High-Level Tetracycline Resistance in Neisseria gonorrhoeae Is Result of Acquisition of Streptococcal tetM Determinant

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Recently, strains of Neisseria gonorrhoeae have been isolated which are highly resistant to tetracycline (MICs of 16 to 64 μg/ml). This resistance was due to the acquisition of the resistance determinant tetM, a transposon-borne determinant initially found in the genus Streptococcus and more recently in Mycoplasma hominis, Ureaplasma urealyticum, and Gardnerella vaginalis. In N. gonorrhoeae, the tetM determinant was located on a 25.2-megadalton plasmid. This plasmid arose from the insertion of tetM into the 24.5-megadalton gonococcal conjugative plasmid. The tetM determinant could be transferred to suitable recipient strains of N. gonorrhoeae by both genetic transformation and conjugation.

Gonorrhea is a major health problem throughout the world. In the United States, gonorrhea is the most frequently reported disease, with approximately 1,000,000 cases reported each year. For more than a decade, tetracycline has been one of the preferred drugs for treating gonococcal infections. Strains of Neisseria gonorrhoeae isolated during the prepenicillin era (1935 to 1948) were highly susceptible to tetracycline, with MICs ranging from 0.02 to 0.2 μg/ml (7). Since the introduction of tetracycline therapy, strains of N. gonorrhoeae with increased levels of resistance (MICs of 0.5 to 4.0 μg/ml) have been isolated. This increased resistance to tetracycline is due to the additive effects of several chromosomal loci designated tet, mtr, and penB (6). The mtr locus also codes for low-level nonspecific resistance to hydrophobic molecules, whereas the penB locus encodes for low-level resistance to both penicillin and tetracycline. More recently, strains of chromosomally mediated resistant N. gonorrhoeae, exhibiting increased resistance to penicillin and tetracycline (MICs up to 8.0 μg/ml), have been isolated in the Far East (38) and the United States (26). However, the genetic basis for this increased tetracycline resistance has not yet been established.

In members of the family Enterobacteriaceae, four different genetic classes (A to D) of tetracycline resistance determinants have been identified by DNA-DNA hybridization (22). All four classes are located on plasmids (22). One of these, class B (Tn10), is also found in the genus Haemophilus (21). At least three tetracycline resistance determinants which are unrelated to the gram-negative determinants have been identified in the genus Streptococcus (3, 5). Two classes, tetN and tetL, are located on plasmids, whereas tetM is often found on a conjugative transposon which codes for both transfer and transposition (3, 13, 15). Insertions of tetM into recipient cell DNA after transfer are rec independent and occur at different sites. Recently, Burdett (4) demonstrated that protein synthesis in extracts prepared from tetM-containing organisms was resistant to tetracycline and suggested that tetM rendered protein synthesis resistant to the antibiotic.

The tetM determinant has recently been found in tetracycline-resistant strains of Mycoplasma hominis (30), an inhabitant of the human genital tract. This observation represented the first description of tetM found in a genus other than Streptococcus. More recently, the tetM determinant has been found in two additional microorganisms which inhabit the human genital tract, Ureaplasma urealyticum and Gardnerella vaginalis (29). In each case, the tetM determinant was integrated into the chromosome. We now report the isolation of 79 strains of N. gonorrhoeae which exhibit high-level resistance to tetracycline (MICs of 16 to 64 μg/ml). These isolates were not chromosomally mediated resistant N. gonorrhoeae, and with the exception of three strains that produced beta-lactamase, all of the isolates were susceptible to <0.5 μg of penicillin per ml (mean, 0.1 μg/ml) and to <0.5 μg of erythromycin per ml (mean, 0.23 μg/ml). High-level resistance to tetracycline was not associated with any known chromosomal gene, but with the presence of the tetM determinant. The tetM determinant was not associated with the chromosome, but with a 25.2-megadalton (MDa) conjugative plasmid.

