ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2005, p. 4999–5006 Vol. 49, No. 12
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.12.4999–5006.2005

Molecular Characterization of Pneumococci with Efflux-Mediated Erythromycin Resistance and Identification of a Novel *mef* Gene Subclass, *mef*(I)

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Received 16 May 2005/Returned for modification 4 August 2005/Accepted 18 September 2005

The molecular genetics of macrolide resistance were analyzed in 49 clinical pneumococci (including an "atypical" bile-insoluble strain currently assigned to the new species *Streptococcus pseudopneumoniae*) with efflux-mediated erythromycin resistance (M phenotype). All test strains had the *mef* gene, identified as *mef*(A) in 30 isolates and *mef*(E) in 19 isolates (including the *S. pseudopneumoniae* strain) on the basis of PCR-restriction fragment length polymorphism analysis. Twenty-eight of the 30 *mef*(A) isolates shared a pulsed-field gel electrophoresis (PFGE) type corresponding to the England^{14}-9 clone. Of those isolates, 27 (20 belonging to serotype 14) yielded multilocus sequence type ST9, and one isolate yielded a new sequence type. The remaining two *mef*(A) isolates had different PFGE types and yielded an ST9 type and a new sequence type. Far greater heterogeneity was displayed by the 19 *mef*(E) isolates, which fell into 11 PFGE types, 12 serotypes (though not serotype 14), and 12 sequence types (including two new ones and an undetermined type for the *S. pseudopneumoniae* strain). In all *mef*(A) pneumococci, the *mef* element was a regular Tn*1207.1* transposon, whereas of the *mef*(E) isolates, 17 carried the mega element and 2 exhibited a previously unreported organization, with no PCR evidence of the other open reading frames of mega. The *mef* gene of these two isolates, which did not match with the *mef*(E) gene of the mega element (93.6% homology) and which exhibited comparable homology (91.4%) to the *mef*(A) gene of the Tn*1207.1* transposon, was identified as a novel *mef* gene variant and was designated *mef*(I). While penicillin-nonsusceptible isolates (three resistant isolates and one intermediate isolate) were all *mef*(E) strains, tetracycline resistance was also detected in three *mef*(A) isolates, due to the *tet*(M) gene carried by a Tn*916*-like transposon. A similar mechanism accounted for resistance in four of the five tetracycline-resistant isolates carrying *mef*(E), in three of which mega was inserted in the Tn*916*-like transposon, giving rise to the composite element Tn*2009*. In the fifth *mef*(E)-positive tetracycline-resistant isolate (the *S. pseudopneumoniae* strain), tetracycline resistance was due to the presence of the *tet*(O) gene, apparently unlinked to *mef*(E).

Constitutively or inducibly expressed *erm*(B)-mediated ribosomal methylation is still the prevalent, albeit not the sole, mechanism of macrolide resistance in pneumococci. Apart from other target site modification-dependent mechanisms, such as the one mediated by the *erm*(A) subclass *erm*(TR) (35), another methylase gene very common in *Streptococcus pyogenes* (15) but only occasionally reported in *Streptococcus pneumoniae* (19), and the one due to mutations in 23S rRNA or ribosomal proteins (19), the current focus of attention is on a worldwide emerging active efflux-dependent mechanism. This is due to a drug pump belonging to the major facilitator superfamily class with efflux driven by the proton-motive force (19) that is associated with a usually low-level resistance pattern affecting, among macrolide, lincosamide, and streptogramin B antibiotics, only 14- and 15-membered macrolides (M phenotype) (40).

M-type resistance is mediated by the *mef* gene. The *mef* variant discovered in *S. pneumoniae* was initially called *mef*(E) (41), whereas the one discovered in *S. pyogenes* was called *mef*(A) (6). Because the *mef* gene was mostly detected using a PCR method that was unable to distinguish between the two variants (39) and since *mef*(A) and *mef*(E) have 90% identity (41), they have been recommended to be regarded as a single gene class, designated *mef*(A) (35). However, it has subsequently been shown that the two variants are carried by two different nonconjugative elements in *S. pneumoniae*: the *mef*(A) gene by a ca. 7.2-kb transposon (Tn*1207.1*) containing eight open reading frames (ORFs), of which *mef*(A) is the fourth (38), and the *mef*(E) gene by a ca. 5.5-kb element (macrolide efflux genetic assembly [mega] element) containing five ORFs, of which *mef*(E) is the first, having related sequences with the last five ORFs of Tn*1207.1* (13). Whereas various nucleotide differences have been found among the sequences deposited as *mef*(A), the five deposited *mef*(E) gene sequences are 100% identical to the one described initially (GenBank accession no. U83667) (17, 41). Downstream of the *mef* gene, both Tn*1207.1* and mega carry an ORF, originally named *orf5* (38) or *mel* (13) and recently collectively given the designation *msr*(D) (M. C. Roberts, personal communication), showing homology to *msr*(A), an ATP-binding cassette gene associated with macrolide efflux in *Staphylococcus aureus* (36). While the Tn*1207.1* transposon has a unique insertion site on the pneumococcal

