Evidence for Natural Horizontal Transfer of tetQ between Bacteria That Normally Colonize Humans and Bacteria That Normally Colonize Livestock

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Though numerous studies have shown that gene transfer occurs between distantly related bacterial genera under laboratory conditions, the frequency and breadth of horizontal transfer events in nature remain unknown. Previous evidence for natural intergeneric transfers came from studies of genes in human pathogens, bacteria that colonize the same host. We present evidence that natural transfer of a tetracycline resistance gene, tetQ, has occurred between bacterial genera that normally colonize different hosts. A DNA sequence comparative approach was taken to examine the extent of horizontal tetQ dissemination between species of Bacteroides, the predominant genus of the human colonic microflora, and between species of Bacteroides of the distantly related genus Prevotella, a predominant genus of the microflora of the rumens and intestinal tracts of farm animals. Virtually identical tetQ sequences were found in a number of isolate pairs differing in taxonomy and geographic origin, indicating that extensive natural gene transmission has occurred. Among the exchange events indicated by the evidence was the very recent transfer of an allele of tetQ usually found in Prevotella spp. to a Bacteroides fragilis strain.

The use of antibiotics as additives in livestock feed has continued to generate controversy about whether this practice increases the incidence of antibiotic resistance genes among human clinical isolates. In a previous paper, we reported that Prevotella ruminicola, one of the numerically predominant genera of bacteria in the rumens and intestinal tracts of livestock animals, could exchange DNA with human colonic Bacteroides species under laboratory conditions (20). Not only is the genus Bacteroides one of the numerically predominant genera of bacteria in the human colon, species of Bacteroides are also opportunistic human pathogens. We showed that a plasmid, pRR14, found originally in an isolate of P. ruminicola obtained from a sheep, could be transferred by conjugation to Bacteroides thetaeauamnonicus. Similarly, a conjugative transposon, Te' Em' 12256, found originally in the clinical isolate Bacteroides fragilis 12256, could be transferred from Bacteroides species to P. ruminicola (20). Demonstrating that two distantly related bacterial genera can exchange DNA under laboratory conditions that have been optimized for conjugal transfer does not prove that such events actually occur in nature. However, the possibility that this type of exchange might have occurred in nature was raised by our finding that the tetracycline resistance (Te') gene on Te' Em' 12256, tetQ, cross-hybridized on Southern blots at high stringency with the tetracycline resistance region of pRR14.

The problem with the use of Southern hybridization data as an indication of similarity between tetQ genes in different natural isolates is that genes which differ by as much as 15% at the DNA sequence level can cross-hybridize under high-stringency conditions. Given an approximate rate of silent nucleotide substitution in bacteria of 1% per million years (13), the fact that two genes cross-hybridize under conditions of high stringency does not provide sufficiently detailed information to ascertain whether recent gene transfer has occurred.

Few antibiotic resistance dissemination studies have actually compared the DNA sequences of resistance genes found in different isolates (9). These studies have been done primarily with human clinical isolates and have focused on bacteria such as Escherichia coli and the facultative gram-positive cocci that account for less than 0.1% of the resident microflora. To assess the possibility that relatively recent transfer events may have occurred between the resident microflora of livestock animals and humans, we used tetQ as an indicator gene. tetQ was chosen because it has been found on virtually all Bacteroides conjugative transposons, a family of broad-host-range chromosomal elements, and was also found on the P. ruminicola plasmid pRR14.

No information has been obtained previously about the distribution of tetQ in ruminal species such as P. ruminicola. It is not easy to obtain isolates of P. ruminicola that are tetracycline resistant, because P. ruminicola is very oxygen sensitive and difficult to cultivate and because most investigators interested in this species have tended to focus on a few well-characterized strains rather than isolate new strains from animals. Those who have isolated P. ruminicola from farm animals have rarely tested for tetracycline resistance, because they are more interested in the contribution of ruminal and intestinal anaerobes to feed efficiency than in antibiotic resistance patterns. Nevertheless, we have managed to obtain several isolates of tetracycline-resistant P. ruminicola from widely separated geographical locations. We were also able to obtain a tetracycline-resistant strain of Prevotella intermediar, a species of Prevotella found in the human oral cavity and thought to play a role in periodontal disease. So far, tetracycline-resistant isolates of P. intermediar have been rare and tetracycline is used widely as part of the treatment of periodontal disease, but there is concern that transfer of tetracycline resistance genes from the colonic Bacteroides species may introduce tetQ into the oral microflora and thus make tetracycline less useful for treatment. We have compared the sequences of an interior region of tetQ alleles found in the various Prevotella and Bacteroides strains surveyed. Our results

