Within-Host Competition Drives Selection for the Capsule Virulence Determinant of Streptococcus pneumoniae

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Summary
For many opportunistic pathogens, it is unclear why their virulence determinants and expression of pathogenic behavior have evolved when damage or death of their host offers no obvious selective advantage to microbial growth or survival [1–3]. Many pathogens initiate interactions with their host on mucosal surfaces and must compete with other members of the microflora for the same niche. Here we explore whether competitive interactions between microbes promote the acquisition of virulence characteristics. During model murine nasal colonization, Haemophilus influenzae outcompetes another member of the local flora, Streptococcus pneumoniae, by recruiting neutrophils and stimulating the killing of complement-opsonized pneumococci [4]. For S. pneumoniae, resistance to opsonophagocytic killing is determined by its polysaccharide capsule [5, 6]. Although there are many capsule types among different S. pneumoniae isolates that allow for efficient colonization, virulent pneumococci express capsules that confer resistance to opsonophagocytic clearance. Modeling of interspecies interaction predicts that these more virulent S. pneumoniae will prevail during competition with H. influenzae, even if production of a capsule is otherwise costly. Experimental colonization studies confirmed the increased survival of the more virulent S. pneumoniae type during competition. Our findings demonstrate that competition between microbes during their commensal state may underlie selection for characteristics that allow invasive disease.

Results and Discussion

Theoretical Modeling
One of the most basic and important evolutionary questions posed about pathogens is why they harm the very sources of their livelihoods, their hosts. Theoretical models (reviewed in [7]) frequently assume that traits contributing to virulence (reduced host fitness) provide a net selective advantage to the pathogen within the host (e.g., increasing growth rate) and/or in transmission to another host (e.g., increasing propagule production). However, for many pathogens, these proposed countervailing advantages of virulence are difficult to identify either on the within- or among-host level [1–3]. For example, in the case of the leading pathogen Streptococcus pneumoniae, its most common disease states (pneumonia, otitis media, and sepsis) are not contagious conditions and therefore represent a dead end for the organism, especially when the result is the rapid demise of the host [8]. Rather, transmission occurs from the reservoir of pneumococci residing asymptomatically in the nasopharynx during the organism’s commensal state [9]. However, among the >92 types of pneumococci expressing structurally distinct capsular polysaccharides, only a few are potentially virulent [10, 11]. So why has the pneumococcus evolved or maintained the capacity for virulent, invasive behavior through the expression of certain thick capsular polysaccharide coats?

To answer this question, we analyzed a simple model for the within-host dynamics of two strains of pneumococcus (resistant P_R and susceptible P_S) together with Haemophilus influenzae (H) (Equations 1; see also Supplemental Results available online). In the absence of the resistant strain (Equations 1 with P_R = 0), we find that the immunomanipulative ability of H. influenzae (captured by x > 1) increases the within-host market share of H up to a point (when x > 1/h) where H can completely outcompete a susceptible lineage of the pneumococcus (Figure S1). The analysis of Equations 1 with P_R = 0 is consistent with a number of previous theoretical and experimental studies suggesting that immunomanipulation can aid a focal lineage (in our case, H) to exclude competitors [4, 12–16] and is more generally consistent with the broad phenomenon of interference competition in bacteria, exemplified by bacteriocins [17].

We now ask, what happens if members of the susceptible lineage (P_S) become resistant to the killing (allelopathic) trait of H? “Resistant nonkillers” are widely reported in studies of bacteriocin-mediated interactions [18], and in our focal system some pneumococcal types play the same role, being largely resistant to the immunomanipulative generated by H. influenzae. We analyzed the full model (Equations 1) to allow for two distinct lineages of pneumococci, the sensitive lineage P_S, and the resistant lineage P_R. To describe the competitive interactions among the two pneumococcal strains, we introduced the competitive impact parameter a and the competitive sensitivity parameter y of P_R. When y and a equal 1, the two pneumococcal strains are competitively equivalent in the absence of H. If, however, the acquisition and maintenance of the capsule come at some cost (relative to P_S), then y > 1 > a, and in the absence of H, P_S will always replace P_R (a < 1 implies an attenuated competitive impact of P_R, and y > 1 implies an increased susceptibility to competition in P_R).

