Published in final edited form as:

Plasmid. 2010 November ; 64(3): 150–155. doi:10.1016/j.plasmid.2010.06.003.

Multiple copies of functional, Tet(M)-encoding Tn916-like elements in a clinical *Enterococcus faecium* isolate

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Abstract

Tn916 and similar elements are very common in clinical enterococcal isolates, and are responsible for transmission of a variety of resistance determinants. It is commonly assumed that clinical strains carrying Tn916 have a single copy, although the actual number of copies in clinical isolates has never been systematically studied. We report a clinical isolate of *Enterococcus faecium* in which three distinct and excision-proficient copies of Tn916-like elements are present in the genome. All of the elements contain *tet(M)* genes, at least one of which confers resistance to tetracycline and minocycline. Two elements (Tn6085a, Tn6085b) are indistinguishable, containing an inserted 2758 bp Group II intron at the start of open reading frame Tn916ORF_06. The third (Tn6084) also contains the intron, but also has an *ISEfa11* integrated upstream of *tet(M)*. All three copies are able to excise from plasmid vectors when cloned in *E. coli*, and at least two of the elements can transfer to an *E. faecium* recipient strain. These data indicate that nearly identical Tn916-like elements encoding Tet (M)-mediated tetracycline/minocycline resistance can coexist in clinical *E. faecium* isolates.

Conjugative transposons form a subgroup of a larger class of mobile elements known as Integrating Conjugative Elements (ICEs)(Burrus et al., 2002). The prototype conjugative transposon is Tn916, originally described from *Enterococcus faecalis* DS16(Franke and Clewell, 1980). Tn916, 18 kb in length, is most commonly chromosomally-encoded. It transposes using a non-replicative lambda-like integration-excision mechanism and has the capacity to transfer to a wide range of hosts using its own conjugation genes(Clewell et al., 1995). Transfer to a recipient strain is generally accompanied by integration into the recipient chromosome, although integration into recipient plasmids has also been demonstrated (Scott et al., 1994).

Study of conjugative transfer of Tn916 in the laboratory has demonstrated multiple integrations into the recipient genome(Rice et al., 1992). The mechanism for these multiple integrations is not clear, nor is it clear whether multiple integrations occur in vivo with any frequency. Although the copy number of conjugative transposons found in specific strains has not been systematically studied in any species, most strains within which Tn916 (as well as most other conjugative elements) has been identified appear to have only a single copy of the transposon. One potential exception was reported by Spigaglia and colleagues(Spigaglia et al., 2006), who

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noted hybridization of two different sized *tet(M)-int* HincII fragments in 8 of 19 *Clostridium difficile* isolates examined. The authors concluded that these eight isolates each contained two different copies of Tn916-like elements, although they did not demonstrate that any of the transposons was either complete or functional.

We report an *Enterococcus faecium* strain that contains three variants of Tn916. All of these variants contain *tet(M)* genes in the typical location, along with functional excision mechanisms.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in these studies are listed and described in Table 1.

PCR amplification experiments

Primers used in PCR amplifications experiments are listed and described in Table 1. PCR amplification protocols were performed as previously described (Rice and Carias, 1994). Extension times were prolonged to 12 to 20 minutes when using GeneAmp XL PCR kit (Applied Biosystems) for amplification of the entire Tn6085b. Smaller amplification of segments of the cloned elements were column purified prior to digestion with HincII followed by separation on 1% agarose gels

Cloning of chromosomal fragments and sequencing

Genomic DNA was isolated from *E. faecium* C68 and digested with restriction enzymes as previously described (Rice and Carias, 1994). Digested fragments were then separated on 0.7% agarose gels and transferred to nylon membranes. Membranes were hybridized with digoxigenin-labelled probes according to the specifications of the manufacturer (Roche Diagnostics). After stringent washes hybridized fragments were detected using an anti-digoxigenin antibody and a chemiluminescent substrate. In subsequent gels, appropriate sized fragments were removed and cloned into an appropriate *E. coli* cloning vector. Colonies containing plasmids with appropriate inserts were identified by colony hybridization as described elsewhere (Dahl et al., 2000). Sequence of selected inserts was accomplished using services of Cogenics, Houston TX.

