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Characterization of IS*1515*, a Functional Insertion Sequence in *Streptococcus pneumoniae*

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We describe the characterization of a new insertion sequence, IS*1515*, identified in the genome of *Streptococcus pneumoniae* I41R, an unencapsulated mutant isolated many years ago (R. Austrian, H. P. Bernheimer, E. E. B. Smith, and G. T. Mills, J. Exp. Med. 110:585–602, 1959). A copy of this element located in the $cap1E_{I41R}$ gene was sequenced. The 871-bp-long IS*1515* element possesses 12-bp perfect inverted repeats and generates a 3-bp target duplication upon insertion. The IS encodes a protein of 271 amino acid residues similar to the putative transposases of other insertion sequences, namely IS*1381* from *S. pneumoniae*, ISL*2* from *Lactobacillus helveticus*, IS*702* from the cyanobacterium *Calothrix* sp. strain PCC 7601, and IS*112* from *Streptomyces albus* G. IS*1515* appears to be present in the genome of most type 1 pneumococci in a maximum of 13 copies, although it has also been found in the chromosome of pneumococcal isolates belonging to other serotypes. We have found that the unencapsulated phenotype of strain I41R is the result of both the presence of an IS*1515* copy and a frameshift mutation in the $cap1E_{I41R}$ gene. Precise excision of the IS was observed in the type 1 encapsulated transformants isolated in experiments designed to repair the frameshift. These results reveal that IS*1515* behaves quite differently from other previously described pneumococcal insertion sequences. Several copies of IS*1515* were also able to excise and move to another locations in the chromosome of *S. pneumoniae*. To our knowledge, this is the first report of a functional IS in pneumococcus.

Insertion sequences (ISs) are bacterial mobile DNA elements that cause genome rearrangements, such as deletions, inversions, duplications, and replicon fusions, by their ability to transpose (24). Furthermore, a critical role of ISs in bacterial virulence is currently being recognized. Virulence genes may be located on transmissible genetic elements and form part of particular regions on the bacterial chromosomes (pathogenicity islands), which, in many cases, are flanked by ISs (14). The frequent occurrence of deletions and/or amplifications associated with pathogenicity islands may be ascribed to ISs and other transmissible genetic elements.

Streptococcus pneumoniae (pneumococcus) is an important human pathogen that has been studied intensively for many decades. Morbidity and mortality from pneumococcal infections remain high, even in regions where efficient antibacterial therapy is freely available. The role of ISs in the natural population dynamics of pneumococcus is entirely unknown, although transposable elements have been identified in the form of conjugative transposons (5, 13, 25) and ISs, namely, IS*1202* (21), IS*1167* (36), and IS*1381* (31). Pneumococcal virulence depends upon the presence of capsular polysaccharide, since unencapsulated (rough) mutants are avirulent (12). Interestingly, recent results indicate that the genetic loci (*cap*) responsible for the synthesis of the pneumococcal capsules are frequently associated with ISs or IS-like sequences (1, 21, 22, 34). In particular, the *cap1* locus involved in the type 1 capsule biosynthesis of *S. pneumoniae* is flanked by nonfunctional copies of IS*1167* (22), suggesting a role of this IS element in the horizontal transfer of the capsular genetic determinants (11). Nevertheless, the transposition capacity of the ISs described in *S. pneumoniae* has not been demonstrated so far.

During our investigations of the *cap* loci of *S. pneumoniae*, we have characterized the mutation responsible for the unencapsulated phenotype of strain I41R, a type 1 derivative (3), and identified a segment of pneumococcal DNA that exhibited all of the hallmarks of a bacterial IS. One of the copies of this element, designated IS*1515*, is inserted into the pneumococcal capsular *cap1E* gene of the strain I41R. The sequence and characterization of IS*1515*, the first functional IS found in *S. pneumoniae*, are reported.

MATERIALS AND METHODS

Bacterial strains and plasmids. I41R (originally designated as $S\text{-}I_1$) (3), an unencapsulated ($S1^-$) mutant of the type 1 pneumococcal strain I41S, was kindly provided by S. Lacks (Brookhaven National Laboratory, N.Y.). The encapsulated pneumococcal strains have been described in a previous publication (22). Strains N and L are type 1 strains that were isolated in the United States in the late 1930s, whereas strain 519/43, also a type 1 strain, was isolated in Denmark in 1943; these three strains were provided by J. Henrichsen (Statens Seruminstitut, Copenhagen, Denmark). A. Fenoll (Pneumococcus Reference Laboratory, Madrid, Spain) provided all other encapsulated *S. pneumoniae* isolates. In particular, the type 1 pneumococci were isolated in different Spanish hospitals between 1990 and 1996. In some experiments, the pneumococcal laboratory strain M22 (28) was used as a DNA source. *Escherichia coli* DH5α (30) was also used. Plasmids pLSE1 (28); pGL32 (17); and pCMM6, pCMM9, pCMM10, pRMM9, and pRMM10 (22) have been described in previous publications. The insert locations, as reported in the DNA sequence of *cap1* (accession no. Z83335), are as follows: pCMM6, 7864 to 9906; pCMM9, 5902 to 8732; pCMM10, 4661 to 9039; pRMM9, 9522 to 14962; and pRMM10, 10020 to 15900. The construction of pRMM34 is described below.

