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# The human microbiome harbors a diverse reservoir of antibiotic resistance genes

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**The increasing levels of multi-drug resistance in human pathogenic bacteria are compromising our ability to treat infectious disease. Since antibiotic resistance determinants are readily exchanged between bacteria through lateral gene transfer, there is an increasing interest in investigating reservoirs of antibiotic resistance accessible to pathogens. Due to the high likelihood of contact and genetic exchange with pathogens during disease progression, the human microflora warrants special attention as perhaps the most accessible reservoir of resistance genes. Indeed, numerous previous studies have demonstrated substantial antibiotic resistance in cultured isolates from the human microflora. By applying metagenomic functional selections, we recently demonstrated that the functional repertoire of resistance genes in the human microbiome is much more diverse than suggested using previous culture-dependent methods. We showed that many resistance genes from cultured proteobacteria from human fecal samples are identical to resistance genes harbored by human pathogens, providing strong support for recent genetic exchange of this resistance machinery. In contrast, most of the resistance genes we identified with culture independent metagenomic sampling from the same samples were novel when compared to all known genes in public databases. While this clearly demonstrates that the antibiotic resistance reservoir of the large fraction of the human microbiome recalcitrant to culturing is severely under sampled, it may also suggest that barriers exist to lateral gene transfer between these bacteria and readily cultured human pathogens. If we hope to turn the tide against multidrug resistant infections, we must urgently commit to quantitatively characterizing the resistance reservoirs encoded by our diverse human microbiomes, with a particular focus on routes of exchange of these reservoirs with other microbial communities.**

The seemingly unchecked spread of multiple-antibiotic resistance in clinically-relevant pathogenic microbes has begun to significantly dull the hue of the Golden Era of antibiotics, ostensibly launched in the 1940s with the industrial production of penicillin. A diverse set of molecular mechanisms of microbial antibiotic resistance have been elucidated, along with strong evidence for transfer of these mechanisms between different pathogenic microbes.[1] Selection of antibiotic-resistant strains has been accelerated by the imprudent prescription and overuse of these life-saving chemotherapeutics, particularly exacerbated by the widespread use of sub-therapeutic amounts of antibiotics as growth-promoters in farm animals.[2] Of further concern, the genetic and biochemical mechanisms which govern the evolution and dissemination of drug resistance can be engineered into or be naturally acquired by many putative bioterror agents, effectively annulling our primary chemotherapeutics against these biological threats. For instance, a conjugative plasmid mediating multidrug resistance in *Vibrio cholera* was recently isolated, with 99.99% sequence identity

to a conjugative plasmid previously isolated from a multidrug-resistant strain of the bubonic plague bacterium *Yersinia pestis*.[3] This mobile element conferred resistance to 6 different drugs including tetracycline and chloramphenicol, the two first-line drugs against the plague bacterium. Consequently, there is an increasing interest in identifying and characterizing microbes from communities that may be accessible reservoirs of antibiotic resistance machinery.

Most antibiotics used in the clinic today are structural derivatives of compounds isolated from natural sources. Since antibiotics in nature represent one of the oldest forms of biochemical warfare, environmental bacteria would be expected to possess intrinsic mechanisms to detect, resist, and neutralize these threats to their existence, and hence represent a reservoir of accessible resistance machinery. Indeed, a significant reservoir of antibiotic resistance determinants have been highlighted in the soil microbiota, recently collectively termed the *antibiotic resistome*.[4-6] While a majority of these antibiotic resistant organisms in the soil are likely non-pathogenic, their biochemical mechanisms of resistance have been found to be identical to those of resistant clinical pathogens,[7] which suggests the soil resistome may constitute an important source of resistance mechanisms for clinical pathogens. A key subset of the soil antibiotic resistome is microbes which produce antibiotics, since they must, at the minimum, harbor self-immunity elements against the antimicrobial toxins which they produce. Since a significant majority of antibiotic chemicals are natural products or their semi-synthetic derivatives, it follows that producer microbes harbor microbial resistance mechanisms against a majority of antibiotics.[7] This hypothesis was elegantly supported by D'Costa et al. who, recognizing that soil-dwelling Actinomycetes produce over 50% of known antibiotics, profiled the antibiotic resistance of a library of 480 Streptomyces strains (an Actinomycete genus), and found them to be on average resistant to 7–8 out of a set of 21 antibiotics tested, which encompassed every known class of antibiotic bacterial target, including completely synthetic antibiotics.[5] We recently helped significantly expand the concept of this environmental antibiotic resistome by discovering hundreds of bacteria from diverse US soils that could consume a startling array of antibiotics as their sole carbon source, including both natural and entirely synthetic compounds.[8] These antibiotic-metabolizing isolates are phylogenetically diverse, and many are closely related to known human pathogens, including members of the Burkholderiales, Pseudomonales and Enterobacteriales. We also found these bacteria to be extensively resistant to most antibiotics tested at over 50 times the concentrations used to define clinical drug resistance—indeed, over a quarter of our isolates were resistant to all 18 compounds tested at 1 mg/mL.

