ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2007, p. 3983–3987 Vol. 51, No. 11
0066-4804/07/$08.00+0 doi:10.1128/AAC.00790-07

Composite Structure of *Streptococcus pneumoniae* Containing the Erythromycin Efflux Resistance Gene *mef*(I) and the Chloramphenicol Resistance Gene *catQ*▽

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Received 19 June 2007/Returned for modification 29 July 2007/Accepted 12 August 2007

In recent years *mef* genes, encoding efflux pumps responsible for M-type macrolide resistance, have been investigated extensively for streptococci. *mef*(I) is a recently described *mef* variant detected in particular isolates of *Streptococcus pneumoniae* instead of the more common *mef*(E) and *mef*(A). This study shows that *mef*(I) is located in a new composite genetic element, whose sequence was completely analyzed and the left and right junctions determined, demonstrating a unique genetic organization. The new composite structure (30,505 bp), designated the 5216IQ complex, consists of two halves: a left one (15,316 bp) formed by parts of the known transposons Tn*5252* and Tn*916*, and a right one (15,115 bp) formed by a new fragment, designated the IQ element. While the defective Tn*916* contained a silent *tet*(M) gene, the IQ element, ending with identical transposase genes on both sides and containing the *mef*(I) gene with an adjacent new *msr*(D) gene variant and a *catQ* chloramphenicol acetyltransferase gene, was completely different from the genetic elements carrying other *mef* genes in pneumococci. This is the first report demonstrating *catQ* in *S. pneumoniae* and showing its linkage with a *mef* gene. Analysis of the chromosomal region beyond the left junction revealed an organization more similar to that of *S. pneumoniae* strain TIGR4 than to that of strain R6. The 5216IQ complex was apparently nonmobile, with no detectable transfer of erythromycin resistance being obtained in repeated transformation and conjugation assays.

In streptococci, active efflux-mediated erythromycin resistance was established in 1996 and associated with the M phenotype, i.e., a low-level resistance pattern affecting only 14- and 15-membered macrolides among macrolide-lincosamide-streptogramin B antibiotics (28). Recently, a trend towards increasing rates of erythromycin resistance, noted in many countries among *Streptococcus pyogenes* (13) and *Streptococcus pneumoniae* (20) populations as well as with other streptococci (4, 18, 23, 31), has been correlated with a worldwide emergence of this efflux mechanism.

The efflux pump responsible for M-type resistance is encoded by the *mef* gene. The first *mef* variant to be discovered in *S. pneumoniae* was called *mef*(E) (29) and was subsequently shown to be carried by a ca. 5.5-kb nonconjugative element (macrolide efflux genetic assembly [mega] element) containing five open reading frames (ORFs), of which *mef*(E) is the first (16). Another *mef* variant, originally described for *S. pyogenes* and called *mef*(A) (8), has been shown to be carried (i) in *S. pneumoniae* by a ca. 7.2-kb nonconjugative transposon (Tn*1207.1*) containing eight ORFs, of which *mef*(A) is the fourth (25), and (ii) in *S. pyogenes* by a variety of larger mobile elements, where it is part of an element identical (6, 24) or related (17) to Tn*1207.1*. In all such instances, an *msr* gene encoding an ATP-dependent efflux pump—now designated *msr*(D), albeit different variants received different appellations—was found downstream of *mef*. It has been suggested that, *msr*(D) being cotranscribed with *mef*, the proteins encoded by the two genes might act as a dual efflux system (16) in which the expression of both genes would be inducible by erythromycin (2) and that the *msr*(D)-encoded pump would be capable of functioning independently of the one encoded by *mef* (2, 14).

More recently, new *mef* alleles have been found in *S. pyogenes* (23), in group G beta-hemolytic streptococci (4), and in *S. pneumoniae* (12). In particular the last, designated *mef*(I), exhibited comparable homologies with the *mef*(E) gene of the mega element and the *mef*(A) gene of the Tn*1207.1* transposon and was associated with no PCR evidence of the other ORFs of either mega or Tn*1207.1* (12). In the present study, aimed at investigating the genetic location of *mef*(I), we show that this new *mef* variant—adjacent to a new *msr* variant undetected with the primers used originally (12) and linked to a *catQ* chloramphenicol resistance gene—is contained in a new 15-kb genetic element (designated IQ) inserted into a defective Tn*916* transposon (10), which in turn is inserted into a defective Tn*5252* transposon (1, 5) to form a new composite structure (30.5 kb in size) designated the 5216IQ complex.

