

Does a Tetracycline Resistance Determinant of Class N Exist?

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pMV120 was reported to carry the tetracycline resistance (Tc^r) determinant of class N. We obtained tetracycline-susceptible transconjugants harboring plasmids with restriction enzyme profiles indistinguishable from those of pMV120 isolated from tetracycline-resistant clones. We conclude that pMV120 is a cryptic plasmid and that class N of Tc^r determinants does not exist.

Tetracycline resistance determinants in streptococci and enterococci have been found to belong to four classes distinguishable primarily by DNA-DNA hybridization: Tet L, Tet M, Tet N and Tet O (12).

The Tet L determinant encodes resistance to all tetracyclines except minocycline. Tet L was first identified on two small nonconjugative plasmids, pMV158 and pMV163, isolated from *Streptococcus agalactiae* (group B) (2, 3).

The Tet M determinant confers resistance to tetracyclines, including minocycline (Tc^r - Mn^r). Tet M was first identified in *S. agalactiae* (3) and is located in most cases on the chromosome. Tet M is widely dispersed among diverse bacterial genera (12), a dispersion perhaps facilitated by its frequent association with conjugative transposons, notably Tn916 (6).

Tet N was described by Burdett et al. (3, 4) as the Tc^r - Mn^r determinant carried by pMV120, a 45.0-kilobase conjugative plasmid originally identified in *S. agalactiae* MV120 B (2). The designation of Tet N was based on the absence of detectable DNA homology between pMV120 and probes corresponding to Tet L and Tet M determinants (3).

Several lines of evidence, briefly reviewed below, led us to suspect that pMV120 does not encode Tc^r and that the designation of class N was erroneous.

When pMV120 transfers from an *Enterococcus faecalis* donor into an *E. faecalis* recipient, cell aggregates appear on the filter during mating (1, 13). Cell aggregates are observed during conjugative transfer of a number of cryptic and R plasmids harbored by *E. faecalis* and are thought to be indicative of a plasmid transfer mechanism mediated by bacterial sex pheromones (5). Cell aggregates have so far been observed only in matings between *E. faecalis* strains (1, 13; T. Horaud, unpublished data). Furthermore, cell aggregation is associated either with an efficient transfer of antibiotic resistance markers in broth matings and a high transfer frequency (10^{-1} to 10^{-4} transconjugants per donor cell) in filter matings or with a low transfer frequency (10^{-6} to 10^{-9} per donor) of resistance markers on filters and no detectable transfer in broth matings (1, 13; T. Horaud, unpublished results). In the first situation, antibiotic resistance markers are plasmid borne; in the second, they are carried by the chromosome, but a plasmid is invariably found in the transconjugant. Such plasmids, which cotransfer with chromosomal markers but which do not themselves encode antibiotic resistance, have been designated cryptic conjugative (Tra^+) plasmids (13); the sole marker that sig-

nals their presence is the appearance of cell aggregates on the mating filter.

In a study of the host range specificity (9) of various streptococcal and enterococcal R plasmids, we observed that the Tc^r - Mn^r marker of an *E. faecalis* transconjugant harboring pMV120 transferred at low frequencies (10^{-6} to 10^{-9} per donor) into both *E. faecalis* and streptococcal (groups A, B, C, and G and *Streptococcus sanguis*) recipients. pMV120 could be isolated only from the *E. faecalis* transconjugants. In retrospect, these results indicated that while the host range of the Tc^r - Mn^r marker was broad, that of pMV120 itself was restricted to *E. faecalis*. pMV120 is unusual in this regard, since, despite its reported origin in *S. agalactiae* (2), it does not transfer from *E. faecalis* back to an *S. agalactiae* recipient. Usually, those R plasmids originating from *S. agalactiae* that transfer by conjugation into an *E. faecalis* recipient also transfer back to an *S. agalactiae* recipient from which they can then be isolated (7).

More recently (13) we have identified, in an *E. faecalis* transconjugant resistant to tetracyclines, including minocycline, and harboring pMV120, a chromosomal element which carries Tet M and is structurally similar to Tn916 (6).

