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The *mef*(E)-Carrying Genetic Element (mega) of *Streptococcus pneumoniae*: Insertion Sites and Association with Other Genetic Elements

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The structure of the macrolide efflux genetic assembly (mega) element, its genomic locations, and its association with other resistance determinants and genetic elements were investigated in 16 *Streptococcus pneumoniae* isolates carrying *mef*(E), of which 1 isolate also carried *tet*(M) and 4 isolates also carried *tet*(M) and *erm*(B). All isolates carried a mega element of similar size and structure that included the operon *mef*(E)-*msr*(D) encoding the efflux transport system. Among tetracycline-susceptible isolates, six different integration sites were identified, five of which were recognized inside open reading frames present in the R6 genome. In the five isolates also carrying *tet*(M), mega was inserted in different genetic contexts. In one isolate, it was part of previously described Tn*916*-like element Tn*2009*. In another isolate, mega was inserted in a transposon similar to Tn*2009* that also included an *erm*(B) element. This new composite transposon was designated Tn*2010*. Neither Tn*2009* nor Tn*2010* could be transferred by conjugation to pneumococcal or enterococcal recipients. In the three isolates in which mega was not physically linked with *tet*(M), this gene was associated with *erm*(B) in transposon Tn*3872*, a Tn*916*-like element. Homologies between the chromosomal insertions of these composite transposons and sequences of multidrug-resistant pneumococcal genomes in the databases indicate the presence of preferential sites for the integration of composite Tn*916*-like elements carrying multiple resistance determinants in *S. pneumoniae*.

Macrolide resistance in *Streptococcus pneumoniae* is mediated by two main mechanisms: target modification due to a ribosomal methylase encoded by the *erm*(B) gene, which confers high-level resistance to macrolides, lincosamides, and streptogramin B (21), and an efflux transport system associated with the *mef* gene, which confers resistance to 14- and 15-membered macrolides only (6, 35). Molecular epidemiology studies have shown that the prevalence of these two mechanisms among macrolide-resistant pneumococci varies in different geographical areas. In the United States (18), Canada (15), and the United Kingdom (2), the efflux system is more common, while in Spain (34), Italy (24), and other European countries (29), the ribosomal modification is prevalent. In addition, isolates carrying both resistance determinants are becoming increasingly common in several areas of the globe (12, 19, 23) and have also been found, although rarely, in Italy (24, 25).

mef, originally considered the necessary and sufficient determinant of the efflux mechanism, appears to be a component of an efflux system that includes *mef* and the associated *msr*(A) homolog, recently renamed *mrs*(D) (8, 35). *mef* encodes two transmembrane domains, and *msr*(D) encodes two ATP-binding domains, likely constituting an efflux transport system of the ATP-binding cassette superfamily (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. 1188, 2004) (1). Two subclasses of the *mef* gene have been found in pneumococci, *mef*(A) and *mef*(E), which share 90% nucleotide identity. Recently, a new variant designated *mef*(I) has been described in two pneumococcal isolates from Italy (7).

In Italy, *mef*(A) is the predominant efflux-associated gene, while *mef*(E) is found in approximately 15% of *S. pneumoniae* strains carrying the efflux pump (unpublished data). In other geographical areas, such as North America and Asia, *mef*(E) is by far the most common gene associated with macrolide efflux in pneumococci (8).

In *S. pneumoniae*, *mef*(A) and *mef*(E) are carried by two similar but distinct elements, designated, respectively, Tn*1207.1* and the mega (macrolide efflux genetic assembly) element (13, 33). Tn*1207.1* is a defective transposon of 7.2 kb that corresponds to the left (L) end of the larger Tn*1207.3* transposon that carries *mef*(A) in *Streptococcus pyogenes* (32). In pneumococci, Tn*1207.1* is always found inserted at the same specific chromosomal site, corresponding to the competence gene *celB* (2, 9, 33). The population structure of isolates carrying Tn*1207.1* is clonal, and almost all of the isolates belong to serotype 14 and to the internationally disseminated clone England^{14}-9 (9, 38). The mega element, 5.5 kb in size, is similar to Tn*1207.1* but lacks the region upstream of *mef*. The mega element can be found inserted at different chromosomal sites (9, 13) in strains belonging to different serotypes and clonal groups (9, 31, 38). Recently, we reported that mega can be part of Tn*2009*, a composite element where mega is integrated in *orf6* of a Tn*916*-like transposon carrying *tet*(M) (10).