Excision experiments

All PCR primers used in these experiments are listed in Table 1. Circularization of Tn6084, Tn6085a and Tn6085b was assayed from *E. coli* clones as previously described (Rice and Carias, 1994).

Conjugation experiments

Transferability of tetracycline resistance was tested by performing matings between *E. faecium* C68 and *E. faecium* TX1330 (*fus^r*, *rif^r*). Matings were performed overnight on BHI agar plates by cross-streak technique as previously described (Rice and Carias, 1998). Transconjugants were selected on BHI agar plates containing tetracycline (15 µg/ml) fusidic acid (25 µg/ml) and rifampin (100 µg/ml). Transconjugant colonies were streaked to single colonies and retested for resistance to tetracycline. The presence of Tn6084 and Tn6085A or Tn6085b in the transconjugants was confirmed by PCR using primers designed to amplify the HincII fragment containing the *tet(M)* determinant and additionally with primers designed to amplify from upstream of *tet(M)* to within the *ISEfa11*.

RESULTS

Identification of three copies of Tn916-like elements in *E. faecium* C68

We have previously identified the Tn916-like VanB-type glycopeptide resistance transposon Tn5382 in *E. faecium* C68 (Carias et al., 1998). Resistance to tetracycline and minocycline in C68 suggested the presence of a *tet(M)* gene, which was confirmed by PCR amplification (data not shown). In an effort to determine whether the *tet(M)* gene was incorporated into a Tn916-like element, and if so whether Tn916 and Tn5382 were in close proximity within the C68 genome, we performed BstZI digestions of C68 genomic DNA and hybridized with internal fragments of *tet(M)* and the integrase (*int*) genes from Tn916 and Tn5382. (data not shown) To our surprise, three different bands were identified that hybridized with the Tn916 *int* internal fragment, suggesting the possibility that there were three copies of a Tn916-like element (in addition to Tn5382, whose *int* gene does not have sufficient homology with Tn916 *int* to hybridize) present in the genome of C68. HincII digestions of genomic DNA from C68 and *E. faecium* D344R (with a single copy of Tn916) hybridized with an internal fragment of *int*₉₁₆, confirming three copies, are shown in Figure 1.

Two of the resident Tn916-like elements were cloned directly into *E. coli* DH10B by ligating *int*-hybridizing BglII restriction fragments to the pIndigoBAC cloning vector. HincII digestion of these inserts suggested that they contained all of the elements of Tn916 (data not shown), although with some variations discussed below. In order to clone the final Tn916 copy in *E. coli*, the above *int*₉₁₆ probe was used to identify the HincII fragment containing this end and its junction with the C68 genome (distinguishable by size from HincII fragments derived from the other copies). This 3kb HincII fragment was cloned into *E. coli* by ligation to cloning vector pACYC184 and the sequence of the junction region was determined. This junction sequence was used to query the publicly available *E. faecium* DO database and identify the target sequence within which the transposon inserted, since a Tn916-like transposon is not present at this location in *E. faecium* DO (Genbank NZ_ACIY00000000). This sequence was used to create an additional primer that would amplify the opposite junction when used with a primer directed outward from the nt 1 end of Tn916. Sequence analysis confirmed that this amplification product included the transposon-C68 junction. Finally, in order to confirm that this insertion represented a functional Tn916-like element, PCR primers were synthesized based on the flanking sequences and the entire transposon with flanking sequences was PCR amplified and cloned into *E. coli* by ligating it to cloning vector pCC1.