What happens when we allow the presence of all three lineages? The simplest scenario occurs if H is strongly immunomanipulative and is able to entirely exclude P_S (x > 1/h; see Figure S1). In this case, the resulting pure H equilibrium can be invaded by rare P_R if y < 1/h (Figure 1A), leading to a stable coexistence between H and P_R (Figure 1B). The second scenario occurs if H is only moderately immunomanipulative, leading first to a coexistence of H and P_S (x < 1/h; see Figure S1). If this scenario, the resulting (H, P_S) equilibrium can then be invaded by rare P_R if x > (y(1 + h(1 - p)) - 1)/(h(y - p)) (i.e., P_R invades more readily as x increases; Figure 1A), resulting in either (H, P_R, P_S) or (H, P_R) coexistence (Figure 1B). Thus,
Despite a potential competitive disadvantage with $P_S$ in the absence of $H$, $P_R$ has an increasing advantage over $P_S$ as the density and/or immunompanipulative behavior of $H$ increases, because it can resist the inflammatory response generated by $H$. In general, selection favors $P_R$ over $P_S$ whenever $H(x-y) > P_S(y-1) + P_R(1-\alpha)$; thus, sufficient $H$ can always drive selection toward $P_R$, given $x>y$ (for a more complete analysis, see Supplemental Results). In the limiting case in which $P_R$ is rare and the cost of capsule is negligible ($y$ tends to 1), the more resistant lineage can invade for any amount of immunomanipulation, i.e., whenever $x>1$.

### Pneumococcal Strains for Experimental Comparison

We tested the model by determining whether pneumococcal capsule type affects the outcome of immunomanipulation by $H. influenzae$ during experimental cocolonization. Pneumococcal isolates of two capsular types were compared. Both type 4 and type 23F pneumococci efficiently colonize the upper airway of mice and induce a similar mild acute inflammatory response characterized by the influx of neutrophils into the nasal spaces following intranasal challenge [19].

To address this question, we generated isogenic strains in which the capsule type was switched (type 4 switched to type 23F to generate $P_{R\rightarrow S}$ and type 23F switched to type 4 to generate $P_{S\rightarrow R}$) to control for the potentially confounding effect of genetic background. A sensitive capture ELISA was used to confirm that $P_{R\rightarrow S}$ and $P_{S\rightarrow R}$ produce equivalent levels of capsular polysaccharide to the isotypic strain (data provided in Table S1). When tested for the ability to cause bacteremic infection, the competitive index (CI) for $P_{S\rightarrow R}/P_S$ was >6.0, confirming the contribution of capsule type rather genetic background to virulence.

The density of colonizing $H. influenzae$ ($H$) is not affected by the presence of $S. pneumoniae$. However, cocolonization of $H$ with $P_S$ results in a steep decline in density of $P_S$ in the upper airways within 24 hr postinoculation (Figure 2B). In contrast, although $P_R$ alone colonized at similar levels to $P_S$ alone in the absence of the competitor, coinoculation with $H$ had no significant effect on its colonization. Switching of the capsule type from $P_{S\rightarrow R}$ was sufficient to eliminate its susceptibility to competition, whereas switching the capsule type from $P_{R\rightarrow S}$ was sufficient to render it sensitive to competition by $H$. These results confirmed that capsule type alone is sufficient to dictate the outcome of a competitive interaction with another species.

### Loss of Pneumococci during Competition Requires Opsonophagocytosis

Next, we explored the host mechanisms selecting for capsule type during interspecies competition. In C57BL/6 mice, systemic depletion of neutrophils is sufficient to eliminate the competitive effect of $H. influenzae$ on type 23F pneumococci.