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suggest that horizontal transfer of antibiotic resistance genes is occurring in nature among bacteria in the human colon, bacteria in the human oral cavity, and bacteria in the intestines and rumens of cattle.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacteroides isolates used in this study were B. thetaiotaomicron DOT2, B. fragilis CEST, and B. fragilis ERL (obtained from Terry England, isolated at the University of Illinois-Chicago, Chicago, Ill.; [19]), B. fragilis V479 (obtained from M. Sebald, isolated at Institut Pasteur, Paris, France [19]), B. fragilis 12256 (obtained from the VPI Anaerobe Laboratory culture collection, isolated at Wadsworth VA Hospital, Los Angeles, Calif. [18]), and B. thetaiotaomicron 7853 (obtained from S. Finegold, isolated at Wadsworth VA Hospital). P. ruminicola isolates used in this study were P. ruminicola 223 (carries pRR4, obtained from Harry Flint, isolated from a sheep at Rowett Institute, Aberdeen, Scotland [3]), P. ruminicola 2063 and 2078 (obtained from Mill Allison, isolated from the same pig at the National Animal Disease Center, Ames, Iowa [14]), and P. ruminicola ISP3 (obtained from Jeff Smith, isolated from a cow at North Carolina State University, Raleigh). Human oral isolate P. intermedia ISS-B128 was provided by Alessandra Arzese (isolated in Rome, Italy). Bacteroides strains and P. ruminicola strains were grown as described previously (6, 20). P. intermedia ISS-B128 was grown in modified Bryant medium (6). E. coli DH5α MCR was used for cloning. E. coli was grown in Luria broth or on Luria broth agar.

DNA cloning, sequencing, and analysis. A 5.5-kbp HincII fragment from pRR4 and a 7.5-kbp PstI chromosomal fragment from B. thetaiotaomicron 7853 were cloned into pFD160 (21) to produce pRTC1 and pMPN78, respectively. The chromosomal fragment was cloned by using tetQ as a hybridization probe. To test for expression of these alleles of tetQ in B. thetaiotaomicron, pRTC1 and pMPN78 were transferred to B. thetaiotaomicron 4001 by conjugation as described previously (12). PCR-amplified DNA was cloned into either pTZ18U (10) or pCRII (Invitrogen Corp., San Diego, Calif.). Cloning, gel electrophoresis, Southern hybridization, and other DNA manipulations were done as described previously (12, 15, 16). The entire tetQ gene from pRR4 and internal 400-bp regions of other tetQ alleles were sequenced by first generating nested deletions (Erase-a-Base system; Promega Corp., Madison, Wis.) (5) and then using the Sequenase version 2.0 kit (U.S. Biochemicals, Cleveland, Ohio) (17) to obtain the DNA sequence.

Genetics Computer Group software (1) was used on a Micro VAX system to compile and analyze nucleotide and deduced amino acid sequences. The GAP program, which utilizes the algorithm of Needleman and Wunsch (11), was used to make pairwise comparisons of sequences. The LINEUP program was used to construct multiple sequence alignments. For each isolate examined by PCR, the sequences of the different clones of the amplified tetQ internal segment were compiled into a multiple sequence alignment. A consensus of the internal 407-bp sequence was then determined from the alignment for each isolate in order to discard point mutations in individual PCR product clones created by Taq polymerase errors. The sequence of the corresponding internal 407 bp from the cloned B. thetaiotaomicron 7853 tetQ gene, the P. ruminicola pRR4 tetQ sequence, and the previously reported Tc'-Em' DOT in B. thetaiotaomicron DOT and B. fragilis 1126 tetQ sequences (7, 12) were aligned with the PCR consensus sequences by using LINEUP. A maximum parsimony tree of the aligned sequences was created by using PAUP (22).

