

# Resident Microbiota Affect *Bordetella pertussis* Infectious Dose and Host Specificity

Laura S. Weyrich,<sup>1,2,a</sup> Heather A. Feaga,<sup>1,2</sup> Jihye Park,<sup>1,3</sup> Sarah J. Muse,<sup>1,2</sup> Chetan Y. Safi,<sup>1</sup> Olivier Y. Rolin,<sup>1,4</sup> Sarah E. Young,<sup>1</sup> and Eric T. Harvill<sup>1</sup>

<sup>1</sup>Department of Veterinary and Biomedical Sciences, <sup>2</sup>Graduate Program in Biochemistry, Microbiology, and Molecular Biology, <sup>3</sup>Graduate Program in Bioinformatics and Genomics, and <sup>4</sup>Graduate Program in Immunology and Infectious Disease, The Pennsylvania State University, State College

Before contacting host tissues, invading pathogens directly or indirectly interact with host microbiota, but the effects of such interactions on the initial stages of infection are poorly understood. *Bordetella pertussis* is highly infectious among humans but requires large doses to colonize rodents, unlike a closely related zoonotic pathogen, *Bordetella bronchiseptica*, raising important questions about the contributions of bacterial competition to initial colonization and host selection. We observed that <100 colony-forming units (CFU) of *B. bronchiseptica* efficiently infected mice and displaced culturable host microbiota, whereas 10 000 CFU of *B. pertussis* were required to colonize murine nasal cavities and did not displace host microorganisms. Bacteria isolated from murine nasal cavities but not those from the human lower respiratory tract limited *B. pertussis* growth in vitro, indicating that interspecies competition may limit *B. pertussis* colonization of mice. Further, a broad-spectrum antibiotic treatment delivered before *B. pertussis* inoculation reduced the infectious dose to <100 CFU, and re-introduction of single *Staphylococcus* or *Klebsiella* species was sufficient to inhibit *B. pertussis* colonization of antibiotic-treated mice. Together, these results reveal that resident microorganisms can prevent *B. pertussis* colonization and influence host specificity, and they provide rationale for manipulating microbiomes to create more-accurate animal models of infectious diseases.

**Keywords.** microbiome; *Bordetella*; antibiotics; whooping cough; pathogen evolution; host adaptation; interspecies competition; bacterial competition.

*Bordetella pertussis* is the major causative agent of whooping cough, which continues to persist in both highly vaccinated and unvaccinated populations worldwide. The World Health Organization estimates that 20–40 million whooping cough cases occur globally per year, resulting in >400 000 deaths each year [1, 2]. Although *B. pertussis* vaccines have been highly effective [3], the annual incidence of *B. pertussis* has recently increased even in highly vaccinated populations [4]. In

the United States, >25 000 cases have been reported every year since 2005 [5, 6]. However, efforts to better control and eliminate whooping cough have been hindered by the lack of experimental models to investigate how *B. pertussis* initially colonizes individuals and efficiently transmits between susceptible hosts.

*B. pertussis* is a member of the classical bordetellae, a group of closely related respiratory pathogens that also includes the widespread zoonotic pathogen *Bordetella bronchiseptica*. Although *B. pertussis* appears to be host restricted to humans, *B. bronchiseptica* has a very diverse host range [7] and can cause a wide range of diseases, from asymptomatic colonization to lethal pneumonia [8]. Interestingly, *B. pertussis* appears to have evolved from a *B. bronchiseptica*-like progenitor strain through genome rearrangements and degradation [9, 10]. In a murine infection model, both species colonize the lower respiratory tract when administered in a high-dose, high-volume regimen of  $5 \times 10^5$  colony-forming units (CFU) in 50  $\mu$ L [11–13]. However, even

Received 14 May 2013; accepted 23 September 2013; electronically published 13 November 2013.

<sup>a</sup>University of Adelaide, Australia (L. S. W.).

Presented in part: 15th International Congress of Mucosal Immunology, Paris, France, 5–9 July 2011.

Correspondence: Eric T. Harvill, PhD, Department of Veterinary and Biomedical Sciences, The Pennsylvania State University, W-213A Millennium Sciences Complex, University Park, PA 16802 (harvill@psu.edu).

The Journal of Infectious Diseases 2014;209:913–21

© The Author 2013. Published by Oxford University Press on behalf of the Infectious Diseases Society of America. All rights reserved. For Permissions, please e-mail: journals.permissions@oup.com.

DOI: 10.1093/infdis/jit597

though these closely related pathogens share about 98.6% sequence similarity among orthologous genes (J. Park and E. T. Harvill, personal communication) and have been referred to as subspecies, their median infectious doses (ID<sub>50</sub>) in mice differ significantly, from <5 CFU of *B. bronchiseptica* to >10 000 CFU of *B. pertussis* [7]. Additionally, *B. bronchiseptica* remains in the murine upper respiratory tract for the life of the animal [11, 14], while even high doses of *B. pertussis* are cleared from the nasal cavity within 1 month after inoculation [11, 15, 16].

In this study, we initially aimed to understand why *B. pertussis* fails to colonize the murine nasal cavity as effectively as *B. bronchiseptica*. Because both *B. pertussis* and *B. bronchiseptica* have been shown to adhere to murine epithelial cell lines in vitro and both species can colonize the nasal cavity at high levels following high-dose inoculation, we considered that differences in colonization were likely not exclusive to effective adherence to nasal cavity tissue [17–21]. Next, we considered that distinct interactions with the host immune system could affect initial colonization. However, both species have been observed to effectively colonize a similar array of immunodeficient mice, suggesting that differential immune responses may not mediate these differences [12, 13, 22–24]. Because the nasal cavity is known to harbor a complex community of microbiota, we also considered the possibility that interactions with resident microbes could contribute to these differences in colonization. Both immune-mediated and direct mechanisms of competition can impact pathogen success within a host [25–28]. Recent studies have indicated that *Bordetella* species contain several mechanisms for interbacterial competition, including bacteriophages, a type VI secretion system, and, potentially, contact-dependent growth inhibition (CDI) [29–31]. On the basis of these observations, we hypothesized that the ability to compete with resident host organisms may mediate the ability of pathogens to successfully colonize a host. Here, we demonstrate that removing resident microorganisms from the nasal cavity allows *B. pertussis* to efficiently colonize the murine nasal cavity and that reintroduction of a single nasal cavity bacterial species was sufficient to block *B. pertussis* colonization in mice, demonstrating that interactions with nasal cavity flora can significantly contribute to infection dynamics.

