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The role of conjugative transposons in spreading antibiotic resistance between bacteria that inhabit the gastrointestinal tract

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Abstract. There is huge potential for genetic exchange to occur within the dense, diverse anaerobic microbial population inhabiting the gastrointestinal tract (GIT) of humans and animals. However, the incidence of conjugative transposons (CTns) and the antibiotic resistance genes they carry has not been well studied among this population. Since any incoming bacteria, including pathogens, can access this reservoir of genes, this oversight would appear to be an important one. Recent evidence has shown that anaerobic bacteria native to the rumen or hindgut harbour both novel antibiotic resistance genes and novel conjugative transposons. These CTns, and previously characterized CTns, can be transferred to a wide range of commensal bacteria under laboratory and in vivo conditions. The main evidence that gene transfer occurs widely in vivo between GIT bacteria, and between GIT bacteria and pathogenic bacteria, is that identical resistance genes are present in diverse bacterial species from different hosts.

Key words. Anaerobic; commensal bacteria; conjugative transposons; Tn*B1230*; tetracycline resistance; gene transfer; gastrointestinal tract; rumen.

Introduction

As will have become clear from the other chapters in this multi-author review, conjugative transposons (CTns) are now recognised to be as important for the dissemination of antibiotic resistance genes as conjugative plasmids. Not all CTns encode antibiotic resistance; those identified in environmental bacteria often encode resistance to aromatic compounds (toluene, phenols, chlorobenzene), heavy metals (e.g. mercury), or contain mobile catabolic genes responsible for the degradation of xenobiotic compounds [1]. In general, CTns encode functions that are essential for survival of bacteria under specific environmental conditions, e.g. antibiotic resistance. Horizontal gene transfer events involving CTns contribute to both long-term bacterial evolution and short-term adaptation, enabling rapid responses to environmental changes. The elucidation of more and more complete bacterial genome sequences will undoubtedly add to our knowledge of the extent of gene transfer between related and unrelated bacterial species.

Virtually every CTn encoding antibiotic resistance (ab^R) described in the literature has been identified in pathogenic bacteria, including pathogenic strains of *Bacteroides*. There is a corresponding dearth of information on the incidence of transposons in environmental or commensal anaerobic gut bacteria, and it is this latter group that this article will focus on. It was previously thought that carriage of ab^R genes slowed bacterial growth and consequently that they would be lost in the absence of any selective pressure. However, it has since been recognised that bacteria can adapt and overcome any disadvantage imposed by the presence of ab^R genes [e.g. 2, 3; see article by Rice, this issue], a fact contributing to their persistence. Adaptations may include introducing compensatory mutations in genes, or controlling expression of the resistance genes so that they are activated only in the presence of the antibiotic [e.g. 4]. Consequently, CTns often encode ab^R genes linked to sensitive regulatory systems.

Conditions in the gastrointestinal tract (GIT) strongly favour intra- or interspecies gene transfer. Both the rumen

and monogastric hindgut contain dense microbial populations which exist in close proximity, and which are often immobilized in biofilms. Gut environments are frequently exposed to low levels of antibiotics (as growth promoters, therapeutic agents or even residues contained in food) which are known to stimulate the transfer of CTns such as Tn*916* [5] and CTnDOT [6]. Therefore, it is clear that transfer of CTns under gut conditions is likely to be a widespread phenomenon. Any such transfer events would tend to increase the number of antibiotic-resistant bacteria in the GIT. The broad host range of most CTns means that they are unlikely to be confined to particular species, and could potentially transfer between unrelated bacteria, including between commensal and pathogenic bacterial species. Indeed, several studies have illustrated the presence of similar genetic elements in the resident commensal microflora and transient inhabitants, including clinical isolates [7–9]. Thus, ignoring the incidence of CTns in the commensal microflora equates to ignoring a substantial part of the bacterial gene pool.

In this article, the evidence that exists for the presence of CTns in commensal anaerobic gut bacteria, excluding *Bacteroides* spp. [see article by Whittle et al., this issue], will be presented. Examples will be used to show the relationships between these transposons, and the antibiotic resistance genes they encode, and those found in pathogens, illustrating that an open route for gene flow does exist. Finally, the potential of CTns as tools for the genetic manipulation of commensal anaerobes will be considered.

Conjugative transposons initially identified in obligately anaerobic commensal bacteria

Identifying the original host for any conjugative transposon is difficult, as a bacterial species initially shown to contain a new CTn may not be the species where the CTn evolved. However, for the sake of this review, the 'original host' shall be considered to be the bacterium in which the CTn was first characterised. Several transposons, including the prototypical CTn, Tn*916* [10, 11], discussed elsewhere in this multi-author review, have been found among the oxygen-tolerant inhabitants of the GIT. In this chapter we will concentrate on CTns carried by members of the commensal GIT microflora that are obligate anaerobes.

Recent work in our laboratory investigating tetracycline resistance among GIT anaerobes has identified two conjugative transposons among the commensal GIT microflora. Rumen *Butyrivibrio fibrisolvens* strain 1.230 was able to transfer Tc^R to a rifampicin-resistant mutant (2221^R) of the type strain of *B. fibrisolvens* 2221 at high frequencies in anaerobic filter matings [12]. Analysis of the transconjugants showed that Tc^R transfer was associ-