MATERIALS AND METHODS

Detection and isolation of tetracycline-resistant N. gonorrhoeae. Cases of gonorrhea caused by tetracycline-resistant N. gonorrhoeae were identified from three groups of specimens submitted to the Centers for Disease Control from local and state health departments. These were gonococcal isolates from treatment failures that are routinely submitted to the Centers for Disease Control for confirmation, isolates submitted specifically for confirmation of tetracycline resistance, and isolates obtained from prospective surveillance to detect tetracycline-resistant N. gonorrhoeae. In Boston and Philadelphia, tetracycline-resistant N. gonorrhoeae strains were detected with disk susceptibility tests. Tetracycline disks (30 μg) were placed on the inoculated plate, and isolates showing zones of inhibition of ≤20 mm in diameter were saved for further studies. Cultures obtained from health departments in DeKalb and Fulton County, Ga., were subcultured onto chocolate agar medium containing tetracycline. Medium containing 2.5 μg of tetracycline-hydrochloride per ml was initially used to detect all tetracycline-resistant isolates, including those with chromosomally mediated tetracycline resistance. More recently, however, medium containing 10.0 μg of tetracycline-hydrochloride per
ml was used to select for isolates of tetracycline-resistant *N. gonorrhoeae*. After MIC testing, strains having MICs of ≥16.0 μg/ml were saved for further studies.

**Strains and media.** Strains of *N. gonorrhoeae* used in this study and their relevant properties are listed in Table 1. *Escherichia coli* HB101 (3) was obtained from W. R. Romig, University of California, Los Angeles. Organisms were stored at −70°C in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) containing 20% (vol/vol) glycerol. Strains of *N. gonorrhoeae* were routinely grown on GC agar (Difco Laboratories, Detroit, Mich.) containing 1% (vol/vol) IsoVitalex enrichment (BBL) and 1% (vol/vol) fetal calf serum. Cultures were incubated at 37°C in an atmosphere containing 4% CO₂. *E. coli* HB101 was grown on meat extract agar plates (25) incubated at 35°C.

**Auxotyping.** Auxotyping was performed by the method of Short et al. (31). Strains were tested for their requirement for proline (Pro⁻), arginine (Arg⁺), hypoxanthine (Hxy⁺), uracil (Ura⁻), methionine (Met⁻), isoleucine (Ile⁻), leucine (Leu⁻), valine (Val⁺), serine (Ser⁻), alanine (Ala⁻), tyrosine (Tyr⁻), histidine (His⁻), glutamic acid (Glu⁻), phenylalanine (Phe⁻), lysine (Lys⁺), threonine (Thr⁻), and tryptophan (Try⁻).

**Serologic classification.** Strains were tested by coagglutination by using monoclonal antibodies as described previously (19, 36). Monoclonal antibodies were provided by Syva Co. (Palo Alto, Calif.). A standardized suspension of protein A-containing staphylococci was obtained from Behring Diagnostics (La Jolla, Calif.). The serovar nomenclature of Knapp et al. (19) was used.

**Antimicrobial susceptibility patterns.** Antimicrobial susceptibilities to ampicillin, cefotaxime, cefoxitin, cefuroxime, doxycycline, penicillin G, spectinomycin, tetracycline hydrochloride, and trimethoprim-sulfamethoxazole were determined by agar dilution tests as described previously (1).

**Plasmid content.** The plasmid content of isolates was determined by agarose gel electrophoresis as described previously (23).

### TABLE 1. Strains of *N. gonorrhoeae* used in this study and their relevant properties

<table>
<thead>
<tr>
<th>Strain</th>
<th>Tetracycline MIC (μg/ml)</th>
<th>Relevant phenotype</th>
<th>Serovar</th>
<th>Plasmid (MDa)</th>
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<tr>
<td>83.022650</td>
<td>32</td>
<td>Tet' Kan² Pro⁻</td>
<td>IB-3</td>
<td>25.2, 2.6</td>
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<tr>
<td>85.022523</td>
<td>32</td>
<td>Tet' Pro⁺</td>
<td>IB-1</td>
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**Preparation of DNA.** Preparations of chromosomal DNA used for transformation were obtained as described elsewhere (34), except that proteins were removed from the DNA solutions by extraction with phenol followed by chloroform-isooamyl alcohol (24:1). Plasmid DNA was prepared by dye-buoyant density centrifugation as described previously (9). The covalently closed, circular DNA fraction was subsequently centrifuged through 5 to 20% neutral sucrose gradients in an SW60 Ti rotor (Beckman Instruments, Inc., Fullerton, Calif.) at 50,000 rpm for 120 min. The gradients were collected from the bottoms of the tubes, and fractions enriched for either the 24.5- or the 25.2-MDa plasmids were identified by agarose gel electrophoresis. Fractions containing these plasmids were pooled and further purified by electrophoresis through 0.8% agarose. Plasmid DNA was recovered by electroelution, extracted twice with phenol, once with chloroform-isooamyl alcohol (24:1), and then precipitated at −20°C with 0.5 volume of 7.5 M ammonium acetate and 2.5 volumes of ethanol.