MATERIALS AND METHODS

Bacteria. As reported elsewhere (12), the two strains originally shown to share *mef*(I) also shared an identical susceptibility pattern (resistance to erythromycin and azithromycin; susceptibility to rokitamycin, telithromycin, clindamycin, tetracycline, penicillin, and levofloxacin), an identical serotype (11A) and pulsed-field gel electrophoresis type, and a new sequence type (ST1774). Although isolated in different areas of Italy, one (Spn529) from the upper respiratory tract

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▽ Published ahead of print on 20 August 2007.

TABLE 1. Oligonucleotide primer pairs used[k]

Gene or region	Primer designation	Primer sequence (5′–3′)	Reference or source	Product size(s) (bp)
orf24	TN6	GCTGAATGAATGTTTGATGG	15	858/5,208[a]
*spr*1199	GS13	GACTTGACAACCTGAAGTAG	15	
*spr*1206	GD1	CAGGAACCATTCTAGCTTGG	15	194/11,639[b]
IR[c]	TN4	AGGCTTTACGAGCATTTAAG	15	
orf9	SG3	GAATCTTTAGCCAGCGGTATC	15	1,778/10,104[d]
mef(I)	MEFA2[e]	TTCTTCTGGTACTAAAAGTGG	27	
mef(I)	MEFA2inv	CCACTTTTAGTACCAGAAGAA	27	3,663[f]
IR[g]	M2[h]	TCATAGTTTCGGTTCCTC	This study	
IR[i]	DR3	TAGAAATCGTCCATAACC	This study	ca. 2,500
nf10	INV4	GACAACGGAAACTCTATGCC	This study	
nf10	DR4	GCATAGAGTTTCCGTTGTCC	This study	ca. 8,000
IR[j]	INV5	AGGCTTTCCGTGATTTGAG	This study	

[a] The smaller product size was expected from the reported right junction in transposon Tn*2009* (15); the larger was consistent with the organization of the 5216IQ complex (EMBL accession no. AJ971089).
[b] The smaller product size was expected from the reported left junction in transposon Tn*2009*; the larger was consistent with the organization of the 5216IQ complex.
[c] From the intergenic region between *orfI* and the left end of Tn*916*.
[d] The smaller product size was expected from the reported organization of transposon Tn*2009*; the larger was consistent with the organization of the 5216IQ complex.
[e] Originally proposed for *mef*(A) and *mef*(E) (27) and likewise targeting *mef*(I) (12).
[f] Consistent with the genetic organization of the composite 5216IQ complex.
[g] From the intergenic region upstream of the 5′ end of the *mef*(I) gene.
[h] Designed from the region upstream of the *mef*(E) coding sequence in the mega element (16) (GenBank accession no. AF274302).
[i] From the intergenic region between *nf9* and *nf10*.
[j] From the intergenic region between *catQ* and the right *tnp1*.
[k] The last three primer pairs were used in inverse-PCR assays.

in 2003 and one (Spn832) from bronchial aspirate in 2004, the two isolates were indistinguishable. The present study was essentially conducted with Spn529, Spn832 being used in control or confirmatory experiments.

PCR amplification experiments. The primer pairs used in PCR experiments are listed in Table 1. DNA preparation and amplification and electrophoresis of PCR products were carried out by established procedures and following recommended conditions for the use of individual primer pairs. The Ex *Taq* system (TaKaRa Bio, Shiga, Japan) was used in amplification experiments expected to yield PCR products exceeding 3 kb in size.

Inverse PCR. Inverse PCR was carried out as described by Sambrook and Russell (22) to analyze unknown DNA regions. HindIII-, NcoI-, or MunI-restricted genomic DNA (the endonucleases were from Roche Applied Science, Basel, Switzerland) were ligated and used as templates in PCR assays using primer pair MEFA2inv/M2, INV4/DR3, or INV5/DR4, respectively (Table 1).

DNA sequence analysis. All PCR products used for sequence analysis were purified using Montage PCR filter units (Millipore Corporation, Bedford, MA). Amplicons were sequenced (bidirectionally or by primer walking) using ABI Prism (Perkin-Elmer Applied Biosystems, Foster City, CA) with dye-labeled terminators. Sequences were analyzed using the Sequence Navigator software package (Perkin-Elmer Applied Biosystems). ORF analysis was performed using the online available software NEB cutter V2.0 (http://tools.neb.com/NEBcutter2/index.php), and sequence similarity and conserved domain searches were carried out using tools (BLAST and CDART) available online at the National Center for Biotechnology Information of the National Library of Medicine (Bethesda, MD) (http://www.ncbi.nlm.nih.gov).