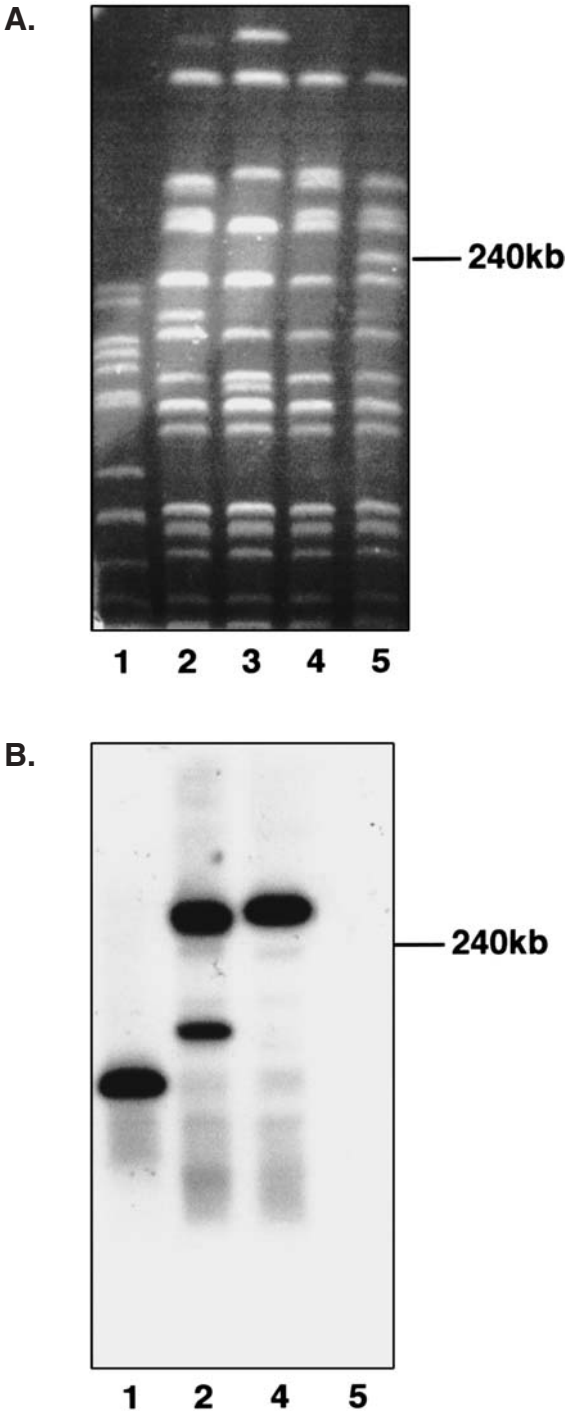

Figure 1. Pulsed-field gel electrophoresis (PFGE) analysis of transconjugants of 2221^R containing Tn*B1230.* (*A*) PFGE gel image. Lane 1, *B. fibrisolvens* 1.230 donor; lanes 2–4, 2221^R transconjugants Tc1, Tc2, Tc3; lane 5, 2221^R recipient strain. (*B*) Representative Southern blot of a PFGE gel hybridised to a polymerase chain reaction amplified fragment of *tet*(W). Lane 1, *B. fibrisolvens* 1.230 donor; lanes 2, 4, 2221^R transconjugants Tc1, Tc3; lane 5, 2221^R recipient strain. The preferred insertion site (a 240-kb fragment) is indicated.

ated with acquisition of a large chromosomal element, 45–50 kb in size, which inserted preferentially at a single site in the recipient genome, although secondary insertions were sometimes observed [13] (fig. 1). Tn*B1230* contains the novel ribosome protection type Tc^R gene, *tet*(W), which is only 68% identical at the amino acid level to its closest relatives Tet(O), Tet(M) and Tet(32) (fig. 2). Initial sequence analysis of Tn*B1230* [14] has identified several open reading frames (ORFs) with sequence identity ranging from 47 to 68% to ORFs on the 34 kb *Enterococcus faecalis* CTn Tn*1549,* known to encode proteins involved in excision and conjugative transfer [15] (fig. 3). Tn*1549* contains 11 contiguous ORFs and two in the opposite orientation, all involved in conjugal transfer. The arrangement of the ORFs in Tn*B1230* differs in that the conjugal transfer genes are interrupted by the resistance gene *tet*(W) (fig. 3). Tn*1549* confers vancomycin (Vm) resistance, and the *vanB* operon follows the genes for conjugal transfer. *B. fibrisolvens* strains containing Tn*B1230* are sensitive to vancomycin. The presence of ORFs on Tn*B1230* similar to others encoding proteins involved in conjugation provides compelling evidence that Tn*B1230* is indeed a novel conjugative transposon, the first to be identified in a rumen anaerobe. A lower level of identity (30%) was found between ORF3 and a transfer protein, traA, encoded on the lactococcal plasmid pMRC01 [14, 16]. The *tra*A gene is the first of a 19-kb operon encoding 16 proteins involved in conjugal transfer. Interestingly pMRC01 is composed of three distinct 20-kb regions, each with a different function, separated by insertion sequences (IS) [16]. The *tet*(W) gene is flanked by directly repeated sequences (DR1, DR2; fig. 3) capable of encoding small proteins with a possible catalytic core found on transposases encoded by IS elements [K. P. Scott et al., unpublished observations]. These regions may have been important in the evolution of the transposon Tn*B1230.* With the exception of the *tet*(W) gene (DNA % G + C = 53%), the other ORFs on Tn*B1230* have % G + C contents similar to that of the *B. fibrisolvens* host bacterium (36–41%) [14]. This reinforces the theory that the *tet*(W) gene integrated into an existent transposon, between some of the genes encoding conjugative transfer functions (fig. 3).

