Heterogeneity of Tn5253-Like Composite Elements in Clinical Streptococcus pneumoniae Isolates

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Several drug resistances in Streptococcus pneumoniae are associated with mobile genetic elements, which are loosely subdivided into a group of smaller (18- to 27-kb) and a group of larger (>50-kb) elements. While the elements of the former group, which typically carry the tetracycline resistance determinant tet(M) and whose prototype is Tn916 (18 kb), have been studied extensively, the larger elements, whose prototype is Tn5253 (~65.5 kb), are not as well explored. Tn5253 is a composite structure consisting of two independent conjugative transposons, Tn5251 (which is virtually identical to Tn916) and Tn5252 (~47.5 kb), with the former inserted into the latter. Tn5252, which so far has only partially been sequenced, carries an integrase gene, driving its site-specific insertion into the host cell genome, and the chloramphenicol resistance cat\textsubscript{pc194} determinant. This study investigated 20 clinical isolates of S. pneumoniae, which were selected on the basis of cat\textsubscript{pc194}-mediated chloramphenicol resistance. All 20 isolates harbored a Tn5253-like element. The composite elements (some of which have been completely sequenced) demonstrated considerable heterogeneity that stemmed from a dual variability: in the Tn5252-like element, due primarily to differences in the integrase gene but also to differences in cargo genes and in the overall genetic organization, and in the Tn916-like element, with the possible involvement, besides Tn916, of a number of Tn916 family pneumococcal elements carrying different erythromycin resistance genes. In mating experiments, only one composite element, containing a less typical Tn916 family element, appeared to be nonmobile. Being part of a Tn5253-like composite element may confer on some Tn916-like transposons, which are apparently nontransferable as independent genetic elements, the ability to be mobilized.

A unique recombination-mediated genetic plasticity is a distinctive feature of Streptococcus pneumoniae (3) and a key to its success as a pathogen. In this context, a cause for serious concern is the emergence and increasing spread of multidrug-resistant clinical pneumococci (28), where multiple resistance is generally associated with mobile genetic elements. Although the nomenclature for such elements is evolving, they are often referred to as transposons or as integrative and conjugative elements (ICEs) (21, 33). Pneumococcal mobile elements (which are also found in other streptococci and relatives (22)) make up of two independent conjugative transposons, Tn5251 and Tn5252, with the former inserted into the latter (2). Tn5251 (18 kb) is virtually identical to Tn916 in structure and size, with only a few differences occurring, chiefly in the tet(M) gene sequence (19, 25). Tn5252 (~47.5 kb) is a conjugative transposon containing a chloramphenicol (CHL) resistance gene (cat), namely, the cat\textsubscript{pc194} determinant from the linearized pC194 Staphylococcus aureus plasmid (32), and an integrase gene (\textit{int\textsubscript{5252}}) driving its site-specific insertion into the host cell genome (30). Only three Tn5252 regions, representing functional modules, have so far been studied and sequenced: the left terminal (LT) region (accession no. L29324), which contains integration (15), mobilization (26, 27), and UV resistance (18) genes; the conjugal transfer-related (CTR) region (accession no. AF295925) (1); and the right terminal (RT) region (accession no. L29325), which contains the DNA cytosine methyltransferase gene (24). Other genetic and functional properties of Tn5252 are still largely unknown.

Recently, variability of some genes of the Tn5252 LT region in clinical isolates of pneumococci has been reported (14). Moreover, analysis of the complete chromosome sequences of multiresistant S. pneumoniae isolates has disclosed new larger

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genetic elements, such as ICE\textit{Sp}23FST81 (\textsim 81 kb; accession no. FM211187) (8) and \textit{Tn}2008 (\textsim 67 kb; accession no. CP001033) (11), both carrying \textit{cat}_{\textit{pc}194} in their \textit{Tn}5252-like transposon and sharing an identical genome integration site that is different from that of \textit{Tn}5253.

The present study of 20 isolates, selected on the basis of \textit{cat}_{\textit{pc}194}-mediated CHL resistance, was aimed at extending our knowledge of the presence, genetic organization, and transferability of \textit{Tn}5253-like composite elements in clinical pneumococci. While all isolates harbored a \textit{Tn}5253-like element, such elements demonstrated a substantial heterogeneity resulting from combined variabilities in the \textit{Tn}5252-like and the \textit{Tn}916-like elements.