## MATERIAL AND METHODS

### Ethics Statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at The Pennsylvania State University, University Park [protocol 31 297, *Bordetella*-Host Interaction]). All animals were anesthetized using isoflurane or euthanized using carbon dioxide inhalation, to minimize animal suffering.

### Bacterial Strains

*B. pertussis* strain 536, a streptomycin-resistant derivative of the sequenced strain Tohama I and *B. bronchiseptica* strain RB50 are described elsewhere [32, 33]. *Bordetella* were maintained on Bordet-Gengou agar (Difco) with 10% sheep blood (Hema Resources) and 20 µg/mL streptomycin (Sigma). Host bacterial isolates were obtained by culture on lysogeny broth agar or blood agar (BD Biosciences) supplemented with 5% sheep blood (Hema Resources). *B. bronchiseptica* and host microbiota species were grown on agar for 48 hours at 37°C, while *B. pertussis* was grown for 5 days at 37°C. To obtain liquid cultures, *Bordetella* were grown overnight to exponential phase in Stainer-Scholte broth while shaking at 37°C, whereas host microbiota isolates were similarly grown in lysogeny broth (BD Biosciences).

### Mouse Experiments

C57BL/6 wild-type mice were obtained from Jackson Laboratories (Bar Harbor, ME) and were housed and bred in a specific-pathogen-free facility at The Pennsylvania State University. All animal experiments were performed according to institutional guidelines and completed essentially as previously described [14, 33, 34]. Briefly, groups of 4 mice were inoculated by gently pipetting the indicated number of *Bordetella* species or host microorganisms suspended in 10 µL of sterile phosphate-buffered saline onto the external nares of anesthetized mice to mimic natural infection. Three days after inoculation, mice were euthanized using CO<sub>2</sub>, and the nasal cavity was excised, homogenized, and plated on Bordet-Gengou agar, to identify quantities of *Bordetella*, or blood agar and lysogeny broth agar, to identify quantities of host microorganisms. Antibiotic treatment was achieved by gently pipetting 45 µg of enrofloxacin (Baytril) in 10 µL of phosphate-buffered saline on 3 separate occasions 12 hours apart onto the external nares of anesthetized mice, and mice were then inoculated with bacteria at least 8 hours later.

### Host Species Identification

All nasal cavity host microorganisms cultured on agar were described morphologically, isolated in pure culture, and preserved in 20% glycerol (Sigma) at –80°C. DNA extraction using the QIAmp DNA Mini Kit (Qiagen) was performed on each distinct organism, and the 16S ribosomal RNA (rRNA) V2 region was amplified using 16SF (AGAGTTTGATCATGGCTCAG) and 16SR (AAGGAGGTGATCCAACC) primers under the following conditions: 94°C for 5 minutes; 95°C for 30 seconds, 56°C for 1 minute, and 72°C for 1.5 minutes, 30 times; and 72°C for 8 minutes [35]. Following amplification, the approximately 1500-bp polymerase chain reaction products were isolated and purified using MinElute Gel Extraction Kit (Qiagen) as per the manufacturer's instructions. Sequencing of each 16S rRNA-encoding region was completed at The Pennsylvania State University Genomics Core Facility. Both forward and reverse

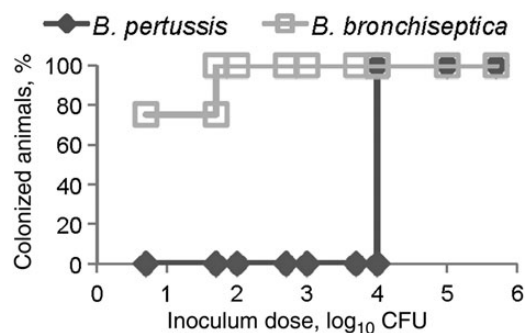
sequences were aligned via MEGA4 [36], and the consensus sequence was submitted to the National Center for Biotechnology Information BLAST (Basic Local Alignment Search Tool) for identification [37]. The top known hit was used for identification with a cutoff of 70% homology.

### 16S rRNA Amplicon Sequencing

Animal dissection and DNA isolation was performed from whole nasal cavities in a UV-sterilized hood with DNA-zap (Ambion)-treated instruments and DNA- and RNA-free filtered pipette tips. The BiOstatic formalin-fixed paraffin-embedded tissue DNA isolation kit (Mo Bio Laboratories) was used to isolate total DNA, with the following modifications: the samples were left at 55°C overnight rather than for 1 hour (step 3), and step 4 was omitted. At The Pennsylvania State University Core Genomic facility, 454 amplicon amplification, library preparation, and sequencing were performed. Bar-coded samples were pooled and sequenced together, achieving approximately 20 000 reads/sample. Sequences were initially analyzed by mothur [38] and identified by BLAST according to the Greengenes database [38]. Sequences were then analyzed via MEGAN (MEtaGenome ANalyzer), producing phylogenetic classification and relationships to known National Center for Biotechnology Information taxonomic trees [39].