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Much of the immense and diverse reservoir of antibiotic resistance genes present in the environment has not yet been observed in human pathogenic bacteria.[5,9] However, the direct and continuous contact between farm animals and the soil increases the possibility of genetic exchange between their associated microbiomes, allowing for the transfer and selection of potentially novel soil resistance genes in farm animal associated microbiomes. It is clear that the large quantities of antibiotics currently used in agriculture selects for resistance genes in microbes associated with farm animals,[10] and these antibiotic resistant microbes can be directly transferred to human associated microbiomes.[11] Thus, an environmental reservoir of microbial genes of particular relevance to human health is that harbored within the human-associated microbiome.[12-15] Outnumbering human cells ten to one, and human genes a hundred to one, this microbial community significantly impacts human health, including beneficial roles in dietary processing and prevention of pathogen intrusion.[16,17] Given the widespread use of antibiotics in human medicine and their broad spectrum activities, the human microbiome has likely undergone substantial responsive changes to this therapy. This prompts the investigation of the extent to which the human associated microbiome in the antibiotic era has evolved to become a reservoir of antibiotic resistance genes.

During the progression of a bacterial infection in the human body, a multiplying pathogen must interact with the commensal microflora at the site of infection, giving opportunity for genetic exchange between these microbes. Hence a virulent pathogen might acquire antibiotic resistance through lateral transfer of antibiotic resistance genes harbored by antibiotic resistant commensal organisms. If antibiotic resistance genes have been exchanged between members of the human microbiome and human pathogens, the evolutionary timeline of this exchange can be assayed by comparing the sequence identity of the relevant genetic machinery between the two groups.[18]

A common method for identifying genes in microbiomic samples that are homologous to previously identified resistance genes is PCR amplification using primers based on the sequences of the known genes. This has been successfully applied to study antibiotic resistance genes from cultured isolates and metagenomic samples from animals,[19] humans[20] and soil.[21] However, this approach requires complete sequence identity between primer regions of the templates queried and the known sequences, and hence has limited utility in the discovery of new genes. Given the sequence diversity of known resistance genes, this approach also requires the design of large numbers of primer sets and concomitantly large numbers of amplification reactions. Genes identified with a PCR-based method that differ in their encoded protein sequences from the known resistance genes must then be experimentally verified to explicitly confirm their function. A complementary technique for elucidating antibiotic resistance genes is metagenomic functional selections[4,22] in which DNA is extracted from a source of interest and cloned directly into a susceptible indicator strain. Indicator strains harboring DNA fragments encoding antibiotic resistance genes can be selected for by subjecting the library of clones to particular antibiotics. Following the selection of antibiotic resistant clones, the metagenomic DNA insert can be sequenced to identify the causative antibiotic resistance gene. Standard metagenomic functional selections allow the interrogation of more than $10^9$ base pairs of genetic information in a single selection and are consequently ideally suited for discovery of antibiotic resistance genes that are functionally compatible with the indicator strain in