Growth conditions and transformation of bacteria. *S. pneumoniae* was grown in liquid C medium (16) containing 0.08% yeast extract (C+Y) without shaking by procedures previously described (33) or on reconstituted tryptose blood agar base plates (Difco Laboratories) supplemented with 5% defibrinated sheep blood. *E. coli* cells were grown in Luria-Bertani medium (30). The preparation of pneumococcal DNA, plasmid purification, and transformation of *S. pneumoniae* and *E. coli* were carried out as described elsewhere (10). When required, capsulated transformants of *S. pneumoniae* were enriched by successive transfers of the transformed culture on C medium containing 0.08% bovine serum albumin and 1 μl of anti-R antiserum per ml before plating. Anti-R (antisomatic) antiserum contains group-specific agglutinins that at a convenient dilution agglutinate only rough pneumococci and was raised in rabbits as previously described (26). Lincomycin- and streptomycin-resistant *S. pneumoniae* transformants were selected with 0.6 and 200 μg of the antibiotic per ml, respectively. Serotyping was carried out by coagglutination (32) with a cell suspension of formalin-treated

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Staphylococcus aureus (Cowan strain) (Sigma Chemicals Co., St. Louis, Mo.) sensitized with type-specific pneumococcal antisera provided by the Staten Seruminstitut.

DNA techniques. Restriction endonucleases, T4 DNA ligase, and the Klenow fragment of DNA polymerase were obtained commercially and used according to the recommendations of the suppliers. Gel electrophoresis of plasmids, restriction fragments, and PCR products was carried out in agarose gels as described previously (30). DNA was recovered from gel slices with the Gene Clean II kit (Bio 101). The NEBlot Phototope kit (Millipore) was used to construct biotin-labeled probes, and the Phototope 6K detection kit (Millipore) was used for chemiluminescent detection. Southern blotting, dot blotting, and hybridizations were carried out according to the manufacturer's instructions. DNA sequencing was carried out with an AbiPrism377 DNA sequencer (Applied Biosystems).

PCR amplifications were performed with 2 U of AmpliTaq DNA polymerase (Perkin-Elmer), 1 μg of chromosomal (or plasmid) DNA, 1 μM (each) synthetic oligonucleotide primer, 200 μM (each) deoxynucleoside triphosphates, and 2.5 mM $MgCl_2$ in the buffer recommended by the manufacturer. Conditions for amplification were chosen according to the G+C content of the corresponding oligonucleotide. The following primers were used: P9A (7258), 5′-GCGGTTA ATTAagcTTTAGGAAG-3′; P9B (8463/c), 5′-ATTTGCACGAAGgAtcCCAA C-3′; P64 (456), 5′-CAACTCATGCTAGAACACCT-3′; and P65 (989/c), 5′-G CAGGAATGAAAGTATTCTC-3′. The numbers in parentheses indicate the positions of the first nucleotide of the primer in the sequence shown in Fig. 2, and c means that the corresponding sequence is on the complementary strand. Lowercase letters indicate nucleotides introduced to construct the appropriate restriction sites, which are shown underlined. An internal fragment of IS*1515* to be used as a probe was cloned as follows: I41R chromosomal DNA was amplified with oligonucleotides P64 and P65, the PCR product was digested with *Apo*I and ligated to *Eco*RI-digested pUC18, and the ligation mixture was used to transform *E. coli* DH5α. The recombinant plasmid was named pRMM34 (see below).

Data analysis. DNA and protein sequences were analyzed with the Genetics Computer Group software package (version 9.0) (7). Amino acid sequence homology searches were performed with the BLAST program at the National Center for Biotechnology Information server (Bethesda, Md.).

Nucleotide sequence accession number. The nucleotide sequence for IS*1515* has been deposited in the EMBL, GenBank, and DDBJ databases under accession no. Z86112.

RESULTS

Identification of IS*1515*. We have recently described the molecular organization of the genes required for the synthesis of type 1 capsular polysaccharide of pneumococcus (22). *cap1* is a cluster that contains 11 genes (*cap1A* to *cap1K*) arranged as a single transcriptional unit. In the course of this research, we wanted to characterize the mutation responsible for the inability of *S. pneumoniae* I41R to synthesize capsular polysaccharide. This strain is a rough derivative of a clinical strain isolated in the United States during the 1950s (2, 3). To determine the genetic defect responsible for the $S1^-$ phenotype, some of the plasmids constructed to sequence the type 1 locus (Fig. 1A) were tested for their ability to transform I41R to the $S1^+$ phenotype. Encapsulated transformants were recovered only when pCMM6, pCMM9, and pCMM10 were used as donor DNAs (not shown). These findings indicated that the I41R mutation(s) mapped between positions corresponding to nucleotides 7864 and 8732 of the $cap1_{13868}$ locus, i.e., either in *cap1E* (positions 7290 to 8426) or in *cap1F* (positions 8419 to 8970). The genes $cap1E_{13868}$ and $cap1F_{13868}$ appear to code for a glycosyl transferase and a galacturonosyl acetylase, respectively (22).

The chromosomal region containing the $cap1E_{I41R}$ gene was amplified by PCR with oligonucleotides P9A and P9B (Fig. 1). Interestingly, a 2.1-kb DNA fragment was amplified instead of the 1.2-kb PCR product expected on the basis of the location of the oligonucleotide primers. Direct sequencing of the 2.1-kb PCR product confirmed the presence of an extra DNA fragment within the $cap1E_{I41R}$ gene (Fig. 1B). Analysis of the nucleotide sequence (Fig. 2) suggested that the foreign DNA corresponds to an IS element that was designated IS*1515* (see below). Furthermore, the gene $cap1E_{I41R}$ showed five point mutations, i.e., four nucleotide changes and a 1-bp deletion, compared with the $cap1E_{13868}$ gene previously described (22). The nucleotide changes were ^{7296}T to G, ^{7554}A to G, ^{7558}A to T, and ^{8086}C to T. The first three mutations would produce amino acid replacements in the corresponding $Cap1E_{I41R}$ protein, that is, ^{3}Leu to Val, ^{89}Ile to Val, and ^{90}Asp to Val, respectively. In addition, a TAG stop codon (between nucleotides 334 and 336 of the $cap1E_{I41R}$ gene) was introduced by the presence of the IS and would lead to a truncated protein (Fig. 2). The finding that transformation of competent I41R cells with pCMM6 (Fig. 2) gave rise to $S1^+$ transformants (see above) ruled out the possibility that those three mutations were responsible for the rough phenotype of *S. pneumoniae* I41R. On the other hand, even in the case of a precise excision of the IS (see below), the deletion mutation located at nucleotide 1635 of the $cap1E_{I41R}$ gene would also encode a defective Cap1E polypeptide as a consequence of frameshifting. Moreover, the ^{8086}C-to-T transition together with the deletion would produce a TGA stop codon and, consequently, a prematurely truncated protein. In summary, either one of the two features, i.e., the presence of the IS*1515* or the frameshift mutation, could be responsible for the rough phenotype characteristic of the I41R *S. pneumoniae* strain.