\section*{MATERIALS AND METHODS}

\subsection*{Bacterial strains.}
Twenty isolates were selected from a collection of 166 clinical isolates of \textit{S. pneumoniae} recovered in several Italian laboratories in 2005 to 2008 and representing geographical and chronological diversities. The inclusion criterion was CHL resistance (MIC, \textsim 8 \mu g/ml) mediated by the \textit{cat}_{\textit{pc}194} gene, determined by PCR using specific primers R3 and F2 (32) (see Table S1 in the supplemental material).

\subsection*{Typing.}
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 Smal endonuclease (Roche Applied Science, Basel, Switzerland) and pulsed-field gel electrophoresis (PFGE) analysis were performed by established methods. If a PFGE pattern was shared by at least two isolates, the type was designated with a capital letter in order of size, as described elsewhere (20).

\subsection*{Antibiotic and susceptibility tests.}
CHL, ERY, and TET were purchased from Sigma Chemical Co., St. Louis, MO. MICs were determined by a standard agar dilution method and interpreted according to current breakpoints (4).

\subsection*{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). Open reading frame (ORF) analysis was performed using the online software ORF finder (http://www.ncbi.nlm.nih.gov/) and NEB cutter V2.0 (New England Biolabs, Ipswich, MA). 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).

\subsection*{Conjugation experiments.}
Mating assays were performed as described elsewhere (7). Transconjugants were selected on plates containing CHL (5 \mu g/ml) in addition to rifampin (10 \mu g/ml) and fusidic acid (10 \mu g/ml). ERY (1 or 5 \mu g/ml) or TET (5 \mu g/ml) was also used as a selecting agent with some donors, according to their resistances. \textit{S. pneumoniae} isolates with different genotypic characteristics were used as donors. Two CHL-, ERY-, and TET-susceptible recipients were used: \textit{S. pneumoniae} R6RF, a rifampin- and fusidic acid-resistant derivative obtained from the well-known laboratory strain R6, and \textit{Streptococcus pyogenes} 12RF, a rifampin- and fusidic acid-resistant derivative obtained from a clinical isolate (13).

\subsection*{Nucleotide sequence accession numbers.}
The sequences of three new genetic elements, \textit{Tn}6058, \textit{Tn}3311, and \textit{ICE}6094, were submitted to the EMBL/GenBank sequence database and assigned accession no. FM201786, FN667862, and FR670347, respectively.

\section*{RESULTS AND DISCUSSION}

\subsection*{Characterization of the isolates.}
The 20 isolates were characterized for a number of phenotypic and genotypic attributes (Table 1).

Typing data included serotyping (with 18 isolates falling into nine serotypes and two being nontypeable) and PFGE typing (with the 20 isolates presenting 13 different PFGE patterns).

Antibiotic resistance studies indicated that, in addition to CHL, 15 isolates [12 bearing the \textit{erm}(B) gene and the \textit{mef}(E) gene] were resistant to ERY and 19 [all bearing the \textit{tet}(M) gene] were resistant to TET [the only TET-susceptible, \textit{tet}(M)-negative isolate (Pn6)] was also the only one to be positive for the kanamycin resistance gene \textit{aphA-3}.

All 20 isolates were positive for both the integrase and the excisionase genes of the \textit{Tn}916 family elements (\textit{int}_{916} and \textit{xis}_{916}). The particular \textit{Tn}916-like pneumococcal element involved was identified by amplification assays. The five \textit{ERY}-susceptible isolates harbored \textit{Tn}916. Of the 12 \textit{erm}(B)-positive isolates, 9 harbored \textit{Tn}0002 (6, 31), 2 \textit{Tn}3872 (16), and 1 (Pn6) an element resembling a deleted form (with an incomplete macrolide-amnoglycoside-streptothricin [MAS] fragment containing \textit{aphA-3} as the sole resistance determinant) of the one previously designated \textit{SpnRi3erm}(B) (6).

All three \textit{mef}(E)-positive isolates harbored \textit{Tn}2009 (10).