DNA amplification. An internal 460-bp region of tetQ was amplified from strains CEST, ERL, 12256, V479, ISS-B128, JSP3, 2063 and 2078 by PCR. Primers used were PCR1, 5'CA TGGATCAGCAATGTCCATATCGG3', and PCR2, 5'CC TGGATCCACAGTATTTGACAGCGG3'. Approximately 1 μl of the earliest accessible frozen stock of each isolate was mixed with 200 ng of each primer in 100 μl of the appropriate reaction buffer (10 mM Tris-HCl [pH 8.8], 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 2.0 mM deoxynucleoside triphosphate mixture) and amplified with Taq DNA polymerase. Amplification conditions consisted of an initial denaturation step of 95°C for 5 min followed by addition of the Taq polymerase and then 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. In the case of V479, the annealing step of the cycle had to be lowered to 45°C in order to obtain detectable levels of amplification product. Bands containing desired PCR products were excised from SeaPlaque low-gelling-temperature agarose (FMC BioProducts, Rockland, Maine) gels and melted at 65°C. Aliquots were mixed with Smal-digested pTZ18U and equilibrated to 37°C, and T4 DNA ligase with reaction buffer was added. The ligation reaction was allowed to proceed at 25°C for 16 h, after which the mixture was transformed into E. coli (4). In later experiments, the TA Cloning protocol was used as specified by the manufacturer (Invitrogen) to construct PCR product clone banks.

Nucleotide sequence accession number. The tetracycline resistance region of pRR14 has been sequenced and assigned GenBank accession number L33696.

RESULTS

Comparison of tetQ regions on pRR14, Tc'-Em' DOT, and B. fragilis 1126. In order to determine the degree of similarity in the tetracycline resistance region on pRR14 and one of the Bacteroides conjugative transposons, Tc'-Em' DOT, we sequenced the tetracycline resistance region of pRR14 and compared it with the sequence of the tetracycline resistance region of Tc'-Em' DOT (12). The sequences of the two regions were nearly identical in a region that extended from 533 bp upstream of the start codon of the tetracycline resistance gene, tetQ, to 143 bp downstream of its stop codon (Fig. 1). In this 2.65-kbp region of virtual identity, only 66 (2.5%) nucleotide differences were found. All but two of these differences were nucleotide substitutions. The two exceptions were length variations of 11 and 11 bp upstream of the start of tetQ (Fig. 1). The sequences of the two tetQ open reading frames were 97% identical. Within the tetQ open reading frame (1,926 bp), there were 52 nucleotide differences (2.7% difference) resulting in 21 differences in the deduced amino acid sequences. Of the 21 amino acid differences, 11 were conservative differences; the amino acids found at the same position in the deduced sequence had chemically similar R groups. Silent or synonymous differences accounted for 31 of the 52 nucleotide differences in the open reading frames, or about 60%.

By comparing the sequences of tetQ from pRR14 and Tc'-Em' DOT (Fig. 1), we observed that a 407-bp segment near the middle of the tetQ coding region contained a cluster of 20 nucleotide differences in the two sequences and was flanked by conserved stretches. Thus, the percent sequence difference in this region was 4.9%, nearly twice the percent difference for the full-length open reading frame. We chose this putative hypervariable region as a reference sequence for comparison of the tetQ genes from different human and animal isolates of
species of Bacteroides and Prevotella. While this work was under way, a new sequence of tetQ from B. fragilis 1126 appeared in the database (7). This sequence had 49 differences when compared with the DOT tetQ sequence and 51 differences when compared with the pRR14 sequence, 2.7 and 2.8% difference, respectively (Fig. 1). The 407-bp internal segment of the strain 1126 sequence had 19 differences when compared with the DOT internal region and 16 differences when compared with the pRR14 internal region (4.0 and 4.8%, respectively).

