through phases of development: initial attachment, irreversible attachment, early aggregation, maturation, and dispersal [60]. While it is clear that adhesins, such as the aforementioned CbpA, are important in initial adhesion to human cell line substrates [58,61], the pneumococcal factors responsible for aggregation and matrix maturation are less well understood. The mature pneumococcal biofilm matrix is composed of extracellular DNA, proteins, and polysaccharides [62,63]. LytC, a lysozyme implicated in lysis of non-competent neighbors, may be an important component of the matrix, binding

DNA and protein complexes [63]. However, it is not yet clear which molecular components bind pneumococcal cells in a biofilm to each other and to the biofilm matrix.

Regulation of pneumococcal biofilm assembly is another area of ongoing research. Biofilm assembly is at least partially regulated through the LuxS and Com quorum sensing (QS) systems. Quorum sensing – the ability to detect and respond to extracellular levels of secreted signals from neighboring bacteria – is a common regulatory mechanism for biofilm formation and other bacterial group behaviors [64,65]. Two recent papers have demonstrated that strains deficient in LuxS (the synthase of the autoinducer-2 signaling molecule) are unable to form biofilms at early time points, release less extracellular DNA, and have decreased genetic competence [66,67]. However, these papers did not pinpoint which downstream mechanism regulated by LuxS was responsible for the biofilm phenotype. Competence stimulating peptide (CSP), the signal secreted by the Com QS system, also has the ability to regulate pneumococcal biofilm formation [68,69]. A recent study from our lab demonstrated the importance of both the LuxS and Com QS in pneumococcal biofilms grown on human cell substrates inside a continuous flow bioreactor [70]. Exactly how these two QS systems work together in biofilm formation is unknown, but LuxS may be necessary for attachment and aggregation in early biofilms and the Com system may be critical to biofilm maturation by regulating the release of eDNA required for matrix development.

Other actors in the nasopharynx

On entering the nasopharynx, the pneumococcus becomes a member of a diverse and dynamic microenvironment. While there may or may not be other strains of pneumococcus present during colonization, there are invariably other bacteria, respiratory viruses, and host immune defenses present. Interactions among pneumococcal strains and between the pneumococcus and other pathogens are extensively reviewed in Chapter 2. Here, I will briefly outline the suspected roles of viruses and the human immune system during pneumococcal colonization.

The interplay between the pneumococcus and respiratory viruses has been extensively studied, with associations observed between the pneumococcus, rhinovirus, respiratory syncytial virus, influenza, parainfluenza, and adenovirus [71]. It has long been recognized that viral infections alter the respiratory epithelium [72] as experiments in cell culture [73,74], animal models [75], and humans [76] have all shown that viral pre-infection increases bacterial adherence to respiratory tract cells. Viral infection may also facilitate bacterial adherence by exposing receptors through altered host regulation, deglycosylation of host cell receptors, or direct display of viral proteins on the host cell surface [72]. Viral neuraminidase can increase pneumococcal colonization and invasion [77] whereas administration of oseltamivir (a neuraminidase inhibitor) improved survival in a mouse model of influenza/pneumococcal co-infection [78]. In addition, viral infections can lead to impaired ciliary action in the respiratory tract, interfering with the movement of particulate matter and mucous [79]. The decrease in mucociliary velocity

mediated by influenza infection decreases pneumococcal clearance in a murine trachea model [80].

Viral infection is also known to precipitate bacterial disease. Epidemiological studies clearly demonstrate that viral upper respiratory infections often precede acute otitis media (AOM) and bacterial pneumonia. Up to 94% of pediatric patients with a new diagnosis of AOM exhibit symptoms of an upper respiratory infection prior to the onset of AOM symptoms [81-83]. These epidemiological findings are supported by animal studies that show pre-infection with influenza virus was associated with increased pneumococcal transmission [84]. Furthermore, an extensive retrospective histopathologic study of lung tissue of soldiers who died during the 1918 influenza pandemic revealed convincing evidence that bacterial pneumonia was a major contributor to mortality during this pre-antibiotic viral pandemic [85].

As human viruses do not interact directly with bacterial cells, it is generally thought that most effects of viruses on pneumococcus are host-mediated. There is evidence that influenza co-infection increases concentrations of the opaque pneumococcal variant in comparison to the transparent variant in a chinchilla otitis media model [86] through a host-mediated mechanism. Plasma levels of interferon gamma peak approximately seven days following infection with the influenza virus. Interestingly, this peak coincides with observed mortality from bacterial pneumonia during influenza pandemics [87]. Experimental studies of influenza/pneumococcal co-infection demonstrate improved survival for interferon gamma knockout mice compared to wild-type mice [88].

However, other studies of interferon gamma knockout mice have found precisely the opposite: interferon gamma protects against pneumococcal invasion [89,90]. Nevertheless, interferon gamma remains an attractive explanatory mechanism because of its ability to suppress phagocytosis of bacteria in the lung [88]. Dysregulation of other immune factors such as type I interferons, IL-12, and TNF alpha are also being investigated [91,92], but no single immune factor has emerged as the definitive mediator of viral potentiation of pneumococcal infections.

Sequelae of colonization

While carriage is most commonly asymptomatic, there can be a mild suppurative rhinitis which presumably assists in transmission to new hosts [47]. Local spread of the pneumococcus to nearby sites most commonly results in sinusitis and otitis media (Figure 2). Invasion of the maxillary, frontal, ethmoid, or sphenoid sinuses leads to acute or chronic sinusitis [93,94], whereas travel through the Eustachian tubes leads to otitis media [95]. *S. pneumoniae* has long been the leading etiological agent for pediatric otitis media, which is an exceedingly common reason for healthcare utilization [96]. However, the efficacy of PCV in preventing acute otitis media may be changing the balance in bacterial etiologies of otitis media cases [97,98].

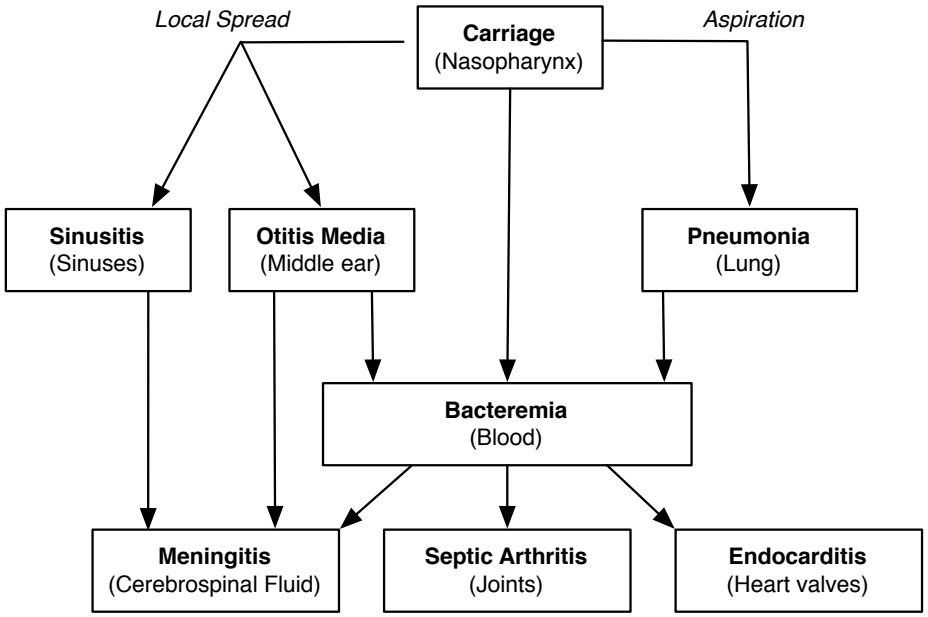

Figure 2. Routes of spread from the nasopharynx to pneumococcal disease sites. Figure inspired by Obaro and Adegbola, 2002 [99].

Progression from nasopharyngeal colonization to pneumonia occurs with aspiration down the respiratory tract along with the proper mix of bacterial invasiveness and host susceptibility. Established risk factors for developing pneumococcal pneumonia or invasive disease include cigarette smoking, chronic obstructive pulmonary disease, HIV, diabetes, and alcoholism [100]. The combination of bacterial proliferation and host immune response within the lung leads to a lobar consolidation, which can be observed radiographically. Invasion of the host endothelial and epithelial layers can occur through the binding of pneumococcal phosphorylcholine (ChoP; Figure 1) to platelet-activating factor (PAF) receptor [101] or through the binding of PspC to epithelial plgR [102]. Once in the blood stream, the pneumococcus can cross the blood-brain barrier to cause meningitis [103] or invade less common disease sites such as joints, heart valves, or the pericardium (Figure 2) [104-106].

Outline of the dissertation

We tend to label the microbes that surround us as either friend or enemy, but this dichotomy often fails us. The pneumococcus is an exceedingly important pathogen that spends the majority of the time as an asymptomatic resident of the nasopharynx. The traditional models of why and when microorganisms cause disease can overlook the importance of the carrier state itself. The focus of this dissertation is to better understand the population dynamics important to nasopharyngeal colonization by the pneumococcus.

Chapter 2 examines the current knowledge of the bacterial interactions occurring in the nasopharynx. By better understanding how different bacterial populations cooperate and interfere in this important anatomical niche, we gain a deeper understanding of the nasopharyngeal ecosystem.

Chapter 3 investigates the role of the pneumolysin in the assembly of biofilms. Long appreciated for its role in pneumococcal diseases, our work is the first to show that pneumolysin is crucial in the early assembly of pneumococcal biofilms. This work has important consequences for how we view bacterial toxins and reveals that a hemolytic toxin can also have an important role during asymptomatic colonization.

Chapter 4 explores what can be learned about the presence and density of other bacterial species when pneumococcus is added to the human nasopharynx in a controlled way. By studying samples from an experimental human pneumococcal colonization study, we

were able to assess presence and density of four clinically-important bacterial species in relation to pneumococcal colonization.

Ultimately, a better understanding of within- and between-species population dynamics during pneumococcal colonization will lead to an increased understanding of the biology of this clinically important microbe and provide insight into why and when it transitions from commensal to pathogen.

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Chapter 2: Influence of bacterial interactions on pneumococcal colonization of the nasopharynx

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"Intraspecies and interspecies interactions modulate the timing and nature of nasopharyngeal colonization by *Streptococcus pneumoniae* (the pneumococcus). The cover image shows pneumococcal cells forming a multicellular biofilm on top of human A549 lung cells *in vitro*. As discussed by Shak, Vidal, and Klugman on pages 129–135, bacterial interactions have implications for biofilm formation, co-colonization with multiple pneumococcal strains, vaccine serotype replacement, and the epidemiology of carriage of the pneumococcus and other nasopharyngeal species."

Review

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Influence of bacterial interactions on pneumococcal colonization of the nasopharynx

Joshua R. Shak[1], Jorge E. Vidal[2], and Keith P. Klugman[2]

[1] Molecules to Mankind Program and Graduate Program in Population Biology, Ecology, and Evolution, Laney Graduate School, Emory University, Atlanta, GA 30322, USA
[2] Hubert Department of Global Health, Rollins School of Public Health, Emory University, Atlanta, GA 30322, USA

Streptococcus pneumoniae **(the pneumococcus) is a common commensal inhabitant of the nasopharynx and a frequent etiologic agent in serious diseases such as pneumonia, otitis media, bacteremia, and meningitis. Multiple pneumococcal strains can colonize the nasopharynx, which is also home to many other bacterial species. Intraspecies and interspecies interactions influence pneumococcal carriage in important ways. Co-colonization by two or more pneumococcal strains has implications for vaccine serotype replacement, carriage detection, and pneumonia diagnostics. Interactions between the pneumococcus and other bacterial species alter carriage prevalence, modulate virulence, and affect biofilm formation. By examining these interactions, this review highlights how the bacterial ecosystem of the nasopharynx changes the nature and course of pneumococcal carriage.**

Streptococcus pneumoniae: a commensal and a pathogen

The Gram-positive bacterium *Streptococcus pneumoniae* (the pneumococcus) is a common asymptomatic resident of the human nasopharynx [1] and is also a primary etiologic agent in pneumonia, otitis media, meningitis, and sepsis. Pneumococcal pneumonia is the leading cause of childhood death in developing countries [2] and imposes a significant burden of morbidity, mortality, and healthcare costs in developed countries such as the USA [3]. Colonization of the nasopharynx is a necessary – but not sufficient – step along the path to pneumococcal disease [4,5]. On entering the nasopharynx, and during its residence there, the pneumococcus shares this anatomical and physiological niche with an array of other viral and bacterial inhabitants. Although there have been recent reviews of host–pneumococcal [6,7] and viral–pneumococcal interactions [8], there has been less written on the direct bacterial–bacterial interactions that influence pneumococcal carriage. This review highlights recent advances in our understanding of the bacterial interactions that alter the nature of pneumococcal carriage and its potential to progress to pneumococcal disease.

Corresponding author: Klugman, K.P. (keith.klugman@emory.edu).
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The nasopharynx lies at a vital crossroads connecting the nose, ears, mouth, and lower respiratory tract (Figure 1). Directly posterior to the nasal cavity and superior to the oropharynx, the nasopharynx is also close to the paranasal sinuses and directly connected to the middle ear by the Eustachian tubes. A dynamic environment with a constant flow of air, blood, and mucus, the nasopharynx modulates incoming air quality and temperature for the host, while also providing a home to millions of microbes. Colonization by the pneumococcus may occur at any time during a person's life, but is most common during infancy, with acquisition even documented in the first day of life [9]. The prevalence of pneumococcal carriage increases in the first few years of life, peaking at approximately 50% to >70% in hosts 2–3 years of age [4,10] and decreasing thereafter until stabilizing at 5–10% in hosts over 10 years of age in developed countries [4,11,12] but as high as 25–60% in some developing countries [13,14]. The median duration of carriage is estimated to be 31 days in adults and 60.5 days in children, with length of carriage dependent on capsular serotype and previous immunologic exposure, as well as the host's age and immunocompetence [15,16]. However, a recent study using intensive sampling of African children found a shorter duration of pediatric carriage: an overall mean of 31.3 days, with serotype-specific means ranging from 6.7 to 50 days [17]. Similar studies in adults may also reveal that the duration of carriage is shorter than previously deduced from studies using monthly assessments.

Once carriage is established in the nasopharynx, the pneumococcus can migrate through the Eustachian tubes to cause otitis media [18], descend the respiratory tract to cause pneumonia [19], or invade the bloodstream through the respiratory epithelium to cause bacteremia or meningitis (Figure 1) [20]. The success of the pneumococcus as both a commensal and a pathogen can be attributed to a repertoire of cellular components that are useful during both carriage and disease [21]. Ultimately, regulation of these dual-use cellular components and changing conditions in the nasopharynx mediate if and when the pneumococcus progresses from a commensal resident to a pathogen. In this review, we argue that the abilities of the pneumococcus to persist and harm are influenced by within-species and cross-species population dynamics.

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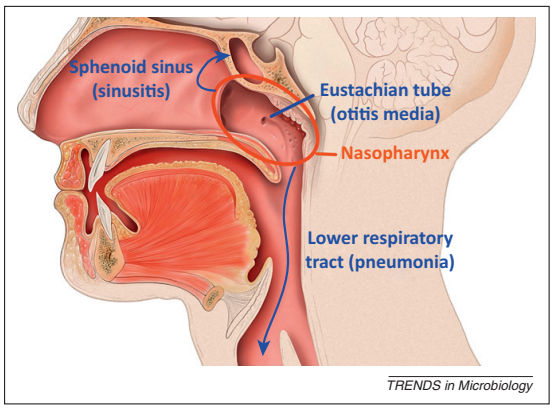

Figure 1. Diagram of the nasopharynx and pathways to pneumococcal diseases. The nasopharynx lies directly posterior to the nasal cavity and superior to the oropharynx. The pneumococcus can spread from its home in the nasopharynx through the Eustachian tube to cause otitis media, to the sinus cavities to cause sinusitis, through the larynx to the lower respiratory tract to cause pneumonia, and then in some cases, bacteremia. Image adapted from an illustration by Patrick J. Lynch distributed under Creative Commons Attribution 2.5 License.

Effects of co-colonization on serotype distribution, genetic exchange, and density

Pneumococcal strains interact directly when they co-colonize a single host, an occurrence more prevalent than previously believed. Co-colonization is clinically and epidemiologically important because pneumococcal strains interfere with or aid in subsequent colonization by additional strains [22–24], and the pneumococcus is highly prone to homologous recombination and transformation of exogenous DNA [24–28]. In fact, it has recently been demonstrated that strains with poor colonization efficiency are better at colonizing a mouse model when a second strain with high colonization efficiency is already present [24]. Furthermore, this phenomenon appears to occur without genetic exchange, possibly through biofilm formation [24].

Recent work has begun to explore specific molecular mechanisms of interaction, including bacteriocin production and competence-induced fratricide in inter-strain dynamics. The *blp* locus in the pneumococcal genome encodes an antimicrobial bacteriocin peptide and corresponding immunity peptide. A strain harboring the *blp* locus has a competitive advantage in a mouse colonization model [22], and the importance of this locus in the wild is supported by a comparative genomic study that demonstrated extensive variation at the *blp* locus, indicative of diversifying selection [23]. Autolysis is another important mediator of inter-strain dynamics; long recognized for its ability to rapidly release the toxin pneumolysin, autolysis may actually be a form of competence-induced fratricide regulated by the Com quorum-sensing (QS) system [29]. In addition to upregulating factors necessary for uptake and transformation of DNA, the Com system activates the two-peptide bacteriocin CibAB and its immunity factor CibC; these bacteriocins target non-competent pneumococci that do not produce CibC, causing LytA-, LytC-, and CbpD-mediated lysis and DNA release from sister cells [30,31]. The role of bacteriocins and competence-induced fratricide *in vivo* is not yet clear, but it has become increasingly important to understand the mechanisms of strain interaction because it is now apparent that co-colonization is far more common than previously believed.

The original study of co-colonization by Gundel and Okura used a technique involving serial intraperitoneal saliva injections into mice to detect multiple serotypes [32]. The cost of this animal work led to a standard assay with streaking out of enriched nasopharyngeal specimens and serotyping of a handful of colonies. However, this approach yielded low rates of co-colonization; only 1.3–1.9% of samples from pediatric populations in South Africa were found to have more than one serotype using this laborious and costly method [33]. Testing of enriched cultures directly with the capsular reaction test using pooled and type-specific serum proved more sensitive, detecting co-colonization in 9.9% of pediatric Danish carriers [34]. However, a more cost-effective approach using PCR amplification of a non-coding region adjacent to the *ply* gene, followed by restriction fragment length polymorphism (RFLP) analysis yielded similar rates of co-colonization, 7.9% and 9.5% of pediatric carriers in two studies [35,36]. More recently, studies have demonstrated the utility of sweep techniques in which nasopharyngeal specimens are plated onto blood agar plates and incubated before all growth is swept up and assayed by multiplex PCR or latex agglutination [37]. A study of indigenous children in Venezuela using the sweep approach followed by multiplex PCR revealed a co-colonization rate of 20% [38]. In a careful comparison of the sweep technique and a microarray-based approach against traditional WHO culture methods, sweep–agglutination and microarray resulted in co-colonization rates of 43.2% and 48.8%, respectively, compared to 11.2% by the WHO-recommended culture [39]. Clearly, multiplex PCR, sweep, and microarray techniques demonstrate that co-colonization is more common than previously believed, and our ability to accurately detect co-colonization will only improve with the refinement of new techniques such as metagenomic sequencing and sequence-based serotype identification [40].

New studies are beginning to examine the theoretical and practical aspects of co-colonization on serotype-specific interactions and the implications for pneumococcal vaccine development and deployment. Current formulations of the pneumococcal conjugate vaccine (PCV) protect against 7, 10, or 13 capsular vaccine types (VTs), but have no direct effect on the more than 80 non-vaccine types (NVTs). Introduction of PCV unmasks the presence of NVTs that may have been at lower density or under less scrutiny during pre-PCV epidemiological studies [41]. In addition, NVTs are likely to gain a competitive advantage, both within hosts and within populations, when strains capable of competition are removed by vaccination [42,43]. PCV introduction has also precipitated a shift towards more NVT-caused invasive pneumococcal disease (IPD) [43]. In general, even with the increase in NVT-caused IPD, the rates of pneumococcal disease are still far below the pre-PCV era [44]. However, in certain populations, namely in Native Alaskan children and adults, IPD has actually increased [45], and in the USA, NVT 19A emerged prior to the introduction of PCV13 as a more virulent and drug-resistant cause of IPD [46,47]. This increase in IPD

due to serotype 19A is likely to be because of its increased carriage prevalence in a population immunized with PCV7.

Co-colonization is also important because the pneumococcus has a significant propensity for genetic exchange through transformation and homologous recombination. A longitudinal study of chronic otitis media demonstrated extensive genomic rearrangements *in vivo* [26], and animal models of pneumococcal disease have demonstrated that even minor genetic differences can significantly alter virulence [23]. A multi-locus sequence typing (MLST) study of pediatric strains in Gambia found extensive recombination and serotype switching among carriage strains; the authors suggested this finding may be due to high rates of co-colonization in this population [48]. Furthermore, a study of 240 full genome sequences from the PMEN1 lineage revealed more than 700 recombination events and 10 capsule-switching events [28], suggesting extensive diversity within a relatively contemporary highly related clone and thus a high frequency of recombination events. More studies using whole-genome sequencing of isolates obtained sequentially from carriers are needed to pinpoint the frequency of these recombination and capsule-switching events, especially in relation to the changing flora of the nasopharynx post-vaccination.

It has recently been suggested that co-colonization with multiple strains of *S. pneumoniae* increases the density of nasopharyngeal carriage [36]. Brugger and colleagues found that subjects co-colonized with two or more strains had higher carriage density than subjects colonized with one strain [36]. Lower density carriage is less likely to be detected by culture methods than by real-time quantitative PCR (qPCR) [49,50], so the findings of previous, culture-based studies may be distorted by the relative frequency or rarity of co-colonization. Nasopharyngeal pneumococcal density is also showing promise as a diagnostic tool for pneumonia [51,52]. If co-colonization is correlated with nasopharyngeal pneumococcal density, then this factor will need to be controlled for when exploring density-based pneumonia diagnostics.

Interactions between nasopharyngeal bacterial species

The nasopharyngeal microbial community is established in the first year after birth and varies throughout a person's lifetime [53]. The five most common bacterial families in the nasopharynx are Moraxellaceae, Streptococcaceae, Corynebacteriaceae, Pasteurellaceae (including the genus Haemophilus), and Staphylococcaceae (Table 1) [10,54–56]. The first microbiome studies to analyze 16S rRNA sequences have revealed high inter-individual variability in nasopharyngeal communities, as well as possible correlations with season [55,56]. In addition to bacteria, viruses and the host immune system are also major determinants of nasopharyngeal dynamics. The synergy between viral co-infections and bacterial pneumonia has been an ongoing area of research [8,57] and a recent review by Lijek and Weiser examines the evidence that mucosal immunity mediates interactions between *S. pneumoniae, Haemophilus influenzae*, and *Staphylococcus aureus* [7]. Here we consider how direct bacterial–bacterial interactions may impact clinically important nasopharyngeal dynamics.

Table 1. Average frequency of 16S rRNA sequences present in nasal swabs of children

Operational taxonomic unit (OTU)[a]	Frequency (%)[b]
Unclassified Moraxellaceae	19.0
Streptococcus	17.9
Corynebacterium	7.0
Moraxella	6.5
Haemophilus	4.7
Unclassified Pasteurellaceae	4.1
Staphylococcus	3.8
Acinetobacter	3.4
Dolosigranulum	3.2
Propionibacterium	3.1
Unclassified Proteobacteria	2.6
Lactococcus	2.6

[a]Adapted with permission from Laufer *et al.* [55].

[b]Average frequency, among 108 children, of 16S rRNA sequences in nasal swab samples. Only OTUs with an average frequency >2% are listed.

With the widespread deployment of PCV and a seemingly concurrent rise in community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) infections, there has been speculation as to whether these two trends may be related [54,58,59]. Epidemiological studies have indeed revealed an inverse correlation between carriage of *S. pneumoniae* and carriage of *S. aureus* [50,54,60,61]. The presence of the pneumococcus in the nasopharynx appears to inhibit *S. aureus* colonization. *In vitro* studies demonstrated that hydrogen peroxide (H_2O_2) produced by the enzyme pyruvate oxidase (SpxB) of *S. pneumoniae* kills *S. aureus* strains. In addition, targeted mutagenesis of staphylococcal catalase results in decreased survival of *S. aureus* in the presence of the pneumococcus [62]. The mechanistic explanation is that without catalase to degrade hydrogen peroxide, the staphylococcal SOS response is triggered, leading to bacteriophage-induced lysis [63,64]. However, the *in vivo* relevance of hydrogen peroxide-mediated killing is in question because a neonatal rat study using an SpxB-deficient *S. pneumoniae* strain or a catalase-deficient *S. aureus* strain demonstrated no significant difference in co-colonization densities [65]. The finding that carriage of *S. aureus* was less likely in individuals carrying piliated strains of *S. pneumoniae* suggests that the pneumococcal pilus may somehow reduce the likelihood of staphylococcal colonization or sustained carriage [66].

Regardless of mechanism, for PCV to contribute to the rise in MRSA there would need to be an inverse correlation specifically between VT the pneumococcus and MRSA. Cross-sectional studies have demonstrated an inverse association between carriage of *S. aureus* and vaccine-type *S. pneumoniae* [54,60]. However, interventional studies that assessed bacterial carriage before and after PCV introduction are divided. Some studies found that carriage prevalence of *S. aureus* remained steady [67–69] whereas others found increased prevalence of *S. aureus* following PCV [61,70,71]. Importantly, although some have searched for it [58], no study has demonstrated an association between pneumococcal carriage and MRSA carriage. Given that the inverse correlation between *S. aureus* and *S. pneumoniae* is absent in immunodeficient HIV-positive populations [61,72], an immune-mediated mechanism

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may be responsible for this relationship. Thus, although there does appear to be a link between carriage of *S. aureus* and carriage of VT *S. pneumoniae*, there is not sufficient evidence to assert that the increase in MRSA is a result of PCV rollout and no increase in community-acquired MRSA has been seen in the years following PCV rollout in Europe [73].

Pneumococcal carriage in the nasopharynx has also been associated with carriage of two other major nasopharynx-based pathogens, *H. influenzae* and *Moraxella catarrhalis*. Although there are exceptions, most studies find a positive association in humans between the presence in the nasopharynx of *S. pneumoniae* and *H. influenzae* and between *S. pneumoniae* and *M. catarrhalis* [74–76]. The epidemiologic association between *S. pneumoniae* and *H. influenzae* was recently extended by the observation that their carriage densities are also positively correlated [50]. Animal studies concur with the human epidemiology, with a neonatal rat study demonstrating that resident *S. pneumoniae* facilitated colonization of *H. influenzae* [77]. In contrast to epidemiologic data showing a positive correlation between the pneumococcus and *H. influenzae*, *in vitro* studies by Pericone and colleagues demonstrated that *S. pneumoniae* inhibits the growth of *H. influenzae*, *M. catarrhalis*, and *Neisseria meningitidis* via hydrogen peroxide production [78]. This apparent paradox suggests that cultivation of the two pathogens together *in vitro* does not accurately reflect their physiological interactions *in vivo*. In a longitudinal study of PCV introduction in the Netherlands, researchers found that whereas overall pneumococcal prevalence remained steady (with VTs decreasing and NVTs increasing), *H. influenzae* prevalence increased [71], suggesting that if there is an association between colonization with *S. pneumoniae* and *H. influenzae*, the relationship may be serotype-specific. Ongoing and future human studies of pneumococcal colonization [79] have the potential to help us better understand the relationship between the pneumococcal colonization event and the nasopharyngeal carriage of other bacterial species.

Although the mechanisms behind inter-species interactions are still being investigated, it is likely that bacteriocins play a role. It has been shown that *S. pneumoniae* bacteriocins inhibit the growth of other streptococcal species [80], and bacteriocins produced by *Streptococcus mitis* and *Streptococcus salivarius* inhibit the growth of the pneumococcus [81]. In addition to growth inhibition via secreted signals, there is also a growing body of evidence that cross-species pheromones alter bacterial gene expression. Recent studies have shown that cross-species signals can modify the expression of pneumococcal virulence genes. In contrast to the findings of Pericone *et al.* [78], Cope and colleagues demonstrated that co-culture of clinical strains of *S. pneumoniae* with *H. influenzae* does not change planktonic growth rates [82]. However, co-culture of these two strains did upregulate expression of SpxB and downregulate expression of *ply*, the gene that encodes pneumolysin, and *pav*A, the gene that encodes pneumococcal adherence and virulence factor A [82]. These results suggest that bacteria sharing the nasopharynx have the potential to alter one another's gene expression. The mechanisms of interspecies gene regulation are just beginning to be explored, but the well-studied QS system LuxS/autoinducer-2 (AI-2) may be partly responsible for interspecies communication. AI-2, synthesized by the enzyme LuxS, is a molecule secreted and recognized by many bacterial species and has the potential to alter growth and virulence [83]. As reviewed in the next section, pneumococcal LuxS/AI-2 plays an important role in biofilm formation.

Pneumococcal biofilms in the nasopharynx

There is mounting evidence that the nasopharynx hosts biofilms created by many bacterial species, including the pneumococcus [84,85], *S. aureus*, *Staphylococcus epidermidis* [86], *N. meningitidis* [87], and *H. influenzae* [88]. Pneumococcal biofilms form in stages, starting with attachment to the substratum mediated by pneumococcal adhesion factors such as CbpA, PcpA, and PspA [85,89], and followed by bacterial aggregation and matrix maturation [90,91]. A mature matrix is mainly composed of extracellular DNA (eDNA), proteins, and polysaccharides [91,92] linking pneumococcal cells and attaching them to host cells in a tight mesh (Figure 2). Recent studies suggest that biofilm formation, cellular autolysis, and genetic competence are all intertwined and regulated by the Com and LuxS/AI-2 QS systems.

