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Antibiotics and the resistant microbiome

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GLOSSARY:

Human microbiota – the collection of microbes living in and on the human body
Human microbiome – the collective genomic content of the human microbiota
Resistome – the complement of all antibiotic resistance genes encoded by a microbial community
Metagenomic functional selection – a culture-independent method for capture of selectable functions from microbial metagenomic DNA through heterologous expression in an indicator host strain.

SUMMARY:

Since the discovery and clinical application of antibiotics, pathogens and the human microbiota have faced a near continuous exposure to these selective agents. A well-established consequence of this exposure is the evolution of multidrug-resistant pathogens which can become virtually untreatable. Less appreciated are the concomitant changes in the human microbiome in response to these assaults and their contribution to clinical resistance problems. Studies have shown that pervasive changes to the human microbiota result from antibiotic treatment and that resistant strains can persist for years. Additionally, culture independent functional characterization of the resistance genes from the microbiome has demonstrated a close evolutionary relationship between resistance genes in the microbiome and in pathogens. Application of these techniques and novel cultivation methods are expected to significantly expand our understanding of the interplay between antibiotics and the microbiome.

INTRODUCTION:

Antibiotic compounds work to either stop bacteria from growing (bacteriostatic agents) or to kill them outright (bacteriocidal agents). The effectiveness of these compounds "against life" stems from their ability to block critical bacterial cellular processes [1,2]. Since these cellular targets are often highly genetically and structurally conserved across diverse members of the bacterial kingdom, deployment of an antibiotic compound against a specific bacterium (e.g. a pathogen) is quite likely to impart collateral damage to the bacterial community which shares the environment of the intended target organism. The spectrum of unintended effects of antibiotic therapy is likely to be considerably larger than simple growth inhibition of susceptible strains, especially considering the role of these bioactive organic compounds as multi-activity signaling molecules [3]. Almost every surface of the human body after birth is colonized by a rich and diverse community of commensal microbes [4-6], and this microbiota has substantial and continuous effects on human health and physiological development, including dietary and nutritional processing, prevention of pathogen invasion, and immune system maturation [7-9]. Accordingly, antibiotic treatment to prevent or eradicate a pathogenic infection at virtually any body site is likely to produce both short-term and long-term impacts on the commensal microbiota and its encoded microbiome [10]. These effects can be broadly categorized into two interrelated categories(Figure 1): (1) ones which change the relative proportions of different species in the microbiota, with introduction of a new species or complete eradication of an existing species as extreme scenarios [11-16], and (2) ones which alter the antibiotic resistome encoded by members of the microbiota [17,18]. In the first case, changes in the architecture of the microbiota can dramatically perturb the carefully evolved homeostasis of microbiota-host mutualism, which can lead to both transient and persistent changes in host physiology and health [19,20]. In the latter case, enrichment and exchange of resistance genes within the microbiota increase the accessibility of these elements to potentially pathogenic organisms, challenging our ability to treat their subsequent infections [21,22].

MAIN TEXT:

Antibiotic induced changes in microbial community structure

Dramatic advances over the past few years in next-generation DNA sequencing technology have enabled a series of recent high-resolution molecular characterizations of the change in microbiota architecture in humans and model organisms in response to antibiotic treatment. Deep 16S rDNA pyrosequencing was used to investigate the effect of two courses of ciprofloxacin on the gastrointestinal microbiota of three healthy individuals, which revealed dramatic and immediate antibiotic-induced decreases in phylogenetic diversity of the previously stable microbial ecosystem [12,15]. While the microbiota began to recover to a relatively stable state within a week after antibiotic insult, the pre-treatment architecture had not fully recovered within 6-10 months, including the apparent complete loss of low-abundance members of the community. Similarly, a combination of 16S sequencing and terminal-restriction fragment length polymorphism (T-RFLP) was used to measure the short- and long-term effects of clarithromycin and metronidazole, which are standard treatment for *Helicobacter pylori* eradication, on the throat and gastrointestinal microbiota of three treated human subjects compared to three controls [16]. Like the ciprofloxacin study, the combination antibiotic treatment caused transient decreases in microbiota diversity in both the throat and the gut, and the overall community architecture had only partially recovered one and four years after treatment. Interestingly, the throat microbiota was found to be more stable after antibiotic treatment and over long periods than the intestinal microbiota.

