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Identification of the Conjugative *mef* Gene in Clinical *Acinetobacter junii* and *Neisseria gonorrhoeae* Isolates

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The *mef* gene, originally described for gram-positive organisms and coding for an efflux pump, has been identified in clinical isolates of *Acinetobacter junii* and *Neisseria gonorrhoeae*. These strains could transfer the *mef* gene at frequencies ranging from 10^{-6} to 10^{-9} into one or more of the following recipients: gram-negative *Moraxella catarrhalis*, *Neisseria perflava*/*sicca* and *Neisseria mucosa* and gram-positive *Enterococcus faecalis*. Three *Streptococcus pneumoniae* strains could transfer the *mef* gene into *Eikenella corrodens*, *Haemophilus influenzae*, *Kingella denitrificans*, *M. catarrhalis*, *Neisseria meningitidis*, *N. perflava*/*sicca*, and *N. mucosa* at similar frequencies. The *mef* gene can thus be transferred to and expressed in a variety of gram-negative recipients.

The *mef* gene encodes a membrane-bound efflux protein and confers resistance to macrolides but not to lincosamides or streptogramin B (24, 25). From geographically diverse areas, two gene variants with 90% nucleotide sequence identity, *mefA* and *mefE*, have been identified in gram-positive organisms, including *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Corynebacterium* spp., *Enterococcus* spp., *Micrococcus luteus*, and viridans group streptococci (2, 5, 9, 20, 23–25). Both genes have now been combined as *mef*(A) (20).

Recently, we have shown that both *Neisseria gonorrhoeae* and oral commensal *Neisseria* spp. carry known rRNA methylase genes (20). However, we have also identified several of these *Neisseria* spp. and oral gram-negative isolates that were macrolide resistant yet did not carry Erm determinants (ErmA, -B, -C, or -F) (21). We selected a gram-negative *Acinetobacter junii* strain and screened two groups of *N. gonorrhoeae* in order to determine if the *mef* gene was present. Because we have shown that the *mef* gene can be transferred by conjugation in various gram-positive genera, we also wanted to determine the mobility of the *mef* gene in these gram-negative isolates as well as the ability of gram-positive donors to move the *mef* gene to gram-negative species (9).

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MATERIALS AND METHODS

Bacteria. Erythromycin-resistant (Ery^r) *Acinetobacter junii* strain 329 was isolated from the oral gumline of a child in Portugal (Table 1). The organism was identified by D. Stroman (Alcon Laboratories, Inc., Fort Worth, Tex.) by sequencing the 16S rRNA. Sixteen *N. gonorrhoeae* isolates with variable susceptibilities to erythromycin (MIC = 0.25 to 4 μg/ml) were collected from urethral specimens from adults in Seattle, Wash., and identified by the *Neisseria* Reference Laboratory at the University of Washington (Table 1). Thirteen *N. gonorrhoeae* isolates carrying the *tetM* 25.2-MDa plasmid were collected in Seattle and the eastern United States during 1983 through 1986 and have been previously described (7). Of the three Ery^r *S. pneumoniae* strains carrying the *mef* gene, two (n011 and 970146) were isolated in Washington State and the third (02J1048) was supplied by Pfizer Inc. (Groton, Conn.) (Table 1) (9, 26). Strains used as recipients are listed in Table 2. Antibiotic susceptibilities were determined using either disk diffusion or agar dilution as described by the NCCLS (13).

Media. *S. pneumoniae* isolates were grown on brucella blood agar (Difco, Detroit, Mich.) supplemented with 5% sheep red blood cells and incubated with 5% CO_2 at 36.5°C (8). The *Haemophilus influenzae* isolate was grown on brain heart infusion (BHI) agar (Difco) supplemented with 2 μg of NAD (Sigma Chemical Co., St. Louis, Mo.) per ml and 10 μg of hemin–L-histidine (Sigma Chemical Co.) per ml (16). *Eikenella corrodens*, *Kingella denitrificans*, *Moraxella catarrhalis*, *N. gonorrhoeae*, *Neisseria meningitidis*, *Neisseria perflava*/*sicca*, and *Neisseria mucosa* were grown on gonococcus (GC) medium agar (Difco) supplemented with Kellogg's supplement solution (0.22 M D-glucose, 0.03 M L-glutamine, 0.001 M ferric nitrate, and 0.02 M cocarboxylase) (Sigma-Aldrich Chemical Co., St. Louis, Mo.) as previously described (6, 15). *Enterococcus faecalis* JH2-2 was grown on BHI agar without supplements (9). For DNA dot blots and DNA extractions, the gram-positive bacteria were grown overnight at 36.5°C in BHI broth (Difco) supplemented with 0.03 M D-glucose and 0.04% DL-threonine (8, 9). The gram-negative bacteria, for DNA dot blots and DNA extractions, were grown in either BHI broth with no supplements (*A. junii*), BHI broth supplemented with 2 μg of NAD per ml and 10 μg of hemin–L-histidine per ml (*H. influenzae*), or GC broth supplemented with 1% sodium bicarbonate and Kellogg's supplement (see above) (*E. corrodens*, *K. denitrificans*, *M. catarrhalis*, and *Neisseria* spp.) as described previously (11, 12, 15).

