JOURNAL OF BACTERIOLOGY, July 1994, p. 4437–4443
0021-9193/94/$04.00+0

Vol. 176, No. 14

NOTES

Isolation, Characterization, and Nucleotide Sequence of IS*1202*, an Insertion Sequence of *Streptococcus pneumoniae*

JUDY K. MORONA,[1] ANGELO GUIDOLIN,[1] RENATO MORONA,[2] DAVID HANSMAN,[1] AND JAMES C. PATON[1]*

Department of Microbiology, Women's and Children's Hospital, North Adelaide, South Australia, 5006,[1] and Department of Microbiology and Immunology, University of Adelaide, Adelaide, South Australia, 5005,[2] Australia

Received 6 December 1993/Accepted 30 April 1994

A comparative hybridization protocol was used to isolate a small segment of DNA present in the *Streptococcus pneumoniae* type 19F strain SSZ but absent from strain Rx1, a nonencapsulated derivative of the type 2 strain D39. This segment of DNA is a 1,747-bp insertion sequence, designated IS*1202*, flanked by 23-bp imperfect inverted repeats and containing a single open reading frame sufficient to encode a 54.4-kDa polypeptide. A 27-bp target sequence is duplicated at either end of the element. IS*1202* is not related to any of the currently known insertion elements and is the first reported for *S. pneumoniae*. Although found predominantly in type 19F strains in up to five copies, it has also been shown to be present in the chromosomes of pneumococci belonging to other serotypes. One of the four IS*1202* copies in the encapsulated strain SSZ is located 1,009 bp downstream of the *dexB* gene, and transformation studies reveal that it is also closely linked to the type 19F capsular polysaccharide synthesis (*cps*) locus.

Streptococcus pneumoniae (the pneumococcus) is an important human pathogen, causing invasive diseases such as pneumonia, bacteremia, and meningitis. Morbidity and mortality from pneumococcal infections remain high, even in regions where effective antibacterial therapy is freely available (3, 21).

An important feature of *S. pneumoniae* is its capacity to produce a polysaccharide capsule, which is structurally distinct for each of the 84 known serotypes of the organism. The polysaccharide capsule is considered to be the sine qua non of pneumococcal virulence (3). This is based on the observations that virtually all fresh clinical isolates of *S. pneumoniae* are encapsulated and that spontaneous nonencapsulated (rough) derivatives of such strains are almost completely avirulent. Moreover, an early study demonstrated that enzymic depolymerization of the capsule of a type 3 pneumococcus increased its 50% lethal dose approximately 10^6-fold (4). More recently, a similar effect on virulence of type 3 *S. pneumoniae* was achieved by transposon mutagenesis of a gene essential for capsule production (28). The precise manner in which the pneumococcal capsule contributes to virulence is not fully understood, although it is known to have strong antiphagocytic properties in nonimmune hosts (3, 21).

During our investigations of the capsular polysaccharide synthesis (*cps*) locus of the *S. pneumoniae* type 19F strain SSZ, we have identified a segment of pneumococcal DNA that exhibits all the hallmarks of a bacterial insertion sequence. One of the four copies of this element, designated IS*1202*, has inserted directly downstream of the pneumococcal *dexB* gene and is closely linked to the type 19F *cps* locus in strain SSZ. Here, we describe the sequence and characterization of IS*1202*, the first insertion sequence to be isolated from *S. pneumoniae*.

Bacterial strains and cloning vectors. The *S. pneumoniae* strains used were Rx1, a nonencapsulated, highly transformable derivative of type 2 strain D39 (26), and SSZ, a type 19F strain obtained from Chi-Jen Lee, Center for Biologics, Food and Drug Administration, Bethesda, Md. A derivative of Rx1 expressing type 19F capsule was constructed by transformation (see below) of Rx1 with DNA from strain SSZ. Production of type 19F capsule by one of the smooth transformants was confirmed by Quellung reaction, and DNA was extracted from this strain and used to transform Rx1 a second time. One of the type 19F encapsulated secondary transformants was designated Rx1-19F and used in further studies. Clinical isolates belonging to types 19A, 19B, and 19C were also obtained from Chi-Jen Lee; other clinical isolates were from the Women's and Children's Hospital, Adelaide, South Australia, Australia. Pneumococci were routinely grown in Todd-Hewitt broth with 0.5% yeast extract (THY) or on blood agar.