Transformation and conjugation experiments. Transfer experiments were carried out as described elsewhere (12), using well-known laboratory strains as recipients: *S. pneumoniae* Rx1 in transformation assays and *S. pneumoniae* R6 and *Enterococcus faecalis* JH2-2 in conjugation assays.

Nucleotide sequence accession number. The complete nucleotide sequence of the 5216IQ complex (30,505 bp) has been submitted to the EMBL sequence database and assigned accession no. AJ971089.

RESULTS AND DISCUSSION

Analysis of the DNA regions flanking *mef*(I). Inverse-PCR experiments aimed at analyzing DNA regions flanking *mef*(I) using primer pair MEFA2inv/M2 yielded no amplification following HindIII-restricted DNA fragment ligation, whereas an amplicon was obtained with undigested DNA. This indicated the unpredicted occurrence of a sequence targeted by primer M2 downstream of *mef*(I). Sequencing demonstrated that the amplicon (3,663 bp) contained the last 816 bp of *mef*(I) (EMBL accession no. AJ971089), followed by an *msr*(D) gene variant with 96.2% and 95.9% homologies with the *msr*(D) pneumococcal genes of the mega element and of Tn*1207.1*, respectively (Table 2). The incomplete homology between the new *msr*(D) variant and the previous ones involved the sequences targeted by the primers used originally (3, 14), thus accounting for the lack of PCR evidence of the *msr*(D) gene initially noted for strain Spn529 (12). In contrast, sequencing confirmed the lack of *orf3*, *orf4*, and *orf5* of the mega element, in line with the negative results of PCR assays (12).

Finding of part (6,003 bp) of the Tn*916* transposon upstream of the DNA region containing *mef*(I). In previous unrelated PCR experiments aimed at investigating the *tet*(M) gene in streptococcal populations, strain Spn529, being tetracycline susceptible (MIC, 0.5 μg/ml), was run among negative controls but unexpectedly yielded a positive reaction. By analogy to Tn*2009*, a composite element recently discovered in *S. pneumoniae* and formed by a Tn*916*-like transposon containing the mega element (15), we investigated whether the DNA fragment carrying *mef*(I) and *mrs*(D) in strain Spn529 was also inserted in a Tn*916*-like element.

By pairing primers SG3 (targeting the *orf9* gene of Tn*916*) and MEFA2, an amplicon (10,104 bp) was obtained, which confirmed that the new DNA fragment was inserted into Tn*916* but in a different fashion compared to Tn*2009*. Its se-

TABLE 2. Characteristics of the sequence of the IQ element from strain Spn529[a]

Coordinates (bp)	ORF	Description and homology
15,434–16,579	*tnp1*	Domain structure similarity with transposase proteins belonging to the ISL*3* family (74.2% homology with IS*Sha1* of *Staphylococcus haemolyticus*, DDBJ accession no. AP006716)
16,940–17,359	*hol*	Belonging to phage holin family proteins (70.1% homology with the corresponding sequence of *Dehalococcoides ethenogenes*, REFSEQ accession no. YP_181786)
17,361–17,930	*nf3*	Structure homology with *N*-acetylmuramoyl-L-alanine amidase family proteins (76.0% homology with the corresponding sequence of *Bacillus cereus* ATCC 10987, GenBank accession no. AE017194)
18,715–20,267	*rec1*	Putative DNA recombinase (90.1% homology with the corresponding sequence of *B. cereus* ATCC 10987)
20,231–20,680	*nf5*	Hypothetical protein (92.8% homology with the corresponding sequence of *B. cereus* ATCC 10987)
20,684–22,255	*rec2*	Putative DNA recombinase (92.2% homology with the corresponding sequence of *B. cereus* ATCC 10987)
22,687–23,904	*mef*(I)	Homologies: 93.6% with *mef*(E) of the mega element (GenBank accession no. AF274302), 91.4% with *mef*(A) of Tn*1207.1* (GenBank accession no. AF227520), 92.3% with a *mef* variant detected in *S. pyogenes* in Norway (GenBank accession no. DQ016305), and 92.1% with a *mef* variant of group G streptococci (EMBL accession no. AJ617704)
24,019–25,482	*msr*(D)	Homologies: 96.2% with *msr*(D) (*mel*) of the mega element, 95.9% with *msr*(D) (*orf5*) of Tn*1207.1*, and 93.0% with an *msr*(D) variant of group G streptococci (EMBL accession no. AM084232)
25,614–26,081	*nf9*	Hypothetical protein
26,199–26,780	*nf10*	Domain structure similarity with oxidoreductase family proteins
26,767–27,267	*nf11*	Hypothetical protein belonging to an uncharacterized protein family (UPF0157) (92.4% homology with the corresponding sequence of *Bacteroides ovatus*, EMBL accession no. AJ557257)
27,589–28,248	*catQ*	Chloramphenicol acetyltransferase (96.5% homology with the corresponding *catQ* gene of *Clostridium perfringens*, GenBank accession no. M55620)
28,952–30,097	*tnp1*	Domain structure similarity with transposase proteins of the ISL*3* family