### In Vitro Bacterial Competition Experiments

*Staphylococcus saprophyticus*, *Staphylococcus cohnii*, *Staphylococcus xylosus*, *Kytococcus* species, *Klebsiella* species, *Rhizobium* species, *Streptococcus oralis*, *Streptococcus mitis*, *Neisseria subflava*, *Corynebacterium* species, and *B. pertussis* cultures were grown to exponential phase. By use of the optical densities to estimate culturable CFUs, all cultures were then all diluted to  $1 \times 10^7$  CFU/mL in Stainer-Scholte liquid medium. Competition between each host microorganism and *B. pertussis* was



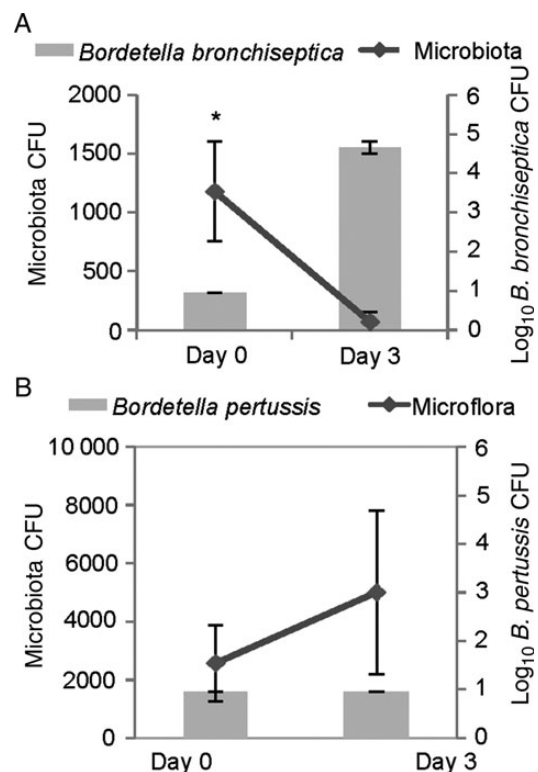
**Figure 1.** A total of  $10^4$  *Bordetella pertussis* are required to colonize the murine nasal cavity. Ten microliters of phosphate-buffered saline containing the indicated colony-forming units (CFU) of either *Bordetella bronchiseptica* (□) or *B. pertussis* (◇) was inoculated into groups of wild-type C57BL/6 mice. Nasal cavity colonization was assessed 3 days later and is presented as the percentage of colonized animals 3 days after inoculation.

created by mixing each host species with an equal number of *B. pertussis* organisms and incubating the mixture in Stainer-Scholte at 37°C for 32 hours while shaking. *B. pertussis* and each microbiota species were additionally grown alone in Stainer-Scholte medium, starting with  $1 \times 10^7$  CFU/mL as a control. Each coculture and control was performed in triplicate and plated on Bordet-Gengou agar with 60 ng/mL streptomycin and blood agar at 0, 2, 4, 8, 24, and 36 hours. The plates were incubated for 48 hours (microbiota) or 5 days (*B. pertussis*) at 37°C, and resulting colonies were counted. Statistical analysis was performed by completing a Student *t* test, and a *P* value of  $\geq .05$  was considered statistically significant.

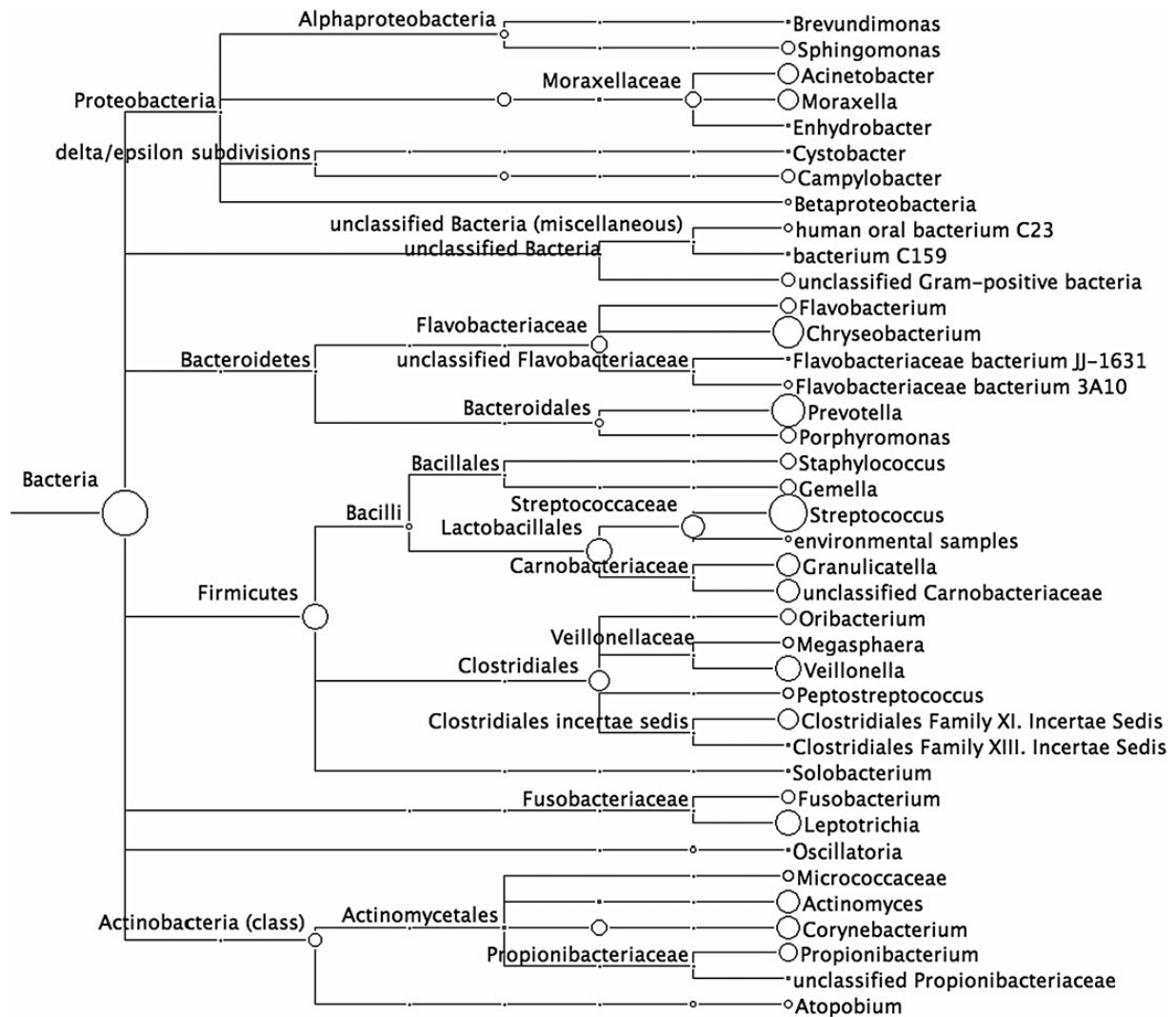
## RESULTS

### *B. pertussis* Requires 10 000 CFU to Colonize the Murine Nasal Cavity

Although high-dose, high-volume delivery of *B. bronchiseptica* and *B. pertussis* leads to efficient growth in the murine nasal cavity [16], these pathogens have not been compared at lower, more natural doses necessary to study the mechanisms of



**Figure 2.** The widespread zoonotic pathogen *Bordetella bronchiseptica* can displace murine flora. Wild-type C57BL/6 mice were inoculated with 100 colony-forming units (CFU) in 10  $\mu$ L of *B. bronchiseptica* strain RB50 (A) or *Bordetella pertussis* strain 536 (B). Host microbiota (lines) and bordetellae (bars) colonization were assessed before inoculation and 3 days after inoculation. Error bars represent SDs. \**P* < .05.



