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Presence of the *tet*(O) Gene in Erythromycin- and Tetracycline-Resistant Strains of *Streptococcus pyogenes* and Linkage with either the *mef*(A) or the *erm*(A) Gene

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Sixty-three recent Italian clinical isolates of *Streptococcus pyogenes* resistant to both erythromycin (MICs ≥ 1 μg/ml) and tetracycline (MICs ≥ 8 μg/ml) were genotyped for macrolide and tetracycline resistance genes. We found 19 isolates carrying the *mef*(A) and the *tet*(O) genes; 25 isolates carrying the *erm*(A) and *tet*(O) genes; and 2 isolates carrying the *erm*(A), *tet*(M), and *tet*(O) genes. The resistance of all *erm*(A)-containing isolates was inducible, but the isolates could be divided into two groups on the basis of erythromycin MICs of either >128 or 1 to 4 μg/ml. The remaining 17 isolates included 15 isolates carrying the *erm*(B) gene and 2 isolates carrying both the *erm*(B) and the *mef*(A) genes, with all 17 carrying the *tet*(M) gene. Of these, 12 carried Tn*916*-Tn*1545*-like conjugative transposons. Conjugal transfer experiments demonstrated that the *tet*(O) gene moved with and without the *erm*(A) gene and with the *mef*(A) gene. These studies, together with the results of pulsed-field gel electrophoresis experiments and hybridization assays with DNA probes specific for the *tet*(O), *erm*(A), and *mef*(A) genes, suggested a linkage of *tet*(O) with either *erm*(A) or *mef*(A) in erythromycin- and tetracycline-resistant *S. pyogenes* isolates. By amplification and sequencing experiments, we detected the *tet*(O) gene ca. 5.5 kb upstream from the *mef*(A) gene. This is the first report demonstrating the presence of the *tet*(O) gene in *S. pyogenes* and showing that it may be linked with another gene and can be moved by conjugation from one chromosome to another.

Since the mid-1990s Italy has seen an increase in erythromycin-resistant *Streptococcus pyogenes* isolates (MICs ≥ 1 μg/ml) (30). In past studies, a correlation between the pulsed-field gel electrophoresis (PFGE) pattern, macrolide phenotypes (inducible versus constitutive), and tetracycline resistance was observed (8, 22); and it was concluded that the spread of erythromycin-resistant *S. pyogenes* in Italy is not due to the spread of a single clone (22). In the study described in this report, 63 recent Italian clinical strains of *S. pyogenes* resistant to both erythromycin and tetracycline were genotyped for the presence of *erm*(A) [subclass *erm*(TR) (27); hereafter designated *erm*(A) according to the present nomenclature (24)] and *erm*(B), which encode rRNA methylases; *mef*(A), which encodes a macrolide efflux protein (2); *tet*(M) and *tet*(O), both of which encode tetracycline resistance ribosomal protection proteins; and *tet*(K) and *tet*(L), both of which encode efflux proteins. The erythromycin resistance genes, *erm*(A), *erm*(B), and *mef*(A), are well known to occur in *S. pyogenes* (15). Both tetracycline resistance genes *tet*(M) and *tet*(O) have previously been reported in *Streptococcus pneumoniae*, but *tet*(O) has been reported from only two geographic areas (17, 31). While the *tet*(M) gene has been found in *S. pyogenes* (1), in which it was first detected in the prototype composite element Tn*3701* (14), which contains a Tn*916*-like transposon (3), the *tet*(O) gene has not previously been identified in this species.

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MATERIALS AND METHODS

Bacterial strains. Sixty-three distinct clinical strains of *S. pyogenes*, collected from several Italian laboratories between 1997 and 2000 and isolated from throat swab cultures of symptomatic patients, were used. Strain identification was confirmed by using bacitracin disks (Difco Laboratories, Becton Dickinson, Sparks, Md.) and a latex agglutination assay (Streptex; Wellcome Diagnostics, Dartford, United Kingdom). Two inclusion criteria were used: all isolates were resistant to both erythromycin (MIC ≥ 1 μg/ml) and tetracycline (MIC ≥ 8 μg/ml), and all isolates were different strains on the basis of their phenotypic and genotypic characteristics.

Antibiotics and susceptibility tests. Erythromycin, tetracycline, minocycline, and clindamycin were purchased from Sigma Chemical Co., St. Louis, Mo. Clarithromycin was obtained from Abbott Laboratories (Abbott Park, Ill.), azithromycin was obtained from Pfizer Inc. (New York, N.Y.), and josamycin was obtained from ICN Biomedicals (Costa Mesa, Calif.). MICs were determined by the broth microdilution method according to the protocols of the National Committee for Clinical Laboratory Standards (19). *S. pneumoniae* ATCC 49619 was used for quality control. Kanamycin and chloramphenicol susceptibilities were determined by a standard NCCLS agar diffusion test (20) with commercial disks (Oxoid Ltd., Basingstoke, United Kingdom) containing 30 μg of either antibiotic. The following zone diameter breakpoints were used for kanamycin: susceptible, ≥18 mm; intermediate, 14 to 17 mm; and resistant, ≤13 mm. The following zone diameter breakpoints were used for chloramphenicol: susceptible, ≥21 mm; intermediate, 18 to 20 mm; and resistant, ≤17 mm.