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chromosome corresponding to the *celB* gene (38), the mega element is integrated at other sites of the bacterial chromosome (10, 13). In some clinical pneumococci resistant to both tetracycline and erythromycin, mega has also been found to be inserted into a Tn*916*-like transposon, typically containing *tet*(M) (7), giving rise to a new composite transposon (Tn*2009*) (11).

The rationale of the present study was to explore the molecular genetics of erythromycin resistance in a collection of extensively typed clinical pneumococci with the M phenotype of macrolide resistance recently isolated in Italy. The responsible genes and the gene-carrying elements were extensively analyzed, and molecular investigations of associated tetracycline resistance were performed. While some previously reported differences between *mef*(A)- and *mef*(E)-containing pneumococcal populations were confirmed, others were not. A novel *mef* gene subclass, identified as *mef*(E) by PCR-restriction fragment length polymorphism (RFLP) analysis, was detected and designated *mef*(I).

MATERIALS AND METHODS

Bacterial strains. Forty-nine pneumococcal isolates with efflux-mediated erythromycin resistance (MIC range, 2 to 16 mg/liter) were examined. All were clinical isolates recovered in different areas of Italy between 2002 and 2004 from a variety of clinical specimens (upper respiratory tract material, sputum, bronchial aspirate, blood, and cerebrospinal fluid). Multiple isolates from the same patient were avoided. Conventional tests, such as susceptibility to optochin and solubility in bile, and the API 20 Strep system (BioMérieux, Marcy-l'Etoile, France) were performed in our laboratory to confirm strain identification. These tests revealed that one bronchial aspirate isolate was not a typical member of *S. pneumoniae* but belonged to a recently recognized subset of bile-insoluble "atypical" pneumococci (29, 44) recently described as a new species called *Streptococcus pseudopneumoniae* (2). This strain was positive for the *ply* gene encoding pneumolysin, and its API profile (0060400) was identical to the one reported previously for other *S. pseudopneumoniae* strains (2). This isolate was investigated together with the other 48 typical pneumococci. All 49 test strains were confirmed to belong to the M phenotype of macrolide resistance on the basis of their patterns of susceptibility to macrolide, lincosamide, and streptogramin B antibiotics and the triple-disk (erythromycin plus clindamycin and rokitamycin) test (27).

Antibiotics and susceptibility tests. Erythromycin, clindamycin, tetracycline, and penicillin were purchased from Sigma-Aldrich (Milan, Italy). The other antibiotics were obtained from the following sources: azithromycin was from Pfizer Italiana (Rome, Italy), rokitamycin was from Formenti Grünenthal (Milan, Italy), and telithromycin and levofloxacin were from Aventis Pharma (Lainate, Italy). Broth microdilution MICs were determined as recommended by the National Committee for Clinical Laboratory Standards (28). *S. pneumoniae* ATCC 49619 was used for quality control.

Typing methods. Serotyping was performed by the capsular swelling test using specific antisera (Statens Seruminstitut, Copenhagen, Denmark). Serotypes were indicated with conventional capsular type designations.

Macrorestriction with SmaI endonuclease (New England Biolabs, Beverly, Mass.) and pulsed-field gel electrophoresis (PFGE) analysis were performed, and PFGE types (designated with a capital letter) and subtypes (if any, designated with the same capital letter followed by an Arabic numeral) were separated as described elsewhere previously (34).

Multilocus sequence typing (MLST) was carried out using the procedure and the seven housekeeping loci described previously by Enright and Spratt (12). The alleles of the seven housekeeping loci and the resulting sequence types were obtained from the MLST database (http://www.mlst.net). Isolates were clustered using the program for tree building provided by the MLST database.

PCR amplification experiments. All primer pairs used in PCR experiments to detect individual ORFs, disclose gene linkages, or assess the insertion of genetic elements into the chromosome or other elements or for sequencing purposes are listed in Table 1. DNA preparation and amplification and electrophoresis of PCR products were carried out by established procedures following the reported conditions for the use of individual primer pairs. The Ex *Taq* system (TaKaRa Bio, Shiga, Japan) was used in the amplification experiments expected to yield PCR products exceeding 3 kb in size. Differentiation between *mef*(A) and *mef*(E) was achieved by PCR-RFLP analysis of an undiscriminating 348-bp amplicon (39) using BamHI endonuclease (New England Biolabs), which has no restriction site in *mef*(E) and one in *mef*(A) generating two fragments of 284 and 64 bp (26). To determine whether the *mef* and *tet* genes were linked, the four primer combinations associated with the possible reciprocal orientation of the two genes were used in cross-PCR assays with the chromosomal DNAs of the strains resistant to both erythromycin and tetracycline. If amplicons spanning *mef* and *tet* genes were obtained, the PCR products were purified using Montage PCR filter units (Millipore Corporation, Bedford, Mass.) and sequenced.

