

Within-Host Evolution for the Invasiveness of Commensal Bacteria: an Experimental Study of Bacteremias Resulting from *Haemophilus influenzae* Nasal Carriage

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Background. Many bacteria responsible for clinically relevant disease reside harmlessly in a large fraction of humans. Three explanations have been proposed to account for why these normally commensal bacteria occasionally cause invasive disease: host susceptibility, stochasticity in the host-bacteria interaction, and the evolution of invasive mutants in colonized hosts. Here we test the third of these hypotheses for the rare invasiveness of commensal bacteria: within-host evolution.

Methods and Results. Using neonatal rats intranasally colonized with pairs of marked *Haemophilus influenzae* type b strains, we demonstrate that the resulting bacteremias are derived from single organisms. To test the within-host evolution hypothesis we explored the relative ability of bacteria isolated from the blood and nasal passages of bacteremic rats to colonize the nasopharynx and invade the bloodstream.

Conclusions. Our results provide support for within-host evolution as one but not the sole explanation for the invasiveness of these bacteria. We discuss the implications of these results for both the rare invasiveness of commensal bacteria and the general observation that bacteria isolated from the sites of human invasive disease are almost invariably monoclonal.

A number of bacteria (e.g., *Staphylococcus aureus*, *Escherichia coli*, *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*) colonize and persist for extended periods of time in substantial fractions of the human population without causing symptomatic disease and thereby are considered commensals. However, these same species of bacteria are responsible for significant morbidity and mortality due to the occasional invasive infection [1–3]. Although there is genetic variability in these species and some variants are more likely to cause invasive disease than others, even for the most virulent strains colonization rarely results

in disease [3–7]. How do these bacteria, which are maintained and transmitted from sites where they are harmless, pass through the host defenses and proliferate in sites where they cause symptoms but are unlikely to be transmitted? And why do they do so in only a small minority of colonized hosts?

There are 3 not-mutually-exclusive answers to these questions. First is host susceptibility; at any given time the immune defenses of a small fraction of hosts are not sufficient to prevent colonizing bacteria from invading. This variation in susceptibility can be physiological—due to age, concomitant infections (e.g., viral infections [8, 9]), or chronic conditions (e.g., diabetes mellitus [10])—or genetic (e.g., immune deficiencies [11–13]). The second explanation is stochasticity; as a consequence of chance alone, even in hosts with perfectly functional defenses there would be a low probability that bacteria enter and replicate in sites where they cause disease. The third explanation is within-host evolution [14, 15]; the colonizing population of bacteria may include members that are selected within colonized hosts because they have or acquire heritable modifi-

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cations that enable them to invade and replicate in new sites.

Although the first explanation is widely accepted [16], the latter 2 appear to have been given less consideration, despite evidence that can be interpreted in their support. Natural populations of the commensal bacteria responsible for rare invasive disease are genetically very diverse [17–20], and at any given time humans are commonly colonized with multiple types [21, 22]. Nevertheless, the bacteria isolated from sites of invasive disease in individual patients are almost invariably monoclonal, and these invasive clones differ among patients [23]. Furthermore, in experiments in which bacteremias occur in rodents that are colonized with pairs of marked strains of bacteria, only one or the other marked strain can be isolated from the blood [24–28]. These 2 observations suggest that the low frequency of invasive disease is due to rare events occurring in the bacterial population. Here, we ascertain whether these rare events are due to random chance encounters between the colonizing bacteria and holes in the host defenses or to the generation and ascent of invasive mutants in the colonizing population.

We used an *H. influenzae*–neonatal rat model similar to that developed by Richard Moxon and colleagues [26, 29] and demonstrate, through statistical analysis, that single organisms are responsible for founding the bacteremias that occur from nasal colonization. We test whether bacteria isolated from the blood of these bacteremic rats were more likely than those from nasal-wash isolates to establish populations in the blood of new rats. We interpret our results as evidence that within-host evolution is one but not the unique reason for the rare invasiveness of commensal bacteria and the monoclonality of invasive disease. We discuss the limitations of these findings and their implications for understanding the mechanisms responsible for invasive disease.