One of the challenges in fully understanding the mechanisms of antibiotic-induced microbiota perturbations is the inherent baseline variability of the microbiota of different human individuals, which is confounded by the small sample size employed in human studies to date. One approach to address this variation is to use animal models, where host genotype, diet and other variables, which may impact baseline microbiota composition can be controlled, while still employing modern high-resolution molecular methods to characterize the microbiota [13,14]. A major finding of such studies is that different antibiotic regimens cause reproducible distinct short-term and long-term impacts on the microbiota in murine models. Mice treated with a combination of amoxicillin, metronidazole and bismuth had significant changes in abundance and structure of their cecal microbiota following treatment, but there was also significant rapid recovery of the community structure towards baseline after removal of the treatment. In contrast, treatment with the broad-spectrum antibiotic cefoperazone caused persistent depression in overall community diversity up to six weeks after treatment withdrawal, though community composition was 'normalized' when the treated cohort was co-housed with a non-treated animal, presumably via coprophagy [14]. In another experiment, mice treated with the glycopeptide vancomycin, which exclusively targets gram-positive organisms, showed similar effects to the combination treatment, where the antibiotic-induced community

perturbations recovered after removal of treatment but differed from both the previous treatments in that vancomycin did not result in a major decrease in overall bacterial biomass [13]. Of note, all treatments caused transient increases in the normally low-abundance Proteobacteria at the expense of the normally dominant Firmicutes and Bacteroidetes, which is particularly intriguing given the recent rapid global proliferation of extensively drug resistant strains of Protoeobacterial human pathogens, including *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and extended-spectrum beta-lactamase producing *Enterobacteriaceae* [23].

The simplest mechanistic explanation for short-term antibiotic-induced changes in the microbiota is differences in the activity spectrum of the drug; drug resistant or insensitive bacteria proportionally increase at the expense of susceptible ones. For instance, the relative enrichment of the Proteobacteria and Tenericutes in mice cecal microbiota in response to Vancomycin is rationalized by innate resistance to this drug by these groups [13]. Vancomycin targets a peptidoglycan precursor needed for normal cell-wall synthesis, but is ineffective against these bacteria due to its inability to penetrate the Proteobacterial outer membrane, and the lack of a cell wall in Tenericutes comprised of peptidoglycan. However, antibiotics can also perturb the complex architecture of the microbiota through indirect or multilayered effects; for example, bacteria which are insensitive to direct action of an antibiotic might nevertheless still be affected if other bacteria or environmental factors which they are dependent on are changed by antibiotic treatment [19]. For instance, mice treated with the broad-spectrum combination metronidazole, neomycin and vancomycin significantly down-regulated intestinal expression of RegIIIγ, a secreted C-type lectin which is induced by commensal microbes and kills gram-negative bacteria [20]. This down-regulation of the host innate immune defense in turn significantly decreased *in vivo* killing of vancomycin-resistant enterococci, demonstrating the indirect effects of antibiotic treatment on a pathogenic microbe.

Persistent effects on the antibiotic resistome following antibiotic treatment

A major expectation from repeated antibiotic exposure of the microbiota is an increasing abundance of antibiotic resistance determinants, caused by the growth advantage of resistant organisms during antibiotic treatment and further exacerbated through the lateral exchange of resistance determinants between diverse bacteria. Growth of this resistant population and their associated resistance genes is obviously dependent on the existence of a basal level of resistance in the microbiota; however, even in absence of direct anthropogenic treatment with antibiotics such populations are generally found present in human, animal and even 'pristine' environmental microbiota [24]. Studies using anaerobic and aerobic cultivation methods have shown that the oral microbiota of children that have not received antibiotics contain a subpopulation of different species that are resistant to tetracycline and other antibiotics [25]. Aerobic

cultivation of enterobacteria from the gut of human volunteers free of antibiotics for over one year documented mean levels of resistance in excess of 20% to 13 different antibiotics [17]. Antibiotic treatment is expected to selectively increase the abundance of these organisms, leading to increased prevalence of their resistance genes and hence increasing the likelihood of their dissemination via lateral gene transfer. Indeed, a cultivation-based study documented significant increases in amoxicillin resistance levels in children exposed to this antibiotic in the three months after exposure, compared to a non-exposed control group [26].