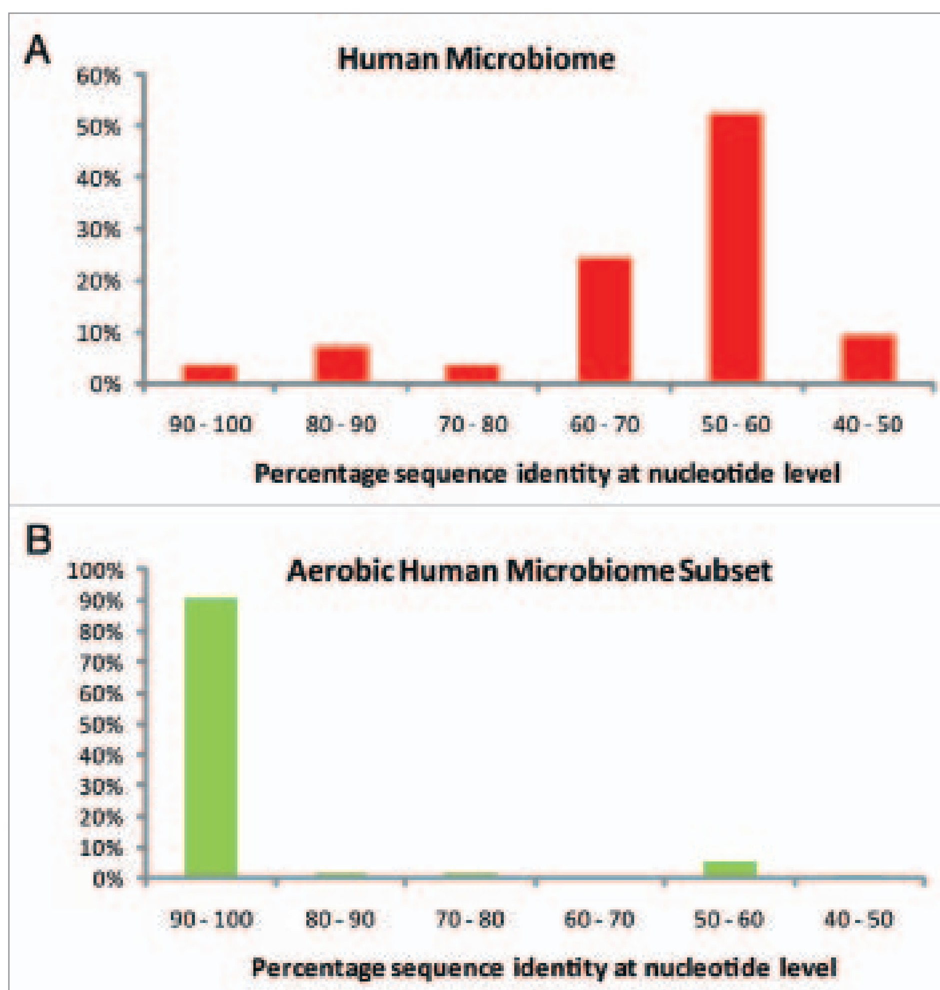


**Figure 1.** Distribution of nucleotide identities for (A) 93 resistance genes identified from DNA extracted directly from saliva and fecal samples and (B) 114 resistance genes identified from cultured aerobic gut isolates to the most similar resistance gene harbored by a pathogenic isolate in NCBI GenBank.

absence of any prior knowledge of their DNA sequence. However, metagenomic functional selections for characterization of resistance reservoirs have certain limitations as well. These primarily involve the requirement for (1) host susceptibility to the antibiotics assayed, (2) appropriate translation and functional compatibility of heterologous gene products in the host, and (3) the resistance gene to encode a dominant phenotype that overcomes the host mechanism of susceptibility. Methods to overcome these limitations include (1) use of multiple host strains which span the spectrum of antibiotic susceptibility mechanisms assayed, (2) engineering of new host strains with an expanded repertoire of translational machinery including rare codon transfer RNAs, and (3) the use of conditional host strain mutants which suppress specific dominant antibiotic susceptibility loci allowing complementation by a transferred resistance gene that is otherwise recessive. Despite current limitations, metagenomic functional selections are useful for the identification of numerous known as well as novel resistance genes, since their success is independent of prior knowledge of the sequence of previously identified genes.

We recently used metagenomic functional selections to identify several hundred resistance genes harbored by the microbiome of healthy individuals.[22] First, we used microbiome-wide culture independent functional selections to identify the prevalent antibiotic resistance genes of the human microbiome. Interestingly these genes had low sequence identity (on average 60.7% at the nucleotide and 54.9% at the amino acid level) to previously characterized genes harbored by pathogens (**Fig. 1A**). Amongst the genes encoding resistance to beta-lactam antibiotics, we identified 10 new sequence families (HGA to HGI and HOA), each with sequence identity less than 61% at the amino acid level to previously characterized resistance gene products. The DNA inserts containing antibiotic resistance genes appeared to predominantly originate from the phyla Bacteroidetes and Firmicutes, based on phylogenetic analysis using PhyloPythia.[23] Previous studies have demonstrated that the gut microbiome is indeed dominated (>90%) by these two phyla, from which the minority (~20%) of phylotypes are cultured obligate anaerobes, while the majority (~80%) remain uncultured.[16] While Firmicutes and