**Figure 3.** Over 80 different bacterial species are identified in the murine nasal cavity. Nasal cavities from uninfected C57BL/6 mice were obtained from our pathogen-free breeding facility. Total DNA was extracted from excised nasal cavities, and the 16S ribosomal RNA encoding region was amplified and sequenced via 454 sequencing to obtain >10 000 reads per animal. Sequences were identified by homology to known sequences in the Greengenes database, and evolutionary relationships between species were analyzed by creating phylogenetic trees in MEGAN, based on the National Center for Biotechnology Information bacterial database.

efficient initial colonization. To compare the infectious doses of these 2 organisms, C57BL/6 wild-type mice were inoculated with 10  $\mu$ L of phosphate-buffered saline containing 5, 50, 100, 500,  $1 \times 10^3$ ,  $5 \times 10^3$ ,  $1 \times 10^4$ ,  $1 \times 10^5$ , or  $1 \times 10^6$  CFU of *B. pertussis* strain 536 or *B. bronchiseptica* strain RB50, and bacterial numbers were determined 3 days after inoculation, capturing several of the important stages during the early colonization phase of infection. A dose of 5 CFU of *B. bronchiseptica* in a 10- $\mu$ L inoculum was sufficient to colonize the nasal cavity of two-thirds of the mice, whereas 10 000 CFU of *B. pertussis* in 10  $\mu$ L was required to recover any *B. pertussis* 3 days after inoculation (Figure 1). A recent human isolate of *B. pertussis*, strain

STO1-CHOC-0008 (obtained from a Collaborative Pediatric Critical Care Research Network), also failed to colonize mice at a low dose (data not shown). These results suggest that *B. bronchiseptica*, a natural murine pathogen, has mechanisms to efficiently colonize the murine nasal cavity that *B. pertussis*, a human-restricted pathogen, lacks.

### ***B. bronchiseptica* Can Displace Murine Microbiota In Vivo, Whereas *B. pertussis* Cannot**

To examine *Bordetella* interactions with host microbiota in vivo, C57BL/6 littermates were cohoused for 5 weeks, and then half of the mice were inoculated with 100 CFU of *B. bronchiseptica*,



whereas the other half were left uninfected. The same experiment was performed with *B. pertussis*. As before, *B. bronchiseptica* was able to colonize the upper respiratory tract at this low dose, whereas *B. pertussis* was not detected 3 days later in mice inoculated with this low dose (Figure 2A and 2B). Interestingly, *B. bronchiseptica* displaced culturable microbiota from the nasal cavities within 3 days after inoculation (Figure 2A), but *B. pertussis* did not (Figure 2B). This experiment identifies a key difference between these closely related species that correlates with their different ability to colonize mice, therefore presenting the hypothesis that competition with resident microbiota may mediate these differences.

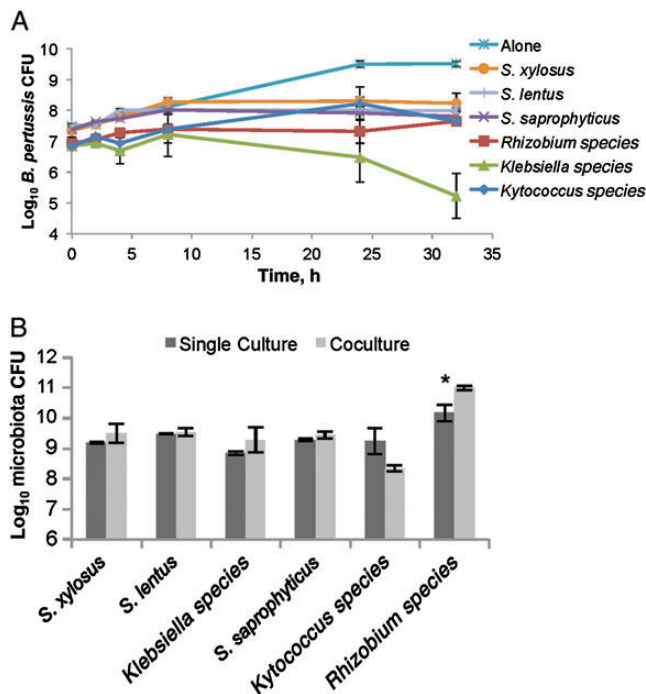
### Murine Nasal Cavity Microbes Can Limit *B. pertussis* Growth In Vitro

To identify bacterial species that interact with *B. pertussis* within the upper respiratory tract, microbiota were cultured from murine nasal cavities on blood agar and identified by sequencing the 16S rRNA-encoding genome region. In these untreated and uninfected mice, 8 different culturable microorganisms were identified, including *Staphylococcus*, *Kytococcus*, *Klebsiella*, and *Rhizobium* species (Supplementary Table 1). A 16S rRNA amplicon sequencing approach was also used to identify all unculturable bacterial microorganisms present in the nasal cavity (Figure 3). In all, >80 different bacterial genera were identified using this approach, indicating that the nasal cavity of specific-pathogen-free mice is a diverse and microbe-rich environment.