DNA sequence analysis. Amplicon sequencing was performed bidirectionally using ABI Prism (Perkin-Elmer Applied Biosystems, Foster City, Calif.) with dye-labeled terminators. Sequences were analyzed using the Sequence Navigator software package (Perkin-Elmer Applied Biosystems). ORF analysis was performed with the DNA Star software package (Lasergene, Madison, Wis.), and sequence similarity was analyzed using the tools (BLAST and CDART) available online from the National Center for Biotechnology Information of the National Library of Medicine (Bethesda, Md.) (http://www.ncbi.nlm.nih.gov).

Transformation and conjugation experiments. Transformation experiments were carried out as described previously by Pozzi et al. (32) using *S. pneumoniae* Rx1, a well-known unencapsulated laboratory strain, as the recipient. Transformants were selected by plating the transformation mixture onto selective plates containing erythromycin (1 μg/ml) and/or tetracycline (5 μg/ml). The transforming DNA was a pneumococcal crude lysate purified using Montage PCR filter units. The transformation frequency was expressed as the number of CFU of the transformants divided by the number of the recipients. After preliminary selection for resistance to rifampin (25 mg/liter) and fusidic acid (25 mg/liter), *S. pneumoniae* R6, a well-known laboratory strain susceptible to erythromycin and tetracycline, was used as the recipient in conjugation experiments. The mating procedure was performed as described previously by Luna and Roberts (22), with the exception that a 1:10 donor-recipient ratio was used. Transconjugants were selected by using multilayer selection plates that contained rifampin (10 μg/ml) and fusidic acid (10 μg/ml) plus erythromycin (1 μg/ml) and/or tetracycline (5 μg/ml). The frequency of transfer was expressed as the number of transconjugants per donor.

RESULTS AND DISCUSSION

***mef* gene.** All 49 test strains had the *mef* gene. Based on PCR-RFLP analysis, the *mef* gene was identified as *mef*(A) in 30 isolates and *mef*(E) in 19 isolates (including the *S. pseudopneumoniae* strain).

Antibiotic susceptibilities. Both *mef*(A) and *mef*(E) isolates were resistant to azithromycin, with an MIC range (2 to 32 μg/ml) similar to that of erythromycin (2 to 16 μg/ml). Remarkably, our results were not consistent with the reported finding of significantly higher erythromycin MICs for *mef*(A) than for *mef*(E) strains (1). All isolates were susceptible to rokitamycin, telithromycin, and clindamycin. Eight isolates, three carrying *mef*(A) and five, including the *S. pseudopneumoniae* strain, carrying *mef*(E), were resistant to tetracycline. All 30 *mef*(A) isolates were susceptible to penicillin, while 4 nonsusceptible isolates (3 resistant and 1 intermediate) were detected among *mef*(E) strains. No test strain was resistant or intermediate to levofloxacin.

While our finding of penicillin-nonsusceptible strains only among *mef*(E) pneumococci is consistent with findings of previous studies (3, 10), the detection of tetracycline-resistant strains among both *mef*(A) and *mef*(E) pneumococci is in contrast to previous reports of tetracycline resistance detected solely among the latter (1, 3, 10). A tetracycline-resistant *mef*(A) pneumococcus has only been described in a previous study by our group (25).

Typing. PFGE types, MLST sequence types, and serotypes are summarized in Table 2. The MLST-based dendrogram of