Determination of macrolide resistance phenotype. Test strains were assigned to the constitutive, the inducible, or the efflux-mediated macrolide resistance phenotypes on the basis of their patterns of susceptibility to macrolide-lincosamide-streptogramin B antibiotics and the triple-disk (erythromycin, clindamycin, and josamycin) test, as described previously (8).

PFGE and random amplified polymorphic DNA analysis. *Sma*I PFGE patterns were determined and analyzed as described recently (22). Random amplified polymorphic DNA analysis was performed by established methods (9) with primers M13 and H2 (26) with 21 of the isolates to distinguish strains that fell into single groups on the basis of their phenotypic or genotypic characteristics and PFGE analysis.

TABLE 1. Sequences of primers and a *tet*(O)-specific probe used

Gene	Primer or probe designation	Sequence (5′-3′)	Product size (bp)	Reference
erm(A)	III_8[a] III_{10}	GCATGACATAAACCTTCA AGGTTATAATGAAACAGA	208	27
erm(A)	TR_1[b] TR_2	ATAGAAATTGGGTCAGGAAAAGG TTGATTTTTAGTAAAAAG	530	12
erm(B)	ERMB1 ERMB2	GAAAAGGTACTCAACCAAATA AGTAACGGTACTTAAATTGTTTAC	639	28
mef(A)	MEFA1[c] MEFA2	AGTATCATTAATCACTAGTGC TTCTTCTGGTACTAAAAGTGG	348	28
tet(K)	tetK-up tetK-rev	TATTTTGGCTTTGTATTCTTTCAT GCTATACCTGTTCCCTCTGATAA	1,159	29
tet(L)	tetL-up tetL-rev	ATAAATTGTTTCGGGTCGGTAAT AACCAGCCAACTAATGACAATGAT	1,077	29
tet(M)	TETM2 TETM3	GAACTCGAACAAGAGGAAAGC ATGGAAGCCCAGAAAGGAT	740	21
tet(O)	TETO1[d] TETO2	AACTTAGGCATTCTGGCTCAC TCCCACTGTTCCATATCGTCA	519	21
int-Tn	*int* forward *int* reverse	GCGTGATTGTATCTCACT GACGCTCCTGTTGCTTCT	1,046	5
orf3[e]	ORF3rev	GACCTACCTGAACAATACC		16
orf4[e]	ORF4	AATATGGGCAGGGCAAGCA		4
orf4[e]	OMI8	TGCTTGCCCTGCCCATATT		4
orf7[e]	ORF7	GAATCTTTGGTCAGACTTGG		16
orf8[e]	ORF8	CCCTCCAATCCACCAGCG		16
tet(O)	TO1[f]	CAACGATTGCAGTAAAGAAATCTG		17

[a] The III_8–III_{10} primer pair was used to detect the *erm*(A) gene.
[b] The TR_1-TR_2 primer pair was used to obtain an *erm*(A)-specific probe.
[c] The MEFA1-MEFA2 primer pair was used to detect the *mef*(A) gene and obtain a *mef*(A)-specific probe.
[d] The TETO1-TETO2 primer pair was used to detect the *tet*(O) gene and obtain a *tet*(O)-specific probe.
[e] Gene of transposon Tn*1207.1*, containing eight open reading frames and carrying *mef*(A) as *orf4* (25).
[f] *tet*(O)-specific probe.

Gene detection and amplification experiments. PCRs with specific primer pairs (Table 1) were used to detect erythromycin resistance genes *erm*(A), *erm*(B), and *mef*(A); tetracycline resistance genes *tet*(K), *tet*(L), *tet*(M), and *tet*(O); and the integrase gene *int-Tn*, associated with the Tn*916*-Tn*1545* family of conjugative transposons. DNA preparation and amplification and electrophoresis of PCR products were carried out by established procedures (10) and according to the conditions indicated for the use of the individual primer pairs. PCR assays for determination of the nature of the *tet*(O)-*erm*(A) linkage were performed with one primer specific for a region within the *tet*(O) gene and a second primer specific for a region within the *erm*(A) gene (Table 1). Other PCR assays for determination of the nature of the *tet*(O)-*mef*(A) linkage were performed with one primer specific for a region within the *tet*(O) gene and a second primer specific for a region within the *mef*(A) gene or upstream of the *mef*(A) gene in *orf3* (25) (Table 1). The Ex *Taq* system (TaKaRa Bio, Shiga, Japan) was used in these amplification experiments.

Mating experiments. *S. pyogenes* strains iB21, iB27, iC38, and iC41 were used as donors for the *erm*(A) and the *tet*(O) genes; strains m46 and m49 were used as donors for the *mef*(A) and the *tet*(O) genes; and strains c7 and c13 were used as donors for the *erm*(B) and the *tet*(M) genes. *S. pyogenes* 12RF, a clinical strain selected in the laboratory for rifampin (25 μg/ml) and fusidic acid (25 μg/ml) resistance and susceptible to both erythromycin (≤0.015 μg/ml) and tetracycline (≤0.015 μg/ml), and *Enterococcus faecalis* JH2-2, a laboratory strain resistant to rifampin and fusidic acid, were both used as recipients of the genes from the *S. pyogenes* donors. Conjugal transfer was performed on a membrane filter (32) or directly on the agar surface as described previously (17). The frequency of transfer was expressed as the number of transconjugants per recipient. JH2-2 transconjugants carrying only the *tet*(O) gene from the *S. pyogenes* donors were used as donors, and *E. faecalis* OG1-10 (pPD1), which is resistant to 1,000 μg of streptomycin per ml (23), was used as a recipient in additional matings. Similarly, the *S. pyogenes* transconjugants were used as donors to the recipient *S. pyogenes* strain 12RF, which had been selected for resistance to streptomycin (500 μg/ml) and nalidixic acid (10 μg/ml). Selected transconjugants and donors were verified as carrying the *tet*(O) gene by the PCR assay (Table 1). Mating experiments were done a minimum of three times.