**Restriction enzymes.** Restriction enzymes were purchased from Bethesda Research Laboratories, Inc. (Bethesda, Md.) and were used according to the directions of the manufacturer.

**Detection and localization of tetM.** Strains to be tested and known positive and negative controls were inoculated on GC agar and incubated overnight at 37°C with 4% CO₂. After incubation, the growth was transferred onto nitrocellulose membranes (type BA85; Schleicher & Schuell, Keene, N.H.). The cells were lysed, and the DNA was denatured by incubating the membrane on a filter pad saturated with 0.5 M NaOH. The membranes were then neutralized by incubating each membrane on four successive filter pads saturated with 2 M ammonium acetate containing 0.05 M NaOH. The membranes were air dried and then fixed by heating at 84°C for 2.5 h under vacuum. *tetM* was localized as follows. Whole plasmid DNA or DNA digested with restriction endonucleases was transferred from agarose gels to nitrocellulose membranes by the method of Southern (32). Hybridization of radiolabeled probes to membrane-bound DNA was carried out at 68°C in 5× SSC with Denhardt solution (10).

**Preparation of pJ12.** pJ12 consists of the cloning vector pVH2124 and a 20-kilo base pair fragment which confers tetracycline resistance cloned from whole-cell DNA of *Streptococcus galactiae* B109 (5). pJ13 consists of the vector pACYC177 and a 5-kb *HincII* fragment subcloned from the original 20-kb fragment of pJ12. This 5-kb fragment contains the 2.8-kb region required for the expression of tetracycline resistance as determined by deletion analysis (5, 16; J. Inamine, Ph.D. thesis, Duke University, Durham, N.C., 1983). There are few restriction sites within this 5-kb fragment, and attempts to reduce the size of the fragment have so far failed. This fragment hybridizes neither with any known gram-negative tetracycline resistance determinant nor with other streptococcal tetracycline resistance determinants (5, 30). Radiolabeled probes prepared by nick translation (27) were made from pJ13, the 5-kb *HincII* fragment, and the vector pACYC177.

**Transformation.** Piliated gonococcal recipients (34) were suspended in gonococcal genetic medium broth (20) to a density of 10⁸ CFU/ml. A 250-μl volume of this cell suspension was mixed with 10 μl of a DNA solution containing 0.5 to 5 μg of nucleic acid. After 10 min was allowed for uptake, 30 μl of a DNase I (Sigma Chemical Co., St. Louis, Mo.) solution (1 mg/ml) was added, and the mixture was incubated...
for 10 min to digest free DNA. After DNase I treatment, 150 μl of the transformation mixture was transferred to the surface of a GC agar plate and then incubated at 35°C in 4% CO₂ for 4 h to allow for phenotypic expression. After this incubation, the cells were harvested and suspended in 0.5 ml of gonococcal genetic medium broth, and appropriate dilutions were spread on GC agar containing 15 μg of tetracycline per ml. For comparison, the transformation frequency of rifampin and streptomycin resistance from strain 28BLRS to 85.022395 was determined by spreading appropriate dilutions of the transformation mixture on GC agar containing 100 μg of streptomycin per ml or 50 μg of rifampin per ml, respectively. Dilutions of the transformed cells were also plated on GC agar without antimicrobial agents to determine the number of viable recipients.

Conjugation. Separate suspensions of nonpenilated donor and recipient cells (10⁵ CFU/ml) were prepared in gonococcal genetic medium broth containing 200 μg of DNase I per ml. Donor and recipient cell suspensions (100 μl each) were mixed at a 1:1 ratio to give 2 × 10⁵ CFU. The cells were then transferred to a 0.22-μm pore filter (Millipore Corp., Bedford, Mass.) which was placed on a GC agar plate and incubated for 6 to 20 h at 35°C in 4% CO₂. Cells were harvested with a cotton swab and suspended in 1 ml of gonococcal genetic medium broth. Volumes of 0.1 ml were spread into GC agar containing tetracycline (10 μg/ml) and streptomycin (75 μg/ml). Tet’ Str’ colonies were scored for Rif’ and proline dependence. Colonies that had acquired Tet’ and had retained the recipient Rif’ and Pro’ phenotype were considered to be transconjugants.