TABLE 1. Oligonucleotide primer pairs used

Gene	Primer		Reference	Product size (bp)
	Designation	Sequence (5′–3′)		
orf1[a]	ORF1	TGATGAAGAGGAAAATTAG	21	266
	ORF1R	TACATCAACATTACCATCTG	1	
orf1-orf2[a]	ORF1	TGATGAAGAGGAAAATTAG	21	1,066
	ORF2R	GATTGATGTTCCTGATGC	21	
orf2-orf3[a]	ORF2	GCATCAGGAACATCAATC	21	1,119
	ORF3R	GACCTACCTGAACAATACC	21	
orf3-mef(A)[a]	ORF3	GGTATTGTTCAGGTAGGTC	21	1,050
	OM18	TGCTTGCCCTGCCCATATT	10	
mef(A)[a]	MEFA1	AGTATCATTAATCACTAGTGC	39	348
	MEFA2	TTCTTCTGGTACTAAAAGTGG	39	
mef(A)[a]	MEF3	GCGTTTAAGATAAGCTGGCA	10	1,743
	MEF4	CCTGCACCATTTGCTCCTAC	10	
mef(A)-*msr*(D)[a]	MF4AR	TTCTTTGCTGATAAAATCGGTGT	21	440
	ORF5F-R	GGCAAGTTCACCCAGATG	21	
msr(D)[a]	MSRD1	TGCCTATATTCCCCAGTT	1	708
	MSRD2	TTAATTTCCGCACCGACTA	1	
msr(D)-*orf6*[a]	MSRD2F	TAGTCGGTGCGGAAATTAA	1	893
	ORF6R	CTACCGCTACTCCAACATG	21	
orf6-orf7[a]	ORF6	CATGTTGGAGTAGCGGTAG	21	202
	ORF7R	CCAAGTCTGACCAAAGATTTC	21	
orf7-orf8[a]	ORF7	GAAATCTTTGGTCAGACTTGG	21	297
	ORF8R	CGCTGGTGGATTGGAGGG	21	
celB	CELB1	GAGAAAGTATTTTCTACGGGATGT	37	430
	CELB2	CCATACAGAACTAGGGTATCATCGTG	37	
orf9-mef	MEFA2	TTCTTCTGGTACTAAAAGTGG	39	1,795
	SG3	GAATCTTTAGCCAGCGGTATC	11	
int	INT1	GCGTGATTGTATCTCACT	31	1,046
	INT2	GACGCTCCTGTTGCTTCT	31	
xis	XIS1	AAGCAGACTGACATTCCTA	1	194
	XIS2	GCGTCCAATGTATCTATAA	1	
tet(K)	TETK1	TATTTTGGCTTTGTATTCTTTCAT	42	1,159
	TETK2	GCTATACCTGTTCCCTCTGATAA	42	
tet(L)	TETL1	ATAAATTGTTTCGGGTCGGTAAT	42	1,077
	TETL2	AACCAGCCAACTAATGACAATGAT	42	
tet(M)	TETM3	GAACTCGAACAAGAGGAAAGC	30	740
	TETM4	ATGGAAGCCCAGAAAGGAT	30	
tet(O)	TETO1	AACTTAGGCATTCTGGCTCAC	30	2,002
	TETO3	TGCTCCCACAAACAGGACACAATATC	4	

[a] Designed from the reported sequence of Tn*1207.1* (38) (GenBank accession no. AF227520). However, the primers specific for the last five ORFs of Tn*1207.1* are also applicable to the corresponding ORFs of the mega-element (13).

the genetic relationships among the 48 test strains of *S. pneumoniae* is illustrated in Fig. 1.

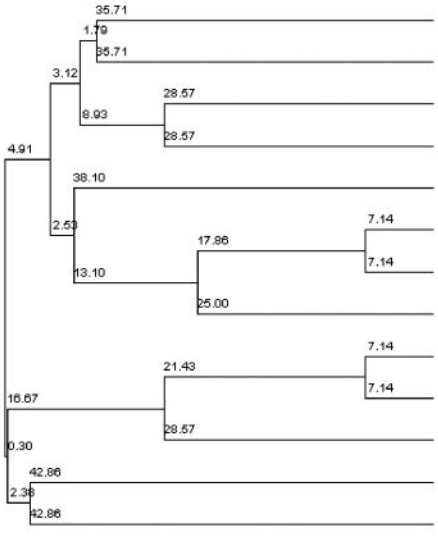
Of the 30 isolates carrying *mef*(A), 28 (93%), including 6 isolates assigned to closely related subtypes A1 ($n = 5$) and A2 ($n = 1$), shared the same PFGE fingerprint pattern (type A) of SmaI restriction digests, exactly corresponding to the England[14]-9 clone of the Pneumococcal Molecular Epidemiology Network (23) reported in the United Kingdom (16), Italy (10), and Spain (3). Of these 28 isolates, 20 belonged to serotype 14 and shared the same sequence type (ST9); the other eight type A isolates grouped into five different serotypes, but seven still yielded sequence type ST9, whereas the eighth iso-

TABLE 2. Typing and *mef* element of 49 clinical pneumococci with efflux-mediated erythromycin resistance subdivided according to the *mef* gene carried