It has long been hypothesized that resistance elements confer a fitness disadvantage to the host, and hence resistant strains should be outcompeted by susceptible counterparts once antibiotic pressure is removed [27]. Unfortunately, there is a growing appreciation that antibiotic resistance can persist for long periods in absence of treatment, as highlighted by the basal levels of resistance in the microbiota described above. Cultivation based studies have shown that treatment with clarithromycin leads to high level resistance within intestinal *Enterococcus sp.* during treatment and that the highly resistant *Enteroccocus sp.* persisted in several patients for 1-3 years after treatment ended [28]. Similar results have been obtained for *Staphyllococcus epidermis* isolated from the nostrils of antibiotic treated patients [29] and *Bacteriodes sp.* isolated from the gut [30]. Furthermore, a culture independent PCR assay showed that the abundance of specific macrolide resistance genes had increased up to 10,000 fold compared to pre-treatment levels, and persisted up to two years after cessation of antibiotic treatment [31].

Mechanisms underlying the persistence of antibiotic resistance are complex and include compensatory adaptations and co-selection [32-35]. An elegant demonstration of how compensatory mutations lead to a reduced fitness cost associated with antibiotic resistance gene carriage was shown for two lineages of *Bacteroides thetaiotaomicron* from the human gut microbiota [36]. In individuals exposed to clindamycin over seven days, a dominant clone of *B. thetaiotaomicron* acquired the *erm*G or F resistance gene, initially leading to a fitness cost in absence of antibiotic selection. However, resistant clones from this lineage isolated two weeks later had significantly reduced fitness cost associated with the carriage of this resistance gene, highlighting the rapid adaptations that can occur in vivo [36]. Interestingly studies on the persistence of antibiotic resistance after cessation of antibiotic therapy in livestock microbiota document more drastic reductions in the prevalence of antibiotic resistance compared to those shown in the human microbiota [37]. The larger reductions in antibiotic resistance levels in livestock microbiota may result from the turnover of the sampled individuals; yet even under these conditions of rapid turnover, antibiotic resistance genes are expected to persist in life stock microbiota for decades after abolishment of antibiotic use [32].

Transfer of antibiotic resistance determinants

The dissemination of antibiotic resistance genes necessitates the physical transfer or acquisition of the genetic element encoding antibiotic resistance. The mechanisms responsible for this transfer are conjugation, transformation or transduction, all of which are fully capable of transferring antibiotic resistance determinants *in vitro* from resistant donor organisms to susceptible recipient organisms [21,38]. Such transfer events have been documented for strains isolated from virtually any reservoir *in vitro* under laboratory conditions. More complex *in vitro* systems mimicking the biofilm community structure of the oral microbiota have also been used to demonstrate transfer of antibiotic resistance genes between distant genera through both conjugation as well as transformation [39]. *In vitro* fermentation systems simulating the human intestinal system have also been used for the investigation of the horizontal transfer of extended spectrum beta-lactamases. While transfer of resistance genes was accelerated greatly by the administration of cefotaxime to which the donor strain harbored resistance, a base line of transconjugants were present shortly after introduction of the resistant donor strain even in absence of antibiotic selection [40].

To extend these *in vitro* findings, a number of groups have studied the *in vivo* transfer of antibiotic resistance genes in animal models. Germ free mice colonized with enterococcal donor and recipient strains were used to demonstrate the conjugal transfer of vancomycin resistance with appreciable rates and resulting in a stable population of transconjugants [41]. However, transfer occurred at much lower rates in mice colonized with the complex microbiota from feces recovered from healthy human subjects. Indeed transfer events were only documented between strains of the same species and these transconjugants did not stably colonize the microbiota [41]. The strong preferential transfer of vancomycin resistance to closely related strains was also recently confirmed for a larger array of clinical gram positive isolates *in vitro* [42]. Recently, transfer of the extended spectrum beta lactamase CTX-M-9 was shown in rats colonized with human microbiota between strains of *Salmonella enterica* and *Escherichia coli*; however, the transconjugants were again only transiently present in the rat microbiome and only during treatment with selective antibiotics [43].

