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Acquired Macrolide Resistance Genes in Pathogenic *Neisseria* spp. Isolated between 1940 and 1987

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Seventy-six *Neisseria gonorrhoeae* isolates, isolated between 1940 and 1987, and seven *Neisseria meningitidis* isolates, isolated between 1963 and 1987, were screened for the presence of acquired *mef*(A), *erm*(B), *erm*(C), and *erm*(F) genes by using DNA-DNA hybridization, PCR analysis, and sequencing. The *mef*(A), *erm*(B), and *erm*(F) genes were all identified in a 1955 *N. gonorrhoeae* isolate, while the *erm*(C) gene was identified in a 1963 *N. gonorrhoeae* isolate. Similarly, both the *mef*(A) and *erm*(F) genes were identified in a 1963 *N. meningitidis* isolate. All four acquired genes were found in later isolates of both species. The *mef*(A) gene from a 1975 *N. gonorrhoeae* isolate was sequenced and had 100% DNA and amino acid identity with the *mef*(A) gene from a 1990s *Streptococcus pneumoniae* isolate. Selected early isolates were able to transfer their acquired genes to an *Enterococcus faecalis* recipient, suggesting that these genes are associated with conjugative transposons. These isolates are the oldest of any species to carry the *mef*(A) gene and among the oldest to carry these *erm* genes.

Erythromycin, the prototype macrolide antibiotic, was introduced over 50 years ago. Macrolide use has increased during the past decade, after introduction of the semisynthetic erythromycin derivatives clarithromycin and azithromycin. These compounds are used extensively to treat community-acquired pneumonia and chlamydial infection (4, 12). Although erythromycin and azithromycin, in the 1-g dose, are not recommended for treatment of gonococcal infection, azithromycin has been used in some parts of the world to treat gonorrhea (7, 30). Increased gonococcal resistance to erythromycin has been noted since the 1960s (19), and resistance to azithromycin has been recently identified (7, 13). Gonococcal resistance to erythromycin has been linked to resistance to killing by fecal lipids, and such resistant strains are more likely to be recovered from men who have sex with men than from heterosexual men (9).

Macrolide resistance in most gram-positive and gram-negative bacteria is often due to the acquisition of rRNA methylase genes. Thirty-one of these genes, which add one or two methyl groups to a specific adenine (A2058 in *Escherichia coli*) in the 23S rRNA (23), have been identified. It has been shown that some recently recovered *Neisseria gonorrhoeae* and commensal *Neisseria* spp. carry, individually or in combination, the *erm*(B), *erm*(C), or *erm*(F) gene (6, 21). These genes are associated with conjugative transposons that can be transferred to both gram-negative and gram-positive recipients and often code for other antibiotic-resistant genes (3, 14, 15, 21, 23). The earliest known isolates of organisms other than *Neisseria* spp. that carry *erm* gene(s) were originally recovered in the 1950s (2, 3). More recently, macrolide resistance due to active efflux encoded by the *mef*(A) gene has been described (5, 15, 16, 24–27). This gene has also been found in recent gonococcal and commensal *Neisseria* spp. isolates (14).

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Finding two different types of acquired genes in recently isolated gonococci led us to question how long the *erm* and *mef*(A) genes have been present in *N. gonorrhoeae* and if these genes could also be found in the related pathogen *Neisseria meningitidis*. Also, we examined the promoter region of the *mtr*(R) gene for sequence changes in the 13-bp repeat, since the loss of an adenine has been shown to alter macrolide susceptibilities in *N. gonorrhoeae* (29, 30), though recently mutations in the 23S rRNA have also been found to change macrolide susceptibility (18). Thus, the study examined three genotypes, acquired *erm*(B), *erm*(C), *erm*(F), and *mef*(A) genes, and changes in the sequence in the promoter region of the *mtr*(R) in 76 *N. gonorrhoeae* isolates isolated between 1940 and 1987 and 7 *N. meningitidis* isolates isolated between 1963 and 1987 by using DNA-DNA hybridization, PCR analysis, and sequencing.