MATERIALS AND METHODS

Microorganism. *H. influenzae* Eagan strain and its spontaneous streptomycin-resistant mutant Rm154 (Str-R) were provided by R. Moxon (Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, UK). Em6 was selected as a spontaneous nalidixic acid-resistant mutant (Nal-R) of Eagan.

Inocula preparation and storage. Inocula for all of the infant rat experiments were prepared by resuspending a frozen aliquot of a midexponential-phase culture in 250-mL flasks that contained 50 mL of brain-heart infusion (BHI) broth supplemented with 10 μ g of hemin and 2 μ g of β NAD/mL. Shaking cultures that reached the midexponential phase were centrifuged (at 5000 *g* for 3 min) and resuspended in PBS with 0.1% gelatin (PBSG).

Bacteremia studies. All experiments were performed under the guidelines of and approved by the Emory University Institutional Animal Care and Use Committee. Two-day-old pups, born to timed-pregnant Sprague-Dawley rats (Charles

River Laboratories), were pooled and randomly reassigned to dams. Groups of 30 five-day-old rats were intranasally inoculated by touching a 10- μ L drop of 10^7 or 10^8 bacteria of a mixture of equal densities of Str-r and Nal-r to the external nares [29, 30]. These doses were higher than that used in previous studies, because this hanging-drop method resulted in more initial loss of inoculum than did the scalp-vein needle method used by Moxon and Murphy [26]. Two days after the infection, 0.5 mL of blood was collected by cardiac puncture; nasal-wash samples were collected from 200 μ L of PBSG instilled into a 5-cm Intramedic polyethylene tubing (PE50) placed into the thorax; and nasal epithelium was scraped from the nasal passages after a second wash with 200 μ L of PBSG and removal of the frontal bones. The nasal epithelium, which has been suggested to contain a distinct, potentially invasive population [31], was homogenized in 1 mL of PBSG. Then, 100 μ L of the blood, nasal-wash, and nasal epithelium samples were plated directly and serially diluted onto BHI agar plates with bacitracin (0.3 g/L) with either streptomycin (4 mg/L) or nalidixic acid (5 mg/L). Plates were incubated overnight at 37°C with 5% CO₂. The limit for detection at any site was 10 cfu/mL. The density of bacteria in bacteremic rats ranged from 500 to 4×10^5 cfu/mL. Bacteremias were considered to be of only one type if at least 50 colonies were observed on one of the antibiotic plates (Nal or Str) but no colonies on the other.

Rare invasion model. Details of the model and the statistical analysis can be found in the Appendix.

Invasiveness studies. For the experiments testing the within-host evolution hypothesis, single colonies were picked at random from antibiotic-containing plates of blood and nasal-wash samples from rats that were bacteremic 48 h after nasal inoculation. These colonies were grown to the midexponential phase in 2 mL of BHI, after which 200 μ L of glycerol was added, and they were frozen at -80°C as 100- μ L aliquots. In each of 3 independent replicates, 10–20 neonatal rats received intranasal inoculation (prepared as indicated above) of mixtures of blood and nasal isolates; invasiveness was determined from blood samples of these rats at 48 h.

The following statistical procedure was used to determine whether the blood isolates were significantly more invasive than the ancestral strains. The invasiveness of the ancestral strains (Em6 and Rm154) was determined from the relationship between the density of a mixed inoculum (10^5 – 10^9 cfu) and the frequency of bacteremias where [32]

$$\text{Fraction with bacteremia} = \frac{e^{-27.84 + 3.52 \log(\text{dose})}}{1 + e^{-27.84 + 3.52 \log(\text{dose})}} .$$

From this function we calculated the anticipated fraction of

invasions for the ancestral strain with the dose set for that used in each replicate, the null hypothesis. We then compared (using the χ^2 test) the observed number of bacteremias for each of the 6 blood isolates in each replicate to that anticipated from this null model. Each replicate of the same pair of blood and nasal isolates were analyzed separately because they represented different inoculum densities given their independent culturing from frozen aliquots. The frequency of bacteremias should be no different from that observed with the ancestral strains if the blood and nasal isolates were equally likely to invade. If the blood isolates were inherently more likely to invade, then the fraction of rats with bacteremias would differ from the null.