Plasmid isolation. Plasmid isolation was performed as described previously (23).

Southern blotting and hybridization. Total DNA was electrophoresed through a 1% agarose gel, transferred to a Zeta-Probe nylon membrane (Bio-Rad Laboratories, Richmond, Calif.) by capillary transfer, and hybridized with an [α-^{32}P]dCTP-labeled DNA probe specific for the *tet*(O) gene (Table 1). DNA fragments generated by PFGE analysis were transferred to nylon membranes and hybridized with [α-^{32}P]dCTP-labeled probes specific for the *erm*(A), *mef*(A), and *tet*(O) genes. These probes were obtained by PCR with the oligonucleotide primers reported in Table 1.

TABLE 2. Associations of erythromycin resistance genes *erm*(A), *erm*(B), and *mef*(A) and tetracycline resistance genes *tet*(M) and *tet*(O) in erythromycin- and tetracycline-resistant *S. pyogenes* strains, also characterized for their susceptibilities to clindamycin, clarithromycin, azithromycin, josamycin, and minocycline

No. of strains tested	Macrolide resistance genotype	No. of strains with the tetracycline resistance gene:			MIC range (μg/ml)[a]								
		tet(M)	*tet*(O)	*tet*(M) + *tet*(O)	ERY	TET	CLI	CLI (ind.)[b]	CLR	AZM	JOS	JOS (ind.)[b]	MIN
15	*erm*(B)	15			>128	32–64	>128	>128	>128	>128	>128	>128	4–16
2	*erm*(B) + *mef*(A)	2			>128	32–64	>128	>128	>128	>128	>128	>128	8
17	*erm*(A)		15	2	>128	32–64	0.03–0.25	>128	>128	>128	≤0.03–0.12	>128	2–8
10	*erm*(A)		10		1–4	64–>128	≤0.03–0.06	>128	0.5–8	4–8	≤0.03–0.12	4–16	2–8
19	*mef*(A)		19		2–16	32–128	≤0.03–0.12	≤0.03–0.12	2–16	2–16	≤0.03–0.12	≤0.03–0.12	8–16

[a] ERY, erythromycin; TET, tetracycline; CLI, clindamycin; CLR, clarithromycin; AZM, azithromycin; JOS, josamycin; MIN, minocycline.
[b] ind., after induction by pregrowth in 0.05 μg of erythromycin per ml.

DNA sequence analysis. Amplicon sequencing was performed bidirectionally by using the ABC Prism sequencer (Perkin-Elmer Italia, Monza, Italy) with dye-labeled terminators. Sequences were analyzed by using the Sequence Navigator software package (Perkin-Elmer).

RESULTS

Phenotypic and genotypic characterization of the strains. The phenotypic and genotypic characteristics of the 63 *S. pyogenes* are listed in Table 2. The isolates carrying the *erm*(A) gene were inducibly resistant and could be divided into two groups on the basis of erythromycin MICs of >128 or 1 to 4 μg/ml. Twenty-five of the 27 *erm*(A)-positive isolates carried the *tet*(O) gene, and the remaining 2 isolates carried both the *tet*(O) and the *tet*(M) genes. Erythromycin MICs were 2 to 16 μg/ml for the 19 isolates carrying the *mef*(A) gene as the only erythromycin resistance determinant; they remained susceptible to clindamycin and josamycin also after induction, and all carried the *tet*(O) gene. The erythromycin MICs for the isolates carrying the *erm*(B) gene or both the *erm*(B) gene and the *mef*(A) gene were high (>128 μg/ml), and all carried the *tet*(M) gene. Of the 17 *erm*(B)-positive isolates, all were chloramphenicol susceptible; 12 isolates were kanamycin resistant and carried the *int-Tn* gene, indicating the presence of a conjugative transposon related to the Tn*916*-Tn*1545* family. None of the 63 isolates carried either the *tet*(K) or the *tet*(L) gene, and plasmids were not observed.