Escherichia coli K-12 DH1 (12) and DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) were grown in Luria-Bertani broth (19) with or without 1.5% Bacto-agar (Difco Laboratories, Detroit, Mich.). Where appropriate, chloramphenicol, ampicillin, or kanamycin was added to the growth medium at concentrations of 25, 50, and 50 μg/ml, respectively.

The low-copy-number, amplifiable cosmid vector pOU61*cos* has been described previously by Knott et al. (16). Plasmids pK194 (15) and pVA891 (18) have also been described previously. Phagemid pBluescript SK$^+$ was obtained from Stratagene, La Jolla, Calif.

DNA techniques. *S. pneumoniae* chromosomal DNA used in Southern hybridization experiments was extracted and purified as described previously (22). Southern hybridization analysis was carried out with digoxigenin-labelled probes, as described previously (6). When the DNA was to be used for the

* Corresponding author. Mailing address: Department of Microbiology, Women's and Children's Hospital, North Adelaide, S.A., 5006, Australia. Phone: 61-8-204-6302. Fax: 61-8-204 6051.

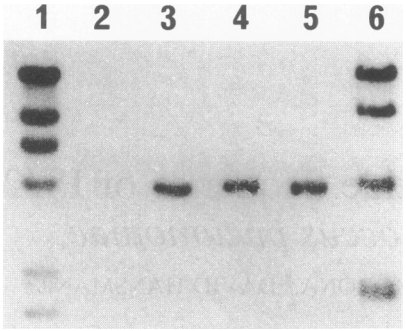

FIG. 1. Southern hybridization analysis of Rx1 transformants expressing type 19F capsule. Chromosomal DNA was digested with *Cla*I, electrophoresed, blotted, and hybridized with a digoxigenin-labelled insert of pJCP451. Lanes: 1, DNA size markers (digoxigenin-labelled lambda DNA digested with *Hin*dIII; fragment sizes, 23.1, 9.4, 6.6, 4.37, 2.3, and 2.0 kb); 2, Rx1; 3 and 4, two independent primary Rx1 transformants expressing type 19F capsule; 5, Rx1-19F; 6, type 19F strain SSZ.

construction of cosmid libraries or for PCR walking, the final ethanol precipitation step was replaced by overnight dialysis at 4°C against 10 mM Tris-HCl–1 mM EDTA (pH 8.0) (TE). For *S. pneumoniae* transformation experiments, the DNA extraction procedure was as described previously (8).

Plasmid DNA was isolated by the alkaline lysis method as described by Morelle (20). Cosmid DNA was purified from cells grown at 30°C for 16 h and subjected to heat induction at 42°C for 2 h.

Transformation of *E. coli* with plasmid DNA was carried out with $CaCl_2$-treated cells as described by Brown et al. (10). *S. pneumoniae* Rx1 and the encapsulated strain Rx1-19F were transformed with chromosomal or plasmid DNA as described previously for Rx1 and D39, respectively (8). Where appropriate, transformants were selected on blood agar containing 0.2 μg of erythromycin per ml.

Construction and screening of cosmid libraries by comparative hybridization. The original purpose of this study was to isolate DNA sequences determining type 19F *S. pneumoniae* capsule production. Strain Rx1 is a nonencapsulated derivative of the type 2 strain D39, and type 2 polysaccharide is structurally distinct from that of type 19F. Thus, the genes which encode their biosynthesis are likely to differ. A derivative of Rx1 expressing type 19F capsule (Rx1-19F) was constructed as described above. Rx1-19F contains SSZ-derived DNA sufficient to confer type 19F capsule production. Thus, clones from an SSZ library which hybridize to labelled chromosomal DNA from Rx1-19F more strongly than to labelled Rx1 chromosomal DNA would be expected to contain DNA sequences associated with 19F capsule production.