[a] The IQ element (15,115 bp) forms the right portion of the 5216IQ complex (accession no. AJ971089).

quencing showed that in strain Spn529 the DNA fragment was integrated upstream of *tet*(M), at nucleotide 11905 of the Tn*916* sequence (GenBank accession no. U09422) (Fig. 1), causing the loss of the *tet*(M) promoter. A 125-bp deletion (at positions 12087 to 11963) included the ribosome-binding site and 67 of the 87 nucleotides of the *tet*(M) leader peptide (*orf12*). The *tet*(M) coding sequence was complete and had 98.1% homology with the *tet*(M) gene of Tn*916* and 99.1% with the *tet*(M) gene of Tn*2009* (GenBank accession no. AY466395). Based on the well-known regulation of *tet*(M) expression in Tn*916* (26), the lack of the promoter and of most of the *tet*(M) regulatory region is consistent with tetracycline susceptibility of strain Spn529. All of the eight ORFs described downstream of *tet*(M) in Tn*916* (*int*, *xis*, *orf5*, *orf8*, *orf7*, *orf10*, *orf9*, and *orf6*) were detected in other PCR and sequencing experiments, whereas the Tn*916* ORFs upstream of *tet*(M) were not (Fig. 1).

Finding of part (9,313 bp) of the Tn*5252* transposon between the Tn*916* region and the left junction of the 5216IQ complex. To define the insertion of the new element containing the defective Tn*916* transposon and the new efflux gene variants [*mef*(I) and *msr*(D)] into the chromosome, we used primer pairs GD1/TN4 and TN6/GS13 (15), where TN4 and TN6 target the two ends of Tn*916* and GD1 and GS13, respectively, *spr*1206 and *spr*1199, i.e., two chromosomal ORFs located approximately 10 kb apart in the genome of strain R6 (GenBank accession no. AF008493 and AF008492). Sequence assays showed that the right end of amplicon TN6/GS13 (5,208 bp)