Notably, a series of experiments have directly investigated the transfer of antibiotic resistance genes within the gut microbiota of healthy human subjects. In one study healthy human volunteers ingested cultures of *Enterococcus faecium* resistant to either glycopeptide or streptogramin antibiotics. Fecal samples from the individuals were sampled using cultivation-based methods and enterococcal isolates resistant to virginiamycin or vancomycin were enumerated. It was found that the ingested resistant strains were able to multiply and persist in the human gut environment for 7 days; however, no transfer events were detected as all resistant isolates were identical to the ingested strains based on the pulsed field gel electrophoresis pattern [22]. These experiments were

followed up with a study demonstrating the *in vivo* transfer of the *van*A resistance determinant from a donor *Enterococcus faecium* strain of animal origin to a recipient *Enterococcus faecium* strain of human origin in the intestine of healthy human subjects [44]. Similar results were shown for the sulfonamide resistance gene *sul*2 within strains of *E. coli* in the human intestine [45].

The *in vivo* studies on antibiotic resistance transfer highlight the importance not only of the transfer event but also the subsequent ability of the strain to establish within the microbiota. While there is a growing body of research documenting persistent effects on the microbiome community structure and the resistance levels within the microbiota, there is limited support yet for this from controlled *in vivo* studies in both animal and human microbiota. This apparent discrepancy may result from the somewhat artificial conditions of controlled *in vivo* studies where donor and recipient strains from a different microbiota are introduced to an already established microbiota. The stability of the established microbiota and the foreign origin of donor and recipient strains may be an important factor explaining the contrasting results. Another consequence of focusing the attention on predefined donor and recipient strains in controlled *in vivo* experiments so far is that the dissemination of antibiotic resistance genes within the total microbial community is not quantified.

Microbiome wide characterization of the antibiotic resistome

Previous culture based studies have thus clearly established that the human commensal microbiota harbors many multidrug resistant isolates, whose load of antibiotic resistance genes have increased steadily over the past decades. However, the majority of constituents in the microbiota have remained inaccessible to traditional culturing techniques, and molecular methods have estimated the gastrointestinal tract is home to nearly 1000 different species and over 7000 different strains [4,10], indicating our picture of the antibiotic resistome of the human microbiota from culturing-studies is incomplete. In collaboration with Gordon and colleagues, we (Dantas) recently demonstrated that use of strict anaerobic conditions and a specially formulated rich gut microbiota medium enabled culturing of over 70% of genus level OTUs observed in corresponding uncultured "complete" microbiota [46]. While this is undoubtedly a dramatic improvement over past estimates of microbiota "culturability", the need for culture-independent methods for complete characterization of the resistome remains acute.

The most commonly used culture-independent method for detection and quantification of antibiotic resistance genes in polymicrobial samples is PCR with primers homologous to known resistance gene sequences [47] (Fig 2B). For example, quantitative PCR (qPCR) screens were recently used to quantify the levels of *tet* and *erm* genes, conferring resistance to tetracycline and macrolide, lincosamide, and streptogramin B antibiotics respectively, in animal and human

waste water [48], highlighting potential routes of dissemination of resistance genes between human, animal and environmental microbiota. However, the extraordinary specificity of PCR based studies is also an important limitation of the technique: because PCR can only be used to interrogate a sample for known genes, it is an ineffective method for identifying novel resistance genes.

A complementary culture-independent method which enables characterization of both known and novel resistance genes is termed 'metagenomic functional selection' (Fig 2B), wherein total community microbial (metagenomic) DNA is extracted from any arbitrary source (e.g. a fecal microbiota sample) and cloned into an antibiotic susceptible indicator strain (e.g. *E. coli*) [17,47]. Indicator strains transformed with DNA that encode resistance genes can be functionally selected by plating the recombinant library on antibiotic media. Functionally selected metagenomic DNA inserts are amplified and sequenced to identify the captured resistance genes. We use the phrase *metagenomic functional selections* to specifically refer to the idea of subjecting metagenomic DNA to an experimental functional assay, generally through shot-gun expression in a heterologous host [49], in an attempt to distinguish this approach from broader definitions of "*functional metagenomics*", which include sophisticated computational approaches for annotation of functions in shot-gun metagenomic sequence data, but without direct experimental validation [50]. As with PCR-based screens, metagenomic functional selections were first developed and applied for resistome characterization of environmental microbial communities [24,47], and have subsequently been successfully applied to identify a variety of enzymatic functions from cultured and uncultured microbes, including genes and pathways for degradation of or resistance towards numerous xenobiotics [51,52].