To examine competitive interactions between *B. pertussis* and isolated murine nasal cavity microbes, we assayed the in vitro growth of *B. pertussis* over time in the presence and absence of individual nasal cavity microorganisms. Stainer-Scholte medium was inoculated with  $10^7$  CFU of *B. pertussis* alone or together with either *S. saprophyticus*, *Staphylococcus cohnii*, *S. xylosum*, *Klebsiella* PAMU, *Kytococcus* species, or *Rhizobium* species and incubated with shaking at 37°C for 32 hours. *B. pertussis* alone grew to approximately  $5 \times 10^9$  CFU/mL, but numbers were reduced by at least 90% when it was cocultured with *Staphylococcus*, *Kytococcus*, or *Rhizobium* species and by >99.9% when incubated with *Klebsiella* PAMU (Figure 4A). However, the growth of each host species was mostly unaffected by the presence of *B. pertussis* (Supplementary Figure 1A–F), although coculture with *B. pertussis* appeared to provide *Rhizobium* a slight growth advantage at 32 hours (Figure 4B). Together, these results suggest that murine nasal cavity bacteria are capable of inhibiting *B. pertussis* growth in vitro.

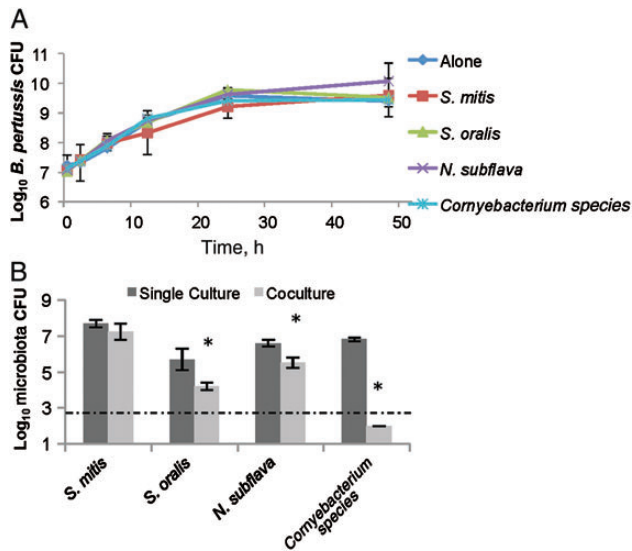
### Human Lower Respiratory Tract Bacterial Isolates Do Not Limit *B. pertussis* Growth

Although earlier experiments demonstrated that murine nasal cavity flora can limit *B. pertussis* growth, *B. pertussis* is highly infectious and successfully colonizes the human lower



**Figure 4.** Murine nasal cavity flora inhibits *Bordetella pertussis* growth. *B. pertussis* was grown alone (star) or cocultured with either *Staphylococcus saprophyticus* (cross), *Staphylococcus xylosum* (circle), *Staphylococcus lentus* (line), *Rhizobium* (square), *Kytococcus* (diamond), or *Klebsiella* PAMU (triangle) in Stainer-Scholte media for 32 hours, and surviving *B. pertussis* colony-forming units (CFU) were determined by plating on Bordet-Gengou agar with 60 ng/mL streptomycin. \* $P > 0.05$ .

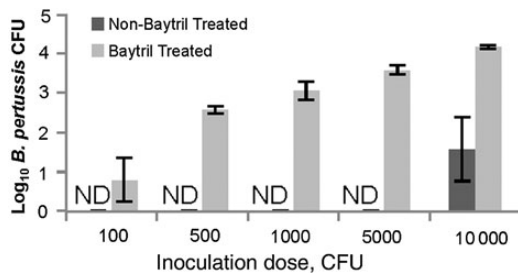
respiratory tract, indicating that there may not be competitors there that can efficiently block its invasion and colonization. Therefore, we hypothesized that *B. pertussis* is able to successfully compete with microorganisms isolated from its only known environmental niche, the human lower respiratory tract. To test this hypothesis, lower respiratory tract microorganisms were cultured from human sputum, obtained from healthy, uninfected human subjects (Supplementary Table 1). These isolates were recovered on blood agar and later grown in the presence of *B. pertussis* in vitro, as done with the murine nasal cavity isolates. Interestingly, *B. pertussis* growth was unaffected by the presence of *S. mitis*, *S. oralis*, *N. subflava*, or *Corynebacterium* species for up to 48 hours, compared to *B. pertussis* grown alone (Figure 5A). However, *S. oralis*, *N. subflava*, and *Corynebacterium* species growth was lower after 48 hours when grown with *B. pertussis* than when each species was grown alone (Figure 5B), even though each species had a different growth rate (Supplementary Figure 2). *S. mitis* growth was unaffected by *B. pertussis* (Figure 5B). These data indicate that *B. pertussis* can successfully compete with these human lower respiratory tract isolates, suggesting that *B. pertussis* has retained mechanisms necessary to compete with bacterial species within its ecological niche.



**Figure 5.** *Bordetella pertussis* outcompetes lower respiratory tract human isolates. *B. pertussis* was grown alone (diamond) or in the presence of *Streptococcus mitis* (square), *Streptococcus oralis* (triangle), *Neisseria subflava* (cross), and a *Corynebacterium* (star) isolate for up to 48 hours. Surviving *B. pertussis* were detected by plating on Bordet-Gengou agar with 60 ng/mL streptomycin (A). Remaining host microbiota from the cocultures (light) or when grown alone (dark) at 48 hours after inoculation were determined by plating on blood agar (B). \* $P > 0.05$ .

### Murine Nasal Cavity Microbiota Inhibit *B. pertussis* Nasal Cavity Colonization

The observed competition with mouse nasal cavity microorganisms could potentially explain the failure of low doses of *B. pertussis* to colonize the mouse nasal cavity. To test this possibility, C57BL/6 mice were first intranasally treated with a broad-spectrum antibiotic, enrofloxacin, a bactericidal compound that affects both Gram-positive and Gram-negative species. Both antibiotic-treated and control mice were then



**Figure 6.** Baytril treatment reduced the *Bordetella pertussis* median infectious dose (ID<sub>50</sub>). Wild-type C57BL/6 mice were either treated 3 times with Baytril (light) or left untreated (dark). All mice were then inoculated with indicated doses of *B. pertussis* strain 536 in 10  $\mu$ L, and levels of *B. pertussis* nasal cavity colonization were assessed 3 days after inoculation. Error bars represent SDs. Abbreviations: CFU, colony-forming units; ND, not detected.