These experiments, however, did not allow us definitively to rule out the possibility that pMV120 itself carries a Tc^r determinant different from those of classes L, M, and O (12). The purpose of the present report is, therefore, to determine whether pMV120 actually encodes Tc^r .

The *E. faecalis* strains used in this study are listed in Table 1. Mating experiments were performed as follows. Donor and recipient strains were grown in broth for 18 h and mated by mixing 0.1 ml of each strain with 0.1 ml of broth directly on a sterile filter membrane (HAEP; 0.45- μ m pore size; 47-mm diameter; Millipore Corp., Bedford, Mass.) which was placed on 5% horse blood agar. After 18 h at 37°C, the filter was washed with 0.5 ml of broth, resulting in either cell aggregates or homogeneous bacterial suspensions. The bacterial suspensions of the controls (donors or recipients alone) were homogeneous. Rifampin, fusidic acid, and streptomycin, used for counterselection of the donor strain, were at final concentrations of 100, 25, and 2,000 μ g/ml, respectively. The MIC of tetracycline was determined as described previously (13), by using a range of tetracycline concentrations from 0.03 to 128 μ g/ml. Plasmid DNA was isolated by dye-buoyant density centrifugation as we described previously (11). Electrophoresis of plasmid DNA digested by restriction endonucleases was done on 0.7 and 1.2% agarose gels.

In order to obtain transconjugants which acquired pMV120 but not the chromosomal Tc^r - Mn^r determinant, strain BM5298 was mated with strain JH2-2, with only

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TABLE 1. *E. faecalis* strains

Strain	MIC of tetracycline ($\mu\text{g/ml}$)	Antibiotic resistance marker(s) ^a	Plasmid	Origin or reference
JH2-2	0.25	Fus ^r Rif ^r	Plasmid free	10
BM133	0.5	Str ^r	Plasmid free	8
BM5297	64	Fus ^r Rif ^r Tc ^r -Mn ^r ^b	pMV120	MV120B \times JH2-2 (2)
BM5298	64	Str ^r Tc ^r -Mn ^r ^b	pMV120	BM5297 \times BM133 (9)
BM5299	0.25	Fus ^r Rif ^r	pMV120	BM5298 \times JH2-2 (this study)
BM5300	0.25	Fus ^r Rif ^r	pMV120	BM5298 \times JH2-2 (this study)
BM5801	0.25	Fus ^r Rif ^r	pMV120	BM5298 \times JH2-2 (this study)
BM5802	0.25	Fus ^r Rif ^r	pMV120	BM5298 \times JH2-2 (this study)

^a Fus^r, Fusidic acid resistance; Mn^r, minocycline resistance; Rif^r, rifampin resistance; Str^r, high-level streptomycin resistance; Tc^r, tetracycline resistance.

^b Tc^r-Mn^r, considered a single marker, is carried by a Tn916-like chromosomal element (13).

fusidic acid and rifampin used for counterselection of the donor strain. The resulting clones, obtained in the absence of tetracycline as a selective agent, comprised a mixture of transconjugants and plasmid-free recipients. The sole possibility of distinguishing the former from the latter was to reveal the capacity to produce cell aggregates in retransfer experiments. For this, 50 of 580 clones obtained at a 10^{-6} dilution of the mating BM5298 \times JH2-2 were mated with BM133. The cell suspensions on the filters were homogeneous for six clones, which were considered to be recipients. For the other 44 clones, the cell suspensions contained cell aggregates. These clones were considered to be transconjugants, since they had acquired the identifiable plasmid marker. The frequency of transfer of the plasmid harbored by these transconjugants was calculated to be 3×10^{-1} per donor, which is significantly higher than that of the Tc^r-Mn^r marker when transferred between *E. faecalis* strains (2×10^{-6} per donor).