In this study, we have examined the mega element in several *S. pneumoniae* isolates to characterize its structure, termini, and insertion sites. We have found new mega insertion sites and a new composite element, designated Tn*2010*, that is similar to Tn*2009* but also contains the *erm*(B) gene.

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TABLE 1. Oligonucleotide primers used in this study to characterize mega and its chromosomal insertion sites

Primer use and name	Sequence (5′-3′)	Nucleotide positions	GenBank accession no. (reference)
mega mapping			
SG6	ACGCAGACAGAACGGAGATA	366–385	AF376746
OM18	TGCTTGCCCTGCCCATATT	1428–1410	AF376746 (9)
MEF3	GCGTTTAAGATAAGCTGGCA	1092–1111	AF376746 (9)
MS37	AAAGCCTCTAAACTTGGTATG	4048–4028	AF376746
OM15	TATCTGTTTTAAGCGGTGGA	3925–3944	AF376746
JL2	GGAATCGTTGAAACAGCAAC	5205–5186	AF376746 (10)
iPCR for mega junctions			
SG5	CACTCTGATACTTACCCACC	501–482	AF376746
MEF5	GCCAAATGATAACTGAGGTTAGAA	2549–2572	AF376746
OM1	ACCGACTATAGGGTATGGATT	3605–3585	AF376746
SG1	CTCACTGCACCAGAGGTGTA	5530–5549	AF376746 (10)
Chromosomal insertions of mega[a]			
spr0166, 3-methyladenine DNA glycosylase I			
U-SQ10	AAAGACCAAAGATAGGCATC	175127–175108	NC_003098
D-SQ11	TATCATGATGAGGAGTGGG	174817–174835	NC_003098
spr0092, CapD protein			
U-SQ12	TAAGATTTTCGTTGGTAAGG	100929–100948	NC_003098
D-SQ13	GATACCAAGTTTGATATGC	101612–101594	NC_003098
spr1450, phosphomethylpyrimidine kinase			
U-SQ5	TCCCTTGATAATGACTGCTG	1434234–1434253	NC_003098
D-SQ6	TCGTATTTTAGCACTTTCTGG	1434741–1434721	NC_003098
spr0144, hypothetical protein			
U-SQ14	AGGCAACTAGAAGAAATAGC	153525–153525	NC_003098
D-SQ15	TCCAGTTGCTTCTTCTAG	153247–153264	NC_003098
spr0246, hypothetical protein			
U-SQ19	ACAGGTGGTCATGTCGAATC	242762–242781	NC_003098
D-SQ20	CACCTGATCTGGATTGCC	243109–243092	NC_003098
spr0932, RNA methyltransferase			
D-SQ18	AGCATCCATGATGGCTTC	918866–918849	NC_003098

[a] Primers annealing upstream (U) or downstream (D) of the insertion sites of mega.

MATERIALS AND METHODS

Bacterial strains. Fifteen *S. pneumoniae* isolates carrying *mef*(E) were derived from a collection of about 1,000 strains obtained in the course of different surveillance programs carried out in Italy from 1997 to 2004 (24). These isolates represented all of the *mef*(E)-positive strains in the collection, with the exception of two strains previously described (10). One additional isolate, PGX1416, was originally obtained from Glaxo, Verona, Italy (24). PN150, carrying Tn*2009* (10), was the control strain in the PCR mapping assays. *S. pneumoniae* FP10 and *Enterococcus faecalis* JH2-2 were used as recipients (16, 17) and DP1322, carrying Tn*5251* (28), was used as a control in conjugation experiments.

Characterization of isolates. Antibiotic susceptibilities, serotyping, and detection of *mef*(E), *tet*(M), and *erm*(B) were performed as previously described (14, 24). Genetic relatedness among the isolates was studied by pulsed-field gel electrophoresis (PFGE) of SmaI-digested bacterial DNAs. PFGE types and subtypes were defined as previously described (14).

Mapping of mega and detection of mega insertion sites. Genomic DNA of the pneumococcal isolates was prepared with a commercial kit (MasterPure DNA purification kit; Epicenter, Madison, Wisconsin).