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**Figure 1.** Manipulation by Resident $H. influenzae$ Increases Vulnerability to Host Invasion by Resistant Pneumococci $P_R$  
(A) Per capita growth rate of rare $P_R$ in a host at $(H, P_S)$ equilibrium (Figure S1), as a function of the strength of H immunomanipulation ($x$) and the cost of $P_H$ capsule ($y$). The growth rate $(dP_R/dt)/P_R$ is positive below the black line; darker shades indicate higher growth rates.  
(B) The equilibrium (long-term trend) occupants of the nasal mucosa as a function of $x$ and $y$. The region supporting nasal establishment by $P_R$ (dark gray shades) is increasing with $x$. Lightest gray: costly capsule and effective immunomanipulation, only $H$ is present. Light gray: costly capsule and weak immunomanipulation, all three strains are present. Darkest gray: cheaper capsule and effective immunomanipulation, $H$ and $P_H$ are present. Results are derived from Equations 1 in the Supplemental Results, with parameters $h = 0.5$, $p = 0.4$, and $a = 0.6$. The exact invasion and equilibrium conditions are detailed in the Supplemental Results.
The effect of capsule type (S and R) in this assay was dependent on the capsule type rather than the presence of P. Provided evidence that the loss of the requirement for neutrophils, complement, and the Mac-1 receptor (CD11b/CD18) mice. Values represent the mean ± standard deviation (SD) for 6–17 animals per condition. *p < 0.05, **p < 0.01, ***p < 0.001. NS denotes nonsignificant.

(P) [Figure 3A] [4]. Additionally, this competitive effect was no longer observed in mice in which complement activity was depleted by prior treatment with cobra venom factor (CoVF). There was also no competitive effect in mice lacking the requirement for neutrophils and complement. In contrast, no killing was detected in strains expressing the virulent type 4 capsule type (P). Thus, the outcome of interspecies competition was dependent on opsonophagocytic clearance.

To test whether capsule type could account for differences in opsonophagocytic clearance, we compared each of the strains in an ex vivo killing assay using elicited murine neutrophils and serum as a source of complement (Figure 3B). Killing in this assay was dependent on the capsule type rather than genetic background. Strains expressing the avirulent type 23F capsule type (P and P) were sensitive to the effects of neutrophils and complement. In contrast, no killing was detected in strains expressing the virulent type 4 capsule type (P and P). Thus, the outcome of interspecies competition requires opsonophagocytosis and correlates with relative resistance to killing by this mechanism. Because capsule type impacts sensitivity to complement deposition and subsequent phagocytosis, it may have a profound effect on the outcome of mucosal competition and, consequently, the selection for more resistant or virulent types [6].

The Effect of H. influenzae on Capsule-Dependent Survival during Experimental Competition

Next, we tested our model by directly competing the isogenic virulent (P) and avirulent (P) pneumococcal strains in the presence of the immunomodulator, H. influenzae (H) (three-strain experiment, Figure 4A). In comparison to controls without H, colonization density was significantly enhanced for P (p < 0.05 and p < 0.01, respectively). The mean competitive index (strain ratio after in vivo competition) was determined for P at a higher (>10⁵ cfu/ml) density, the mean competitive index for P was significantly >1 (p < 0.001, t test compared to null expectation of CI = 1). To test the model prediction that increasing densities of H will change the magnitude of selection on capsule type, we stratified mice by their colonization density (P) or type 4 (black bars, P and P capsules). (Controls P and P shown in A are also shown here to allow for comparison to groups with H). Values represent the mean ± SD for 4–11 animals per condition. *p < 0.05, **p < 0.01, ***p < 0.001. NS denotes nonsignificant.
Microbial Competition Promotes Virulence

Within-Host Ecology and the Evolution of Virulence

The impact of within-host competition on the evolution of virulence has been the subject of a diverse range of models, offering contrasting explanations for either an increase or a decrease in virulence as within-host diversity increases [22]. A key distinction separating our work from nearly all theoretical studies of virulence evolution in multiple infected hosts is that we examine multispecies infections (as opposed to multiple strains differing only in a focal virulence trait [22]). A major consequence of this distinction is that the direction of selection on virulence traits no longer simply depends on the number of coinfecting strains but now depends very much on their identity. In the context of our current study, we show that more invasive—and therefore more virulent—pneumococcal capsule variants can be selected for within the mucosal environment, but only if there is sufficient burden of an immunomaniupulative coinfecting microbe (Figure 4A).

