The \textit{Butyrivibrio fibrisolvens} \textit{tet}(W) Gene Is Carried on the Novel Conjugative Transposon Tn\textit{B1230}, Which Contains Duplicated Nitroreductase Coding Sequences

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Received 11 November 2003/Accepted 13 February 2004

The \textit{Butyrivibrio fibrisolvens} \textit{tet}(W) gene is located on the conjugative transposon Tn\textit{B1230}. Tn\textit{B1230} encodes transfer proteins with 48 to 67% identity to some of those encoded by Tn1549. \textit{tet}(W) is flanked by directly repeated sequences with significant homology to oxygen-insensitive nitroreductases. The 340 nucleotides upstream of \textit{tet}(W) are strongly conserved and are required for tetracycline resistance.

Tetracyclines are the second most widely used group of antibiotics worldwide, and tetracycline resistance (\textit{Te}c) is extremely common among bacteria (17). Many \textit{Te}c genes are transmissible, being carried by plasmids or transposable chromosomal elements. \textit{Te}c genes have mainly been described for pathogenic bacteria, but the new resistance genes \textit{tet}(W) (2, 20) were first identified in anaerobic commensal bacteria from the rumen and from porcine and human feces. These genes are distinct from previously described ribosome protection genes, with the highest identities being 68% [to \textit{Tet}(W)] and 76% [to \textit{Tet}(32)] at the corresponding amino acid level. \textit{tet}(W) is one of the most widespread tetracycline resistance genes in environmental samples (1, 23).

Copies of \textit{tet}(W) found in environmentally and phylogenetically distinct bacterial isolates (2, 20) have remarkable sequence conservation, which argues for the extensive rapid transfer of this gene in nature. Previous work showed that \textit{tet}(W) could be transferred at high frequencies (10^{-2} to 10^{-5}) in vitro between strains of the rumen anaerobe \textit{Butyrivibrio fibrisolvens} (19) and that resistance transfer was accompanied by acquisition of a large chromosomal fragment of 40 to 50 kb (19). The mobile chromosomal DNA integrated at a preferred site in the recipient genome, and Southern blots of pulsed-field gels showed that the new band hybridized to a \textit{tet}(W) probe (2). The object of the present study was to investigate the organization and distribution of the putative element involved in the transfer of \textit{tet}(W) between \textit{B. fibrisolvens} strains, Tn\textit{B1230}.

\textit{TnB1230} has sequence similarity to Tn1549. Genomic DNA was purified from \textit{B. fibrisolvens} 1.230 grown overnight in anaerobic M2GSC medium (14) containing 10 \mu g of tetracycline/ml. Tetracycline-resistant clones (resistant to a 5-\mu g ml^{-1} concentration) containing \textit{tet}(W) were identified in Lambda ZAPII and SuperCos 1 libraries (Stratagene Europe, Amsterdam, The Netherlands). Assembly of 12 kb of the possible 40- to 50-kb Tn\textit{B1230} sequence revealed the presence of eight unidirectional open reading frames (ORFs) (Fig. 1). The \textit{tet}(W) gene has a higher G+C content (53%) than the other genes (Fig. 1), due to the preference of \textit{tet}(W) for a G or C as the third nucleotide base in the codon (2). \textit{B. fibrisolvens} itself has a low DNA G+C content (36 to 41%) (5).

