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Homology Among *tet* Determinants in Conjugative Elements of Streptococci

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A mutation to tetracycline sensitivity in a resistant strain of *Streptococcus pneumoniae* was shown by several criteria to be due to a point mutation in the conjugative Ω(*cat-tet*) element found in the chromosomes of strains derived from BM6001, a clinical strain resistant to tetracycline and chloramphenicol. Strains carrying the mutation were transformed back to tetracycline resistance with the high efficiency of a point marker by donor deoxyribonucleic acids from its ancestral strain and from nine other clinical isolates of pneumococcus and by deoxyribonucleic acids from group D *Streptococcus faecalis* and group B *Streptococcus agalactiae* strains that also carry conjugative *tet* elements in their chromosomes. It was not transformed to resistance by *tet* plasmid deoxyribonucleic acids from either gram-negative or gram-positive species, except for one that carried transposon Tn*916*, the conjugative *tet* element present in the chromosomes of some *S. faecalis* strains. The results showed that the *tet* determinants in conjugative elements of several streptococcal species share a high degree of deoxyribonucleic acid sequence homology and suggested that they differ from other *tet* genes.

The *tet* determinant found in a number of tetracycline-resistant strains of *Streptococcus pneumoniae* (pneumococcus) is in the chromosome as part of a large insertion of foreign DNA (Ω*tet*) that is often linked closely to an insertion (Ω*cat*) conferring chloramphenicol resistance (20). The most interesting property of Ω*tet* and other insertions carrying *tet* is that some of them transfer by a novel conjugation process to the chromosomes of wild-type recipients and that the transconjugants can retransfer the elements, all in the absence of detectable plasmids (21, 23; manuscripts in preparation). They can also be transferred by transformation, but only at a low efficiency (20). Transfer of an insertion into wild-type cells by transformation is analogous to putting wild-type DNA into deletion recipients, requiring entry into the cell of a donor strand that is long enough to span the entire region of nonhomology and to integrate by use of the flanking regions of homology. Random cuts of the donor DNA associated with binding and entry (16) make this a rare event for long insertions. The frequency of Ω*tet* transformation was about 10^{-3} of that for point mutations when the donor preparation was a gently handled crude lysate and fell rapidly with moderate shear of the lysate. From these data, the length of Ω*tet* was estimated to be at least 30 kilobases (kb) (20).

With respect to the conjugative transfers, other laboratories are reporting similar transfers of what appear to be chromosomal elements both within and between species of pneumococcus (1), group B streptococci (9), and *Streptococcus faecalis* (5). In each case, the conjugative element carries a *tet* determinant and may carry up to three other resistance genes. In *S. faecalis*, a conjugative *tet* has been shown to be part of a transposon, Tn*916* (5).

In this paper we report that the *tet* genes in all but one of these conjugative elements share a high degree of DNA sequence homology. However, they do not appear to have homology to *tet* genes found on plasmids in either gram-positive or gram-negative species, with the exception of pAM211, which is pAD1 to which Tn*916* has transposed (5).

The evidence for these conclusions came from transformation experiments in which the recipient strain was tetracycline sensitive because of a point mutation, called *tet-3*, in the Ω*tet* region derived from the clinical strain BM6001. In contrast to the low and shear-sensitive transformation of wild type to tetracycline resistance as described above, this strain was transformed to tetracycline resistance with the same high efficiency as reference point markers by DNAs from its Tc^r parent and from other Tc^r streptococci of three species and varied origins, but not by DNAs from a number of other Tc^r strains. Be-

cause efficient transformation of this kind requires a high degree of homology between donor and recipient, the transformation assay provides a sensitive probe for in vivo hybridization of test DNAs to the *tet* region found in BM6001.

Below, we describe the origin of the *tet-3* mutation and the evidence establishing that it is a point mutation in Ω*tet* and then present data on the tests of homology by the transformation criterion.

MATERIALS AND METHODS

Bacterial strains. Strains relevant to the origin and characterization of *tet-3* are listed in Table 1. Other strains are listed in Tables 5 and 6.

Growth. All strains were grown in a rich casein hydrolysate-yeast extract medium (17). For clinical strains of pneumococcus, this medium was supplemented with an extra 0.5% filter-sterilized yeast extract. *Escherichia coli* strains were grown in the presence of 10 μg of tetracycline (Sigma Chemical Co., St. Louis, Mo.) per ml.