MATERIALS AND METHODS

Bacterial strains. The isolates (n = 76) were reconstituted from lyophilized ampoules and from stocks frozen at −70°C and included 7 strains isolated from 1940 to 1969 (Denmark, 5; United States, 1; and Ethiopia, 1), 26 from 1970 to 1979 (England, Singapore, Asia, Belgium, Kenya, New Zealand, and the United States), and 43 from 1980 to 1987 (United States). Twenty-one isolates carrying β-lactamase plasmids and 16 isolates carrying a *tet*(M) plasmid (20, 22) were included. Seven *N. meningitidis* isolates (United States, 6; and Denmark, 1) from 1963 to 1987 were studied. The identity of the isolates was confirmed, and susceptibilities to erythromycin, azithromycin, penicillin, and tetracycline were determined for each species by using methods recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (17) on samples of 37 of the gonococcal isolates and all 7 *N. meningitidis* isolates.

Detection of acquired genes. The isolates were initially screened by using DNA-DNA hybridization of whole-cell dot blots and/or DNA dot blots, as previously described (6, 14, 21). The presence of all genes was confirmed by PCR assays, as previously described (14, 16, 21). The primers used are listed in Table 1.

Sequencing. The *mef*(A) gene from a 1975 *N. gonorrhoeae* isolate was sequenced as previously described (6, 14). The *mef*(A) sequence was compared to sequences from *Streptococcus pneumoniae* (GenBank accession no. U83667) and *S. pneumoniae* Tn*1207.1* (GenBank accession no. AF227520) by using Genetics Computer Group software (University of Wisconsin, Madison). The GenBank accession no. for the *N. gonorrhoeae mef*(A) gene is AY319932.

TABLE 1. Primers used for PCR and DNA-DNA hybridization studies

Gene	Primer	Sequence[a]
erm(B)	$ermB_F$	GAA AAG GTA CTC AAC CAA ATA
	$ermB_R$	AGT AAC GGT ACT TAA ATT GTT TAC
erm(C)	$ermC_F$	TCA AAA CAT AAT ATA GAT AAA
	$ermC_R$	GCT AAT ATT GTT TAA ATC GTC AAT
erm(F)	ermF1	CGG GTC AGC ACT TTA CTA TTG
	ermF2	GGA CCT ACC TCA TAG ACA AG
mef(A)	MF4A	ACC GAT TCT ATC AGC AAA G
	MF6	GGA CCT GCC ATT GGT GTG
mtr(R) promoter	mtrF1	GCC AAT CAA CAG GCA TTC TTA
	MTR13r1	GTT GGA ACA ACG CGT CAA AC

[a] The primers read from the 5′ end to the 3′ end.

Analysis of *mtr*(R) region. A 380-bp PCR fragment which included the promoter region of the *mtr(R)* genes was amplified and sequenced as previously described by using a GenBank sequence (accession no. Z25796) to represent the wild type (6). The isolates were grouped as wild type, loss of an adenine, or other, which included other variations of sequences in this region (6, 13, 29, 30).

Conjugation experiments. Donors included the 1963 *N. meningitidis* isolate and three *N. gonorrhoeae* isolates from the 1970s recovered from various geographic locations. The recipient was the erythromycin-susceptible *Enterococcus faecalis* strain JH2-2, for which the MIC is <0.5 μg/ml. Matings were performed on agar plates, and transconjugants were identified as previously described (14, 21). The transconjugants were selected on 5 or 10 μg of erythromycin/ml (14, 15). The presence of acquired *erm* and/or *mef* genes was determined by DNA-DNA hybridization and PCR. Erythromycin MICs for selected transconjugants were determined by using standard NCCLS protocols for agar dilution susceptibility (17).

Analyses. The Kruskal-Wallis test was utilized to assess the relationship between acquired erythromycin resistance genes and erythromycin susceptibilities.

RESULTS

Distribution of the acquired genes. Among the isolates from 1940 through 1969, one 1955 Danish isolate carried the *erm*(B), *erm*(F), and *mef*(A) genes; a second Danish strain from 1963 carried the *erm*(C) gene; and a 1960s United States isolate carried the *mef*(A) gene (Table 2). All seven isolates from this time period had wild-type *mtr*(R) promoter 13-bp inverted repeat sequences.