The Com system is well known for its ability to control the genetic competence of the pneumococcus and mediate killing of non-competent sister cells [30]. Competence-stimulating peptide (CSP), the signal secreted by the Com QS system, is strain-specific and regulates autolysis and genetic competence, which allows for release and uptake of DNA from other streptococci [93]. Recent studies have shown that gene transference among streptococci is more efficient in early biofilm structures [24,94], and LytC, a lysozyme implicated in ComC-mediated lysis of non-competent neighbors, is a common component of DNA–protein complexes in pneumococcal biofilms [92]. Two recent studies have also demonstrated that strains deficient in LuxS are unable to form biofilms at early time points and have decreased ability for eDNA release and competence [90,95]. How these two QS systems work together in biofilm formation is unknown, but perhaps LuxS/AI-2 is necessary for attachment of early biofilms and pneumococcal biofilm maturation can only be completed with activation of the Com system and release of the eDNA required for matrix development.

The pneumococcus is also capable of participating in multi-species biofilms. Co-incubation of *H. influenzae* and *S. pneumoniae* increases the biomass of pneumococcal biofilms [96]. In addition, these interspecies biofilms are able to confer protection against antibiotics to strains of susceptible *S. pneumoniae* through passive transference of β-lactamase produced by resistant *H. influenzae* [97]. Biofilm formation in *S. pneumoniae* and *H. influenzae* is in part regulated by the LuxS/AI-2 QS mechanism [90,95,98]. Accordingly, animal studies also demonstrate that the LuxS/AI-2 system regulates persistence of the pneumococcus in the mouse nasopharynx [99] and persistence of *H. influenzae* biofilms in the ear epithelium of chinchillas [98].

AI-2 is produced by most bacterial pathogens and normal flora strains, so it is quite possible that the pneumococcus

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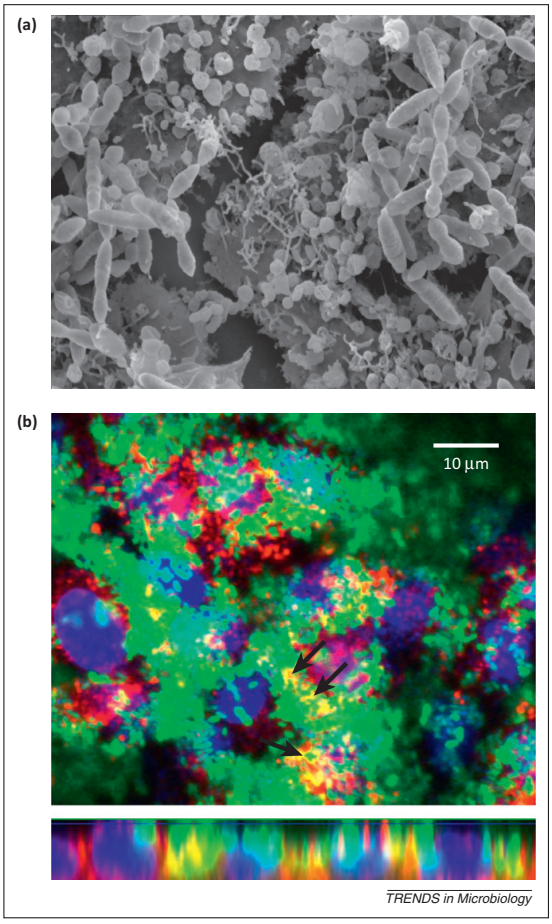

Figure 2. *Streptococcus pneumoniae* forms multicellular structures *in vitro* on top of human lung cells. **(a)** Scanning electron micrograph of pneumococcal cells in 8-h biofilms grown on top of human lung epithelial cells show bacterial cells connected by an extracellular matrix. **(b)** Confocal images of GFP-expressing *S. pneumoniae* biofilms demonstrate cells co-localizing with sialic acid residues (stained red) present on human lung epithelial cells (nuclei stained blue). The top image is an optical *xy* section and the bottom is an *xz* section.

secretes AI-2 in the nasopharynx to modify the behaviors of other bacterial species or that the pneumococcus responds to AI-2 secreted by other colonizers. In support of this theory, exogenous addition of purified AI-2 significantly increased the biofilm biomass of *S. pneumoniae* strain D39 [90]. Furthermore, *M. catarrhalis*, which does not possess a luxS homolog, produced more robust biofilms in response to AI-2 secreted by *H. influenzae* strains [100]. A number of other streptococcal species that colonize the pharynx, such as *S. intermedius*, *S. oralis*, *S. gordonii*, and *S. mutans*, utilize LuxS/AI-2 to control biofilms [83]. Whether AI-2 is used for competition or cooperation among species in the pharynx requires further investigation.

Concluding remarks

Our understanding of how microbes and humans live together in health and disease is rapidly evolving. The pneumococcus is a clinically important and biologically fascinating organism that we can better understand by observing its interactions with the human host and with other microbes. Additional pneumococcal strains and other bacterial species play key roles in pneumococcal colonization. Metagenomic sequencing of all DNA in the nasopharynx will not only allow us to take a better census of the microbes and viruses, but will also help us to understand the full genetic and transcriptional activity of these populations [101]. Furthermore, experimental human studies of pneumococcal colonization of the nasopharynx have the potential to distinguish cause and effect when a correlation or change in bacterial composition is observed [79,102]. Ultimately, the challenge will be to identify biological and clinical relevance when examining large complex networks of intraspecies and interspecies interactions inside the human host.

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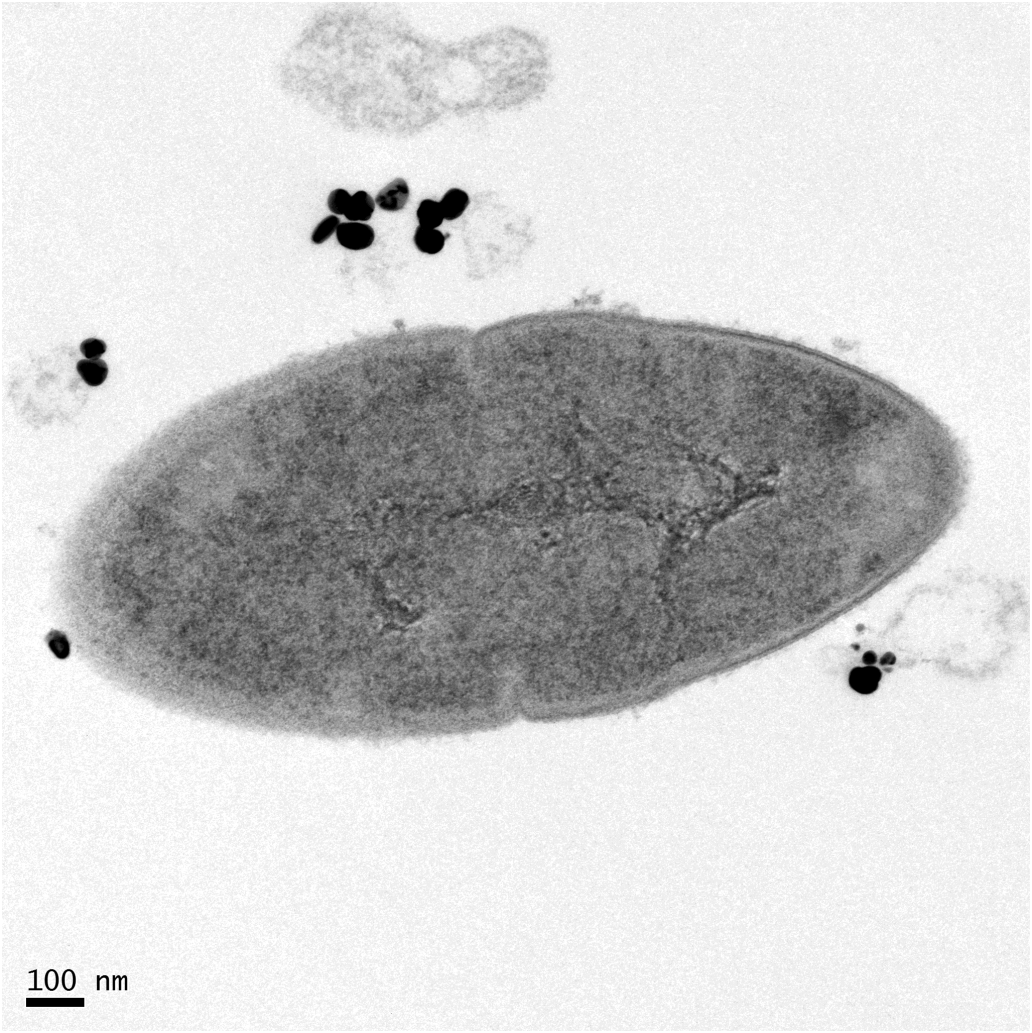
Chapter 3: Novel Role for the *Streptococcus pneumoniae* Toxin Pneumolysin in the Assembly of Biofilms



RESEARCH ARTICLE

Novel Role for the *Streptococcus pneumoniae* Toxin Pneumolysin in the Assembly of Biofilms

Joshua R. Shak,[a] Herbert P. Ludewick,[a,b] Kristen E. Howery,[a] Fuminori Sakai,[a] Hong Yi,[c] Richard M. Harvey,[d] James C. Paton,[d] Keith P. Klugman,[a] Jorge E. Vidal[a]

Hubert Department of Global Health, Rollins School of Public Health, Emory University, Atlanta, Georgia, USA[a]; Center for Immunology & Microbial Disease, Albany Medical College, Albany, New York, USA[b]; Robert P. Apkarian Integrated Electron Microscopy Core, Emory University, Atlanta, Georgia, USA[c];Research Centre for Infectious Diseases, School of Molecular and Biomedical Science, University of Adelaide, Adelaide, Australia[d]

J.R.S. and H.P.L. contributed equally to this article.

ABSTRACT ***Streptococcus pneumoniae* is an important commensal and pathogen responsible for almost a million deaths annually in children under five. The formation of biofilms by *S. pneumoniae* is important in nasopharyngeal colonization, pneumonia, and otitis media. Pneumolysin (Ply) is a toxin that contributes significantly to the virulence of *S. pneumoniae* and is an important candidate as a serotype-independent vaccine target. Having previously demonstrated that a *luxS* knockout mutant was unable to form early biofilms and expressed less *ply* mRNA than the wild type, we conducted a study to investigate the role of Ply in biofilm formation. We found that Ply was expressed in early phases of biofilm development and localized to cellular aggregates as early as 4 h postinoculation. *S. pneumoniae ply* knockout mutants in D39 and TIGR4 backgrounds produced significantly less biofilm biomass than wild-type strains at early time points, both on polystyrene and on human respiratory epithelial cells, cultured under static or continuous-flow conditions. Ply's role in biofilm formation appears to be independent of its hemolytic activity, as *S. pneumoniae* serotype 1 strains, which produce a nonhemolytic variant of Ply, were still able to form biofilms. Transmission electron microscopy of biofilms grown on A549 lung cells using immunogold demonstrated that Ply was located both on the surfaces of pneumococcal cells and in the extracellular biofilm matrix. Altogether, our studies demonstrate a novel role for pneumolysin in the assembly of *S. pneumoniae* biofilms that is likely important during both carriage and disease and therefore significant for pneumolysin-targeting vaccines under development.**

IMPORTANCE The bacterium *Streptococcus pneumoniae* (commonly known as the pneumococcus) is commonly carried in the human nasopharynx and can spread to other body sites to cause disease. In the nasopharynx, middle ear, and lungs, the pneumococcus forms multicellular surface-associated structures called biofilms. Pneumolysin is an important toxin produced by almost all *S. pneumoniae* strains, extensively studied for its ability to cause damage to human tissue. In this paper, we demonstrate that pneumolysin has a previously unrecognized role in biofilm formation by showing that strains without pneumolysin are unable to form the same amount of biofilm on plastic and human cell substrates. Furthermore, we show that the role of pneumolysin in biofilm formation is separate from the hemolytic activity responsible for tissue damage during pneumococcal diseases. This novel role for pneumolysin suggests that pneumococcal vaccines directed against this protein should be investigated for their potential impact on biofilms formed during carriage and disease.

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Address correspondence to Jorge E. Vidal, jvidalg@emory.edu.

Streptococcus pneumoniae (the pneumococcus) is a Gram-positive bacterium annually responsible for 14.5 million cases of disease and 800,000 deaths in children under 5 years of age (1). The pneumococcus colonizes the nasopharynx in up to 90% of children and approximately 15% of adults and can spread from there to other anatomic sites to cause sinusitis, otitis media, pneumonia, bacteremia, and meningitis (2). Biofilm formation is important, both for colonization of the human nasopharynx and in pathogenesis (3). In particular, the role of pneumococcal biofilms in otitis media has been clearly demonstrated in humans (4) and investigated in a chinchilla model (5, 6). Despite the relevance of biofilms in commensalism and pathogenesis, the mechanisms of pneumococcal biofilm formation are not yet fully understood. Our laboratories have previously demonstrated that LuxS, the autoinducer 2 (AI-2) synthase, is essential for biofilm formation at early time points (7, 8) and observed that transcription of *ply*, the gene that encodes pneumolysin, is down-regulated approximately 35-fold in a *luxS*-null mutant (7).

Pneumolysin (Ply) is a well-established virulence factor (9, 10) that has been extensively studied for its hemolytic activity and cytotoxic properties. Ply, a 53-kDa surface protein (11), is a cholesterol-dependent cytolysin that binds cholesterol in eukary-

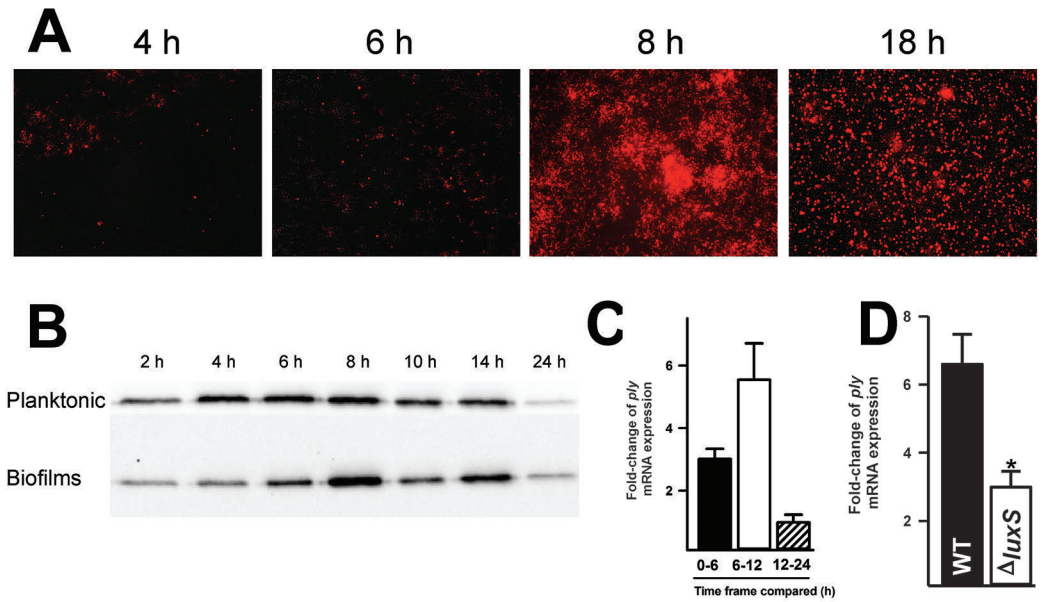

FIG 1 Pneumolysin is expressed in *S. pneumoniae* biofilms. (A) Fluorescence microscopy of wild-type D39 biofilms visualized with an anti-Ply antibody and a secondary conjugated antibody demonstrates Ply expression at 4, 6, 8, and 18 h. (B) Cell lysates from wild-type D39 grown in planktonic cultures or biofilms grown on polystyrene plates were probed by Western blotting. (C) To examine pneumolysin expression over time qPCR of cDNA was used to compare levels of *ply* mRNA at different time points of biofilm formation on polystyrene. Levels of *ply* mRNA were compared between the inoculum (0 h) and the 6-h biofilm, between 6-h and 12-h biofilms, and between 12-h and 24-h biofilms. (D) qPCR comparing levels of mRNA in 6-h biofilms to 12-h biofilms, showing that those formed by wild-type D39 have significantly more *ply* mRNA than those formed by D39Δ*luxS*. Error bars indicate standard errors of the means, and the asterisk indicates a *P* value of 0.01.

otic cell membranes and forms 400-Å pores that lead to cell lysis (12). Through its activity at the bacterial cell surface, or upon release through pneumococcal autolysis, Ply is responsible for almost all pneumococcal hemolytic activity (13). As anti-Ply antibodies are generated following carriage or otitis media (14–16) and TLR4-mediated recognition of pneumolysin stimulates a profound immune response (17, 18), a detoxified derivative of pneumolysin is under investigation as a serotype-independent vaccine candidate (19–21). However, it is currently unclear what role Ply plays during asymptomatic carriage that exposes this protein to the host immune system.

Though prior studies have demonstrated that the hemolytic alpha-toxin of *Staphylococcus aureus* is essential for biofilm formation on plastic and *ex vivo* porcine mucosa (22, 23), we are unaware of any other hemolysins being directly implicated in biofilm formation. That said, some preliminary data suggest that Ply has a role in pneumococcal biofilm development. A study of *S. pneumoniae* ATCC 6303 biofilms in a continuous-flow system demonstrated more Ply present in 3-day-old biofilms than in planktonic cultures (24), and more recently, Marks et al. reported that 48-h biofilms formed by a Ply-deficient mutant, grown on NCI-H292 epithelial cells with medium replacement every 12 hours, differed in appearance and biomass from wild-type D39 (25). Furthermore, our previous study of the role of LuxS/AI-2 in biofilm development suggests that genes regulated by LuxS, including *ply* (7, 26), the putative hemolysin SP1466 (26), and the *cbpD* gene, encoding choline-binding protein D (CbpD), may be important in biofilm development. While it has been shown that CbpD is required for biofilm formation (8), the role of pneumolysin in biofilm development has not been thoroughly investigated.

To assess whether Ply has a part in pneumococcal biofilm formation, we examined the timing and distribution of Ply expression in pneumococcal biofilms and evaluated biofilm formation of Ply knockout mutants under static and continuous-flow conditions, on both abiotic and human cell substrates. We found that Ply is expressed in pneumococcal biofilms, localizing to the earliest aggregates of cells, and knockout mutants deficient in Ply are impaired in their ability to form biofilms. While biofilm formation appears to mask the hemolytic activity of pneumococcal cells, pneumolysin's role in biofilm formation appears to be separate from its hemolytic activity. Altogether, our results demonstrate a novel and important role for Ply in the early development of pneumococcal biofilms.

RESULTS

Pneumolysin is expressed in pneumococcal biofilms. We first examined whether Ply was expressed in wild-type D39 during biofilm development in static cultures on a polystyrene substrate. To visualize Ply in biofilms, we employed a mouse monoclonal anti-Ply antibody followed by a fluorescently labeled anti-mouse antibody. Immunofluorescence images revealed low levels of Ply expression in biofilms 4 h and 6 h postinoculation, followed by maximal expression 8 h postinoculation and decreased expression

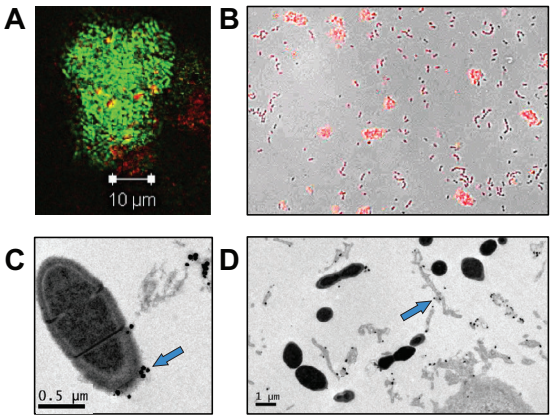

FIG 2 Location of pneumolysin in pneumococcal biofilms. (A) Confocal microscopy images of D39 (antipneumococcal antibody, green) and Ply (anti-Ply antibody, red) indicate that pneumococcal cells and pneumolysin colocalized in D39 biofilms at 24 h. (B) An image of differential interference contrast (Nomarski) with a fluorescent anti-Ply overlay of 4-h wild-type D39 biofilms revealed that pneumolysin localized to the earliest cellular aggregates in biofilms grown on polystyrene. (C) Transmission electron microscopy of an 8-h biofilm grown on A549 cells and treated with anti-Ply primary antibody and a gold-conjugated secondary antibody reveals that Ply is located on the bacterial surface (arrow) and in the extracellular matrix. (D) Another TEM image demonstrates Ply located throughout the extracellular matrix (arrow) between pneumococcal biofilm cells.

by 18 h (Fig. 1A). As a control, we probed 18-h biofilms of a *ply* knockout mutant (see Fig. S1 in the supplemental material) with the anti-Ply antibody and observed minimal fluorescence (see Fig. S2 in the supplemental material). Western blotting confirmed that pneumolysin is present in both planktonic and biofilm cells as early as 2 h postinoculation and reaches maximal expression 6 to 8 h following inoculation (Fig. 1B). Using reverse transcriptase and qPCR to quantify pneumolysin mRNA in biofilm cells, we found that *ply* expression increased from 0 to 6 h and from 6 to 12 h but there was little change in mRNA levels between 12 and 24 h (Fig. 1C). In line with our previous finding that *ply* is downregulated in planktonic cultures of D39Δ*luxS* (7), we found that the change in *ply* expression between 6 and 12 h in biofilms formed by D39Δ*luxS* was significantly less than in biofilms formed by wild-type D39 (Fig. 1D).

Pneumolysin localizes to cellular aggregates and is present in the cell wall and extracellular matrix. Using confocal microscopy to examine the location of Ply in three dimensions using an anti-Ply antibody and green fluorescent protein (GFP)-expressing wild-type D39 biofilms, we found that Ply colocalized with aggregates of biofilm cells (Fig. 2A). To confirm that the pneumolysin signal was specific to aggregates and not simply coincidental with the largest mass of cells, we examined sparse 4-h biofilms. Using differential interference contrast (Nomarski) images overlaid with images obtained using an anti-Ply antibody and a fluorescently labeled secondary antibody, we found that the Ply signal localized to nascent biofilm structures (i.e., early bacterial aggregates) (Fig. 2B). We then used immunogold transmission electron microscopy (TEM) to better localize Ply within 8-h biofilm structures and found that Ply localized to the bacterial cell well (arrow, Fig. 2C) and to the extracellular matrix (arrow, Fig. 2D). Altogether, these results confirm the specific localization of Ply to cellular aggregates and suggest a role as a matrix protein.

Early biofilm assembly on polystyrene is impaired in a Ply-deficient knockout. To examine whether Ply is essential for assembly of pneumococcal biofilms, we constructed a Ply-deficient knockout in D39 (see Fig. S1 in the supplemental material) and examined the biofilm formation of this mutant as well as a TIGR4 derivative provided by Andrew Camilli (Table 1). While the planktonic growth of the Ply-deficient mutants did not differ from that of the corresponding wild-type strains (see Fig. S3 in the supplemental material), the ability of the Δ*ply* derivatives to form biofilms was drastically impaired. Fluorescence microscopy of GFP-expressing derivatives of D39, D39Δ*ply*, AC2394 (TIGR4), and AC4037 (TIGR4Δ*ply*) demonstrated that biofilm formation ability of the Δ*ply* mutants was inferior to that of the wild-type

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
S. pneumoniae strains		
D39	Avery strain, clinical isolate, capsular serotype 2	46
SPJV01	D39/pMV158GFP, Tet^r	7
SPJV05	D39Δ*luxS* Ery^r	7
SPJV08	D39Δ*luxS*/pMV158GFP Ery^r Tet^r	7
SPJV10	D39Δ*comC* Ery^r	27
SPJV14	D39Δ*ply* Ery^r	This study
SPJV15	D39Δ*ply*/pMV158GFP Ery^r Tet^r	This study
Pn+	Reconstituted ply mutant	13
Ply306	D39 with ST 306 *ply* allele	This study
AC2394	Acapsular TIGR4, invasive clinical isolate	47
AC2394gfp	AC2394/pMV158GFP Tet^r	This study
AC4037	AC2394Δ*ply* Spc^r	47
AC4037gfp	AC2394Δ*ply*/pMV158GFP Spc^r Tet^r	This study
CDC_881	Serotype 1 strain, ST306	L. McGee, CDC
CDC_1403	Serotype 1 strain, ST306	L. McGee, CDC
CDC_4829	Serotype 1 strain, ST306	L. McGee, CDC
Plasmid		
pMV158GFP	*S. pneumoniae* mobilizable plasmid encoding GFP; confers resistance to tetracycline	48

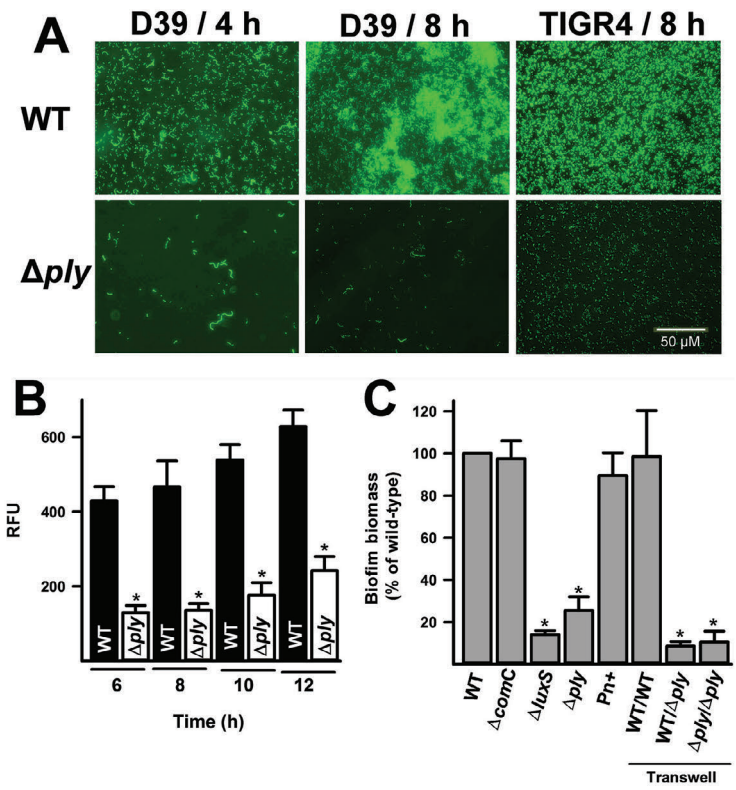

FIG 3 Ply knockout mutants form inferior biofilms on polystyrene at early time points. (A) GFP-expressing D39, D39Δ*ply*, AC2394 (TIGR4), and AC4037 (TIGR4Δ*ply*) were incubated on glass slides for 4 or 8 h, and biofilms were imaged with fluorescence microscopy. (B) Wild-type D39 and D39Δ*ply* biofilm biomasses on polystyrene plates at 6, 8, 10, and 12 h were quantified using a polyclonal FITC-conjugated anti-pneumococcal antibody. Asterisks indicate *P* values of <0.05. (C) Biofilm biomass of D39Δ*comC*, D39Δ*luxS*, D39Δ*ply*, and the complemented strain Pn+ (13) after 8 h of incubation. In addition, biofilm biomass formed on the bottom of the well of Transwell experiments with the wild type above and below, the wild type above and the *ply* mutant below, and the *ply* mutant above and below the barrier, after 10 h of incubation. Values from both the 8-h single-well experiments and 10-h Transwell experiments are expressed as percentages of the biofilm biomass of wild-type D39 at the respective time points. Error bars indicate standard errors of the means, and asterisks indicate *P* values of <0.01.

strains at 4 and 8 h (Fig. 3A). When absolute biofilm biomass was examined using a fluorescein isothiocyanate (FITC)-conjugated antipneumococcal antibody, we found that D39Δ*ply* produced significantly less biomass than the wild type (WT) at 6, 8, 10, and 12 h ($P < 0.05$; Fig. 3B). While a *comC* knockout mutant formed wild-type levels of biofilm biomass on abiotic surfaces, the *luxS* and *ply* knockout mutants formed significantly less biofilm biomass than the wild type ($P < 0.01$) (Fig. 3C). Similar results were obtained when biofilm assays were conducted using polystyrene substrates and Dulbecco's modified Eagle medium (DMEM) (data not shown). As a control, we examined the previously characterized complemented *ply* mutant Pn+ (13) and found that its ability to form biofilms did not differ significantly from that of the wild type (Fig. 3C).

To ascertain whether Ply released into the medium could be sufficient to restore biofilm formation capability to cells lacking endogenous Ply, we conducted experiments using Transwell permeable supports to separate strains of bacteria while allowing the free flow of nutrients and proteins. We grew three combinations of strains in wells with Transwell supports (given in the format top/bottom), WT/WT, WT/Δ*ply* mutant, and Δ*ply* mutant/Δ*ply* mutant. These wells were incubated at 37°C for 10 h, and biofilms on the bottom of the wells were quantified using GFP fluorescence. We found that the Δ*ply* strain with wild-type D39 above the Transwell barrier grew no more biofilms than the Δ*ply* strain with Δ*ply* organisms above the Transwell barrier (Fig. 3C).