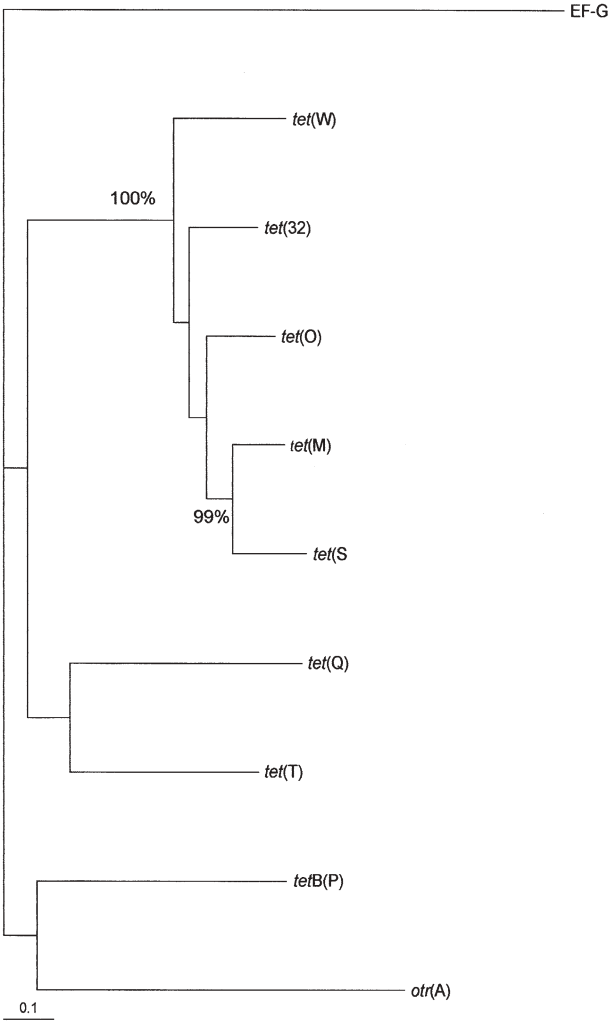

Figure 2. Phylogenetic tree illustrating the sequence relationships between the ribosome protection type of tetracycline resistance genes. The amino acid sequence of the *Aquifex aeolicus fus*A gene for translation factor EF-G was used to root the tree. Figures beside nodes indicate bootstrap values when greater than 95% (based on 500 trials). The scale bar refers to amino acid substitutions per position. Genbank accession numbers are as follows: *fus*A, AE000669; Tet(O), Y07780; Tet(M), U58986; Tet(S), X92946; Tet(W), AJ222769; Tet(32), AJ295238; Tet(Q), X58717; Tet(T), L42544; TetB(P), L20800; and Otr(A), X53401.

The rumen *B. fibrisolvens* strain 1.230 also contained a nonmobile *tet*(O) gene [13], identical in nucleotide sequence to *tet*(O) from *Streptococcus pneumoniae*. Both *tet*(O) and *tet*(W) are ribosome protection-type Tc^R genes. This is not the only case of a bacterium containing two genes encoding resistance to the same antibiotic by the same mechanism, but the advantage to the bacterium is unclear. Sequence analysis of the upstream regions of the *tet*(O) gene identified an ORF with 41% identity [K. P. Scott et al., unpublished observations] to the *tnp*V gene from the *C. perfringens* transposon Tn*4451* [17] (fig. 4). The function of TnpV is not known [17; see article by Adams et al., this issue] but Tn*4451* is mobilisable although not self-mobile, and it is interesting to speculate whether this is also the case for the *B. fibrisolvens tet*(O) gene.

Tetracycline resistance was transferable from a human gut anaerobe related to *Clostridium* spp. to the rumen *B. fibrisolvens* 2221^R in anaerobic laboratory matings [18], illustrating transfer across both the bacterial species and the host barrier. Transfer of Tc^R involved another new mobile chromosomal element that did not cross-hybridise to Tn*916* or Tn*B1230* sequences. Full characterisation of this element Tn*K10*, potentially a new conjugative transposon,

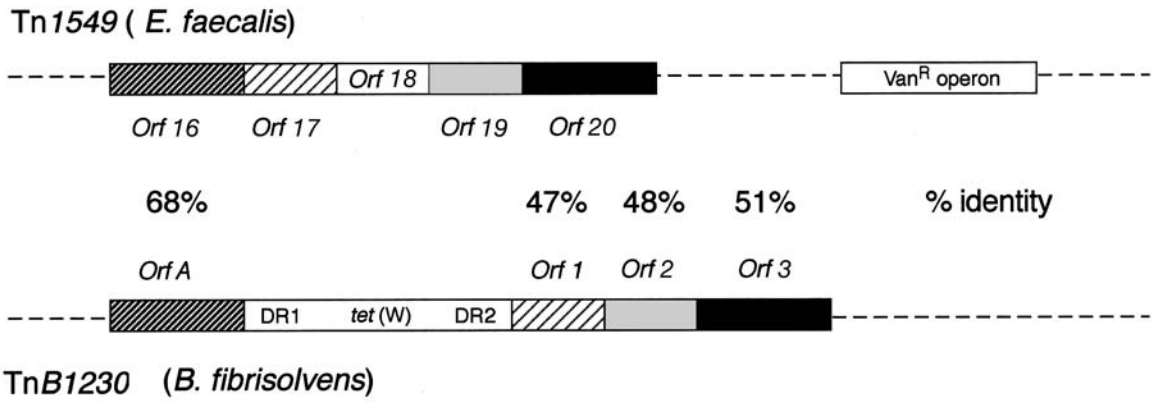

Figure 3. Diagrammatic arrangement of ORFs encoded by Tn*B1230* and Tn*1549*. The % identity between similar ORFs (illustrated by shading) is indicated, and the relative positions of the resistance genes are shown. DR1 and DR2 are directly repeated DNA sequences flanking *tet*(W), 713 nucleotides in length.

which contains the novel Tc^R gene *tet*(32) [18] is in progress. Tet(32) is 76% identical to Tet(O), its closest relative among the ribosome protection type of Tc^R proteins (fig. 2). *tet*(32) confers a much higher level of resistance in *B. fibrisolvens* 2221^R than *tet*(W) (MIC_{90} values for *tet*(32) and *tet*(W) are 200 µg/ml Tc and 90 µg/ml Tc, respectively [18]). It is difficult to compare the resistance level to other ribosome protection type of Tc^R genes due to strain : strain variation and the effect preexposure to tetracycline may have on the resistance level [19].

The isolation of novel conjugative transposons, associated with novel Tc^R genes, among the commensal microflora in both the rumen and human GIT is convincing evidence that commensal bacteria play an important role in gene dissemination, and consequently bacterial evolution.