Mating procedure. Donor and recipient bacteria were grown separately at 36.5°C overnight. Each isolate was suspended in 0.5 to 1.0 ml of BHI or GC broth (Difco) to a density of approximately 10^9 cells per ml (3 McFarland). Donor and recipient bacteria at ratios of 1:20 or 1:40 (donor to recipient) were mixed. The bacterial suspension was then plated directly onto brucella blood agar plates and incubated in CO_2 at 36.5°C for 48 h (8, 9). After incubation, the mating mixture was serially diluted onto antibiotic-supplemented plates as follows: for *H. influenzae* recipients, erythromycin (10 μg/ml) and rifampin (10 μg/ml); for *E. faecalis* recipients, erythromycin (10 μg/ml) and rifampin (10 μg/ml); for *M. catarrhalis*, *N. gonorrhoeae*, and *N. meningitidis* recipients, erythromycin (2 μg/ml) and rifampin (10 μg/ml); and for *E. corrodens*, *K. denitrificans*, *N. perflava*/*sicca*, and *N. mucosa* recipients, erythromycin (2 μg/ml) and tetracycline (10 μg/ml). Gram stains were prepared from all transconjugants, and all transconjugants were identified to species level by established methods (4, 14). Mating experiments were performed a minimum of two times. DNase I (1 mg/ml) (Sigma-Aldrich Chemical Co.) was added to the mating mixture in at least one of the two matings for comparison with mating without DNase to rule out transformation.

Extraction of whole-cell DNA. Whole-cell DNA was prepared from isolates and transconjugants as previously described (1, 8). DNA was separated on a 0.7% agarose gel and Southern blots were prepared (22).

Plasmid analysis of transconjugants. Plasmid extractions were prepared from *K. denitrificans*, *N. perflava*/*sicca*, and their respective transconjugants after 8 h of growth in 40 ml of GC peptone broth supplemented with Kellogg's supplement solution and 1% sodium bicarbonate as previously described (3, 15). DNA was separated on a 0.7% agarose gel in 0.5× Tris-borate-EDTA (TBE) buffer at 100 V for 1.5 h; Southern blots were prepared and hybridized with *mef*-specific ^{32}P-labeled oligonucleotide probe MF5 to examine the location of the *mef* gene.

Dot blots. Two milliliters of overnight bacterial growth in supplemented BHI or GC broth was placed into 1.5-ml sterile Eppendorf tubes and centrifuged at 8,000 × *g* for 2 min, and the supernatant was decanted. The bacterial pellet was resuspended with 1 ml of BHI broth to create a turbid suspension of 3 McFarland. Two hundred microliters of the suspension, in 25-μl aliquots, was spotted onto a GeneScreen Plus membrane (NEN Research, Boston, Mass.), dried, and treated with 0.5 M NaOH for 10 min, 1 M Tris-HCl for 3 min, and 1 M Tris-HCl with 1.5% NaCl, pH 7.5, for 10 min. The membrane was dried, washed in

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TABLE 1. Isolates carrying the *mef* gene