Conjugal transfer of erythromycin and tetracycline resistance genes. Eight isolates with different combinations of macrolide and tetracycline resistance genes were used as donors in mating experiments. The results are summarized in Table 3. Seven of the eight isolates were able to transfer macrolide, tetracycline, or macrolide and tetracycline resistance to *S. pyogenes* and/or *E. faecalis* recipients at detectable frequencies under the conditions used. The frequencies of transfer from the donors with the *erm*(B) *tet*(M) and *erm*(A) *tet*(O) genotypes were lower when tetracycline was used for selection than when erythromycin was used for selection, regardless of which recipient was used; one exception was when donor iB27 and recipient JH2-2 were mated. The two isolates with the *mef*(A) *tet*(O) genotype did not transfer their genes to the *E. faecalis* recipient at measurable frequencies; however, they could transfer these genes to the *S. pyogenes* recipient at virtually the same frequency irrespective of whether tetracycline or erythromycin was used for selection, and all transconjugants carried both the *mef*(A) and the *tet*(O) genes. In contrast, strain iC41 transferred genes only to the *E. faecalis* recipient. With donors iB21 and iB27, segregation between the *erm*(A) and *tet*(O) genes was observed with both *S. pyogenes* and *E. faecalis* recipients. However, of 10 transconjugants obtained with donor iB21 and recipient 12RF, 9 had an *erm*(A) genotype and 1 had an *erm*(A) *tet*(O) genotype. Selected *E. faecalis* and *S. pyogenes* transconjugants carrying only the *tet*(O) gene were used as donors to examine whether they could transfer the *tet*(O) gene without the presence of the *erm*(A) gene. Both types of transconjugants could retransfer the *tet*(O) gene to the same species of recipient at the same frequencies shown in Table 3 (data not shown). Plasmids were not seen in the *S. pyogenes* transconjugants, and *tet*(O)-specific probe TO1 (Table 1) hybridized to the chromosomal DNA. No additional plasmids were seen in the *E. faecalis* OG1-10(pPD1) recipient, which carries a cryptic plasmid.

PFGE analysis and hybridization experiments. Donor iB21, recipient 12RF, and a 12RF transconjugant that was selected on erythromycin and that had an *erm*(A) *tet*(O) genotype were chosen for PFGE analysis. Compared with the PFGE profile of the recipient, the transconjugant exhibited a two-band difference resulting from the disappearance of a ca. 230-kb fragment and the appearance of a new one of ca. 260 kb (Fig. 1A). An *erm*(A)-specific probe (Fig. 1B) and a *tet*(O)-specific probe (Fig. 1C) both hybridized with an identical fragment from the donor and with an identical fragment (the new one) from the transconjugant. A similar experiment was done by mating donor m46 carrying the *mef*(A) and the *tet*(O) genes and recipient 12RF by using two 12RF transconjugants obtained by selecting one for erythromycin resistance and one for tetracycline resistance, but with both showing a *mef*(A) *tet*(O) genotype. The two transconjugants had identical PFGE patterns that differed from the PFGE pattern of the recipient by two bands resulting from the disappearance of a ca. 230-kb fragment (the same fragment described above) and the appearance of a new one of ca. 290 kb (Fig. 2A). A *mef*(A)-specific probe (Fig. 2B) and a *tet*(O)-specific probe (Fig. 2C) both hybridized with an identical fragment of the donor and with an identical fragment (the new one) of the transconjugants.

DNA amplification and sequencing. The PCR assay did not yield products with the primers specific for the *tet*(O) and the

TABLE 3. Conjugal transfer of erythromycin resistance genes *erm*(A) and *mef*(A) and/or tetracycline resistance gene *tet*(O) from erythromycin- and tetracycline-resistant *S. pyogenes* donors to susceptible *S. pyogenes* or *E. faecalis* recipients

Donor		Recipient	Selection for resistance to:[a]	Transfer frequency	Transconjugants		
Strain	Genotype				Genotype	MIC (μg/ml)[a]	
						ERY	TET
c7	*erm*(B) *tet*(M)	12RF	ERY	1.3×10^{-7}	*erm*(B) *tet*(M)	>128	32
c7	*erm*(B) *tet*(M)	12RF	TET	ND[b]			
c7	*erm*(B) *tet*(M)	JH2-2	ERY	7.1×10^{-8}	*erm*(B) *tet*(M)	>128	32
c7	*erm*(B) *tet*(M)	JH2-2	TET	ND			
c13	*erm*(B) *tet*(M)	12RF	ERY	7.4×10^{-9}	*erm*(B) *tet*(M)	>128	32
c13	*erm*(B) *tet*(M)	12RF	TET	1.8×10^{-10}	*erm*(B) *tet*(M)	>128	32
c13	*erm*(B) *tet*(M)	JH2-2	ERY	1.9×10^{-7}	*erm*(B)	>128	0.25
c13	*erm*(B) *tet*(M)	JH2-2	TET	ND			
iB21[c]	*erm*(A) *tet*(O)	12RF	ERY	9×10^{-9}	*erm*(A)	>128	≤0.125
				1×10^{-9}	*erm*(A) *tet*(O)	>128	64
iB21	*erm*(A) *tet*(O)	12RF	TET	ND			
iB21	*erm*(A) *tet*(O)	JH2-2	ERY	3.8×10^{-7}	*erm*(A)	>128	0.25
iB21	*erm*(A) *tet*(O)	JH2-2	TET	5×10^{-9}	*tet*(O)	≤0.125	64
iB27	*erm*(A) *tet*(O)	12RF	ERY	2.5×10^{-6}	*erm*(A)	>128	≤0.125
iB27	*erm*(A) *tet*(O)	12RF	TET	7×10^{-7}	*tet*(O)	≤0.125	64
iB27	*erm*(A) *tet*(O)	JH2-2	ERY	5.2×10^{-7}	*erm*(A)	>128	0.25
iB27	*erm*(A) *tet*(O)	JH2-2	TET	5×10^{-7}	*tet*(O)	≤0.125	64
iC38	*erm*(A) *tet*(O)	12RF	ERY	ND			
iC38	*erm*(A) *tet*(O)	12RF	TET	ND			
iC38	*erm*(A) *tet*(O)	JH2-2	ERY	ND			
iC38	*erm*(A) *tet*(O)	JH2-2	TET	ND			
iC41	*erm*(A) *tet*(O)	12RF	ERY	ND			
iC41	*erm*(A) *tet*(O)	12RF	TET	ND			
iC41	*erm*(A) *tet*(O)	JH2-2	ERY	3.6×10^{-7}	*erm*(A)	>128	0.25
iC41	*erm*(A) *tet*(O)	JH2-2	TET	ND			
m46	*mef*(A) *tet*(O)	12RF	ERY	6×10^{-4}	*mef*(A) *tet*(O)	16	64
m46	*mef*(A) *tet*(O)	12RF	TET	5.8×10^{-4}	*mef*(A) *tet*(O)	16	64
m46	*mef*(A) *tet*(O)	JH2-2	ERY	ND			
m46	*mef*(A) *tet*(O)	JH2-2	TET	ND			
m49	*mef*(A) *tet*(O)	12RF	ERY	1.3×10^{-7}	*mef*(A) *tet*(O)	16	128
m49	*mef*(A) *tet*(O)	12RF	TET	2.1×10^{-7}	*mef*(A) *tet*(O)	16	128
m49	*mef*(A) *tet*(O)	JH2-2	ERY	ND			
m49	*mef*(A) *tet*(O)	JH2-2	TET	ND			