Cosmid banks of *S. pneumoniae* SSZ were constructed with pOU61*cos*, as described previously (6). Replicates of the library were grown in microtiter trays at 30°C overnight, and cosmid DNA was then amplified by heat induction at 42°C for 2 h. The plates were centrifuged, and lysates were prepared by resuspending the cell pellets in 10 μl of TE and then adding 5 μl of 10% sodium dodecyl sulfate. After mixing, 50 μl of 0.5 M sodium hydroxide–1.5 M sodium chloride was added to each well and the plates were mixed and centrifuged again. Aliquots of the supernatants (3 μl) were spotted onto nylon membranes.

Duplicate DNA dot blots of clones from the SSZ cosmid library were probed with digoxigenin-labelled chromosomal DNA from strains Rx1 and Rx1-19F. Cosmid clones which appeared to react more strongly with the latter probe were further analyzed by Southern hybridization. One cosmid contained a 1.8-kb *Bam*HI fragment that hybridized with labelled chromosomal DNA from Rx1-19F but not with labelled Rx1 DNA. The remaining cosmid-derived restriction fragments reacted with both chromosomal probes or with a pOU61*cos* probe (data not shown). The 1.8-kb *Bam*HI fragment was subcloned into the *Bam*HI site of plasmid pK194 to give plasmid pJCP451. Southern hybridization analysis using the insert of pJCP451 as a probe (Fig. 1) revealed that this fragment hybridized to DNA from Rx1 transformants expressing type 19F capsule but not to Rx1 DNA. However, the probe reacted with four *Cla*I fragments in digested SSZ DNA, one of which was common to Rx1-19F (Fig. 1). The presence of multiple copies of the reactive sequence in the SSZ genome and its absence from Rx1 suggested that it might constitute part of a repetitive DNA element such as an insertion sequence or a transposon, so it was subjected to further characterization.

Southern hybridization analysis using the digoxigenin-labelled 1.8-kb *Bam*HI fragment was used to generate a physical map of the region of the Rx1-19F chromosome surrounding this SSZ-derived sequence (Fig. 2). One of two *Hin*dIII sites present in the pJCP451 insert was not found in the Rx1-19F chromosome. This could be accounted for by polymorphism

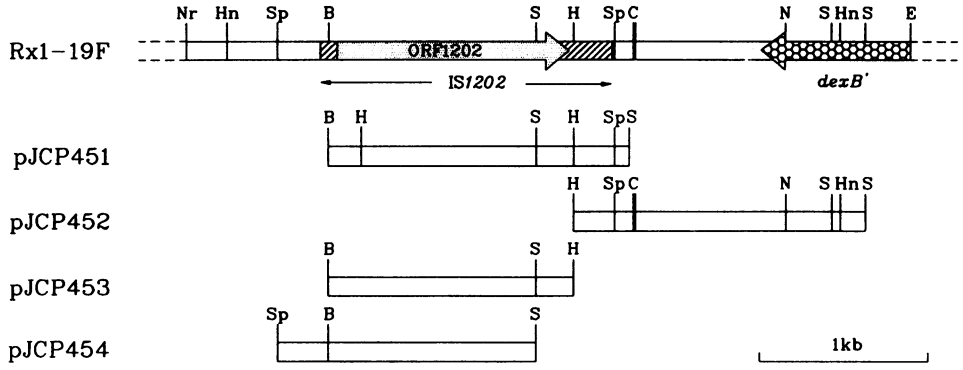

FIG. 2. Genomic restriction map of the Rx1-19F chromosome, generated by Southern hybridization analysis using the insert of pJCP451 as a probe. The positions of the pneumococcal DNA inserts in pCJP451, pJCP452, pJCP453, and pJCP454 are also shown. The location of IS*1202* (cross-hatched region) and the position of ORF1202 and the partial ORF of *dexB* (shaded arrows) are indicated. Restriction sites are indicated as follows: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Hn, *Hin*dII; N, *Nco*I; Nr, *Nru*I; S, *Sau*3A; Sp, *Spe*I.