The genetic structure of mega was examined by PCR mapping with three sets of primer pairs to generate three overlapping fragments (Table 1 and Fig. 1). The intergenic region between *mef*(E) and *msr*(D) was examined with primers MEF5 (Table 1) and MEF4 (9). The sizes of the fragments obtained with the different isolates were compared with those obtained with PN150 (10).

The L junction (LJ) and right (R) junction (RJ) of mega were determined by using two inverse PCR (iPCR) assays. To define the LJ, EcoRI-restricted genomic DNA was ligated and used as the template in an iPCR assay with primer pair SG5-MEF5 (Table 1 and Fig. 1). To define the RJ, PstI-restricted genomic DNA was used in a similar iPCR with primer pair OM1-SG1 (Table 1 and Fig. 1). After sequencing of the amplicons obtained, two primers specific for each insertion site were chosen, annealing, respectively, upstream (U primer) or downstream (D primer) of the insertion of mega (Table 1 and Fig. 1). In each isolate, the junctions of mega were confirmed by direct PCR assays with two pairs

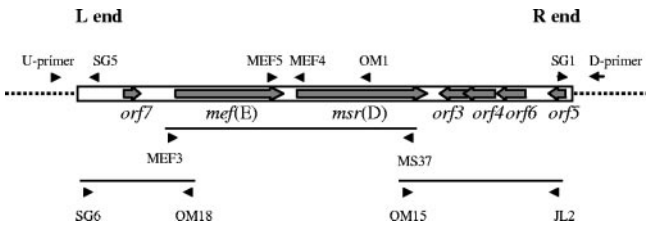

FIG. 1. Genetic structure of mega. The element is boxed, and the chromosome is represented by a dotted line. The positions of the primers used for PCR mapping, for iPCRs, and to define the junctions of mega are indicated by arrowheads. The expected amplicons for PCR mapping are indicated by bars. The primers used to amplify the LJ and RJ of mega for each insertion site are collectively indicated as primers annealing upstream (U primer) and downstream (D primer) of the insertion site.

TABLE 2. Properties of *S. pneumoniae* strains carrying mega or mega and other genetic elements

Strains	Serotype	PFGE type.subtype[a]	Presence of genetic determinant of resistance:			Genetic element(s)	Insertion site(s)[b]
			mef(E)	*tet*(M)	*erm*(B)		
713	6A	7	+	−	−	mega	*spr1450*, phosphomethylpyrimidine kinase
AP163	6B	4	+	−	−	mega	*spr0246*, hypothetical protein
APT186	7F	10	+	−	−	mega	*spr0166*, 3-methyladenine DNA glycosylase I
AP26	9V	5.1	+	−	−	mega	*spr0092*, CapD protein
SP234	9V	5.1	+	−	−	mega	*spr0092*, CapD protein
PT178	9V	5.2	+	−	−	mega	No homology (LJ); *spr0932*, RNA methyltransferase (RJ)
SP355	9V	5.3	+	−	−	mega	*spr0166*, 3-methyladenine DNA glycosylase I
APT136	11A	8	+	−	−	mega	*spr0092*, CapD protein
SP267	12F	6	+	−	−	mega	*spr0144*, hypothetical protein
PT9	14	1.1	+	+	+	mega; Tn*3872*	*spr0166*, 3-methyladenine DNA glycosylase I; *spr1764*, hypothetical protein
PT92	14	1.1	+	+	+	mega; Tn*3872*	*spr0166*, 3-methyladenine DNA glycosylase I; *spr1764*, hypothetical protein
PT124	14	1.2	+	+	+	mega; Tn*3872*	*spr0166*, 3-methyladenine DNA glycosylase I; *spr1764*, hypothetical protein
SP274	19A	9	+	−	−	mega	*spr0166*, 3-methyladenine DNA glycosylase I
PGX1416	19F	2	+	+	+	Tn*2010*	*spr1764*, hypothetical protein
AP104	23F	3	+	+	−	Tn*2009*	No homology (Tn*5252* [LJ], 670-6B genome [RJ])[c]
AP199	23F	11	+	−	−	mega	*spr0166*, 3-methyladenine DNA glycosylase I

[a] See reference 14.
[b] Homologies to ORFs of R6 genome.
[c] Homology with other pneumococcal genomes in GenBank (see text).

of primers: for the LJ, a U primer and OM18, annealing to mega; for the RJ, OM15, annealing to mega and a D primer (Table 1 and Fig. 1).