Comparison of a tetQ internal region among natural isolates. We obtained three additional tetracycline-resistant isolates of P. ruminicola. DNA from these isolates, JSP3, 2063, and 2078, cross-hybridized on Southern blots with a probe containing tetQ, indicating that these isolates carried tetracycline resistance determinants in the Tet Q class. In all three strains, the cross-hybridizing DNA appeared to be chromosomal rather than plasmid encoded. We also included a tetracycline-resistant isolate of human oral species of Prevotella, P. intermedia ISS-B128, in our analysis. We used the PCR-based approach to compare the hypervariable internal region of tetQ from the different Prevotella isolates. Little information was available about the extent of DNA sequence variation among tetQ genes in human colonic Bacteroides strains. Accordingly, the sequences of the tetQ internal region from five other Bacteroides isolates were also obtained. Most of the Bacteroides and Prevotella species were isolated in different geographical locations. An alignment of the PCR product consensus sequences for the 407-bp internal segment is shown in Fig. 2.

DNA sequence analysis. Of the 407 nucleotide positions that were examined, 59 positions were variable across the collective sequences analyzed. Of these 59 positions, 33 varied in only one of the sequences. The V479 sequence possessed 26 of these 33 unique variations. The nucleotide differences at the 59 variable positions can be categorized in three groups: nonconservative, conservative, and synonymous substitutions. At 16 of the positions, nucleotide differences resulted in a nonconservative difference in the deduced amino acid at that codon (a nonconservative substitution). At 13 positions, the differences resulted in conservative amino acid differences (conservative substitutions). At the remaining 30 (51%) varied positions, the nucleotide difference did not affect the deduced amino acid for that codon (synonymous substitutions). If the V479 sequence was excluded from the analysis, of 33 varied positions, 9 had nonconservative substitutions, 5 had conservative substitutions, and 19 (58%) had synonymous substitutions. Though the V479 sequence was the most varied and therefore the obvious outlying sequence in the group, it still demonstrated a high percentage of both silent and conservative substitutions. There appears to be a selection for amino acid sequence conservation in this group of genes.

The relatedness of the sequences analyzed is illustrated in the maximum parsimony trees shown in Fig. 3. The sequences clustered in two groups, one in which Bacteroides species are hosts and one in which Prevotella species are hosts. The exception to this arrangement was B. fragilis 1126, which grouped with the Prevotella sequences with significant confidence. The B. fragilis V479 tetQ internal sequence was very distinct and thus created an outlying branch in the tree that did not fall into either cluster. The most similar sequences were the Bacteroides CEST, ERL, and DOT sequences and the P. intermedia ISS-B128 and B. fragilis 1126 sequences, which were 100% identical in both cases. Other highly similar sequences were B. fragilis 12256 and B. thetaiotaomicron 7853, with 99% identity; P. ruminicola JSP3 and either P. intermedia ISS-B128 or B. fragilis 1126, with 98% identity; and P. ruminicola 2063 and 2078, with 99% identity. The least similar sequences were Bacteroides DOT, CEST, ERL and P. ruminicola pRR14, with 94% identity; Bacteroides DOT, CEST, and ERL and P. ruminicola JSP3, with 94% identity; and Bacteroides DOT, CEST, and ERL and ISS-B128/1126, with 95% identity.
DISCUSSION

Though the tetQ sequences on pRR14 and strain Tc' Em' DOT were similar enough to suggest that horizontal transfer of tetQ had occurred at some point, their divergence (2.8% nucleotide difference) indicated that the transfer event could have occurred millions of years previously. Roughly two-thirds of the nucleotide differences between the two tetQ sequences were synonymous, while 75% of completely random nucleotide changes would result in amino acid changes. The proportion of silent changes was also seen when the 1126 tetQ sequence was compared with that of either pRR14 or DOT (51 and 59% of the total differences, respectively). Thus, there appeared to be a selection for a particular amino acid sequence, and it could be argued that convergent evolution might have given rise to the similarity of the tetQ genes on Tc' Em' DOT and pRR14. Comparable similarity was found among the tetQ internal sequences of the human clinical strains and animal strains included in the survey. Though there appeared to be selective pressure to maintain the tetQ-encoded amino acid sequence, a number of variants of the gene have emerged. However, the high level of nucleotide conservation that was evident in the tetQ internal sequences is unlikely to have arisen from convergent evolution. Amino acid sequence similarity would be the expected product of convergent evolution, not high levels of nucleotide conservation. This divergent evolutionary trend is also supported by the emerging phylogenetic pattern of the
Ribosome protection tetracycline resistance gene family, whose members have a broad distribution of sequences, yet bear similarity to (and presumably have a common origin in) elongation factor genes (8).