Isolate	Yr obtained	Location	Susceptibility pattern[a]	Plasmid size(s) (MDa)	Erm determinant(s)	Reference
S. pneumoniae 02J1048	Unknown	Unknown	Ery^r (*mef*) Rif^s Fus^s Str^s Tet^s	None	None	24
S. pneumoniae n011	1996	Seattle, Wash.	Ery^r (*mef*) Rif^s Fus^s Str^s Tet^s	None	None	9
S. pneumoniae 970146	1997	Seattle, Wash.	Ery^r (*mef*) Rif^s Fus^s Str^s Tet^r	None	None	9
A. junii 329	1999	Lisbon, Portugal	Ery^r (*mef*) Rif^s Fus^s Str^s Tet^s	None	None	This paper
N. gonorrhoeae 98.420	1998	Seattle, Wash.	Ery^r (*mef*) Pen^r Rif^s Str^s Tet^s	2.6	B, F	This paper
N. gonorrhoeae 98.560	1998	Seattle, Wash.	Ery^r (*mef*) Pen^r Rif^s Str^s Tet^s	2.6	B, C	This paper
N. gonorrhoeae 98.737	1998	Seattle, Wash.	Ery^r (*mef*) Pen^r Rif^s Str^s Tet^s	2.6	None	This paper
N. gonorrhoeae 85.022462	1985	DeKalb County, Ga.	Ery^r (*mef*) Tet^r	2.6, 25.2	B, F	7

[a] Ery, erythromycin; Fus, fusidic acid; Rif, rifampin; Str, streptomycin; Tet, tetracycline. *H. influenzae*, *N. meningitidis*, and *E. faecalis* were chromosomally resistant to the antibiotics.

chloroform-isoamyl alcohol (24:1), rinsed in water twice, washed in 1 M Tris-HCl with 1.5% NaCl for 10 min, and baked at 80°C for 1 h. The filters were stored at room temperature until hybridized with labeled oligonucleotide probes (8).

Labeled probes. MF5 (5′ GGT GCT GTG ATT GCA TCT ATT AC 3′) was used as the oligonucleotide probe for the *mef* gene (9). The oligonucleotide probes for the *erm* genes were the following: $ermB_F$ (5′ GAA AAG GTA CTC AAC CAA ATA 3′), $ermC_R$ (5′ GCT AAT ATT GTT TAA ATC GTC AAT 3′), and ermF1 (5′ CGG GTC AGC ACT TTA CTA TTG 3′) (19). A ^{32}P-labeled probe was used for whole-bacterial-cell dots as previously described (9).

PCR. PCR amplification used 40 ng of genomic DNA from *S. pneumoniae* 02J1048 as a positive control and 30 ng of genomic DNA as a template from the transconjugants and *A. junii*. Primers were MF4a (5′ ACC GAT TCT ATC AGC AAA G 3′) and MF6 (5′ GGA CCT GCC ATT GGT GTG 3′). Both primers are in the conserved regions of the *mef* gene. Each reaction mixture contained 2 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), 200 μM deoxynucleoside triphosphates, 1× PCR buffer (1.5 mM $MgCl_2$), and 100 ng of each primer. Using a Perkin-Elmer Cetus thermal cycler, the reactions were carried out by denaturation at 94°C for 1 min, annealing at 37°C for 1 min, and elongation at 72°C for 2 min, for 35 cycles. The PCR products were dried, resuspended in 1/10 volume of sterile water, and separated on a 1.5% agarose gel with 0.5× TBE running buffer. The PCR bands were visualized by ethidium bromide staining, and Southern blots were prepared. The 940-bp PCR product hybridized with a labeled internal *mef* probe, MF5 (9). Positive and negative controls were run with each assay.

DNA-DNA hybridization. Southern blots using uncut whole-cell DNA or the PCR fragments were prepared on Magnagraph nylon (Micron Separation Inc., Westboro, Mass.) and hybridized with ^{32}P-labeled oligonucleotide MF5. DNA dot blots containing 30 to 300 μg of extracted whole-cell DNA from the donor strains and bacterial dot blots from transconjugants were placed on GeneScreen Plus membranes and hybridized with the radiolabeled oligonucleotide probe (8, 9).

Sequencing. Sequencing of selected isolates (*A. junii* 329 and *N. gonorrhoeae* 98.420 and 98.737) was performed using oligonucleotide primers MF7 (5′ ATG CAG ACC AAA AGC GCG AT 3′) and MF8 (5′ CGG TAT CTG TTC TGG TAG CG 3′). Both primers are in the conserved regions of the *mef* gene and within the generated PCR fragment. Each reaction mixture contained 2 U of *Taq* polymerase, 200 μM deoxynucleoside triphosphates, 1× PCR buffer (1.5 mM $MgCl_2$), and 100 ng of each primer. Using a Perkin-Elmer Cetus thermal cycler, the reactions were carried out by denaturation at 94°C for 1 min, annealing at 51°C for 1 min, and elongation at 72°C for 2 min, for 35 cycles. The PCR products were filtered by centrifugation with a 0.5-μm-pore-size separation filter (Micron) by following the manufacturer's directions. A small part of the PCR product obtained was dried and separated on a 1.5% agarose gel with 0.5× TBE running buffer. The 253-bp PCR product was then visualized by ethidium bromide staining. The remainder of the PCR product was then sequenced by following the Big Dye Terminator chemistry protocol (Perkin-Elmer Cetus, Foster City, Calif.). The sequence reaction mixture included 100 ng of the PCR product and 4 pmol of primer MF7, with a total volume of 12 μl. The *mef* sequence was compared to the *mef* sequence from *S. pneumoniae* (GenBank number U83667) using the Genetics Computer Group software (University of Wisconsin, Madison).