In isolates carrying *mef*(E) and *tet*(M), the location of mega in Tn*2009* was investigated by PCR with primer pairs SG3-OM18 and SG1-TetMd (10, 14). Sequencing was performed to confirm the insertion sites at the nucleotide level.

Structure of the composite elements and detection of chromosomal insertion sites. PCR mapping of Tn*916*-like elements was performed as previously described (10). The sizes of the fragments obtained were compared with those obtained by using the genomic DNA of PN150.

The DNA regions flanking the ends of the composite elements were explored by two iPCR assays. The LJ was determined as previously described (10). The RJ was determined by a similar iPCR with NdeI-restricted genomic DNA and primers TN1 (5′-ATAAAGTGTGATAAGTCCAG-3′) and TN2 (5′-GAAG TAGAAGCTAAAGATGG-3′), designed on the basis of the Tn*916* sequence (GenBank accession no. U09422). The results were confirmed by direct amplification of the junctional fragments and sequencing.

In isolates carrying *erm*(B), the location of this gene inside the composite transposons was investigated by PCR with different combinations of primers EB1 and EB2, annealing to *erm*(B) (14), with primers annealing to Tn*916* (27).

Conjugation experiments. To test the transferability of the composite elements, two different recipient strains were used in mating experiments: *S. pneumoniae* FP10 (16) and *E. faecalis* JH2-2 (17). Donor bacteria were grown in the presence of either tetracycline (5 μg/ml) or erythromycin (1 μg/ml), and mating experiments were carried out as previously described (9). Transconjugants were selected by using plates containing the antibiotics suitable to select the respective recipients (10, 17) and either erythromycin (1 μg/ml) or tetracycline (5 μg/ml). The conjugation assays were replicated three times.

Nucleotide sequence accession numbers. The sequences of the LJ and RJ of Tn*2010* in PGX1416 and the RJ of Tn*2009* in AP104 have been assigned GenBank accession no. DQ426906, DQ426907, and DQ426908, respectively.

RESULTS AND DISCUSSION

Typing of isolates. Of the 16 erythromycin-resistant, *mef*(E)-positive isolates, 1 was also resistant to tetracycline and carried *tet*(M) besides *mef*(E) and 4 were resistant to tetracycline and to high levels of erythromycin and were found to carry *mef*(E), *tet*(M), and *erm*(B) (Table 2).

The isolates belonged to 10 different serotypes (Table 2). Three serotypes included multiple isolates: 9V (four isolates), 14 (three isolates), and 23F (two isolates). On the basis of the PFGE macrorestriction profiles, the isolates could be grouped into 11 genetically distinct PFGE types. Nine types included single isolates, while two types, type 1 and type 5, included the three serotype 14 isolates and the four serotype 9V isolates, respectively (Table 2).

Structure and insertion sites of mega. Mapping of mega revealed that both the *mef*(E) and *msr*(D) genes were present in all of the isolates. The overall size, structure, and sequences of the L and R ends of mega were similar to those previously described (9, 13). In seven isolates, the size of the amplicon of the intergenic region between *mef*(E) and *msr*(D) appeared approximately 100 bp smaller than that obtained with control strain PN150. This suggests that the intergenic region was truncated, as already described in mega elements found in North American isolates (13, 37).

A variety of different mega insertion sites were found. In tetracycline-susceptible isolates, five different sites could be recognized inside sequences annotated as open reading frames (ORFs) in the genome of R6 (GenBank accession no. NC_003098), three of which corresponded to already described insertion sites (13) and two of which were novel (Table 2). In one isolate (PT178), only the RJ of mega could be identified since the L end was flanked by a sequence showing no significant homology with pneumococcal or other bacterial genomes (Table 2). Sequence analysis of the mega insertion sites in comparison with the R6 genome showed a 2-bp deletion in the target sites, consisting of CA in three sites and CT or CG in one site each.