[a] ERY, erythromycin; TET, tetracycline. For the selection of transconjugants, erythromycin and tetracycline were used at concentrations of 1 and 10 μg/ml, respectively.
[b] ND, no detectable transfer under conditions used.
[c] Of 10 transconjugants obtained, 9 had the genotype *erm*(A) and one had the genotype *erm*(A) *tet*(O).

erm(A) genes. In contrast, PCR products were obtained by using the primers specific for the *tet*(O) gene and the *mef*(A) or the *orf3* gene (Fig. 3). DNA sequencing confirmed the presence of the *tet*(O) gene at the left ends of both amplicons and of the *orf3* and the *mef*(A) genes, respectively, at their right ends. On the basis of the results of this work, the *tet*(O) gene is ca. 5.5 kb upstream from *mef*(A).

DISCUSSION

The increase in the prevalence of erythromycin-resistant *S. pyogenes* strains carrying the *erm*(A), *erm*(B), and/or *mef*(A) gene has been the subject of a number of recent reports (8, 11, 15, 27). In contrast, less work has been done to characterize the mechanism of tetracycline resistance in this species in which only *tet*(M) has been the commonly identified gene. Nevertheless, other species of streptococci which carry the *tet*(O) gene, which codes for another tetracycline resistance ribosomal protection protein, or the *tet*(K) and the *tet*(L) genes, which code for efflux-mediated tetracycline resistance (1), have been identified. Therefore, the potential exists for *S. pyogenes* to acquire other tetracycline resistance genes. In this study we found that 46 (73%) of 63 tetracycline- and erythromycin-resistant *S. pyogenes* isolates carried the *tet*(O) gene and the *erm*(A) or the *mef*(A) gene. In contrast, all the remaining isolates carried the *tet*(M) and the *erm*(B) genes, with 71% having conjugative transposons related to the Tn*916*-Tn*1545* family on the basis of detection of the *int-Tn* gene.

A linkage between the *erm*(B) and the *tet*(M) genes has been well established in a variety of gram-positive cocci (1, 3), whereas a linkage involving *tet*(O) has not previously been reported. The differential transfer of the *tet*(O) and the *erm*(A) genes in the mating experiments, the ability to transfer genes to both *S. pyogenes* and *E. faecalis*, and the appearance of a single insertion in the transconjugant receiving both *tet*(O) and *erm*(A) suggest that these two genes are associated with conjugative elements. Moreover, Southern blotting analysis indicated that the single extra band hybridized with both a *tet*(O)-specific probe and an *erm*(A)-specific probe. The fact that the acquisition of *erm*(A) and *tet*(O) conferred to the transconjugant a PFGE pattern denoting the insertion of new DNA into an existing restriction fragment is consistent with a chromo-