We have used metagenomic functional selections to demonstrate that the resistome of the human gut microbiota had been profoundly under-sampled using traditional culture-based methods [17,49]. We identified several hundred antibiotic resistance genes within the microbial communities of two healthy, unrelated adults. Over 95 % of the resistance genes in cultured Proteobacterial isolates from these microbiota were highly similar to previously characterized genes (>90 % nucleotide identity), many of which had been identified in pathogens. In sharp contrast, application of the same method to directly interrogate uncultured microbiota from the same fecal samples yielded predominantly novel resistance genes, with low sequence identity to previously characterized genes (<61 % nucleotide identity). Furthermore, while over 65 % of the resistance genes derived from cultured isolates were highly similar between the two individuals, <10 % of the resistance genes from culture-independent sampling were similar between these two individuals. Our results exposed the substantial under-sampling of resistance genes in prior studies of human microbiota, which stemmed from a reliance on culture-dependent methods. Importantly, our data underscore the utility of metagenomic functional selections for resistance gene discovery and obtaining a culture-unbiased depiction of the diverse and personalized resistomes of the microbiota in humans.

With Gordon and co-workers, we (Dantas) recently showed that inter-individual differences in human microbiota antibiotic resistomes could be detected by subjecting both uncultured 'complete' fecal microbiota as well as pools of phylogenetically-representative fecal culture collections to metagenomic functional selections [46]. Notably, the metagenomic detection of specific resistance genes (e.g. encoding amikacin resistance) in complete samples significantly correlated with the fraction of sample-matched cultured isolates phenotypically resistant to those compounds, and the exact genes were then reconfirmed to be present in those cultured isolates by PCR. Additionally, when transplanted into gnotobiotic mice, complete and cultured communities exhibited similar colonization dynamics, biogeographical distribution, and responses to dietary perturbations, and these personalized culture collections could be enriched *in vivo* for taxa suited to specific perturbations. These methods for careful recapitulation and manipulation of microbiota both *in vitro* and *in vivo* hold great promise for quantifying the phylogenetic specificity of specific resistance genes and interrogating correlations between prevalence and dynamics of antibiotic resistomes and specific genetic and environmental variables.

CONCLUSION:

Antibiotic treatment leads to pervasive changes in the microbial community structure and persistent increases in antibiotic resistance amongst sampled cultivatable species. This results from the rapid proliferation of antibiotic resistant strains present in low basal levels in the microbiota and through horizontal transfer of antibiotic resistance genes. While advances in sequencing technology have driven increases in our knowledge of the effects of antibiotic treatment on total community architecture, we still have a limited understanding of the evolutionary pathways through which the genetic elements encoding antibiotic resistance spreads within a community. However, the application of metagenomic functional selections, novel cultivation methods and high throughput sequencing on microbiota exposed to antibiotics should enable a more detailed understanding of the processes governing the interplay between antibiotics and the resistant microbiome and its contribution to clinical problems with antibiotic resistance.

FIGURE LEGENDS

Figure 1: **The effects of antibiotic treatment on the microbiota**. (A) Prior to antibiotic selection the constituents of the microbiota range in sensitivities towards antibiotics from sensitive (blue, green and yellow) to resistant (red). During exposure susceptible organisms will decrease in abundance, unless they acquire resistance determinants from viable resistant organisms. Upon cessation of treatment some of the susceptible organisms may increase in abundance to previous levels, whereas others will be lost from the microbiota. (B) During antibiotic exposure the microbial community architecture is significantly altered due to the differing sensitivities of the constituent microbes. Following cessation of antibiotic treatment community architecture largely re-establishes yet certain changes persist. (C) Schematic depiction of the changes in species diversity and antibiotic resistance levels upon antibiotic treatment followed by partial recovery of microbial community structure and resistance levels upon cessation of treatment.