Relative fitness. Relative fitness of the ancestral strains, nasal isolates, and blood isolates was measured in 3 environments: in vitro, nasal passage, and bloodstream. In vitro fitness was determined by sampling initial and final densities after 8 h of growth from mixed (50:50) liquid cultures. Nasal passage fitness was determined by inoculating sets of 10–25 five-day-old rats with 50:50 inoculum; 48 h later we sampled nasal-wash and epithelium samples as described for bacteremia studies. Because there were no venous access points without surgical manipulation in rats of this age, intraperitoneal inoculation of ~100 bacteria suspended in 100 μ L of PBSG into a set of 3 neonatal rats and subsequent 48-h blood cultures were used to determine the relative fitness in the blood. For relative fitness, we used the selection rate r_{sn} , which is a measure of the relative recovery of the Nal-r and Str-r bacteria inoculated into the nasal passages over the 48 h and is given by

$$r_{sn} = \ln \frac{N_s(48)}{N_s(0)} - \ln \frac{N_n(48)}{N_n(0)},$$

where $N_s(0)$ and $N_n(0)$ are the initial densities in the inoculum of Str-r and Nal-r, respectively, and $N_s(48)$ and $N_n(48)$ are the densities detected at the specific site after 48 h [33]. A selection rate of 0 indicates no fitness difference between the strains and a positive rate indicates an advantage for the Str-r strain.

RESULTS

Bacteremias derived from single organism. Rats were inoculated into the nasal passage with a mixture of 10^7 Nal-r and 10^7 Str-r *H. influenzae* type b Eagan strain. The design of this experiment is illustrated in figure 1A. Of 29 rats with mixed nasal colonizations, 5 were bacteremic at 48 h; of these, 3 yielded only Nal-r colonies and 2 yielded only Str-r colonies (figure 1B). With a 10-fold increase in the initial inoculum, 14 of 30 rats were bacteremic; of these, 4 bacteremias were due to purely Str-r organisms, 6 were due to purely Nal-r organisms, and 4 were due to mixed infections.

We compared these results with the predictions of a statistical model that assumes that bacteria enter and establish a popu-

lation in the blood (invade) at random (table 1). In this model (detailed in the appendix), bacteremias with both Nal-r and Str-r bacteria are generated in 2 ways: by bacteria of both types establishing a population in the blood in a single invasion event or bacteria establishing populations separately in multiple invasion events. The expected number of independent invasions of the bloodstream in each rat for the 10^7 and 10^8 inoculation doses was calculated using Poisson distribution. The assumption in this model that both strains were equally likely to invade was confirmed, because both strains had similar invasion rates (data not shown), and we failed to detect differences in the in vivo fitness between the strains (table 2). By using binomial distribution, we determined for a certain number of bacteria invading in each invasion the expected number of blood infections caused by a single type of bacteria (Nal-r or Str-r) and the expected number caused by both types (Nal-r and Str-r). In table 1, we compared this expected number of rats with a