\subsection*{Comparative analysis of known sequences and amplification experiments to detect \textit{Tn}5252-like elements.}
Comparative analysis of known sequences of the \textit{Tn}5252 LT, CTR, and RT regions and of genetic elements ICE\textit{Sp}23FST81 and \textit{Tn}2008 showed that the three \textit{Tn}5252 regions were present and were identical in the two new elements. While their CTR and RT regions had close identities (around 94%) to the corresponding \textit{Tn}5252 regions, their LT regions differed considerably from the LT prototype, with which they shared only three \textit{Tn}5252 ORFs (orf4, orf9, and orf10). In particular, the integrase gene of ICE\textit{Sp}23FST81 and \textit{Tn}2008, here designated \textit{int}_{\textit{Sp}23FST81} was completely different from \textit{int}_{916}. Moreover, the latter is adjacent to the excisionase gene \textit{xis}_{5252}, whereas an excisionase gene is not found when the integrase gene is \textit{int}_{\textit{Sp}23FST81}.

Testing for the two different integrase genes gave a positive PCR for \textit{int}_{5252} and \textit{xis}_{5252} in 7 isolates [5 harboring \textit{Tn}0002, 1 \textit{Tn}916, and 1 the \textit{SpnRi3erm}(B)-like element] and for \textit{int}_{\textit{Sp}23FST81} in 13 [4 harboring \textit{Tn}916, 4 \textit{Tn}6002, 3 \textit{Tn}2009, and 2 \textit{Tn}3872] (Table 1).

\subsection*{Characterization of \textit{Tn}5253-like composite elements positive for \textit{int}_{5252} and \textit{xis}_{5252}.}
In the seven isolates positive for \textit{int}_{5252} and \textit{xis}_{5252}, PCR experiments with suitable primer pairs were performed to understand the \textit{Tn}5252 structure, seek a linkage with the associated \textit{Tn}916-like transposon, and elucidate the genome integration site. The results indicated that in all seven isolates the \textit{Tn}916-like element was inserted into the \textit{Tn}5252-like element, forming various \textit{Tn}5253-like composite structures (Table 1), all sharing the specific site of \textit{Tn}5252 (Table 1), all sharing the specific site of \textit{Tn}5252.
All isolates, selected for cefuroxime-modified CHL resistance, were positive for the integron gene int916 and the excisionase gene 916b. Eleven of the 13 isolates displayed an organization of the Tn5252-like element, seek a linkage with the associated Tn916-like transposon, and elucidate the genome integration site. The results indicated that in all 13 isolates the Tn916-like element was inserted into a Tn5252-like element forming various Tn5253-like composite structures (Table 1), all sharing the specific site of ICESp23FST81 integration into the pneumococcal genome, flanked by a 16-bp tandem duplication, near the 3′ end of gene rplL (8) (accession no. FM211187).

Eleven of the 13 isolates displayed an organization of the composite element closely resembling that of ICESp23FST81 (8) (Fig. 2A and B). The associated Tn916 family element...
Based on PCR analysis, the Tn5253-like element, designated int5252, was always the same, in the area between the CTR and the RT regions, in ICESp916-like element varying accordingly, but its insertion site varied (four isolates had Tn916, three had Tn6002, three had Tn2009, and one had Tn3872), with the overall size of the Tn5253-like element varying accordingly, but its insertion site was always the same, in the area between the CTR and the RT regions.

The other two isolates (Pn12 and Pn19) shared a distinctive new organization of the composite element, designated ICE6094. Based on PCR analysis, the Tn5252-like element appeared to be identical in the two isolates but substantially different from both ICESp23FST81 and the Tn5252 prototype (Fig. 2C). The only difference between the two isolates was in the Tn916-like element harbored in the ICE6094 structure: Tn872 in Pn12 and Tn6002 in Pn19. Unlike ICESp23FST81 and the composite elements of the 11 isolates carrying intSp23FST81, where the Tn916-like element is inserted between the CTR and the RT regions, in ICE6094 the Tn916-like element is inserted in the LT region, between intSp23FST81 and the mobilization genes, and has an opposite orientation. In particular, in ICE6094 from isolate Pn19, whose Tn5252-like backbone was completely sequenced (61,030 bp; accession no. FR670347), the Tn6002 insertion site was found at nucleotide 10910 of the sequence. An ~13.5-kb area to the right of Tn6002 displayed high identity to corresponding areas of Tn3872 (97.3%) and the genome of S. pneumoniae G54 (99.6%), another multidrug-resistant clinical isolate whose genome has been completely sequenced (accession no. CP001015).