The translated products of five of the Tn\textit{B1230} ORFs have significant identity to proteins encoded by the 34-kb conjugative transposon Tn1549 from \textit{Enterococcus faecalis} E93 (8). Tn1549 is organized into three functional regions. The right end (3 kb) is implicated in the excision-integration process, the central part (7 kb) carries the VanB2 operon conferring vancomycin resistance, and the left extremity (24 kb) comprises a transfer operon of 18 contiguous ORFs (ORFs 13 to 30) (8). The proteins encoded by Tn\textit{B1230} ORFs A, 1, 2, 3, and 4 share sequence identities ranging from 48 to 67% with those encoded by Tn1549 ORFs 16, 17, 19, 20, and 21, respectively (Fig. 1). No homologue of Tn1549 ORF 18 was present in the sequenced region. Several of these Tn1549-encoded proteins have identity to other conjugative proteins: ORF 16 encodes a TrsK-like transfer protein and contains a conserved domain of the TraG/TraD family of bacterial conjugation proteins; the ORF 20 protein is similar to the conjugation protein TrsE encoded on the \textit{Staphylococcus aureus} plasmid pGO1 (15); and ORF 21 is 45% identical to Tn916 ORF 14, which is implicated in intercellular transposition (6). The strong identity of the Tn\textit{B1230} proteins (>48%) to proteins encoded within the Tn1549 transfer operon, themselves homologous to other proteins involved in conjugative transfer, provides compelling evidence that Tn\textit{B1230} is a conjugative transposon.

\textit{tet}(W) is flanked by identical DNA sequences. Two identical, 707-bp DNA direct repeat (DR) sequences flank \textit{tet}(W); DR1 finishes 657 bp upstream of the \textit{tet}(W) start codon, and DR2 starts 35 bp after the \textit{tet}(W) stop codon (Fig. 1). These sequences contain ORFs capable of encoding proteins of 98 amino acids. An alternative start codon is present upstream of DR1, outside the repeated DNA sequence, potentially encoding a longer 168-amino-acid protein. The ORF contained in DR2 is in the same frame as \textit{tet}(W).

Although DR sequences flanking antibiotic resistance genes often correspond to insertion sequence (IS) elements (18), we did not obtain evidence to suggest that the DR sequences in Tn\textit{B1230} were ISs. They have no sequence similarity to previ-
ously characterized IS elements, and they lack terminal inverted repeats and the DDE triad characteristic of the catalytic core of many IS transposases (9, 11), although there is a possible alternative DDD triad. On the other hand, database searches showed that the proteins encoded by the DRs had significant identity to bacterial nitroreductases (38% identity to the nitroreductase [Nrd] from \textit{Clostridium acetobutylicum} [GenBank accession number AE007749]). The longer upstream ORF-reading into DR1 encodes an oxygen-insensitive type I nitroreductase (Nrd1), while DR2 encodes an identical nitroreductase (Nrd2) that is truncated at the N terminus (Fig. 1). Nitroreductase enzymes are involved in the reduction of nitrogen-containing aromatic compounds, often releasing toxic, carcinogenic, or mutagenic by-products. The antimicrobial agent metronidazole is activated following reduction by nitroreductase enzymes. All of the \textit{B. fibrisolvens} strains (1.230, 2221, or 2221\textsuperscript{R} transconjugants) were sensitive to metronidazole (in concentrations ranging from 20 to 80 \mu\text{g} \text{ml}^{-1}), indicating that they contain active Nrd enzymes.

\textbf{FIG. 1.} Diagram showing the genetic organization of 12 kb of the sequenced region of Tn\textit{B1230}. The arrows indicate the location and direction of transcription of each ORF. The positions of the DRs (DR1 and DR2) are indicated by hatched boxes. The number of amino acids (aa) encoded by each ORF is shown, and the DNA G+C content is also indicated. The sequence identity to ORFs encoded by Tn\textit{J549} is indicated as appropriate. This figure is not drawn to scale.