Isolates (total no.) bearing *mef* gene	PFGE type (no. of isolates)	MLST sequence type (no. of isolates)	Serotype (no. of isolates)	*mef* element	
				Tn*1207.1*	mega
mef(A) (30)	A (22)	ST9 (21)	14 (17), 23F (2), 19A (1), 31 (1)	+	−
		ST1733[a] (1)	23F (1)	+	−
	A1 (5)	ST9 (5)	14 (3), 23F (1), 33A (1)	+	−
	A2 (1)	ST9 (1)	3 (1)	+	−
	G (1)	ST1775[a] (1)	3 (1)	+	−
	I (1)	ST9 (1)	23F (1)	+	−
mef(E) (19)	A (2)	ST9 (1)	19F (1)	−	+
		ST448 (1)	6B (1)	−	+
	A1 (1)	ST199 (1)	23A (1)	−	+
	B (2)	ST81 (2)	23F (1), 35 (1)	−	+
	B1 (1)	ST81 (1)	23A (1)	−	+
	C (2)[b]	ST1774[a] (2)	11A (2)	−	−
	D (2)	ST490 (2)	18F (1), 19A (1)	−	+
	E (1)	ST180 (1)	23F (1)	−	+
	E1 (1)	ST180 (1)	9V (1)	−	+
	F (2)	ST156 (2)	23F (2)	−	+
	H (1)	ST218 (1)	12F (1)	−	+
	J (1)	ST1776[a] (1)	3 (1)	−	+
	K (1)[c]	ND[b] (1)	19A (1)	−	+
	L (1)	ST1250 (1)	10F (1)	−	+
	M (1)	ST448 (1)	6B (1)	−	+

[a] New (not previously identified) sequence type.
[b] In the course of this study, these two isolates were found to carry a novel *mef* gene variant, named *mef*(I).
[c] *S. pseudopneumoniae* strain. ND, sequence type not determined; being an atypical pneumococcus, this strain could not be entered into the pneumococcal MLST database.

late (serotype 23F) displayed a new (i.e., not previously identified) sequence type (ST1733). The remaining two *mef*(A) isolates (PFGE types G and I) belonged to serotypes 3 and 23F and yielded a new sequence type (ST1775) and the ST9 type, respectively. Thus, overall, 27 (90%) of the 30 *mef*(A) isolates shared the same sequence type (ST9) detected in Scottish (1) and Italian (24) surveys, and most (n = 20 [74%]) of these 27 isolates belonged to serotype 14. It is worth noting that, while variable rates of *mef*(A) pneumococci belonging to serotypes other than serotype 14 have been reported in some studies (1, 9, 24), this was the sole serotype found in other investigations (3, 10, 46).



Sequence type	No. of isolates	PFGE type
ST156	2	F
ST218	1	H
ST1774	2	C
ST180	2	E (1), E1 (1)
ST199	1	A1
ST490	2	D
ST1776	1	J
ST1250	1	L
ST9	29	A (22), A1 (5), A2 (1), I (1)
ST1775	1	G
ST1733	1	A
ST81	3	B (2), B1 (1)
ST448	2	A (1), M (1)

FIG. 1. MLST-based dendrogram of the genetic relationships among the 48 test strains of *S. pneumoniae*. Genetic distances between sequence types are indicated. PFGE types corresponding to individual sequence types are shown in the third column (relevant numbers of isolates are reported in parentheses in case of more than one PFGE type per sequence type).