MICs. Cultures were grown in the absence of drug and diluted. Portions containing ~100 colony-forming units (CFU) in 50 μl were spotted on the surface of casein hydrolysate-yeast extract-blood agar plates containing twofold increasing concentrations of tetracycline. The minimal inhibitory concentration (MIC) was taken as the lowest concentration on which colonies did not appear after 24 h of incubation at 37°C.

DNA preparations. Pneumococcal lysates were prepared by a deoxycholate-sodium dodecyl sulfate procedure as described previously (19). *S. faecalis* lysates were prepared by treatment with lysozyme followed by sodium dodecyl sulfate (24). Group B (*Streptococcus agalactiae*) lysates were prepared by mutanolysin-induced lysis of cells washed in 10 mM potassium acetate–1 mM EDTA, pH 7 (27). The final

TABLE 1. *Pneumococcal recipient strains and derivatives from BM6001*

Strain	Genotype[a]	Origin (reference)
Wild type with respect to *cat* and *tet*		
Rx1	*hex*[b]	8
DP1001	*ery-2*	Transformant of Rx1 (8)
DP1002	*nov-1*	Transformant of Rx1 (8)
DP1004	*str-1*	Transformant of Rx1 (8)
DP1007	*rif*	Mutant of Rx1
DP1008		Tc[r] mutant of Rx1 (see text)
DP1009	*str-1 rif*	Rif[r] mutant of DP1004
DP1609	*hex*[+] *str-1*	Transformant of DP1600 (7)
DP1617	*hex*[+] *str-1 ery-2 nov-1 sul-d fus stg*	Derivative of DP1600 (N. B. Shoemaker)
BM6001 and derivatives carrying *cat* or *tet* insertions		
BM6001	Ω(*cat-tet*)	Paris clinic (3)
BM6002	Ω*tet*	"Cured" BM6001 (3)
DP1302	*ery-2* Ω(*cat-tet*)	DP1001 × BM6001 DNA[c]
DP1322	Ω(*cat-tet*)	Rx1 × DP1302 DNA (20)
DP1328	Ω(*cat-tet-1*)	Tc[s] mutant of DP1322
DP1329	Ω(*cat-tet-2*)	Tc[s] mutant of DP1322
DP1330	Ω(*cat-tet-3*)	Tc[s] mutant of DP1322
DP1333	Ω*tet-3*	Cm[s] mutant of DP1330
DP1334	*str-1 ery-2 nov-1* Ω(*cat-tet*)	DP1322 × DP1617 DNA
DP1344	*hex*[+] *str-1* Ω(*cat-tet-3*)	DP1609 ×Cg DP1330[d]
DP1345	*str-1* Ω(*cat-tet-3*)	DP1004 ×Cg DP1330
DP1346	*str-1* Ω(*cat-tet-3*)	DP1330 × DP1617 DNA
DP1347	*nov-1* Ω(*cat-tet-3*)	DP1330 × DP1002 DNA
DP1349	*str-1* Ω(*cat-tet*)	Tc[r] revertant of DP1346

[a] *str-1, nov-1, ery-2, rif, sul-d, fus,* and *stg* are point mutations of the normal genome, conferring resistance to streptomycin (Str), erythromycin (Ery), novobiocin (Nov), rifampin (Rif), sulfonamides (Sul), fusidic acid (Fus), or streptolydigin (Stg). Ω(*cat-tet*) and Ω*tet* are insertions of DNA not found in the normal genome (see reference 20 and Fig. 1). The complete designation is Chr:: Ω(*cat-tet*), etc. We have not assigned numbers to the various insertions because it is not yet clear to what extent those of similar *cat* and *tet* content are identical.

[b] Rx1 and all of its derivatives listed here except DP1609, DP1617, and DP1344 are genotype *hex*, phenotype Hex[−], lacking the marker-specific mismatch correction system found in Hex[+] strains (see reference 8). For emphasis, only the *hex*[+] genotype is listed here where it applies. DP1600 was constructed by transformation of Rx1 with R6 DNA (7).

[c] DP1001 × BM6001 DNA indicates transformation.

[d] DP1609 ×Cg DP1330 indicates filter mating of DP1609 recipients with DP1330 donors.

preparation in each case contained about 10 μg DNA per ml in 150 mM NaCl–15 mM sodium citrate–0.0008% sodium dodecyl sulfate. *E. coli* preparations were made by incubating washed cells with lysozyme, adding sodium dodecyl sulfate, and banding the lysates in CsCl. The DNA bands were collected, diluted twofold with water, ethanol precipitated, and redissolved in 150 mM NaCl–15 mM sodium citrate. The final DNA concentrations were near 10 μg/ml, as estimated from intensities of bands in gel electrophoresis.