Sequence analysis of the three transposon variants

The fact that the three cloned transposons represented three separate insertions was confirmed by demonstrating distinct junction sequences for each (Figure 2). HincII digests of amplification products spanning 4 segments of each cloned transposon revealed identical sized internal restriction fragments for two of the elements, with differences observed in one of the elements (Tn6084) for restriction digest of one amplification product (Figure 3). Sequence analysis revealed that this difference was due to insertion of *ISEfa11* (Genbank accession number [FJ866609](#)) 56 nucleotides upstream of *tet(M)* (data not shown). While it is not clear whether this insertion exerted an effect on *tet(M)* transcription, it should be noted that previous work has suggested that the presence of sequences further upstream of the structural *tet(M)* genes in Tn916-like elements enhances transcription (Hill et al., 1988). The newly available genome assembly for C68 [provided through support by the National Institute of Allergy and Infectious Diseases (NIAID), Genome Sequencing Centers] allowed us to determine the sequences of the remainder of the elements. All three of the transposons contained a 2.7 kb putative group II intron inserted at Tn916 nucleotide 3913 (Genbank accession NC_006372). This insertion occurred at the beginning of open reading frame Tn916_06 (formerly *orf19*), whose function remains unknown. One (Tn6084) had the above-described insertion of

ISEfa11. The other two transposons were identical and therefore were given the designations Tn6085a and Tn6085b. Comparison of the remainder of the sequence with the sequence of Tn916 reveal only 7 nucleotide changes in the non-*tet(M)* region of the transposons. The *tet(M)* gene was found to be identical to the *tet(M)* reported in two Tn916 like elements found in human isolates of *E. faecalis* (18854-s-1; 20028-s-1)(Agero et al., 2006), which differs from the Tn916 *tet(M)* sequence by 47 nucleotides. The sequences of the Tn6084, Tn6085a and Tn6085b and their flanking sequences can be found in Genbank under the accession numbers HM243622, HM243621 and HM43623.

Functional analysis of excision of the three transposons

We performed excision assays in *E. coli* to determine whether each element was capable of excision. The excision assay (Rice et al., 2007) measured the formation of the classical circular intermediate by amplifying the transposon termini and the “joint” region that connects the two ends when the transposon has circularized. Joint regions were readily amplified from each of the cloned transposons, indicating that each of the elements had a functional excision mechanism (data not shown).

Transferability of the Tn916-like elements

In order to determine whether any of these transposons was conjugative, we performed matings between C68 and *E. faecium* 1330 (kindly provided by Barbara E. Murray)(Nallapareddy et al., 2006). Matings were performed overnight on filters as previously described (Christie et al., 1987) with selection on BHI agar plates containing tetracycline (15 µg/ml), rifampin (100 µg/ml) and fusidic acid (25 µg/ml). Transconjugants were recovered at a low frequency (ca. 10^{-7} /recipient CFU) indicating that at least one of the transposons was conjugative. Growth of the donor strain in tetracycline (15 µg/ml) increased the frequency of transfer 3-fold. PCR amplification designed to amplify the HincII fragment containing *tet(M)* revealed that all of the transconjugants had received a copy of the either Tn6085a or Tn6085b (Figure 4A). One transconjugant (CV63) revealed a second, fainter band that was larger in size and consistent with the expected amplification product from Tn6084, with the ISEfa11 inserted. Amplification with primers designed to amplify from upstream of *tet(M)* to within the ISEfa11 confirmed that Tn6084 was also present in CV630 but not in CV631 or CV632 (Figure 4B).

Discussion

The wealth of genome sequence data that has accumulated over the past few years has provided an opportunity to better appreciate the variety and prevalence of mobile elements in the bacterial genome. However, the number of repeated IS elements in many of these genomes makes it very difficult to complete and close these sequences, so the majority of them are left as incomplete assemblies. Under these circumstances, it is not possible to determine precisely how many repeated elements exist within the genome. As such, the question of how many copies of a large transposon such as Tn916 exist within a specific genome cannot be answered easily by reference to publicly available genome sequences.