The observation of identical or nearly identical sequences in isolates differing in phylogeny and geographic origin indicates that multiple horizontal transfers of tetQ variants have occurred in the group of bacteria surveyed. The majority of these transfers appear to have occurred within genera, either Bacteroides to Prevotella or Prevotella to Prevotella. This type of exchange is evidenced by the high sequence similarity among the internal tetQ sequences in Bacteroides isolates DOT, CEST, and ERL, in Bacteroides isolates 12256 and 7853, in Prevotella isolates JSP3 and ISS-B128, and in Prevotella isolates 2063 and 2078. Natural transfer within genera is not surprising, since conjugal systems are usually better adapted to phylogenetically related recipients. Also, occupation of shared or neighboring niches, as in the cases of the human colonic Bacteroides species and the animal intestinal Prevotella species, would increase the likelihood of contact and subsequent DNA exchange. However, the high degree of sequence similarity observed in Prevotella ruminicola JSP3, isolated from the rumen of a cow, and Prevotella intermedia ISS-B128, isolated from a human oral cavity, indicates that intrageneric horizontal transfer has occurred between bacteria that normally colonize different hosts. The other examples of evidence for intrageneric transmission were provided by isolates that colonize the same hosts and were isolated in the same geographic regions. The case of Bacteroides isolates DOT, ERL, and CEST, in which 100% sequence identity was observed, indicates that a tetQ variant was transferred in the very recent past between B. thetaiotaomicron and B. fragilis, perhaps in the Chicago area.

The data also supply evidence for a very recent tetQ transfer between species of Prevotella and Bacteroides. The 100% nucleotide sequence identity found in P. intermedia ISS-B128 and B. fragilis 1126 serves as evidence for recent intergeneric transfer of a tetQ variant between bacteria that colonize the human oral cavity and bacteria that are part of the resident colonic flora. This transfer is of interest not only because it occurred very recently and between different genera but also because it provides a recent link in tetQ exchange between Prevotella species from animals and Bacteroides species from humans. The particular variant of tetQ that was transferred in this instance falls into the cluster of Prevotella tetQ sequences (Fig. 3). Thus, the direction of transfer in this case is likely to have been from Prevotella to Bacteroides. A few different schemes can explain the arrangement of tetQ variants within these isolates: (i) The JSP3 variant was transferred to the human oral Prevotella species, accumulated nucleotide changes to become the ISS-B128/1126 variant, and was then transferred to colonic Bacteroides species; (ii) the ISS-B128/1126 variant arose in an animal Prevotella strain either as a decendent of the JSP3 variant or as a close relative derived from a common ancestor and was then transferred to the oral Prevotella and the colonic Bacteroides strains, either directly or successively; or (iii) the ISS-B128/1126 variant arose in an animal Prevotella strain as described above, was carried into the oral cavity by a colonizing Prevotella strain, and was then transferred to a colonic Bacteroides strain. Any of these schemes is consistent with the data, but the net result is the same: the horizontal transmission of a gene has occurred between members of the resident microflora of different hosts. The sequence identity between tetQ internal regions of genes found in human isolates of Bacteroides spp. and animal isolates of P. ruminicola provides the first direct evidence that natural horizontal transfer events between members of the resident bacterial microflora of humans and farm animals can occur and have occurred recently in history.

The ease with which we were able to identify cases of identity between tetQ sequences within human and animal bacterial groups, despite the relatively small number of isolates available for testing, indicates that natural gene exchange takes place between these groups more commonly than had been previously suspected. Our findings do not prove that there is a cause and effect relationship between feeding antibiotics to farm animals and the frequency of gene transfer events between human and animal bacteria, but in the context of our findings it is interesting to note that tetracyclines have been used widely as an additive in livestock feed. It is also important to consider that tetQ was chromosomally located in all but one of the strains tested and is known to be carried on conjugative transposons (12, 18). There has been a tendency in the past to equate broad-host-range transfer with plasmid transfer. Our findings, taken together with recent work on the broad-host-range streptococcal conjugative transposons (9), indicate that conjugative transposons may play as important a role as plasmids in broad-host-range gene transfer in the environment.

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