**Transferability of Tn5253-like composite elements.** Nine isolates, three carrying composite elements positive for int5252 and six carrying composite elements positive for intSp23FST81, were tested as donors in mating experiments. The former included Pn1 (harboring a Tn5253-like element), Pn20 (harboring Tn6058), and Pn6 (harboring Tn3872). The latter included Pn3, Pn8, Pn13, and Pn14 (harboring ICESp23FST81 variants containing Tn2009, Tn916, Tn3872, and Tn6002, respectively, as the Tn916 family transposons) and Pn12 and Pn19 (harboring ICE6094 containing Tn3872 and Tn6002, respectively, as the Tn916 family transposon). The results are summarized in Table 2.

No detectable transfer was obtained from any donor when S. pneumoniae R6RF was used as the recipient. Detectable transfer from eight of the nine donors was obtained, at variable frequencies (10⁻² to 10⁻⁶), with S. pyogenes 12RF as the recipient. All transconjugants exhibited the expected CHL, TET,
and/or ERY resistances, even though the MIC levels expressed in the *S. pyogenes* host were often higher than those expressed in the pneumococcal donors. Transconjugants were obtained from the two donors harboring composite ICEs with Tn3872 as the associated Tn916 family transposon (Pn12 and Pn13) only when CHL was used for the selection. Pn6, harboring the SpnRi3erm(B)-like element, i.e., a deleted form of previously described SpnRi3erm(B) element, which is also nonmobile (6), was the sole donor from which transconjugants could not be obtained.

**TABLE 2. Conjugal transfer of resistance determinants from *S. pneumoniae* donors with different genotypic characteristics to the susceptible recipient *S. pyogenes* 12RF**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Composite element (Tn96 family transposon)</th>
<th>Genotype</th>
<th>Selection for resistance</th>
<th>Transfer frequency</th>
<th>Genotype</th>
<th>MIC (μg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CHL</td>
</tr>
<tr>
<td>Pn1</td>
<td>Tn5235-like (Tn916)</td>
<td>cat₅₉₁₄ tet(M)</td>
<td>CHL</td>
<td>1.6 × 10⁻⁷</td>
<td>cat₅₉₁₄ tet(M)</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Pn3</td>
<td>ICESp23FST81-like (Tn2009)</td>
<td>cat₅₉₁₄ tet(M) mef(E)</td>
<td>CHL</td>
<td>2.8 × 10⁻⁸</td>
<td>cat₅₉₁₄ tet(M) mef(E)</td>
<td>32</td>
</tr>
<tr>
<td>Pn6</td>
<td>Tn1311 (SpnRi3erm(B)-like)</td>
<td>cat₅₉₁₄ erm(B) aph4-3</td>
<td>ERY</td>
<td>5.7 × 10⁻⁸</td>
<td>cat₅₉₁₄ erm(B) aph4-3</td>
<td>32</td>
</tr>
<tr>
<td>Pn8</td>
<td>ICESp23FST81 (Tn916)</td>
<td>cat₅₉₁₄ tet(M)</td>
<td>ERY</td>
<td>3.4 × 10⁻⁵</td>
<td>cat₅₉₁₄ tet(M)</td>
<td>32</td>
</tr>
<tr>
<td>Pn12</td>
<td>ICE6094 (Tn3872)</td>
<td>cat₅₉₁₄ tet(M) erm(B)</td>
<td>TET</td>
<td>3.7 × 10⁻⁷</td>
<td>cat₅₉₁₄ tet(M)</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Pn13</td>
<td>ICESp23FST81-like (Tn3872)</td>
<td>cat₅₉₁₄ tet(M) erm(B)</td>
<td>ERY</td>
<td>8.0 × 10⁻⁸</td>
<td>cat₅₉₁₄ tet(M)</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Pn14</td>
<td>ICESp23FST81-like (Tn6002)</td>
<td>cat₅₉₁₄ tet(M) erm(B)</td>
<td>ERY</td>
<td>1.9 × 10⁻⁸</td>
<td>cat₅₉₁₄ tet(M)</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Pn19</td>
<td>ICE6094 (Tn6002)</td>
<td>cat₅₉₁₄ tet(M) erm(B)</td>
<td>ERY</td>
<td>3.4 × 10⁻⁶</td>
<td>cat₅₉₁₄ tet(M)</td>
<td>32</td>
</tr>
<tr>
<td>Pn20</td>
<td>Tn6058 (Tn6002)</td>
<td>cat₅₉₁₄ tet(M) erm(B)</td>
<td>ERY</td>
<td>2.2 × 10⁻⁸</td>
<td>cat₅₉₁₄ tet(M)</td>
<td>32</td>
</tr>
</tbody>
</table>

*a* No detectable transfer was obtained when *S. pneumoniae* R6RF was used as the recipient.