Gel electrophoresis. Gels were run in 0.5% agarose (Seakem, Marine Colloids Division, FMC Corp., Rockland, Maine) at 1 to 2 V/cm in Tris borate-EDTA buffer as described previously (22). Quantities and mobilities of DNA samples were compared with those of known quantities of lambda DNA and its *Eco*RI digest.

Transformation. Preparation, thawing, and use of cells previously grown to competence and stored at −80°C have been described (8). There were typically about 4×10^7 cells (2×10^7 CFU) per ml at the time of addition of 0.1 volume of donor DNA, and the cells resumed growth at about this time. Pancreatic DNase (Worthington Diagnostics, Freehold, N.J.) was added 30 min after the DNA addition. The cells were then diluted, plated in 6 ml of casein hydrolysate-yeast extract agar on a base of 22 to 25 ml of casein hydrolysate-yeast extract agar, followed by an 8-ml overlay of casein hydrolysate-yeast extract agar, and then incubated at 37°C for 90 min before the addition of drug in an 8-ml agar overlay, followed by further incubation. Drug concentrations were as described previously (20), except that the overlay contained 5 instead of 10 μg of tetracycline per ml.

Tests of curing, transformation, or mutation to drug sensitivity. Resistant strains were made competent and exposed to wild-type or other DNAs to determine whether they would transform the recipients to sensitivity. After exposure to DNA, the cells were incubated in liquid for 4 h to allow segregation before plating without drug. The next day, colonies were transferred with sterile toothpicks to blood agar plates with and without drug, as described previously (20). Cultures exposed to the DNA of the recipient cell and to no DNA were tested as controls.

Conjugation. Matings were on nitrocellulose filters under agar overlays in the presence of 70 μg of DNase per ml, 10 mM $MgSO_4$, and 2 mg of bovine albumin per ml, as described previously (23). Transconjugants were selected as described, and their phenotypes were confirmed by toothpick replication to other drugs (21).

Reversion tests. Dilutions of cultures growing at about 5×10^8 cells per ml were plated as described above for scoring of transformants, except that the selective drug was added immediately after the agar had hardened, in 8 ml containing 10 μg of tetracycline or 15 μg of chloramphenicol per ml, along with controls without drug. Colonies were counted after 24 h of incubation at 37°C.

RESULTS

The work falls into two parts: (i) the origin and characterization of a point mutation to tetracycline sensitivity and (ii) the use of this mutation as a probe for homology of *tet* genes from other strains and species to that in BM6001.

Origin of Tc^s and Cm^s derivatives of BM6001. The mutation of interest arose in experiments designed to look for "curing" of resistance in a Cm^r Tc^r strain by the addition of wild-type DNA as described above. Tc^s derivatives of DP1322 were rare, usually less than 10^{-3}, and none was confirmed to have deleted Ω*tet*. However, one experiment yielded three Tc^s clones, numbered DP1328, DP1329, and DP1330, each of which reverted readily to tetracycline resistance and behaved as if it carried a point mutation rather than a deletion of Ω*tet* (see below).

In contrast, after DP1322 was exposed to wild-type DNA, Cm^s Tc^r colonies appeared at a frequency of 1.9% (23 of 1,196 CFU tested). However, because control experiments then showed that DP1322 spontaneously segregated Cm^s Tc^r colonies at a frequency approaching 1% (41 of 4,771 CFU tested), we could not prove that the wild-type DNA had induced the "cure" of chloramphenicol resistance, and these experiments were set aside. On examining four such Cm^s Tc^r strains derived from these and similar experiments, each behaved as if it had deleted all or part of the Ω*cat* region, by the criteria that (i) they did not revert to chloramphenicol resistance ($<10^{-8}$/CFU), and (ii) as recipients for DNA from Cm^r donors, they were transformed to chloramphenicol resistance at frequencies that were 15 to 20% of those for point markers and were moderately sensitive to shear of the donor DNA (see DP1333 below and DP1304 in reference 20). This result was like that obtained by adding wild-type DNA to a deletion recipient or by adding Ω*tet* to wild-type cells as described above, though it was much less extreme than that for the latter. The numbers suggested that the *cat* region deleted in these strains had a size of a few kilobases, substantially smaller than the estimate of ≥30 kb for Ω*tet* (20).