The data presented in this paper confirm that *E. faecium* C68 has three functional copies of nearly identical Tn916-like elements, all containing *tet(M)*, in its genome. This would seem to be a rather inefficient arrangement if the purpose of acquiring Tn916 is to express resistance to tetracycline. The level of tetracycline resistance expressed by C68 does not differ significantly from enterococcal strains with just one copy of Tn916 (data not shown). The presence of these three elements in a single strain may confer some other selective advantage. It may be that one or more of the insertions activates or inactivates nearby genes in a manner that provides a selective advantage in an environment. Alternatively, the presence of three such elements in the genome may provide for genetic flexibility through interaction between

individual elements. We have previously shown deletion of a large *E. faecium* genome segment that occurred through an interaction between non-identical but related Tn916-like elements (Rice et al., 2005). It is reasonable to presume that the rates of such interactions between identical elements would occur at an even higher frequency than among non-identical elements and that this flexibility could be advantageous in the challenging environment of the mammalian gastrointestinal tract. The presence of multiple elements may also increase the frequency of excision and circularization of each element (as has been shown in *E. faecalis*) (Flannagan and Clewell, 1991) which would then increase the frequency of genetic exchange through read-through transcription of the conjugation genes which are downstream of the integrase and excisionase only in the circular form (Celli and Trieu-Cuot, 1998). Such increased genetic exchange could also be advantageous in a challenging environment.

We can only speculate about the mechanism by which *E. faecium* acquired three copies of Tn916-like element. As noted above, transfer of Tn916 in the laboratory is frequently associated with the appearance of multiple copies in the transconjugant genome (Scott et al., 1994). It is conceivable that the three copies all arrived during a single mating event, with Tn6084 subsequently acquiring the *ISEfa11* from elsewhere in the genome (there are one or two additional copies of *ISEfa11* in the *E. faecium* C68 genome, based on analysis of individual trace files of the whole genome sequence), or that Tn6085a and Tn6085b arrived in one mating event, with the subsequent arrival of the *ISEfa11*-containing transposon during a different conjugation. Previous work in *E. faecalis* suggested that the presence of Tn916 in a genome did not confer immunity to subsequent acquisitions of similar elements (Norgren and Scott, 1991). It seems unlikely that the number was reached by the arrival of a single transposon with subsequent duplication, as Tn916 transposes via a conservative mechanism. However, recent work by Lee, et al (Lee et al.) indicates that some ICE elements may replicate after excision. If, contrary to current beliefs, Tn916 replicates after excision, then the multiplication of insertions could occur. The complete discordance of the junction sequences of the three elements would argue against one element being the immediate progenitor of the other or that the three copies resulted from a mating event from a donor with a single copy.

E. faecium C68 is a member of the highly effective clonal complex 17, a group of ampicillin-resistant *E. faecium* strains that has spread worldwide and is responsible for the majority of vancomycin-resistant *E. faecium* outbreaks around the world (Top et al., 2008). C68 itself is a representative of the only vancomycin-resistant *E. faecium* clone found in all 11 Cleveland hospitals studied in the 1990's, suggesting it is well-adapted to survival in the hospital (Donskey et al., 1999). As noted above, C68 also contains a fourth Tn916-like element, the 30 kb VanB transposon Tn5382 (Carias et al., 1998). Taken together, these data indicate that 90 kb, or roughly three percent of the 3.08 megabase C68 genome consists of Tn916-like elements, constituting a large number of duplicated genes. If this is not an accident, then the presence of these elements must confer significant advantages to these opportunistic pathogens in the clinical setting.

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Figure 1.

Hybridizations of HincII-digested genomic DNA from enterococcal strains using an internal fragment of *int916* as a probe. Lane 1: *E. faecium* C68 digested with HincII; Lane 2: *E. faecium* D344R (with a single copy of Tn916) digested with HincII; Lane 3: λ HindIII size standards – sizes in kb to the left of the figure; Lanes 1'–3': Southern hybridization of the DNA pictured at the left using the internal fragment of *int916*.