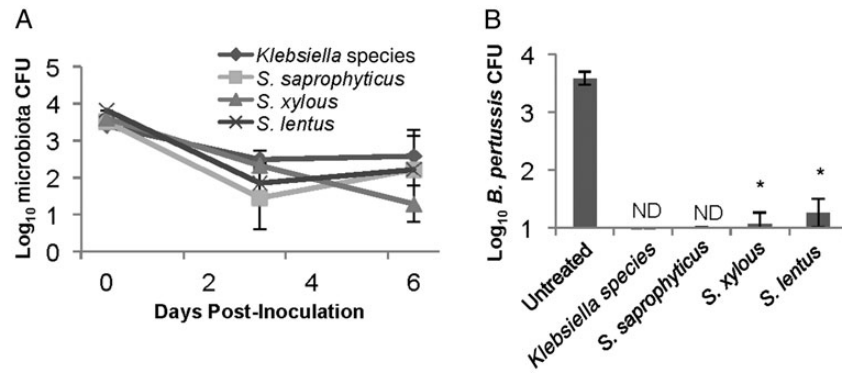
inoculated with 100, 500, 1000, 5000, or 10 000 CFU *B. pertussis*, and bacterial numbers were determined 3 days later. A total of 10 000 CFU of *B. pertussis* were required to colonize mice with their microbiota still intact, as expected, but as few as 100 CFU of *B. pertussis* were sufficient to colonize mice previously treated with enrofloxacin (Figure 6). This finding indicates that bacterial communities present in the upper respiratory tract of mice can inhibit *B. pertussis* nasal cavity colonization.

### *Klebsiella* or *Staphylococcus* Species Are Sufficient to Prevent *B. pertussis* Colonization

To determine whether single bacterial species are sufficient to block *B. pertussis* colonization, wild-type C57BL/6 mice were intranasally treated with enrofloxacin, and 3 days later 1000 CFU of *Klebsiella* PAMU, *S. saprophyticus*, *S. lentus*, or *S. xylosus* were administered to reintroduce individual microorganisms. Each of the other species was recovered from animals at numbers similar to those observed in untreated mice (Figure 7A). Then, 3 mice within these inoculated groups were additionally inoculated with 5000 CFU of *B. pertussis*, >10 times the infectious dose in antibiotic-treated mice. This inoculum resulted in approximately 1000 CFU of *B. pertussis* 3 days after inoculation in all antibiotic-treated mice. However, this dose was not sufficient to colonize antibiotic-treated mice in which *Klebsiella* or *S. saprophyticus* had been reintroduced (Figure 7B). Only tens of *B. pertussis* were detected in mice in which *S. lentus* or *S. xylosus* had been reintroduced (Figure 7B). These data indicate that single bacterial species from the murine nasal cavity are sufficient to inhibit *B. pertussis* colonization of the murine nasal cavity. Furthermore, levels of *Klebsiella* PAMU, *S. saprophyticus*, *S. lentus*, or *S. xylosus* were maintained at similar numbers after *B. pertussis* inoculation (Figure 7A), confirming that this human pathogen does not displace these species under these conditions.

## DISCUSSION

The earliest events in the colonization of a new host are critical limiting aspects of transmission, disease spread, and pathogenesis, but the microscopic scale in which these events occur make them difficult to observe and measure. Often, unnaturally high doses are often used to investigate infectious diseases in experimental models to achieve reproducibility, with very little consideration of how this affects initial colonization mechanisms, such as adherence, immune evasion, competition with other bacteria, and growth rates. The murine model of *B. pertussis* is among the best animal models of human diseases, in that it has been highly effective in revealing aspects of *B. pertussis* infection kinetics, immune response, and vaccination efficacy [40]. However, it has also been criticized for its use of relatively large inocula of approximately  $5 \times 10^5$  CFU. Although its efficient growth within the respiratory tract and eventual clearance by immune mechanisms all closely resemble the infectious process



**Figure 7.** Inoculation with known microbiota inhibits *Bordetella pertussis* colonization. C57BL/6 mice were treated with enrofloxacin and then inoculated with 5000 colony-forming units (CFU) of *Staphylococcus saprophyticus* (square), *Staphylococcus lentus* (cross), *Staphylococcus xylosum* (triangle), or *Klebsiella* (diamond). A, Colonization of these organisms was monitored at 0 and 3 days after inoculation and at day 6 after inoculation, 3 days after inoculation of approximately 5000 CFU of *B. pertussis*. B, *B. pertussis* colonization on day 6 after inoculation of microbiota and 3 days after inoculation of *B. pertussis* was determined for each of the groups initially inoculated with individual host microbiota species. When *B. pertussis* was not detectable, the sample was labeled "ND." \* $P > 0.05$ .

in humans, the limiting steps during the earliest stages of colonization are poorly mimicked by the common high-dose inoculation regimens. In this study, low-dose, low-volume inoculations in mice are used to investigate how competition with microbiota alters host range and infectiousness, ultimately demonstrating that low-dose inoculations with *B. pertussis* can be successfully achieved if animals are first treated with enrofloxacin. Interestingly, large inoculation doses must be used in murine models to study other human pathogens, such as *Pseudomonas*, *Burkholderia*, and *Streptococcus* species. Therefore, it is plausible that host microorganisms also contribute to observed host specificity in other murine infection models.

Although we observed that *B. bronchiseptica* was capable of displacing murine nasal cavity flora while *B. pertussis* is only successful against lower respiratory tract flora, the mechanisms behind these interactions remain unclear. *B. pertussis* may lack factors necessary for competition against murine microbiota, but because several attempts to routinely isolate lower respiratory tract murine microorganisms failed, murine lower respiratory tract microorganisms have yet to be examined. Nevertheless, type VI secretion systems (T6SS) have been found to be required for microbial competition in *Pseudomonas*, *Vibrio*, and *Serratia* species [41–43], and colicins, phages, and CDI systems have been shown to mediate bacterial competition in *Escherichia coli* [31, 44, 45]. Our laboratory recently described a T6SS locus in *B. bronchiseptica* that may be involved in competition against murine nasal cavity flora and observed that this locus is missing in *B. pertussis* [46]. Interestingly, we find that *B. pertussis* contains genes annotated to encode colicins, phages, and potential CDI systems, but these mechanisms are apparently not sufficient to outcompete murine nasal cavity microorganisms, although they may be effective against human lower respiratory

tract isolates [30]. Unique subsets or combinations of competition mechanisms may be required to simultaneously overcome multiple resident bacterial species to efficiently colonize a host.