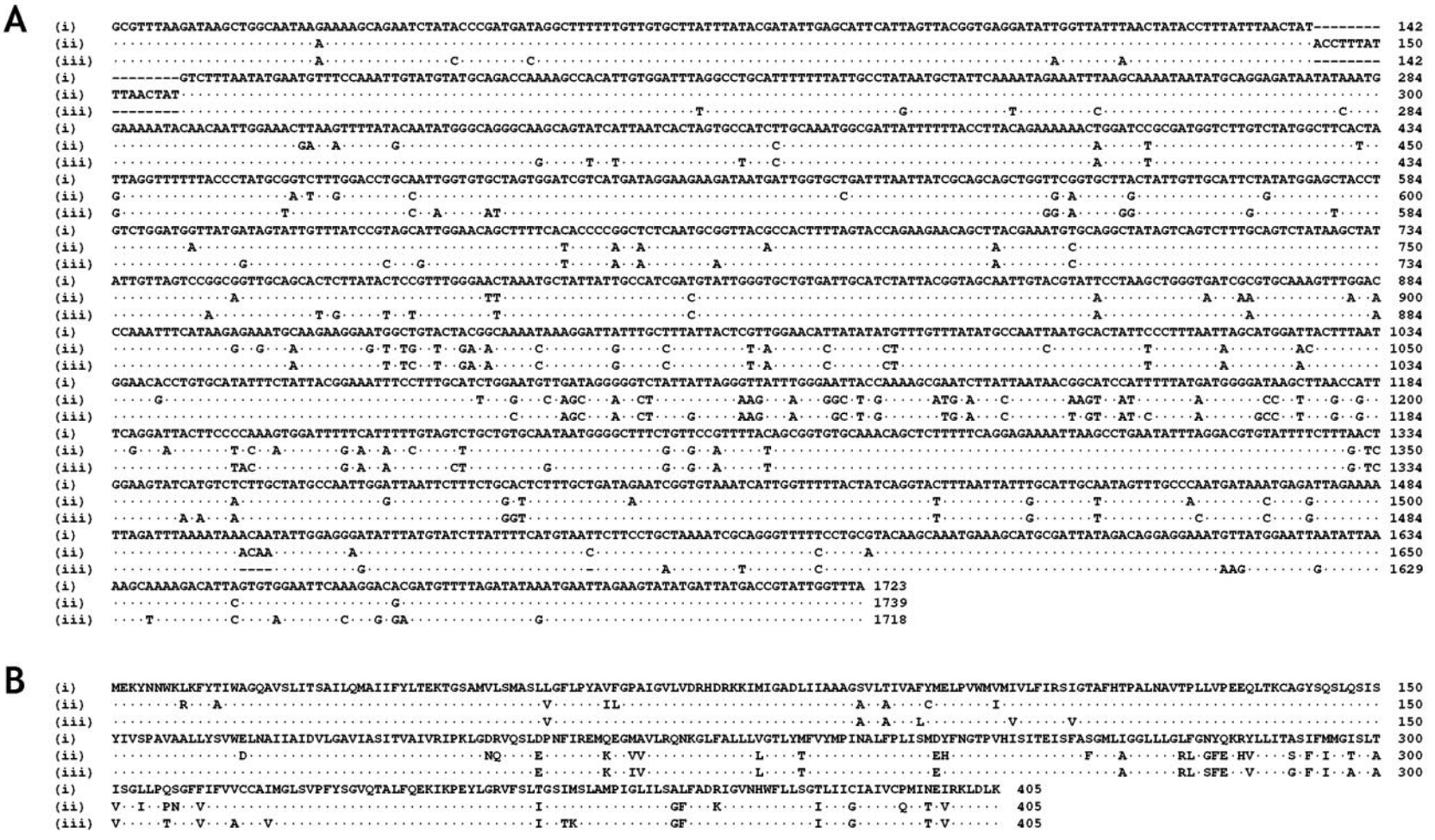


FIG. 2. A, alignment of three *mef* gene-containing nucleotide sequences: (i) amplicon obtained by pairing primers MEF3 and MEF4 (10) from the Tn*1207.1* transposon (GenBank accession no. AF227520); (ii) corresponding sequence, containing the *mef*(E) gene, from the mega element (accession no. AF274302); (iii) corresponding sequence containing the *mef*(I) gene (accession no. AJ971089). B, alignment of the amino acid sequences corresponding to the coding regions of the three above-described nucleotide sequences. Alignments were obtained using the ClustalW program available online from the European Bioinformatics Institute (Cambridge, United Kingdom). Dots represent residues in nucleotide or amino acid sequences of ii and iii identical to those of i.

Far greater heterogeneity was displayed by the 19 isolates carrying *mef*(E), which fell into 11 PFGE types (three of which, A, B, and E, were associated with one subtype each), 12 serotypes, and 12 sequence types, including two new ones and an undetermined type for the *S. pseudopneumoniae* strain (see below). Interestingly, while the isolates fell into the PFGE type A predominant among *mef*(A) isolates, the 12 serotypes did not include serotype 14, which, in contrast, has been found in *mef*(E) isolates from Spain (3), Canada (46), and other regions of the world (9) as well as in one Italian isolate also carrying *erm*(B) (24). The 12 sequence types included ST9, detected in a single PFGE type A, serotype 19F isolate, and two new sequence types (ST1774 and ST1776) corresponding to PFGE types C (two isolates, both belonging to serotype 11A) and J (one isolate belonging to serotype 3), respectively. The same serotype (6B) and sequence type (ST448) were shared by two isolates belonging to different PFGE types (A and M). The *S. pseudopneumoniae* strain was assigned to PFGE type K and serotype 19A; a sequence type could not be obtained because, being an atypical pneumococcus, it could not be entered into the pneumococcal MLST database. However, compared with the pairwise percentage differences in the allelic profiles of the seven housekeeping genes from typical pneumococci, those from this strain fitted within a range (2% to 7%) that was closer to that calculated for typical alleles included in the database than was the range reported for atypical pneumococci in other studies (29, 44).

***mef* elements.** The homogeneity of pneumococci carrying *mef*(A) was also confirmed with regard to the *mef* element which, in PCR experiments, was invariably found to be a regular Tn*1207.1* transposon (38) inserted into the *celB* gene of the pneumococcal chromosome (Table 2).