Because different capsule types are antigenically distinct and the immunodominant constituent of the pneumococcal surface, it has been assumed that the diversity of capsular types arose to escape immune pressure in the host [23]. However, only a few capsule types generally account for the overwhelming majority of both carriage and disease isolates, for unknown reasons [11]. There is a metabolic cost to produce large amounts of capsular polysaccharide, and the capsule may obscure surface molecules, such as adhesins, needed to interact with the host [24–26]. We suggest that the diversity of capsule types will in part be explicable by variation among the flora. Thus, in situations in which the competitor (H) is highly prevalent and/or highly immunomaniupulative (high H and/or high x), we anticipate that selection would favor thicker capsule types that are more resistant to clearance by opsonophagocytosis. Thus, our observations provide an explanation for the evolution of potentially costly capsular types more resistant to acute inflammatory responses that promote the virulent behavior of an organism with an otherwise commensal lifestyle.

Our findings provide a theoretical and experimental demonstration that the capsule virulence determinant confers a selective advantage during colonization by allowing for persistence during mucosal inflammation induced by competitive interactions among the flora. This paradigm offers a possible explanation for the disproportionate prevalence of the few pneumococcal types associated with invasive disease [27]. Like the pneumococcus, many opportunist pathogens must become coccal capsule variants can be selected for within the mucosal flora. Thus, in situations in which the competitor (H) is highly prevalent and/or highly immunomaniupulative (high H and/or high x), we anticipate that selection would favor thicker capsule types that are more resistant to clearance by opsonophagocytosis. Thus, our observations provide an explanation for the evolution of potentially costly capsular types more resistant to acute inflammatory responses that promote the virulent behavior of an organism with an otherwise commensal lifestyle.

Experimental Procedures

Theoretical Model

The results in Figure 1 are derived from the following differential equations, tracking the densities of H. influenzae (H), and two strains of S. pneumoniae (sensitive [P] and resistant [P])

\[
dH/dt = H(1 - H - p P - p a P)
\]

\[
dP/dt = P(1 - h x H - P - a P)
\]

\[
dP/dt = P(1 - h y H - y P - P)
\]

(Equations 1).

For detailed presentation and analysis, see Supplemental Results. The parameters p, a, h, x, and y capture the competitive interactions among the three strains. p is the competitive impact of P on H, and h is the impact of H on P in the absence of immunomaniupulation (p = h = 1 would imply competitive equivalence; we assume p < 1, h < 1, ensuring coexistence between H and P when x = 1). x captures the additional impact of H on P because of immunomaniupulation (x = 1 would imply no immunomaniupulation; we assume x > 1). y and a capture the competitive abilities of P relative to P. We assume y > 1 (P is more sensitive to competition from the other strains) and a < 1 (P has an attenuated competitive impact on the other strains).
Mouse Strains and Model of Nasal Colonization

Six- to eight-week-old mice used in the study were housed in accordance with Institutional Animal Care and Use Committee protocols. Mouse strains used for this study included C57BL/6 (WT) and B6.129S4-Itgamtm1Myl/J (Mac-1 [CD11b/CD18]-deficient mice, Jackson Laboratories), with a targeted mutation in the gene for integrin alpha M or complement receptor type 3 [28]. Neutrophils from these animals are deficient at phagocytosing complement-opsonized particles and in several Fc-mediated functions.

H. influenzae phase 100 μl. Neomycin, 20 μg/ml, or erythromycin, 1 μg/ml, was used to select for S. pneumoniae either P1121 and its derivatives or TIGR4 and its derivatives, respectively.

Statistical Analysis

For single-factor analyses, statistical comparisons of colonization between groups were made using either the Kruskal-Wallis test with Dunn’s post test or t tests, as appropriate.

For statistical comparisons of two or more variables simultaneously, two-way analysis of variance (ANOVA) was used to determine whether the interaction between these variables was significant. If the interaction between variables was significant by two-way ANOVA, Bonferroni post tests were used to determine each variable’s effect on colonization outcome. If there was no statistically significant interaction between variables, colonization outcomes were compared using one-factor analyses, as described above. For comparisons of competitive indices (calculated as the ratio of strains in nasal lavages compared to the ratio of strains in the inoculum), log-transformed values were analyzed using ratio t tests.

Supplemental Information

Supplemental Information includes Supplemental Data, Supplemental Experimental Procedures, one figure, and one table and can be found with this article online at doi:10.1016/j.cub.2010.05.051.