Evidence that DP1330 carries a point mutation in Ω*tet*. DP1330 and its derivatives were examined for reversion to tetracycline resistance, for ability to transfer either the Tc^s or the revertant Tc^r phenotype by conjugation, and for their properties as recipients for transformation of tetracycline sensitivity to tetracycline resistance. The reversion and transformation tests were done in strains with and without the mismatch-specific correction system that reduces both spontaneous mutation and transformation for some point mutations and not others in cells of Hex^+ phenotype (8, 15, 25).

Some conjugative transfers are presented in Table 2. Transconjugants from DP1330 or its Nov^r derivative DP1347 were selected for chlor-

amphenicol resistance and found to be uniformly tetracycline sensitive but revertible to tetracycline resistance, and the revertant donor DP1349 transferred tetracycline resistance with chloramphenicol resistance. Thus, both the mutation to tetracycline sensitivity and its reversion occurred in the transferable Ω*tet* element rather than elsewhere in the genome. BM6002, a Cm^s Tc^r segregant from BM6001 (3), still transferred tetracycline resistance, and the transconjugants did not revert to chloramphenicol resistance.

Revertant frequencies in cultures of several Hex^- strains were sometimes well above 10^{-5}/CFU plated (Table 3). Subclones grown from small inocula showed frequencies that were lower but still near 10^{-5}/CFU, suggesting that the reversion frequency was near 5×10^{-6}/cell. However, the Hex^+ transconjugant DP1344 showed 10- to 30-fold fewer revertants ($\sim 6 \times 10^{-7}$/CFU) than did the Hex^- parents or the Hex^- transconjugant DP1345.

Transformation data are shown in Table 4 for recipient DP1333, which was a spontaneous Cm^s

TABLE 2. *Conjugative transfer from Tc^s mutant, Tc^r revertant, and Cm^s Tc^r donors*

Donor	Recipient	Transconjugants[a]	Transconjugants per donor[b]
DP1330	DP1609	Str^r Cm^r Tc^s	4×10^{-6}
DP1347	DP1004	Str^r Cm^r Tc^s	3×10^{-5}
DP1349	DP1007	Rif^r Cm^r Tc^r	3×10^{-6}
BM6002	DP1004	Str^r Cm^s Tc^r	4×10^{-5}

[a] Filter matings were performed. Initial selections were for all combinations of one donor and one recipient resistance. Cotransfer of phenotypes was checked by toothpick replication of transconjugant colonies. No transfer of Nov^r from DP1347 or Str^r from DP1349 was detected.

[b] Donors and recipients averaged 10^7 and 10^8 CFU/ml of resuspended cells, respectively.

TABLE 3. *Reversion to tetracycline resistance in strains carrying tet-3*

Strain	No. of Tc^r colonies per 10^5 CFU plated[a]
DP1345	2.6
DP1346	1.5[b]
DP1347	3.1
DP1333	3.1
DP1330	4–6
DP1330 SC[c]	0.8
DP1344 (Hex^+)	0.06
DP1344 SC	0.06

[a] Cultures were tested as described in the text. Numbers of CFU plated ranged from 5×10^6 to 1.5×10^7.

[b] Mean of 10 determinations that ranged from 0.6 to 3.2.

[c] SC, Subcultures grown from small inocula.

TABLE 4. *Transformation of tet-3 strains for tetracycline resistance and other markers*

Recipient	Donor	Resistance scored[a]	Transformants per μl[b]	
			No shear	Sheared[c]
DP1333 (Hex^-)	DP1334	Tc	2,400	990
		Cm	380	11
		Str	2,000	1,000
		Ery	2,200	960
		Nov	1,600	830
	DP1328	Tc	2.7	3.2
		Cm	160	17
	DP1329	Tc	2.7	2.4
		Cm	83	18
	DP1617	Tc	2.5[d]	
		Str	1,700	
	None	Tc	2.3[d]	
		Cm	<0.01	
		Str	<0.01	
DP1344 (Hex^+)	DP1334	Tc	230	
		Nov	160	
		Ery	640	
	None	Tc	~0.05[d]	

[a] Str^r, Ery^r, and Nov^r result from the point markers *str-1*, *ery-2*, and *nov-1*, respectively, which are in DP1334 and DP1617. Transformation for streptomycin could not be scored in DP1344 because it has *str-1*.

[b] To simplify reading, entries are presented per microliter, so that 10^3 here corresponds to 10^6 per milliliter of transformed culture.

[c] For DP1334, DP1328, and DP1329, lysates were used either as prepared or after five passages through a no. 27 needle, which reduced the molecular weight to near 5×10^6. Purified, high-molecular-weight DP1617 DNA was used.