In 17 (including the *S. pseudopneumoniae* strain) of the 19 *mef*(E) isolates, the *mef*(E) gene was carried by the mega element (13), whereas no PCR evidence of *msr*(D) or of the last three ORFs of mega was found in the remaining two isolates (Table 2). The last two isolates, which shared an identical PFGE type (C), serotype (11A), and new sequence type (ST1774) but which had been isolated from different materials in different areas and years, were also analyzed using the *mef* primer pair described previously by Del Grosso et al. (10), which yields a PCR product of 1,743 bp. The amplicons from the two isolates had identical sequences and demonstrated homologies of 93.6% and 91.4% with the corresponding sequences from the mega element and the Tn*1207.1* transposon, respectively (Fig. 2).

***mef*(I), a novel *mef* gene subclass.** The *mef* gene identified as *mef*(E) on the basis of PCR-RFLP analysis in the two isolates apparently lacking the mega element and showing comparable homologies with the *mef*(E) gene of mega and the *mef*(A) gene of Tn*1207.1* appears to be a novel *mef* gene subclass that we propose to call *mef*(I). It is worth noting that divergences in the *mef*(E) gene sequence have never been reported (17). The amino acid sequence corresponding to *mef*(I) showed similarities of 96.5% to the protein encoded by *mef*(E) and of 94.3% to the protein encoded by *mef*(A), which are both greater than the similarity between *mef*(A) and *mef*(E) (93.8%).

Molecular characterization of tetracycline-resistant strains. Tetracycline resistance genes sought using PCR included *tet*(K), *tet*(L), *tet*(M), and *tet*(O). The genes *xis* (excisase) and *int* (integrase) were analyzed to reveal the presence of conjugative transposons of the Tn*916* family (7) (both genes were tested after pneumococci that were positive for only one of them were described [1]). The major findings of the molecular characterization of the eight tetracycline-resistant isolates are summarized in Table 3.

TABLE 3. Molecular characterization of eight tetracycline-resistant isolates detected among 49 clinical pneumococci with efflux-mediated erythromycin resistance subdivided according to the *mef* gene carried

Tetracycline-resistant isolates (total no.) bearing *mef* gene	Tetracycline resistance gene[a]		Genes of Tn*916*-like transposons		Apparent linkage[b] between the relevant *mef* gene and the relevant *tet* gene
	tet(M)	*tet*(O)	*xis*	*int*	
mef(A) (3)	+		+	+	No
	+		+	+	No
	+		+	+	No
mef(E) (5)[c]	+		+	+	Yes[d]
	+		+	+	Yes[d]
	+		+	+	Yes[d]
	+		+	+	No
		+	−	−	No

[a] *tet*(K) and *tet*(L) were not detected in any test strain.
[b] Of course, a linkage could be detected provided that the two genes were not so distant as to prevent PCR products from being obtained.
[c] The fifth, *tet*(O)-positive isolate was the *S. pseudopneumoniae* strain.
[d] The linkage between *mef*(E) and *tet*(M) was due to the integration of the mega element into a Tn*916*-like transposon to form the composite element Tn*2009* (11).

All three tetracycline-resistant isolates bearing *mef*(A) had the *tet*(M) gene and were positive for *xis* and *int*. No apparent linkage between *mef*(A) and *tet*(M) was detected in cross-PCR assays with primers specific for the two genes. Both *mef*(A)- and *tet*(M)-carrying elements could be separately transferred: Tn*1207.1* in transformation assays to the Rx1 recipient (frequency, 2×10^{-5}) and Tn*916* in conjugation assays to the R6 recipient (frequency, 8×10^{-8}).

In four of the five tetracycline-resistant isolates bearing *mef*(E), PCR assays showed the presence of genes *tet*(M), *xis*, and *int*. The *tet*(M) genes of these four isolates had identical sequences and were identical to the *tet*(M) gene carried by Tn*5251* (GenBank accession no. X90939), a transposon of the Tn*916* family previously described in *S. pneumoniae* (33). Pairing of a *mef*-specific primer (MEFA1) with a primer specific for *tet*(M) (TETM3) (Table 1) yielded an amplicon of approximately 5.5 kb in three isolates, indicating linkage and opposite and convergent orientations of the genes *mef*(E) and *tet*(M). In these three isolates, PCR assays performed by pairing primers SG3 and MEFA2 (Table 1) confirmed the integration of the mega element into a Tn*916*-like transposon, and sequencing of the PCR product identified this composite element as Tn*2009* (GenBank accession no. AF376746) (11). No amplicon was obtained with the fourth isolate using the four combinations of primer pairs associated with the possible reciprocal orientation of *mef*(E) and *tet*(M), showing that the two genes were unlinked and that the mega and Tn*916*-like elements were not integrated to form a composite element in this strain. When transformation and conjugation experiments were performed