The pneumolysin deficient mutant forms comparable biofilm biomass by 24 h, and cellular autolysis is delayed. Quantification of biofilms formed at 18 and 24 h using a fluorescently conjugated antipneumococcal antibody revealed that comparable biofilm biomass was found in wild-type and Δ*ply* strains (Fig. 4A). However, when biofilms were resuspended in phosphate-buffered saline (PBS) and plated on blood agar plates (BAPs), we found that more CFUs were recoverable from the wild-type strain at 8 h and 18 h postinoculation, but at 24 h postinoculation, there were no CFUs recoverable from wild-type D39 biofilms, while D39Δ*ply* and D39Δ*luxS* biofilms still yielded ~1×10^7 CFU/ml (Fig. 4B). Fluorescence microscopy of GFP-expressing D39 and D39Δ*ply* at 24 h revealed that the Δ*ply* strain was more metabolically active (as evidenced by expression of GFP) than the wild-type strain (Fig. 4C). Electron microscopy images directly illustrated pneumococcal autolysis; while wild-type D39 cells in 8-h biofilms contained electron-dense material, wild-type D39 cells in 24-h biofilms have disrupted cellular membranes and exhibit an absence of electron-dense material (Fig. 4D). TEM of the *ply* knockout mutant demonstrated healthy electron-dense cells at both 8 h and 24 h and also showed more surface-associated polysaccharide than in the wild type at 8 h (Fig. 4D).

Ply-deficient D39 forms less biofilm than the wild type on human cell line substrates. To verify that pneumolysin is necessary for early biofilm formation under more physiologically relevant conditions, we examined biofilm formation and *ply* expression in biofilms grown on human cell substrates under static and continuous-flow conditions. When biofilm formation was assessed using an A549 human lung cell substrate in static growth conditions, we observed that wild-type D39 formed confluent biofilms (Fig. 5A), while D39Δ*ply* formed very little biofilm (Fig. 5B). Quantitatively, the biofilm biomass of D39Δ*ply* was significantly less than that of the wild type 6 h ($P < 0.05$) and 8 h ($P < 0.01$) postinoculation (Fig. 5C). In addition, when the strains

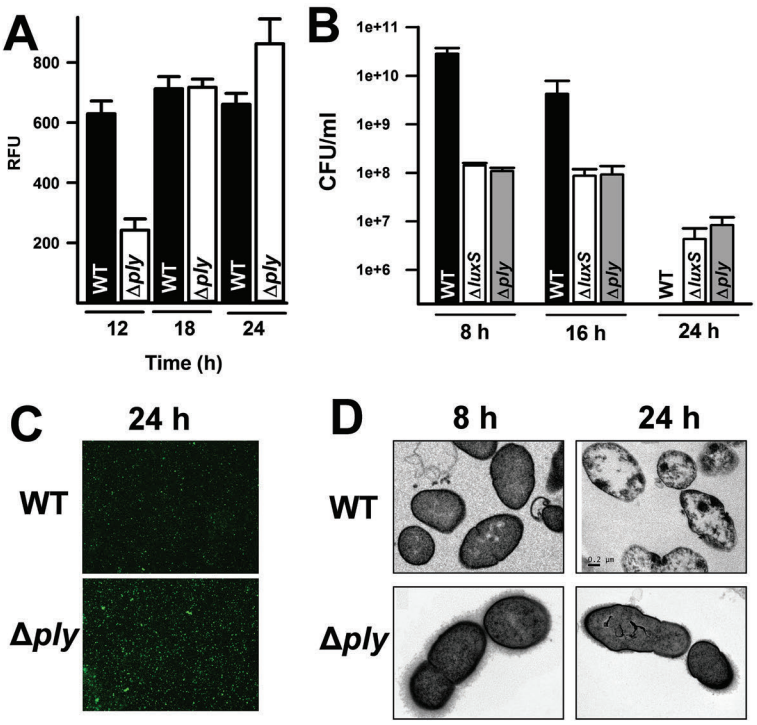

FIG 4 Ply-deficient D39 biofilm formation is comparable to that of the wild type by 24 h, but cellular autolysis is delayed. (A) Biofilm biomasses of D39 and D39Δ*ply* grown on polystyrene for 12, 18, and 24 h, quantified using a FITC-conjugated antipneumococcal antibody. (B) Biofilms formed by D39, D39Δ*luxS*, and D39Δ*ply* on polystyrene after 8, 16, or 24 h of incubation were resuspended in 1 ml of PBS and plated on BAP to estimate CFU. The limit of detection of this assay was 10 CFU/ml. (C) Fluorescence microscopy of GFP-expressing D39 and D39Δ*ply* confirms that the number of enzymatically active cells is greater in D39Δ*ply* biofilms than in wild-type biofilms following 24 h of static incubation. (D) Transmission electron microscopy of wild-type D39 and the Δ*ply* mutant grown on A549 cells demonstrates healthy D39 at 8 h and autolyzed cells at 24 h and healthy Δ*ply* cells at both 8 and 24 h. Error bars indicate standard errors of the means.

were grown on top of Detroit 562 pharyngeal cells for 8 h, we found that D39Δ*ply* and D39Δ*luxS* formed significantly less biofilm biomass than the wild type ($P < 0.01$) (Fig. 5D).

To better simulate *in vivo* conditions of pneumococcal biofilm growth, we grew D39 biofilms on an A549 substrate in a continuous flow bioreactor previously described (27). Confocal microscopy studies demonstrated that Ply coated the bacterial cell wall, with the top section showing pneumococcal chains surrounded by Ply (Fig. 6A) and a middle section showing abundant localization of Ply within aggregated bacteria (Fig. 6B). In relation to the log-phase planktonic inoculum, *ply* expression was upregulated ~6-fold in cells grown on immobilized A549 under static conditions for 8 h, ~2-fold in biofilms grown for 8 h in a bioreactor, and ~6-fold in biofilms grown for 24 h in a bioreactor (Fig. 6C).

Pneumococcal hemolytic activity is not essential for biofilm formation. To examine whether the biofilm formation function of pneumolysin was related to its hemolytic function, we examined the biofilm formation capability of serotype 1 strains belonging to sequence type (ST) 306 that produce a variant of pneumolysin with minimal hemolytic activity (28). Although Western blot analysis confirmed expression of Ply in the serotype 1 strains (see Fig. S4 in the supplemental material), hemolytic activity assays demonstrated the absence of hemolytic activity in D39Δ*ply* and the serotype 1 isolates CDC_881, CDC_1403, and CDC_4829 (Fig. 7A). However, these three serotype 1 strains displayed no statistically significant difference in biofilm biomass at 8 h compared to wild-type D39 (Fig. 7B). Biofilm formation capacity is known to vary markedly between pneumococcal strains. Thus, to ensure that the different genetic background of the serotype 1 strains did not affect the experiment, we constructed Ply306, a D39 derivative expressing the nonhemolytic *ply* allele from ST 306, using methods described previously (29). We found that the biofilm biomass formed by the Ply306 D39 strain did not differ significantly from that of wild-type D39 (Fig. 7B). Taken together, these results suggest that Ply's role in early biofilm formation is independent of hemolytic activity.

As pneumococcal biofilms are present in the nasopharynx with no apparent epithelial damage, the incorporation of Ply into the biofilm matrix may interfere with its hemolytic and cytotoxic activity. We therefore examined the hemolytic activity of wild-type D39 harvested from planktonic cultures in comparison to wild-type D39 harvested from biofilms. While D39 harvested from planktonic cultures maintained constant hemolytic activity over time (Fig. 7C and D), the hemolytic activity of D39 in biofilms

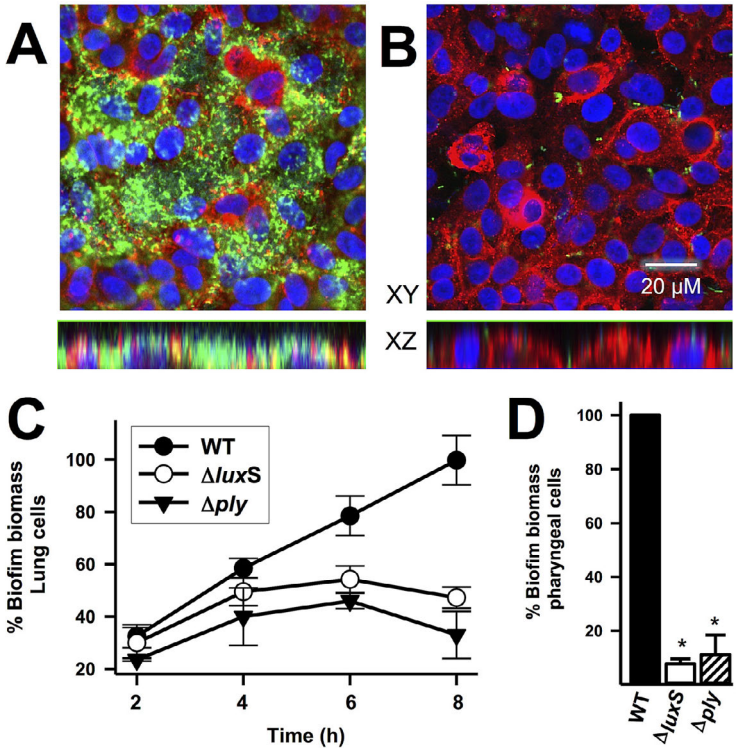

FIG 5 Ply-deficient D39 forms inferior biofilms when grown in static conditions on human lung cells. Wild-type D39 (A) and D39Δ*ply* (B) biofilms were grown under static conditions for 8 h on A549 human lung cell substrates. GFP-expressing bacteria are green, eukaryotic nuclei are blue, and eukaryotic membranes are red. (C) Wild-type D39, D39Δ*luxS*, and D39Δ*ply* were grown on human lung A549 cells under static conditions for 2, 4, 6, and 8 h, and biofilm biomass was quantified using GFP fluorescence. The amount of biofilm biomass is shown as a percentage of that formed by wild-type D39 at 8 h. At 6 and 8 h postinoculation, wild-type D39 formed significantly more biofilm biomass than D39Δ*luxS* or D39Δ*ply* ($P < 0.05$). (D) D39Δ*luxS* and D39Δ*ply* form significantly less biofilm than wild-type D39 when grown on Detroit 562 human pharyngeal cells for 8 h under static conditions. Error bars indicate standard errors of the means, and asterisks indicate *P* values of <0.01.

decreased from 128 hemolytic units (HU)/mg at 6 h postinoculation (Fig. 7C) to 64 HU/mg 8 h postinoculation (data not shown) and further to 32 HU/mg at 10 h postinoculation (Fig. 7D). This difference suggests that incorporation of pneumococcal cells into biofilms is correlated with a decrease in hemolytic activity.

DISCUSSION

This study is the first to demonstrate that pneumolysin is essential for the production of early pneumococcal biofilms. Not only was Ply expressed in pneumococcal biofilms produced under static and continuous-flow conditions on abiotic and human cell substrates, but Ply knockout mutants produced significantly less biofilm biomass than the wild type at early time points. Early aggregates of pneumococci were the first to express pneumolysin, and expression peaked with maximal biofilm formation at approximately 8 to 10 h postinoculation. Incorporation of cells into biofilms led to a decrease in hemolytic activity, but the hemolytic function of Ply appears to be separate from its role in biofilm formation. In total, we have demonstrated that pneumolysin has a previously unrecognized role, key in the early stages of the biofilm formation process.

Previous studies have alluded to a connection between pneumolysin and biofilm formation. Most evidence has been circumstantial, demonstrating that biofilm formation results in changes in *ply* expression. In 2006, Oggioni et al. reported extensive regulatory differences between planktonic and sessile phases, including the finding that in comparison to mid-exponential-phase liquid cultures, pneumococcal cells on agar and in biofilms expressed 100-fold and 5-fold less *ply*, respectively (30). Lizcano et al. concluded that pneumolysin was not important in biofilm formation in TIGR4; however their study only examined biofilm biomass by measuring crystal violet staining at 18 h postinoculation (31). Our results indicate that after 18 h of growth, cells in wild-type biofilms may have already begun to undergo autolysis, while the Ply knockout mutant continues to form more biofilm (7, 32). By examining biofilm formation of wild-type strains and isogenic mutants using various growth conditions, multiple substrates, and a variety of time points, we have shown that there is an important role for pneumolysin in the early stages of pneumococcal biofilm development.

Confocal and electron microscopy localized pneumolysin to the pneumococcal cell surface and extracellular matrix, suggesting a linking role for this protein during the aggregation phase of biofilm formation. While altered degradation of GFP may be responsible for the difference in autolysis witnessed by fluorescence, our CFU measurements and TEM results clearly demonstrate that the *ply* knockout mutant has delayed autolysis. This may be a function of metabolic changes, perhaps resulting from altered proximity to other bacterial cells. Previous studies have indicated that presence of a capsule impairs pneumococcal biofilm formation (33, 34) and that genes in the capsule operon are downregulated during biofilm formation (35). The polysaccharide halo surrounding the *ply* knockout cells imaged by TEM is consistent with altered capsular regulation, but further studies are needed to understand the role of capsular expression in pneumococcal biofilm formation.

Pneumolysin has been recognized as a virulence determinant for decades (36), and animal and human studies have indicated that the hemolytic activity of pneumolysin is largely responsible for pneumococcal virulence (37). However, the amount of hemolytic activity necessary for virulence is reportedly just 0.1% of the wild-type levels (13). Nevertheless, it was surprising when Kirkham et al. reported the isolation of nonhemolytic serotype 1 strains from invasive pneumococcal disease (28). This result led

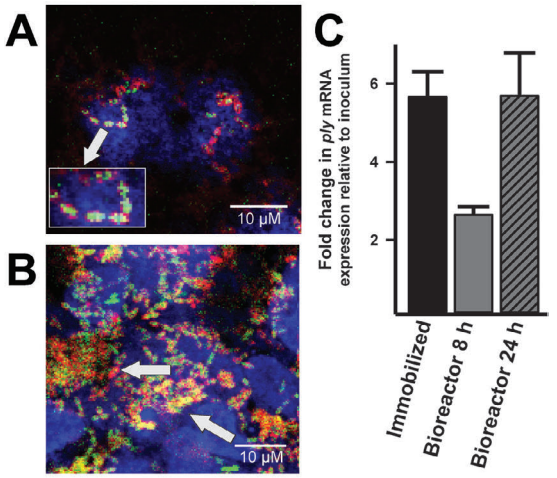

FIG 6 Pneumolysin is expressed in biofilms produced on human lung cells in a continuous-flow bioreactor. (A) The optical top section from a bioreactor with wild-type D39 biofilm cells on an A549 human cell substrate after 8 h of growth. Cells and biofilms were fixed, nuclei were fluorescently stained with TO-PRO-3 (blue), and Ply was stained with an anti-Ply antibody (red). Arrows indicate GFP-expressing pneumococci (green) surrounded by Ply, enlarged in the inset. (B) The middle section from the same 8-h bioreactor demonstrates abundant Ply expressed within pneumococcal biofilm aggregates grown in this model system. (C) Reverse transcription and qPCR of RNA extracted from biofilms grown on immobilized cells in static culture for 8 h or in a continuous-flow bioreactor for 8 or 24 h demonstrate that *ply* is upregulated relative to the inoculum when the bacteria are grown on A549 cells. Error bars indicate standard errors of the means.

those authors to speculate that an immune activation function of pneumolysin may contribute to virulence; this hypothesis is supported by the findings of Malley et al. showing that pneumolysin induces a substantial immune response by macrophages (17). The results of our study raise the possibility that pneumolysin's contribution to pneumococcal virulence may be partially attributed to its role in biofilm formation. At the same time, our finding that pneumococcal hemolytic activity of cells decreases as biofilms are formed may indicate that incorporation of cells into the biofilm matrix may mask the hemolytic epitope and prevent pneumolysin from provoking a clearing immune response during carriage.

Recently we demonstrated that LuxS/AI-2 regulates early pneumococcal biofilm formation (7, 8) and observed that a *luxS* null mutant expressed lower mRNA levels of the pneumolysin gene than the wild type. In a follow-up study, we demonstrated that both the LuxS/AI-2 and Com quorum-sensing systems regulate biofilm production in a bioreactor with living cultures of human respiratory cells (27). Our finding that pneumolysin is downregulated in a *luxS* knockout mutant but not in a *comC* knockout mutant appears to indicate that pneumolysin is predominantly under the regulatory control of the LuxS/AI-2 system. From these results we can hypothesize that LuxS-regulated pneumolysin plays a critical role in early aggregation, while other factors regulated by Com contribute to biofilm maturation. The components of biofilm assembly and maturation, and the regulation of those components, are a subject in need of further study.

Our results suggest that pneumolysin directly contributes to early aggregation of pneumococcal cells into early biofilms. Given that pneumolysin is an important candidate antigen for the development of a serotype-independent vaccine (19–21, 38, 39), our

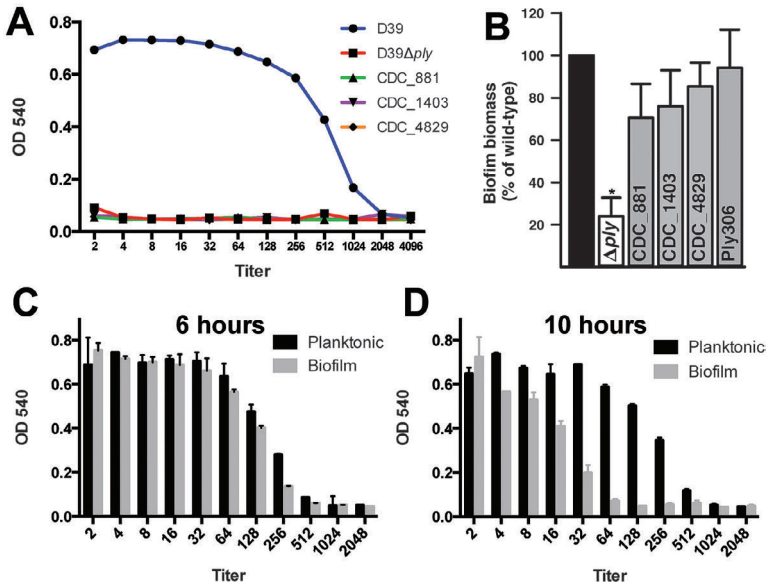

FIG 7 Hemolytic activity and biofilm phenotypes. (A) D39, D39Δ*ply*, and three serotype 1 isolates were examined for hemolytic activity. Wild-type D39 possessed hemolytic activity, while all other strains examined had almost none. (B) When biofilm formation capability was assessed, CDC_881, CDC_1403, CDC 4829, and Ply306 exhibited biofilm biomasses similar to that of wild-type D39. (C) Wild-type D39 cells harvested from planktonic and biofilm cultures assayed for hemolytic activity did not differ in hemolytic activity at 6 h postinoculation. (D) Planktonic and biofilm cultures of D39 harvested 10 h postinoculation demonstrate a marked difference in hemolytic activity. Error bars indicate standard deviations, and the asterisk indicates a P value of <0.01.

characterization of pneumolysin's role in biofilm formation has important implications. Carriage of the pneumococcus is a necessary precursor to pneumococcal diseases (40) and if Ply is routinely expressed in nasopharyngeal biofilms, its potential as a vaccine antigen is more easily understood. These results highlight the urgent need for further exploration of pneumolysin and the immune response against it, using both *in vivo* and *in vitro* models of biofilm formation.

MATERIALS AND METHODS

Strains and bacterial culture methods. The *S. pneumoniae* reference and derivative strains used in this study are listed in Table 1. Strains were cultured on Trypticase soy agar BAPs or in Todd-Hewitt broth containing 0.5% (wt/vol) yeast extract (THY). When biofilm formation was visualized or quantified using GFP-expressing strains, 2% (wt/vol) maltose was added to the culture medium. Inocula for all biofilm assays were prepared using growth on an overnight BAP to prepare a cell suspension in THY broth at an optical density at 600 nm (OD_{600}) of 0.05. This suspension was incubated at 37°C in a 5% CO_2 atmosphere until the culture reached an OD_{600} of 0.2 to 0.3 (early log phase); glycerol was added to a final concentration of 10% (vol/vol), and the suspension was stored at −80°C until used.

Growth of biofilms on abiotic and biotic surfaces. For experiments conducted on abiotic surfaces, 24-well Costar polystyrene plates (Corning, Tewksbury, MA) were used for quantification experiments, and 8-well Lab-Tek II glass chamber slides (Thermo, Fisher Scientific, Rockford, IL) were used for visualization experiments. Wells or chambers were filled with THY medium, inoculated 1:10 with inocula prepared as described above, and incubated at 37°C for various lengths of time. When indicated, experiments used Transwell permeable supports (Corning, Tewksbury, MA) with 0.4-μm pores to separate strains of bacteria within wells of 24-well plates while allowing the free flow of nutrients and secreted proteins.

To simulate the environment in the human respiratory tract, a human cell line substrate was employed, in both static and continuous-flow cultures, as recently described (27). Briefly, human-derived lung A549 cells (ATCC CCL-185) or human-derived pharyngeal Detroit 562 cells (ATCC CCL-198) were grown on polystyrene plates or Snapwell filters (Corning, Tewksbury, MA) until confluent (4 to 5 days). For static experiments, human cells were fixed with 2% paraformaldehyde (Sigma) for 15 min. Following several washes with PBS, immobilized human cells were inoculated with prepared bacterial inocula (~7×10^5 CFU/ml) and incubated in DMEM at 37°C for various times. For continuous-flow experiments, confluent cells on Snapwell filters were inoculated as detailed above and immediately placed in a sterile vertical diffusion chamber (27). Both basolateral and apical sides (inner chamber) were perfused with sterile DMEM with no antibiotics using a Master Flex L/S precision pump system (Cole-Parmer, Vernon, IL) at a low flow rate (0.20 ml/min). Bioreactor chambers containing *S. pneumoniae* and lung cells were incubated at 37°C in a sterile environment. At the end of the incubation period, inserts containing biofilms were removed, and biomass was analyzed qualitatively and quantitatively.

Visualizing pneumococcal biofilms. When GFP-expressing strains were used, biofilms on microtiter plates were washed with PBS and visualized by fluorescence microscopy, or biofilms produced on 8-chamber slides were washed with PBS, fixed with 2% paraformaldehyde, and mounted with Vectashield (Vector Laboratories, Burlingame, CA), and fluorescence was visualized with an inverted Evos FL microscope (Advanced Microscopy Group, Carlsbad, CA) or confocal microscopy. When non-GFP-expressing strains were used, biofilms were fixed with 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) for 15 min, washed with PBS, blocked with 2% bovine serum albumin, and stained for 1 h at room temperature with a polyclonal anti-*S. pneumoniae* antibody (~40 μg/ml) coupled to fluorescein isothiocyanate (FITC; ViroStat, Portland, ME). Where indicated, Ply was detected using 2.0 μg/ml unconjugated mouse monoclonal anti-Ply antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by an Alexa Fluor 568 goat anti-mouse IgG secondary antibody (Molecular Probes, Invitrogen, Carlsbad, CA). For preparations from the human cell bioreactor system, sialic acid residues present on the plasma membranes were stained with 5 μg/ml wheat germ agglutinin conjugated with Alexa Fluor 555 (Molecular Probes, Invitrogen, Carlsbad, CA) for 30 min (41), and nucleic acids were stained with TO-PRO-3 (1 μM), a carbocyanine monomer nucleic acid stain (Molecular Probes, Invitrogen, Carlsbad, CA) for 15 min. Finally, preparations were washed three times with PBS, mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and analyzed with a Zeiss LSM510 confocal microscope. Confocal images were analyzed with an LSM image browser, version 4.0.2.121.

For immunogold localization of Ply, 8-h biofilms grown on top of A549 lung cells were fixed with either 2 or 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) overnight at 4°C. Cells were then washed and treated with 0.05% Triton X-100 for 10 min before blocking with PBS containing 5% bovine serum albumin (BSA) and 0.1% cold-water-fish gelatin. Mouse anti-Ply primary antibody was diluted to 8 μg/ml in PBS containing 0.1% acetylated BSA for overnight incubation. After washes, cells were incubated overnight with ultrasmall-colloidal-gold-conjugated goat anti-mouse IgG secondary antibody (Aurion, Wageningen, the Netherlands), followed by washes and postfixation in 2.5% buffered glutaraldehyde. Silver enhancement of ultrasmall gold particles was carried out using R-gent SE-EM silver enhancement kit (Aurion, Wageningen, the Netherlands) following the manufacturer's instruction. Cells were then fixed with 0.5% osmium tetroxide, dehydrated, and embedded in Eponate 12 resin. Ultrathin sections were counterstained with 5% uranyl acetate and 2% lead citrate and examined on a JEOL JEM-1400 transmission electron microscope (JEOL Ltd., Japan) equipped with a Gatan UltraScan US1000 charge-coupled device (CCD) camera (Gatan, Inc., Pleasanton, CA).

Quantification of biofilm biomass. Biofilm biomass was quantified by fluorescence methods as previously described (7, 27) or by serial dilution followed by plating to obtain cell counts (CFU/ml). For GFP-expressing strains and biofilms stained with a FITC-conjugated antibody, arbitrary relative fluorescence units (RFU) were obtained using a Victor X3 multilabel plate reader (PerkinElmer, Waltham, MA). The number of arbitrary fluorescence units of wild-type D39 was set to a biofilm biomass of 100% and used to calculate the biofilm biomass percentages of all of the other *S. pneumoniae* strains tested and those at different time points. Results of repeated experiments were plotted using GraphPad Prism or SigmaPlot and examined for statistical significance with two-tailed *t* tests for normal data and Mann-Whitney *U* tests for nonparametric data.

Western blot assays. To compare the expression of Ply in biofilm and planktonic cells, bacterial cells were treated with the B-PER bacterial protein extraction reagent (Thermo, Fisher Scientific, Rockford, IL) according to the manufacturer's protocol and quantified using the Bradford protein assay (42). A 5-μg portion of extracted protein was loaded into each well on a 12% polyacrylamide gel, subjected to SDS-PAGE, and transferred onto a nitrocellulose membrane. Those membranes were blocked with PBS with 0.05% (vol/vol) Tween 20 and 5% (wt/vol) nonfat dry milk for 1 h and then probed with a 1:200 dilution (final concentration, 0.5 μg/ml) of mouse anti-Ply monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (43). Bound antibody was detected with a horseradish peroxidase-conjugated secondary anti-mouse antibody diluted 1:10,000 and addition of Pierce ECL Western blotting substrate (Thermo, Fisher Scientific, Rockford, IL).

Construction of D39 derivatives with *ply* knocked out or modified. A *ply* knockout mutant was generated by PCR ligation mutagenesis as previously described (44) (see Fig. S1 in the supplemental material). Briefly, the construct was generated by PCR amplification of a 5′ segment of the *ply* gene with primers HPL1 and HPL2 and a 3′ segment with primers HPL5 and HPL6. Primers HPL3 and HPL4 were used to amplify the *ermB* gene, encoding erythromycin resistance, and to add XbaI and

TABLE 2 Primers used in this study

Primer	Target gene	Sequence[a]	RE site	PCR product size (bp)
HPL1L	SPD1728-*ply*	TTGGCGACAAGCATTTTGTA		800
HPL2R	*ply*-SPD1728	CAGTCTAGACCACTACGAGAAGTGCTCCA	XbaI	
HPL3L	*ermB*	CAGTCTAGAAAAAATTTGTAATTAAGAAGGAGT	XbaI	796
HPL4R	*ermB*	CAGCTCGAGCCAAATTTACAAAAGCGACTCA	XhoI	
HPL5L	*ply*-flank	CAGCTCGAGCTTTAAAAGGGAATGTTCGTAATCTCT	XhoI	800
HPL6R	Flank-*ply*	GCGACAAAAACAATCATACTGC		
JVS35L	16S rRNA	AACCAAGTAACTTTGAAAGAAGAC		126
JVS36R	16S rRNA	AAATTTAGAATCGTGGAATTTTT		
JVS59L	*ply*	TGAGACTAAGGTTACAGCTTACAG		225
JVS60R	*ply*	CTAATTTTGACAGAGAGATTACGA		
luxS-L	*luxS*	ACATCATCTCCAATTATGATATTC		257
luxS-R	*luxS*	GACATCTTCCCAAGTAGTAGTTTC		

[a] Underlining indicates the restriction enzyme (RE) site used for cloning.

XhoI restriction sites to the ends (7). The 5′ and 3′ segments were ligated to the gene encoding erythromycin resistance using T4 DNA ligase, and the cassette was generated by amplification with primers HPL1 and HPL6. A 100-ng portion of the cassette was then transformed into competent D39 cells, and the SPJV14 (D39Δ*ply*) recombinants were selected for on BAP containing 0.5 μg/ml of erythromycin. Knockouts were confirmed by PCR (see Fig. S1 in the supplemental material), sequencing (data not shown), and Western blotting (see Fig. S4 in the supplemental material). A derivative of D39 expressing the nonhemolytic *ply* allele from an *S. pneumoniae* type 1 ST 306 strain was constructed by allelic replacement using an analogous protocol and the primers employed for construction of a derivative expressing the ply_{4496} allele, which has significantly less hemolytic activity (29).

Hemolytic activity assay. Pneumococcal cells were prepared for assay by growth in THY to mid-log phase (OD_{600}, 0.3 to 0.4) and 10× concentration before resuspension in PBS. Bacterial cells were lysed with a 10-min incubation at 37°C in 0.1% sodium deoxycholate solution, and then protein was quantified using the Bradford assay. Sheep red blood cells (RBCs) were washed three times using PBS. RBCs (2 ml) were added to 32 ml PBS and 50 μl β-mercaptoethanol to make a 3% RBC preparation. Serial 2-fold dilutions of 17 μg of each protein preparation were made in PBS in microtiter plates before addition of 50 μl of the 3% RBC preparation and incubation for 30 min at 37°C. Unlysed RBCs were pelleted by centrifugation, and supernatant was transferred to a new microtiter plate for measurement at A_{540}. A_{540} values were plotted against the dilution factor. Hemolytic units are defined as the lysate dilution factor when hemolysis is 50% of that of wild-type D39.