[d] Spontaneous revertants (see text).

segregant from DP1330, and for the Hex^+ DP1344 (results similar to those for DP1333 were seen for Tc^r DNAs transforming DP1330). Significant observations were that (i) for donor DP1334 (the parental Cm^r Tc^r DP1322 with three point markers added), transformation to tetracycline resistance was comparable to that for the point markers both before and after shear of the donor DNA; (ii) transformation of DP1333 to chloramphenicol resistance was substantially lower and moderately sensitive to shear, as with other Cm^s derivatives; (iii) DP1328 and DP1329 Tc^s donors did not transform DP1333 to tetracycline resistance at a frequency detectable above a high background of revertants seen with no DNA or DP1617 DNA; and (iv) in DP1344 recipients, tetracycline resistance behaved as a low-efficiency marker, giving transformants at numbers near those for *nov-1* and well below those for *ery-2*, which are known to be low and intermediate efficiency markers, respectively, in Hex^+ cells (7). Transformation of DP1344 to

tetracycline resistance was also hypersensitive to UV irradiation of the donor DNA (data not shown), another property of low-efficiency markers in Hex$^+$ cells (7), and the background of Tc^r revertants was lower in this recipient, consistent with the data shown in Table 3.

The Hex system is known to act to reduce spontaneous mutation (25) and to reject some mismatches, but not others, in the donor-recipient heteroduplexes formed during transformation (8). The data therefore implied that the *tet-3/tet*$^+$ mismatch was of the low-efficiency class strongly attacked by the Hex system. Because all such cases so far characterized involve missense (11, 25) or frameshift (6) mutations, these results reinforced the evidence that *tet-3* was a point mutation blocking expression of tetracycline resistance.

Homology of *tet-3* to other *tet* genes. Along with previous data (20), the above results lead to the schematic diagrams of the *tet* region shown in Fig. 1a. A key point was that, in contrast to transformation of wild-type cells, *tet-3* strains were transformed to *tet*$^+$ by relatively short segments of donor DNA from the Tc^r parents (Fig. 1b). Therefore, they should also be transformed by DNA from any Tc^r donor that has a segment with homology to the region surrounding *tet-3*. Interspecies transformation for most markers is low even for related species (18), and efficient transformation may be taken to reflect rather good homology. Because donor DNA is single stranded after uptake and forms a donor-recipient heteroduplex, in vivo restriction is neither observed in intraspecies transformation (12 and data not shown) nor expected for DNA from another species.

DP1333 was exposed to DNAs from a number of Tc^r strains of pneumococcus, *S. faecalis*, and *S. agalactiae* and from other species carrying a variety of *tet* plasmids (Table 5). The donors fell into two cleanly resolved groups, those for which transformation was high and comparable to that for point markers and those for which it was indistinguishable from the background reversion. For each negative donor, the presence of high-molecular-weight DNA in the donor preparation was confirmed by gel electrophoresis, and the transformation was repeated with an added reference DNA carrying *str-1* to insure absence of an inhibitor of transformation (data not shown).

Transformation of DP1333 to tetracycline resistance occurred with high efficiency with DNAs from all 10 clinical isolates of pneumococcus tested, from transconjugants or transformants from two of these strains, from *S. faecalis* strains that carried Tn*916*, and from three of four strains of *S. agalactiae* (group B) that

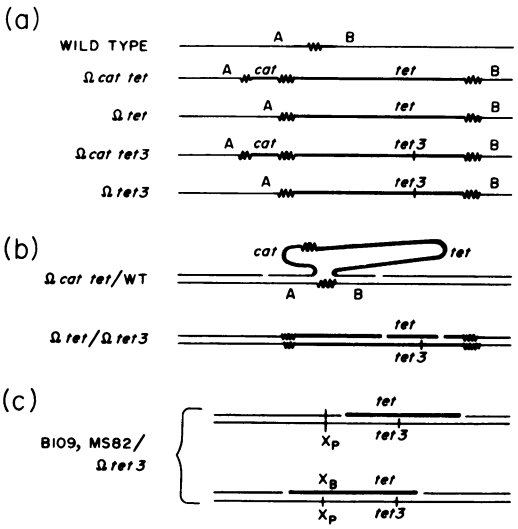

FIG. 1. *The Ω(cat-tet) region in the pneumococcus chromosome and transformation reactions involving it. (a) Schematic maps of Ω(cat-tet) or Ωtet inserted between A and B, a specific but unknown region linked to nov by transformation assay (20). The zigzags indicate sequences of unknown character and extent, previously called H and H', that were invoked because crossover to wild type Rx and R6 strains can occur between cat and tet but at a frequency suggesting that homology is poor (20). (b) Intermediates in the transformation of wild type (WT) or the tet-3 recipient by DNA carrying Ω(cat-tet). (c) Intermediates in the transformation of tet-3 by a donor that differs at a second site, X, linked to tet-3 (see text). The scale of this sketch is expanded relative to the others.*