Conjugative transposons have also been found in bacteria used as starter cultures in food preparation, particularly lactic acid bacteria. These bacteria are facultative anaerobes and will not be discussed further. However, any bacteria ingested with food are potential transient inhabitants

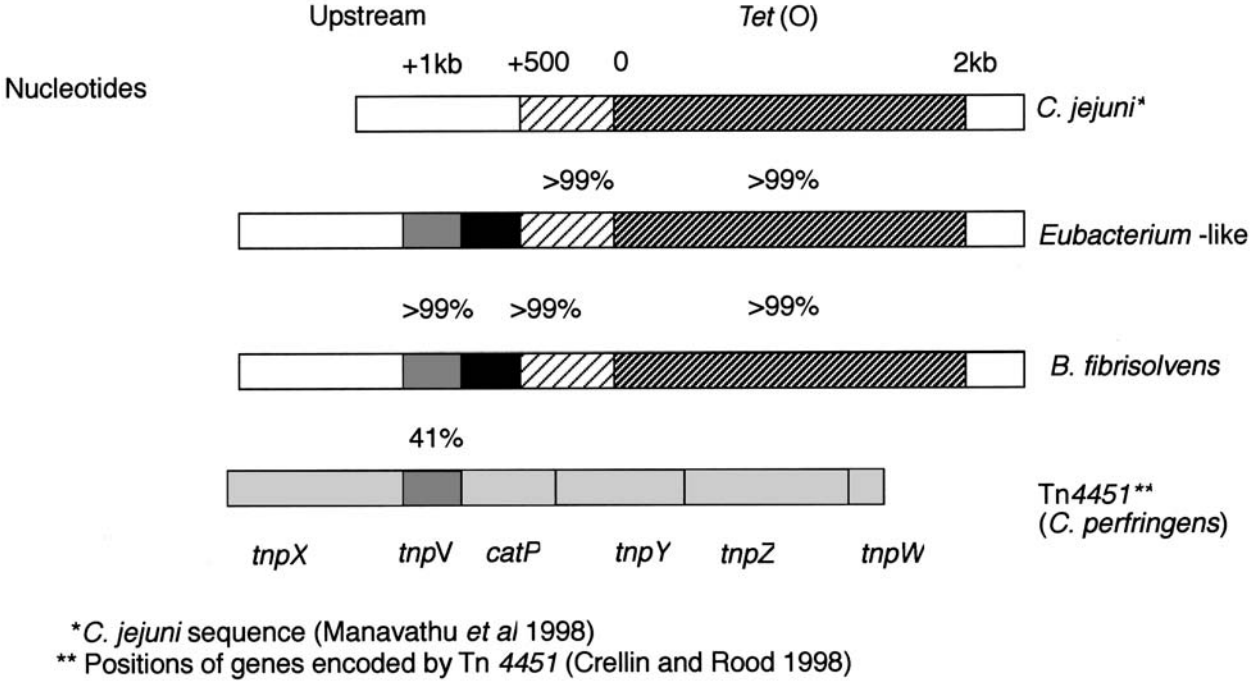

Figure 4. Comparison of the DNA sequence adjacent to and including the *tet*(O) gene in various bacterial species. The % identity between similar regions (illustrated by shading) is indicated. The references for the *C. jejuni* (Manavathu et al. [69]) and Tn*4451* (Crellin and Rood [17]) sequences are given.

of the GIT, and it should be borne in mind that all bacteria passing through the GIT contribute to the gene pool available to resident bacterial species.

Conjugative transposons capable of transfer into commensal bacteria in anaerobic filter matings

Although there is little information on the natural incidence of CTns among the native microflora of the GIT, there have been many studies investigating the host ranges of CTns, and this includes transfer to and from commensal bacteria. Generally, transfer frequencies associated with the transfer of conjugative transposons are as high as those obtained for conjugative plasmids under comparable conditions, specifically anaerobic filter matings (table 1). The host range of Tn*916* has been studied most extensively and is reviewed in Clewell et al. [20]. Hespell and Whitehead found that Tn*916* could be transferred by conjugation in anaerobic filter matings from *E. faecalis* to the rumen anaerobes *B. fibrisolvens* [21] and *Streptococcus bovis* [22] (table 1). The transferred resistance was stable in *S. bovis* in the absence of selective pressure [23]. Although *S. bovis* transconjugants subsequently acted as donors for transfer into *B. fibrisolvens*, the transposon could not be transferred out of *B. fibrisolvens* [21]. *L. lactis* MG1363 also acts as a recipient for Tn*916* but is unable to act as a donor in subsequent mating experiments [24, 25]. Thus, although broad host range transfer of Tn*916* is possible, indicating widespread recognition of conjugative proteins, additional host specific functions appear to be required for successful gene transfer to occur.

Tn*1545* is a 25.3-kb conjugative transposon related to Tn*916*, originally identified in the Gram-positive pathogen *S. pneumoniae* [26]. Tn*1545* was transferable in both directions between *Clostridium beijerinckii* and the rumen anaerobe *Eubacterium cellulosolvens* [27] (table 1). A single copy of the transposon transferred into the recipient chromosome, where it was stably maintained in the absence of antibiotic selection. Tn*1545*, in common with Tn*916*, targets AT-rich regions in the recipient genome [28]. Such sequences must be widely distributed in *E. cellulosolvens* since many different insertion sites were observed [27].

In filter matings of rumen *B. fibrisolvens* strains 1.230 and 2221^{R}, Tn*B1230* transferred at high frequencies that were dependent on the growth stage of the donor and recipient cells [12] (table 1). Transconjugants of 2221^{R} acted as donors of tetracycline resistance in onward transfer experiments. Tn*B1230* also transferred to the *B. fibrisolvens* strain D6/1, but transfer to *E. faecalis* was not detected [K. P. Scott et al., unpublished results].

Tn*K10* transferred from the human colonic anaerobe *Clostridium* sp. strain. K10 to the rumen *B. fibrisolvens*

Table 1. Transfer frequencies obtained in vitro in matings involving commensal bacteria.

