

Human intestinal bacteria as reservoirs for antibiotic resistance genes

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Human intestinal bacteria have many roles in human health, most of which are beneficial or neutral for the host. In this review, we explore a more sinister side of intestinal bacteria; their role as traffickers in antibiotic resistance genes. Evidence is accumulating to support the hypothesis that intestinal bacteria not only exchange resistance genes among themselves but might also interact with bacteria that are passing through the colon, causing these bacteria to acquire and transmit antibiotic resistance genes.

Until recently, bacterial pathogens were the primary focus of studies of antibiotic resistance genes and their spread. Now, scientists are starting to wonder whether this focus is too narrow. Could the microflora of the human colon, normally considered innocuous or beneficial, be playing a more sinister role in human health as reservoirs for antibiotic resistance genes? The reservoir hypothesis is depicted in Figure 1. According to this view, human intestinal bacteria not only share resistance genes among themselves but can also acquire from or donate resistance genes to bacteria that are just passing through the intestine [1–3]. The possibility that resistance gene spread in the human colon might be a serious threat to human health was first raised in connection with post-surgical infections, which are usually caused by the normal microflora of the patient or the patient's caretakers [4,5]. Recently, concern about resistance gene transfers in the human colon has expanded to include agriculture [6,7]

Farm to fork and beyond

There is no question that feeding antibiotics to livestock to enhance an animal's growth selects for antibiotic resistant bacteria in the animal's intestine [8–10], but to what extent are such bacteria a threat to human health? After all, farms are located at a considerable distance from places, such as cities, where high concentrations of people are found. Nonetheless, there is a very significant link between farm and city: the food supply. It is now well established that antibiotic resistant bacteria from chickens, pigs and cattle enter the food supply and can be found in meat offered for sale in supermarkets [11–13].

If these foods are not properly cooked, the resistant bacteria will enter the intestinal tracts of consumers and will have the opportunity to commingle with members of the resident human microflora [5,14]. Also passing

through the human colon on a regular basis are pathogens such as *Streptococcus pneumoniae* and *Staphylococcus aureus*; these bacteria are normally found in the nose or throat but can pass through the colon if swallowed [15]. Until recently, such bacteria were thought to be transients that spent little time in the human colon, but some recent reports suggest that *S. aureus* might transiently or persistently colonize the human colon in low numbers, especially in hospitalized patients [16,17]. In addition to such pathogenic transients, there are potentially pathogenic members of the intestinal microflora itself, such as *Escherichia coli*, *Enterococcus* species, *Clostridium* species and *Bacteroides* species [18,19]. Is it possible that such diverse bacteria can and do regularly exchange DNA under conditions found in the human colon?

Assessing the extent to which resistance gene transfer actually occurs in the human colon

How can the actual extent of resistance gene transfer in the human colon be assessed? One approach would be to feed people resistant animal bacteria, then determine whether genes carried by these bacteria enter human colonic bacteria. This approach has not been taken for two obvious reasons. First, such an experiment would be considered unethical in most countries. Second, such a study would be prohibitively expensive, especially in view of the fact that it is not clear how long the duration of the sample collection period should be. Such a study could alternatively be done in laboratory animals. Notably,

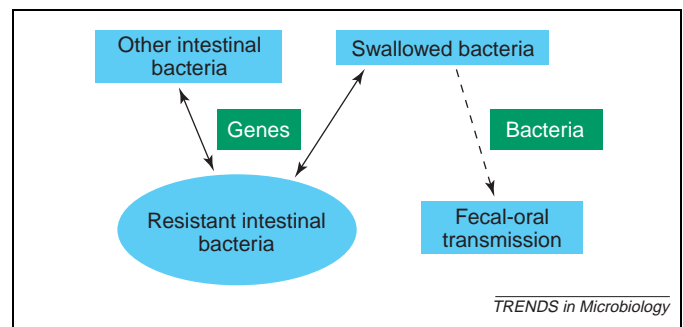


Figure 1. The resistance gene reservoir hypothesis. Bacteria that normally reside in the human colon, most of which are normally benign, transfer resistance genes among themselves. This type of transfer becomes a problem if the commensals, many of which are opportunistic pathogens, go on to cause post-surgical infections. Bacteria that are merely passing through the human colon will be in transit through the colon long enough to transfer or acquire genes by conjugation. These bacteria might return to the sites where they are usually found (e.g. the mouth and skin) by contamination of these sites with excreted bacteria. Gene transfer could be occurring in the mouth, where thick biofilms are found, but here we focus on the colon for simplicity.