The difference in transfer frequency between the plasmid marker (formation of cell aggregates) and the chromosomal antibiotic resistance marker (Tc^r-Mn^r) is in itself evidence of two separate conjugative elements present in BM5298. Furthermore, because the transfer frequency of the plasmid is much higher than that of the chromosomal Tc^r-Mn^r marker, it is virtually impossible, by intraspecies (*E. faecalis* to *E. faecalis*) conjugative transfer, to obtain a transconjugant resistant to tetracyclines, including minocycline, that does not harbor the plasmid. However, it would be possible to obtain a plasmid-free *E. faecalis* transconjugant carrying the chromosomal element by an indirect route involving transfer from an *E. faecalis* donor to a streptococcal recipient which is outside the plasmid host range (9) and then retransfer to an *E. faecalis* recipient. Such an experiment has been performed for the conjugative transposon Tn3702 of *E. faecalis* origin, which encodes Tc^r-Mn^r (T. Horaud, F. Delbos, and G. de Cespédès, submitted for publication).

Four tetracycline-susceptible clones (Table 1) which formed cell aggregates in retransfer experiments were examined further. Each harbored a plasmid having the same electrophoretic migration as that of the plasmid isolated from a tetracycline- and minocycline-resistant clone. Comparison of *EcoRI*, *HindIII*, *HpaI*, and *HincII* restriction patterns of the plasmids isolated from strains BM5299, BM5300, and BM5801 with those of the plasmid isolated from strain BM5297 indicated that there was no detectable modification in their profiles, regardless of the enzyme used (Fig. 1). The MICs of tetracycline for the strains resistant and susceptible to tetracyclines, shown in Table 1, indicate that the plasmid did not encode a level of tetracycline resistance above that of the plasmid-free host strain.

The experiments performed in the present study confirm

the supposition that pMV120 is a cryptic Tra⁺ plasmid which cotransfers in filter matings along with the Tc^r-Mn^r chromosomal marker of its bacterial host (13). Likewise, pIP685 (9), initially considered on the basis of conjugation experiments to be a Tc^r-Mn^r plasmid, is in fact a cryptic Tra⁺ plasmid. D366, the *E. faecalis* wild-type host of pIP685, carries Tc^r-Mn^r on a Tn916-like chromosomal element (K. Pepper and T. Horaud, unpublished results). Cryptic Tra⁺ plasmids, including pMV120, harbored by antibiotic-resistant strains may be mistakenly supposed to encode antibiotic resistance if the determinants are not precisely localized by DNA-DNA hybridization.

In conclusion, we consider that the class N of Tc^r determinants, such as it has been described (3), does not exist. To avoid confusion, we propose that the letter N be eliminated from future designations of new classes of Tc^r determinants.

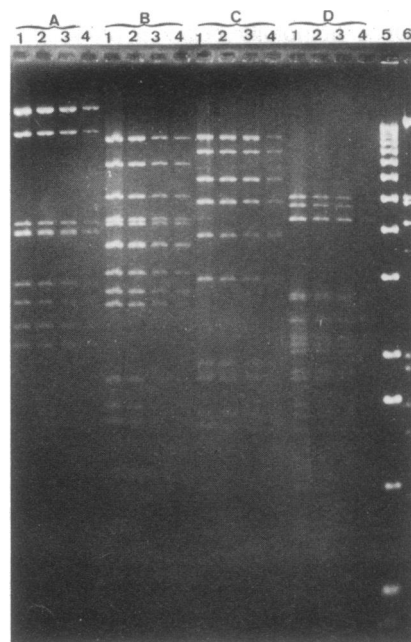


FIG. 1. Restriction endonuclease profiles of pMV120 isolated from transconjugants resistant or susceptible to tetracyclines. The restriction fragments were separated on 1.2% agarose gels. Lanes: 1, pMV120 isolated from strain BM5297; 2, pMV120 isolated from BM5299; 3, pMV120 isolated from BM5300; 4, pMV120 isolated from BM5801; 5, 1-kilobase DNA ladder; 6, λ digested by *EcoRI* and *HindIII*. Plasmid DNA was digested by *EcoRI* (A), *HindIII* (B), *HpaI* (C), and *HincII* (D).

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