On the basis of previous results (10), linkage between mega and *tet*(M) was explored in the five tetracycline-resistant isolates but could be demonstrated in two isolates only, AP104

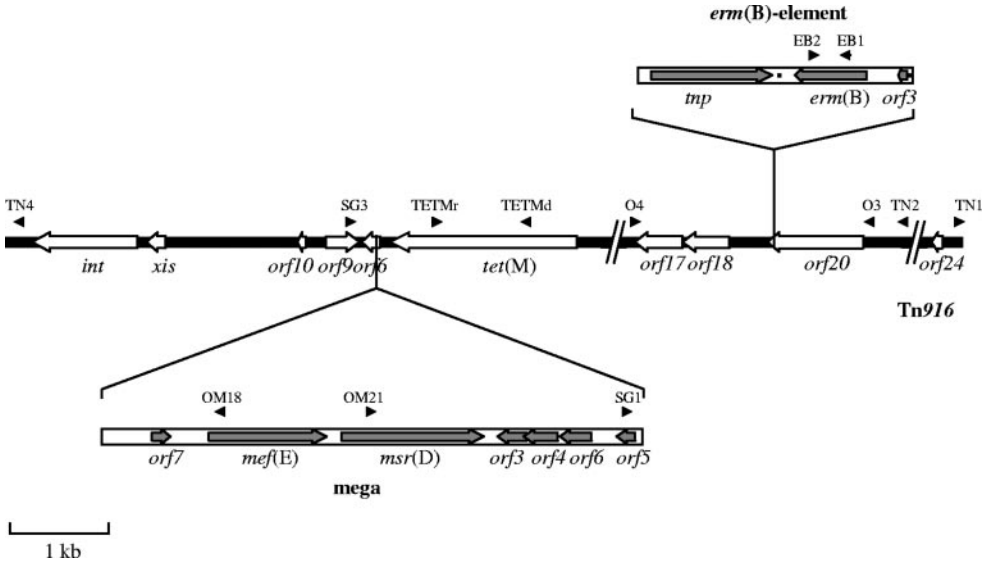

FIG. 2. Schematic representation of Tn*2010*, a novel Tn*916* family transposon harboring mega and an *erm*(B) element. The black bar represents Tn*916*, and the white arrows indicate the locations of the relevant ORFs. The structures of the two inserted elements are boxed, and gray arrows indicate the ORFs. Black arrowheads indicate the positions of the primers used to amplify and sequence relevant portions of Tn*2010* and its chromosomal junctions.

and PGX1416. Sequencing confirmed that in both isolates mega was inserted in *orf6* of Tn*916*, the same site occupied by mega in Tn*2009* (Fig. 2) (10).

In the other three tetracycline-resistant isolates, all belonging to serotype 14, no linkage between *mef*(E) and *tet*(M) was demonstrated, since mega was found inserted in *spr0166*, which has been predicted to encode 3-methyladenine DNA glycosylase I in R6 (Table 2). This mega insertion site was the most common one in the isolates examined, being found in 7 out of 16 isolates. In these same isolates, mega showed a common structural feature, i.e., the intergenic-region deletion already described. Interestingly, in *Streptococcus oralis* we found a mega element with the same intergenic deletion that was inserted in a sequence similar to *spr0166* (data not shown). The mega element lacks enzymes required for DNA transposition and is nonconjugative in pneumococci (9, 13). Evidence of common insertion sites in pneumococci and other streptococcal species supports the hypothesis that mega could spread by transformation among these species.

Composite elements. On the basis of previous investigations, the tetracycline-resistant isolates were examined by PCR mapping targeting Tn*916*-like elements (10).

In AP104, the amplicons obtained were of the same size as the corresponding fragments obtained with control strain PN150, confirming that AP104 harbors Tn*2009*.

In PGX1416, the amplicons were of the same size as the corresponding fragments obtained with control strain PN150, with the exception of the amplicon spanning the region from *orf17* to *orf20*, which was larger than expected. With primer pairs EB2-O3 and O4-EB1, amplicons were produced indicating that *erm*(B) was located inside the *orf17-orf20* fragment, oriented in the same direction as *tet*(M) and opposite to *mef*(E). Sequence analysis showed that *erm*(B) was contained in a fragment of 2,841 bp that included a transposase (*tnp*) (Fig. 2) and was integrated in *orf20* of Tn*916*, at position 3,847 of the sequence of Flannagan et al. (GenBank accession no. U09422). This *erm*(B) element and the adjacent Tn*916* regions showed identity to a sequence found in multidrug-resistant *S. pneumoniae* strains 670-6B (The Institute for Genomic Research, microbial genomes in progress at http://www.tigr.org) and G54 (GenBank accession no. AL449927) (11). In addition, the same *erm*(B) element was part of a transposon designated Tn*916*Erm (GenBank accession no. AY898750) found in *Streptococcus cristatus*, an oral streptococcal species.