Gene expression studies. Suspensions of biofilm cells or planktonic cells were combined with an equal volume of RNA Protect (Qiagen Inc., Valencia, CA). Total RNA was extracted with an RNeasy minikit (Qiagen Inc., Valencia, CA) and treated with 2 U of DNase I (Promega, Madison, WI) as previously described (7). The integrity of RNA preparations and the concentrations of samples were assessed using a NanoDrop ND-1000 spectrophotometer (Thermo, Fisher Scientific, Wilmington, DE). Total RNA was reverse transcribed into cDNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), using the manufacturer's instructions. Quantitative PCR (qPCR) was performed with generated cDNA using SYBR green (Bio-Rad, Hercules, CA) and a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA). qPCRs were performed in duplicate with 30 ng of total RNA, a 250 nM concentration of the primers JVS59L and JVS60R to quantify *ply* transcripts (Table 2), and the following conditions: 1 cycle of 95°C for 3 min, 40 cycles of 95°C for 30 s and 55°C for 30 s, and 1 cycle of 72°C for 1 min. Melting curves were acquired on SYBR green channel from 65°C to 95°C with 0.5°C increments. Relative quantities of mRNA expression were normalized to the constitutive expression of the housekeeping 16S rRNA gene (7) calculated by the comparative $2(-\Delta\Delta C_T)$ method (45). Error bars in the figures represent the standard errors of the means calculated using data from three independent experiments.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00655-13/-/DCSupplemental.

Figure S1, EPS file, 1.2 MB.
Figure S2, EPS file, 14.8 MB.
Figure S3, EPS file, 1 MB.
Figure S4, EPS file, 1.2 MB.

ACKNOWLEDGMENTS

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SUPPLEMENTAL MATERIAL

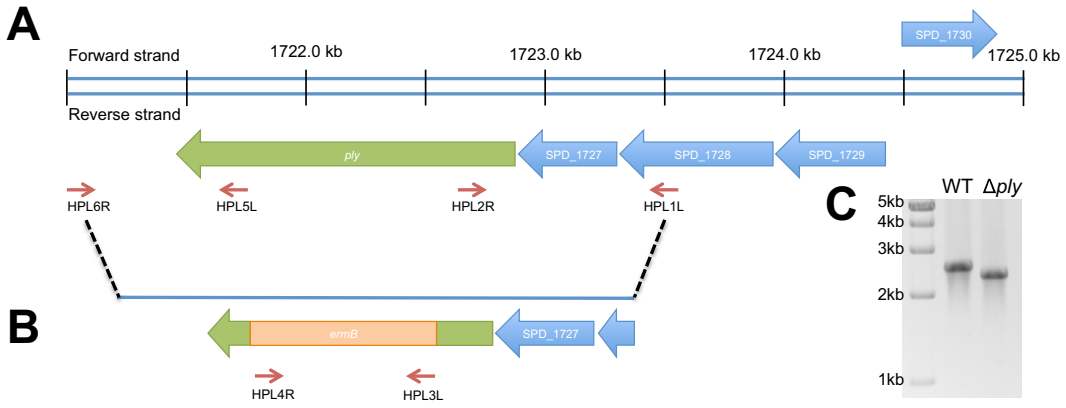


Figure S1. Schematic of the *ply* locus in *S. pneumoniae* D39 and the Ply-deficient mutant. (A) The *ply* gene is immediately downstream from three putative coding regions (SPD_1727, SPD_1728, and SPD_1729); together these four genes constitute a putative operon. (B) Ply-deficient mutants were constructed through PCR ligation mutagenesis, replacing the central segment of the *ply* gene with the *ermB* gene, conferring erythromycin resistance. Red arrows indicate the locations of primers used for mutant construction. (C) PCR using HPL1L and HPL6R confirm different sized products in the wild type and the *ermB*-containing mutant.

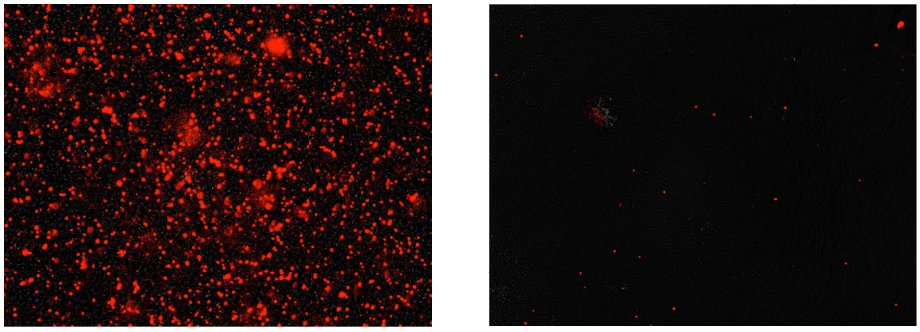


Figure S2. Anti-Ply antibody is specific for pneumolysin. To verify that the anti-Ply antibody used was specific to pneumolysin, we probed paraformaldehyde-fixed 18-h biofilms grown on polystyrene with the monoclonal antibody, followed by a goat anti-mouse IgG secondary antibody. Biofilms formed by wild-type D39 appeared red on fluorescence microscopy, whereas biofilms formed by the D39 *ply* knockout exhibited minimal fluorescence.

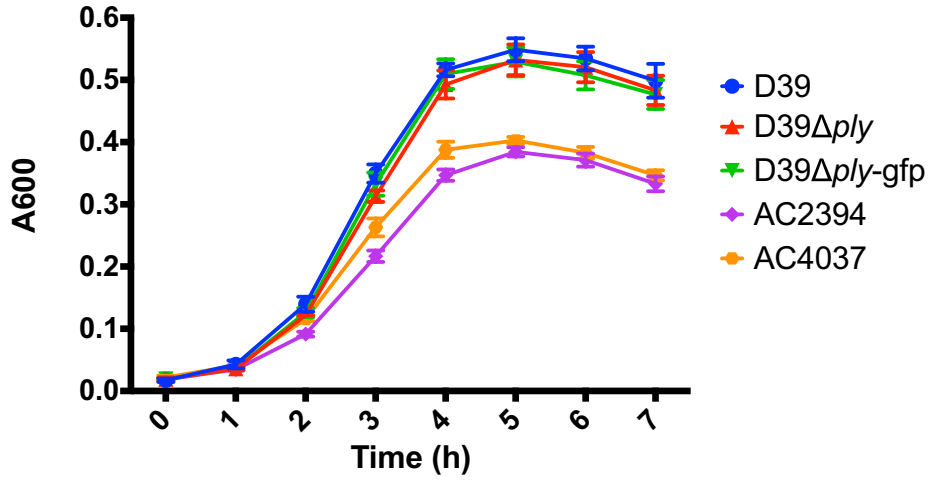


Figure S3. Planktonic growth rates of wild-type and derivative strains do not significantly differ. Wild-type D39, the Ply-deficient mutant, the GFP-containing derivative, AC2394, and AC4037 were grown in liquid THY for 7 h, and optical density was measured hourly. Experiments were performed in triplicate and on three different days; error bars indicate standard errors of the means from three different days.

Figure S4. Pneumolysin is expressed in hemolysis-deficient serotype 1 isolates. Cell lysates of wild-type D39, D39Δ*ply*, and three nonhemolytic serotype 1 isolates were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and probed with an anti-Ply antibody. The resulting Western blot displayed the presence of the pneumolysin protein in D39 and the three serotype 1 strains but not in D39Δ*ply*.

ADDENDUM TO CHAPTER 3

The preceding article contained two curious findings regarding the *Δply* knockout that merited further study: the delayed autolysis in biofilms and the apparent extracellular capsule noted on electron microscopy. To better understand these phenotypic findings, we conducted gene expression studies of *lytA* and *cps4A* in wild-type and *Δply* samples grown in biofilms on polystyrene plates as well as the log-phase planktonic inocula. The autolysin gene *lytA* is the primary mediator of pneumococcal cell death but other genes are involved in cell lysis including *lytC* and *cpbD*. The capsular gene *cps4A* is thought to be a transcriptional regulator of the capsular polysaccharide synthesis locus.

Expression studies were conducted according to the protocol noted in the Materials and Methods section, with 16S gene expression used to normalize the data and all results expressed in relation to the gene expression of planktonic wild-type cells. The forward and reverse primers used for *cps4A* amplification were 5'-CGTCTAAGAGTCAGTCTTTCAATA-3' and 5'-ATTGATATCCACTCCATAGAGATT-3', respectively. Experiments were conducted in duplicate on two separate days.

We found that *lytA* expression was 1.7 times greater in the *Δply* planktonic inoculum than in the WT planktonic inoculum (Figure A1); this was not a significant difference when using the standard cutoff of 2-fold change for significance (Livak and Schmittgen, 2001). However, the expression of *cps4A* was significantly higher (4.9-fold) in the *Δply* inoculum relative to the wild-type inoculum (Figure A1).

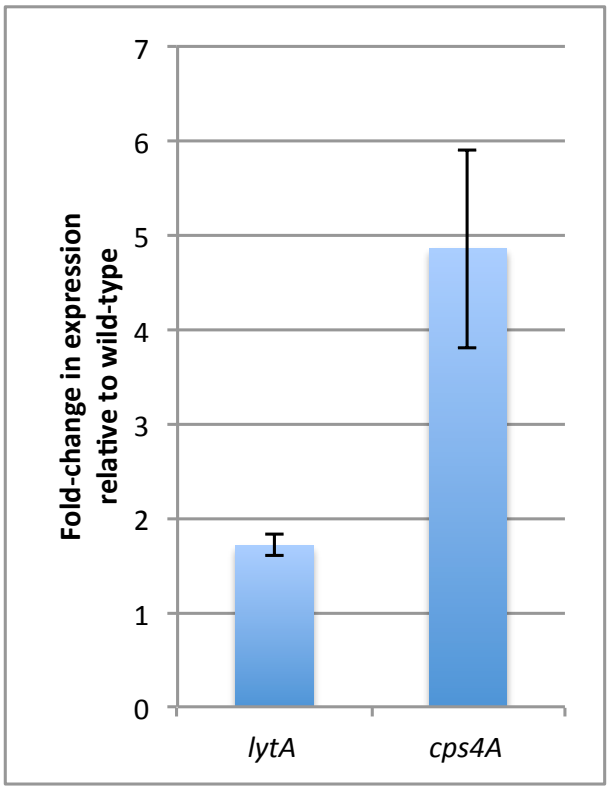


Figure A1. Gene expression of *lytA* and *cps4A* in log-phase planktonic D39*Δply* cells relative to log-phase planktonic wild-type cells. Expression is normalized to 16S and data shown are mean values with error bars showing the standard error of the mean.

When expression of these genes were examined in biofilm cells, *lytA* expression was significantly higher (8.6 – 17.8-fold) as compared to the planktonic inoculum, while relative expression of *cps4A* in wild-type biofilms did not exceed the 2-fold level of significance (Figure A2).

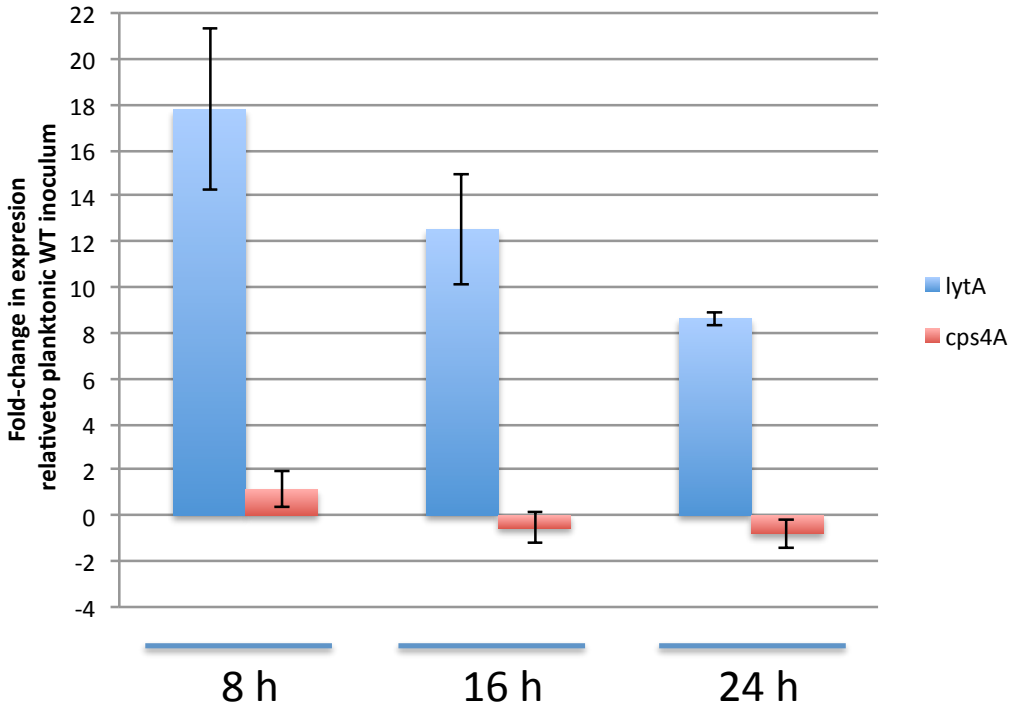


Figure A2. Normalized expression of *lytA* and *cps4A* mRNA in wild-type biofilms grown for 8 h, 16 h, and 24 h relative to planktonic WT inoculum. Expression is normalized to 16S and data shown are mean values with error bars showing the standard error of the mean.

When expression was analyzed in *Δply* biofilms relative to *Δply* planktonic cells, *lytA* was up-regulated and *cps4A* was down-regulated at 16 and 24 hours (Figure A3). At the 8 hour time point examined by transmission electron microscopy (Figures 2 and 4), expression of *cps4A* did not differ significantly from planktonic *Δply* cells. While *cps4A* expression decreases at 16 hand 24 h relative to the high level seen in the *Δply* inoculum (Figure A3), the level of expression at 24 h *Δply* biofilms is roughly equivalent to that seen in the wild-type inoculum (data not shown).

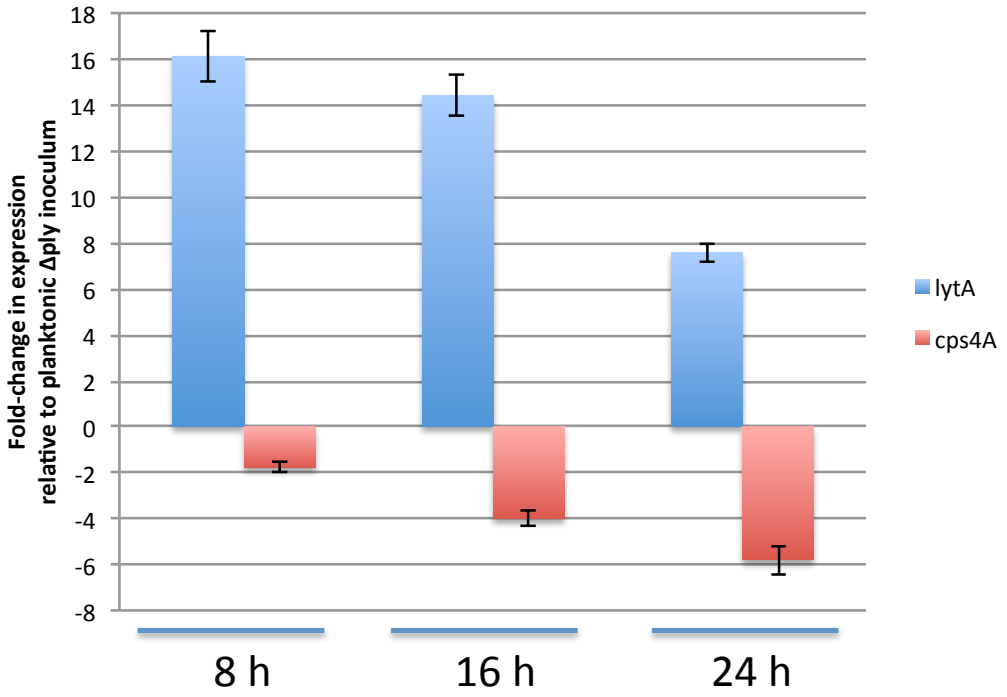


Figure A3. Normalized expression of *lytA* and *cps4A* mRNA in *Δply* biofilms grown for 8 h, 16 h, and 24 h relative to planktonic *Δply* inoculum. Expression is normalized to 16S and data shown are mean values with error bars showing the standard error of the mean.

In total, these experiments reveal a few interesting findings. First, when examining log-phase planktonic cells, expression of the capsular gene *cps4A* is significantly higher in *Δply* cells than in wild-type cells. This high level of *cps4A* expression is still present in 8 h *Δply* biofilms and may explain the extracellular polysaccharide we observed by electron microscopy. Second, *lytA* expression is up-regulated in both wild-type and *Δply* biofilms relative to their respective planktonic inocula. This is somewhat surprising given that we witnessed delayed cell-death in the *Δply* biofilms and may indicate that other factors such as *lytC* and or *cpbD* may be responsible for cell death inside biofilms (Guiral *et al.*, 2005). These additional results reinforce the assertion that capsular expression and cell death in pneumococcal biofilms are promising areas for future research.

Chapter 4: Nasopharyngeal densities of common bacterial pathogens in relation to experimental human pneumococcal carriage

Joshua R. Shak, Jorge E. Vidal, and Keith P. Klugman
Department of Global Health, Rollins School of Public Health, Emory University, Atlanta, Georgia, USA

Amelieke Cremers, Aldert Zomer, and Peter W. Hermans
Laboratory of Pediatric Infectious Diseases, Radboud University Medical Centre, Nijmegen, Netherlands

Jenna F. Gritzfeld and Stephen B. Gordon
Respiratory Infection Group, Liverpool School of Tropical Medicine, Liverpool, UK

ABSTRACT

Colonization of the nasopharynx by *Streptococcus pneumoniae* (Sp) is a necessary precursor to pneumococcal diseases that result in morbidity and mortality worldwide. The nasopharynx is host to other bacterial species, including the common pathogens *Staphylococcus aureus* (Sa), *Haemophilus influenzae* (Hi) and *Moraxella catarrhalis* (Mc). To better understand how these bacteria interact with Sp during pneumococcal colonization, we examined bacterial densities using quantitative PCR before and after controlled inoculation of healthy human adults with Sp serotype 6B. Of the 52 subjects challenged with Sp, 33 (63%) carried Sp at 2, 7, or 14 days post-inoculation. While the baseline presence or density of Sa, Hi, and Mc were not associated with likelihood of successful Sp colonization at a statistically significant level, there were trends in the data that suggest a possibly protective effect of baseline Sa colonization and a facilitative effect of Hi colonization. While carriage prevalence of Sa, Hi, and Mc were not significantly different between those colonized at 2 and 7 days post-inoculation, at 14 days post-inoculation, the proportion carrying Sa was significantly lower among those colonized with Sp as compared to those not colonized with Sp ($p = 0.008$). These data on bacterial associations are the first to be reported surrounding experimental human pneumococcal colonization and provide questions and estimated effect sizes that will be useful in future studies.

INTRODUCTION

Streptococcus pneumoniae (commonly called the pneumococcus) is a resident of the human nasopharynx as well as an important pathogen responsible for 14.5 million episodes of disease annually [1]. Colonization of the nasopharynx is a necessary precursor to pneumococcal diseases including pneumonia, otitis media, and sepsis [2]. *S. pneumoniae* (Sp) shares the nasopharyngeal niche with other bacterial species including the pathogens *Staphylococcus aureus* (Sa), *Haemophilus influenzae* (Hi) and *Moraxella catarrhalis* (Mc). Hi is a common etiologic agent of otitis media, sinusitis, and pneumonia [3], Mc causes otitis media as well as lower respiratory tract infections [4], and Sa is a common etiologic agent of skin infections, sinusitis, bacteremia, and pneumonia [5]. The mechanisms by which these bacteria interact in the human nasopharynx are an ongoing area of research.

With the introduction of pneumococcal conjugate vaccine (PCV) worldwide, the relationship between nasopharyngeal carriage of vaccine-type Sp and other bacterial pathogens is of special interest. Cross-sectional studies have demonstrated an inverse association between carriage of *S. aureus* and vaccine-type *S. pneumoniae* [6-8] and studies of PCV introduction have found increased prevalence of *S. aureus* following PCV roll-out [9-11]. Other cross-sectional studies have found positive correlations between Sp and Hi [8,12,13], and between Sp and Mc [14-16]. However, no study to date has examined the ecological effect of controlled inoculation with Sp on other bacterial residents of the nasopharynx.

The Experimental Human Pneumococcal Carriage (EHPC) study [17] is a model of pneumococcal carriage in healthy human adults with the potential for testing vaccine candidates using prevention of carriage as a surrogate endpoint. Early results have indicated that Sp challenge provokes mucosal immunity even when Sp carriage is not established [18] and induced carriage offers protection against carriage when re-challenged with the same serotype [19]. In addition to immunological responses produced by experimental carriage, there is also the possibility of altered nasopharyngeal bacterial carriage. We hypothesized that successful colonization with Sp may be affected by the bacteria present in the nasopharynx at baseline and that the addition of pneumococcus to the nasopharynx could alter the carriage of other bacterial pathogens. Using DNA extracted from nasal wash samples from the EHPC study and real-time quantitative PCR (qPCR), we sought to determine whether the presence of Sa, Hi, or Mc prior to inoculation could predict successful Sp colonization and if carriage density of these three bacterial species was altered by successful Sp colonization.

METHODS

Study Population and Sample Collection. Healthy adults recruited in Liverpool, UK. This study was approved by the United Kingdom's National Research Ethics Service (NRES) and informed consent was obtained from all subjects enrolled. Nasal wash samples were collected as previously described [20,21] from subjects 7 days before, and 2, 7, and 14 days after inoculation with 60k , 80k , 160k , or 320k CFUs of *S. pneumoniae* 6B. 2ml of a 20ml nasal wash was mixed and incubated with 4ml RNAprotect Bacteria Reagent (Qiagen Inc., Valencia CA). It was stored at -80°C until genomic DNA extraction. The remaining volume of recovered nasal wash not combined with RNAprotect was centrifuged for 10 min at 3345 x g. The supernatant was removed and the pellet resuspended in skim milk-tryptone-glucose-glycerol (STGG) medium before plating on blood and chocolate agar plates for identification of Sp, Hi, Sa, and Mc.

DNA Extraction. The nasal wash RNAprotect mixture was pelleted by centrifugation and the pellet was resuspended in phenol and 300 ul lysis buffer (AGOWA mag Mini DNA Isolation Kit, AGOWA, Berlin, Germany). Then 25-50 mg zirconium beads were added and the sample disrupted by TissueLyser (Qiagen Inc., Valencia CA) for 2 min, twice. The supernatant containing the released DNA was then purified according to the protocol included with the AGOWA mag Mini DNa isolation Kit. Samples were eluted in 63 µl of elution buffer and DNA yield was measured with 16S rRNA qPCR.

Quantification of Bacterial DNA. Real-time quantitative PCR (qPCR) was used to quantify bacterial DNA densities in the nasal wash samples. Reactions were conducted in a volume of 20 µl containing the TaqMan Universal PCR Master Mix (Invitrogen by Life Technology, CA, USA), 1.0 µl of sample DNA, forward and reverse primers and fluorogenic probes (Table 1). For all reactions, the qPCR conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All samples were run in duplicate; if duplicates mismatched, samples were considered negative if the positive duplicate had Ct > 38.

Table 1. Primers and probes used for qPCR assays.

Species (gene)	**Primer**	**5'-3' nucleotide sequence[a]**	**Size**	**Source**
S. pneumoniae (lyta)	lytaF	ACGCAATCTAGCAGATGAAGC	101 bp	[22]
	lytaR	TGTTTGGTTGGTTATTCGTGC		
	lytaPr	TTTGCCGAAAACGCTTGATACAGGG		
S. aureus (nuc)	nucF	GTTGCTTAGTGTTAACTTTAGTTGTA	154 bp	[23]
	nucR	AATGTCGCAGGTTCTTTATGTAATTT		
	nucPr	AAGTCTAAGTAGCTCAGCAAATGCA		
H. influenzae (hpd)	hpdF729	AGATTGGAAAGAAACACAAGAAAAAG A	113 bp	[24]
	hpdR819	CACCATCGGCATATTTAACCACT		
	hpdPr762i	AAACATCCAATCG"T"AATTATAGTTTA CCCAATAACCC		
M. catarrhalis (copB)	copbF	CGTGTTGACCGTTTTGACTTT	125 bp	[16]
	copbR	TAGATTAGGTTACCGCTGACG		
	copbPr	ACCGACATCAACCCAAGCTTTGG		

[a] All probes were labeled with Hex at 5′-end and Black Hole Quencher (BHQ) at the 3′-end with the exception for hpdPr762, which was labeled with BHQ at an internal "T" and SpC6 at the 3'-end.

Samples from the dose-ranging study were analyzed using an Applied Biosystems 7500 Fast Real-Time PCR System and samples from the reproducibility study were analyzed on a Bio-Rad CFX96 Real-Time System. Primer and probe concentrations were

optimized for each target and each machine. For the ABI 7500, final primer/probe concentrations were 100/100 nM for lytA, 200/200 nM for nuc, 300/300 nm for hpd, and 100/100 nm for copB. For the Bio-Rad CFX96, final primer/probe concentrations were 100/100 nM for lytA, 400/200 for nuc, 300/300 nM for hpd, and 200/200 nm for copB.

Standard curves were created using purified genomic DNA extracted from laboratory strains and quantified using the NanoDrop ND-1000. For *S. pneumoniae,* DNA was extracted from TIGR4. For *S. aureus,* DNA was extracted from ATCC 29213. For *H. influenzae,* DNA was extracted from R2866. And for *M. catarrhalis,* DNA was extracted from BBH18.

Back-calculating genomes/ml from DNA quantities. Since it has long been standard to report nasopharyngeal densities in colony forming units (CFUs) per ml, we determined the number of bacterial genomes per ml of nasal wash using the mass of bacterial DNA in the qPCR reactions as shown in the following equation:

$$number\ of\ genome\ copies = \frac{mass\ in\ ng * Avagadro^{'}\ s\ number}{genome\ length * 1e9 * 650}$$

where 650 is the average weight of a DNA basepair. This number was then multiplied by 31.5 to account for the difference between the volume of nasal wash used in the extraction (2 ml) and the volume of extracted DNA resulting from the reaction (63 ul) in order to determine genome copies per ml of nasal wash. For example, for *S. pneumoniae*:

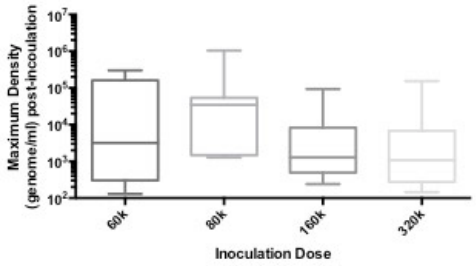
$$\frac{genomes}{ml} = 31.5 * \frac{mass\ in\ ng * 6.022e23}{2160842 * 1e9 * 650}$$

Statistical Analyses. Given the non-normal distribution of the data (as assessed by the Shapiro-Wilk test) numbers are reported as median (interquartile range) except when noted. Differences in proportions were assessed by the Fisher exact test and differences in continuous variables (such as density) were assessed using the Mann-Whitney U test. When assessing difference in density of multiple groups, a Kruskall-Wallis analysis of variance was employed. G*Power 3.1 [25] was used for power calculations and all other statistical analyses were conducted in GraphPad Prism 6 or RStudio 0.97.

RESULTS

Inoculation dose of Sp does not change colonization rate or density. To increase the sample size of this analysis, subjects receiving different inoculation doses of Sp were grouped together. To verify that all subjects receiving 60k to 320k CFU were experiencing biologically similar challenges, we compared the frequency and density of colonization among these groups. The maximum post-inoculation colonization log densities of the 60k, 80k, 160k, and 320k CFU groups were 3.5 (2.5-5.0), 4.5 (3.7-4.7), 3.1 (2.9-3.8), and 3.0 (2.7-3.5), respectively (Figure 1).

We defined successful Sp colonization as a positive qPCR result at any time point post-inoculation. Using this definition, 55% of subjects in the 60k CFU group became colonized with Sp while 70% of subjects in the 80k, 160k, and 320k groups became colonized (Figure 1). There was no statistically significant differences between the four groups in density (p = 0.30) or colonization rate (p = 0.47).



	60k (repro)	80k (DR)	160k (DR)	320k (DR)
Colonized	55%	70%	70%	70%
Not Colonized	45%	30%	30%	30%

Figure 1. Maximum post-inoculation density and colonization rate segregated by inoculation dose.

Carriage rates and densities over time. While all subjects were negative for *S. pneumoniae* by culture at baseline, 3 subjects (6%) carried Sp at baseline as assessed by

qPCR. Following inoculation, carriage of Sp increased to 50% at 2 days post-inoculation and then fell to 46% and 34% at 7 and 14 days post-inoculation, respectively (blue line, Figure 2). In all, 33 subjects (63%) carried Sp at ≥1 time point post-inoculation; this subset was our successful colonization group.

At baseline, 16 subjects (31%) carried Hi, 14 subjects (27%) carried Sa, and 10 subjects (19%) carried Mc (Figure 2). Hi was carried by 33%, 25% and 21% of subjects 2, 4, and 7 days post-inoculation, respectively (red line, Figure 2). Sa was carried by 27%, 27% and 25% of subjects 2, 4, and 7 days post-inoculation, respectively (green line, Figure 2). Mc was carried by 10%, 15%, and 12% of subjects 2, 4, and 7 days post-inoculation, respectively (purple line, Figure 2). Overall, while the rate of Sp carriage increased dramatically after inoculation, the carriage rates of Sa, Hi, and Mc stayed relatively constant.