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there have been a few reports of resistance gene transfer in laboratory animals. For example, transfer of antibiotic resistance genes among enterococci has been demonstrated in germfree rodents [16]. In another study, a species of *Lactococcus* was eliminated from the normal microflora of rodents and replaced by an antibiotic resistant strain of the same species [20]. Transfer of a plasmid carrying an antibiotic resistance gene was demonstrated in this model. A problem with interpreting the results of studies that use laboratory rodents is that the normal microflora of rodents is different from that of humans. Also, the use of germfree animals eliminates the bulk of the microflora that is normally present and focuses attention on normally minor populations that in the germfree animal become the predominant bacteria.

Given the problems inherent in such prospective studies, a second, retrospective approach has been taken, where DNA sequences were determined for resistance genes found in different bacteria in the human colon or in other sites and were compared. The assumption is that if genes found in two different bacterial species are at least 95% identical, then this gene must have been transferred horizontally by one means or another. The 95% cutoff is somewhat arbitrary, but finding nearly identical sequences in different species rules out the possibility of convergent evolution, where selective pressures on different bacteria produce proteins that are virtually identical even though they evolved separately. Because selection is for a specific amino acid sequence and not for a specific DNA sequence, third base differences enable the DNA sequences that encode identical proteins to differ by more than 90%.

Getting the goods on intestinal anaerobes

In our studies, we chose to focus on *Bacteroides* species. *Bacteroides* species account for ~25% of the bacteria in the human colon [21,22]. Because of their high concentration, they appear most likely to be involved in horizontal gene transfer events. *Bacteroides* species harbor two types of conjugative elements: conjugative plasmids [23–28] and conjugative transposons [29–31]. Initially, two resistance genes were monitored in natural isolates of *Bacteroides* species: *tetQ* and *ermF* [32,33]. To date, *tetQ* has only been found on conjugative transposons in *Bacteroides* spp. The *ermF* gene has been found on both conjugative transposons and conjugative plasmids [34–37]. A survey of 289 strains, representing more than 10 different *Bacteroides* species, revealed that in the period before 1970, 20–30% of isolates carried *tetQ*, whereas *ermF* was found only rarely. By contrast, in the 1990 s, over 80% of strains carried *tetQ* and 15% carried *ermF* (Table 1) [35]. DNA sequence analysis of a selected

subgroup of these strains revealed that all the *tetQ* genes were at least 94% identical and the *ermF* genes had even fewer sequence differences. The increased carriage of these genes over the past three decades indicated that horizontal transfer was occurring frequently enough to have spread these genes widely over a time period that is small in evolutionary terms [35]. In addition, the fact that the genes were found in people with no recent history of antibiotic use (community isolates) indicates that these genes are maintained stably once they are acquired. This is bad news for those who have accepted as an article of faith that susceptible strains are inherently more fit than resistant ones and that stopping use of an antibiotic would inevitably result in the disappearance of the resistant strain. Importantly, stably maintained antibiotic resistance genes are fairly common and in many cases resistant bacteria hold their own quite well when faced with competition with resistant strains [38].

To determine what type of element was transferring *tetQ*, DNA from some of the strains carrying *tetQ* was probed with DNA from a conjugative transposon known to carry *tetQ*, known as CTnDOT. The digest pattern on a Southern blot was consistent with *tetQ* being part of a conjugative transposon in the CTnDOT family [39]. CTnDOT and related conjugative transposons have an unusual feature. Their transfer is stimulated 100–1000 fold by tetracycline [40,41]. Therefore, it is possible that the extensive transfer of *tetQ*, which has occurred during the past three decades, was triggered by the use of tetracycline.

However, there could be other stimulatory conditions. A surprising finding of this survey was that even during the pre-1970 years, when tetracycline had not yet been widely used, the carriage of *tetQ* was as high as 20–30% (Table 1). Similar to the strains found in the 1990 s, *tetQ* was carried on a CTnDOT type element in the earlier isolates. Consequently, horizontal gene transfer was occurring even before heavy use of tetracycline was begun [35].