appear to carry *tet* in their chromosomes (9). It was not transformed at a detectable frequency by DNA from one of the group B strains or by *tet* genes on plasmids from either gram-positive (pMV158, pAMα1, pT181) or gram-negative organisms, except for pAM211, which carries Tn*916* transposed from the chromosome of *S. faecalis* (5). The gram-negative group included each of the four classes of *tet* genes defined by DNA hybridization tests (13).

The Tc^r strain DP1008 was isolated while attempting to transform Rx1 cells with pT181, where controls and experimentals each gave a few Tc^r colonies. DP1008 DNA transformed both Rx1 (data not shown) and DP1333 to the low-level tetracycline resistance seen in DP1008 (MIC, 1.5 μg/ml) at frequencies comparable to that of a point marker, but did not transform DP1333 to the level of resistance conferred by *Ωtet*$^+$. It had no detectable plasmid and instead appeared to have a spontaneous mutation in a chromosomal gene.

Levels of tetracycline resistance. Table 6

TABLE 5. *Transformation of DP1333 (Ωtet-3) to tetracycline resistance by DNAs from various Tc^r donors*[a]

Donor	Resistances or plasmids[b]	Origin or source (reference)[c]	No. of Tc^r CFU/μl
S. pneumoniae			
BM6001	Cm Tc	Paris clinic (3)	600
BM4200	Cm Tc MLS Km Sul Tmp Pen	Paris clinic (1)	1,100[d]
11	Cm Tc (serotype 1)	Paris clinic	950
234	Tc (serotype 23)	Paris clinic	1,000
447	Cm Tc (serotype 5)	Paris clinic	690
568	Tc (serotype 18)	Paris clinic	1,300
B381	Cm Tc MLS Str Sul Pen	South Africa	1,500[d]
B135	Str Tc	South Africa	1,500[d]
16626/74	Cm Tc	Australia	880
N77	Cm Tc	Japan (14)	750
DP1302	Ery Cm Tc	DP1001 × BM6001 (20)	1,900
DP1402	Str Cm Tc MLS Km	DP1004 ×Cg BM4200	350[d]
DP1008	Tc	Mutant of Rx1 (see text)	3[e]
DP3214	Str pMV158 (Tc)	DP1004 ×Cg DP3201 (24)	3[f]
S. faecalis (group D)			
DS16	pAD1, pAD2, Chr::Tn*916*	Clinical isolate (26)	660
DS16C3	Chr::Tn*916*	Cured DS16 (5)	1,500
DJ-2	Rif Fus Chr:Tn*916*	Transconjugant to JH2-2 (5)	1,100
DT-11	Rif Fus pAMα1	Transconjugant to JH2-2 (4)	2
pAM211[g]	pAD1::Tn*916*	Derivative of pAD1 (5)	>2,000[g]
S. agalactiae (group B)			
B109	Cm Tc MLS	Paris (10)	2,300
B117	Cm Tc	Paris (10)	5
B119	Tc	Paris (10)	1,100
B121	Tc MLS	Paris (10)	2,200
E. coli strains[h] with plasmids carrying *tet* genes[i]			
D15-9	RP1 (A)	S. Levy	1
C600/R100-1	R100-1 (B, Tn*10*)	V. Burdett	7
RC709J53F	R27 (B1)	E. Lederberg	2
—	pACYC184 (C, pSC101 type)	S. Gross	—[j]
TP123 Nal^r	TP123	E. Lederberg	2
D7-5	RA1 (D)	S. Levy	3
Proteus sp. SLH1299	Tc	S. Levy	6
Staphylococcus aureus RN2424	pT181	R. Novick	6
No donor DNA			7

[a] Competent cells from a single batch of DP1333 were exposed to an estimated 1 μg of DNA per ml for 30 min.

[b] Abbreviations: Cm, chloramphenicol; Tc, tetracycline; Sul, sulfonamides; Str, streptomycin; Ery, erythromycin; MLS, macrolides, lincosamides, and streptogramin B group (gene *erm*); Km, kanamycin and related aminoglycosides (gene *aphA*); Tmp, trimethoprim; Pen, penicillins. In the clinical pneumococci listed here, sulfonamides and streptomycin are due to chromosomal point mutations rather than to resistance determinants (manuscript in preparation). Penicillin appears also to be due to chromosomal mutations (28).