Figure 2: **Schematic representation of methods for characterizing antibiotic resistance from human fecal microbiota.** (A) The traditional method for characterizing antibiotic resistant isolates from the fecal microbiota begins with phenotypic growth assays of individual cultured clones on antibiotic-containing media to determine the minimum inhibitory concentration (MIC). Resistance genes are detected by either PCR amplification based on the sequence of known genes or by whole genome sequencing followed by computational annotation of resistance genes using homology to known genes. (B) Culture-independent resistome approaches enable microbiota-wide characterization of resistance genes. Detection and quantification of known resistance genes can be accomplished by PCR. Characterization of both known and novel resistance genes can be accomplished through metagenomic functional selections, wherein metagenomic DNA is directly extracted from the fecal sample and cloned into an expression system in a cultivable, genetically-tractable host strain (e.g. E. coli). Metagenomic transformants harboring DNA fragments that encode antibiotic resistance genes are selected by subjecting the library of clones to specific antibiotics at concentrations which inhibit the growth of the untransformed indicator strain. Selected DNA fragments can then be sequenced to identify the specific resistance genes.

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(**) High-throughput anaerobic culturing techniques are coupled with gnotobiotic animal husbandry and metagenomics to show that the human fecal microbiota consists largely of taxa and predicted functions that are represented in its readily cultured members. Metagenomic functional selections were used to detect inter-individual differences in the resistomes of both uncultured 'complete' fecal microbiota as well as pools of phylogenetically-representative fecal culture collections.