Three-party crosses were performed in a similar fashion. In the first cross, the Tet’ donor (83.022650) was mated with a gonococcal strain (86.014312) containing the 4.4-MDa R factor which codes for beta-lactamase. Filters were incubated as above for 3 h, the cells were harvested, and a portion of the intermediate cross was spread onto GC agar containing tetracycline (20 μg/ml) and penicillin (0.5 μg/ml). The remaining mixture was incubated with 10⁶ CFU of E. coli HB101 for 6 h. All the cells from this final cross were harvested as described above, and appropriate dilutions were spread onto meat extract agar (25) containing streptomycin (100 μg/ml) with either penicillin (200 μg/ml) or tetracycline (10 μg/ml). Pen’ transconjugants were tested for beta-lactamase production with the chromogenic cephalosporin nitrocefin (24). Several beta-lactamase-positive colonies from the intermediate and final crosses were selected, lysed, and subjected to agarose gel electrophoresis to confirm the presence of the 4.4-MDa R factor.

**RESULTS**

Characteristics of tetracycline-resistant strains. A total of 79 isolates of *N. gonorrhoeae* from 11 states were confirmed within the past year as exhibiting high-level resistance to tetracycline (MIC ≥ 16.0 μg/ml). These tetracycline-resistant strains were isolated from anogenital and pharyngeal sites and were associated with treatment failure in all patients who were treated with tetracycline alone. Isolates were obtained from homosexual men as well as heterosexual men and women. The epidemiology of tetracycline-resistant *N. gonorrhoeae* in the United States will be described elsewhere (manuscript in preparation). Nine of the isolates had a tetracycline MIC of 16 μg/ml; the remainder had MICs between 24 and 64 μg/ml. With the exception of the three isolates which contained the 3.2-MDa beta-lactamase plasmid, all were susceptible to penicillin G (MIC ≤ 0.5 μg/ml).

All of the isolates were also susceptible to spectinomycin (MIC = 12.0 μg/ml) and cefotaxime (MIC ≤ 0.004 μg/ml) but were moderately resistant to cefoxitin (0.25 to 1.0 μg/ml). The isolates were also resistant to doxycycline (MIC = 8 to 24 μg/ml) and minocycline (MIC = 12 to 32 μg/ml).

The auxotype and serovar of the tetracycline-resistant *N. gonorrhoeae* strains were determined to ascertain whether this phenomenon represented the spread of a single strain. The results (Table 2) indicated that there were 19 auxotype-serovar classes among the 79 isolates, suggesting that this was a widely distributed phenomenon. Nevertheless, the majority of the isolates (53.2%) belonged to the Pro’-IB-1 auxotype-serovar class. However, analysis by genetic transformation (Table 3) showed that there was more than one genetic lesion responsible for the Pro’ phenotype of the Pro’-IB-1 isolates. The large number of recombinants obtained when DNA from six tetracycline-resistant *N. gonorrhoeae* isolates was used to transform strain F62 (proA-like) to proline independence suggested that the defect in proline biosynthesis was not in the same gene as that in the recipient strain F62. The finding that some of the tetracycline-resistant *N. gonorrhoeae* strains gave recombinants when transformed with DNA isolated from other tetracycline-resistant *N. gonorrhoeae* strains indicated that the mutations which caused the proline requirement were not identical (Table 3).

Transformation of all the recipients to Pro’ at high frequency by DNA purified from the prototroph, 85-044571, showed that all of the recipients were adequately competent.

Plasmid analysis indicated that two plasmids were common to all of the tetracycline-resistant *N. gonorrhoeae* isolates. These were the 2.6-MDa cryptic plasmid (6) and a plasmid with a size similar to that of the 24.5-MDa conjugative plasmid (6). Previous studies (28) suggested that it was highly unlikely that all strains would contain the 24.5-MDa conjugative plasmid. Consequently, a closer examination revealed that the size of this plasmid was slightly larger than

<table>
<thead>
<tr>
<th>TABLE 2. Auxotype-serovar classes of 79 tetracycline-resistant <em>N. gonorrhoeae</em> isolates from the United States</th>
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<tbody>
<tr>
<td><strong>Auxotype</strong></td>
</tr>
<tr>
<td>Arg’</td>
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<td>Pro’</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Pro’ Val’</td>
</tr>
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<td>Pro’ Val’ Ser’</td>
</tr>
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</table>

* Isolates were from Arizona (1), California (2), Georgia (33), Louisiana (1), Massachusetts (23), Maryland (3), Michigan (1), New Hampshire (1), Oregon (9), Pennsylvania (4), and Texas (1).

a Number of strains which also contained the 3.2-MDa beta-lactamase plasmid is in parentheses.
that of the 24.5-MDa conjugative plasmid. We estimated that the size of this new plasmid was 25.2 MDa.