[c] Pneumococcal strains were received from the following people: BM6001 and BM6002, D. Bouanchaud via V. Burdett; BM4200, P. Courvalin; 11, 234, 447, and 568, F. Goldstein and A. Buu-Hoi; B381 and B135, H. Bernheimer; 16626/74, D. Hansman; N77, V. Burdett. *S. faecalis* strains were received from D. Clewell, and *S. agalactiae* strains were obtained from T. Horodniceanu.

[d] These donors all carried point markers which transformed at about the same frequency as tetracycline resistance in these particular DNA preparations. The lower levels for some of the positive preparations appeared to reflect the quality of the preparation rather than lower homology.

[e] When the overlay had only 3 instead of 5 μg of tetracycline per ml, transformants were seen at about 5 × 10^5/ml.

[f] This number included both revertants and plasmid transformants.

[g] Purified DNA of plasmid pAM211 was examined in separate experiments from the main body here.

[h] Total cell DNA was prepared from the strains listed with the designations as received.

[i] The *tet* gene group as reported by Mendez et al. (13) is indicated in parentheses.

TABLE 6. *MICs of tetracycline*

Strain	MIC (μg/ml)	*tet* element
S. agalactiae		
B109, B117, B119, B121	25–50	Chr::Ω*tet* and others
S. faecalis		
DS16, DS16C3, DJ-2	50	Tn*916*
DT11	6	pAMα1
JH2-2	~0.1	
S. pneumoniae		
BM6001, N77, B135, B381, DP1302, and others	25–50	Ω*tet* and related insertions
BM4200 and its transconjugants	12	Ω(*cat-tet-erm-aphA*)[a]
Xg31, Xg32	6–12	Tn*916*[b]
DP1450	12	Ω(*cat-tet-erm*) from B109[b]
MS81	12	Ω*tet-3* × B109 DNA (see text)
MS82	6	Ω*tet-3* × B109 DNA (see text)
DP3214	3	pMV158
DP1008	1.5	Mutation in Rx1
Rx1, DP1004	<0.1	
DP1330, DP1346	<0.1	Ω*tet-3*

[a] The genes *erm* and *aphA* encode resistance to the macrolides, lincosamides, and streptogramin B group and to kanamycin and related aminoglycosides, respectively.

[b] Tn*916* and the inserted Ω(*cat-tet-erm*) segment of B109 were transferred by filter mating to DP1004 and DP1009, respectively (to be described elsewhere).

lists MICs for a number of strains and shows some potentially interesting differences among them. In these strains, chromosomally inserted *tet* genes conferred higher levels of resistance in streptococci than did plasmid *tet* genes. Both Tn*916* and Ω(*cat-tet-erm*) from B109, when transferred to pneumococcus by conjugation (to be described elsewhere), conferred lower resistance than in their parent strains. Among the pneumococcal strains, BM4200 and its transconjugants were two- to fourfold less resistant than BM6001 and other strains, suggesting either some difference in the product(s) of *tet* or their expression or perhaps unknown modifying factors.

One case of differing levels of resistance was informative. When DP1333 was transformed by *S. agalactiae* B109 DNA, both large and small Tc^r colonies appeared. Clone MS81, from a large colony, had an MIC of 12 μg/ml, and MS82, from a small colony, had an MIC of only 6 μg/ml. Lysates of each clone were prepared and used as donors again to DP1333. MS81 gave only large-colony transformants, whereas MS82 gave both large and small colonies. Shearing the MS82 lysate increased the porportion of large-colony transformants from about 20 to 45%. These results suggested that B109 and MS82 differed from DP1333 at both *tet-3* and a linked site which lowered the level of resistance in pneumococcus and frequently cotransferred with the wild-type allele of *tet-3* (Fig. 1c). The results were consistent with the second site being 1.5 to 2 kb from *tet-3*, if the mean length of the strand integrated were 7 kb from the unsheared donor and 3 to 4 kb from the needle-sheared donor, as estimated from previous data on intracellular strand length distributions (16).

Continued growth of strains DP1302 and DP1402 in a subinhibitory concentration of tetracycline (2 μg/ml) did not lead to a detectable change in the MICs, in contrast to the results found for pAMα1 (2).