Donor	Recipient	Element	Transfer frequency[a]		Reference
			Donor	Recipient	
E. faecalis	*S. bovis*	Tn*916*	1.4×10^{-6}	6.9×10^{-6}	22
		pAMβ1	8×10^{-6}	1×10^{-6}	
S. bovis	*B. subtilis*	Tn*916*	2×10^{-9}	1×10^{-7}	
		pAMβ1	5.5×10^{-10}	5×10^{-8}	
S. bovis	*B. fibrisolvens*	Tn*916*	3.5×10^{-7}	2.5×10^{-7}	
		pAMβ1	6×10^{-6}	1.2×10^{-5}	
E. faecalis	*B. fibrisolvens*	Tn*916*	6.6×10^{-6}	3.2×10^{-6}	21
E. faecalis	*S. bovis*	Tn*916*	ND	4.6×10^{-8}	23
B. thuringiensis	*R. albus*	pAMβ1	ND	7.5×10^{-8}	66
E. faecalis	*E. coli*	Tn*916*	1.4×10^{-8}	6.6×10^{-7}	49
E. coli	*E. faecalis*	Tn*916*	4.6×10^{-4}	1.1×10^{-5}	
E. coli	*B. subtilis*	Tn*916*	5.5×10^{-6}	6.8×10^{-6}	
C. beijerinckii	*Eu. cellulosolvens*	Tn*1545*	ND	1×10^{-6}	27
Eu. cellulosolvens	*C. beijerinckii*	Tn1545	ND	1×10^{-7}	
L. monocytogenes	*E. faecalis*	Tn*916*	5.9×10^{-6}	ND	67
B. subtilis	*L. lactis*	Tn*916*	8×10^{-6}	ND	24
B. fibrisolvens	*B. fibrisolvens*	Tn*B1230*	10^{-2}–10^{-5}	10^{-3}–10^{-5}	12
Clostridium sp.	*B. fibrisolvens*	Tn*K10*	10^{-4}	ND	18
Clostridium sp.	*Roseburia* sp.	Tn*K10*	ND	1×10^{-6}	Scott et al.[b]
B. uniformis	*P. ruminicola*	Tn12256[c]	ND	4×10^{-7}	30
P. ruminicola	*B. uniformis*	Tn12256[c]	ND	4×10^{-8}	
B. thetaiotamicron	*P. ruminicola*	Tn12256[c]	ND	0.9×10^{-8}	
P. ruminicola	*B. thetaiotamicron*	Tn12256[c]	ND	3.3×10^{-7}	

[a] Transfer frequency given is the number of transconjugants per donor/recipient cell. Value stated is the average for each particular transfer experiment. ND, not determined.

[b] K. P. Scott et al., unpublished data.

[c] *B. fragilis* chromosomal element CTn Tc^{r}Em^{r}12256.

strain 2221^{R} [18] and the human colonic *Roseburia* sp. strain A2-194 [K. P. Scott et al., unpublished results]. These three bacteria are all members of the Cluster XIVa *Clostridium* subphylum of low % G+C content Gram-positive bacteria based on 16S rRNA sequencing [29].

Transfer of the chromosomal element Tc^{r}Em^{r}12256, originating in a clinical isolate of *Bacteroides fragilis,* was possible in both directions between closely related human colonic commensal *B. uniformis* and *B. thetaiotaomicron* and rumen *Prevotella ruminicola* strains [30] (table 1). The *P. ruminicola* conjugative plasmid pRRI4 transferred to the colonic *Bacteroides* strains at 100-fold higher frequencies (1.5×10^{-6} per recipient).

These in vitro experiments, investigating the transfer of different CTns between diverse rumen and human bacterial species, illustrate the high potential for conjugal gene transfer to occur between bacterial species normally resident in different hosts. Even if direct transfer is not possible between two bacterial species, it is possible that intermediary hosts could be involved. Ultimately, this means that all bacteria may have access to the same gene pool, facilitating rapid evolution.

Transfer of conjugative transposons in the GIT

There has been much less investigation into the transfer of CTns into random, unknown members of the GIT microflora under in vivo conditions, despite the fact that the abundance of bacteria, and their close proximity, means that the GIT forms an ideal location for gene transfer.

One of the few studies of this type investigated transfer of the *C. difficile* transposon Tn*5397* from *Bacillus subtilis* in a simulated oral biofilm [31]. The biofilm was allowed to establish for 21 days before inoculation with the donor *B. subtilis*. A Tc^{R} Gram-positive coccus identified as *Streptococcus acidominimus,* subsequently confirmed to have acquired Tn*5397*, was isolated within 6 h of donor inoculation. This transconjugant bacterium was still present after 24 h, even though by this time the donor strain was no longer detectable. This experiment demonstrates transfer from a transient inhabitant to an unrelated resident oral bacterium [31].

A major concern in terms of gene transfer is the possible spread of antibiotic resistance from commensal bacteria to incoming pathogens resulting in failure of antibiotic therapy. Transfer of Tn*1545* from *E. faecalis* (a GIT bacterium) to *Listeria monocytogenes* (a food-borne pathogen) occurred at frequencies of 2.5×10^{-7} per donor in filter matings, and a 20-fold increase in transfer frequency was observed if tetracycline selective pressure was applied [32]. The experiment was repeated in vivo using gnotobiotic mice in which the recipient was allowed to establish before the donor was added. Transfer frequencies were lower (1.1×10^{-8}) but again increased 10-fold under selective pressure [32]. Increases in transfer frequencies of several transposons in the presence of subinhibitory antibiotic concentrations have been noted [e. g. 5]. This important point may increase the probability of the spread of antibiotic resistance genes during drug therapy.