Among 26 isolates from the 1970s, 13 (50%) did not carry any of the four acquired genes examined. Nine (35%) isolates carried one of the four genes, three (12%) carried two of the genes, and one (4%) carried three of the acquired genes (Table 2). One isolate had a deletion at position A2058 (delA2058), also described as −A (13, 18, 29). The other isolates (96%) carried wild-type sequences in the *mtr*(R) promoter region.

TABLE 2. Acquired genes and variability in *mtr*(R) promoter region

Organism and year(s) isolated	No. of isolates	No. (%) of isolates with indicated gene:				
		erm(B)	*erm*(C)	*erm*(F)	*mef*(A)	Wild type
N. gonorrhoeae						
1940–69	7	1 (14)[a]	1 (14)	1 (14)	2 (28)	7 (100)
1970s	26	2 (8)	2 (8)	6 (23)	8 (30)	25 (96)
1980s	43	2 (5)	5 (13)	6 (13)	3 (8)	32 (74)
N. meningitides						
1963–1987	7	1 (14)	1 (14)	2 (28)	2 (28)	2 (28)[a]

[a] Two isolates had the same sequence as wild-type *N. gonorrhoeae*; sequences of the other five were 96 to 97% identical with wild-type *N. gonorrhoeae* and the *N. meningitides* genome.

Of the 43 isolates from the 1980s, 32 (74%) carried no acquired genes, 8 carried a single acquired gene, 3 carried two genes, and 1 carried three genes. In this group, 32 (74%) carried a wild-type *mtr*(R) promoter sequence, 8 (17%) had an adenine deletion, and 3 (7%) carried other changes in the 13-bp inverted repeat region.

Four (57%) of the seven *N. meningitidis* isolates carried acquired genes, one carried *mef*(A) and a single *erm* gene, two carried one or more *erm* genes alone, and one carried *mef*(A) alone. Both *mef*(A) and *erm*(F) were identified in a Danish strain (NRL 5041) isolated in 1963. Two of the these seven isolates had wild-type 13-bp *mtr*(R) sequences, and the remaining five strains had three base pair differences from the wild-type sequence that had previously been described when the genome of *N. meningitidis* serogroup A strain Z2491 was sequenced (25).

Among gonococci tested for antimicrobial susceptibility, 12 of 37 isolates carried a single acquired resistance gene and 5 carried multiple acquired genes. None of these 37 isolates had mutations in the *mtr*(R) promoter region. The geometric mean MIC for strains carrying the *erm*(B) gene was 1.0 μg/ml, compared to a geometric mean of 0.31 μg/ml for all other strains (P, 0.04). The carriage of *erm*(C) (MIC, 0.71 μg/ml versus 0.31 μg/ml), *erm*(F) (MIC, 0.51 μg/ml versus 0.31 μg/ml), or *mef*(A) (MIC, 0.38 μg/ml versus 0.33 μg/ml) was not significantly associated with decreased gonococcal susceptibility to erythromycin. The limited number of *N. meningitidis* strains did not permit analysis of the effect of the carriage of acquired genes on antimicrobial susceptibilities.

***mef*(A) sequence.** There was 100% identity at the nucleotide and amino acid level between the *mef*(A) gene from a 1975 gonococcal isolate from the United States and that from *S. pneumoniae* U83667. In contrast, there was only 90% identity with the *mef*(A) gene from *S. pneumoniae* Tn*1207.1* AF227520 (data not shown).

Conjugal transfer studies. The donors were three *N. gonorrhoeae* isolates from the 1970s carrying *erm*(F) plus *mef*(A) or the *erm*(F) or *mef*(A) gene alone and one 1963 *N. meningitidis* isolate carrying *erm*(F) plus *mef*(A). From 3.2×10^{-7} to 5.4×10^{-8} macrolide resistance genes per recipient were transferred to *E. faecalis* JH2-2 organisms from each of the four donors examined. The *N. gonorrhoeae* and *N. meningitidis* strains carrying both *erm*(F) and *mef*(A) genes transferred each gene separately at similar frequencies (the number of transconjugants carrying one versus the other acquired gene was indistinguishable).

The MIC of erythromycin was determined for selected transconjugants carrying the *erm* or *mef*(A) genes from matings with each of three different donors. The MIC for all the transconjugants was >64 μg/ml, while the MIC for the parental *E. faecalis* strain was <0.5 μg/ml.