\begin{table}[h]
\centering
\begin{tabular}{llll}
\hline
\textbf{Strain} & \textbf{Source} & \textbf{Drug resistance} & \textbf{Gene(s) present} & \textbf{Reference or strain designation} \\
\hline
\textit{Butyrivibrio fibrisolvens} & & & & \\
1.230\textsuperscript{b} & Bovine rumen; RRI & Tc\textsuperscript{r} & tet(W), tet(O) & 19 \\
1.210 & Bovine rumen; RRI & Tc\textsuperscript{r} & tet(O) & 19 \\
2221 & Type strain of \textit{B. fibrisolvens} & Te\textsuperscript{r} & tet(W) & ATCC 19171 \\
2221\textsuperscript{R} & Spontaneous Rif\textsuperscript{r} mutant of 2221 & Te\textsuperscript{r} & & 19 \\
\textit{Selenomonas ruminantium} FB322\textsuperscript{b} & Bovine rumen; RRI & Te\textsuperscript{r} & tet(W) & 7 \\
\textit{Mitsuokella multiacidus} P208-58 & Pig feces; Japan & Tc\textsuperscript{r} & tet(W) & 13 \\
\textit{Clostridium} sp. strain K10 & Human feces; RRI & Tc\textsuperscript{r} & tet(W), tet(32) & 20 \\
\textit{Bifidobacterium longum} F5\textsuperscript{b} & Human feces; RRI & Tc\textsuperscript{r} & tet(W) & 20 \\
\textit{Roseburia} sp. strain A2-183\textsuperscript{b} & Human feces; RRI & Tc\textsuperscript{r} & tet(W) & 3 \\
\hline
\end{tabular}
\caption{General characteristics of anaerobic bacteria used in this study\textsuperscript{a}}
\end{table}

\textsuperscript{a} Abbreviations: RRI, Rowett Research Institute, Aberdeen, United Kingdom; Rif\textsuperscript{r}, rifampicin-resistant mutant of type strain 2221; Te\textsuperscript{r}, tetracycline sensitive.
\textsuperscript{b} Strains in which the 340 bp immediately upstream of \textit{tet(W)} are identical.
carried by the opportunistic livestock pathogen *Arcanobacte-
rium pyogenes* (GenBank accession number AY049983) (4) are
only 64% identical to those of the commensal anaerobes de-
scribed here (data not shown).

The sequence conservation includes two 17-bp inverted re-
peat sequences capable of forming a stable stem-loop with a
\[\left\{\text{G} \atop \text{H}11002\right\} \text{H9004}\] of \[\left\{\text{G} \atop \text{H11021}\right\} \text{H11021}\] kcal/mol (2, 22) (Fig. 2), culminating in
a poly(U) sequence. This organization is characteristic of atten-
uation terminator sequences (http://www.bork.embl-heidelberg.
de/Docu/attenuation/index.html). Similar inverted repeat se-
dquences are located upstream of the ribosome protection genes
tet(M), tet(O), tet(S), tet(T), and tet(32), and they are important
for regulating gene expression in tet(O) (24) and tet(M) (21). The
expression of tet(M) depends on the synthesis of a 28-amino-acid
leader peptide encoded immediately upstream of the tet(M) ri-
osome binding site (21). A similar sequence potentially encoding
a 14-amino-acid peptide (MLYMCMSATYNPWQ) is present
upstream of the tet(W) gene in the commensal anaerobes (Fig. 2).
Each leader peptide contains a large proportion of rare amino
acids; 50% of the amino acids in the tet(W)-encoded leader pep-
tide correspond to the five rarest amino acids, which normally
comprise <14% of the total residues in a protein (10). Sequences
downstream of tet(W) diverge within 120 bp of the stop codon in
the different isolates (data not shown).

*Escherichia coli* cells transformed with pGEM-T vector (Pro-
mega) clones containing amplified fragments of the tet(W)
gene plus either 27 or 340 bp of the upstream sequence (Fig. 2)
were grown in concentrations of tetracycline ranging from 0 to
\[\text{g} \atop \text{ml}^{-1}\] (optical density at 650 nm was 0.1 after 24 h of growth in
a concentration of 15 \[\mu\text{g} \atop \text{ml}^{-1}\]), whereas those containing the