DISCUSSION

The results indicated that *tet-3* was a point mutation within the conjugative Ω*tet* element found in the chromosomes of BM6001 and derivative strains. As such, it was transformable back to *tet*$^+$ with the high efficiency of a point marker by relatively short fragments of DNAs carrying the parental *tet*. Donor DNAs from other Tc^r bacteria fell into two groups: those that transformed *tet-3* with efficiencies near that of the parent and those that did not transform it at a detectable level. The factor of discrimination exceeded 100-fold.

On the background of revertants, two points need comment. In transformation experiments, the apparent reversion frequency was elevated relative to transformation frequencies because of time needed for phenotypic expression of the transformants. During the 90 min after plating before addition of drug, the cells replicated and mutants could increase without an increase in the number of transformed colonies. The numbers were consistent with there being about 5 × 10^7 cells per plate (for 0.1 ml of culture plated)

when the tetracycline acted, reverting at a frequency about 5×10^{-6} per cell (Table 4). Statistical fluctuations ("jackpots") and other variables could affect these results, and the background has been both higher and lower than this (Table 5), limiting the detection of low numbers of true transformants. Thus, the failure of DP1328 and DP1329 to transform DP1333 to tetracycline resistance at a level above the background may or may not imply that they carry mutations at the same site, although the fact that they arose in the same culture is consistent with their being sisters of DP1330.

For those donors that transformed *tet-3*, the high efficiency implies that their DNAs were largely homologous to that surrounding the *tet-3* mutation. Data on transformation as a function of size of homologous donor DNA suggest that the region of homology exceeded 2 kb, because donors shorter than this in the cell are, at most, half as efficient as the 6- to 8-kb segments that enter from high-molecular-weight DNA (15). The simplest interpretation for the negative donors is that they have little or no homology to the *tet-3* region, but this cannot be stated rigorously in the absence of in vitro hybridization data. Streptococcal *tet* genes are currently being examined by DNA-DNA hybridization criteria by V. Burdett of Duke University (personal communication). That Tn*916* DNA transformed *tet-3* at high efficiency from either plasmid or chromosomal sites (Table 5) supports the expectation that the negative result with other plasmid *tet* DNAs was not due to their being on a different physical carrier.

Several further points need comment. (i) No information is available on the mechanism of tetracycline resistance in streptococci. (ii) Our results do not distinguish between a structural or regulatory locus for *tet-3*. (iii) Both revertants and transformants of *tet-3*, particularly by DNAs of nonparental origin, may be pseudo-wild, differing in some respects from the original Ω*tet* sequence. This is clearly the case for MS82, the strain with a lower MIC that resulted from transformation with B109 DNA, where at least one genetic difference was identifiable. More extreme effects of this kind could lead to a negative result by the criteria used here. (iv) Reversion to recovery of resistance at greater than 10^{-6} seems high for true reversion of a point mutation that inactivated a function. It raises the question of whether *tet-3* is a frameshift and the reversions are nearby suppressor frameshifts, in which case the revertants may differ in various properties. These questions are being examined further, along with more examples of transformants from nonancestral DNAs.

The spontaneous deletion of *cat* from Ω(*cat-tet*) strains was interesting in relation to other observations. Such deletions left behind a conjugative (Tra^+) Ω*tet* element, for example, in strains BM6002 (Table 2) and DP1304 (21). However, Ω*cat* and Ω*tet* single transformants that arose from Ω(*cat-tet*) donors by crossover between *cat* and *tet* (see Fig. 1a and b) were not able to transfer either element by conjugation (for example, DP1301 and DP3209 [21]; other such strains have also been Tra^-). These results suggest that a sequence to the left of *cat* is needed for transfer as well as functions encoded within Ω*tet* and that *cat* is inserted within and deletes from a basic conjugative *tet* element (perhaps by pairing of flanking direct repeats, as in Tn*9*) without disturbing the necessary sequence at the left end. Proof of this hypothesis will have to await molecular mapping of these regions in the various strains.

It is significant that a variety of conjugative elements found in the chromosomes of streptococcal species all have *tet* and that all but one of these *tet* determinants have been shown to share a high degree of homology. The exception so far is B117, for which the negative transformation result has been found repeatedly. Whether it carries a totally different *tet* sequence or differs only in small respects that prevent its function in the pneumococcal background remains to be seen.

The *tet-3* mutation is a useful tool for a variety of other studies on the organization of the resistance determinants in these strains. For example, it allows interspecies transformation to be used as an assay for experiments on the physical behavior of *tet* genes from any donor that will transform it. These and other applications of *tet-3* will be described elsewhere.

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