The new composite element of approximately 26.3 kb that carries *mef*(E), *tet*(M), and *erm*(B) and is found in *S. pneumoniae* PGX1416 was designated Tn*2010* (Fig. 2).

Tn*2010* resembles previously described transposons with the addition of mega, such as Tn*1545* described in *S. pneumoniae* by Caillaud and coworkers, which also includes the kanamycin resistance gene *aphA-3* (3), or Tn*3703*, an *S. pyogenes* transposon derived from Tn*916* by insertion of a 3-kb sequence carrying *erm*(B) (20).

Neither AP104, carrying Tn*2009*, nor PGX1416, carrying Tn*2010*, was able to transfer macrolide or tetracycline resistance by conjugation (transfer frequency, $<10^{-9}$), confirming previous observations (10).

In the three isolates belonging to serotype 14 in which mega was not linked to *tet*(M), a linkage between *tet*(M) and *erm*(B) was found. PCR mapping and sequencing indicated the presence of another Tn*916*-like transposon, Tn*3872*, a composite element consisting of Tn*917*, carrying *erm*(B), inserted in *orf9* of Tn*916* (22).

Insertion sites of composite elements. Tn*2009* of AP104 appeared to be inserted in a chromosomal site different from that previously described in PN150 and PN34 (10). By iPCR and sequencing, the LJ of Tn*2009* in AP104 was found to be identical to the LJ of Tn*5251* with Tn*5252* (GenBank accession no. X90940). The R end was flanked by a sequence almost identical to a sequence of 670-6B. At the nucleotide level, the