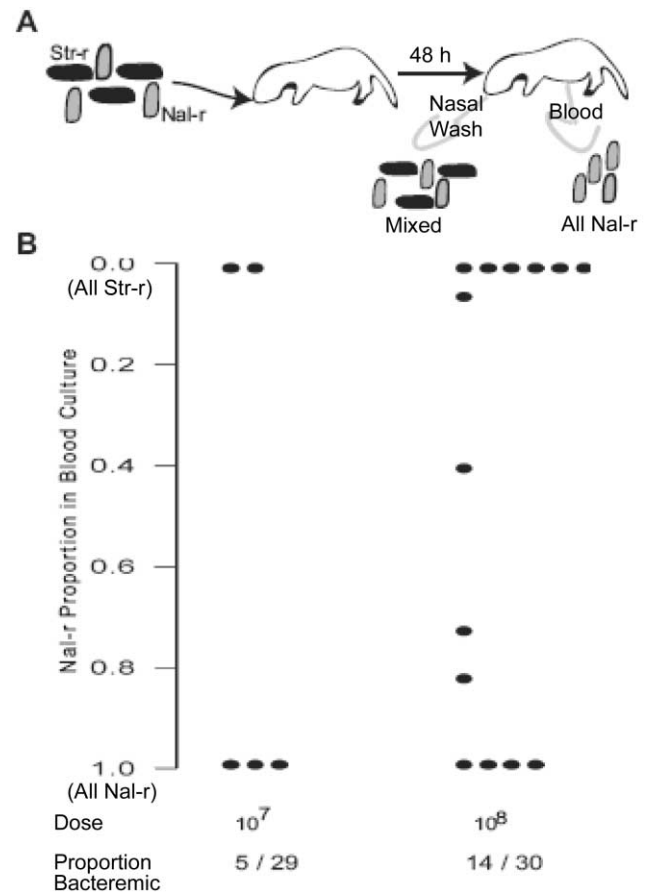


Figure 1. Mixed-culture colonization experiments. A, Experimental design. Equal densities of nalidixic acid-resistant mutant (Nal-r) and streptomycin-resistant mutant (Str-r) *Haemophilus influenzae* bacteria were inoculated into nasal passages of 5-day-old rats. Then, 48 h later, nasal washes and blood were sampled. B, Results. The Nal-r bacteria fraction in positive blood cultures for 2 different inoculation densities (10^7 and 10^8) is shown.

Table 1. Comparison of the no. of blood cultures with 1 or 2 types of bacteria observed and expected from a statistical no.

Bacteria	10 ⁸ dose (14 bacteremic rats)			10 ⁷ dose (5 bacteremic rats)		
	Rats with 1 type	Rats with 2 types	<i>P</i>	Rats with 1 type	Rats with 2 types	<i>P</i>
Observed	10	4		5	0	
Expected from the statistical model if the no. of bacteria is						
1 bacterium	11.82	2.18	.33	4.76	0.24	.52
2 bacteria	5.45	8.55	.006	2.33	2.67	.014
3 bacteria	2.62	11.38	>.001	1.15	3.85	>.001
4 bacteria	1.28	12.72	>.001	0.57	4.43	>.001

NOTE. Rats with 1 type have either nalidixic acid-resistant mutant or streptomycin-resistant mutant bacteria in their blood cultures. *P* values where the model does not significantly differ from observed values are in bold type.

bacteremia of a single strain for different numbers of bacteria establishing blood populations to the observed numbers of bacteremias (with 10⁷ and 10⁸ inoculation doses). For both the 10⁷ and 10⁸ inoculation doses, the most probable number of bacteria establishing blood infections is 1.

Evidence for within-host evolution. To test whether the bacteremias were caused by a mutant in the bacterial population with a greater propensity to invade the blood, we isolated single colonies of bacteria (3 Nal-r and 3 Str-r) from the blood and nasal-wash samples of 6 separate bacteremic rats. These isolates were grown to the midexponential phase and stored in aliquots at -80°C. Each of the blood isolates was paired with nasal isolates with the alternative marker (from the same bacteremic rat) and introduced in equal frequency into the nasal passages of a new set of rats. The design of this experiment is illustrated in figure 2A.

Five of the 6 blood isolates provided no evidence for an increase or decrease in the likelihood of blood invasion relative to the nasal isolates (figure 2C). However, 1 blood isolate (Em091) showed significant increases in relative invasiveness in 3 independent trials (*P* > .001, *P* > .031, and *P* > .004, calculated from a comparison with the ancestor's invasiveness for that inoculation density) (figure 2B).