Another surprising outcome of this survey was the finding that two other *erm* genes in addition to *ermF* – *ermB* and *ermG* [35,42] – had moved into *Bacteroides* species during the period between 1970 and the 1990 s (Table 1). These *erm* genes had previously been associated almost exclusively with Gram-positive bacteria, not with the Gram-negative *Bacteroides* species [43–45]. Subsequent studies have shown that *ermB* and *ermG* are carried on conjugative transposons that are unrelated at the DNA sequence level to any conjugative transposons found in *Bacteroides* species to date [46,47]. This raises the possibility that *ermB* and *ermG* have entered *Bacteroides* species from Gram-positive bacteria.

Table 1. Prevalence of *tetQ*, *ermF*, *ermG* and *ermB* genes in colonic *Bacteroides* spp.

Isolates	<i>ermB</i> (%)	<i>ermG</i> (%)	<i>ermF</i> (%)	<i>tetQ</i> (%)
Community (pre-1970) ^a	0	0	0	32
Clinical (pre-1970) ^b	0	0	9	22
Community (1996–1997) ^a	3	8	15	81
Clinical (1980–1995) ^b	3	18	30	86

^a*Bacteroides* isolates from the colon of people who were healthy and did not have a history of recent antibiotic use.

^bIsolates from people with *Bacteroides* infections.

Transactions between major populations of intestinal Gram-positive and Gram-negative bacteria

As already mentioned, *Bacteroides* species account for ~20–30% of bacteria isolated from the human colon. Most of the remaining 70–80% of colonic isolates consists of poorly characterized Gram-positive anaerobes. The well-studied facultative species, such as *E. coli* and the enterococci, are numerically minor, constituting less than 1% of colonic isolates [21]. A question that needs to be answered is what types of conjugative elements are found in the Gram-positive anaerobes, and are these bacteria participating in a significant way in the exchange of antibiotic resistance genes among bacteria in the human or animal intestinal tract?

The original question raised in this article was whether members of the normal flora of the human intestine could exchange genes with bacterial pathogens that might be present in low numbers or just passing through the intestine. Evidence that such transfers can and do happen is summarized in Figure 2, where several genes are shown along with the genera in which they have been found. In the case of the *erm* genes, the sequence identity of the genes found in different species is usually 99% or higher. The *ermB* gene has been found in a variety of pathogenic Gram-positive bacteria, including *Streptococcus pneumoniae* and *Clostridium perfringens* [45,48]. The *ermB* and *ermG* genes were found in more than one species, therefore these genes appear to have entered *Bacteroides* species more than once in the past [35,47]. One of the few reports that implicates the human colonic Gram-positive anaerobes in resistance gene transfer is a recent report of vancomycin resistance genes, such as those found in pathogenic enterococci, in the colonic anaerobe *Clostridium innocuum* [49].

It is also worth noting that bacteria from different sites appear to be exchanging genes. For example, *Porphyromonas gingivalis* is an oral anaerobe and *Prevotella ruminicola* is normally found in the rumen of cattle and the intestines of pigs. It is easy to imagine how *P. gingivalis* might be involved in gene transfers because it is constantly being swallowed, and strains from the colon could be reintroduced into the mouth by the fecal-oral route. How a rumen anaerobe, which is very sensitive to oxygen, made contact with human colonic bacteria (if that is where *P. ruminicola* picked up *tetQ*) and then made it back into the animal is harder to imagine.

A caveat is in order. The strategy of using virtually identical genes found in different genera and species to deduce that there is some genetic conduit open between those species has a couple of limitations. First, it is usually not possible to ascertain the direction of the transfer. The only reason we feel comfortable about saying that *ermG* and *ermB* appear to have entered *Bacteroides* species from some other species of bacteria is that the pre-1970 strains did not carry these genes. A second limitation is that there is no way to ascertain how many transfers it took for a gene to move between two of the species shown in Figure 2. Moreover, there are almost certainly unknown players, such as the Gram positive colonic anaerobes and even soil bacteria.