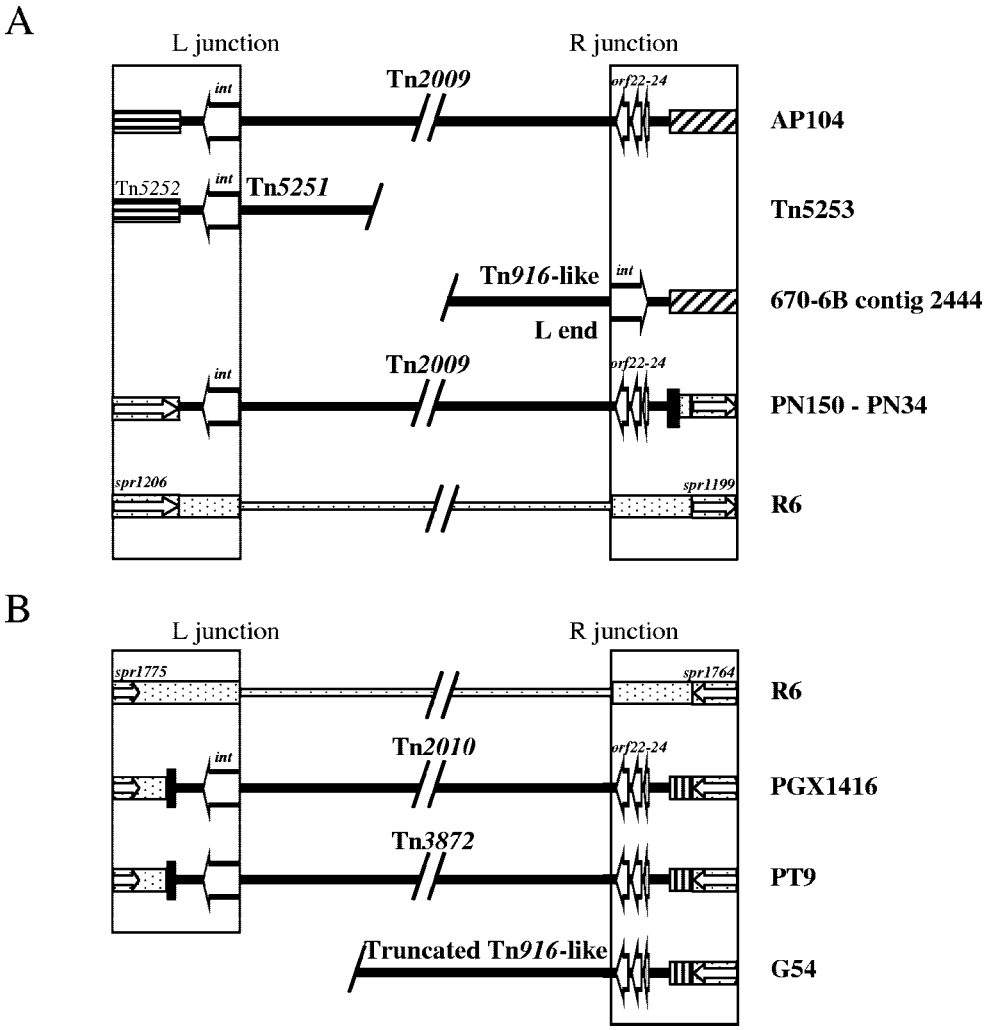

FIG. 3. Homology of the chromosomal LJ and RJ of composite transposons Tn*2009* (A), Tn*2010*, and Tn*3872* (B) with other pneumococcal sequences. The *S. pneumoniae* strains from which the sequences were derived are identified on the right. Transposons are represented by black lines. Boxes show magnified details of the junctions. Homologous regions are indicated by the same pattern. Black bars at the R end of Tn*2009* and at the L end of Tn*2010* and Tn*3872* represent exogenous sequences showing no homology with other bacterial sequences. The insertion sites of Tn*2009* in PN150 and PN34 have been described previously (10).

R end appeared to be integrated at nucleotide 114,074 of contig 2444 (The Institute for Genomic Research, microbial genomes in progress at http://www.tigr.org) at the same position where the L end of a Tn*916*-like transposon is integrated in 670-6B (Fig. 3). This complex context suggests that Tn*2009* of AP104 might be part of a larger element.

Analysis of the junctions of Tn*2010* in the chromosome of PGX1416 revealed the presence of six nucleotides at the L end, likely representing the coupling sequence (4). The L end of Tn*2010* appeared to be inserted in a region homologous to the R6 genome at base 1,743,232 (GenBank accession no. NC_003098), adjacent to *spr1775*. The R end appeared to be inserted at nucleotide 1,731,928 at the 3′ end of *spr1764*. On the basis of the R6 genome, insertion of Tn*2010* appeared to cause a deletion of approximately 11,300 bp. Both the L and R ends appeared to be flanked by brief sequence stretches (24 and 66 nucleotides, respectively) that did not show homology with the R6 genome, although the 66-bp sequence flanking the R end was identical to a sequence flanking the R end of a truncated Tn*916*-like transposon in G54 (Fig. 3) (11). The presence of exogenous sequences flanking the transposon's ends and of large chromosomal deletions occurring at the insertion site are features of Tn*2010* already observed in Tn*2009* (10).

Surprisingly, the insertion site of Tn*3872* in the serotype 14 isolates was the same, at the nucleotide level, as that of Tn*2010* in PGX1416 (Fig. 3). This and other similarities observed in the sequences flanking the Tn*916*-like transposons suggest the presence of preferential insertion sites for these elements in the pneumococcal chromosome.

Conclusions. Our findings confirm a tendency to the formation of modular aggregation of resistance determinants in mobile elements (36) that, in *S. pneumoniae*, appear to be centered on a core structure represented by Tn*916*. The mega element is one of the functional modules present in Tn*2009* and Tn*2010*, and this characteristic differentiates it from the

similar element Tn*1207.1*, which has not been found to associate with other resistance determinants in pneumococci. The Tn*916* family of transposons shows a very broad host range, including more than 40 bacterial genera (30). This characteristic could contribute to the diffusion of Tn*2009* and Tn*2010* in pneumococci and other bacterial species. Tn*2009* has been found in several pneumococcal isolates in Italy (5, 7) and recently has also been identified in a gram-negative species, *Acinetobacter junii* (26). Tn*2010*, which carries both *erm*(B) and *mef*(E), could be responsible for the spreading of the double macrolide resistance genes that have been recognized in particular pneumococcal clones in recent years (12, 19).

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