Parkhill et al suggested that *B. pertussis* adaptation to humans correlates with a 25% loss of its genome, compared with the evolutionary progenitor-like strain *B. bronchiseptica* [10]. Until now, it has remained unclear how genome loss could result in host adaptation between 2 closely related strains that still maintain similar virulence factors. We speculate that *B. pertussis*, which appears to have a closed life cycle involving only acute infections and direct transmission from one human to another, may have lost many genes involved in interspecies competition outside of the human lower respiratory tract. In contrast, the ability of *B. bronchiseptica* to outcompete numerous host species is likely a critical factor to persistently colonize a wide range of mammals. The differential ability to compete with host microbiota could potentially explain the differences in host range, infectiousness, persistence, and/or infection strategy observed between these 2 closely related pathogens. Identifying differences in microbiota from different host species and organs and understanding how pathogens interact with these flora will elucidate key factors that contribute to initial colonization events that, in turn, will suggest novel ways to combat disease.

In this study, the resident species identified in the murine nasal cavity are wildly diverse, but many of these species are also commonly isolated from the upper respiratory tract in humans. *Staphylococcus*, *Kytococcus*, and *Klebsiella* species are routinely cultured from the human nasal cavity [47, 48], and therefore the interactions identified in this study between *B. pertussis* and nasal cavity flora may also occur in humans. Interestingly, *B. pertussis* can be difficult to culture from nasopharyngeal swab specimens from patients with pertussis-like



symptoms, and polymerase chain reaction and serology must then be used to efficiently and reproducibly identify whooping cough-associated infections. Competition with nasal cavity organisms could limit *B. pertussis* growth in and isolation from the nasal cavity. Alternatively, *B. pertussis* could be more quickly displaced from the nasal cavity than from the lower respiratory tract, making it difficult to culture after symptoms present. Furthermore, our results suggest that disruption of resident nasal cavity microbiota may increase susceptibility to *B. pertussis* nasal cavity colonization. This could contribute to observations that infants, who likely lack a well-established nasal cavity microbiota, may be more susceptible than healthy adults to *B. pertussis* infections. Furthermore, these results may also suggest that administering broad-spectrum antibiotics to people could facilitate nasal cavity colonization of *B. pertussis* [47]. Nevertheless, this work provides a tractable experimental system in which to investigate the mechanisms that drive microbiome contributions to infectious diseases susceptibility.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org/>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

**Acknowledgments.** We thank The Pennsylvania State University Core Genomics Facility, for completing the 454 amplicon sequencing presented in this article, and Laura Goodfield, for additional help with experimentation and interpretation of results.

**Disclaimer.** The of this article contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health.

**Financial support.** This work was supported by the National Institutes of Health (grant GM083113 to E. T. H. and training grant T32AI074551 to S. J. M.) and the National Science Foundation Graduate Research Fellowship Program (to L. S. W.).