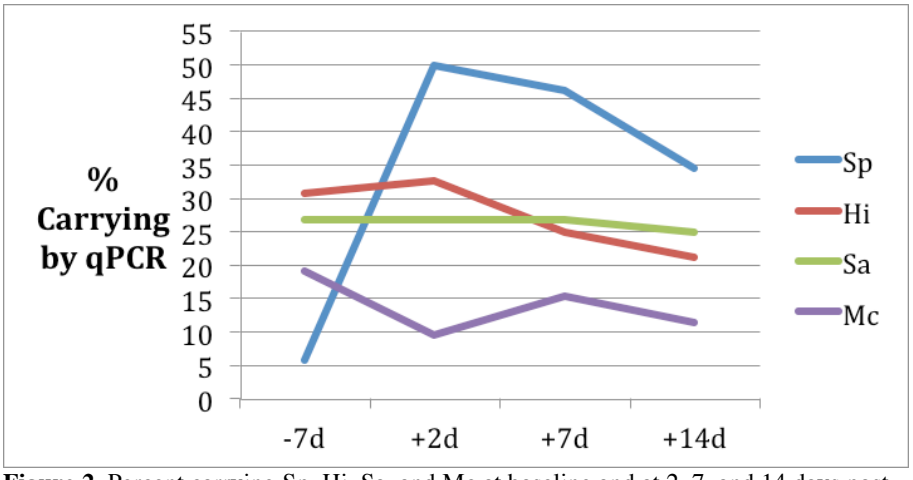


Figure 2. Percent carrying Sp, Hi, Sa, and Mc at baseline and at 2, 7, and 14 days post-inoculation.

When carriage densities were examined for each bacterium over the four time points (Figure 3), only Sp showed a significant change over time, with median log density increasing from 2.3 at baseline to 3.7 at 14 days post-inoculation (p = 0.003).

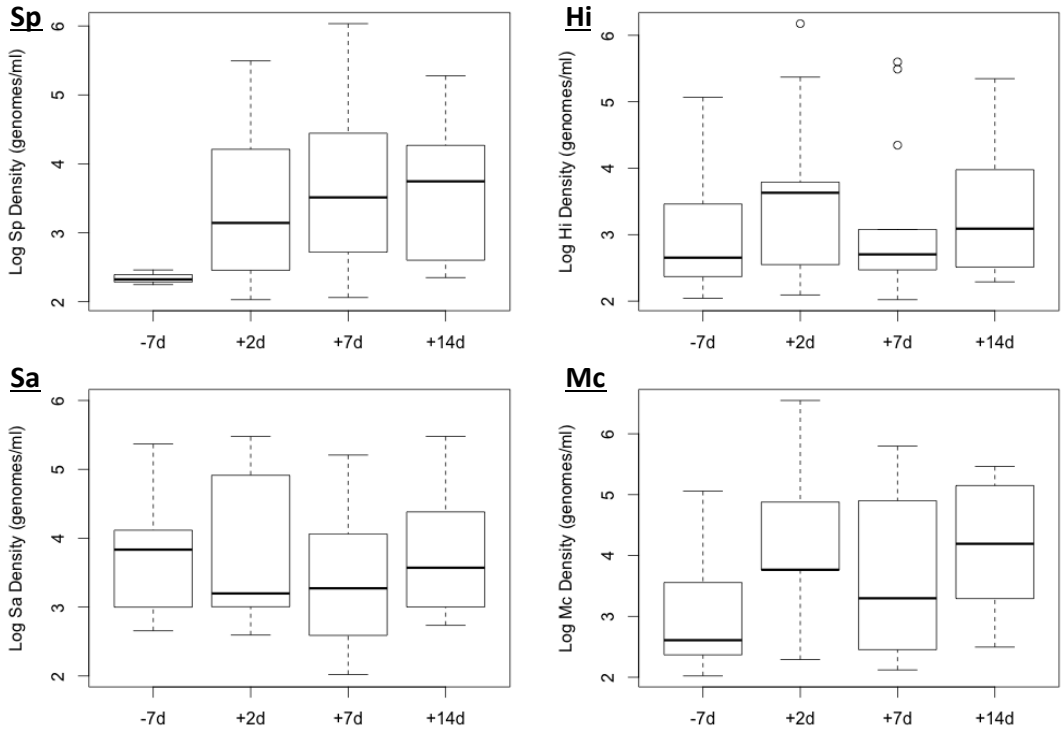


Figure 3. Densities of Sp, Hi, Sa, and Mc at four time points. Boxplots of log density (genomes/ml) of all samples with genomes/ml > 100 at each time point.

Baseline bacterial carriage as a predictor of Sp colonization. To assess whether carriage of Hi, Sa, or Mc was associated with likelihood of Sp colonization post-inoculation, we examined carriage as both a binary variable and a continuous variable. While 75% of those Hi+ at baseline would go on to become colonized with Sp, only 58% of those Hi- would become Sp colonized; this difference in proportions was not significant at this sample size (p = 0.71). Conversely, 50% of Sa+ became colonized with Sp while 68% of Sa- became colonized with Sp; this difference in proportions was not

statistically significant (p = 0.33). Individuals carrying Mc at baseline became colonized with Sp 50% of the time while individuals not carrying Mc at baseline became colonized with Sp 57% of the time; a difference not statistically significant (p = 0.47). Similarly, Mann-Whitney U tests revealed no statistically significant difference in baseline densities of Mc, Sa, or Hi when comparing those colonized with Sp to those not colonized by Sp (data not shown).

Table 2. Contingency tables of carriage of Hi, Sa, and Mc at baseline against colonization with Sp post-inoculation for 52 subjects.

Carriage at baseline	Sp Col+ (n=33)	Sp Col- (n=19)	Fisher exact p-value
Hi+ (n=16)	12 (75%)	4 (25%)	0.71
Hi- (n=36)	21 (58%)	15 (42%)	
Sa+ (n=14)	7 (50%)	7 (50%)	0.33
Sa- (n=38)	26 (68%)	12 (32%)	
Mc+ (n=10)	5 (50%)	5 (50%)	0.47
Mc- (n=42)	28 (67%)	14 (33%)	

Hi, Sa, and Mc carriage post-inoculation. While the proportion of subjects carrying Hi decreased from 31% pre-inoculation to 21% post-inoculation (Figure 2), to determine if there was a change in Hi related to successful Sp colonization, we segregated the data by Sp colonization status. Among those colonized by Sp, carriage of Hi decreased from 36% pre-inoculation to 15% 2 days post-inoculation (red line; Figure 4A). Among those not colonized by Sp, carriage of Hi increased from 21% pre-inoculation to 42% 2 days post-inoculation (blue line; Figure 4A). However, the difference in proportions carrying Hi

between the Sp colonized group and the Sp uncolonized group was not statistically significant at any of the 4 time points measured.

Among those colonized by Sp, carriage of Mc decreased from 15% pre-inoculation to 9% post-inoculation (red line; Figure 4B). Among those not colonized by Sp, carriage of Mc decreased from 26% pre-inoculation to 16% post-inoculation (blue line; Figure 4A). The difference in proportions between the Sp colonized group and the Sp uncolonized group was not statistically significant at any of the 4 time points.

For *S. aureus*, 37% of the Sp uncolonized group carried Sa at baseline, while 21% of the Sp-colonized group carried Sa at baseline; this difference in proportions was not statistically significant ($p = 0.33$). Similarly the carriage rates of Sa were not significantly different between the two groups at 2 and 7 days post-inoculation. However, at 14 days post inoculation, those colonized with Sp had a significantly lower rate of Sa carriage (12%) than those uncolonized with Sp (47%; $p = 0.008$; Figure 4C). To further characterize the difference in Sa carriage at 14 days post-inoculation, we compared the density of carriage among those colonized by Sp to those uncolonized by Sp. Among those carrying Sa at this time point, the log median density was lower among those colonized by Sp (3.2) than those uncolonized by Sp (4.1; Figure 4D); however this difference was not statistically significant ($p = 0.71$).

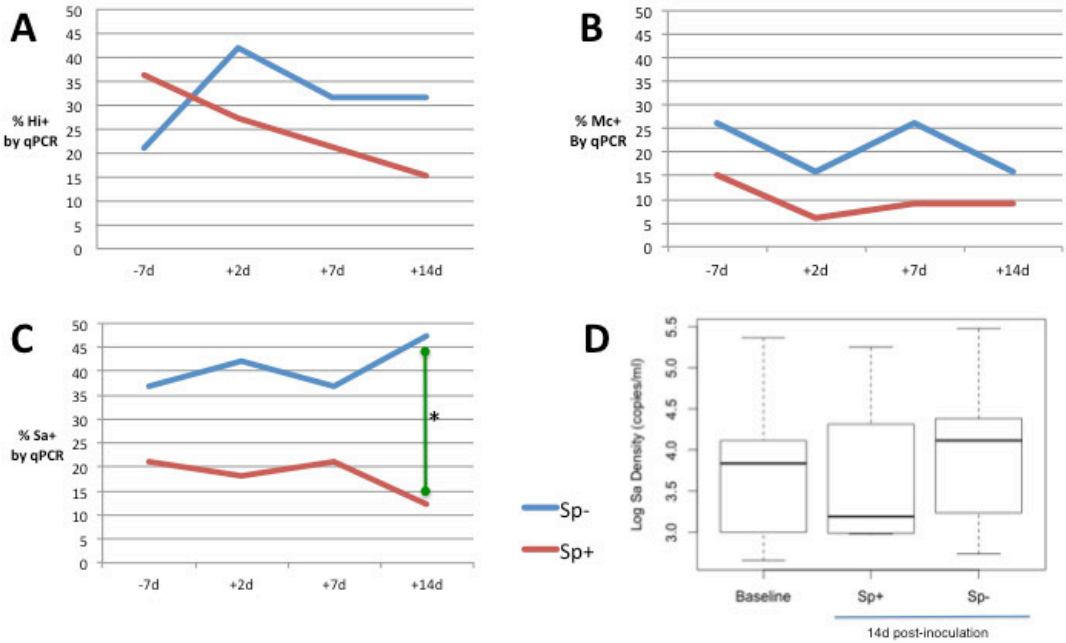


Figure 4. Percent carrying Hi (A), Mc (B), and Sa (C) at 4 time points and density of Sa colonization at baseline and 14d (D) segregated by success of pneumococcal colonization.

DISCUSSION

This is the first study to examine bacterial densities of *S. aureus*, *H. influenzae*, and *M. catarrhalis* before and after controlled addition of *S. pneumoniae* to the human nasopharynx. Furthermore, 6B is a clinically important serotype of *S. pneumoniae*. A recent meta-analysis found that pneumonia patients infected with 6B were at an increased risk of death [26]. The prevalence of 6B in carriage and disease led to its inclusion in all formulations of the pneumococcal conjugate vaccine. While a smaller, previous study examined the immune response to experimental human pneumococcal carriage, that study did not characterize the bacterial load in the nasopharynx before and after inoculation [27]. Therefore, the current study of pneumococcal colonization of healthy adults offers the first chance to estimate effect size for two separate questions. First, do baseline bacterial densities correlate with likelihood of Sp colonization? And second, does successful pneumococcal colonization lead to a change in the presence or density of other nasopharyngeal species?

On examining 52 subjects, 33 (63%) of whom were successfully colonized by Sp, we found no statistically significant association between baseline presence or density of Hi, Sa, or Mc and likelihood of colonization with Sp. However, there were tendencies in the data which suggest that successful pneumococcal colonization may be more likely in those carrying Hi at baseline and less likely in those carrying Sa; these are associations consistent with previous studies. While the current study only had a power of 0.22 to

detect a difference in proportions of 17%, a future study could enroll approximately 250 subjects to achieve a power of 0.8 now that the estimated effect size is better described.

Successful colonization with Sp was associated with an absence of Sa in samples 14 days post-inoculation. This difference in proportions was due to a decrease in prevalence of Sa in subjects colonized with Sp and an increase in prevalence of Sa in those uncolonized with Sp. While these data are not sufficient to prove that colonization with Sp always decreases prevalence of Sa at 14 days post-colonization, a time-delayed effect on *S. aureus* carriage would implicate an immunologic mechanism in the antagonistic relationship between Sp and Sa. Notably, studies of HIV-positive children have demonstrated a lack of antagonism between Sa and Sp [9,28] and a recent study has demonstrated that pneumococcal colonization in mice elicits cross-reactive antibodies that protect against *S. aureus* [29]. Future studies may want to measure levels of the cross-reactive antibodies identified by Lijek *et al*., staphylococcal P5CDH and pneumococcal SP_1119. However, the difference in proportions we observed at 14 days was an exacerbation of a pre-existing difference in proportions at baseline, 2 and 7 days post-inoculation. Therefore, repeated studies are needed to very this observed delayed effect on Sa carriage.

The primary limitations of this study include sample size and the lack of sham-inoculated controls. *A priori*, it was difficult to estimate the effect size of bacterial carriage on pneumococcal colonization and *vice versa*. From this study, it appears that the effect size of baseline carriage of Sa and Hi on likelihood of pneumococcal colonization is in the

range of 15-20%. Similarly, the examination of carriage post-inoculation was limited by the small number of subjects carrying Hi, Sa, or Mc; with only 19-31% of subjects carrying these microbes, the comparison of those Sp-colonized to those uncolonized with Sp is very limited. Future studies may want to examine a population in which carriage of these bacterial species is more prevalent. While this study examined bacterial population dynamics in adults, colonization with Sp and other nasopharyngeal pathogens is most common in children; however, ethical considerations may preclude experimental pneumococcal challenge studies from being conducted in a pediatric population. Finally, the design of this study enabled the comparison of successful colonization with unsuccessful colonization without examining the effect of repeated nasal washes on nasopharyngeal bacterial population dynamics. While nasal washes are an effective way to determine nasopharyngeal carriage [20], it is unknown how this procedure may disturb the nasopharyngeal microbial community.

This study is the first to examine nasopharyngeal bacterial densities in healthy human adults inoculated with Sp. While children are the primary reservoirs of pneumococcal carriage, experimental studies in adults will likely be the best approximation we can achieve for observing pneumococcal colonization in a controlled environment. The difference we found in prevalence of Sa carriage 14 days post-pneumococcal colonization is an intriguing finding that lends support to the hypothesis that Sp and Sa antagonism is mediated by the host immune system. Larger, future studies will be able to examine the relationship between pneumococcal colonization and Sa, Hi, and Mc carriage, and microbiome studies using 16S sequencing data may reveal associations with

species that are unculturable or are not known pathogens. Furthermore, the experimental human pneumococcal carriage model offers future opportunities to examine vaccine efficacy and observe the changes in nasopharyngeal microbiology that occur near the time of vaccination.

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Chapter 5: Summary and Outlook

This thesis investigated the role of intra- and inter-species population dynamics on pneumococcal nasopharyngeal carriage by synthesizing the existing literature, investigating the molecular mechanisms of pneumococcal biofilm assembly, and measuring the effect of pneumococcal colonization on other nasopharyngeal species. Through multiple scientific approaches and in cooperation with international collaborators, this dissertation has shed light on the cooperation and competition between bacterial populations during their residence in the nasopharynx.

In the second chapter we examined the published literature on co-colonization with multiple pneumococcal serotypes, interactions between multiple bacterial species in the nasopharynx, and the role of pneumococcal biofilms in carriage. We found that newer laboratory techniques revolutionized the exploration of the nasopharyngeal ecosystem. These techniques have demonstrated that the simultaneous carriage of multiple pneumococcal strains is more common than previously believed, that the data on nasopharyngeal bacterial interactions are suggestive (but not definitive) of clinically-relevant associations, and that the study of biofilms needs more physiologically-relevant, multispecies models. Despite all the work yet to be accomplished, the existing literature supports the argument that bacterial interactions are a critical part of pneumococcal carriage and a better understanding of these interactions will lend insight into the transition from commensal to pathogen.

The third chapter of this dissertation explored one of the mechanisms crucial to pneumococcal biofilm development. Prior research had identified the LuxS/AI-2 quorum sensing system as an important regulator of pneumococcal biofilm formation, however, the downstream mechanism by which LuxS controlled biofilm assembly was not known. Since expression of pneumolysin was down-regulated in *luxS* knockouts, we examined whether pneumolysin might be the downstream effector involved in biofilm formation. In fact, we not only found that pneumolysin was expressed in biofilms, but that pneumolysin knockout strains had greatly impaired biofilm formation capabilities and pneumolysin was present on both the surface of pneumococcal cells and in the extracellular matrix. While it was suspected that the pneumococcus harbors surface-associated proteins that facilitate biofilm assembly, the identification of pneumolysin as one of those proteins was unexpected. Pneumolysin is a well-characterized cytotoxin, recognized as a primary mechanism of tissue damage in pneumococcal diseases. If pneumolysin is shown to be critical biofilm component in the human nasopharynx, this would explain why humans generate anti-pneumolysin antibodies following carriage and strengthen the argument that a non-toxic derivative of pneumolysin is a good choice for a serotype-independent vaccine target. While more detailed *in vitro* and *in vivo* studies are needed, our findings suggest that pneumolysin may be one of the most critical proteins for pneumococcal commensalism in addition to its well-known role in pathogenesis.

The analysis of bacterial densities before and after experimental human pneumococcal carriage in the fourth chapter was the first step towards designing future studies that can pinpoint causal relationships between bacterial species carried in the nasopharynx. A

dynamic environment with a constant flow of air, mucus, and blood, the nasopharynx is never static. The analysis of bacterial densities measured by qPCR of nasal wash samples taken before and after addition of *S. pneumoniae* 6B was an effort to capture snapshots of the bacterial composition of the nasopharynx in relation to the introduction of the pneumococcus. There were a number of intriguing trends and findings that support what has been observed in cross-sectional and vaccine studies. Of particular note was the finding that subjects successfully colonized by the pneumococcus were significantly less likely to be carrying *S. aureus* 14 days post-inoculation than those subjects uncolonized by the pneumococcus. The real value in this analysis is that it provides future studies an estimate of the magnitude of the effect that may be expected when examining bacterial densities in relation to pneumococcal colonization. With these estimates in hand, future studies can be designed to have adequate power to assess the presence of these relationships.

Three larger lessons can be extracted from this work. First, the molecular mechanisms that allow colonization and pathogenesis are inextricably linked and, in fact, sometimes identical. This notion was highlighted in Jeffrey Weiser's 2010 review "The pneumococcus: why a commensal misbehaves" [1]. This paper emphasized that the molecular mechanisms most important to pathogenesis – the polysaccharide capsule, exoglycosides such as neuraminidase, and immune evasion mechanisms like IgA protease – have important roles during nasopharyngeal carriage as well. Weiser postulated that pneumolysin may be important during carriage because of its ability to provoke an inflammatory response which can lead to suppurative rhinitis and increased

transmission. With the work presented in this dissertation, we can confidently say that pneumolysin's role in carriage likely goes beyond provoking mucus production. Pneumolysin appears to be critical in binding pneumococcal cells to one another and to the extracellular matrix of biofilms important to nasopharyngeal carriage. If born out by future studies, this has important implications for pneumolysin-targeting vaccines, which may in fact be effective at clearing carriage in addition to preventing disease. The elimination of carriage of the pneumococcus – as we have witnessed following the adoption of the *H. influenzae* type B vaccine – would undoubtedly have important implications for the bacterial ecosystem of the nasopharynx.

The second major lesson is that measuring changes in dynamic nasopharyngeal bacterial population densities is exceedingly difficult and establishing causal relationships is even more difficult. Our review paper cited numerous studies that found associations in presence or density of *S. pneumoniae* and *S. aureus*, *H. influenzae*, and *M. catarrhalis*. However, the mechanisms of antagonism or facilitation have proven elusive and proving causality almost impossible. By examining specimens from the experimental human pneumococcal colonization study, we endeavored to simplify the number of variables in the model system. Our data suggest that pneumococcal colonization affects the likelihood of carriage of other bacterial species in the range of 15-20%; however, our study was not adequately powered to determine a statistically significant effect of this magnitude. This indicates that either there is no real association to observe, the association is of relatively small magnitude, or the methods we used to sample and quantify bacterial load are deficient. Certainly methods currently employed to sample the nasopharynx (including

nasal swab and nasal wash) are relatively crude. Better sampling methods or new approaches to normalizing observed bacterial density relative to volume recovered or humans cells present may help us quantitatively observe these dynamics more accurately. Regardless, studies that can truly assess the relationships between nasopharyngeal pathogens will take place in the future, and hopefully the results of this thesis can inform the design of such studies.

The third major lesson is that evolution of the pneumococcus may be heavily influenced by events during nasopharyngeal carriage. There are a few hypotheses about why microbes evolve virulence. The first hypothesis is that the action of the virulence factor offers the microbe an opportunity for transmission to a new host. The second hypothesis, championed by the aforementioned review by Weiser, contends that the factors that cause virulence also offer an unrelated fitness advantage. A third hypothesis, synthesized by Levin and Bull [2] based on the earlier observations of Meynell and Stocker [3], and Moxon and Murphy [4], is that evolution of virulence may be advantageous to microbes within hosts even when deleterious for between host transmission. Our finding that the toxin pneumolysin is critical to biofilm development active during carriage strengthens the assertion that virulence factors often have additional, non-virulence-related roles. Whether pneumolysin evolved to damage human tissue or to facilitate biofilm assembly is unknown, but further study of its evolutionary origins may provide insight into commensal pathogens like the pneumococcus become virulent.

In conclusion, the pneumococcus is a commensal and a pathogen that must be understood through the lens of population biology at every level. Isogenic populations of pneumococci work in concert to create biofilms. Different serotypes interact with one another in the nasopharynx and the pneumococcus interacts with other bacterial species during asymptomatic carriage and pathogenesis. By understanding both the mechanisms of interactions (e.g., the role of pneumolysin in biofilm formation) and the greater population trends (e.g., antagonism and synergism between bacterial species), we will be able to understand and manipulate the complex networks of microbes that inhabit our bodies during health and disease.

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Appendix: Other Published Work

During the course of graduate school, I have been presented with research opportunities that, although outside the scope of this dissertation, were still fantastic opportunities to acquire experience in scientific collaboration inside and outside Emory University. This has led to published works that are not about *Streptococcus pneumoniae*, but are related to my general interest in human pathogens that spend the majority of their lifecycle harmlessly in the environment or as human commensals.

The first such opportunity was a clinical study to investigate the connection between anemia and *Helicobacter pylori*. A common resident of the human stomach, *H. pylori* can also cause peptic ulcer disease, gastric cancer, and general mucosal inflammation. Previous studies have reported a link between *H. pylori* and iron-deficiency anemia – presumably due to gastric bleeding. Having observed high rates of anemia in a rural Haitian population I was privileged to work with through Project Medishare, I organized a study to determine whether high rates of *H. pylori* infection might explain the high prevalence of anemia in Haiti's central plateau. Surprisingly, we found that in our study population, *H. pylori* infection was actually protective against anemia (Appendix 1).

The second opportunity presented itself during an infectious diseases clerkship just prior to the beginning of my PhD research. When an unusual case of multi-drug resistant *Aeromonas hydrophila* came on to the service, a literature review yielded few previous reports of aminoglycoside-resistant *A. hydrophila*. As aminoglycosides such as amikacin

are a recommended first-line therapy for *A. hydrophila* wound-infections, we wrote a case report to bring this new kind of resistance to the attention of clinical microbiologists and infectious disease physicians (Appendix 2).

The third opportunity came with my first graduate rotation in the genomics laboratory of Timothy Read. Having just seen this case of *A. hydrophila* in the clinic, I wanted to understand further what made this strain of *Aeromonas* so virulent. Tim indulged my curiosity and we sequenced two separate strains isolated from the clinical case. Shortly after publication of the case report, I received a telephone call from Amy Horneman, who was passionately interested in the newly isolated strains. This began a fruitful collaboration with genomicist Chris Grim and *Aeromonas* pathogenesis expert Ashok Chopra which yielded a multidisciplinary report on the clinical, genetic, and laboratory characteristics of these novel strains of *A. hydrophila* (Appendix 3).

In total, these papers reflect my broader interest in microbes that are usually harmless, but can be very harmful on occasion. Similar to the pneumococcus, in addition to virulence factors and host susceptibility, *H. pylori* and *A. hydrophila* are strongly influenced by the microbes that surround them.

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Anemia and *Helicobacter pylori* Seroreactivity in a Rural Haitian Population

Joshua R. Shak,* Jamie B. Sodikoff, Rebecca A. Speckman, Francois G. Rollin, Marie P. Chery, Conrad R. Cole, and Parminder S. Suchdev
Emory University School of Medicine, Atlanta, Georgia; Project Medishare, Thomonde, Haiti

Abstract. Anemia is a significant health concern worldwide and can be the result of nutritional, environmental, social, and infectious etiologies. We estimated the prevalence of anemia in 336 pre-school children and 132 adults in the rural Central Plateau of Haiti and assessed associations with age, sex, household size, water source, sanitation, and *Helicobacter pylori* seroreactivity using logistic regression analysis; 80.1% (269/336) of children and 63.6% (84/132) of adults were anemic. Among children, younger age was associated with increased prevalence of anemia (adjusted odds ratio [aOR] = 4.1, 95% confidence interval [CI] = 1.5–11.1 for children 6–11 months compared with children 48–59 months). Among adults, 50.8% were *H. pylori*-seropositive, and seropositivity was inversely associated with anemia (aOR = 0.4, 95% CI = 0.2–0.9). Anemia prevalence in this region of Haiti is very high and not attributable to sanitary conditions or a high prevalence of *H. pylori* infection.

INTRODUCTION

Anemia is a serious global health concern that affects one-quarter of the world's population and 40% of pre-school–aged children.[1–3] It is associated with increased susceptibility to infection, decreased work productivity, and delayed physical and cognitive development.[3–6] In the developing world, the most common causes of anemia are micronutrient deficiencies, parasitic diseases, and inherited disorders of hemoglobin.[7] *Helicobacter pylori*, an inflammatory gastric bacterial infection especially prevalent in developing countries,[8–10] has been implicated as a possible etiology for anemia.[11,12] Haiti's Central Plateau is a particularly impoverished region with a high prevalence of anemia.[13,14] Because the identification of etiological factors would aid anemia interventions, we conducted a cross-sectional study of pediatric and adult subjects visiting mobile clinics in the rural Central Plateau of Haiti to estimate the prevalence of anemia and look for associations with sanitary conditions and *H. pylori* infection.

METHODS

Study population. This study was a cross-sectional study conducted at mobile clinics organized by Project Medishare in November 2007, 2008, and 2009 in the Central Plateau of Haiti. In 2007 and 2008, children 6–59 months of age were recruited. Preliminary analyses found a high prevalence of anemia in these children, and therefore, in November of 2009, data collection was expanded to include adult subjects and examine *H. pylori* seroreactivities as a potential risk factor for anemia. Project Medishare is a non-profit organization based in Miami, FL and Thomonde, Haiti, that shares human and technical resources between countries to deliver healthcare and development services to rural Haitian communities.[15] The Institutional Review Board of Emory University and the Director of the Ministry of Public Health for the Central Plateau of Haiti approved the study. Kreyol speaking interpreters informed all participants of the purpose, procedures, risks, and benefits of the study, and we obtained written consent from all adults and from guardians of all subjects less than 18 years of age. No compensation was provided to the patients for participation in the study.

A convenience sample of participants was recruited during 4-day free mobile clinics organized by Project Medishare for Haiti. In 2009, the most common reasons for presentation to the adult clinic were generalized pain, well-woman check-up, vision problems, hypertension, and malnutrition. In the same year, the most common reasons for presentation to the pediatric clinic were intestinal parasites, anemia, rash, and upper respiratory infection (Leeds I and Zaeh S, unpublished data). Each mobile clinic took place within the La Hoye area of the Central Plateau and provided basic medical screening and treatment, with referrals to local hospitals when necessary. All subjects arriving for care at the mobile clinics were approached for enrollment. Exclusion criteria included refusal of consent, presentation with severe medical conditions that necessitated urgent care, and age less than 6 months. Using a standard questionnaire, Kreyol-speaking staff conducted interviews with adult participants and caretakers of minor participants. Information was collected on village of residence, number of persons per household, source of drinking water, water purification practices, and location of defecation. For children, anthropometric measurements included height or length using a wooden measuring board accurate to 0.1 cm (Irwin Shorr Productions, Olney, MD) and weight to the nearest 0.1 kg using a digital scale (Seca Corp, Hanover, MD).

Laboratory analysis. Non-fasting blood samples were obtained by finger stick in the sitting position. Hemoglobin concentrations were measured directly from the finger using calibrated HemoCue B-Hemoglobin photometer (Ängelholm, Sweden). *H. pylori* seroreactivity was measured using the Quidel Quickvue Rapid Whole Blood Antibody Test (San Diego, CA) or the PerMaxim RediScreen *H. pylori* Test Device (Santa Rosa, CA) according to the manufacturers' instructions.[16]

Statistical methods. Data sources for the 6–59 months subgroup included surveys conducted in 2007–2009, and measured exposures included sex, age, water source, water treatment, location of defecation, number in household, stunting, underweight, wasting, and *H. pylori* seroreactivity (2009 only). For this subgroup, age was divided into five categories: 6–11 months, 12–23 months, 24–35 months, 36–47 months, and 48–59 months. *Z* scores for height for age/length

* Address correspondence to Joshua R. Shak, Emory University School of Medicine, 1648 Pierce Drive, Room 374, Atlanta, GA 30322. E-mail: jshak@emory.edu

for age (HAZ), weight for age (WAZ), and weight for height/weight for length (WHZ) were calculated using the 2009 World Health Organization (WHO) growth standards (Anthro macro for SAS). Stunting was defined as HAZ < –2, underweight was defined WAZ < –2, and wasting was defined as WHZ < –2.

Data for the adult subgroup (≥ 16 years) came from the 2009 survey, and exposure variables included sex, age, water source, water treatment, location of defecation, number in household, and *H. pylori* seroreactivity. For this subgroup, age was divided into categories: 16–24 years, 25–34 years, 35–44 years, 45–54 years, 55–64 years, and ≥ 65 years.