While there have been few studies investigating the transfer of CTns in vivo, there have been many more looking at the transfer of conjugative plasmids in gnotobiotic and human faecal microflora-associated mice. The most successful experiments involve inoculating gnotobiotic animals with both donor and potential recipient bacterial strains [33, 34]. Attempts to look for transfer in conventional animals, even following inoculation with donor and recipient strains, have largely proved unsuccessful, possibly because the introduced strains are unable to become established against the background resident microflora [34]. Transfer of the conjugative plasmid, pAMβ1, was observed from *Lactococcus lactis* to the *E. faecalis* component of minimally associated mice, and frequencies increased by the order of 10^{4} if antibiotic selective pressure was applied [35]. Transfer of pAMβ1 was also detected in the GIT of chickens inoculated with donor and recipient *E. faecium* strains, but any possible transfer to native GIT bacteria could not be determined due to inadequate selection techniques [36]. Transfer of native plasmids between rumen *E. coli* strains in the presence of the complete rumen microflora has also been demonstrated, at reduced frequencies compared with the corresponding in vitro filter transfers [37].

Circumstantial evidence for the widespread transfer of resistance genes, and the importance of CTns in transfer events

In the previous sections the evidence for the presence and mobility of conjugative transposons in the GIT has been presented. This provides a very biased picture of gene transfer events as, with a few exceptions, it deals with easily cultivable bacteria that are often minor GIT populations. However, if the evidence for gene transfer in the GIT is considered from a different angle, specifically the incidence of identical genes in multiple, geographically distinct and bacteriologically diverse GIT samples, the evidence for widespread gene transfer in the GIT is overwhelming. This is despite the fact that the DNA base substitution rate in bacteria is estimated as 1% in 1 million years [38], which means that two genes that are 99% identical in base sequence could have evolved up to 1 million years ago. Evidence that a gene has been acquired by gene transfer and is not native to a bacterial species can be indicated from a DNA % G+C content distinct to that of the rest of the bacterial genome, and a different codon usage pattern. The distribution of Tc^{R} genes provides a model for

Table 2. Characteristics of strains harbouring tet(W) (DNA % G + C content 53%).

Strain	Source	Year	No. nt changes from 1.230 *tet*(W) (1919 nt)	Genome % G+C
Butyrivibrio fibrisolvens				36–41%
1.230, 1.23	bovine rumen (UK)	1993	0	
JK51, JK214	ovine rumen (Australia)	1996	0, 0	
Selenomonas ruminantium				54–61%
FB32, FB34	bovine rumen (UK)	1989	0, 0	
FB322	bovine rumen (UK)	1989	1	
Mitsuokella multiacidus				56–58%
46/5(2)	ovine rumen (UK)	1987	0	
P208–58	pig faeces (Japan)	1974	7	
Clostridium sp.				~40%
K10	human faeces (UK)	1999	0	
Bifidobacterium longum				58%
F5, F8, F10	human faeces (UK)	1999	1	

gene flow, and exemplifies the mechanisms by which gene transfer may occur between diverse bacterial species. Tetracycline is one of the most widely used antibiotics worldwide; thus, bacteria have been under intense, long-term selective pressure to acquire Tc^R. The resistance mechanisms utilised, mainly drug efflux or ribosome protection, have been extensively reviewed [e.g. 39]. All ribosome protection type Tc^R genes have sequence similarities to elongation factor G (fig. 2), from which they are thought to be derived, but sequence diversity and distribution within classes of these Tc^R genes implicates gene transfer events in their spread. The sequence diversity within four classes of ribosome protection type Tc^R genes is summarised in table 3.

The complete DNA sequences of *tet*(W) genes isolated over a 30-year period from various rumen, human and pig GIT anaerobes were 99–100% identical [13, 40] (tables 2, 3). Convergent evolution would not result in such strong nucleotide sequence conservation, and the extremely high sequence identity implies that transfer events have been recent. The presence of such identical genes in the human, pig and rumen GIT indicates that there is no obstacle to gene transfer between these communities. Comparison of the % G + C content of the *tet*(W) gene (53%) with that of the original host *B. fibrisolvens* (40%) strongly infers that the gene was acquired by *B. fibrisolvens* from a different, higher % G + C content bacterial species. The codon usage pattern supports this; there is a strong preference for a G or C residue as the third base in the codon, compared with other *B. fibrisolvens* genes [13].

A separate study illustrated the strong conservation between *tet*(W) genes from steer and pig samples [41]. In this case specific primers were used to amplify 170 nucleotide fragments of the genes from total bacterial DNA samples, and divergence in the PCR products was analysed by DGGE. No differences were observed. A similar result was obtained for *tet*(O) genes, again isolated from steer and pig samples [41] (table 3). These DGGE comparisons are, however, only of very short fragments of the total gene length (<10%), and thus do not illustrate total sequence conservation or variation. Since genes often contain both highly variable and highly conserved regions, in order to infer gene transfer events from sequence similarities, it is important to compare full-length sequences. Our group previously found that the complete sequences of *tet*(O) genes from rumen *B. fibrisolvens* strains were identical to those from pathogenic *S. pneumoniae* [13] (table 3), and only 3/1920 nucleotides were different to *tet*(O) genes in pathogenic *C. jejuni* and human commensal *Eubacterium* species (K. P. Scott et al., unpublished observations]. The sequence upstream of the *tet*(O) gene in these bacteria was also highly conserved (fig. 4). The identity between *B. fibrisolvens* and *Eubacterium* isolates continued to at least 1 kb upstream, while the sequences of *C. jejuni* and *S. pneumoniae* diverged 500 nt upstream of the start codon. In *S. pneumoniae* the immediate upstream region has a role in regulating gene expression [42]. Presumably, the expression of *tet*(O) is regulated in the same way in all these diverse bacterial species. Full-length *tet*(O) sequences of several pathogenic bacteria were also virtually identical (nine nucleotide changes [43]), although another study comparing shorter sequences found only 91–95% identity between *S. pneumoniae* and *S. mutans* isolates [44] (table 3), reinforcing the importance of comparing full-length sequences.

The degree of sequence conservation found between *tet*(W) and *tet*(O) genes is convincing evidence that recent gene transfer events have occurred between diverse bacteria, although the transfer mechanisms have not been fully described. The *tet*(W) gene has been associated with the highly mobile CTn Tn*B1230*, but this CTn is not present in all bacteria that contain *tet*(W) [14]. Plasmid-encoded *tet*(O) genes are known to be transferable, while those encoded on the chromosome were thought

Table 3. Sequence conservation within classes of tetracycline resistance genes.