Structural analysis of IS*1515*. The comparative analysis of the nucleotide sequences of $cap1E_{13868}$ and $cap1E_{I41R}$ revealed an 871-bp DNA fragment in the latter, which was inserted after position 334 and flanked by a 3-bp (AAT) duplication (Fig. 2), that showed all of the features characteristic of prokaryotic ISs (9). The IS element contains 12-bp perfect inverted repeat sequences at the ends and was named IS*1515*. It consists of one open reading frame (ORF [provisionally designated tnp_{1515}]) with a potential ATG start codon at positions 375 to 377 and extending to a TAG termination codon at positions 1188 to 1190. The G+C content of the tnp_{1515} gene (41.1%) is similar to the average G+C content of the *S. pneumoniae* genes (39.1%) (23). An extended −10 site (TGtTAT AcT) characteristic of many *S. pneumoniae* promoters (29) is located 6 bp upstream of the ATG initiation codon. Eighteen base pairs upstream of the TATAcT box, a possible -35 region (TTGttc) was found. As already reported for some pneumococcal genes (29), no apparent ribosome-binding site for translation of tnp_{1515} was observed. The tnp_{1515} gene putatively encodes a 271-amino-acid peptide with a predicted pI value (9.53) characteristic of bacterial transposases (9).

Computer searches of the major databases revealed that Tnp_{1515} was similar to several putative transposases. Figure 3 shows a multiple alignment of the predicted amino acid sequences of the transposase of IS*1515* with those of ISL*2* from *Lactobacillus helveticus* (37), IS*702* from *Calothrix* sp. strain PCC 7601 (19), IS*1381* from *S. pneumoniae* (31), and IS*112* from *Streptomyces albus* G (27). Tnp_{1515} was nearly 40% identical to the transposases of ISL*2*, IS*702*, and IS*1381*, whereas that of IS*112* was the most divergent (about 25% identity). In addition, the lengths of the four transposases were very similar, ranging from 257 to 276 amino acid residues. There were no further sequence homologies to other reported IS elements, transposons, or structural genes. All of the features described above strongly suggested that the 871-bp pneumococcal DNA fragment corresponded to a new IS element.

Frequency and distribution of IS*1515* among pneumococcal isolates. To study the distribution of IS*1515* among different pneumococcal isolates, we cloned an internal PCR fragment of IS*1515*, to be used as a probe in hybridization experiments. The recombinant plasmid pRMM34 (Fig. 1B) harbors the 490-bp *Eco*RI/*Apo*I-*Apo*I fragment from IS*1515* (Fig. 2). To determine the number of IS*1515* copies, *Eco*RI-digested chromosomal DNAs from several *S. pneumoniae* isolates of different serogroups were hybridized with pRMM34. Since one