*b* NDT, no detectable transfer.
Concluding remarks. All 20 clinical isolates of S. pneumoniae, selected on the basis of cat\textsubscript{PC}C194-mediated CHL resistance, proved to harbor a Tn5253-like element. These composite elements demonstrated substantial heterogeneity, consistent with the belief that element recombination is frequent and that module swapping can result in the emergence of new elements (33). The only correlation between typing data and particular composite elements was the fact that four of the five isolates harboring Tn6058 belonged to serotype 3 and PFGE type A. Such heterogeneity confirms and affords further insights into the diversity of putative Tn5253-like pneumococcal elements, which has recently been reported by Henderson-Begg et al. (14) on the basis of combined analysis of selected genes and antibiogram data. Remarkably, that study also underscored that these composite elements appear to be prevalent among internationally recognized pandemic clones of S. pneumoniae. The present findings clarify that the overall heterogeneity of Tn5253-like pneumococcal elements results from at least two levels of variability: in the Tn5252-like and in the Tn916-like transposons.

At the first level, at least two variants of the Tn5252-related transposon can be distinguished to begin with, depending on the presence of the int\textsubscript{5252} or the int\textsubscript{SP23FST1} gene. The two integrate genes drive the integration of their elements into the pneumococcal genome at specific sites, i.e., into the spr\textsubscript{1043} gene (R6 genome) and near the 3′ end of gene rplL, respectively. Moreover the Tn5252-like elements show further variability in their genetic organization and structure regardless of the int gene. While the CTR and RT regions seem to be stabilizer modules, the LT region, aside from the int gene, seems to be more changeable (e.g., it lacks the UV resistance gene cluster in Tn1311 and is split by the insertion of the Tn916-like element between the integration and the mobilization genes in ICE6094). Furthermore, a variety of different cargo genes make the intermodular areas largely unpredictable.

A second level of variability is related to the Tn916 family transposon, which is inserted (at variable sites, consistent with the poor specificity of int\textsubscript{916}) into the Tn5252-like transposon. The Tn916-like transposon may be Tn916 or another element from a number of Tn916 family pneumococcal relatives carrying a variety of insertions containing erythromycin resistance genes such as erm(B) or mef(E) in addition to the tet(M) tetracycline resistance determinant typical of the family (22, 29). It is noteworthy that Tn916 family pneumococcal transposons such as Tn545 and Tn6003, containing a complete MAS fragment with a second erm(B) gene lacking the stop codon (5), were not found in Tn5253-like elements in this study.

While Tn916 family transposons can be found both as independent elements and inserted into Tn5252-related transposons to form a Tn5253-like composite element, Tn5252-related transposons, whether carrying int\textsubscript{5252} or int\textsubscript{SP23FST1}, are not known to occur naturally outside a composite element, i.e., as independent genetic elements. It is worth noting that S. pneumoniae SP1000, a historical strain used in early studies of the Tn5252 transposon (15, 18, 24, 26, 27, 30), was a laboratory-derived organism obtained by deleting Tn5251 from Tn523 (2).

Our failure to obtain detectable transfer in mating experiments using S. pneumoniae R6RF as the recipient probably reflects the poor propensity of pneumococci to acquire foreign genetic material via conjugation. In contrast, conjugal transfer was mostly successful when S. pyogenes 12RF was used as the recipient. The sole nonmobile composite element (Tn1311, isolate Pn6) contains a Tn916-related element, the SprR3erm(B)-like element, which, despite the occurrence of int\textsubscript{916} and x\textsubscript{916}, appears to be a less typical member of the Tn916 family. In some instances, being part of a composite element may give some Tn916 family transposons, which as independent genetic elements are apparently nontransferable in conjugation experiments, an opportunity to be mobilized. This might be the case for Tn2009, which is non-transferable by conjugation in both inter- and intraspecific mating assays (9, 10), and for Tn3872, whose nontransferability by conjugation, despite exceptions with streptococcal donors other than pneumococci, is extensively documented (16, 29).

Remarkably, both composite Tn5253-like elements bearing Tn3872 as the Tn916-like transposon (ICE6094 in isolate Pn12 and the ICESp23FST1-like element in isolate Pn13) turned out to be transferable only when selection was for CHL resistance, whose determinant is located on the Tn5252-like transposon. TET or ERY (clearly not CHL) was used for selection in previous studies reporting nontransferability of Tn3872.

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