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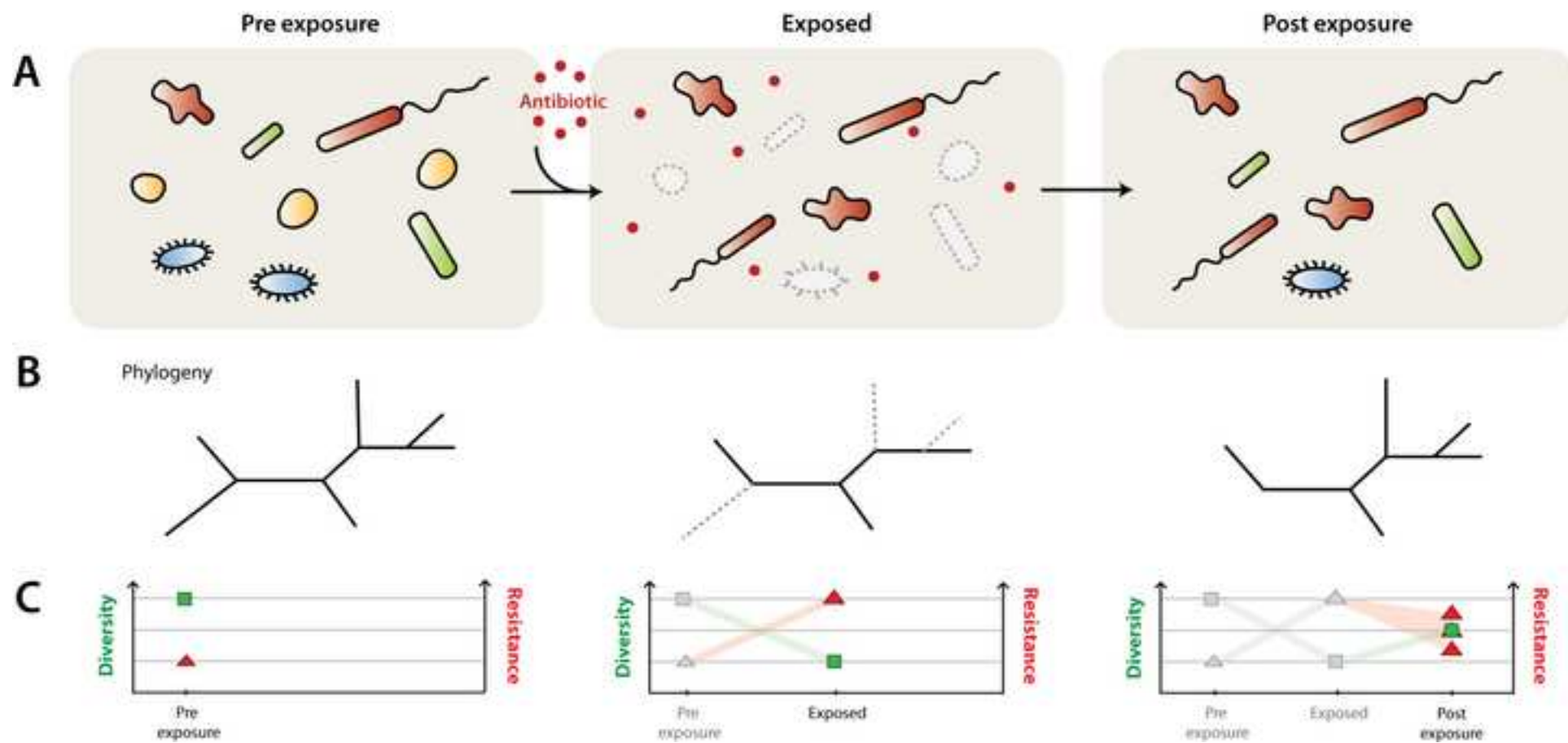51. Uchiyama T, Miyazaki K: **Functional metagenomics for enzyme discovery: challenges to efficient screening**. *Curr Opin Biotechnol* 2009, **20**:616-622.
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Highlights:
1) Evolution of antibiotic resistance in pathogens has become a global public health threat
2) Microbiome wide characterization of resistance genes supports recent genetic exchange between the resistant microbiota and pathogens
3) Treatment with antibiotics leads to pervasive changes in the composition of the human microbiota and its encoded resistome
4) In vivo studies have demonstrated transfer of antibiotic resistance genes within the microbiota even in absence of exposure
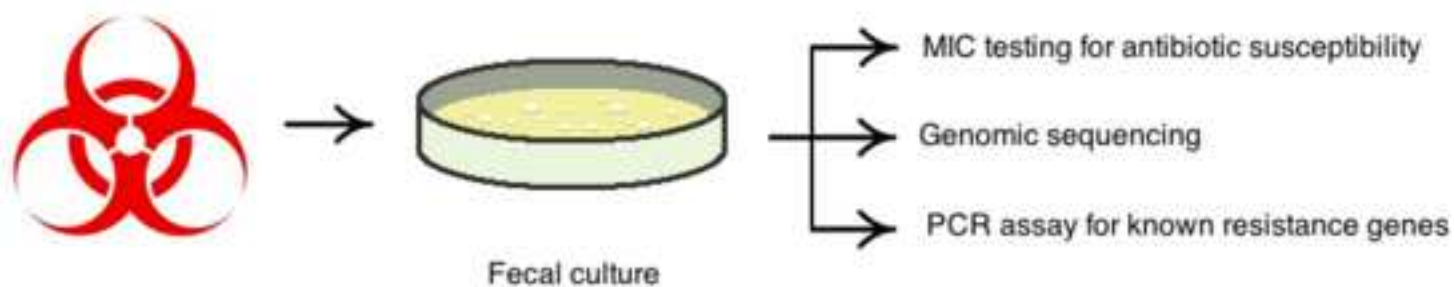5) Enrichment of resistance genes in the microbiota is likely to exacerbate problems with pathogenic resistance

Pre exposure
Exposed
Post exposure

A

Antibiotic

B   Phylogeny

C   Diversity   Resistance   Pre exposure   Exposed   Pre exposure   Exposed   Post exposure

Culture-dependent

Fecal culture

- MIC testing for antibiotic susceptibility
- Genomic sequencing
- PCR assay for known resistance genes

Culture-independent

Fecal sample

Extracted genomic DNA

PCR assay for known resistance genes

~2kb fragments of genomic DNA

Genomic DNA fragments ligated into plasmid vector

Transfection of indicator host strain (e. g. *E. coli*)

Functional selection on antibiotic-containing growth media

PCR amplification of selected resistance genes

CCAGTAC

Sequencing and annotation