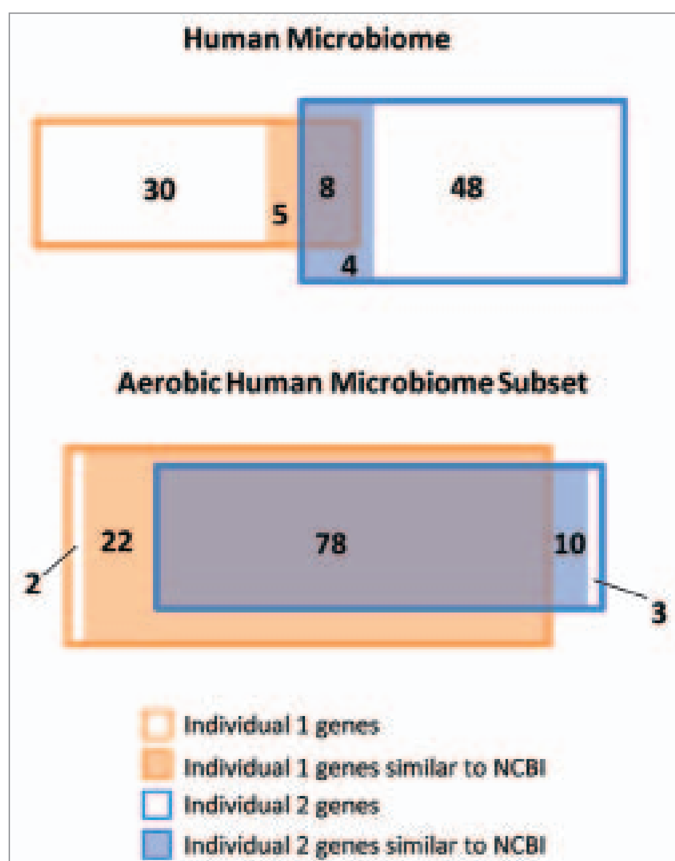


**Figure 2.** Overlap of resistance genes identified from the human microbiomes of two healthy unrelated individuals. Resistance genes from two individuals are compared to each other and defined as being shared if they have greater than 90% sequence identity at the nucleotide level. Resistance genes identified using metagenomic functional selections (top) are shared between two individuals to a much lesser extent than resistance genes identified from cultured gut isolates (bottom). Notably, all resistance genes that are shared between the two individuals also have high sequence identity (>90% at the nucleotide level) to previously characterized genes in NCBI GenBank.

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Bacteroidetes include important human pathogens such as *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Bacteroides fragilis*, we found that the majority of our genes have low sequence identity to resistance genes previously identified in these closely related pathogens. A number of human pathogens are also readily culturable facultative anaerobic Proteobacteria, including epidemic strains of *Klebsiella pneumoniae, Serratia marcescens, Shigella sonnei, Salmonella typhimurium, Vibrio cholerae* and *Haemophilus influenzae*. While Proteobacteria constitute less than 1% of the human gut microbiome,[16] they are seen to dramatically increase in abundance during antibiotic treatment at the expense of the normally abundant gut microbes,[24,25] indicating that they have increased relative fitness during antibiotic treatment. As a consequence of their normal low abundance in healthy individuals, they are not well represented in libraries made using an unbiased functional metagenomic approach. Given their potential importance as a reservoir of antibiotic resistance genes readily accessible to closely related human pathogens, we assayed for the capacity of culturable aerobic bacteria to grow in the presence of antibiotics and elucidated their resistance genes using functional selections. Notably, the antibiotic resistance genes harbored by this subset of the human microbiome were highly similar to previously characterized resistance genes and included several genes such as *TetA*, a tetracycline efflux pump, and *CTX-M-15*, an epidemic beta-lactamase, frequently found in pathogenic isolates. In fact, over 95% of the resistance genes harbored by human gut microbes capable of growing aerobically on rich media were more than 90% identical at the nucleotide level to resistance genes in pathogenic isolates (**Fig. 1B**).