The recent discovery of tetM in several genitourinary tract microorganisms suggests that this determinant might also be responsible for high-level tetracycline resistance in N. gonorrhoeae. This possibility was initially examined by using dot blots hybridized to the radiolabeled 5-kb HincII fragment isolated from pJI3, the entire pJI3 plasmid, or the vector pACYC177. For the beta-lactamase-producing tetracycline-resistant N. gonorrhoeae isolates, only the radiolabeled 5-kb HincII fragment was used. Hybridization to all of the tetracycline-resistant N. gonorrhoeae isolates was observed when the probe was either the 5-kb fragment or the entire pJI3 plasmid, but not when the vector pACYC177 was used, indicating that DNA homology occurred specifically with the 5-kb fragment (data not shown).

The radiolabeled 5-kb HincII fragment was also used as a probe for Southern blots of whole-cell DNA or in an in situ filter blot hybridization with purified plasmid DNA from 25 tetracycline-resistant N. gonorrhoeae isolates. The results (Fig. 1) show that the tetM sequence was present in the 25.2-MDa plasmid; no hybridization was observed with either chromosomal DNA or with the 2.6-MDa plasmid. In addition, no hybridization was observed with whole-cell DNA from eight tetracycline-susceptible strains of N. gonorrhoeae that possessed 24.5-MDa conjugative plasmids (data not shown). It was of considerable interest to determine (i) whether the 25.2-MDa plasmids from various tetracycline-resistant N. gonorrhoeae isolates were similar and (ii) whether the 25.2-MDa plasmid resulted from the insertion of tetM into the 24.5-MDa conjugative plasmid. To address these questions, we isolated 25.2-MDa plasmids from strains 83.022650 (New Hampshire, 1983), 85.000921 (Georgia, 1985), 85.038493 (Georgia, 1985), and 85.034676 (Pennsylvania, 1985), and the 24.5-MDa plasmid from strain 76.072389 by dye-buoyant density ultracentrifugation followed by sucrose density centrifugation and preparative agarose gel electrophoresis. The purified plasmids were digested with either Smal or HincII and analyzed by agarose gel electrophoresis (Fig. 2). The four 25.2-MDa plasmids obtained from strains isolated from three states over a 2-year period showed identical restriction profiles when digested separately with Smal (Fig. 2, lanes B to E) and HincII (Fig. 2, lanes G to J). The restriction profiles of Smal digests of the 25.2-MDa plasmid were also similar to that of the Smal digest of the 24.5-MDa conjugative plasmid (Fig. 2, lane A). Two Smal fragments of the 24.5-MDa plasmid were missing from the Smal profiles of the 25.2-MDa plasmids; the Smal profiles of the four 25.2-MDa plasmids contained a new fragment with a molecular size of ca. 7.9 MDa. A Southern blot of this gel was probed with the radiolabeled 5-kb HincII fragment from pJI3. The results (Fig. 2) indicated that the tetM determinant was located on the 7.9-MDa Smal fragment and on a 5.0-MDa HincII fragment. Additional studies showed that the DNA from strains containing the 25.2-MDa plasmid hybridized to a radiolabeled 24.5-MDa plasmid probe; no hybridization was observed with strains lacking the 25.2- and 24.5-MDa plasmids (data not shown).

Genetic transfer of tetM. Tetracycline resistance could not be transferred by transformation from any of 10 tetracycline-resistant N. gonorrhoeae isolates into recipient gonococcal strains F62RS, 28BLRS, and 82.072374RS, all of which lack the 24.5-MDa plasmid (transformation frequency, <10^-8 transforms per CFU). Subsequent experiments with a recipient strain containing a 24.5-MDa plasmid (strain 85.022395) yielded tetracycline-resistant transformants at a frequency of 2.5 x 10^-7 transforms per CFU. This

![Fig. 1: Localization of tetM in N. gonorrhoeae by Southern transfer and hybridization.](image-url)
frequency was much lower than the frequency of transformation of streptomycin or rifampin resistance (2.0 × 10⁻³ transformants per CFU) from donor strain 28BLRS to strain 85.022395. The critical difference in the crosses involving tetracycline resistance appeared to be the presence of the 24.5-MDa plasmid in the recipient, suggesting that marker rescue was necessary for transformation of tetracycline resistance (2).