The primary study outcome was anemia. Anemia was defined according to WHO hemoglobin cutoffs: < 11.0 g/dL for children 6–59 months, < 12.0 g/dL for women, and <13.0 g/dL for men.[4] Unadjusted association of exposures with anemia was assessed using χ^2 or Fisher exact tests for contingency tables. Unadjusted odds ratios with 95% Wald confidence intervals were obtained using unadjusted logistic regression models. Adjusted odds ratios were obtained using a multivariate logistic regression model for each study group (6–59 months and ≥16 years). Potential confounders of variables crudely associated with anemia were identified by assessing crude associations between variables that were considered to be possible confounders.

Variables that were associated with anemia in the crude analysis either by statistical significance or meaningful magnitude of association (including potential confounders) and variables previously shown to be associated with anemia (sex and anthropometric measures of nutritional status) were retained in the multivariate models. The multivariate model for the pediatric group included sex, age, household size, and malnutrition indicators. A multivariate model including *H. pylori*, which was collected only in 2009, was performed on the 2009 subgroup. The multivariate model for the adult group included sex, age, and *H. pylori*. Colinearity among covariates in the multivariate models was evaluated using linear regression and was not found to be present. Interaction terms for sex with other covariates were dropped from the adult multivariate model by backward elimination. Interaction terms for study year with other covariates were dropped from the pediatric multivariate model by backward elimination. In secondary analyses, linear regression models with hemoglobin as a continuous outcome and polytomous logistic regression models with two categories of anemia (mild = hemoglobin from 9.0 to age- and sex-specific cutoff; moderate to severe = hemoglobin less than 9.0) as outcomes were evaluated. Statistical analyses were conducted using SAS version 9.2. A P value ≤ 0.05 was considered statistically significant.

RESULTS

Children. Of 336 children 6–59 months of age included in the analysis (Figure 1A), 67 (19.9%) were not anemic (Hb ≥ 11), 174 (51.8%) were mildly anemic (9 ≤ Hb < 11), 84 (25.0%) were moderately anemic (7 ≤ Hb < 9), and 11 (3.3%) were severely anemic (Hb < 7) (Table 1). The mean age of those children without anemia (32.5 months) was significantly greater than the mean age of subjects with anemia (25.6 months; P = 0.001). Among children, 250 (74.4%) reported using unpiped water as their primary drinking source, 97 (28.9%) reported drinking untreated water, 226 (67.3%) reported regularly defecating in a bush or on the ground, 119 (35.4%) reported living in a household with greater than six people, and 4 (8.2% of 49 subjects tested for *H. pylori*) were *H. pylori*-seropositive (Table 1). In addition, malnutrition was common in children, with 22.0% stunted, 14.9% underweight, and 6.3% wasted.

There was no meaningful difference in the prevalence of anemia between male (81.1%) and female (79.1%) children (P = 0.64) (Table 2). In the unadjusted analysis, children 6–11 months were more likely to be anemic with an unadjusted

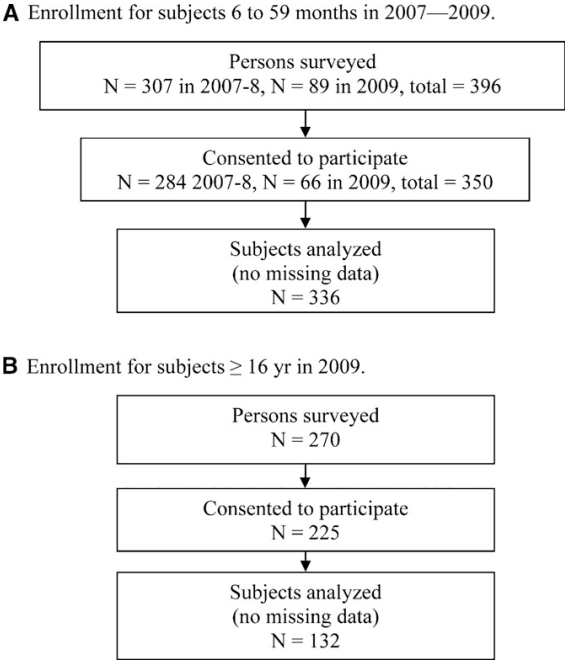

FIGURE 1. Flowcharts of enrollment for pediatric (**A**) and adult (**B**) study groups.

TABLE 1

Prevalence of anemia, sanitary conditions, and *H. pylori* seroreactivity in pediatric and adult populations

	Children 6–59 months (N = 336)		Adults ≥ 16 years (N = 132)	
	n	Percent (95% CI)*	n	Percent (95% CI)*
Individual factors				
Male	164	48.8 (43.4, 54.3)	39	29.6 (21.9, 38.1)
No anemia	67	19.9 (15.8, 24.6)	48	36.4 (28.2, 45.2)
Anemia total	269	80.1 (75.4, 84.2)	84	63.6 (54.8, 71.8)
Mild (> 9.0)†	174	51.8 (46.3, 57.2)	71	53.8 (44.9, 62.5)
Moderate (7.0–9.0)	84	25.0 (20.5, 30.0)	11	8.3 (4.2, 14.4)
Severe (< 7.0)	11	3.3 (1.7, 5.8)	2	1.5 (0.2, 5.4)
H. pylori positive‡	4	8.2 (2.3, 19.6)	67	50.8 (41.9, 59.6)
Stunted	74	22.0 (17.7, 26.8)	NA	
Underweight	50	14.9 (11.3, 19.1)	NA	
Wasted	21	6.3 (3.9, 9.4)	NA	
Household factors				
Unpiped water	250	74.4 (69.4, 79.0)	123	93.2 (87.5, 96.8)
Untreated water	97	28.9 (24.1, 34.0)	73	55.3 (46.4, 64.0)
Defecation outside latrine	226	67.3 (62.0, 72.3)	77	58.3 (49.4, 66.9)
More than six in household	119	35.4 (30.3, 40.8)	58	43.9 (35.3, 52.8)

* Exact confidence intervals for proportions.
† Upper cutoff was 11.0 for children, 12.0 for adult females, and 13.0 for adult males.
‡ *H. pylori* data was collected only for 2009. For children, number is of 49 with *H. pylori* values recorded in 2009.

TABLE 2
Risk factor frequencies by category and unadjusted and adjusted associations with anemia in 336 pediatric subjects (6–59 months) in 2007–2009

Risk factor	*n*	Anemic *n* (%)	χ^2 *P* value	Unadjusted OR (95% CI)	Adjusted OR (95% CI)*
Sex					
Male	164	133 (81.1)	0.642	1.14 (0.66, 1.94)	1.20 (0.69, 2.08)
Female	172	136 (79.1)		Reference	Reference
Age					
6–11 months	77	70 (90.9)	0.016†	4.12 (1.52, 11.14)‡	4.47 (1.62, 12.35)‡
12–23 months	78	66 (84.6)		2.27 (0.94, 5.43)	2.39 (0.98, 5.85)
24–35 months	71	54 (76.1)		1.31 (0.57, 2.99)	1.28 (0.55, 2.97)
36–47 months	62	45 (72.6)		1.09 (0.47, 2.51)	1.12 (0.48, 2.62)
48–59 months	48	34 (70.8)		Reference	Reference
Water source					
Pond/river/lake/well	172	136 (79.1)	0.795	0.80 (0.41, 1.56)	
Other	78	62 (79.5)		0.82 (0.37, 1.79)	
Piped water	86	71 (82.6)		Reference	
Water					
Untreated	97	78 (80.4)	0.918	1.03 (0.57, 1.87)	
Treated	239	191 (79.9)		Reference	
Defecation					
Bush or ground	226	184 (81.4)	0.372	Reference	
Latrine or toilet	110	85 (77.3)		0.78 (0.44, 1.36)	
Number in household					
≤ 6 (median)	217	169 (77.9)	0.177	Reference	Reference
> 6	119	100 (84.0)		1.50 (0.83, 2.69)	1.52 (0.83, 2.80)
Stunted					
Yes	74	59 (79.7)	0.936	0.97 (0.51, 1.85)	1.18 (0.51, 2.73)
No	262	210 (80.2)		Reference	Reference
Underweight					
Yes	50	40 (80.0)	0.991	1.00 (0.47, 2.11)	1.05 (0.37, 2.95)
No	286	229 (80.1)		Reference	Reference
Wasted					
Yes	21	17 (81.0)	0.916	1.06 (0.35, 3.27)	0.81 (0.23, 2.88)
No	315	252 (80.0)		Reference	Reference
H. pylori rapid test§					
Positive	4	3 (75.00)	0.431¶	0.75 (0.07, 8.09)	
Negative	45	36 (80.0)		Reference	
Study year					
2007	145	115 (79.3)	0.861	Reference	
2008	135	110 (81.5)		1.15 (0.64, 2.07)	
2009	56	44 (78.6)		0.96 (0.45, 2.03)	

*Adjusted model includes sex, age, number in household, stunted, underweight, and wasted.
†χ^2 *P* value < 0.05.
‡Wald *P* value for category OR < 0.05.
§*H. pylori* data collected only for 2009.
¶Fisher exact test *P* value.

odds ratio of 4.1 (95% confidence interval [CI] = 1.5–11.1) compared with children 48–59 months. Subjects 12–23 months were also more likely to be anemic, but this association was not statistically significant (odds ratio [OR] = 2.3, 95% CI = 0.9–5.4, $P = 0.07$). There were no statistically significant differences based on sex, water source, water treatment, defecation location, household size, anthropometric measurements, *H. pylori* seropositivity, or study year. In the multivariate analysis, children 6–11 months and 12–23 months were still more likely to be anemic than those children 48–59 months, with adjusted ORs of 4.5 (1.6–12.4) and 2.4 (1.0–5.9, $P < 0.05$), respectively. As in the unadjusted models, no variable besides age was significantly associated with anemia. In a multivariate regression model of the 2009 subsample, there remained no statistically significant association of *H. pylori* with anemia. Models using hemoglobin as a continuous outcome or multiple anemia categories as outcomes did not change the main findings.

Adults. During the 4-day study period in 2009, a total of 225 of 270 persons ≥ 16 years of age were enrolled (enrollment rate = 83.3%); 93 of these subjects had missing data, leaving 132 subjects in the final analysis (Figure 1B). Of those subjects in the analysis, 48 (36.4%) were not anemic (Hb > 13 for men and Hb > 12 for women), 71 (53.8%) were mildly anemic (9 ≤ Hb < upper limit for sex), 11 (8.0%) were moderately anemic (7 ≤ Hb < 9), and 2 (1.5%) were severely anemic (Hb < 7) (Table 1). There was no statistically significant difference between the mean ages of subjects with anemia (43.1 years) and those subjects without anemia (45.2 years; $P = 0.05$). Among adults, 123 (93.2%) reported using unpiped water as their primary drinking source, 55 (55.3%) reported drinking untreated water, 77 (58.3%) reported regularly defecating in a bush or on the ground, 58 (43.9%) reported living in a household with greater than six people, and 67 (50.8%) were *H. pylori*-seropositive (Table 1).

Of 39 males, 24 (61.5%) were anemic compared with 60 of 93 females (64.5%). Sex, water source, water treatment, location of defecation, and number in household were not significantly associated with anemia in unadjusted or adjusted analyses (Table 3). Those subjects in the 45–54 year age group were significantly less likely to be anemic, with an unadjusted

TABLE 3
Risk factor frequencies by category and unadjusted and adjusted associations with anemia in 132 adult subjects (≥ 16 years) in 2009

Risk factor	*n*	Anemic *n* (%)	χ^2 *P* value	Unadjusted OR (95% CI)	Adjusted OR (95% CI)*
Sex					
Male	39	24 (61.5)	0.746	0.88 (0.41, 1.91)	0.79 (0.33, 1.88)
Female	93	60 (64.5)		Reference	Reference
Age					
16–24 years	29	20 (69.0)	0.118	0.74 (0.22, 2.49)	0.52 (0.14, 1.92)
25–34 years	25	19 (76.0)		1.06 (0.29, 3.88)	0.93 (0.23, 3.70)
35–44 years	11			0.58 (0.13, 2.71)	0.41 (0.08, 2.10)
45–54 years	28	13 (46.4)		0.29 (0.09, 0.95)†	0.24 (0.07, 0.82)†
55–64 years	15	7 (46.7)		0.29 (0.07, 1.15)	0.24 (0.06, 1.00)
≥ 65 years	24	18 (75.0)		Reference	Reference
Water source					
Pond/river/lake/well	59	41 (69.5)	0.397	1.14 (0.26, 5.07)	
Other	64	37 (57.8)		0.69 (0.16, 2.99)	
Piped water	9	6 (66.7)		Reference	
Water treatment					
Untreated	73	44 (60.3)	0.372	0.72 (0.35, 1.48)	
Treated	59	40 (67.8)		Reference	
Defecation					
Bush or ground	77	51 (66.2)	0.463	Reference	
Latrine or toilet	55	33 (60.0)		0.77 (0.37, 1.57)	
Number in household					
≤ 6 (median)	74	46 (62.2)	0.691	Reference	
> 6	58	38 (65.5)		1.16 (0.57, 2.37)	
H. pylori rapid test					
Positive	67	37 (55.2)	0.041†	0.47 (0.23, 0.98)†	0.42 (0.19, 0.91)†
Negative	65	47 (72.3)		Reference	Reference

*Adjusted model includes sex, age, and *H. pylori* as covariates.
†χ^2 *P* value < 0.05.
‡Wald *P* value for category OR < 0.05.

OR of 0.3 (0.09–0.95) and an adjusted OR of 0.2 (0.07–0.82) compared with those subjects ≥ 65 years ($P = 0.04$) (Table 3). *H. pylori* seropositivity was negatively associated with anemia, with an unadjusted OR of 0.5 (95% CI = 0.2–1.0) and adjusted OR of 0.4 (95% CI = 0.2–0.9) (Table 3). Models using hemoglobin as a continuous outcome or mild and severe anemia categories as outcomes did not change the main findings.

DISCUSSION

The prevalence of anemia in this rural Haitian population was alarmingly high; four of five children and nearly two of three adults were anemic. This rate is higher than reported in other developing countries[3,17] and higher than reported in previous surveys of Haiti. In 2005–2006, the Demographic and Health Surveys (DHS) of Haiti estimated a national 61% prevalence of anemia in Haitian children 6–59 months, 46% prevalence of anemia in adult women, and 24% prevalence of anemia in adult men.[13] Within the Central Plateau region of Haiti, the DHS reported a 64% prevalence of anemia in children 6–59 months, a 43% prevalence in adult women, and a 22% prevalence in adult men. Our finding of higher prevalence of anemia in people from the Central Plateau compared with those people reported by the DHS survey could be attributable to selection bias inherent in our study design; subjects visiting mobile clinics are presumably less healthy than the general population. That said, several natural disasters occurring even before the January 2010 earthquake, including Hurricane Hanna in 2008, may have increased anemia prevalence because of resulting food insecurity, disruption of existing nutrition programs, and spread of infectious diseases.[18]

The higher prevalence of anemia in the 6- to 23-month group was particularly concerning, because the cognitive effects of anemia during this period of critical brain development may be irreversible.[4,19] This age group is especially vulnerable to anemia because of increased iron metabolism and the shift from breastfeeding to table foods.[3,7,20,21] A 2004 study of the nutrient content of traditional complementary table food in Haiti found inadequate densities of iron, zinc, or vitamin A.[22] Although we found a high prevalence of malnutrition and poor sanitation among both the pediatric and adult populations studied, we did not find an association of anemia with stunting or wasting. This result contrasts with previous studies that found associations between severe forms of anemia and poor growth,[23–25] and it may implicate a non-nutritional etiology for anemia in our study population, such as infection, inflammation, or inherited blood disorders.

Although the prevalence of *H. pylori* antibodies in adults was similar to other studies,[26,27] it was surprising that there was an inverse association between anemia and *H. pylori* seroreactivity. This finding contrasts with previous studies that have reported a positive association.[12,28] In addition, meta-analyses of randomized control trials of *H. pylori* eradication have indicated that eradication can increase hemoglobin levels.[12,28–30] However, one study of pregnant women on Pemba Island, Zanzibar, found a positive association between hemoglobin concentration and *H. pylori* bacteria load.[31] Farang and others[31] suggest that high bacterial load infection enhances hemoglobin concentrations by increasing the production of stomach acid, and low bacterial load infections correspond to advanced

gastric atrophy, resulting in impaired iron absorption. It is possible that the majority of subjects in our study had high bacterial load infection, resulting in the observed correlation between anemia and *H. pylori* seroreactivity. It is also possible that we observed an inverse association because of an unmeasured confounding variable (e.g., socioeconomic status, location, or inherited disease). Additional investigation of our Haitian population, including measurements of bacterial load and iron status indicators, would expand our understanding of the relationship between anemia and *H. pylori* infection.

Our study had several limitations that restrict the generalizability of the results. The population examined was a convenience sample composed of patients likely less healthy than those people in the general population of the Central Plateau. The small pediatric sample size may have limited this study's power to detect associations in the logistic regression models. In addition, the rapid *H. pylori* antibody test used indicated current or past infection, which could possibly overestimate the prevalence of infection, thus minimizing the association between *H. pylori* and anemia. Some studies indicate that stool antigen tests are more sensitive and specific for current *H. pylori* infection, especially in children.[32] Finally, the number of subjects recruited was significantly larger than the number with complete data because of the logistical challenges of data collection at a mobile clinic (Figure 1). As a result, we cannot make wider conclusions about the prevalence of anemia in the Central Plateau of Haiti or the causes of anemia. Nevertheless, this study is a unique glimpse at an understudied and underserved population.

In developing countries, the impact of anemia may be significantly diminished through public health interventions such as deworming programs, food assistance, iron-fortified complementary foods,[22] iron supplementation,[33,34] and home food fortification with micronutrient powders.[14,35–39] Given the high prevalence of anemia and malnutrition in this population, it is obviously appropriate to study the effects of public health interventions such as micronutrient fortification powders,[37] AK-1000 (Akamil) fortified home supplement,[40] deworming,[41] and maternal education on infant nutrition.[20]

Finally, the devastating January 2010 earthquake and the subsequent cholera epidemic have likely exacerbated the situation and increased the prevalence of anemia and malnutrition in this rural Haitian population. Although there have been many efforts in Haiti surrounding infectious disease and disaster management, these findings highlight the urgent need for additional study of the etiologies of malnutrition and anemia in rural populations and targeted public health interventions.

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Authors' addresses: Joshua R. Shak, Jamie B. Sodikoff, Rebecca A. Speckman, Francois G. Rollin, and Parminder S. Suchdev, Emory University School of Medicine, Atlanta, GA, E-mails: jshak@emory.edu, jsodiko@emory.edu, speckman.rebecca@gmail.com, frollin@gmail.com, and parminder.suchdev@emory.edu. Marie P. Chery, Project Medishare for Haiti, Miami, FL, E-mail: mariechery@gmail.com. Conrad R. Cole, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, E-mail: conrad.cole@cchmc.org.

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Aminoglycoside-Resistant *Aeromonas hydrophila* as Part of a Polymicrobial Infection following a Traumatic Fall into Freshwater▽

Joshua R. Shak,[1]* Jennifer A. Whitaker,[2] Bruce S. Ribner,[2] and Eileen M. Burd[3]

Medical Scientist Training Program,[1] Division of Infectious Diseases,[2] and Department of Pathology and Laboratory Medicine,[3] Emory University School of Medicine, Atlanta, Georgia

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Amikacin is a first-line treatment for *Aeromonas* infection due to high efficacy. There are few reports of aminoglycoside-resistant *Aeromonas* spp. We report a soft tissue infection containing multiple pathogens, including a strain of *Aeromonas hydrophila* resistant to amikacin, tobramycin, and multiple cephalosporins.

CASE REPORT

A 35-year-old man presented to the emergency department of our academic tertiary care facility after suffering a right posterior leg laceration. He was walking on a wooden dock over the Chattahoochee River in Georgia when the structure collapsed beneath him. A wooden shard or a metal pipe underneath the dock lacerated his leg before he fell into the river, and the wound was exposed to water. Shortly after the fall, a physician friend cleaned the wound with povidone-iodine and sutured the laceration closed. He also received a tetanus booster and cephalexin at a local hospital. Past medical and surgical histories were unremarkable.

One day after the accident, the patient presented to the emergency department with rapidly advancing erythema, low-grade fevers, nausea, severe right leg pain, and purulent malodorous drainage from the wound. On presentation, his temperature was 38.0°C, blood pressure was 137/74 mm Hg, heart rate was 105 beats per minute, and respiratory rate was 20 breaths per minute. There was an 8-cm laceration to the right popliteal fossa, repaired with a nylon suture. Cellulitis extended approximately 10 cm proximally and distally to the wound. Laboratory values were remarkable for a white blood cell count of 22,500 cells/μl (93% granulocytes).

Fluid was obtained from the wound, and a Gram stain of the fluid revealed many Gram-negative rods. Aerobic and anaerobic cultures were conducted using standard microbiologic procedures. Species identification and antibiotic susceptibility tests were conducted using the MicroScan WalkAway Plus system (Siemens Healthcare Diagnostics, Deerfield, IL). The wound culture subsequently grew many colonies of *Escherichia coli*, few colonies of *Enterococcus* species, few colonies of alpha-hemolytic *Streptococcus* species, few colonies of *Clostridium perfringens*, and 2 strains (many colonies isolates each) of *Aeromonas hydrophila* (species identification later confirmed by conventional biochemical and cell wall fatty acid analysis at the Georgia Public Heath Laboratory). The *E. coli* and *Enterococcus* isolates were sensitive to all antibiotics tested. Strain 1 of *Aeromonas* was resistant to ampicillin and tetracycline. Strain 2 was resistant to the same antibiotics as strain 1. In addition, strain 2 was resistant to amikacin, aztreonam, cefepime, cefoxitin, ceftazidime, ceftriaxone, cefotaxime, ertapenem, gentamicin, and tobramycin (Table 1). Per the microbiology laboratory's policy, susceptibilities were not ascertained for the *C. perfringens* and alpha-hemolytic *Streptococcus* species; however, these bacteria are generally susceptible to the empirical antibiotic regimen selected for our patient, vancomycin and piperacillin-tazobactam.

Due to the rapidly advancing cellulitis, the patient was taken emergently to the operating room for surgical debridement of the wound. Deep tissue cultures obtained in the operating room grew the same pathogens as the cultures obtained in the emergency department. When preliminary culture results the following day suggested possible *Aeromonas* infection, empirical treatment with vancomycin and piperacillin-tazobactam was supplemented with amikacin and levofloxacin. On hospital day 2, due to advancing cellulitis and purulent drainage from the wound despite intensive antibiotic therapy, a second surgical debridement was performed. Cultures of specimens from this surgery revealed only oxidase-positive Gram-negative rods, implicating *Aeromonas* as the primary pathogen in this infection. Due to the confirmed susceptibilities of the *Aeromonas* species (Table 1), the patient's antibiotic regimen was narrowed to vancomycin and piperacillin-tazobactam.

The patient's wound improved rapidly over the next 5 days, and a split-thickness skin graft was placed. After the grafting, the patient was discharged on oral levofloxacin and amoxicillin-clavulanate and has fully recovered.

Aeromonas hydrophila is a Gram-negative, oxidase-positive bacillus that is a common freshwater and food-borne pathogen that can cause enterocolitis, bacteremia, meningitis, and soft tissue infections (3, 4). Here we report a polymicrobial soft tissue infection after a traumatic laceration and freshwater exposure that included two strains of *A. hydrophila*. We believe that *Aeromonas* played a major role in this patient's disease process, since it was most numerous on the Gram stain and the

* Corresponding author. Mailing address: Emory University School of Medicine, 1648 Pierce Drive, Room 374, Atlanta, GA 30322. Phone: (413) 297-1525. Fax: (404) 727-0045. E-mail: jshak@emory.edu.

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TABLE 1. Antibiotic susceptibilities of the *Aeromonas hydrophila* strains[a]

Strain[b]	MIC (μg/ml)[c]																
	AMP	TZP	FOX	CAZ	CTX	CRO	FEP	IPM	ETP	ATM	GEN	AMK	TOB	CIP	TET	CHL	POB
Aero1	**>16**	≤16	≤8	≤1	≤2	≤8	≤8	≤4	≤2	≤8	≤4	≤16	≤4	≤1	**>8**	ND	ND
Aero2	**>16**	≤16	**16**	**>16**	**>32**	**32**	**>16**	≤4	**>4**	**>16**	**8**	**>32**	**>8**	≤1	**>8**	ND	ND
ATCC 7966	**>256**	2	4	<0.5	<0.5	<0.5	<0.5	0.5	ND	<0.5	2	4	4	<0.5	0.5	<0.5	2
MB443	**>256**	**>256**	**>256**	**>256**	**>256**	**>256**	**32**	**32**	ND	<0.5	2	8	**16**	**8**	**>256**	2	16

[a] Antibiotic susceptibilities of the two *Aeromonas hydrophila* strains recovered in this study, the completely sequenced prototype strain ATCC 7966 (12), and the VIM metallo-β-lactamase-producing strain MB443 (7).

[b] Aero1 and Aero2, *Aeromonas hydrophila* strains 1 and 2 from this study. MIC data for ATCC 7966 and MB443 are reprinted with permission from Libisch et al. (7).

[c] Boldface indicates resistance according to current Clinical and Laboratory Standards Institute guidelines (2). AMP, ampicillin; TZP, piperacillin-tazobactam; FOX, cefoxitin; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; FEP, cefepime; IPM, imipenem; ETP, ertapenem; ATM, aztreonam; GEN, gentamicin; AMK, amikacin; TOB, tobramycin; CIP, ciprofloxacin; TET, tetracycline; CHL, chloramphenicol; POB, polymyxin B; ND, not determined.

only bacterium cultured from specimens taken during repeated surgical debridements. One of the strains of *Aeromonas* exhibited an unusual resistance to amikacin and tobramycin as well as aztreonam, ertapenem, ceftazidime, and cefepime.

Heretofore, reports of aminoglycoside resistance in *Aeromonas* species have been very limited. We are aware of only four strains of demonstrated aminoglycoside-resistant *Aeromonas hydrophila* in the literature: one strain resistant to gentamicin and amikacin but susceptible to tobramycin, isolated at the Massachusetts General Hospital in the mid-1970s (9), and three strains of amikacin-resistant *A. hydrophila* from a survey conducted in Taiwan in 1996 (5). In 2008, Libisch et al. first reported a VIM metallo-β-lactamase-producing *Aeromonas hydrophila* strain with extensive resistance to beta-lactams and carbapenems but not aminoglycosides (7). To our knowledge, our report is the first to present a clinical description of an *A. hydrophila* infection with resistance to both aminoglycosides and expanded-spectrum cephalosporins.

Known antimicrobial resistance patterns can affect the choice of empirical antibiotic regimens used to treat specific infections. A 1996 study of antibiotic susceptibility in clinical isolates of *Aeromonas* in Taiwan (5) concluded that certain cephalosporins (moxalactam, ceftazidime, and cefepime), amikacin, aztreonam, imipenem, and quinolones were reasonable choices for empirical treatment. A 2009 study of *Aeromonas* in France recommended that a cephalosporin or a quinolone be prescribed along with an aminoglycoside in cases of severe infection (6). We believe that the reported emerging resistance of *Aeromonas* to quinolones (1, 10) and carbapenems (7, 11), as well as aztreonam, cephalosporins, and aminoglycosides (this report), might influence physicians to employ polytherapy for empirical treatment of severe *Aeromonas hydrophila* infections. However, comprehensive studies are needed to examine broader trends in *Aeromonas* antibiotic resistance.

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RESEARCH ARTICLE

Characterization of *Aeromonas hydrophila* Wound Pathotypes by Comparative Genomic and Functional Analyses of Virulence Genes

Christopher J. Grim,[a] Elena V. Kozlova,[b] Jian Sha,[b] Eric C. Fitts,[b] Christina J. van Lier,[b] Michelle L. Kirtley,[b] Sandeep J. Joseph,[c] Timothy D. Read,[c] Eileen M. Burd,[c,d] Ben D. Tall,[a] Sam W. Joseph,[e,f] Amy J. Horneman,[g] Ashok K. Chopra,[b] Joshua R. Shak[c]

Food and Drug Administration, Laurel, Maryland, USA[a]; Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas, USA[b]; Department of Medicine, Division of Infectious Diseases, Emory University School of Medicine, Atlanta, Georgia, USA[c]; Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia, USA[d]; Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, Maryland, USA[e]; Maryland Institute of Applied Environmental Health, School of Public Health, University of Maryland, College Park, Maryland, USA[f]; Pathology and Laboratory Medical Services, VA Maryland Health Care System, Baltimore, Maryland, USA[g]

A.J.H., A.K.C., and J.R.S. contributed equally to this article.