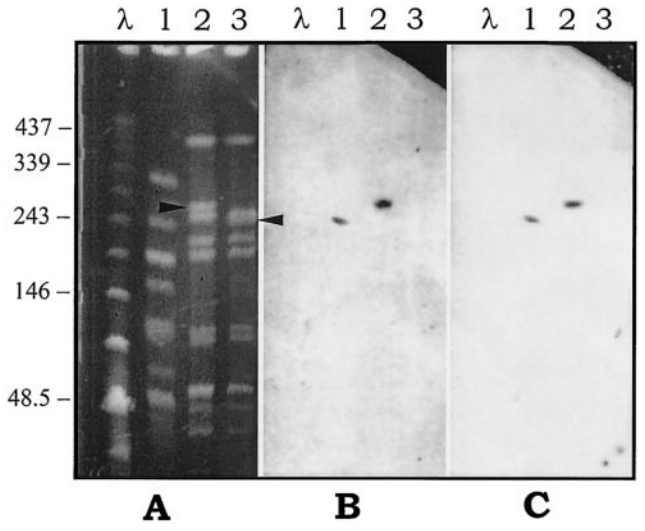

FIG. 1. PFGE patterns of *Sma*I-digested genomic DNA of the *S. pyogenes* strains involved in the intraspecific mating iB21 × 12RF (A) and hybridization with the *erm*(A) specific probe (B) or the *tet*(O)-specific probe (C). Lanes 1, donor strain iB21; lanes 2, transconjugant selected for erythromycin resistance with an *erm*(A) *tet*(O) genotype; lanes 3, recipient strain 12RF. Bacteriophage lambda DNA concatemers (Bio-Rad) were used as molecular size markers (lanes λ). The arrowheads indicate a ca. 230-kb fragment of the recipient (lane 3) that disappeared and a ca. 260-kb new fragment that appeared in the transconjugant (lane 2). The numbers on the left are molecular sizes (in kilobases).

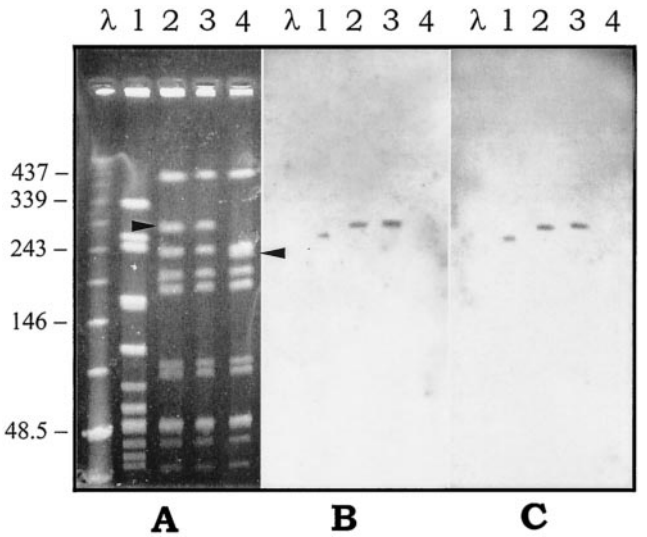

FIG. 2. PFGE patterns of *Sma*I-digested genomic DNA of the *S. pyogenes* strains involved in the intraspecific mating m46 × 12RF (A) and hybridization with the *mef*(A)-specific probe (B) or the *tet*(O)-specific probe (C). Lanes 1, donor strain m46; lanes 2, transconjugant selected for erythromycin resistance with a *mef*(A) *tet*(O) genotype; lanes 3, transconjugant selected for tetracycline resistance with a *mef*(A) *tet*(O) genotype; lanes 4, recipient strain 12RF. Bacteriophage lambda DNA concatemers (Bio-Rad) were used as molecular size markers (lanes λ). The arrowheads indicate a ca. 230-kb fragment of the recipient (lane 4) that disappeared and a ca. 290-kb new fragment that appeared in the transconjugants (lanes 2 and 3). The numbers on the left are molecular sizes (in kilobases).

somal location of the two genes. The *erm*(A) gene has previously been described on transposon Tn*554* (24) and has more recently been shown to transfer from *S. pyogenes* into a variety of recipients (6), while the *tet*(O) gene has been found on plasmids or in the chromosome (1, 13, 31, 33). These strains are also interesting because of a recent report showing an association between the *erm*(A) and the *erm*(B) genes and the *prtF1* gene, which encodes a protein required for streptococcal invasion of eukaryotic cells (6).

The association between the *mef*(A) gene and the *tet*(O) gene is suggested by the mating experiments and by PFGE and hybridization studies. Again, the PFGE patterns of the transconjugants with a *mef*(A) *tet*(O) genotype showed a single insertion of new DNA, consistent with an association and a chromosomal location of the two genes. Likewise, Southern blotting analysis indicated that the single extra band hybridized with both a *tet*(O)-specific probe and a *mef*(A)-specific probe. The *mef*(A) gene has been shown to be associated with both conjugative and nonconjugative elements as well as composite elements (15, 18, 25).

The fact that amplification and sequencing experiments ruled out a close proximity of *tet*(O) and *erm*(A) was not surprising, considering that the two genes were successfully cotransferred in a single mating experiment, with selection only for erythromycin resistance, and to only 1 of 10 transconjugants. In contrast, the finding of a linkage between *tet*(O) and *mef*(A), which is the first documented linkage between *tet*(O) and another gene, is consistent with the fact that the two genes were always cotransferred to the *S. pyogenes* recipient at the same frequency whether tetracycline or erythromycin was used for selection. The *mef*(A) gene has been shown to be carried by Tn*1207.1*, a chromosomal, defective, nonconjugative transposon of ca. 7.2 kb (25). In clinical isolates of *S. pyogenes*, Tn*1207.1* has been reported to be part of a larger conjugative