Tn6085a ctttttatttttaAAAATAGCATAAAAATCTA...AGAAATTTATACTTTGTTTattttataaaaatt

Tn6084 tctttttataaatAAAATAGCATAAAAATCTA ...AGAAATTTATACTTTGTTTctatataaaaaa

Tn6085b atttttaaataaaAAAATAGCATAAAAATCTA ...AGAAATTTATACTTTGTTTataataaaaata

Figure 2.

Termini and junction sequences of each of the three *E. faecium* C68 transposon insertions. Transposon nucleotides are in upper case. Junction regions (underlined) and genomic nucleotides are in lower case.

Figure 3.

HincII restriction digestions of PCR amplifications of four segments cloned into *E. coli*.

Identical bands are seen for the three transposons for the first, second and fourth amplification products. Digestion of the third PCR product of Tn6084 yields two bands due to the insertion of *ISEfa11* (Lane H). Size standard sizes are listed to the right of the figure. Lanes A, D, G, J: Tn6085a; Lanes B, E, H, K: Tn6084; Lanes C, F, I, L: Tn6085b. Lane SS: 1 kb size standard. PCR amplifications Lanes A,B,C: nt 7 TO 4449; Lanes D,E,F: nt 4228 to 10083; Lanes G,H,I: nt 9961 to 14956; Lanes J,K,L: nt 14865 to 17978.

Figure 4A

Figure 4B

Figure 4.

A. PCR amplification of *tet(M)* region of 12 transconjugants resulting from a mating between *E. faecium* C68 and *E. faecium* TX1330. Lane 1 is the recipient strains (negative control). LANES 2–13: Transconjugants CV621–632. Lane 14: *E. faecium* C68 grown without tetracycline; Lane 15: C68 grown with tetracycline; Lane 16: 1 kb size standard. Sizes are marked to the right of the figure. The arrows indicate the additional band resulting from amplifying this segment in Tn6084, which has the insertion of *ISEfa11* in the region. B. PCR amplification using primers ACJQ_149_7990 and 6773_B designed to amplify a segment from within *ISEfa11* to a flanking region. The results confirm that transconjugant CV630 has a copy of Tn6084 while transconjugants CV631 and CV632 do not.