RESULTS

The *mef* gene in gram-negative bacteria. We examined 29 *N. gonorrhoeae* isolates for the presence of the *mef* gene using DNA-DNA hybridization of bacterial dot blots. Four (14%) were positive and verified as carrying the *mef* gene by hybridization of Southern blots and positive PCR assays. Three of the *mef*-positive isolates were from Seattle and one was from Georgia. The *mef* gene was also identified by the same means in an oral *A. junii* isolate from Portugal. The PCR fragments were sequenced from the *A. junii* isolate and from two of the *N. gonorrhoeae* isolates. The first 9 nucleotides of the PCR product could not be adequately sequenced; however, the three isolates had 97 to 98% nucleotide identity with the GenBank sequence of the pneumococcal *mef* gene over the remaining 244 nucleotides and had 98% amino acid identity over 81 amino acids.

All four *N. gonorrhoeae* isolates carried the gonococcal 2.6-MDa plasmid, and strain 85.022462 carried a 25.2-MDa plasmid as well. When the *mef* probe was hybridized with Southern blots containing both chromosomal and plasmid DNA, only the chromosomal band hybridized in all four isolates. The *A. junii* isolate had no visible plasmids, and the *mef* probe hybrid-

TABLE 2. Characterization of recipients used

Isolate	Location	Susceptibility pattern[a]	Reference
H. influenzae RD8	Laboratory	Ery^i Rif^{r}Fus^{r}Str^r	15
E. corrodens CTM 1.6	DeKalb County, Ga.	Ery^{s}Tet$^{r b}$	6
N. meningitidis NRL 9205[c]	Laboratory	Ery^s Rif^{r}Fus^{r}Str^r	16
N. perflava/*sicca* 86.000348	DeKalb County, Ga.	Ery^{s}Tet$^{r d}$	6
N. mucosa CTM 1.1	DeKalb County, Ga.	Ery^{s}Tet$^{r d}$	6
K. denitrificans 87.023461	DeKalb County, Ga.	Ery^{s}Tet$^{r b}$	6
M. catarrhalis NRL 30018	Laboratory	Ery^s Rif^r Str^r	15
E. faecalis JH2-2	Laboratory	Ery^s Rif^{r}Fus^r	19

[a] Ery, erythromycin; Fus, fusidic acid; Rif, rifampin; Str, streptomycin; Tet, tetracycline. *H. influenzae*, *N. meningitidis*, *M. catarrhalis*, and *E. faecalis* were chromosomally resistant to the antibiotics.
[b] Carries a 25.2-MDa plasmid with the *tetM* gene (16).
[c] Serotype A.
[d] The *tetM* gene is carried on the chromosome (6).

TABLE 3. Conjugal transfer of the *mef* gene[a]

Donor	Recipient	Frequency (per donor cell)
A. junii 329	*E. faecalis* JH2-2	9.5×10^{-6}
	M. catarrhalis NRL 30018	5.0×10^{-8}
	N. mucosa CTM 1.1	8.5×10^{-8}
	N. perflava/sicca 86.000348	1.0×10^{-9}
N. gonorrhoeae 98.737	*E. faecalis* JH2-2	4.1×10^{-9}
	M. catarrhalis NRL 30018	1.6×10^{-8}
	N. mucosa CTM 1.1	1.2×10^{-8}
S. pneumoniae 02J1048	*N. meningitidis* 9205	1.3×10^{-8}
	N. perflava/sicca 86.000348	1.4×10^{-8}
	E. corrodens CTM 1.6	1.7×10^{-6}
S. pneumoniae n011	*N. mucosa* CTM 1.1	4.0×10^{-8}
	K. denitrificans 87.923461	1.0×10^{-7}
	H. influenzae RD8	3.0×10^{-7}
S. pneumoniae 970146	*H. influenzae* RD8	1.6×10^{-8}
	M. catarrhalis NRL 30018	1.3×10^{-9}

[a] Mating experiments were performed a minimum of two times.

ized with the chromosomal band in this isolate (data not shown).