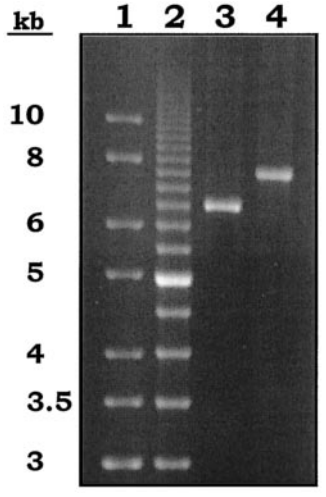

FIG. 3. Gel electrophoresis of long PCR products obtained from strain m46 [genotype *mef*(A) *tet*(O)] by using TETO1 as the *tet*(O)-specific primer (left end) and ORF3rev (lane 3; product size, ca. 6.5 kb) or OM18 (lane 4; product size, ca. 7.5 kb) to target *orf3* or *mef*(A) at the right end. GeneRuler (M-Medical Genenco, Cornaredo, Italy) (lane 1) and a 500-bp molecular size ruler (Bio-Rad) (lane 2) were used as molecular size markers.

transposon, Tn*1207.3* (M. Santagati, F. Iannelli, C. Messina, M. R. Oggioni, S. Stefani, and G. Pozzi, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1-2014, 2001). The reported size of Tn*1207.3* (ca. 52 kb) appears to be consistent with the disappearance of a ca. 230-kb fragment of the recipient and the appearance of a new fragment of ca. 290 kb in the transconjugants (Fig. 2).

The 63 Italian *S. pyogenes* isolates characterized in this study illustrate the ongoing evolution that is occurring as these bacteria cope with the ever-changing landscape in the clinical environment. This is the first study showing that the *tet*(O) gene can be mobile also when it is found in the chromosome. One can predict that if *tet*(O) is now associated with a conjugative element, then this gene is likely to spread to other streptococci and other gram-positive and gram-negative species, similar to what has been found with the host range of *tet*(M) (1).