Back to the farm

Research of the sort described in this review has been controversial because it can be interpreted as support for concerns about possible effects of agricultural use of antibiotics on bacteria that cause human infections. People in the animal agricultural field are quick to point out that currently there is no 'smoking gun' linking the use of antibiotics on the farm with the appearance of resistance genes in human pathogens and subsequent treatment failures resulting from agriculture-associated resistance gene transfer. It is important to remember, however, that absence of evidence is not the same as evidence of absence. The diversity of bacteria found in the various microfloras of the human and animal body is staggering. Most of these bacteria and the resistance genes they carry have not been studied a great deal. The fact that conjugative transposons, which were unknown until recently, appear to be a driving force in the transfer of antibiotic resistance genes in the human body is unexpected and illustrates the principle that scientists know a lot less than they think they do about mechanisms of horizontal gene transfer in nature [29,50,51]. It is important that we establish what selective or stimulatory pressures are driving the spread of antibiotic resistance genes so that we can assess the relative contributions of different types of antibiotic use or other human activities to this spread. Only then will it be possible to design effective strategies for preventing further increases in the incidence of antibiotic-resistant bacteria.

Beyond antibiotic resistance genes

This article has focused on the transfer of antibiotic resistance genes in nature, but gene transfer among

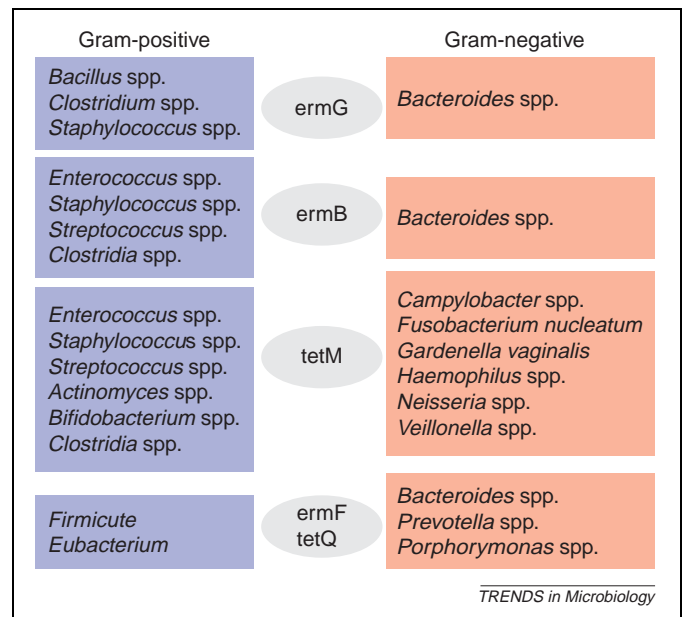


Figure 2. Evidence that transfer of resistance genes occurs between Gram-positive and Gram-negative bacteria in the mammalian colon and in other environmental sites. This evidence consists of finding virtually identical resistance genes in natural isolates representing different bacterial species. The resistance gene is contained in the oval that connects the Gram-positive (left boxes) and Gram-negative (right boxes) bacterial species, in which virtually identical copies of the resistance gene have been found. Examples of possible transfer events are shown here, however, this is not meant as a complete listing of all cases found in the literature.

bacteria has broader reaching consequences. Plasmids and conjugative transposons carry genes in addition to antibiotic resistance genes, such as nitrogen fixation genes that can alter the metabolic potential of a bacterial cell [52,53]. Conjugal elements can also carry virulence factors, such as toxin genes. For example, plasmids found in *Bacillus anthracis* (pOX1 and pOX2) have made this species much more pathogenic than its very close relative *Bacillus cereus* [54]. *Yersinia pestis*, the cause of plague, and *Salmonella typhimurium* strain LT2 have also acquired plasmids that make them virulent for humans. Perhaps the most spectacular example of horizontal gene transfer to date is the 500 kbp conjugative transposon of *Mesorhizobium loti* strain R7A that carries genes important for symbiosis between the rhizobia and plants [55,56]. There are bacteria that have chromosomes that are only ~500 bp in size [57], therefore the transfer of this large conjugative transposon in soil is equivalent to the transfer of an entire bacterial chromosome. However, despite all of this rampant 'bacterial sex', horizontal gene transfer does not appear to have homogenized bacteria. Genetic diversity and a well-defined phylogenetic tree for bacteria are still the rule rather than the exception [58].

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