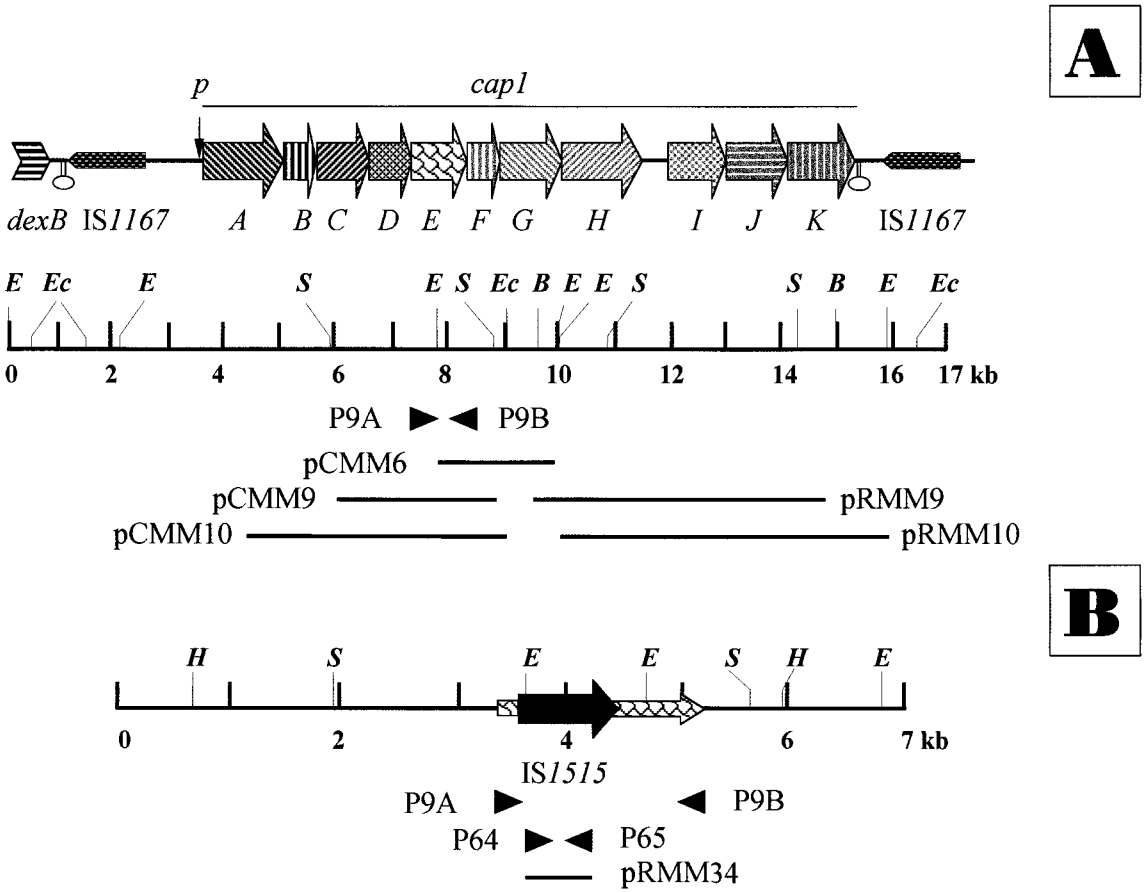

FIG. 1. Genetic organization of the *cap1* cluster of *S. pneumoniae* 13868 containing the genes coding for type 1 capsular polysaccharide synthesis (A) and the *cap1E*$_{I41R}$ gene (B). The partial restriction maps of the corresponding regions are also shown. Thick and thin arrows represent complete or interrupted ORFs, respectively. Some of the plasmids used in this study are indicated, as are relevant restriction sites (*B*, *Bgl*II; *E*, *Eco*RI; *Ec*, *Eco*47III; *H*, *Hin*dIII; and *S*, *Sca*I). The location of the promoter of the *cap1* cluster is also shown (*p*). Ovals represent putative transcription terminators (22). Facing solid triangles indicate the locations and directions of pairs of oligonucleotide primers.

cleavage site for *Eco*RI is present in the IS element but not in the probe (Fig. 2), the number of hybridization bands should reflect the copy number of IS*1515*. This IS (or portions of it) was found in strains of serogroups 1, 5, 19, and 25 (Fig. 4) but not in isolates of serogroups 2, 3, 4, 6, 7, 8, 9, 12, 14, 16, 17, 18, 22, 23, 31, 33, or 37 (not shown). However, since only one isolate of each group has been analyzed so far, these results cannot be taken as a proof of an association between serotype and IS carriage. The apparent copy number in the IS*1515*-positive strains ranged from 2 to 13. It should be mentioned that the common laboratory strain R6 (a type 2 rough derivative) and its descendants (e.g., the M22 strain) did not appear to contain the IS*1515* element.

We have recently proposed that most (if not all) type 1 pneumococcal strains are of clonal origin (22). This suggestion came from studies showing that the molecular organizations of the *cap1* gene cluster and its surrounding regions were virtually identical on otherwise unrelated type 1 *S. pneumoniae* isolates. Southern blot hybridization revealed that 17 of 19 DNAs prepared from *S. pneumoniae* type 1 clinical isolates contained several copies of the insertion sequence (Fig. 4) and that most of them shared a common pattern of bands. Only strains N and L that were isolated in the United States in the late 1930s did not hybridize with the probe. Evidence suggesting the presence of IS*1515* in other gram-positive or gram-negative bacteria analyzed so far was not found (not shown).

Precise excision of IS*1515*. Insertion of a transposon or IS element within a gene prevents the formation of an active gene product. The activity of such a gene can be restored at a low frequency by precise excision of the IS element (9). As documented above, the *cap1E*$_{I41R}$ gene contains, in addition to the IS*1515* element, a frameshift mutation located downstream of the IS copy. Consequently, to restore the S1$^+$ phenotype in the I41R strain, both a precise excision of the IS element and the correction of the mutation are required. When competent I41R cells were transformed with pCMM6, encapsulated transformants were readily isolated (see above), indicating that this double event had been achieved. To provide insights into the detailed mechanisms underlying this finding, we constructed a *lytA* mutant of I41R by transformation of this strain with pGL32, a plasmid that harbors a frameshift mutation in the *lytA* gene coding for the *S. pneumoniae* major autolysin (17). The availability of such a strain (designated I41RΔ*lytA32* hereafter) allows long periods of incubation at 37°C by preventing the autolytic process characteristic of *S. pneumoniae*, thus facilitating the screening of capsule production among the transformed clones. The I41RΔ*lytA32* strain was transformed with pCMM6, and 10 independently isolated S1$^+$ transformants