To determine whether the apparent increased invasiveness of Em091 was due to a competitive advantage in either the nasal passage or blood, we estimated its fitness relative to a nasal isolate in these sites. As can be seen in table 2, there was no evidence for an increase (or decrease) in Em091's fitness in the nasal wash or epithelium after nasal inoculation or in the blood after intraperitoneal inoculation. This was also the case for the 5 blood-isolated clones, for which we detected no enhanced ability to invade the blood (data not shown). We were unable to detect a trade-off between invasiveness and colonization in the strains tested.

DISCUSSION

We interpret the results of these experiments as evidence that the rare invasiveness of commensal bacteria and the monoclinality of the invasive populations can be attributed to rare events in the colonizing bacteria, either by the random success of one bacterium in establishing an invasive population or a random mutation in the colonizing population producing an invasive clone.

The results of our experiments are consistent with those of previous rodent model studies with *H. influenzae* as well as

Table 2. Selection rate of ancestral strains and the evolved blood isolate (Em091).

Strain (Str-r/Nal-r)	Nasal wash (<i>n</i> = 25)	Nasal epithelium (<i>n</i> = 25)	Blood (<i>n</i> = 6)	In vitro (<i>n</i> = 6)
Rm154/Em6 (ancestral)	-0.199 ± 0.159	-0.087 ± 0.205	0.070 ± 0.615	-0.151 ± 0.262
Em091/Em092 (blood/nasal)	0.129 ± 0.189	0.256 ± 0.190	0.069 ± 0.599	

NOTE. The selection rate is a measure of fitness calculated from

$$r_{sm} = \ln \frac{N_s(48)}{N_s(0)} - \ln \frac{N_m(48)}{N_m(0)},$$

where $N_s(t)$ is the density of the streptomycin-resistant mutant (Str-r) strain at t h and $N_m(t)$ is the density of the nalidixic acid-resistant mutant (Nal-r) strain. A selection rate of 0 indicates no difference in the Str-r and Nal-r strain fitness. Data are mean ± SE. The blood isolate is in bold type.

ulations. This would have been the case even if all rather than just 1 of the 6 blood isolates that we tested were inherently more invasive. That we were unable to detect heritable increases in invasiveness for 5 of the 6 clones points to the importance of stochastic processes other than mutation in the bacteria as responsible for the rare invasiveness of commensal bacteria and the monoclonality of the invasive populations.

We do not interpret the results of these experiments as evidence against the conventional wisdom that the rare invasiveness of commensal bacteria can be attributed to variation in host susceptibility [16]. There is good evidence for inherited variation in human susceptibility to infectious disease [43, 44] and a plethora of noninherited mechanisms, including age and underlying disease [45, 46], that could account for how colonizing populations of bacteria become invasive. Although host variation may play a prominent role in whether colonizing bacteria will or will not lead to invasive disease, in itself variation in host susceptibility cannot account for the monoclonality of invasive bacterial populations. It seems unlikely that, when confronted with millions of bacteria, only a single bacterium would pass through the hole(s) in the defenses of a host with physiologically or genetically compromised immune defenses. The invasion process is likely to involve a number of steps: the bacteria must adhere to and pass through the epithelium and replicate within the bloodstream, all the while avoiding opsonization and evading the host's other immune responses. It is easy to imagine why at each of these successive steps there are fewer and fewer bacteria; this may well explain why bacteria normally do not succeed in establishing populations in the blood. But if the invasion of the blood is due to an even transitory defect in one of these barriers, it is hard to explain why many bacteria would not exploit this defect.

That is not to say that the monoclonality of infections excludes host susceptibility from playing a prominent role in the rare invasiveness of commensal bacteria. The monoclonality in the invasive population of bacteria could be due to secondary processes, such as selective or random purging of the variation from the invading population. Another possibility for this secondary process is a host response to the invasion. For example, it has been proposed that inflammation induced during the invasion of one clone may preclude the invasion of others [47].

We set out to test the hypothesis that the rare virulence of commensal bacteria can be attributed to selection for mutants within the colonizing populations that are capable of invading sites where they cause disease: within-host evolution. Although the results of these experiments provide some evidence in support of this hypothesis, they also point to the importance of alternative mechanisms. Most important, the results of our experiments indicate that whatever mechanisms are responsible

for commensal bacteria becoming invasive, they must account for the monoclonality of the invasive population.