Gene	% identity	No. of nucleotides compared	Source and bacterial genera		Reference
tet(W)	>99%	full-length	***B. fibrisolvens*** (R) *S. ruminantium* (R) *M. multiacidus* (Pig)	*Bi. longum* (H) *Clostridium* sp. (H)	13, 40
tet(W)	100%	170 nt	**total DNA** (R and Pig)		41
tet(O)	100%	170 nt	**total DNA** (R and Pig)		41
tet(O)	>99%	full-length	***B. fibrisolvens*** (R) *Eubacterium*-like (H)	*C. jejuni* (P) *S. pneumoniae* (P)	13, Scott et al. unpublished
tet(O)	>99%	full-length	*C. jejuni* (P) *C. coli* (P)	***S. pneumoniae*** (P) *S. mutans* (P)	43
tet(O)	91 – 95%	300 nt	*S. pneumoniae* (P)	***S. mutans*** (P)	44
tet(Q)	97.2%	full-length	*P. ruminicola* (R)* ***B. thetaiotaomicron*** (H)	*B. fragilis* (H)	46
tet(Q)	100%	407 nt	***P. intermedia*** (H oral)	*B. fragilis* (H colon)	
tet(Q)	96 – 100%	758 nt	*Bacteroides spp.* (H) (33 strains)		8
tet(M)	92%	full-length	*E. faecalis* (H) *Staphylococcus aureus* (H) *Ureaplasma urealyticum* (P)	***S. pneumoniae*** (P) *Neisseria gonorrhoeae* (P)	47

R, rumen; H, human; P, pathogen. Bacterial strain shown in bold contains the reference Tc^R gene sequence to which the others were compared. * *tet*(Q) carried by plasmid pRRI4 [68].

not to be [44]. In the case of *Campylobacter* spp. and *S. pneumoniae,* genes could be acquired by natural transformation. However, the presence of *tet*(O) genes on the chromosome of rumen and human gut bacteria that are not known to be transformable implies that a transfer mechanism exists which has yet to be elucidated. The identification of gene sequences similar to those on the mobilisable CTn, Tn*4451* [17], in both *B. fibrisolvens* and *Eubacterium* spp. may provide the answer to this conundrum.

Studies on sequence conservation in *tet*(Q) and *tet*(M) imply that the sequences diverge much more than *tet*(W) and *tet*(O). *tet*(Q) genes are widespread among human colonic *Bacteroides* spp., and are generally located on large mobile chromosomal elements (see article by Whittle et al., this issue). Human colonic *Bacteroides* spp. fall into the same phylogenetically diverse group of Gram-negative bacteria as rumen (and human) *Prevotella* spp. [45], and include both commensal and pathogenic isolates. Several studies have compared full-length or partial sequences of *tet*(Q) genes carried by rumen *P. ruminicola*, human oral *P. intermedia* and human colonic *Bacteroides* spp. [e. g. 8, 46]. The sequence identity varied from 96 to 100% depending on which part of each gene from the different bacteria was compared was used in comparing bacteria (table 3). However, the degree of homology again illustrates intergeneric gene transfer between different bacterial species normally resident in different hosts. Some of these transfer events may have occurred long ago on an evolutionary time scale, and the genes have subsequently diverged.

Many different *tet*(M) genes from clinical bacterial isolates, located on various plasmids and CTns, have been sequenced and shown to diverge by between 1 [39] and 8% [47] (table 3). The nonrandom distribution of nucleotide changes illustrates the existence of mosaic genes, derived from two distinct alleles, which appear to have arisen by recombination after the gene integrated into Tn*916* [47]. It appears that *tet*(W) has much less sequence variation than *tet*(Q), *tet*(M) and even *tet*(O) genes. This might indicate that *tet*(Q), *tet*(M) and *tet*(O) evolved first, and have disseminated among bacterial species over a longer time period. These genes would thus have had more time to adapt to particular bacterial genera. Alternatively the CTns they are associated with may have a broad host range resulting in greater potential for two genes to enter the same host, leading to homologous recombination and sequence variation. This is almost certainly the case for Tn*916*-encoded *tet*(M) genes [47]. However, *tet*(W) genes have not yet been so widely studied, and it may be that as more sequences are elucidated, more variation will be observed. Indeed, *tet*(W) genes have recently been identified in a human *Bifidobacterium* sp. (EMBL accession no. AF202986) and the animal pathogen *Arcanobacterium pyogenes* (GenBank accession no. AY049983; [48]). These genes are only 98 and 92% identical to the *B. fibrisolvens tet*(W) gene, respectively.

The evidence for horizontal transmission of Tc^R genes is more convincing than the alternative possibility of convergent evolution, which would result in amino acid sequence identity without necessarily the same level of nucleotide sequence conservation. The location of many of these genes on CTns illustrates the important role CTns play in disseminating genetic information between diverse bacterial species, isolated from different ecological niches.

Conjugative transposons as tools for genetic manipulation

In addition to their role in contributing to the spread of ab^R between commensal and pathogenic bacteria, CTns are also potentially important for the genetic manipulation of gut anaerobes for which vector systems do not exist. The broad host range of CTn Tn*916*, encompassing both Gram-positive and Gram-negative bacterial species [49], has meant that it has been used extensively in random mutagenesis studies and as a cloning vehicle. Suicide insertion vectors containing specific active gene sequences have been designed to integrate into Tn*916* by homologous recombination. The resultant conjugative Tn*916*-derivatives provide a mechanism to introduce new genes into bacterial strains where transformation is not possible, or where vector systems are unavailable [50–53]. Tn*916* can also be used to mobilise nonmobile plasmids into nontransformable hosts, e.g. the mobilisation of *Staphylococcus aureus* plasmid pUB110 into *B. fibrisolvens* [54]. The success of genetically modified gut anaerobes often depends on reintroduction and establishment in the GIT, in competition with a huge background population. Although native GIT anaerobes may be difficult to manipulate, non-GIT bacteria are unlikely to be able to adapt and thrive under conditions prevailing in the GIT. Even a native GIT bacterium may fail to colonise after reintroduction since, even among the most numerous GIT anaerobes, individual strains may only comprise 0.1% of the total bacterial microflora. Consequently, it may be preferable to carry out the manipulation and reintroduction simultaneously on more than one bacterial strain.