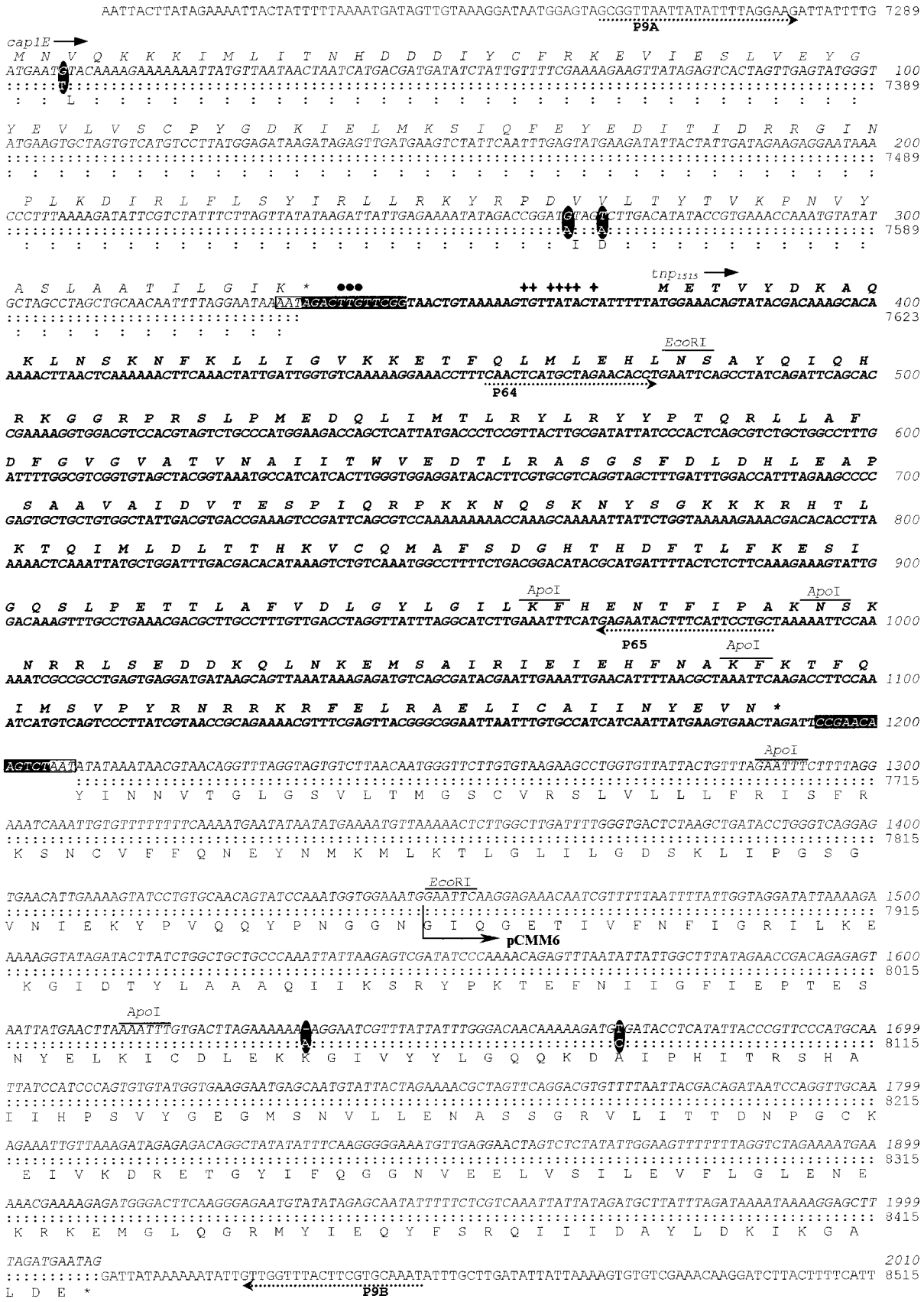
P9A
cap1E
tnp1515
EcoRI
P64
ApoI
P65
pCMM6
P9B

FIG. 2. Nucleotide and deduced amino acid sequences of the $cap1E_{I41R}$ gene containing the IS*1515* element. The sequence of the $cap1E_{13868}$ gene (22) is shown for comparison. The sequence corresponding to the I41R strain is shown in italics, whereas that for the IS*1515* element is represented in boldface italics. When nucleotides (or amino acid residues) coincide, that corresponding to strain 13868 is substituted for by a colon. The nucleotide differences between both *cap1E* genes are highlighted as boldface ellipses. The repeated target sequence AAT is shown inside a white box, whereas the terminal inverted repeat sequences of IS*1515* are inside black boxes. Upstream of the tnp_{1515} gene coding for the IS*1515* transposase, putative extended −10 (✚) and −35 promoter (●) regions are located. The locations and directions of oligonucleotide primers are indicated, as are *Eco*RI-*Apo*I restriction sites. One of the ends of the *Eco*RI insert of plasmid pCMM6 is also shown. Asterisks indicate stop codons.

were studied in detail. Hybridization experiments with DNA prepared from these transformants as the template and pRMM34 as the probe showed that both a 5.2-kb *Hin*dIII band (Fig. 5A) and a 3.7-kb *Sca*I band (Fig. 5B), corresponding to the IS*1515* copy located within the $cap1E_{I41R}$ gene (Fig. 1), were missing in all of the transformants tested, whereas new IS*1515* copies could be observed. This finding indicated that the IS*1515* element is indeed a mobile genetic insertion se-

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IS1381   --MNDEATKQLTDARFKRLVGVQRTTFEEMLAVLKTAYQLKHAKGGRKPK   48
ISL2     --MKYETAKNLNNTRFKRLIGVAKPVFDEMVKVLKAEYQVKHARGGRKPK   48
IS702    --MKIEKAKQLTARKFKRMSGVSRQTFNYMVDVVKAD-EKKKKKPGRRPK   47
IS1515   METVYDKAQKLNSKNFKLLIGVKKETFQLMLEHLNSAYQIQHRKGGRPRS   50
IS112    ---VAGVITASEPSWIAPFSGLSPRQFGKLVTVLRRE-GADAVRKGRPWS   46

IS1381   LSLEDLLMATLQYVREYRTYEEIAADFGIHESNLIRRSQWVEVTLV---Q   95
ISL2     LAIEDLLLATLQYLKEYRTYEQIAADYGVHDSNLIRRSHWAEETLV---K   95
IS702    LIIEDQVLMVIQYWREYRTYYHIGLDWGLSESAVCRTVYKIENILISSRK   97
IS1515   LPMEDQLIMTLRYLRYYPTQRLLAFDFGVGVATVNAIITWVEDTLRASGS  100
IS112    LPLEDRALLVAAYWRTNLTMRQLAPLFGVSKSAADRIIDHLGPMLALQPR   96