**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

## References

- World Health Organization. The world health report 2004—changing history. Geneva: WHO, 2004. <http://www.who.int/whr/2004/en/>. Accessed 12 January 2012.
- Tan T, Trindade E, Skowronski D. Epidemiology of pertussis. *Pediatr Infect Dis J* 2005; 24:S10–8. [http://journals.lww.com/pidj/Fulltext/2005/05001/Epidemiology\\_of\\_Pertussis.3.aspx](http://journals.lww.com/pidj/Fulltext/2005/05001/Epidemiology_of_Pertussis.3.aspx).
- Cherry JD. Historical review of pertussis and the classical vaccine. *J Infect Dis* 1996; 174:S259–63.
- Mielcarek N, Debrie A, Raze D, et al. Live attenuated *B. pertussis* as a single-dose nasal vaccine against whooping cough. *PLoS Pathog* 2006; 2:e65.
- Centers for Disease Control and Prevention. Pertussis vaccines. In: The pink book: course textbook (May 2012). 2nd ed. Updated 20 September 2013. <http://www.cdc.gov/vaccines/pubs/pinkbook/pert.html>. Accessed 15 November 2013.
- Pam D. Whooping cough makes global comeback. *Lan Infect Dis* 2002; 2:322.
- Mattoo S, Cherry JD. Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other *Bordetella* subspecies. *Clin Microbiol Rev* 2005; 18:326–82.
- Goodnow RA. Biology of *Bordetella bronchiseptica*. *Microbiol Rev* 1980; 44:722–38.
- Diavatopoulos DA, Cummings CA, Schouls LM, et al. *Bordetella pertussis*, the causative agent of whooping cough, evolved from a distinct, human-associated lineage of *B. bronchiseptica*. *PLoS Pathog* 2005; 1:e45.
- Parkhill J, Sebahia M, Preston A, et al. Comparative analysis of the genome sequences of *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. *Nat Genet* 2003; 35:32–40.
- Wolfe DN, Kirimanjswara GS, Harvill ET. Clearance of *Bordetella parapertussis* from the lower respiratory tract requires humoral and cellular immunity. *Infect Immun* 2005; 73:6508–13.
- Mann PB, Wolfe D, Latz E, et al. Comparative toll-like receptor 4-mediated innate host defense to *Bordetella* infection. *Infect Immun* 2005; 73:8144–52.
- Andreasen C, Powell DA, Carbonetti NH. Pertussis toxin stimulates IL-17 production in response to *Bordetella pertussis* infection in mice. *PLoS One* 2009; 4:e7079.
- Kirimanjswara GS, Mann PB, Harvill ET. Role of antibodies in immunity to *Bordetella* infections. *Infect Immun* 2003; 71:1719–24.
- Heininger U, Cotter PA, Fescemyer HW, et al. Comparative phenotypic analysis of the *Bordetella parapertussis* isolate chosen for genomic sequencing. *Infect Immun* 2002; 70:3777–84.
- Harvill ET, Cotter PA, Miller JF. Pregenomic comparative analysis between *Bordetella bronchiseptica* RB50 and *Bordetella pertussis* Tohama I in murine models of respiratory tract infection. *Infect Immun* 1999; 67:6109–18.
- Gueirard P, Ave P, Balazuc AM, et al. *Bordetella bronchiseptica* persists in the nasal cavities of mice and triggers early delivery of dendritic cells in the lymph nodes draining the lower and upper respiratory tract. *Infect Immun* 2003; 71:4137–43.
- Tuomanen E, Weiss A. Characterization of two adhesins of *Bordetella pertussis* for human ciliated respiratory-epithelial cells. *J Infect Dis* 1985; 152:118–25.
- Weiss AA, Hewlett EL, Myers GA, Falkow S. Genetic studies of the molecular basis of whooping cough. *Dev Biol Stand* 1985; 61:11–9.
- Mattoo S, Foreman-Wykert AK, Cotter PA, Miller JF. Mechanisms of *Bordetella* pathogenesis. *Front Biosci* 2001; 6:E168–86.
- Tuomanen EI, Nedelman J, Hendley JO, Hewlett EL. Species specificity of *Bordetella* adherence to human and animal ciliated respiratory epithelial cells. *Infect Immun* 1983; 42:692–5.
- Zhang X, Hester SE, Kennett MJ, et al. Interleukin-1 receptor signaling is required to overcome the effects of pertussis toxin and for efficient infection- or vaccination-induced immunity against *Bordetella pertussis*. *Infect Immun* 2011; 79:527–41.
- Wolfe DN, Mann PB, Buboltz AM, Harvill ET. Delayed role of tumor necrosis factor- $\alpha$  in overcoming the effects of pertussis toxin. *J Infect Dis* 2007; 196:1228–36.
- Pilione MR, Harvill ET. The *Bordetella bronchiseptica* type III secretion system inhibits gamma interferon production that is required for efficient antibody-mediated bacterial clearance. *Infect Immun* 2006; 74:1043–9.
- Read AF, Taylor LH. The ecology of genetically diverse infections. *Science* 2001; 292:1099–102.
- Lina G, Boutite F, Tristan A, et al. Bacterial competition for human nasal cavity colonization: role of staphylococcal agr alleles. *Appl Environ Microbiol* 2003; 69:18–23.
- Lysenko ES, Ratner AJ, Nelson AL, Weiser JN. The role of innate immune responses in the outcome of interspecies competition for colonization of mucosal surfaces. *PLoS Pathog* 2005; 1:e1.
- Griffin AS, West SA, Buckling A. Cooperation and competition in pathogenic bacteria. *Nature* 2004; 430:1024–7.



29. Hood RD, Singh P, Hsu F, et al. A type VI secretion system of *Pseudomonas aeruginosa* targets a toxin to bacteria. *Cell Host Microbe* **2010**; 7:25–37.
30. Joo J, Gunny M, Cases M, et al. Bacteriophage-mediated competition in *Bordetella* bacteria. *Proc Biol Sci* **2006**; 273:1843–8.
31. Aoki SK, Pamma R, Hernday AD, et al. Contact-dependent inhibition of growth in *Escherichia coli*. *Science* **2005**; 309:1245–8.
32. Stibitz S, Yang MS. Subcellular localization and immunological detection of proteins encoded by the *vir* locus of *Bordetella pertussis*. *J Bacteriol* **1991**; 173:4288–96.
33. Cotter PA, Miller JF. BvgAS-mediated signal transduction: analysis of phase-locked regulatory mutants of *Bordetella bronchiseptica* in a rabbit model. *Infect Immun* **1994**; 62:3381–90.
34. Pishko EJ, Kirimanjeswara GS, Pilione MR, et al. Antibody-mediated bacterial clearance from the lower respiratory tract of mice requires complement component C3. *Eur J Immunol* **2004**; 34:184–93.
35. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **1991**; 173:697–703.
36. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **2007**; 24:1596–9.
37. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* **1990**; 215:403–10.
38. Schloss PD, Westcott SL, Ryabin T, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* **2009**; 75:7537–41.
39. Huson DH, Auch AF, Qi J, Schuster SC. MEGAN analysis of metagenomic data. *Genome Res* **2007**; 17:377–86.
40. Mills KH. Immunity to *Bordetella pertussis*. *Microbes Infect* **2001**; 3: 655–77.
41. Russell AB, Hood RD, Bui NK, et al. Type VI secretion delivers bacteriolytic effectors to target cells. *Nature* **2011**; 475:343–7.
42. MacIntyre DL, Miyata ST, Kitaoka M, Pukatzki S. The *Vibrio cholerae* type VI secretion system displays antimicrobial properties. *Proc Natl Acad Sci* **2010**; 107:19520–4.
43. Murdoch SL, Trunk K, English G, et al. The opportunistic pathogen *Serratia marcescens* utilizes type VI secretion to target bacterial competitors. *J Bacteriol* **2011**; 193:6057–69.
44. Majeed H, Gillor O, Kerr B, Riley MA. Competitive interactions in *Escherichia coli* populations: the role of bacteriocins. *ISME J* **2011**; 5: 71–81.
45. Brown SP, Inglis RF, Taddei F. Evolutionary ecology of microbial wars: within-host competition and (incidental) virulence. *Evol Appl* **2009**; 2: 32–9.
46. Weyrich LS, Rolin OY, Muse SJ, et al. A type VI secretion system encoding locus is required for *Bordetella bronchiseptica* immunomodulation and persistence in vivo. *PLoS One* **2012**; 7:e45892.
47. Normal flora. In: Baron S, ed. *Medical microbiology*. Galveston, TX: University of Texas Medical Branch at Galveston, **1996**. <http://www.ncbi.nlm.nih.gov/books/NBK7617/>. Accessed 8 January 2012.
48. Turnbaugh P, Ley RE, Hamady M, et al. The human microbiome project. *Nature* **2007**; 449:804–10.