DISCUSSION

erm(A), *erm*(B), *erm*(C), and *erm*(F) genes from a 1950s *Bacteroides thetaiotaomicron* isolate, an *erm*(F) gene from a 1950s *Bacteroides fragilis* isolate (3), and an *erm*(B) gene from a 1950s enterococcus isolate (2) have previously been identi-

fied. The *mef*(A) gene has been identified in *Streptococcus pyogenes* and *S. pneumoniae* strains isolated in the 1990s (5, 26, 27) and in viridans group streptococci isolated between 1988 and 1995 (1). In this study, the earliest strains identified with acquired genes included a 1955 *N. gonorrhoeae* strain carrying *erm*(B), *erm*(F), and *mef*(A) genes, a 1963 *N. gonorrhoeae* strain carrying an *erm*(C) gene, and a 1963 *N. meningitidis* strain carrying both *erm*(F) and *mef*(A) genes. These isolates are the oldest identified to date that carry the *mef*(A) gene and are among the oldest isolates to carry the *erm*(B), *erm*(F), and *erm*(C) genes. All the transconjugants were selected on erythromycin with a concentration that was at least 10-fold higher than the MIC for the recipient. The MIC of erythromycin for all of the transconjugants tested was >64 μg/ml, clearly suggesting that the *erm* and *mef*(A) genes are able to confer erythromycin resistance to the transconjugants. Differences in susceptibilities of the transconjugants carrying the *mef*(A) versus the *erm* gene were not observed, though this may be evident at higher concentrations of erythromycin than those we tested.

The *mef*(A) gene from a 1975 isolate was sequenced and had 100% amino acid identity with the *mef*(A) gene from a *S. pneumoniae* strain isolated in the 1990s. This *S. pneumoniae* strain also carried the *orf3* to *orf8* genes, most of which have unknown functions, though they have been found in conjugative transposon Tn*5252* and have previously been described in Tn*1207.1* (24). This finding suggests that this type of element has been in the nonstreptococcal population for at least 20 years prior to its identification and study in streptococci in the 1990s.

In streptococci, carriage of the *mef*(A) gene confers a lower level of resistance to erythromycin than does carriage of the *erm*(B) gene (16), but these differences have not always been found (14, 15). Therefore, it was not unexpected that the MICs for the transconjugants carrying the *mef*(A) gene were indistinguishable from those for strains not carrying the *mef*(A) gene. However, the number of strains studied was small, and the sample was not meant to represent the gonococcal population. Additionally, because of the small sample size we were unable to control for the effects of other resistance determinants, such as chromosomal mutations, that have been shown to influence macrolide susceptibilities (8). The influence of these acquired genes on macrolide susceptibilities awaits larger studies of isolates selected in an unbiased way.

Previously, *N. meningitidis* strains have been used as recipients (14); however, this is the first time that *N. meningitidis* has been shown to transfer both *erm* and *mef*(A) genes to a recipient, suggesting that these genes were functional in the genus and associated with mobile elements for 40 to 50 years. In addition, the 1970s *N. gonorrhoeae* strains were able to act as donors for both *erm*(F) and *mef*(A) genes, indicating their presence on mobile elements. It has previously been demonstrated that *Bacteroides* conjugative chromosomal elements, such as Tc^r Em^r DOT, Tc^r Em^r 12256, and Tc^r Em^r CEST, which contain both *erm*(F) and the *tet*(Q) genes, are able to mobilize *Bacteroides* nonconjugative plasmids in *cis* (11, 28). More recently, it has been shown that the *mef*(A) gene in *S. pyogenes* is linked upstream with *tet*(O), which encodes a ribosomal protection tetracycline resistance protein highly related to the Tet(M) protein found in *Neisseria* spp. This linkage allows both the *tet*(O) and the *mef*(A) genes to be transferred as a single unit, and for the first time the *tet*(O) gene can be moved between chromosomes of different species and genera (10). Given the potential that these mobile elements present in influencing movement of themselves and other antibiotic resistance genes, it will be of interest to learn if these elements have had an impact on, or could impact, the evolution of pathogenic *Neisseria* in this time of increasing antibiotic use of macrolides.

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