IS1381   SGVTISRTPL---SSEDTVMIDATEVKINRPKKELA-NDSGKKKFHAMKA  141
ISL2     HGFNIGKQEI---KPDDVVLIDATEVKIQRPKKDKQLIIPARKSSTVLKA  142
IS702    FSLPGKKELLKMPSQENLVVMDVTESPIERPKKSQKKFFSGKAGEHTLKT  147
IS1515   FDLD------HLEAPSAAVAIDVTESPIQRPKKNQSKNYSGKKKRHTLKT  144
IS112    KRF----------AKDTVLIVDGTLV----PTRDHTIAERSKNYRYSTNH  132

IS1381   QAIV-TSQGRIVSLDIT-VNYCHDMKLFKMS-RRNIGQAGKILADSGYQG  188
ISL2     QAIT-DTTGRIIHLDSC-QAYRHDMRLLRES-RRSLHRSGLILADSGYQG  189
IS702    QLVIHQKTSQIICLGHG-KGRIHDFRLFKTS-GVKFSELLKVIADKGYQG  195
IS1515   QIMLDLTTHKVCQMAFS-DGHTHDFTLFKESIGQSLPETTLAFVDLGYLG  193
IS112    QVVIDADTRLVVVVGRPLAGNRNDCKAWEESGAKAAVGKTLTIADGGYPG  182

IS1381   LMKIYPQAQTPRKSSKLKPLTVEDKACNHALSKERSKVENIFAKVKTFKM  238
ISL2     LDKIYFQAKTPVKSSKKKPLTQQDRELNHLISSIRIKVEHVFGKVKAYKI  239
IS702    ITKIHKLSETPIKKPKGKKLAKEQKEYNRELNRLRIVVEHVNRRLKIFNI  245
IS1515   ILKFHENTFIPAKNSKNRRLSEDDKQLNKEMSAIRIEIEHFNAKFKTFQI  243
IS112    TGLVIPHRRERGQAG----LPDWKEEHNKSHKQVRARVEHVFARMKTWKI  228

IS1381   FSTTYQNHRKRFGLRMNLSAGIINHELGF                       267
ISL2     FSTTYRNHRRRFNLRMNLICGIINQELAI                       268
IS702    LSNQYRNRHRRFGLRSNLIAGIYNYELALKA                     276
IS1515   MSVPYRNRRKRFELRAELICAIINYEVN                        271
IS112    LRDC---RLKGDGVHHAMLGIARMHNLALTG                     257
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	IS*1381*	ISL*2*	IS*702*	IS*112*
IS*1515*	38.0 (53.2)	39.2 (56.9)	39.6 (61.1)	25.4 (44.8)
IS*1381*		58.8 (74.5)	41.3 (62.0)	20.9 (43.8)
ISL*2*			40.4 (63.3)	23.4 (48.4)
IS*702*				27.3 (50.6)

FIG. 3. Alignment of the deduced amino acid sequences of transposases of several IS*1515*-related elements. The multiple alignment was carried out with the PILEUP program. Identical amino acid residues in at least four of the proteins are shown in black boxes, and conserved substitutions in all of the transposases are shown in shaded boxes. Pairwise comparisons of the transposases were done with the BESTFIT program. The percentages of identities and similarities (in parentheses) are indicated.

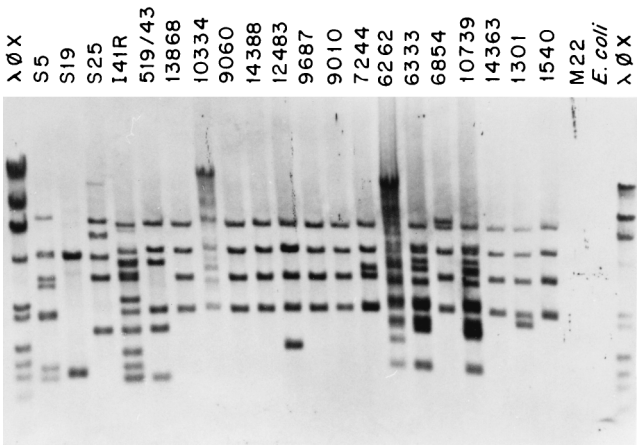

FIG. 4. IS*1515* distribution among strains of *S. pneumoniae* belonging to different serogroups. Southern blotting was performed with *Eco*RI-digested genomic DNAs from the indicated independent isolates, which were electrophoresed on agarose gels. The hybridization was carried out at 65°C with biotin-labeled pRMM34 as the probe. S5, S19, and S25 represent isolates belonging to serogroups 5, 19, and 25, respectively. M22 is a rough derivative of a type 2 strain. All other strains are type 1 isolates. λϕX indicates a mixture of *Hin*dIII-digested λ DNA and the replicative form of ϕX174 DNA digested with *Hae*III.

quence. The selected encapsulated transformants showed at least four different band patterns (Fig. 5). The *cap1E* gene from one of these encapsulated transformants was PCR amplified with oligonucleotides P9A and P9B, and a DNA band of 1.2 kb was obtained (not shown). This size corresponds to that