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**FIG. 2.** The CLUSTALW program was used to align sequences upstream of tet(W) genes from *B. fibrisolvens* 1.230 (GenBank accession number AJ222769), *M. multiacidus* P208 (GenBank accession number AY603069), and Clostridium sp. strain K10 (GenBank accession number AY601650) with tet(M) from *E. faecalis* (GenBank accession number M85225) and tet(O) from *Campylobacter jejuni* (GenBank accession number M18896). Solid arrows indicate the inverted repeats predicted to form secondary stem-loop structures. The DRs present only in *M. multiacidus* P208 are indicated by dashed arrows above the sequence. The putative poly(U) attenuation terminator (TTTTT), ribosome binding sites (GGAXG), and the ATG start codon are boxed. Nucleotides conserved in all five sequences are underscored with asterisks. The positions of the upstream primers used to amplify full-length (PCRB1) or truncated [tet(W)] sequences for expression cloning (indicated by [1] and [2], respectively) are shown by dashed overlining. The sequences in tet(W) and tet(M) encoding the putative leader peptides are shown in bold.
tet(W) gene and additional upstream sequences were able to grow well in the presence of up to 60 μg ml⁻¹ (optical density at 650 nm was 0.35 after 24 h of growth in a concentration of 60 μg ml⁻¹). This finding confirms that the region between 27 and 340 bp upstream of the tet(W) start codon is required for full expression of Tc,

Possible evolution of TnB1230. Primer sets designed for use against specific TnB1230 ORFs flanking tet(W) failed to give products with any of the other commensal bacteria containing tet(W) (Table 1), indicating the presence of different genetic supports for tet(W) in other host bacteria. The A. pyogenes tet(W) gene is sometimes associated with a mob gene related to the tnpZ genes found on the clonalrivial mobilizable transposons (4). The A. pyogenes tet(W) gene transferred at very low frequencies (10⁻⁹ to 10⁻¹¹) (4) compared to those of TnB1230 (10⁻² to 10⁻³) (19), presumably because the element relies on mobilization rather than self-conjugation.

The arrangement of TnB1230 ORFs encoding transfer proteins suggests that this element arose from a precursor lacking both nrd and tet(W) genes. The nrd gene may have integrated into this conjugative transposon, thereby interrupting, but not inactivating, an operon of genes involved in conjugative transfer. Transmissible, plasmid-borne noreductases from other groups of bacteria have been reported (16). This transposable element harboring a noreductase gene may have been the immediate precursor for TnB1230, with a small incoming element carrying tet(W) inserting into the nrd gene. The partial duplication of the nrd gene, found in TnB1230, may have occurred during the initial insertion event or subsequently by homologous recombination between an excised tet(W) fragment with nrd sequences downstream and an intact nrd present on another copy of the precursor transposon. This series of events explains why the sequences of the noreductase genes flanking tet(W) are identical and have a lower DNA G + C content than the surrounding ORFs and also why the DNA G + C content of the tet(W) gene is different from that of other TnB1230 or B. fibrisolvens genes. We noted previously that during conjugative mating experiments, B. fibrisolvens transconjugants occasionally gained more than one copy of TnB1230, illustrated by additional band shifts in the pulsed-field gel electrophoresis profile (19). Not all these bands hybridized to a tet(W) probe, and they may thus be the result of movement of shorter derivatives of TnB1230. It remains possible that TnB1230 is a complex, composite transposon carrying more than one set of transfer genes, but this possibility can be resolved only when the complete sequence of TnB1230 is available. The work reported here establishes for the first time the close linkage of a tet(W) gene with a cluster of transfer genes.

Nucleotide sequence accession number. The DNA sequence of TnB1230 discussed here has been deposited in the GenBank database under accession number AJ222769.

We thank Pauline Young and Katarzyna Kazimierczak for the automated DNA sequencing and Teresa M. Barbosa for construction of the Lambda ZAPII library.

We acknowledge the financial support of SEERAD (Scottish Executive Environment and Rural Affairs Department) and FSA (Food Standards Agency). R.B. was a summer student from the L.U.T., Clermont-Ferrand, France, on placement at RRI.

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