Initially, no direct conjugal transfer of tetracycline resistance from any of the 15 tetracycline-resistant N. gonorrhoeae strains tested was detected in crosses with F62RS, 28BLRS, or 82.072347RS (data not shown). However, results from a three-party cross indicated that the 25.2-MDa plasmid was transferred from strain 83.022650 to a gonococcal intermediate donor (strain 86.014312) containing a 4.4-MDa beta-lactamase plasmid. When a sample of the initial cross, 83.022650 × 86.014312, was spread on medium containing penicillin and tetracycline, transconjugants were observed at a frequency of 3.0 × 10⁻⁴ Pen⁺ Tetr⁺ transconjugants per Tet⁺ donor. In the subsequent step of the cross, the 4.4-MDa R factor was transferred to E. coli HB101 at a frequency of 8.0 × 10⁻³ Pen⁺ transconjugants per Tet⁺ donor. Pen⁺ transconjugants were not observed in two-party crosses between N. gonorrhoeae 86.014312 and E. coli HB101 (frequency, <10⁻⁸).

Twenty Pen⁺ Tetr⁺ colonies that resulted from the cross between N. gonorrhoeae 83.022650 and 86.014312 were picked and found to be identical to the intermediate donor (86.014312) with respect to serovar, auxotype, and resistance to kanamycin. In addition, all of the transconjugants tested possessed a 25.2-MDa plasmid as determined by agarose gel electrophoresis and Southern blots hybridized with the 5-kb HinclI fragment of pJI3 (Fig. 3).

**FIG. 2.** (Left) Photograph of 1.2% agarose gel of restriction endonuclease-digested plasmid DNA from N. gonorrhoeae stained with ethidium bromide and visualized with UV. Lane S contains a HinclI digest of bacteriophage lambda DNA. Lanes A to E contain Smal digests of plasmid DNA. Lanes: A, 24.5-MDa plasmid from the tetracycline-susceptible strain 76.073389; B to E, 25.2-MDa plasmids from the tetracycline-resistant strains 85.000921, 83.022650, 85.038493, and 85.034676, respectively; F to J, HinclI digests of plasmid DNA; F, 24.5-MDa plasmid from strain 76.073389; G to J, 25.2-MDa plasmids from strains 85.000921, 83.022650, 85.038493, and 85.034676, respectively. (Right) Autoradiograph of Southern blot of gel at left hybridized to the 32P-labeled 5-kb HinclI fragment from pH3. Lanes A’ to J’ correspond to lanes A to J.

**DISCUSSION**

Since 1985, isolates of N. gonorrhoeae exhibiting high-level resistance to tetracycline (MICs of 16 to 64 μg/ml) have been isolated from several areas within the United States. These isolates were unlike those previously reported which exhibited low-level tetracycline resistance due to the additive effects of several chromosomal loci designated tet, mtr, and penB (6). This occurrence probably represents a new phenomenon, as a review of tetracycline MICs from over 9,500 gonococcal isolates tested at the Centers for Disease Control between January 1983 and December 1984 revealed only one earlier isolate from New Hampshire (8). It is possible that these isolates represent the spread of a single or limited number of strains. However, auxotype and serovar analyses indicated that the high-level tetracycline resistance phenotype occurred in strains representing 19 auxotype-serovar classes. It was somewhat surprising that 53.2% of the isolates belonged to the Pro⁻IB-1 auxotype-serovar class. However, data from genetic transformation studies indicated that there was heterogeneity in the genetic basis for the proline requirement of these Pro⁻IB-1 strains.