This work exposed the significant under-sampling of antibiotic resistance genes in the human microbiome, which has resulted from the reliance on culture dependent methods, and demonstrates the utility and importance of using metagenomic functional selections for studying antibiotic resistance reservoirs. While we found a large number of microbial DNA fragments encoding resistance genes that have never before been described, our analysis suggests that we have just begun to scratch the surface of the immense diversity of antibiotic resistance machinery in the human microbiome and we estimate that complete sequencing of these libraries would yield hundreds more resistance genes.[22] When we compare the resistance genes derived from the microbiomes of the two different individuals, we find that over 65% of the resistance genes derived from cultured aerobes are highly similar (>90% nucleotide sequence identity) between the two individuals, whereas less than 10% of the metagenomically derived resistance genes are highly similar between the two individuals (**Fig. 2**). Furthermore, all of the genes (metagenomic and cultured gut aerobes) shared between individuals have close homologs (>90% nucleotide sequence identity) in GenBank. Interestingly, while 84% of the genes unique to an individual from cultured aerobic isolates also have close homologs in GenBank, only 11% of the metagenomically derived genes unique to an individual have close homologs in GenBank (**Fig. 2**). These results suggest that the under-sampled antibiotic resistance reservoir accessed by our metagenomic functional selection approach is extremely diverse compared to the well studied subset of cultured gut aerobes and merits application to a larger number of individuals. Also, in spite of the sparse sampling of this work, the results seem to indicate that the antibiotic resistance reservoir sampled using culture independent methods is shared to a lesser extent amongst individuals compared to that of the cultured aerobic isolates.

Given that 45% of the resistance genes from cultured human gut microbiome isolates uncovered in this study are identical at the nucleotide level to resistance genes from human pathogenic isolates underscores their extremely close evolutionary relationship. While this identity provides no information regarding the direction or mechanism of transfer, these results lead to a few formal hypotheses regarding the evolution of an antibiotic resistant human pathogen through interactions with the human microbiome. First, the human microbiome may constitute a mobilizable reservoir of antibiotic resistance genes which are accessed by a pathogenic bacterium to acquire antibiotic resistance. Culturable isolates can transfer antibiotic resistance determinants to previously susceptible strains in vivo in the avian gut[26] as well as in the gut of gnotobiotic mice[27] and rats,[28] but direct experimental proof of in vivo transfer of antibiotic resistance genes within the human microbiome remains to be shown. Second, an antibiotic resistant member of the human microbiome may become pathogenic through the acquisition of virulence genes derived from a pathogen.[29] Finally, despite our selection of samples from healthy humans, the aerobic cultured microbiome isolates containing antibiotic resistance genes that are identical to those harbored by clinical pathogenic isolates may indeed themselves be dormant pathogens inhabiting the human microbiome. In all these possibilities a multidrug resistant human microbiome may act as a reservoir for pathogenesis—either as a donor of antibiotic resistance genes, a recipient of virulence factors, or as a permissive environment for dormant pathogens. For this reason it will be critical to test these hypotheses in vivo, as well as determine the factors that are responsible for the maintenance and transfer of antibiotic resistance in the healthy human microbiome.

In contrast to cultured isolates, the resistance genes uncovered using a culture independent approach are largely novel and distantly related to resistance genes from even closely related pathogenic isolates, which may reflect an unappreciated barrier to lateral gene transfer in vivo between the dominant commensals in healthy humans and disease causing isolates. From the standpoint of a reservoir for pathogens, one interpretation of this finding is that the resistance genes of the human microbiome are inaccessible or infrequently exchanged with human pathogens. However, all the resistance genes identified in this study are expressed and functional in *E. coli*, a readily culturable strain with close pathogenic relatives, which suggests that if a barrier to gene transfer exists between the constituents of the human microbiome and pathogens, it must stem from processes other than functional compatibility.

In light of the ever-increasing problems of antibiotic resistance in pathogens, a deeper and more quantitative understanding of the human microbiome as a reservoir for pathogenesis is critical. Our initial work using metagenomic functional selections highlighted that an unappreciated diversity of antibiotic resistance genes is harbored by the dominant species of the human microbiome which had been missed likely due to reliance on culture and PCR based techniques for interrogating this microbiome. However, this work only scratched the surface of this diverse reservoir and furhter in depth characterization and quantitation across populations is needed to better understand how this reservoir is connected to other resistance reservoirs and the extent to which this reservoir is accessible to pathogens.

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