In the case of rumen bacteria, manipulations have been aimed at improving plant fibre degradation, and ultimately animal production [reviewed by e.g. 55, 56]. Despite the fact that ruminants and their bacterial symbionts have coevolved over thousands of years, recent changes in farming practices mean that the resident bacterial population is no longer optimal to provide maximal energy release from the feed provided. The few conjugative systems that exist for rumen anaerobes involve the use of conjugative plasmids rather than CTns, and there is consequently the additional problem of stability in the absence of selective pressure.

The native rumen bacterium *Streptococcus bovis* is a facultative anaerobe that has been manipulated to express novel genes, including those for polysaccharide degradation, introduced either by electroporation of plasmids [57] or more recently by natural transformation [58]. The most successful experiment involving an obligate anaerobe was the introduction of a fluoroacetate dehalogenase gene into *B. fibrisolvens*, on a shuttle plasmid [59]. Not only was the construct stable in the absence of selective pressure, but the genetically modified (GM) bacteria survived reintroduction into the sheep rumen for at least 5 months with no detectable plasmid loss [60]. The sheep containing the modified constructs were less susceptible to fluoroacetate poisoning. Since Tn*916* transfers conjugatively to *S. bovis* and *B. fibrisolvens* (table 1), Tn*916* derivatives provide an alternative mechanism to introduce new genes into these bacteria, avoiding problematic transformation procedures.

There is an increasing public awareness of the potential benefits of including probiotics in the diet of nonruminant animals and humans. Probiotics are live bacterial supplements claimed to improve gastrointestinal health, either by their own metabolic activities or by preventing colonisation of the GIT by pathogenic bacteria. The possibility of increasing commercial return from probiotic products will undoubtedly result in the development of improved probiotic strains, often by the introduction of additional traits from other bacteria. Target genes may encode degradative enzymes to break down specific substrates available in the colon, releasing beneficial by-products, or be genes known to enhance bacterial survival. The persistence of probiotics in the GIT is crucial if they are to function effectively. Currently, the most widely used probiotic bacteria are *Bifidobacterium* or *Lactobacillus* species, and without continual intake numbers of the probiotic rapidly become undetectable in faeces. Adherent probiotic strains can be detected in biopsy samples for longer [61] although they are eventually eliminated.

Certain bacteria are also being developed as vaccine delivery systems, either by using attenuated strains of pathogenic bacteria (e.g. *Salmonella typhimurium*; [62]) or by expressing antigens derived from pathogenic bacteria in 'safe' bacteria. For example, *L. lactis* MG1363 modified to express a *Helicobacter pylori* protein did elicit an immune response in mice, but there was no protection against *H. pylori* challenge [63]. The ineffective immune response could have been due to the weak antigenic properties of the protein used, but it may also be due to the rapid elimination of *L. lactis* from the GIT [53].

These approaches are currently limited to bacteria that can be readily genetically manipulated, which may be species different from those able to tolerate conditions in the GIT. CTns, including the newly identified CTns Tn*B1230* and Tn*K10,* could be used to manipulate commensal GIT anaerobes to form more effective probiotics or vaccine delivery systems. However, the potential spread of introduced genes, or of ab^R genes present on the CTns, into the native GIT bacterial population is a problem that will have to be prevented before the full potential of GM bacteria can be realised. The ability of CTns to transfer readily between diverse bacterial species would rapidly disseminate any ab^R genes encoded on them into the general bacterial population. The inclusion of a factor immobilising the CTns, rendering them nonconjugative in the GM bacteria, would alleviate this problem. The safety of probiotics is

paramount in the development of new strains [64]. Each new development should be assessed individually, and the potential repercussions for different groups of people, e. g. immunocompromised patients, considered [65].

The immediate benefit of CTns is their use as tools to identify and verify gene function, either by the introduction of specific genes into new hosts, or the interruption of existing genes. In this case, the presence of ab^R genes on CTns aids the selection of manipulated or mutant strains. The discovery of CTns originating in groups of bacteria where manipulation was previously impossible is invaluable and will lead to improved understanding of the function, and thus importance, of key groups of gut anaerobes. For example, the role of *Eubacterium* spp. in starch degradation could be investigated by transposon mutagenesis using either Tn*K10* or Tn*1545*. Alternatively, genes involved in the degradation of carbohydrates in the colon, including inulin and resistant starch, could be identified and introduced into new host bacteria to verify their function. The interruption of such genes in the original host would confirm their role in key degradative pathways. This would both improve our understanding of the interactions between major groups of gut anaerobes, and identify native GIT bacteria with important biological functions. These bacteria could then be developed as probiotics without requiring genetic manipulation to introduce beneficial traits. Since they originated in the GIT, they would be expected to have improved persistence and therefore efficacy.

Conclusions

The scarcity of examples of CTns among the commensal anaerobic microflora of the GIT probably reflects a lack of research rather than any lack of abundance. In the two random studies carried out by our group, two new Tc^R genes were identified; both encoded on novel CTns. Many commensal GIT anaerobic bacteria act as recipients for conjugative transfer of CTns characteristically found in pathogenic species, indicating the lack of any barrier to gene transfer. The incidence of identical resistance genes among commensal anaerobes, often also identical to genes found on pathogenic bacteria, demonstrates that bacteria have access to a single gene pool and that gene flow between bacterial species occurs rapidly, on an evolutionary timescale, and is widespread. The CTns already described, and those still to be discovered, inevitably contribute to this gene flow.

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