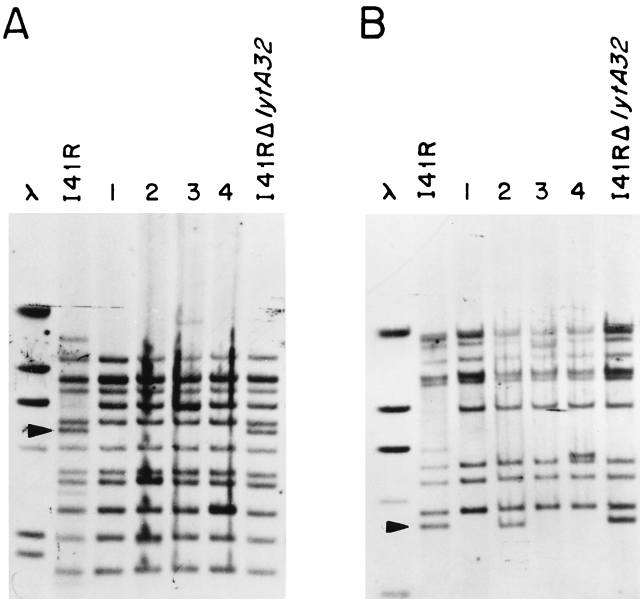

FIG. 5. IS*1515* distribution in encapsulated transformants derived from the I41R strain. Competent cells of I41RΔ*lytA32* were transformed with pCMM6, and encapsulated cells were isolated as described in the text. Chromosomal DNA prepared from four independently isolated transformants (1 through 4) was digested with *Hin*dIII (A) or *Sca*I (B), and the fragments were separated by agarose gel electrophoresis. Southern blotting was performed at 65°C with biotin-labeled pRMM34 as the probe. The profiles of the chromosomal DNAs prepared from the parental strains I41R and I41Δ*lytA32* digested with the same restriction enzymes are also shown. The DNA bands (5.2-kb *Hin*dIII and 3.7-kb *Sca*I fragments, respectively) corresponding to the IS*1515* copy located in the $cap1E_{I41R}$ gene are indicated by arrowheads. λ indicates *Hin*dIII-digested λ DNA.

expected for a *cap1E* gene lacking the IS*1515* element (Fig. 1 and 2). Furthermore, sequencing of the PCR product confirmed the precise excision of the inserted element as well as the elimination of the frameshift mutation (not shown). However, the three $cap1E_{I41R}$ mutations located upstream of the insertion site were still present in the encapsulated transformant, demonstrating that those mutations were not responsible for the unencapsulated phenotype of the I41R strain.

Interestingly, I41RΔ*lytA32*, the strain used as the recipient in the transformation experiment described above, although containing the IS*1515* copy within its *cap1E* gene, showed a band pattern different from that of the parental I41R strain, i.e., the IS copy located in the highest-M_r *Hin*dIII band in I41R disappeared in I41RΔ*lytA32*, whereas this strain exhibited a new copy of the IS located in a DNA fragment with a size of about 6.5 kb (Fig. 5). This observation suggested that apart from the IS*1515* element located in the $cap1E_{I41R}$ gene, at least an additional copy of the IS element is functional in the I41R strain, able to move along the *S. pneumoniae* chromosome, and capable of integrating into different sites of the bacterial genome.

Analysis of excision of IS*1515* by genetic transformation. Since the I41RΔ*lytA32* strain was constructed by transformation of I41R, the results presented above indicated that genetic transformation could be an appropriate tool with which to analyze the excision of IS*1515*. Streptomycin- or lincomycin-resistant isolates were scored after transformation of competent I41R cells with either M22 chromosomal DNA or pLSE1. DNA was purified from several independently isolated transformants, and Southern blot hybridization experiments with pRMM34 as a probe showed that in every transformant, at least one copy of IS*1515* had moved from its original position (Fig. 6). It should be noted that the 1-kb *Eco*RI band corresponding to the IS*1515* copy located in $cap1E_{I41R}$ does not disappear in any of the unencapsulated transformants shown in Fig. 6.

DISCUSSION

The analysis of the nucleotide sequence of a multicopy DNA fragment identified in *S. pneumoniae* showed that this fragment displays the characteristic features of an IS element (24) and has been named IS*1515*. These features included the following. (i) The IS element is bounded by two 12-bp terminal perfect inverted repeats and flanked by two short (3-bp) direct repeats, indicating the duplication of the target sequence. (ii) There is a structure consisting of an ORF (tnp_{1515}) encoding a putative protein with a size of 271 amino acids. This protein is rich in basic amino acid residues (pI 9.53). There is a high degree of similarity with IS*1381*, recently characterized in pneumococcus (31), as well as with two ISs detected in three gram-positive species, i.e., ISL*2* from *L. helveticus* (37), IS*702* from *Calothrix* sp. (19), and IS*112* from *S. albus* (27). In contrast, no significant similarity was detected when IS*1515* was compared to IS*1202* (21) and IS*1167* (36), two ISs previously described in *S. pneumoniae.*

We have shown here the presence of a copy of IS*1515* in the $cap1E_{I41R}$ gene of an unencapsulated strain of pneumococcus. This gene has been identified as part of the *cap1* cluster coding for the polysaccharide capsule of type 1 pneumococcus (22). Hybridization tests revealed that the genome of I41R contains at least 13 copies of IS*1515*, but this finding does not necessarily imply that all of these copies were identical or even functional. The IS reported here was present in multiple copies in most type 1 strains of pneumococcus (17 of 19 isolates tested) but not in the majority of pneumococci of other sero-