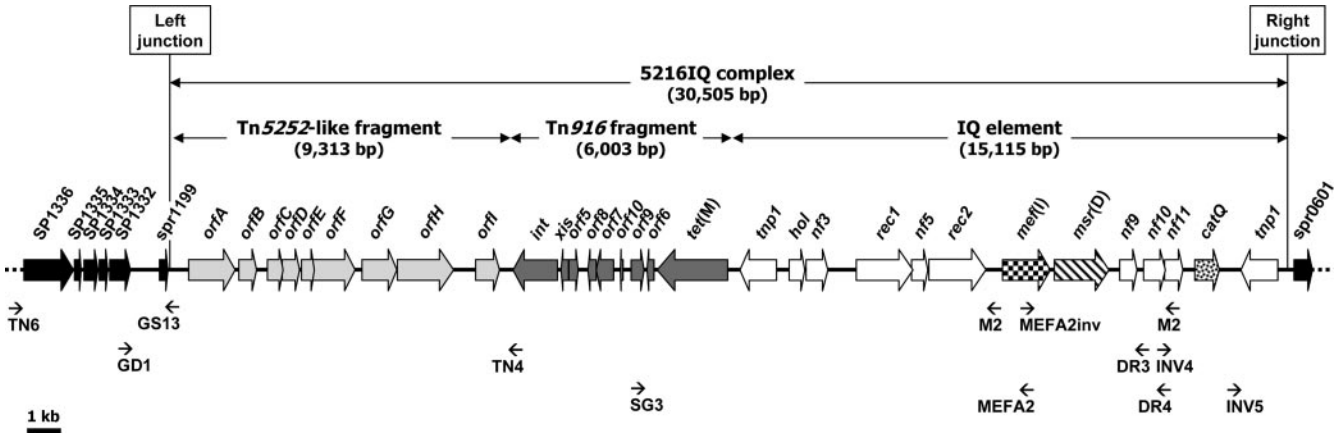

FIG. 1. ORF map of the 5216IQ complex from *S. pneumoniae* strain Spn529 (accession no. AJ971089). Light-gray arrows indicate ORFs from transposon Tn*5252*. Dark-gray arrows indicate ORFs from transposon Tn*916*. White arrows indicate ORFs from the new fragment containing *mef*(I) (checkered), *msr*(D) (striped), and *catQ* (spotted). Black arrows indicate chromosomal ORFs. Thin arrows below the ORF map indicate the positions and directions of the primers used in inverse-PCR experiments and of the major primers used to investigate the genetic organization of the new element.

overlapped with the left end of amplicon GD1/TN4 (11,639 bp) (Fig. 1), thus not only revealing that *spr*1206 and *spr*1199 were both upstream of the 5216IQ complex but also disclosing a chromosomal organization largely different from that of strain R6. Sequencing of the DNA fragment encompassed by the regions targeted by primers TN4 and TN6 (14,664 bp) revealed the presence, downstream of the *int* gene of Tn*916*, of a sequence (9,313 bp) displaying 96.5% homology to a fragment of the streptococcal conjugative transposon Tn*5252* (1, 5), the defective Tn*916* being inserted at nucleotide 239 of the reported sequence of Tn*5252* (GenBank accession no. AF295925). While in the latter sequence this fragment contains nine ORFs (designated *orf28* to *orf20*), the corresponding sequence in our element contains equally nine ORFs (designated *orfA* to *orfI*), apparently resulting from rearrangements (point mutations, premature stops, frameshifts, etc.) of those of Tn*5252* (Fig. 1) (see the deposited sequences of the 5216IQ complex and Tn*5252* for details). The left junction of the 5216IQ complex was found upstream of this sequence, as demonstrated by the detection of an ORF homologous (96.3%) to *spr*1199 of the genome of strain R6 (Fig. 1).

Analysis of the chromosome region upstream of the left junction of the 5216IQ complex. Upstream of the left junction of the 5216IQ complex, sequence analysis confirmed a chromosomal organization quite different from that of strain R6. Upstream of *spr*1199, a DNA fragment (2,960 bp) showing a chromosomal organization similar (93.9% homology) to that of *S. pneumoniae* TIGR4, a well-established virulent isolate (30) (GenBank accession no. AE005672), was detected. This fragment contained five ORFs, homologous to TIGR4 ORFs SP1332 (93.0%), SP1333 (99.1%), SP1334 (93.6%), SP1335 (92.0%), and SP1336 (94.1%). Remarkably, primers GD1 and TN6, designed from the sequences of *spr*1206 of the R6 chromosome and of *orf24* of Tn*916*, respectively (15), matched the TIGR4 chromosomal ORFs SP1332 (93.1% homologous to *spr*1206) and SP1336 (albeit with 4 different bases out of 22 in the targeted sequence), respectively.

IQ element and right junction of the 5216IQ complex. Completing the characterization of the 5216IQ complex still required analysis of its right portion, where early inverse-PCR and sequencing experiments had demonstrated an *msr*(D) gene variant downstream of *mef*(I).

Sequencing of the region encompassed by the last detectable base of Tn*916* and the sequence targeted by primer MEFA2 revealed six ORFs upstream of the *mef*(I) gene, designated *tnp1* (1,146 bp), *hol* (420 bp), *nf3* (570 bp), *rec1* (1,553 bp), *nf5* (450 bp), and *rec2* (1,572 bp) (Fig. 1). *tnp1* encoded a transposase belonging to the ISL*3* family, *hol* a protein belonging to the holin family, *nf3* and *nf5* two hypothetical proteins, and *rec1* and *rec2* two putative DNA recombinases. The complex formed by *rec1*, *nf5*, and *rec2* had 89.8% homology to a sequence occurring in the genome of *Bacillus cereus* (Table 2).