The recent discovery of tetM in genitourinary tract microorganisms such as M. hominis (30), U. urealyticum (29), and G. vaginalis (29) has suggested that this determinant might have spread to N. gonorrhoeae. Hybridization experiments with a radiolabeled 5-kb HinclI fragment from pH3 revealed that all tetracycline-resistant N. gonorrhoeae isolates possessed the streptococcal tetM determinant. Furthermore, this determinant was carried on a 25.2-MDa plasmid. Results obtained by restriction analysis, genetic transformation, and hybridization suggested that the 25.2-MDa plasmid shared considerable homology with the 24.5-MDa conjugative plas-
mid. There were two explanations for this phenomenon. The first was that these gonococcal isolates represented the independent acquisition of \textit{tetM} from an unknown donor(s) with preferential insertion into a 24.5-MDa conjugative plasmid. The second possibility was that there was a one-time transfer event with subsequent dissemination to other gonococcal strains. Electrophoretic analysis of \textit{SmaI} and \textit{HincII}-digested 25.2-MDa plasmid DNA from four tetracycline-resistant \textit{N. gonorrhoeae} strains isolated from different geographic areas over a 2-year period indicated that these plasmids had identical profiles. Southern blots of these gels hybridized to a radiolabeled \textit{tetM} probe demonstrated that the \textit{tetM} determinant was present on a 7.9-MDa \textit{SmaI} fragment and on a 5-MDa \textit{HincII} fragment. Thus, the 25.2-MDa plasmids have been virtually identical for the strains examined to date and suggest that the latter hypothesis is more likely.

High-level tetracycline resistance was transformed or conjugally transferred from tetracycline-resistant \textit{N. gonorrhoeae} strains to suitable recipient strains of \textit{N. gonorrhoeae}. The acquisition of \textit{tetM} by transformation required the presence of a 24.5-MDa plasmid in the recipient strain; the function of this plasmid was apparently to allow marker rescue of \textit{tetM}. Presumably, this resulted from recombination of 25.2-MDa plasmid sequences which flanked \textit{tetM} with the homologous sequences on the 24.5-MDa plasmid (2).

Only one of four strains of \textit{N. gonorrhoeae} tested functioned as a recipient during conjugation with tetracycline-resistant \textit{N. gonorrhoeae} 83.022650. Both physical and genetic evidence indicated that \textit{tetM} was transferred to recipient strain 86.014312 as part of a transmissible 25.2-MDa plasmid. The presence of such a plasmid, which hybridized with a radiolabeled \textit{tetM} probe, was demonstrated in Pen' \textit{Kan'} transconjugants of strain 86.014312. Genetic evidence for the transfer of a conjugative plasmid was obtained from a three-party cross which used strain 86.014312 as the intermediate donor and \textit{E. coli} HB101 as the recipient. Strain 86.014312 was converted to a donor after conjugation with strain 83.022650 as evidenced by the ability of the former strain to transfer its 4.4-MDa beta-lactamase plasmid to \textit{E. coli} HB101. Likewise, four of the Pen' \textit{Kan'} transconjugants transferred Pen' to \textit{E. coli} HB101 in separate matings. No transfer of \textit{tetM} to \textit{E. coli} HB101 was observed, although \textit{tetM} is expressed in \textit{E. coli} (5). This result is consistent with those obtained by other investigators (12, 33), which suggest that the gonococcal conjugative plasmid is not maintained in \textit{E. coli}.

There is considerable evidence that \textit{N. gonorrhoeae} can acquire R factors from other bacterial genera (6). However, the source of the \textit{tetM} determinant found in \textit{N. gonorrhoeae} is unknown at present. The \textit{tetM} determinant has been identified in a number of genitourinary tract microorganisms including group B streptococci (3), \textit{G. vaginalis} (29), \textit{U. urealyticum} (29), and \textit{M. hominis} (30). Although many of these organisms can be simultaneously isolated from the same patient (11, 37), it is not known whether they can donate \textit{tetM} to \textit{N. gonorrhoeae} either in vivo or in vitro.

We demonstrated that \textit{tetM} can be transferred between strains of \textit{N. gonorrhoeae} by both transformation and conjugation. Young et al. (39) presented preliminary data which suggested that as many as 34% of patients are infected with more than one auxotype of \textit{N. gonorrhoeae}. This observation suggests a basis for the dissemination of \textit{tetM} in vivo. Since other \textit{Neisseria} species can acquire DNA from \textit{N. gonorrhoeae} by transformation (17) or by conjugation (14), the possibility also exists that \textit{tetM} will be acquired by these organisms as well.

Recently, a strain of \textit{N. gonorrhoeae} which had a tetracycline MIC of 12 $\mu$g/ml was isolated in the Federal Republic of Germany by Jahn et al. (18). This strain carried a novel plasmid with a molecular mass of 10.5 MDa. At this time, we are uncertain as to the nature of the tetracycline resistance determinant in this strain and whether the determinant is located on the 10.5 MDa plasmid.

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**LITERATURE CITED**


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