using two of these four *tet*(M)-carrying isolates as donors, i.e., one of those carrying Tn*2009* and the one with unlinked *mef*(E) and *tet*(M), neither transformants nor transconjugants were obtained with the former donor, whereas transformants and transconjugants were both obtained with the latter donor (the mega element was transformed at a frequency of 1.8×10^{-6}, and the Tn*916*-like element was transferred at a frequency of 2.6×10^{-7}). The fifth tetracycline-resistant isolate that contained *mef*(E) (in a regular mega element) was the *S. pseudopneumoniae* strain. Its tetracycline resistance was due to the presence of the *tet*(O) gene. This could not be transferred by either transformation or conjugation and exhibited sequence homologies of 98.8% with the *tet*(O) gene from *S. pneumoniae* isolated in South Africa (GenBank accession no. Y07780) (45) and of 98.5% with the same gene from *S. pyogenes* recently described in Italy (GenBank accession no. AJ715499) (4, 14). No apparent linkage between *mef*(E) and *tet*(O) was detected in this strain in cross-PCR assays with primers specific for the two genes. The *tet*(O) gene has so far been reported in *S. pneumoniae* from only two geographic areas: South Africa (45) and Washington State (22). The failure to find it in Europe had been attributed to the requirement for acquisition of the gene and the absence of selective pressure (18). However, the *tet*(O) gene has recently been found to be the customary tetracycline resistance determinant in the erythromycin-resistant *S. pyogenes* strains carrying gene *mef*(A) (4, 14) or *erm*(A) (14).

Conclusions. After initial perplexity (10, 26) about the usefulness of uniting the *mef*(A) and *mef*(E) variants of the *mef* gene under a single class designated *mef*(A) (35), and after several authors continued using the names *mef*(A) and *mef*(E) in spite of this recommendation, a recent minireview concluded that the current knowledge of the properties of the *mef* genes is now in favor of maintaining the original distinction (17). The most straightforward method for molecular discrimination between *mef*(A) and *mef*(E) is based on the differential presence of restriction enzyme recognition sites in the two genes (17), and differentiation can be achieved by means of RFLP analysis of different PCR products (10, 26). However, this method was unable to detect the *mef*(I) gene subclass and differentiate it from *mef*(E). *mef*(I) did not exactly match with either *mef*(A) (91.4% homology) or *mef*(E) (93.6% homology) and occurred in the absence of both the Tn*1207.1* transposon and the mega element. Sequencing experiments are in progress to understand whether the novel *mef* gene is associated with a new (or defective) genetic element, to set up a new PCR-RFLP approach capable of differentiating among the three *mef* gene variants, and to assess the meaning of the lack of PCR evidence of the *msr*(D) gene. Indeed, it cannot be excluded that differences similar to those emerging in the *mef* gene class and resulting in the novel *mef* variant might also be present in the *msr* gene class and result in *msr* variants that are undetectable using the customary primers. Gay and Stephens (13) showed that *msr*(D) not only is consistently found adjacent to *mef*(E) but is also cotranscribed with it and suggested that the proteins encoded by the two genes are a dual efflux system in *S. pneumoniae*. More recently, the ATP-dependent efflux pump encoded by *msr*(D) has been reported to be capable of functioning independently of that encoded by *mef*(E) (9) and has even been reported to be more important in determining M-type macrolide resistance (F. Iannelli, M. Santagati, J. D. Doquier, M. Cassone, M. R. Oggioni, G. Rossolini, S. Stefani, and G. Pozzi, Abstr. 44th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1-1188, 2004). However, the erythromycin MICs for the two *mef*(I)-positive isolates (16 μg/ml) were in the high MIC range (2 to 16 μg/ml) for the *mef*-positive isolates examined in this study. Remarkably, evidence is emerging that the *mef* gene, far from being confined to streptococci, can be associated with a wide range of gram-positive and gram-negative bacteria (8, 20, 21, 43). Whether the *mef* gene is close to an *msr* gene has not generally been investigated in these organisms, but, at least in *Bacteroides* strains, an *msr* gene has been found adjacent to *mef* (43). In contrast, no *msr* gene has been detected in a minority of viridans group streptococci carrying the *mef* gene (5).

ACKNOWLEDGMENTS

We thank Eleonora Giovanetti for helpful discussions. We also thank Angela Brueggemann, curator of the *S. pneumoniae* database in the MLST website, for assessing new allelic variants in some of our isolates.

This work was supported in part by the Italian Ministry of Education, University, and Research.

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