The region downstream of the *msr*(D) gene variant and the right junction of the 5216IQ complex were determined by sequencing the amplicons obtained in inverse-PCR experiments with primer pairs INV4/DR3 and INV5/DR4. Sequencing of amplicon INV4/DR3 revealed the presence of four ORFs: three, designated *nf9* (468 bp), *nf10* (582 bp), and *nf11* (501 bp), encoded hypothetical proteins; and the fourth (660 bp) was a *cat* (chloramphenicol acetyltransferase) gene displaying 96.5% homology with *catQ* of *Clostridium perfringens* (Fig. 1; Table 2). Accordingly, strains Spn529 and Spn832 were chloramphenicol resistant (MIC, 16 μg/ml). It is worth noting that upstream of the *catQ*-like gene an 18-bp sequence that overlapped with that targeted by primer M2 (except for 4 bases) was found, thus accounting for the above-mentioned 3,663-bp amplicon obtained in early PCR experiments with primer pair MEFA2inv/M2. Sequencing of amplicon INV5/DR4 revealed a transposase gene identical to the above-mentioned transposase gene *tnp1* downstream of *catQ*. The new fragment, ending with identical transposase genes on both sides, was designated the IQ element. The right junction of the 5216IQ complex was discovered after the right *tnp1*: next to a no-homology intergenic DNA segment of 408 bp there was a 24-bp sequence that was identical to a chromosomal sequence shared by both *S. pneumoniae* R6 (REFSEQ accession no. NC_003098) and *S. pneumoniae* TIGR4 (GenBank accession no. AE005672) and was followed by an ORF displaying 98.1% homology with the same ORF designated *spr*0601 in R6 and SP0686 in TIGR4 (Fig. 1). The sequence of *tnp1* has been submitted to the ISFinder database (http://www-is.biotoul.fr), where it was designated IS*Spn3*.

Transfer experiments. No detectable transfer of erythromycin resistance was obtained in repeated transformation and conjugation assays using both Spn529 and Spn832 as donors.

Conclusions. This study shows that *mef*(I), a macrolide efflux resistance *mef* gene variant recently detected in *S. pneumoniae* (12), is carried by a new, apparently nonmobile, composite structure that we designated the 5216IQ complex. Its complete sequencing and the analysis of its left and right junctions revealed a unique genetic organization, identical in both strains Spn529 and Spn832. The 5216IQ complex (30,505 bp) is composed of two halves of comparable sizes: (i) a left one (15,316 bp) formed by parts of known transposons, namely, 9,313 bp of Tn*5252* and 6,003 bp of Tn*916*; and (ii) a right one (15,115 bp) formed by the IQ element. In the assembly of the left half, the ORFs of Tn*916* lying upstream of the *tet*(M) gene have apparently been lost, and the same is true of most of Tn*5252*. It is worth noting that the occurrence in Tn*916*-like elements not only of a regular *tet*(M) gene encoding tetracycline resistance, as long established (10), but also of a silent *tet*(M) gene associated with a tetracycline-susceptible phenotype is consistent with recent findings for *erm*(B)-carrying elements from both *S. pneumoniae* (11, 21) and *S. pyogenes* (7). The IQ element, ending with identical transposase genes on both sides, contained the *mef*(I) gene, a new *msr*(D) gene variant, and a *catQ* chloramphenicol resistance gene. To our knowledge, this is the first report demonstrating *catQ* in *S. pneumoniae* and showing its linkage with a *mef* gene. The IQ element—whose genetic organization is totally unlike those of the genetic elements carrying other *mef* genes, i.e., *mef*(E) (15, 16) or *mef*(A) (25), in pneumococci—should probably be considered a novel transposon, although it does not appear to be mobile in the genetic context of the pneumococcus. The right junction of the 5216IQ complex was just upstream of the *spr*0601 gene of the *S. pneumoniae* R6 chromosome (designated SP0686 in TIGR4). The left junction was just downstream of the *spr*1199 gene of the R6 chromosome; however, further sequencing revealed a chromosomal organization more similar to that of strain TIGR4 than to that of strain R6. Such extensive genome rearrangements

are consistent with the well-established recombination-mediated genetic plasticity that is a distinctive feature of *S. pneumoniae* (9). The apparent nonmobility of the 5216IQ complex is consistent with the loss of conjugation-related ORFs of both Tn*916* (10) and Tn*5252* (19). On the other hand, owing to the greater similarity to TIGR4 than to R6, a lack of zones of homology with the pneumococcal chromosome could account for the unsuccessful transformation experiments, considering that the standard Rx1 recipient has the same derivation (from Avery's historical D39) as R6.

ACKNOWLEDGMENT

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

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