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REFERENCES

1. **Chopra, I., and M. Roberts.** 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol. Mol. Biol. Rev. **65:**232–260.
2. **Clancy, J., J. Petitpas, F. Dib-Hajj, W. Yuan, M. Cronan, A. V. Kamath, J. Bergeron, and J. A. Retsema.** 1996. Molecular cloning and functional analysis of a novel macrolide resistance determinant, mefA, from *Streptococcus pyogenes.* Mol. Microbiol. **22:**867–879.
3. **Clewell, D. B., S. E. Flannagan, and D. D. Jaworski.** 1995. Unconstrained bacterial promiscuity: the Tn*916*-Tn*1545* family of conjugative transposons. Trends Microbiol. **3:**229–236.
4. **Del Grosso, M., F. Iannelli, C. Messina, M. Santagati, N. Petrosillo, S. Stefani, G. Pozzi, and A. Pantosti.** 2002. Macrolide efflux genes *mef*(A) and *mef*(E) are carried by different genetic elements in *Streptococcus pneumoniae.* J. Clin. Microbiol. **40:**774–778.
5. **Doherty, N., K. Trzcinski, P. Pickerill, P. Zawadzki, and C. G. Dowson.** 2000. Genetic diversity of the *tet*(M) gene in tetracycline-resistant clonal lineages of *Streptoccus pneumoniae.* Antimicrob. Agents Chemother. **44:**2979–2984.
6. **Facinelli, B., C. Spinaci, G. Magi, E. Giovanetti, and P. E. Varaldo.** 2001. Association between erythromycin resistance and ability to enter human respiratory cells in group A streptococci. Lancet **358:**30–33.
7. **Giovanetti, E., G. Magi, A. Brenciani, C. Spinaci, R. Lupidi, B. Facinelli, and P. E. Varaldo.** 2002. Conjugative transfer of the *erm*(A) gene from erythromycin-resistant *Streptococcus pyogenes* to macrolide-susceptible *S. pyogenes*, *Enterococcus faecalis*, and *Listeria innocua.* J. Antimicrob. Chemother. **50:** 249–252.
8. **Giovanetti, E., M. P. Montanari, M. Mingoia, and P. E. Varaldo.** 1999. Phenotypes and genotypes of erythromycin-resistant *Streptococcus pyogenes* strains in Italy and heterogeneity of inducibly resistant strains. Antimicrob. Agents Chemother. **43:**1935–1940.
9. **Gruteke, P., A. van Belkum, L. M. Schouls, W. D. H. Hendriks, F. A. G. Reubsaet, J. Dokter, H. Boxma, and H. A. Verbrugh.** 1996. Outbreak of group A streptococci in a burn center: use of pheno- and genotypic procedures for strain tracking. J. Clin. Microbiol. **34:**114–118.
10. **Hynes, W. L., J. J. Ferretti, M. S. Gilmore, and R. A. Segarra.** 1992. PCR amplification of streptococcal DNA using crude cell lysates. FEMS Microbiol. Lett. **94:**139–142.
11. **Kataja, J., P. Huovinen, the Macrolide Resistance Study Group, and H. Seppälä.** 2000. Erythromycin resistance genes in group A streptococci of different geographical origins. J. Antimicrob. Chemother. **46:**789–792.
12. **Kataja, J., H. Seppälä, M. Skurnik, H. Sarkkinen, and P. Huovinen.** 1998. Different erythromycin resistance mechanisms in group C and group G streptococci. Antimicrob. Agents Chemother. **42:**1493–1494.
13. **LeBlanc, D. J., L. N. Lee, B. M. Titmas, C. J. Smith, and F. C. Tenover.** 1988. Nucleotide sequence analysis of tetracycline resistance gene *tetO* from *Streptococcus mutans* DL5. J. Bacteriol. **170:**3618–3626.
14. **Le Bouguenec, C., G. de Cespedes, and T. Horaud.** 1990. Presence of chromosomal elements resembling the composite structure Tn*3701* in streptococci. J. Bacteriol. **172:**727–734.
15. **Leclercq, R.** 2002. Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. Clin. Infect. Dis. **34:**482–492.
16. **Luna, V. A., M. Heiken, K. Judge, C. Ulep, N. Van Kirk, H. Luis, M. Bernardo, J. Leitao, and M. C. Roberts.** 2002. Distribution of *mef*(A) in gram-positive bacteria from healthy Portuguese children. Antimicrob. Agents Chemother. **46:**2513–2517.
17. **Luna, V. A., and M. C. Roberts.** 1998. The presence of the *tetO* gene in a variety of tetracycline-resistant *Streptococcus pneumoniae* serotypes from Washington State. J. Antimicrob. Chemother. **42:**613–619.
18. **Luna, V. A., P. Coates, E. A. Eady, J. Cove, T. T. H. Nguyen, and M. C. Roberts.** 1999. A variety of gram-positive bacteria carry mobile *mef* genes. J. Antimicrob. Chemother. **44:**19–25.
19. **National Committee for Clinical Laboratory Standards.** 2000. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 5th ed. Approved standard M7-A5. National Committee for Clinical Laboratory Standards, Wayne, Pa.
20. **National Committee for Clinical Laboratory Standards.** 2000. Performance standards for antimicrobial disk susceptibility tests, 7th ed. Approved standard M2-A7. National Committee for Clinical Laboratory Standards, Wayne, Pa.
21. **Olsvik, B., I. Olsen, and F. C. Tenover.** 1995. Detection of *tet*(M) and *tet*(O) using the polymerase chain reaction in bacteria isolated from patients with periodontal disease. Oral Microbiol. Immunol. **10:**87–92.
22. **Ripa, S., C. Zampaloni, L. A. Vitali, E. Giovanetti, M. P. Montanari, M. Prenna, and P. E. Varaldo.** 2001. *Sma*I macrorestriction analysis of Italian isolates of erythromycin-resistant *Streptococcus pyogenes* and correlations with macrolide-resistance phenotypes. Microb. Drug Resist. **7:**65–71.
23. **Roberts, M. C., and G. E. Kenny.** 1987. Conjugal transfer of transposon Tn*916* from *Streptococcus faecalis* to *Mycoplasma hominis.* J. Bacteriol. **169:** 3836–3839.
24. **Roberts, M. C., J. Sutcliffe, P. Courvalin, L. B. Jensen, J. Rood, and H. Seppälä.** 1999. Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. Antimicrob. Agents Chemother. **43:**2823–2830.
25. **Santagati, M., F. Iannelli, M. R. Oggioni, S. Stefani, and G. Pozzi.** 2000. Characterization of a genetic element carrying the macrolide efflux gene *mef*(A) in *Streptococcus pneumoniae.* Antimicrob. Agents Chemother. **44:** 2585–2587.
26. **Seppälä, H., Q. He, M. Österblad, and P. Huovinen.** 1994. Typing of group A streptococci by random amplified polymorphic DNA analysis. J. Clin. Microbiol. **32:**1945–1948.
27. **Seppälä, H., M. Skurnik, H. Soini, M. C. Roberts, and P. Huovinen.** 1998. A novel erythromycin resistance methylase gene (*ermTR*) in *Streptococcus pyogenes.* Antimicrob. Agents Chemother. **42:**257–262.
28. **Sutcliffe, J., T. Grebe, A. Tait-Kamradt, and L. Wondrack.** 1996. Detection of erythromycin-resistant determinants by PCR. Antimicrob. Agents Chemother. **40:**2562–2566.
29. **Trzcinski, K., B. S. Cooper, W. Hryniewicz, and C. G. Dowson.** 2000. Expression of resistance to tetracyclines in strains of methicillin-resistant *Staphylococcus. aureus.* J. Antimicrob. Chemother. **45:**763–770.
30. **Varaldo, P. E., E. A. Debbia, G. Nicoletti, D. Pavesio, S. Ripa, G. C. Schito, G. Tempera, and the Artemis-Italy Study Group.** 1999. Nationwide survey in Italy of treatment of *Streptococcus pyogenes* pharyngitis in children: influence of macrolide resistance on clinical and microbiological outcomes. Clin. Infect. Dis. **29:**869–873.
31. **Widdowson, C. A., K. P. Klugman, and D. Hanslo.** 1996. Identification of the tetracycline resistance gene, *tet*(O), in *Streptococcus pneumoniae.* Antimicrob. Agents Chemother. **40:**2891–2893.
32. **Willetts, N.** 1988. Conjugation. Methods Microbiol. **21:**49–77.
33. **Zilhao, R., B. Papadopoulou, and P. Courvalin.** 1988. Occurrence of the *Campylobacter* resistance gene *tetO* in *Enterococcus* and *Streptococcus* spp. Antimicrob. Agents Chemother. **32:**1793–1796.