ABSTRACT ***Aeromonas hydrophila*** **has increasingly been implicated as a virulent and antibiotic-resistant etiologic agent in various human diseases. In a previously published case report, we described a subject with a polymicrobial wound infection that included a persistent and aggressive strain of *A. hydrophila* (E1), as well as a more antibiotic-resistant strain of *A. hydrophila* (E2). To better understand the differences between pathogenic and environmental strains of *A. hydrophila*, we conducted comparative genomic and functional analyses of virulence-associated genes of these two wound isolates (E1 and E2), the environmental type strain *A. hydrophila* ATCC 7966^T, and four other isolates belonging to *A. aquariorum*, *A. veronii*, *A. salmonicida*, and *A. caviae*. Full-genome sequencing of strains E1 and E2 revealed extensive differences between the two and strain ATCC 7966^T. The more persistent wound infection strain, E1, harbored coding sequences for a cytotoxic enterotoxin (Act), a type 3 secretion system (T3SS), flagella, hemolysins, and a homolog of exotoxin A found in *Pseudomonas aeruginosa*. Corresponding phenotypic analyses with *A. hydrophila* ATCC 7966^T and SSU as reference strains demonstrated the functionality of these virulence genes, with strain E1 displaying enhanced swimming and swarming motility, lateral flagella on electron microscopy, the presence of T3SS effector AexU, and enhanced lethality in a mouse model of *Aeromonas* infection. By combining sequence-based analysis and functional assays, we characterized an *A. hydrophila* pathotype, exemplified by strain E1, that exhibited increased virulence in a mouse model of infection, likely because of encapsulation, enhanced motility, toxin secretion, and cellular toxicity.**

IMPORTANCE ***Aeromonas hydrophila*** **is a common aquatic bacterium that has increasingly been implicated in serious human infections. While many determinants of virulence have been identified in *Aeromonas*, rapid identification of pathogenic versus nonpathogenic strains remains a challenge for this genus, as it is for other opportunistic pathogens. This paper demonstrates, by using whole-genome sequencing of clinical *Aeromonas* strains, followed by corresponding virulence assays, that comparative genomics can be used to identify a virulent subtype of *A. hydrophila* that is aggressive during human infection and more lethal in a mouse model of infection. This aggressive pathotype contained genes for toxin production, toxin secretion, and bacterial motility that likely enabled its pathogenicity. Our results highlight the potential of whole-genome sequencing to transform microbial diagnostics; with further advances in rapid sequencing and annotation, genomic analysis will be able to provide timely information on the identities and virulence potential of clinically isolated microorganisms.**

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Address correspondence to Joshua R. Shak, jshak@emory.edu, or Ashok K. Chopra, achopra@utmb.edu.

The Gram-negative bacterium *Aeromonas hydrophila*, a ubiquitous inhabitant of fresh and estuarine waters (1), has increasingly been implicated as an etiologic agent in a variety of human diseases (2). The spectrum of disease severity is broad, ranging from mild diarrhea to life-threatening necrotizing fasciitis, septicemia, meningitis, cholera-like illness, and hemolytic-uremic syndrome (3). The mere presence of *A. hydrophila* in an infected wound is an independent predictor of death among patients with necrotizing fasciitis (4). While traditionally regarded as a pathogen of immunocompromised humans, there have been several recently reported *Aeromonas* infections of immunocompetent individuals (5–8).

Recently, a case report by Shak et al. described a human wound infection involving a mixture of Gram-positive and Gram-negative bacteria, including two distinct strains of *A. hydrophila*, Aero 1 and Aero 2 (8), which we refer to here as strains E1 and E2, respectively. Both of these strains were recovered when the patient was initially admitted to the hospital and were identified as *A. hy-*

TABLE 1 Genome characteristics of three *A. hydrophila* strains and four closely related *Aeromonas* species as determined by the RAST annotation pipeline

Organism	Source	No. of contigs	No. of bp	No. of CDSs	No. of RNAs	No. of tRNAs	No. of rRNAs	No. of rRNA operons[b]	G+C content (%)
A. hydrophila E1	Wound infection	249	4,754,562	4,373	76	70	6	10	61.3
A. hydrophila E2	Wound infection	426	4,564,644	4,241	60	56	4	10	61.5
A. hydrophila ATCC 7966^T	Fishy milk	1	4,744,448	4,279	158	127	31	10	61.6
A. aquariorum AAK1	Septicemia, necrotizing fasciitis	36	4,763,532	4,275	100	88	12	10	61.8
A. veronii B565	Pond	1	4,551,783	3,936	133	102	31	10	58.7
A. salmonicida A449	Furunculosis, brown trout	6[a]	5,040,536	4,306	137	109	28	9	58.5
A. caviae Ae398	Diarrhea, child	149	4,439,218	3,912	78[b]	72	6	?	61.4

[a] Includes one chromosome and five plasmids.
[b] rRNA operon numbers for draft genomes were estimated on the basis of the presence of full and partial 16S and 23S rRNA genes and comparison to *A. hydrophila* ATCC 7966^T.

drophila on the basis of cell wall fatty acid analysis and biochemical characterization (8). While strain E1 was resistant to ampicillin and tetracycline, strain E2 exhibited additional antimicrobial resistance, specifically, to aminoglycosides and several expanded-spectrum cephalosporin β-lactams (8). Despite aggressive antibiotic treatment and surgical debridement, *A. hydrophila* strain E1 continued to be cultured from advancing cellulitis. With repeated surgical debridement and treatment with vancomycin and piperacillin-tazobactam, the infection cleared and the patient made a full recovery.

The pathogenic potential of *A. hydrophila* has been related to several virulence factors, including the cytotoxic enterotoxin Act (9), which has hemolytic, cytotoxic, and enterotoxic activities; a variety of proteases (10, 11); cytotonic enterotoxins Ast and Alt (12); type 3 secretion systems (T3SSs) (13); and motility factors such as lateral and polar flagella (14). As in previous case reports of *Aeromonas*-associated human wound infections (15, 16), the report by Shak et al. (8) did not describe the genotypic or mechanistic determinants of virulence and antibiotic resistance. We hypothesized that the persistence of strain E1 in the wound of this patient could be attributed to known *Aeromonas* virulence factors identifiable at the genotypic and phenotypic levels.

To examine this hypothesis and further develop the earlier findings of Joseph et al. (15) and Shak et al. (8), the whole genome of each clinical strain was sequenced and the resulting draft genomes were compared to other *Aeromonas* genomes, including the closed genome of the type strain, *A. hydrophila* ATCC 7966^T (17). Comparative genomics revealed several differences in the genomes of *A. hydrophila* strains E1 and E2 that suggested that strain E1 was more virulent because of the presence of several virulence factor-encoding genes. To complement the genomic findings and investigate the functionality of these virulence traits, bioassays of strains E1 and E2 were conducted alongside environmental isolate *A. hydrophila* ATCC 7966^T (17) and virulent diarrhea isolate SSU (18) to assess motility, cytotoxicity, protease activity, secretion system functionality, the ability to form biofilms, and serum resistance. Finally, a septicemic-mouse model of infection was used to investigate the virulence potential of both strains E1 and E2 in comparison with those of other well-studied clinical and environmental *Aeromonas* isolates. Altogether, we demonstrated that genotypic differences correlated with functional virulence factor assays, strongly suggesting the existence of an identifiable virulent pathotype of *A. hydrophila* that leads to wound infections in humans.

RESULTS

Genomic characteristics of *A. hydrophila* strains E1 and E2. Pyrosequencing of *A. hydrophila* strains E1 and E2 resulted in draft genomes with calculated G+C contents of 61.3 and 61.5%, respectively, similar to those of *A. hydrophila* ATCC 7966^T and the closely related species *A. aquariorum* and *A. caviae* (Table 1). The genomes of strains E1 and E2 contained an estimated 10 rRNA operons, equal to the genomes of *A. hydrophila* ATCC 7966^T, *A. aquariorum* AAK1, and *A. veronii* B565 (Table 1). No plasmids were harbored in the genome of either strain E1 or E2 at the time of sequencing. Average nucleotide identity (ANI) analysis by BLAST confirmed that both strains E1 and E2 belong to *A. hydrophila* (Table 2). Importantly, E1 and E2 were distinct strains, with 96.88 and 96.96% ANIs, and both strains E1 and E2 were equally dissimilar to *A. hydrophila* ATCC 7966^T, with ANIs of approximately 97% (Table 2). Bidirectional BLAST analyses of annotated coding DNA sequences (CDSs) of *A. hydrophila* strains ATCC

TABLE 2 Pairwise ANIs, by BLAST, of three strains of *A. hydrophila* and four closely related *Aeromonas* species

Organism	*A. hydrophila* E1	*A. hydrophila* E2	*A. hydrophila* ATCC 7966^T	*A. aquariorum* AAK1	*A. veronii* B565	*A. caviae* Ae398	*A. salmonicida* A449
A. hydrophila E1		**96.88**[a]	**96.84**	92.8	85.73	86.6	86.49
A. hydrophila E2	**96.96**		**97.16**	92.95	85.79	86.79	86.82
A. hydrophila ATCC 7966^T	**96.81**	**97.06**		92.92	85.5	86.45	86.47
A. aquariorum AAK1	92.93	93.04	93.00		85.24	86.57	85.92
A. veronii B565	85.8	85.77	85.55	85.18		83.74	84.10
A. caviae Ae398	86.63	86.68	86.48	86.54	83.63		83.97
A. salmonicida A449	86.58	86.87	86.54	86.03	84.15	84.22	

[a] Values in bold indicate strains that belong to the same species (i.e., ANI of >95).

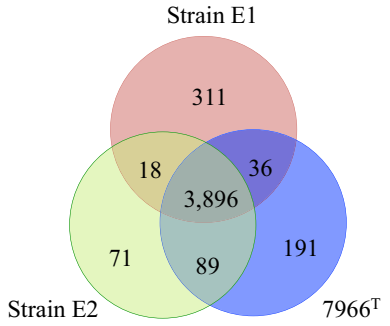

FIG 1 Venn diagram of the distribution of protein CDSs inferred from the genomes of *A. hydrophila* ATCC 7966^T, E1, and E2. Numbers of genes unique to and common to ATCC 7966^T, E1, and E2 are indicated within the Venn diagram. The values are gene counts following manual curation and differ from the gene counts in the automated RAST pipeline results presented in Table 1.

7966^T, E1, and E2, as well as *A. aquariorum* AAK1, *A. caviae* Ae398, *A. salmonicida* A449, and *A. veronii* B565, confirmed the results of the ANI genomic comparison (see Table S1 and Fig. S1 in the supplemental material).

Comparative genomics of *A. hydrophila*. The genome sequences of strains E1, E2, and ATCC 7966^T contained 4,373, 4,241, and 4,279 CDSs, respectively, upon initial annotation by the RAST server (Table 1). Manual curation was performed to reconcile genes that were fragmented because of sequence quality or disrupted by gaps in contigs and a number of small hypothetical protein-encoding genes. Following curation, we found that all three *A. hydrophila* strains shared a core genome of 3,896 genes, which was, on average, 93% of the total CDSs in each genome (see Fig. 1 and Table S1 in the supplemental material).

Several of the core *A. hydrophila* genes were shared with the other *Aeromonas* species analyzed in this study. For example, all seven genomes contained the following notable operons or features: T1SS (*tolC*); Sec and Tat secretion systems; a mannose-sensitive hemagglutinin (MSHA) bundle-forming type IV pilus (BFP; Table 3); polar flagella encoded in four gene clusters; the cytotonic enterotoxin gene *alt*; glycerophospholipid:cholesterol acyltransferase (GCAT); enolase; the vibriolysin/pseudolysin-like extracellular zinc protease/elastase (Table 3); the cephalosporinase β-lactamase gene *cepS*; the adenylate kinase β-lactamase gene *ampS* (or *ampH*); the carbapenemase gene *cphA*; multiple RND and ABC multidrug resistance (MDR) efflux pump genes; an ABC antimicrobial peptide transporter, the *N*-acetylglucosamine (NAG) utilization operon; an *N*,*N*′-diacetylchitobiose [(GlcNAc)$_2$] utilization operon; the phenolate siderophore amonabactin gene cluster; the *fbp* gene for a ferric iron ABC transporter that transports iron from the periplasmic space into the cytosol; a hemin utilization locus, *hut*; ferric iron transporter genes; the ferrous iron transporter-encoding gene *feo* cluster; the *luxS* gene; the QS pair *qseBC*; and the QS regulator of virulence gene *hapR* (data not shown). Interestingly, the Tap type IV pilus, encoded in four clusters, was present in all seven genomes, but the level of amino acid identity between the species (~20 to 60%) was relatively low.

A certain number of *A. hydrophila* core genes were not shared with all other *Aeromonas* species genomes (i.e., absent from one or more non-*A. hydrophila* species; Table 3). Interestingly, this group contained genes that encode an alpha, or class 5, chaperone/usher (CU) fimbrial operon (AHA_0060, usher gene); a π-fimbrial operon (AHA_0521); the *N*-acetylgalactosamine (*aga*) phosphotransferase system (PTS); the arabinose utilization operon; the pore-forming hemolysin/cytolysin gene *hlyA*; the polymyxin B resistance gene cluster *arn*; as well as several other ABC- and RND-type MDR efflux pumps and a T6SS (Table 3; see Table S1 in the supplemental material). Additionally, the cytotonic heat-stable enterotoxin gene *ast* was found only in the three *A. hydrophila* genomes.

As described below, a number of genes present in the genome of strain ATCC 7966^T were present in the genome of either strain E1 or E2 but not in both. Common to *A. hydrophila* ATCC 7966^T and strain E1 were genes that encode the cytotoxic enterotoxin Act, a MATE family MDR efflux pump (Table 3), and five small genomic regions of unknown function (see Table S1). Strains E2 and ATCC 7966^T shared several metabolic operons and transporters, including those for the utilization of *N*-acetylmuramic acid, for maltose (or a maltose homolog), and for the utilization and transport of the sulfonic acid taurine (Table 3). They also shared genes that encoded a second α-fimbriae (AHA_1021, usher gene), an RTX toxin (AHA_1359) and its transporter (Table 3), and operons involved in the degradation of chloroaromatics (dienelactone) (Table 3), arsenical compounds (see Table S1), and quaternary ammonium compounds, as well as ethanolamine (Table 3).

There were only 18 genes that were shared by *A. hydrophila* E1 and E2 but not present in the genome of ATCC 7966^T (Fig. 1). Of interest among these were distinct serogroup-specific capsular polysaccharide operons that contained some homologous genes (four out of seven; see Table S1 in the supplemental material).

There were 191 CDSs present in the genome of *A. hydrophila* ATCC 7966^T, which were absent in the genomes of strains E1 and E2 (Fig. 1), including two large prophage-like integrated regions (see Table S1), the serogroup O:1 antigen lipopolysaccharide (LPS) cluster, and a putative LPS modification gene cluster, along with several small genomic regions of unknown function (see Table S1), the tight adherence (TAD) Flp pilus (Table 3), a phosphonate transporter, and an acriflavin RND transporter (Table 3). Of the three *A. hydrophila* genomes analyzed, that of strain E2 had the fewest unique genes, 71 (Fig. 1), most of which encode proteins of unknown functions. Notable exceptions were a gene cluster that encodes a putative arylsulfatase, the O:18 antigen gene cluster, a type I restriction modification system, several transposons, and a putative MFS xylose transporter (see Table S1).

Virulence factor-encoding genes unique to the genome of *A. hydrophila* E1. The genome of *A. hydrophila* E1 contained a number of virulence factor-encoding genes not found in the genome of *A. hydrophila* ATCC 7966^T or strain E2. These included a T3SS, a lateral flagellar system (Table 3), and several large exoproteins, annotated as hemagglutinins or adhesins/hemolysins, including one that was 64% identical to exotoxin A of *Pseudomonas aeruginosa* (see Table S1). The T3SS found in the genome of E1 was approximately 26,037 bp in length, was contained on contigs 00088 (bp 1710 to the end) and 00128, and was 97% identical (100% coverage) to the T3SS found in *A. hydrophila* strain AH1 (GenBank accession no. AY394563.2) (see Fig. S2 and S3A in the supplemental material). The genes that encode the lateral flagella of E1 were contained in a single gene cluster of 35 kb located on

TABLE 3 Presence or absence of protein CDSs in the genomes of three strains of *A. hydrophila* and four closely related *Aeromonas* species

Putative protein CDS	*A. hydrophila* ATCC 7966^T	*A. hydrophila* E1	*A. hydrophila* E2	*A. aquariorum* AAK1	*A. veronii* B565	*A. salmonicida* A449	*A. caviae* Ae398
Metabolic and related							
pgt utilization operon	+	+	+	+	−	−	−
Maltose homolog PTS, glucosidase	+	−	+	+	+	+	−
N-Acetylgalactosamine, *aga*	+	+	+	+	+	−	−
N-Acetylmuramic acid transporter	+	−	+	−	−	−	+
Xanthosine	+	+	+	−	−	−	−
Uncharacterized hexose-P PTS, *uhpABC*	+	+	+	+	+	+	−
Arabinose utilization	+	+	+	−	−	+	−
2-Aminoethylphosphonate	+	+	+	+	+	−	−
Xanthine, *yge*, *yqe* cluster	+	+	+	+	+	−	−
Mannose transporter	+	+	+	−	−	−	−
L-Lactate utilization	+	+	+	+	−	−	+
L-Cystine transporter	+	+	+	−	−	−	+
Glutamate-aspartate transporter	−	+	−	+	−	−	+
Taurine transporter	+	−	+	+	−	−	−
Cytochrome *o*	+	+	+	+	−	+	+
Methylamine homolog utilization	+	+	+	+	+	−	−
Benzoate, *N*-acetylglucosamine	+	+	+	+	+	−	+
Phosphonate transporter	+	−	−	+	−	+	−
Decaheme cytochrome *c*, *nrf*	+	+	+	+	−	−	−
Cysteine operon, *cysPTWA*	+	+	+	+	−	+	+
Glutamine/glutamate transporter	+	+	+	+	−	+	+
Chemotaxis cluster, AHA_2527-2538	+	+	+	−	−	−	−
N-Ribosylnicotinamide transporter	+	+	+	+	+	+	−
NO reductase	+	+	+	+	−	+	+
Dienelactone hydrolase	+	−	+	−	−	−	−
Quaternary ammonium compound resistance, propanediol, ethanolamine	+	−	+	+	−	−	−
Anaerobic sulfite reductase	+	+	+	−	+	−	−
Tungstate transporter	+	+	+	+	+	+	−
Tetrathionate reductase	+	+	+	+	−	−	+
Nitrate-nitrite reductase	+	+	+	−	−	−	+
Appendages							
CFA/I (α C/U) fimbriae, AHA_0060	+	+	+	+	−	+	+
CFA/I (α C/U) fimbriae, AHA_1021	+	−	+	−	+	+	−
P (π C/U) fimbriae, AHA_0521	+	+	+	+	+	+	−
Tap type IV pilus	+	+	+	+	+	+	+
TAD Flp pilus	+	−	−	−	+	+	−
MSHA BFP type IV pilus	+	+	+	+	+	+	+
Polar flagellum	+	+	+	+	+	+	+
Lateral flagellum	−	+	−	−	−	+	−
Toxins and exoenzymes							
T3SS, *aexU*	−	+	−	−	−	+	−
T6SS, *hcp*	+	+	+	+	−	+	−
Cytotonic enterotoxin/lipase, *alt*	+	+	+	+	+	+	+
Cytotoxic enterotoxin/hemolysin, *act*	+	+	−	+	+	+	−
Cytotonic enterotoxin, *ast*	+	+	+	−	−	−	−
Enolase	+	+	+	+	+	+	+
Elastase	+	+	+	+	+	+	+
RTX toxin, AHA_1359, and transporter cluster	+	−	+	+	−	−	−
FHA family, RTX toxin	−	+	−	−	−	−	−
Pore-forming cytolysin/hemolysin, *hlyA*	+	+	+	+	−	+	−
Phospholipase/lecithinase/hemolysin-GCAT	+	+	+	+	+	+	+
Capsule	−	+	+	+	+	−	−
Antibiotic and multidrug resistance							
Macrolide-specific ABC efflux pump	+	+	+	+	−	+	+
Polymyxin B resistance (*arn*)	+	+	+	+	−	+	−
Acr family RND efflux pump, AHA_2959-60	+	+	+	+	+	+	−
ABC-type multidrug transport system, AHA_0484-6	+	+	+	+	−	+	−
MATE efflux pump	+	+	−	+	+	+	+
OmpK-AmpG	+	+	+	+	+	+	+
NodT family RND efflux pump	−	+	−	−	−	−	+
Acriflavin RND transporter	+	−	−	+	+	−	+

contig 00003 (~bp 93,233 to 127,913). This cluster was highly homologous to that of *A. salmonicida* A449 and *A. hydrophila* AH3, 85% identical, with 93% coverage (see Fig. S3B). In addition, the genome of strain E1 harbored at least two prophage-like elements, a glutamate-aspartate transporter, several transposases and insertion elements, and a number of genomic regions of unknown function (see Table S1).

***A. hydrophila* strains E1 and E2 exhibit motility.** To examine how the genetic differences in motility-associated factors influenced phenotypes, we performed swimming (polar flagellum) and swarming (lateral flagella) motility assays. Strain E1 displayed significantly higher swimming motility than the type strain ATCC 7966^T and the virulent diarrhea isolate *A. hydrophila* SSU, whose motility has been previously demonstrated (10). Strain E2 exhibited less swimming motility than strain SSU but more than ATCC 7966^T (Fig. 2A). Since strain E1 was highly motile, we repeated the swimming motility assay with larger petri dishes to better gauge differences in motility between clinical isolates SSU and E1. Indeed, the differences in motility between these two strains increased further because of the increased surface area available for bacteria to swim (Fig. 2B).

The swarming motility assays were performed as described by Kirov et al. (19), with freshly poured and dried swarming agar plates inoculated from the swimming agar plate culture. Strains E1, E2, and SSU exhibited similar swarming behavior on 0.5% Eiken agar plates at 30°C (Fig. 3A). This was surprising, given the absence of lateral flagella genes in the genome of strain E2. An alternate protocol, detailed in Materials and Methods, was used that gave phenotypic results for strain E2 in agreement with the genomic findings (Fig. 3B) while not affecting the observed swarming phenotypes of strains SSU and E1. Other perturbations of the swarming motility assay protocol had pronounced effects on the outcome, including the temperature of incubation of the plates, the concentration of agar added to the medium, and the brand of agar used, as well as the overall medium formulation (data not shown). For example, when the swarming plates were incubated at 37°C irrespective of the agar used (0.5% Eiken versus 0.8% Bacto agar), none of the bacterial cultures tested (E1, E2, or SSU) exhibited any swarming (data not shown). When a different medium composition was used at 30°C, the swarming pattern changed, with strains E1 and SSU exhibiting swarming, while strain E2 showed a moderate level of motility (data not shown).

Electron microscopy demonstrates lateral flagella of strain E1 and rafting behavior of strain E2. To further investigate the confounding swarming behavior of strain E2, electron microscopy of cultures taken directly from the swarming plates was performed (see Fig. S4 in the supplemental material). In agreement with the alternate swarming assay and genome analysis, *A. hydrophila* strain E1 produced lateral flagella (see Fig. S4A) similar to those of *A. hydrophila* strain SSU (see Fig. S4C). In contrast, *A. hydrophila* strain E2 did not produce lateral flagella but instead exhibited a "rafting"-like behavior that could mimic swarming because of unidentified cell-cell interactions possibly involving extracellular capsule and one or more types of small fimbriae (see Fig. S4B).

***A. hydrophila* strains E1 and E2 produce AHLs.** Quorum sensing (QS)- or cell density-dependent regulation is controlled by the concentration of small signal molecules, *N*-acyl-homoserine lactones (AHLs), termed autoinducers. A common approach used to detect AHLs is via bacterial reporter strains,

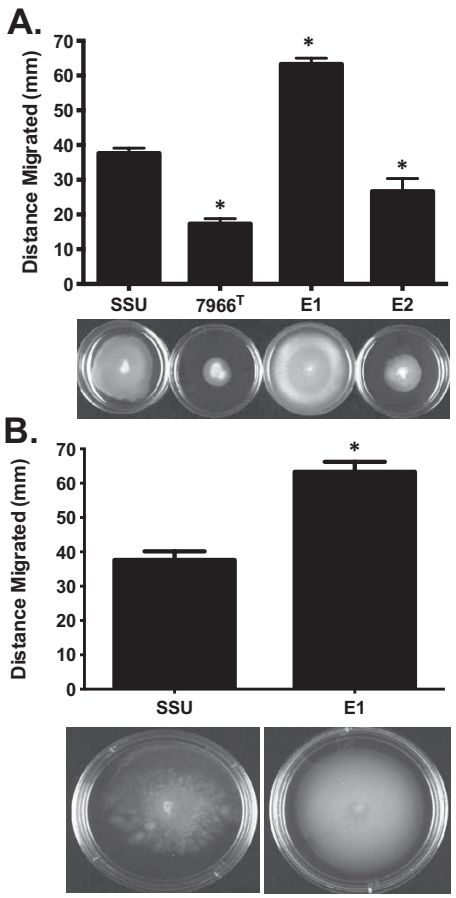

FIG 2 Swimming motility of *A. hydrophila* strains SSU, ATCC 7966^T, E1, and E2. (A) Strain E1 showed greater swimming motility than SSU ($P < 0.001$), while strain E2 exhibited less motility than SSU ($P < 0.001$) (50-mm-diameter petri dishes). (B) To measure exact zones of migration by *A. hydrophila* strain E1, we used 80-mm-diameter petri dishes. Three independent experiments were performed, and the arithmetic means ± the standard deviations were plotted. An asterisk indicates a *P* value of <0.001 as determined by one-way ANOVA.

which do not produce intrinsic AHLs. In the presence of exogenously produced AHLs, these reporter strains display specific, QS-induced phenotypes, such as purple pigment production by *Chromobacterium violaceum* CV026 (20). *A. hydrophila* strains E1, E2, and SSU produced similar levels of AHLs (Table 4). Two negative-control *A. hydrophila* strains, namely, ATCC 7966^T (21) and the Δ*ahyRI* isogenic mutant of strain SSU (10), demonstrated no lactone production in this bioassay.

Strains E1, E2, and ATCC 7966^T form less biofilm biomass than strain SSU does. Biofilm formation represents a characteristic feature of persistent infections, and 30% of *Aeromonas* infections are associated with this virulence trait (22–24). To measure solid surface-associated biofilm formation by *A. hydrophila* strains E1 and E2, we performed a crystal violet (CV) staining assay of biofilms from cultures grown in polystyrene tubes at 37°C after

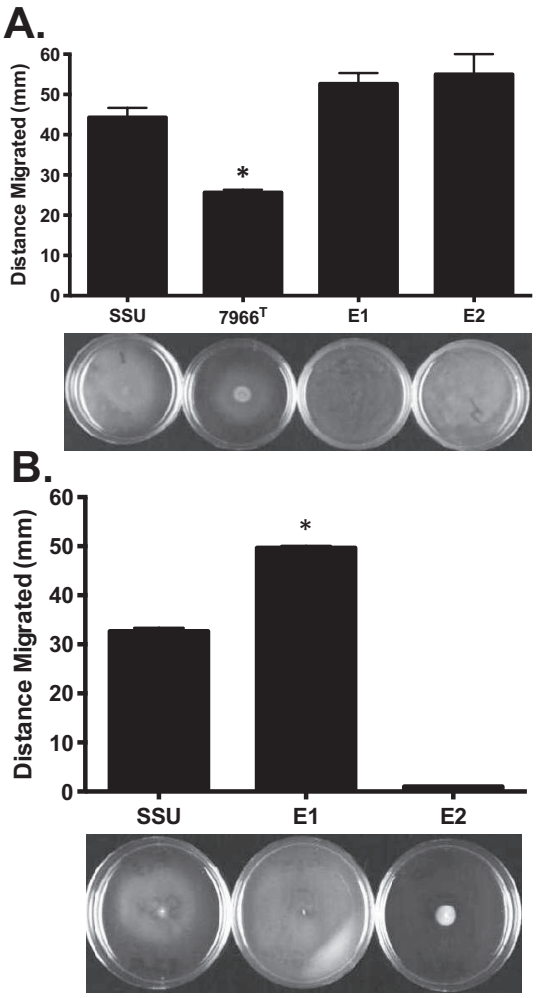

FIG 3 Swarming motility of *A. hydrophila* strains SSU, ATCC 7966^T, E1, and E2. (A) Strains E1 and E2 had swarming motility comparable to that of strain SSU on Difco nutrient agar plates with 0.5% Eiken agar at 30°C when the bacteria were propagated as described previously (19). (B) By an alternative protocol, strains were subcultured from the freezer onto blood agar plates and then inoculated onto swarming plates. In this assay, strain E1 had swarming motility superior to that of strain SSU, and strain E2 exhibited no swarming motility. Three independent experiments were performed, and the arithmetic means ± the standard deviations were plotted. An asterisk indicates a *P* value of <0.001 as determined by one-way ANOVA.

overnight incubation. *A. hydrophila* E1 and E2 formed significantly less solid surface-associated biofilm in polystyrene tubes, as demonstrated by a more-than-3-fold decrease in CV staining, compared to that of the clinical isolate, SSU (see Fig. S5 in the supplemental material). The biofilms formed by strains E1 and E2 were comparable to that of the type strain, ATCC 7966^T.

Hemolytic and proteolytic activities of *A. hydrophila* E1 and E2. We observed that the hemoglobin release from rabbit red blood cells (RBCs) by the hemolysin(s) produced by strain E1 was comparable to that produced by strain SSU (163 ± 2.8 and 115 ± 3.3, respectively), while that produced by strain E2 (24 ± 4.5 U/ml/10^8 CFU) was much lower (Table 4), which was considered baseline activity. The hemolytic activity associated with strain ATCC 7966^T was 52 ± 4.0 U/ml/10^8 CFU. To demonstrate that most of the hemolytic activity of strain E1 was associated with Act, we neutralized the toxin with specific antibodies that were serially diluted (5-fold). These antibodies abrogated the hemolytic activity associated with Act in the culture filtrates of strains E1, SSU, and ATCC 7966^T in a dose-dependent fashion (Fig. 4A). Strain E2 does possess the *hlyA* gene, GCAT, and a number of other putative hemolysins (Table 3; see Table S1 in the supplemental material), which may be responsible for the low baseline hemolytic activity observed in Table 4. As noted in Table 3, strain E2 did not possess the Act-encoding gene. Previous studies have indicated that the pathogenic nature of *A. hydrophila* is, in part, associated with the production of exoenzymes, such as proteases and lipases (25, 26). Consequently, we measured protease production and noted that E1 produced a significantly lower level of protease activity than isolate SSU, while strain E2 had a production level of this enzyme comparable to that of SSU (Fig. 4B).