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APPENDIX

STATISTICAL MODEL FOR NUMBER OF INVADING BACTERIA

To statistically evaluate the number of bacteria involved in establishing blood populations, we constructed a model that assumes that bacteria enter and establish a population in the blood—invalidate—at random. In the first part of our analysis, we calculated the expected number of independent invasions of the bloodstream in the same rat for the 10^7 and 10^8 inoculation doses. The number of independent invasion events, k , can be calculated from the observed fraction of infections that did not yield bacteremias and from that anticipated from a Poisson distribution. Let λ be the expected number of bacteremias in a group of rats inoculated with a given dose of bacteria placed in the nasal passages. In accord with Poisson distribution, the probability of not having bacteremia is $\lambda = -\ln[P(k=0)]$ or $P(k=0) = e^{-\lambda}$. With the 10^7 inoculum, our estimate is $P(k=0) = \frac{24}{29}$ or $\lambda = -\ln(\frac{24}{29}) = 0.19$, and with the 10^8 inoculum, the estimate is $\lambda = 0.63$.

From these estimates of λ and the full Poisson distribution, we can calculate the distribution of rats with different numbers of invasion events ($k = 0, 1, 2$):

$$P(k; \lambda) = e^{-\lambda} \frac{\lambda^k}{k!}.$$

From a binomial distribution, we then calculate the expected number of blood infections caused by a single strain (Nal-r or Str-r) and the expected number caused by both strains (Nal-r and Str-r) for different numbers of bacteria invading in each event. As in the case of heads and tails in a coin toss, we assume that the Nal-r and Str-r strains are equally likely to invade. The parameter w is the number of bacteria that are responsible for establishing the blood population in each invasion event, equivalent to the number of tosses of the coin. From the binomial

distribution, the probability of either all Nal-r or all Str-r (all heads or all tails) in the blood is then

$$P(\text{all Nal-r or all Str-r}; k) = \frac{1}{2} + \frac{1}{2} wkwk .$$

We then can determine the expected number of rats with bacteremia caused by a single (Nal-r or Str-r) strain or both (Nal-r and Str-r) strains for different numbers of bacteria establishing blood populations by multiplying the probability of having a bacteremia due to k invasion events by the probability that k events are all of one strain.