We examined the five isolates for the presence of *erm* genes, which code for rRNA methylase genes (20). Of the four *Neisseria* isolates, 98.420 and 85.022462 hybridized with probes for *erm*(B) and *erm*(F) while 98.560 hybridized with the probes for *erm*(B) and *erm*(C). *N. gonorrhoeae* 98.737 and *A. junii* 329 did not hybridize with any of the *erm* probes examined (Table 1). In addition, *A. junii* 329 was susceptible to clindamycin, suggesting that this isolate did not carry an Erm determinant.

In mixed cultures, we were able to transfer the *mef* gene from *A. junii* 329 and *N. gonorrhoeae* 98.737 to *E. faecalis* at frequencies of 10^{-6} and 10^{-9} per donor cell, respectively, and to *M. catarrhalis* and *N. mucosa* at a frequency of 10^{-8} per donor cell (Table 3). We were able to transfer the *mef* gene by conjugation from *A. junii* to *N. perflava/sicca* at a frequency of 10^{-9} per donor cell (Table 3). DNA-DNA hybridization of bacterial dot and Southern blots of cellular DNA and amplification of PCR products confirmed the presence of the *mef* gene in transconjugants. The recipients did not carry plasmids and the *mef* gene probe hybridized with the chromosomal band in these transconjugants, although the presence of very large plasmids could not be ruled out. The transconjugants grew on media supplemented with 2 to 10 μg of erythromycin per ml, depending upon the recipient, whereas the original recipients did not grow on media supplemented with erythromycin. This corresponds to a six- to eightfold increase in MICs (0.03 μg/ml versus 2 to 10 μg/ml). All of the transconjugants remained susceptible to clindamycin. As in our previous work, no other antibiotic resistance determinants were transferred with the *mef* gene.

Conjugation of the *S. pneumoniae mef* gene with gram-negative bacteria. To examine whether gram-positive *S. pneumoniae* could transfer *mef* to gram-negative recipients, we used three pneumococcal donors and obtained frequencies of transfer of *mef* to *E. corrodens*, *H. influenzae*, *K. denitrificans*, *M. catarrhalis*, *N. meningitidis*, *N. mucosa*, and *N. perflava/sicca* that were similar those obtained with *A. junii* as the donor (Table 3). Although the *K. denitrificans* and *E. corrodens* transconjugants carried the 25.2-MDa *tetM* plasmid, the *mef* probe hybridized with the chromosomal region but not with the 25.2-MDa plasmid.

DISCUSSION

This is the first time the *mef* gene has been identified in clinical gram-negative isolates. These isolates were collected over a 13-year time span and from different geographical areas and ecological niches (oral and genital). We showed that these isolates transfer the *mef* gene to both gram-negative and gram-positive recipients (Table 3). In all cases, the *mef* gene appeared to be localized to the chromosome rather than to indigenous plasmids, although plasmids larger than 25.2 MDa cannot entirely be ruled out. Previous work with the *mef* gene in gram-positive donors and recipients has demonstrated not a specific location in the chromosome but potentially multiple random sites (9, 10). We are currently examining whether this is also true with the gram-negative recipients used in this study. However, we did not find the *mef* gene inserted into the 25.2-MDa plasmid. The *mef* sequence in the *A. junii* and *N. gonorrhoeae* isolates had 97 to 98% nucleotide identity to the *mef* gene found in *S. pneumoniae*, suggesting that these genes originally came from a gram-positive donor. A similar picture has emerged for the *tetM*, *tetO*, and *erm* genes found in the gram-negative flora (18–21).

To examine whether the *mef* genes located in *S. pneumoniae* could be transferred into gram-negative recipients, we examined three randomly chosen isolates and documented their ability to transfer *mef* into a variety of different oral recipient species (Table 3). Our data suggest that the *mef* gene host range may extend to a larger number of species and genera than the *Acinetobacter* and *Neisseria* organisms in this study. A survey of a variety of gram-negative species and genera from different ecological niches and locations should be done to better define the host range of the *mef* gene and its potential impact on macrolide therapy.

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