Expression of T3SS and T6SS. The secretion of hemolysin-coregulated protein (Hcp) has become a reliable indicator of functional T6SS in all bacteria with an intact T6SS structure, even though the gene that encodes Hcp is not always found in T6SS clusters (27). Among the *Aeromonas* strains analyzed in this study, Hcp production (cell pellet) and secretion (supernatant) were noted only in *A. hydrophila* strain SSU (Fig. 5A). While strain ATCC 7966^T did not produce Hcp, strains E1 and E2 did synthesize Hcp, as it could be detected in the cell pellet, but were unable to secrete it into the medium (Fig. 5A). This raises the question of whether the T6SS is functional in strains E1 and E2.

The AexU toxin is a T3SS effector identified in *A. hydrophila* SSU whose secretion can be indicative of a functional T3SS (28, 29). While the positive control, *A. hydrophila* SSU, expressed and produced AexU when grown in Dulbecco's modified eagle medium (DMEM), strains ATCC 7966^T, E1, and E2 were unable to express the gene encoding AexU (Fig. 5B). The results for strain E1 were surprising, given the fact that the genome of strain E1 har-

TABLE 4 Lactone production and hemolytic activities of four *A. hydrophila* isolates

A. hydrophila strain	Lactone production[a]	Mean hemolytic activity ± SD (U/ml/10^8 CFU)
SSU	+++	163 ± 2.8[b]
SSUΔ*ahyRI*	−	NA[c]
ATCC 7966^T	−	52 ± 4.0
E1	+++	115 ± 3.3
E2	+++	24 ± 4.5

[a] Lactone production scored semiquantitatively: − (none), + (weak), ++ (moderate), or +++ (high).
[b] The differences between hemolytic activity titers were statistically significant ($P < 0.001$), as determined pairwise by *t* test.
[c] NA, hemolytic activity of strain SSUΔ*ahyRI* was not measured in this study.

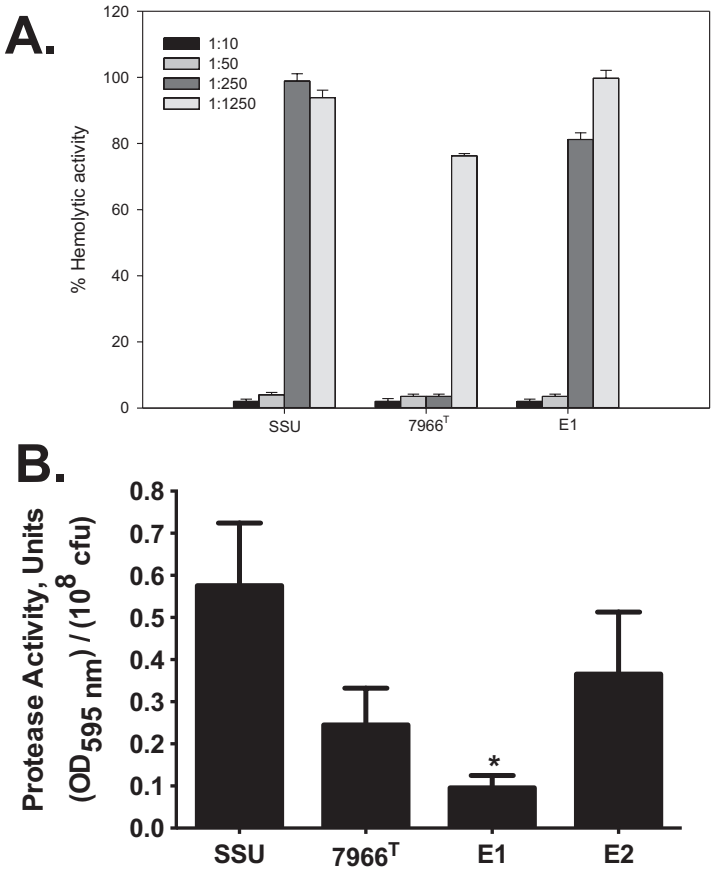

FIG 4 The Act-associated hemolytic activity neutralization assay and the protease activity of *A. hydrophila* strains E1 and E2 compared to that of *A. hydrophila* SSU and/or ATCC 7966^T. (A) The neutralization of hemolytic activity associated with Act in the culture filtrates of *A. hydrophila* E1 and ATCC 7966^T compared to *A. hydrophila* SSU. The culture filtrates of the strains studied were mixed with either preimmune rabbit serum (control) or 5-fold dilutions of hyperimmune rabbit serum (laboratory stock) containing antibodies to Act before the measurement of hemolytic activity. (B) Protease activity in the culture supernatants of *A. hydrophila* E1 and E2 compared to that of *A. hydrophila* SSU and ATCC 7966^T. *A. hydrophila* E1 demonstrated a statistically significant decrease in protease activity compared to that of E2 and the two control strains, SSU and ATCC 7966^T. The data were normalized to 1×10^8 CFU to account for any minor differences in the growth rates. All of the experiments were performed in triplicate, and the data presented are arithmetic means ± standard deviations. OD_{595}, optical density at 595 nm.

bored the T3SS gene cluster, as well as the small accessory *aexU* gene cluster (Table 3; see Table S1 in the supplemental material). We questioned whether bacterium-host contact might be needed for the expression of the *aexU* gene in strain E1, and indeed, interaction of E1 with HeLa cells led to the expression and production of AexU (Fig. 5B).

Serum resistance of strains E1 and E2. The survival of strains E1 and E2 in the presence of naïve-mouse serum was compared with that of *A. hydrophila* ATCC 7966^T (negative control). As expected, no difference in colony counts was noted when strain ATCC 7966^T was incubated with either phosphate-buffered saline (PBS) or naive-mouse serum for 1 h (Fig. 6). In contrast, the colony counts obtained with both strains E1 and E2 after incubation with serum were significantly higher than the counts of samples from PBS, and this difference was greater for the E1 strain than for the E2 strain (Fig. 6). These data indicated that strains E1 and E2 were serum resistant, as demonstrated by continued growth during 1 h of incubation with serum.

***A. hydrophila* E1 is highly virulent in an animal model.** To assess the overall virulence potential of *A. hydrophila* strains E1 and E2, compared to that of strains SSU and ATCC 7966^T, we injected various doses of these bacterial isolates into mice via the intraperitoneal (i.p.) route. While a dose of 5×10^7 CFU of strain SSU killed more than 90% of the mice, ATCC 7966^T killed <20% of the animals at this dose (Fig. 7). Strain E2 had moderate virulence, as it killed more than 70% of the mice at a dose of 5×10^7 CFU. All of the animals injected with strain E1 at a dose of 1×10^7 CFU died within 2 days, while minimal mortality was noted in mice injected with either strain SSU or E2 at this dose (data not shown). When animals were injected with smaller doses of strain E1, for example, 8×10^6, 5×10^6, or 3×10^6 CFU, 85, 60, and 20% (not shown) mortality, respectively, was observed (Fig. 7). These data indicated that strain E1 was much more lethal than the other *A. hydrophila* strains tested in this mouse model. For example, strain E1 was approximately 10 times more virulent than strain SSU.

DISCUSSION

This is the first genomic analysis of a virulent clinical *A. hydrophila* isolate, namely, E1. By analyzing the genome and phenotypic characteristics of E1 alongside those of a less virulent clinical isolate and several additional strains of *Aeromonas*, we have identified key differences that distinguish this disease-causing strain from the ubiquitous environmental isolates common to freshwaters. Among the features that distinguished E1 from the other aeromonads in this study were capsular polysaccharide, enhanced bacterial motility, a functional T3SS, and the presence of Act. Our genomic and phenotypic findings regarding each of these features have important implications for virulence in *Aeromonas*.

Antigen presentation can be a critical determinant of bacterial pathogenicity. While strain ATCC 7966^T is defined as an O:1 serotype, we could deduce from the genomic sequences of the O-antigen and group II capsular polysaccharide genes that strain E2 was an isolate of either the O:18 serogroup (30) or a very closely related one. The serotype of strain E1 was unclear, but it was not O:1, O:18, or O:34, for which we have sequence information for comparison. From genome annotation, we could also deduce that

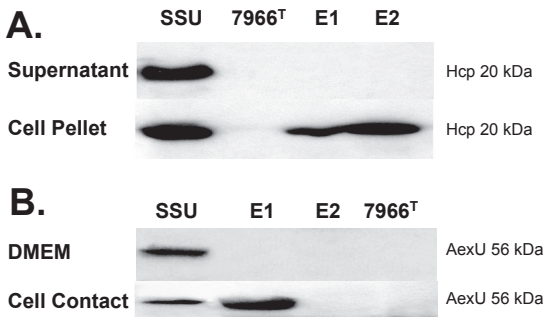

FIG 5 Expression and production of T6SS-associated Hcp and T3SS effector AexU in *A. hydrophila* strains SSU, ATCC 7966^T, E1, and E2. (A) While only SSU secretes Hcp into the supernatant, the Hcp protein is found in the cell pellet of strains E1 and E2. (B) Though SSU is the only strain to express and produce AexU when grown in DMEM, both SSU and E1 expressed and produced AexU when grown in contact with HeLa cells. As expected from the genomic analyses that demonstrated the absence of AexU from strains E2 and ATCC 7966^T, neither of these strains produced AexU under either condition.

strains E1 and E2 were encapsulated, while the capsular polysaccharide gene cluster was absent from the genome of environmental isolate ATCC 7966^T, diarrhea isolate Ae398, and fish isolate A449; however, *A. salmonicida* isolates typically harbor analogous A(S)-layer protein-encoding genes. From these genomic data and other experimental findings, it is clear that capsule production imparts serum resistance to the bacteria (30). Indeed, the higher level of *A. hydrophila* strain E1 and E2 resistance to naive-mouse serum than that of ATCC 7966^T could also be related to the presence of capsular polysaccharide in the wound isolates. Therefore, we conclude that extracellular layers such as the capsule are important for the survival of bacteria in wounds.

Bacterial motility, enabled by lateral or polar flagella, may facilitate the invasion of human and nonhuman host cellular barriers (31, 32). Our study found that the lateral flagellum in the genome of *A. hydrophila* E1 was homologous to that found in *A. salmonicida* A449 (33) and enabled swarming motility (34).

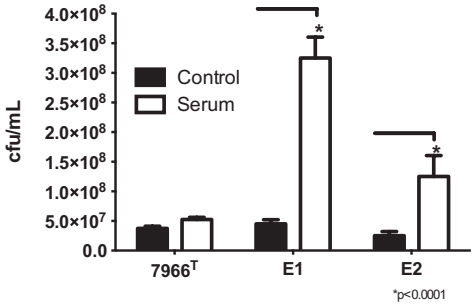

FIG 6 Serum resistance of *A. hydrophila* E1 and E2 isolates compared to that of *A. hydrophila* ATCC 7966^T. *A. hydrophila* 7966^T, E1, and E2 were grown overnight, harvested, and resuspended in an equivalent amount of PBS. An aliquot (50 μl) of the bacterial cells was mixed with either PBS or serum and incubated at 37°C for 1 h. The CFU counts in the samples were then determined as described in Materials and Methods. *A. hydrophila* strains E1 and E2 demonstrated statistically significant increases in serum resistance compared to that of strain ATCC 7966^T.

Surprisingly, strain E2 mimicked swarming motility under certain assay conditions although it lacks genes for lateral flagella. While electron microscopy revealed lateral flagella on strains E1 and SSU, E2 lacked flagella and exhibited a rafting or "bridging" multicellular behavior that may be driven by LPS, capsule, or cell surface-associated factors such as type IV pili (35). E2's phenotype may also be explained by "sliding" motility, in which bacteria synthesize and secrete surfactants that allow them to spread over surfaces (34). In all, we found flagellum-enabled "true" motility in *Aeromonas* strains with enhanced virulence but only rafting or sliding motility in less virulent strains such as E2 and ATCC 7966^T.

The ability to form biofilms, multicellular sessile communities (22), was not a distinguishing feature of *A. hydrophila* E1. Biofilms can facilitate wound chronicity and persistence by creating a barrier against neutrophils, macrophages, and antimicrobials (36). We recently demonstrated that the effect of cyclic di-GMP (c-di-GMP) on motility and biofilm formation in *A. hydrophila* SSU was dependent on the coexpression of three QS systems, AI-1, AI-2, and AI-3 (37–39). Intriguingly, the genomes of E1 and E2 harbored numerous genes that encode proteins with GGDEF and EAL domains (involved in the synthesis and degradation of c-di-GMP, respectively), as well as *N*-acyl-homoserine AI-1, *S*-ribosylhomocysteinase (LuxS)-based AI-2, and QseBC-based AI-3 QS systems. In addition, we examined AHL production, since AHL-mediated QS has been shown to regulate exoprotease production and biofilm formation in *A. hydrophila* (10, 37–41). While AHL-deficient knockout mutants demonstrated attenuated virulence in a mouse model (10), the role of AHL-mediated QS in wound infection isolates of *A. hydrophila* such as E1 and E2 has never been tested before. This study demonstrated AHL production by strains E1 and E2, corroborating the presence of an AI-1 QS system. Taken together, these findings indicate that strains E1 and E2 produce less biofilm biomass or that biofilms from these isolates take longer to mature. Aside from regulatory defects unrelated to QS, one possible explanation is that the presence and expression of capsular polysaccharide genes may have a negative effect on biofilm formation rates, as has been demonstrated in various *Vibrio* species (42, 43). Future studies should include extension of the assay time and the use of static culture for biofilm formation, as well as the examination of other regulatory factors to better understand the role of *Aeromonas* biofilms in virulence.

In contrast, the ability to form and secrete extracellular toxins appears to be a distinguishing feature of *A. hydrophila* E1. For example, Act is a potent virulence factor secreted via the T2SS that functions as a hemolysin, a cytotoxin, or an enterotoxin, depending upon the target cells (12, 25, 44–46). In this study, we demonstrated that the significant hemolytic activity of *A. hydrophila* E1 was due primarily to Act. In contrast, the genome of *A. hydrophila* strain E2 did not harbor the *act* gene and it exhibited baseline hemolytic activity. While both strains E1 and E2 exhibited a functional T6SS, as evidenced by the presence of Hcp in the bacterial pellet, no Hcp was found in the supernatant of strain E1 or E2. Therefore, future studies may desire to determine whether E1 and E2 can translocate Hcp into host cells, since the evasion of host innate immunity by strain SSU has been attributed to the secretion of this effector (47). The presence of T3SSs is more strongly correlated with pathogenicity because of the presence of this factor in the genomes of known pathogens, such as *Yersinia pestis*, *Salmonella enterica*, *Vibrio parahaemolyticus*, and *Shigella* species (48–50). We found T3SS genes only in the genomes of *A. hydro-*

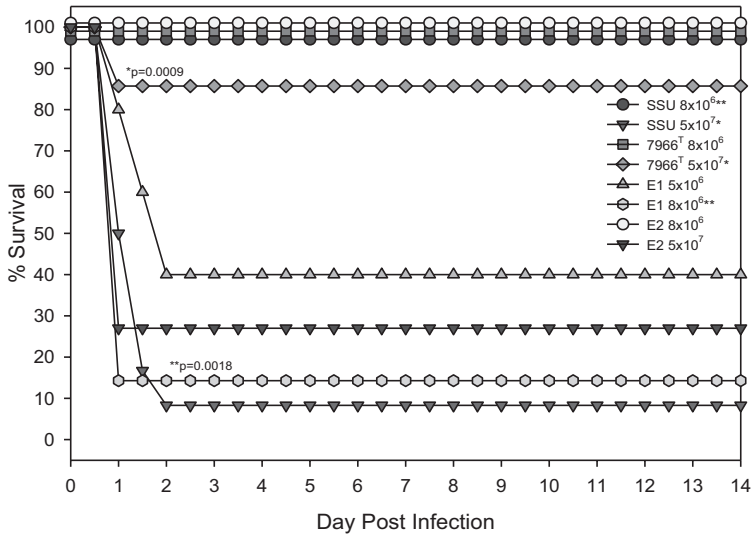

FIG 7 Kaplan-Meier survival curves of mice injected with *A. hydrophila* strains SSU, ATCC 7966^T, E1, and E2. Groups of nine Swiss-Webster mice were injected with doses of 5×10^7, 1×10^7, 8×10^6, 5×10^6, and 3×10^6 CFU by the i.p. route. The animals were observed for death over a period of 14 days. The data were statistically analyzed by using a Kaplan-Meier survival estimate. Single and double asterisks indicate the groups compared for statistically significant differences.

phila strain E1 and *A. salmonicida* A449 and demonstrated the functionality of strain E1's T3SS by detecting AexU in the presence of host cells. As AexU inhibits macrophage phagocytosis of *A. hydrophila* SSU and leads to host cell apoptosis (29, 51), it is likely that the T3SS played a role in the virulence of strain E1

Extracellular proteases have also been suggested as a virulence factor of aeromonads (52), and both temperature-labile serine proteases and temperature-stable metalloproteases have been characterized in *A. hydrophila* (53). In experimental animal models, protease-null mutants of *A. hydrophila* and *A. salmonicida* exhibit less virulence than wild-type bacteria (54, 55), and a role for protease in *Aeromonas*-associated tissue damage has been reported (55). Recently, we showed that the AI-1 QS system positively modulates metalloprotease activity in *A. hydrophila* SSU (10). Interestingly, we observed a lower level of protease activity in the culture filtrate of *A. hydrophila* strain E1 than in that of strains SSU and E2. These results raise the possibility that, in contrast to previous studies (25, 26), protease activity and virulence may be inversely correlated in the *Aeromonas* strains studied—a topic that warrants further study with specific gene knockouts. There were approximately 20 genes annotated as proteases or proteinases in the genome of *A. hydrophila* strains ATCC 7966^T and E2. Only one of these genes was missing from strain E1, specifically, that for a PfpI-like protease. However, it is quite likely that this defect may not be explained by differences in gene content.

When initially isolated, strain E2 exhibited resistance to several aminoglycoside antibiotics, namely, amikacin, tobramycin, and gentamicin, as well as several cephalosporins (8). During the annotation of the genome sequences, we did not find any unique aminoglycoside-modifying enzymes in the genome of strain E2, compared to that of strain E1. Furthermore, upon resuscitation from cold storage, in preparation for whole-genome sequencing, strain E2 was sensitive to the above antimicrobials. Therefore, the loss of a plasmid harboring antibiotic resistance genes is a possible explanation. Previous studies have demonstrated plasmid-borne antimicrobial resistance in *Aeromonas*; for example, plasmid pAsa4 of *A. salmonicida* A449, contains a streptomycin/spectinomycin *O*-adenyltransferase-encoding gene (33). Furthermore, *Aeromonas* strains have been shown to lose plasmids under stressful conditions (56, 57). Alternatively, initial resistance to several aminoglycosides could be explained by adaptive resistance via increased cell impermeability in this strain, which was induced in the clinical setting (58).

Through comparative genomic and functional analyses of wound isolates along with other clinical and environmental strains of *Aeromonas*, we not only demonstrated that strain E1 was more virulent than strains E2 and ATCC 7966^T in a mouse model of infection but also showed the presence of several functional virulence factors that may be related to its enhanced pathogenicity. Previously, Daily et al. (59) and Joseph and Carnahan (60) hypothesized the possibility of subsets of virulent aeromonads within and between the eight species most frequently associated with disease. The findings of the present study provide the foundation for the establishment of distinct pathotypes within the genus *Aeromonas*. Although strain E1 produced less protease and biofilm than diarrhea isolate SSU did, it was much more virulent in a mouse model of infection, possibly because it harbored a capsule, flagella, a functional T3SS, and highly hemolytic Act. Furthermore, we demonstrated the ability to detect these virulence-associated factors through genome sequencing and annotation. More extensive genomic and epidemiological investigations will allow us to ascertain the frequency of core and accessory genes in environmental and clinical *Aeromonas* isolates. In the future, we can aim to detect novel virulence factors through genomic surveillance and estimate the virulence potential of clinical isolates through sequence analysis alone.

MATERIALS AND METHODS

Bacterial strains. Freezer stocks of *A. hydrophila* strains E1 and E2, corresponding to Aero 1 and Aero 2 of Shak et al. (8), were streaked and subcultured on Trypticase soy agar plates with 5% sheep blood agar (SBA; BDMS, Sparks, MD). While E1 colonies were flat and grayish in color with a smooth surface, colonies of E2 were raised and whitish yellow in color with a visible mucoidal exterior, suggestive of the presence of an extracellular capsule. Strains E1 and E2 were tested for seven phenotypic traits by using the Aerokey II dichotomous key as previously described (61) and were identified as *A. hydrophila* subsp. *hydrophila*. Unless otherwise specified, *A. hydrophila* here refers to *A. hydrophila* subsp. *hydrophila*. For reference, additional strains of *A. hydrophila*, ATCC 7966^T, SSU, and SSUΔ*ahyRI* (10, 62), were included in phenotypic experiments.

Genome sequencing and annotation. Genome sequencing of *A. hydrophila* strains E1 and E2 was performed at the Emory Genome Center with a GS Junior pyrosequencer (454 Life Sequencing, Branford, CT). The numbers of reads were 130,920 for strain E1 and 130,449 for strain E2, and the average read lengths were 405 and 338 bp, respectively. The estimated average coverages of the E1 and E2 genomes were 11-fold and 9-fold, respectively. Contigs were assembled by using Newbler (63) and uploaded as multiple-sequence FASTA files to RAST for annotation (64). Further analyses to identify shared and dispensable genetic traits were performed by using the closed genomes of *A. hydrophila* ATCC 7966^T (GenBank accession no. CP000462.1) (17) and *A. salmonicida* A449 (GenBank BioProject PRJNA58631) (33), as well the draft genomes of *A. aquariorum* AAK1 (GenBank accession no. BAFL00000000.1) (65), *A. veronii* B565 (GenBank accession no. CP002607.1) (66), and *A. caviae* Ae398 (GenBank accession no. WGS CACP00000000) (67). To facilitate comparisons, the genomes of the other five *Aeromonas* species were also annotated by using the RAST server with small hypothetical protein-encoding genes accepted from the initial RAST annotation only if they were consistently annotated among the majority of the genomes analyzed. Genomic regions and mobile genetic elements were mapped to the syntenic core on the basis of the homology of conserved flanking genes or sequences.

Comparative genomic analyses. The syntenic core genome of *A. hydrophila* was determined by using the SEED viewer comparative genomic feature (68). To ensure the most accurate syntenic core gene set, the closed genome of *A. hydrophila* ATCC 7966^T (GenBank accession no. CP000462.1) was used to "map" contigs from draft genomes. For the draft genomes of strains E1 and E2, genes at the end of a contig or interrupted by contig gaps were analyzed by using bidirectional BLASTN analysis against all other genomes. ANI by BLAST was computed with Jspecies (69). Evolutionary analyses were conducted in MEGA5 (70). Comparison of the T3SS gene cluster was performed with the Artemis comparison tool (71).

Motility assays. Luria-Bertani (LB) medium with 0.35% Bacto agar (Difco Laboratories, Detroit, MI) was used to characterize swimming motility, while Difco nutrient broth with 0.5% Eiken agar (Eiken Chemical Co., Ltd., Tokyo, Japan) was used to measure the swarming motility of *A. hydrophila* strains. To evaluate swimming motility, the overnight cultures were adjusted to the same optical density and equal numbers (10^8) of CFU were stabbed onto 0.35% LB agar plates. The plates were incubated at 37°C overnight, and motility was measured by examining the migration of bacteria through the agar from the center toward the periphery of the plate (62). Growth from the edge of the swimming zone within the agar of these plates provided the inocula for the swarming assay. The swarming plates were inoculated by streaking bacteria onto the surface of the agar (19, 72) and then incubated at 30°C overnight. For swarming motility, an alternate protocol was also used (J. G. Shaw, personal communication). Briefly, inocula from cryogenically frozen cultures were subcultured onto SBA plates, which were incubated overnight at 30°C. Growth from SBA was used to inoculate the surfaces of swarming plates as described above or to inoculate an alternative formulation containing 0.8% (wt/vol) Bacto agar; 0.5% (wt/vol) glucose; 1.0% (wt/vol) tryptone; 0.5% (wt/vol) NaCl; and 0.002% (vol/vol) Tween 80 (J. G. Shaw, personal communication). Inoculated swarming plates were then incubated at 30°C and examined after 8, 16, and 24 h of growth for the swarming phenotype.

Electron microscopy. Cell suspensions of cultures grown overnight at 30°C on swarming plates were added to 200 μl of 0.5% sodium phosphotungstate (pH 6.8). Subsequently, 15 μl of the sample was applied to the surface of a 300-mesh, carbon-coated, Formvar-coated copper grid. Excess stain was removed, and the grids were air dried. A JEOL 1011 transmission electron microscope (JEOL United States, Inc., Peabody, MA) operating at an accelerating voltage of 80 kV was used to examine the bacterial cells for the presence of lateral flagella.

Production of AHLs. AHL production was detected by cross-streaking *A. hydrophila* strains against the biosensor strain *C. violaceum* CV026 on LB agar plates. Positive assays were judged by the degree of induction of the purple violacein pigment in the biosensor strain (73). Pigment production by *C. violaceum* CV026 was scored on the basis of the intensity of the color after overnight incubation of the plates at 30°C. Lactone production was scored semiquantitatively as follows: −, no lactone production; +, weak; ++, moderate; +++, high. An isogenic Δ*ahyRI* mutant of *A. hydrophila* SSU was used as a negative control, as this mutant does not produce lactones and, hence, no violacein is induced in the biosensor strain (62).

Biofilm formation assays. In a modification of the biofilm ring assay (74), strains of *A. hydrophila* were transferred from fresh LB agar plates into 3 ml of LB medium contained in polystyrene tubes at 37°C overnight with shaking. Biofilm formation was quantified according to procedures previously described (75). Biofilm formation results were normalized to 1×10^9 CFU to account for any minor differences in the growth rates of the various bacterial strains used.

Measurement of hemolytic activity. To examine the lysis of RBCs, culture filtrates from *A. hydrophila* strains grown for 18 h in LB medium at 37°C with shaking (180 rpm) were treated with trypsin (final concentration, 0.05%; to activate Act or other hemolysins) (76, 77) at 37°C for 1 h and then subjected to a hemolytic assay as described previously (12). The number of hemolytic units per milliliter of cell filtrate per 1×10^8 CFU was reported (62). For the neutralization assay, culture filtrates of the strains studied were mixed and incubated for 1 h at 37°C with either preimmune (control) or serially diluted (5-fold) hyperimmune rabbit serum (laboratory stock) containing antibodies to Act before the measurement of hemolytic activity (10).

Measurement of protease activity. Protease activity was measured in filtrates of *A. hydrophila* cultures grown overnight as described previously (78). LB medium was inoculated with fresh growth from LB agar plates and incubated overnight at 37°C. Protease activity was calculated per milliliter of culture filtrate per 10^8 CFU. Hide Powder Azure (Calbiochem, La Jolla, CA) was used as the substrate to measure protease activity because of the sensitivity and rapidity of the assay. The substrate incubated with PBS alone served as a negative control.

Expression and production of AexU. To examine the expression and production of AexU, cultures of *A. hydrophila* isolates grown overnight were either reinoculated (1×10^8 CFU) into 2 ml of DMEM with 4 mM glutamine and incubated at 37°C for 4 h in the CO_2 incubator or used directly to infect HeLa cells at a multiplicity of infection of 10 for 4 h in a six-well plate. The bacterial cells were then harvested from either the DMEM or HeLa cell culture supernatants by centrifugation and subsequently lysed in SDS-PAGE loading buffer for Western blot analysis with anti-AexU antibodies as previously described (51).

Expression and secretion of Hcp. Western blot analysis was used to detect T6SS effector protein Hcp expression in and secretion from *A. hydrophila* isolates. Briefly, the supernatants and cell pellets from overnight broth cultures of *Aeromonas* strains were separated by centrifugation. The cell pellets were directly lysed in SDS-PAGE loading buffer, while the proteins in the supernatants were first precipitated with 10% trichloroacetic acid and then dissolved in the loading buffer. The samples of supernatants and pellets were subjected to SDS-PAGE, followed by Western

blot analysis with specific antibodies to Hcp, according to the procedure described previously (47).

Serum resistance assay. Pooled sera from naive mice were used in the serum resistance assay. Briefly, overnight *A. hydrophila* ATCC 7966^T, E1, and E2 cultures were harvested and diluted in PBS to an optical density at 600 nm of 0.2 (~1×10^8 CFU/ml). Next, 50 μl of the diluted bacteria (~5×10^6 CFU) was mixed with 200 μl of normal mouse serum or PBS. The samples were incubated at 37°C for 1 h. The number of CFU of surviving bacteria in each sample was determined by serial dilutions and plating on LB plates.

Animal virulence model. Groups of nine female Swiss Webster mice (Taconic Farms) were injected via the i.p. route with *A. hydrophila* strains SSU, ATCC 7966^T, E1, and E2 in accordance with an approved Institutional Animal Care and Use Committee protocol. The animals were injected with doses of 5×10^7, 1×10^7, and 8×10^6 CFU of all of the strains. Additional animals were also injected at lower doses of 5×10^6 and 3×10^6 CFU of strain E1. Deaths were recorded daily for 14 days postinfection.

Statistical analyses. All *in vitro* experiments were performed in triplicate, and differences were analyzed for significance by one-way analysis of variance (ANOVA). The animal data were analyzed by using a Kaplan-Meier survival estimate, and P values of ≤ 0.05 were considered significant.

Nucleotide sequence accession numbers. The genome sequences determined in this study were deposited at NCBI under accession numbers SRA063950 (*A. hydrophila* strain E1) and SRA063951 (*A. hydrophila* strain E2).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00064-13/-/DCSupplemental.

Figure S1, EPS file, 1.9 MB.
Figure S2, EPS file, 2.2 MB.
Figure S3, EPS file, 1.4 MB.
Figure S4, EPS file, 8.9 MB.
Figure S5, EPS file, 1.7 MB.
Table S1, XLS file, 1.9 MB.

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