Table 1

Strains, plasmids and primers used in these studies

Strain	Resistance (phenotype)	Origin (reference)
<i>E. faecium</i> C68	Ap ^r , Vm ^r , Tc ^r /Mc ^r	Clinical isolate
<i>E. faecium</i> TX1330	Kan ^r , Rif ^r , Tc ^s	Provided by Barbara E. Murray (Nallapareddy et al., 2006); Used as a recipient in mating experiments with C68
<i>E. faecium</i> CV621 to CV632	Rif ^r , Fus ^r , Tc ^r	transconjugants from mating of C68 with TX1330, selected for Tc ^r transfer
<i>E. coli</i> DH10B	$\Delta M15 \Delta lacX74 deoR$ $recA1 \Delta ara \Delta I39 \Delta(ara$ $leu)7697 galU galK \lambda^-$ $rpsL endA1 nupG$	Transformation-competent <i>E. coli</i> (Invitrogen, Carlsbad, Calif.)
<i>E. coli</i> EPI300	F ⁻ $mcrA \Delta(mrr-hsdRMS-$ $mcrBC) \Phi 80 \Delta lacZ \Delta M15$ $\Delta lacX74 recA1 endA1$ $araD139 \Delta(ara, leu)7697$ $galU galK \lambda^- rpsL nupG$ $trfA dhfr$	Strain used for maintaining copy control of pCC1 plasmids (Epicentre Biotechnologies, Madison, Wis.)
Plasmid		
pACYC184	Cm ^r , Tc ^r	Cloning vector
PCR-XL-TOPO	Km ^r	<i>E. coli</i> vector for cloning PCR products directly (Invitrogen)
pIndigoBAC-5	Cm ^r	<i>E. coli</i> vector for cloning (Epicentre Biotechnologies, Madison, Wis.)
CopyControl pCC1(Blunt)	Cm ^r	<i>E. coli</i> F-factor replicon (maintains one copy per cell) with an inducible high-copy <i>oriV</i> origin of replication (Epicentre Biotechnologies, Madison, Wis.)
pCWR1216	Km ^r , Tc ^r	21 kb Tn6085a-containing BglIII fragment from C68 cloned into BamHI-digested pIndigoBAC cloning vector
pCWR1217	Km ^r , Tc ^r	23 kb Tn6084-containing BglIII fragment from C68 cloned into BamHI-digested pIndigoBAC cloning vector
pCWR1262	Cm ^r	3 kb HincII fragment from C68 chromosome containing the <i>int₉₁₆xis₉₁₆</i> genes and termini from Tn6085b and junction sequence cloned into EcoRV-digested pACYC184
pCWR1782	Cm ^r , Tc ^r	20 kb PCR amplification product containing intact Tn6085b from the C68 chromosome cloned into CopyControl pCC1 vector
Primer name	Primer Sequence	Purpose of Primer
Tn916-1	5'- GTTTGTGACCTTGATAA AGTGTGATAAGTCC-3'	Used to detect circularization of Tn916 construct (Rice and Carias, 1994)

Strain	Resistance (phenotype)	Origin (reference)
Tn916-17890	5'-CACTTCTGACAGCTAA GACATGAG-3'	Used to detect circularization of Tn916 construct (Rice and Carias, 1994)
Tn916INT_A	5'-CGCAAAACAGAACGC TCAAAAGACC-3'	Tn916 integrase amplification
Tn916INT_B	5'-GTGTCAAACCACCAA CTCTGAAATACG-3'	Tn916 integrase amplification
p1262_C68	5'-AGATGATCGAAATCAT AATGGGATGGTAAGT- 3'	derived from <i>E. faecium</i> DO to use with Tn916-1
1266_B	5'-TCTTTTAGATACAGCT TTGAGTC-3'	used to amplify Tn6085b
1262_C	5'-TATTCTTCCCTCATAG CTTCC-3'	used to amplify Tn6085b
Tn916_7	5'-GCATAAAATCTAGTT ATCCGC-3'	used to amplify Tn916 then HincII digest PCR product
Tn916_4449_R	5'-GATACCATTTCCCAT GTCG-3'	used to amplify Tn916 then HincII digest PCR product
Tn916_4228	5'-GGCTTGAATGATGAAT ATGAG-3'	used to amplify Tn916 then HincII digest PCR product
Tn916_10083_R	5'-GCATAACATCTCCGC AG-3'	used to amplify Tn916 then HincII digest PCR product
Tn916_9961	5'-GCTCATAAGCCTATGG TAG-3'	used to amplify Tn916 then HincII digest PCR product
Tn916_14956_R	5'-GATGTACTTCATGGCG ACG-3'	used to amplify Tn916 then HincII digest PCR product
Tn916_14865	5'-TGGTAGTGCTATTAC GCTG-3'	used to amplify Tn916 then HincII digest PCR product
Tn916_17978_R	5'-GTAAAGCCTTATTCTA TGTGC-3'	used to amplify Tn916 then HincII digest PCR product
ACJQ_149_7990	5'-TTA CAA ATA TGC TCT TAC GTG CT -3'	used with 6773_B to amplify Tn6084, distinguishing it from Tn6085
6773_B	5'-AAT GAG CTT TAT TTG CGC AGA TG -3'	Within <i>ISEfa11</i> used with ACJQ_149 to amplify Tn6084, distinguishing it from Tn6085