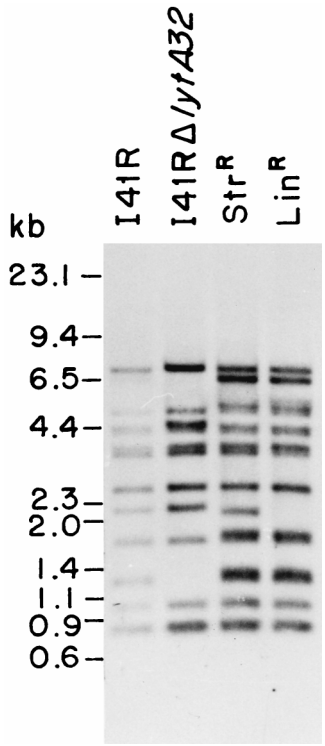

FIG. 6. IS*1515* distribution in streptomycin (Str^R)- or lincomycin (Lin^R)-resistant transformants of the I41R strain. Competent cells of I41R were transformed for the $LytA^-$ phenotype with pGL32, for streptomycin resistance with M22 chromosomal DNA, or for lincomycin resistance with pLSE1. Total DNA from one transformant of each class was digested with *Eco*RI, and the fragments were separated by agarose gel electrophoresis, blotted, and hybridized at 65°C with biotin-labeled pRMM34. The molecular sizes (in kilobases) of the standards (a mixture of *Hin*dIII-digested λ DNA and the replicative form of φX174 DNA digested with *Hae*III) are indicated to the left.

types studied (18 of 21 serotypes analyzed). Fingerprinting analyses of type 1 pneumococci like the one carried out here with IS*1515* as a marker, alone or in combination with other techniques, such as pulsed-field gel electrophoresis, may be of value in the epidemiological survey of type 1 pneumococcal infection.

One of the fundamental points in understanding the transposition is the specificity with which the ISs insert themselves into the target DNA. Specificity may result from the recognition of a specific sequence (hot spots), a structural or functional feature of the DNA, or a combination of these factors (9). The target site for the insertion of IS*1515* in the *cap1E*$_{I41R}$ gene is preceded by the sequence (319) 5′-ATTTTAGGAAT AAAAT-3′ (the underlined bases correspond to the direct repeat) that have a potential to form a stem-loop structure (Fig. 2). On the other hand, the region flanking the IS*1515* element is particularly rich in AT base pairs (86% over 35 bp). It has been reported that the insertion hot spots for IS*1* have high A+T contents (20), although this feature does not appear to be the only factor influencing the IS*1* site selection (35). Very recently, a preliminary nucleotide sequence covering up to 90% of the genome of a type 4 pneumococcal strain has been released (available upon request). Only one copy of the IS*1515* element, located immediately downstream of a putative ORF containing a gene encoding a PhoH homolog (contig no. 4139), appears to be present in the pneumococcal strain studied. This copy differs in two nucleotides from that reported here: a T-to-G transversion and a deletion of a TA pair at positions 418 and 423, respectively, and, thus, it codes for a truncated, putatively inactive transposase. Apart from the 3-bp target duplication (AAT) that is also conserved in this type 4 pneumococcus, no other obvious similarities in the regions flanking the IS could be found. Therefore, additional work should be carried out in order to ascertain the requirements of IS*1515* for insertion into the *S. pneumoniae* chromosome. Precise excision of the IS presumably removes one of the direct repeats together with the inserted material. The molecular analysis of the *cap1E*$_{I41R}$ gene from DNA prepared from a type 1 encapsulated strain obtained by transformation of I41R with pCMM6 revealed that the corresponding copy of IS*1515* underwent a precise excision.

Transposition frequency can be regulated indirectly through the control of transposase level or directly by modulation of the recombination reaction by alterations in transposase activity and the reactivity of the DNA substrates. The regulatory mechanisms used by transposable elements to control transpositions are numerous and act at virtually every level of gene expression (for a recent review, see reference 4). Certain proteins regulate transposase activity by interacting with transposase or by competing with transposase for DNA binding sites. This appears to be the case for the IS1 transposase, which, as reported for several IS elements, is synthesized by translational frameshifting in the −1 direction (24). The N-terminal DNA-binding domain can act as a negative regulator of transposition, presumably by excluding intact transposase from the ends of the element by competitive binding. An atypical +1 translational frameshift has recently been claimed to occur for the *S. pneumoniae* IS*1381* transposase (31). In contrast, the IS*1515* element gene encodes a complete transposase that does not require any frameshifting to be synthesized and that confers full functionality on the IS element. An attractive hypothesis is that transposition is modulated by the cellular environment, there being certain cellular conditions under which transposition will be favored and other conditions under which it will be disfavored (4). It is well known that many host mutations that increase excision are in genes implicated in DNA repair (9). The role of RecA in the transposition process, however, remains unclear, although it has been reported that transposition is a RecA-dependent process in several systems, such as IS*2* (6) and IS*30* (8). Transposition of IS*1515* takes place during genetic transformation (Fig. 5 and 6), although we cannot conclude from these experiments whether transforming DNA or the development of competence itself (or both) is responsible for excision of the IS. It has recently been demonstrated that transcriptional activation of the *S. pneumoniae recA* gene occurs at competence (18). It would be interesting to test for transposition of the IS*1515* element in a *recA* background. Unfortunately, since *recA* pneumococcal strains are not transformable and mutants with conditional mutation of this gene have not been isolated so far, the role of RecA in transposition in *S. pneumoniae* remains an open question. On the other hand, transposition of IS*911*, a member of the IS*3* family of ISs isolated from the enterobacterium *Shigella dysenteriae*, exhibits a temperature-sensitive phenotype; i.e., whereas transposition was optimal at 30°C, it was greatly reduced at 42°C (15). Since the incubation of competent pneumococcal cells with transforming DNA is carried out at 30°C, we cannot discard the possibility that the transposase of the IS*1515* may be also stimulated at this temperature. New experimental tools are to be designed for *S. pneumoniae* in order to achieve conclusive answers to the above questions.

The presence of a large number of IS elements might be expected to have a strong influence on the structure and stability of the genome, since, in addition to their transposition

properties, ISs act as substrates for homologous recombination. Although other ISs have been found in pneumococci, as reported here, IS*1515* represents the first example of a functional IS element in this important human pathogen.

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