References

- Bogaert D, De Groot R, Hermans PW. *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. *Lancet Infect Dis* **2004**; 4: 144–54.
- Schuchat A, Robinson K, Wenger JD, et al. Bacterial meningitis in the United States in 1995. Active Surveillance Team. *N Engl J Med* **1997**; 337:970–6.
- Robinson DA, Edwards KM, Waites KB, Briles DE, Crain MJ, Hollingshead SK. Clones of *Streptococcus pneumoniae* isolated from nasopharyngeal carriage and invasive disease in young children in central Tennessee. *J Infect Dis* **2001**; 183:1501–7.
- Townsend R, Goodwin L, Stevanin TM, et al. Invasion by *Neisseria meningitidis* varies widely between clones and among nasopharyngeal mucosae derived from adult human hosts. *Microbiology* **2002**; 148: 1467–74.
- Brueggemann AB, Griffiths DT, Meats E, Peto T, Crook DW, Spratt BG. Clonal relationships between invasive and carriage *Streptococcus pneumoniae* and serotype- and clone-specific differences in invasive disease potential. *J Infect Dis* **2003**; 187:1424–32.
- Hausdorff WP, Feikin DR, Klugman KP. Epidemiological differences among pneumococcal serotypes. *Lancet Infect Dis* **2005**; 5:83–93.
- Sleman KL, Griffiths D, Shackley F, et al. Capsular serotype-specific attack rates and duration of carriage of *Streptococcus pneumoniae* in a population of children. *J Infect Dis* **2006**; 194:682–8.
- Okamoto S, Kawabata S, Nakagawa I, et al. Influenza A virus-infected hosts boost an invasive type of *Streptococcus pyogenes* infection in mice. *J Virol* **2003**; 77:4104–12.
- McCullers JA, Rehg JE. Lethal synergism between influenza virus and *Streptococcus pneumoniae*: characterization of a mouse model and the role of platelet-activating factor receptor. *J Infect Dis* **2002**; 186:341–50.
- Muller LMAJ, Gorter KJ, Hak E, et al. Increased risk of common infections in patients with type 1 and type 2 diabetes mellitus. *Clin Infect Dis* **2005**; 41:281–8.
- Figueroa JE, Densen P. Infectious diseases associated with complement deficiencies. *Clin Microbiol Rev* **1991**; 4:359–95.
- Emonts M, Hazelzet JA, de Groot R, Hermans PW. Host genetic determinants of *Neisseria meningitidis* infections. *Lancet Infect Dis* **2003**; 3:565–77.
- Hibberd ML, Sumiya M, Summerfield JA, Booy R, Levin M. Association of variants of the gene for mannose-binding lectin with susceptibility to meningococcal disease. *Lancet* **1999**; 353:1049–53.
- Levin BR, Bull JJ. Short-sighted evolution and the virulence of pathogenic microorganisms. *Trends Microbiol* **1994**; 2:76–81.
- Meyers LA, Levin BR, Richardson AR, Stojiljkovic I. Epidemiology, hypermutation, within-host evolution and the virulence of *Neisseria meningitidis*. *Proc Biol Sci* **2003**; 270:1667–77.
- Casanova J-L, Abel L. Inborn errors of immunity to infection: the rule rather than the exception. *J Exp Med* **2005**; 202:197–201.
- Musser JM, Kroll JS, Granoff DM, et al. Global genetic structure and molecular epidemiology of encapsulated *Haemophilus influenzae*. *Rev Infect Dis* **1990**; 12:75–111.
- Maiden MC, Bygraves JA, Feil E, et al. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci USA* **1998**; 95: 3140–5.
- Fitzgerald JR, Sturdevant DE, Mackie SM, Gill SR, Musser JM. Evolutionary genomics of *Staphylococcus aureus*: insights into the origin of methicillin-resistant strains and the toxic shock syndrome epidemic. *Proc Natl Acad Sci USA* **2001**; 98:8821–6.
- Silva NA, McCluskey J, Jefferies JM, et al. Genomic diversity between strains of the same serotype and multilocus sequence type among pneumococcal clinical isolates. *Infect Immun* **2006**; 74:3513–8.
- Cespedes C, Said-Salim B, Miller M, et al. The clonality of *Staphylococcus aureus* nasal carriage. *J Infect Dis* **2005**; 191:444–52.
- St Sauver J, Marrs CF, Foxman B, Somsel P, Madera R, Gilsdorf JR. Risk factors for otitis media and carriage of multiple strains of *Haemophilus influenzae* and *Streptococcus pneumoniae*. *Emerg Infect Dis* **2000**; 6:622–30.
- Sandgren A, Sjöström K, Olsson-Liljequist B, et al. Effect of clonal and serotype-specific properties on the invasive capacity of *Streptococcus pneumoniae*. *J Infect Dis* **2004**; 189:785–96.
- Meynell GG, Stocker BA. Some hypotheses on the aetiology of fatal infections in partially resistant hosts and their application to mice challenged with *Salmonella paratyphi-B* or *Salmonella typhimurium* by intraperitoneal injection. *J Gen Microbiol* **1957**; 16:38–88.
- Meynell GG. The applicability of the hypothesis of independent action to fatal infections in mice given *Salmonella typhimurium* by mouth. *J Gen Microbiol* **1957**; 16:396–404.
- Moxon ER, Murphy PA. *Haemophilus influenzae* bacteremia and meningitis resulting from survival of a single organism. *Proc Natl Acad Sci USA* **1978**; 75:1534–6.
- Pluschke G, Mercer A, Kusecek B, Pohl A, Achtman M. Induction of bacteremia in newborn rats by *Escherichia coli* K1 is correlated with only certain O (lipopolysaccharide) antigen types. *Infect Immun* **1983**; 39:599–608.
- Rubin LG. Bacterial colonization and infection resulting from multiplication of a single organism. *Rev Infect Dis* **1987**; 9:488–93.
- Moxon ER, Smith AL, Averill DR, Smith DH. *Haemophilus influenzae* meningitis in infant rats after intranasal inoculation. *J Infect Dis* **1974**; 129:154–62.
- Moxon ER, Vaughn KA. The type b capsular polysaccharide as a virulence determinant of *Haemophilus influenzae*: studies using clinical isolates and laboratory transformants. *J Infect Dis* **1981**; 143:517–24.
- Briles DE, Novak L, Hotomi M, van Ginkel FW, King J. Nasal colonization with *Streptococcus pneumoniae* includes subpopulations of surface and invasive pneumococci. *Infect Immun* **2005**; 73:6945–51.
- Peto S. A dose-response equation for the invasion of micro-organisms. *Biometrics* **1953**; 9:320–35.
- Travisano M, Lenski RE. Long-term experimental evolution in *Escherichia coli*. IV. Targets of selection and the specificity of adaptation. *Genetics* **1996**; 143:15–26.
- Nilsson AI, Kugelberg E, Berg OG, Andersson DI. Experimental adaptation of *Salmonella typhimurium* to mice. *Genetics* **2004**; 168: 1119–30.
- Zelle MR. Genetic constitutions of host and pathogen in mouse typhoid. *J Infect Dis* **1942**; 71:131–52.
- Smith EE, Buckley DG, Wu Z, et al. From the cover: genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc Natl Acad Sci USA* **2006**; 103:8487–92.
- Israel DA, Salama N, Krishna U, et al. *Helicobacter pylori* genetic diversity within the gastric niche of a single human host. *Proc Natl Acad Sci USA* **2001**; 98:14625–30.
- Gay RT, Belisle S, Beck MA, Meydani SN. An aged host promotes the evolution of avirulent coxsackievirus into a virulent strain. *Proc Natl Acad Sci USA* **2006**; 103:13825–30.
- Sokurenko EV, Hasty DL, Dykhuizen DE. Pathoadaptive mutations:

- gene loss and variation in bacterial pathogens. *Trends Microbiol* **1999**; 7:191–5.
40. Engleberg NC, Heath A, Miller A, Rivera C, DiRita VJ. Spontaneous mutations in the CsrRS two-component regulatory system of *Streptococcus pyogenes* result in enhanced virulence in a murine model of skin and soft tissue infection. *J Infect Dis* **2001**; 183:1043–54.
41. Moxon R, Bayliss C, Hood D. Bacterial contingency loci: the role of simple sequence DNA repeats in bacterial adaptation. *Annu Rev Genet* **2006**; 40:307–33.
42. Corn PG, Anders J, Takala AK, Kayhty H, Hoiseth SK. Genes involved in *Haemophilus influenzae* type b capsule expression are frequently amplified. *J Infect Dis* **1993**; 167:356–64.
43. Sorensen TI, Nielsen GG, Andersen PK, Teasdale TW. Genetic and environmental influences on premature death in adult adoptees. *N Engl J Med* **1988**; 318:727–32.
44. Segal S, Hill AVS. Genetic susceptibility to infectious disease. *Trends Microbiol* **2003**; 11:445–8.
45. Sims RV, Boyko EJ, Maislin G, Lipsky BA, Schwartz JS. The role of age in susceptibility to pneumococcal infections. *Age Ageing* **1992**; 21: 357–61.
46. Schoenmakers MCJ, Hament JM, Fleer A, et al. Risk factors for invasive pneumococcal disease. *Rev Med Microbiol* **2002**; 13:29–36.
47. Pfeiffer JK, Kirkegaard K. Bottleneck-mediated quasispecies restriction during spread of an RNA virus from inoculation site to brain. *Proc Natl Acad Sci USA* **2006**; 103:5520–5.