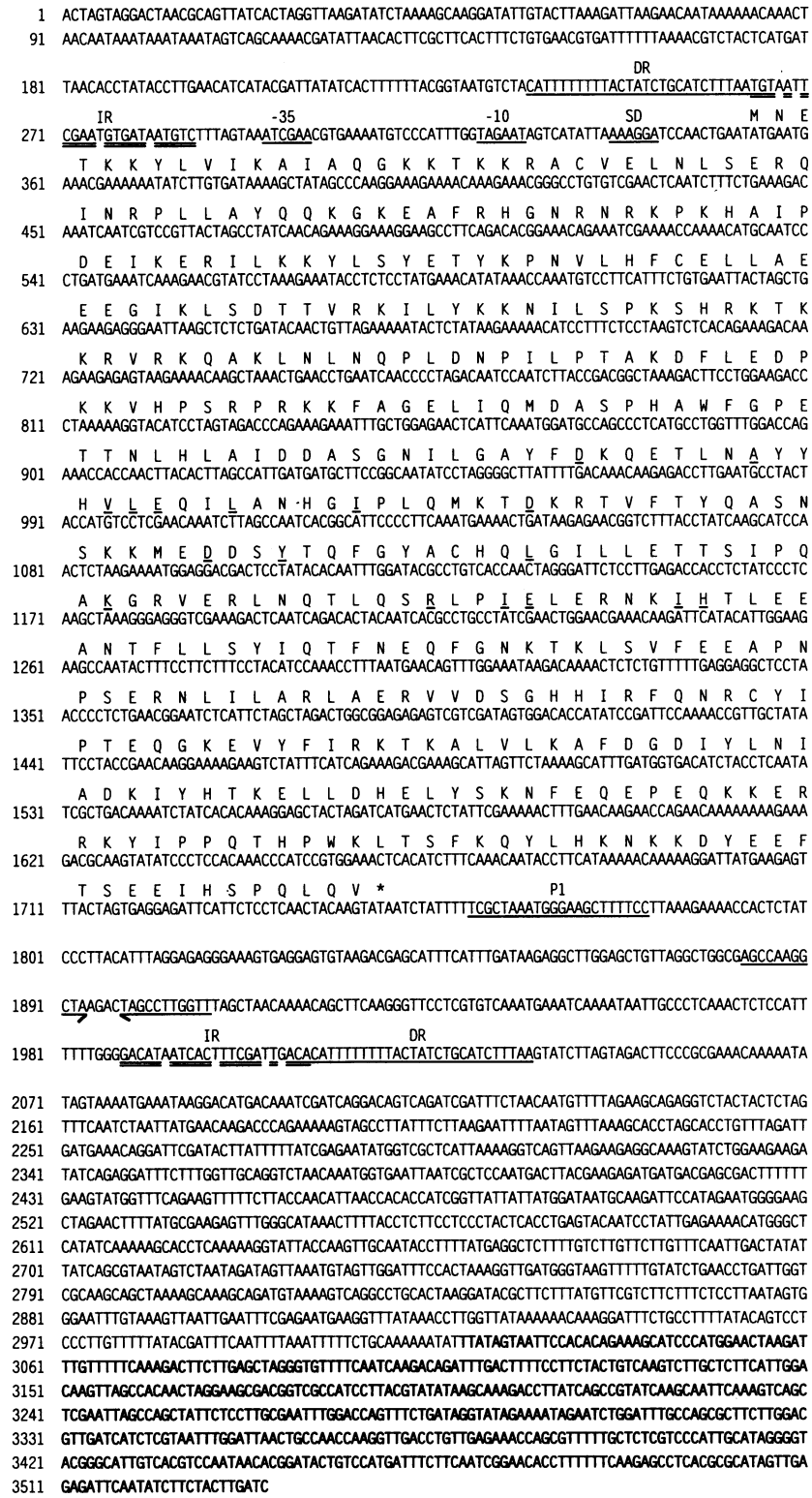

FIG. 3. Nucleotide sequence of IS*1202* and flanking sequences. Nucleotide positions 264 and 2010 define the left and right ends, respectively, of IS*1202*; the left and right terminal inverted repeats (IR) are double underlined except for mismatched nucleotides, and the directly repeated target sequence (DR) is underlined. The amino acid translation of ORF1202 is represented by single-letter code above the first nucleotide of each codon. Residues that are identical or similar to those of the consensus motifs of other transposases (23), a putative ribosome binding site (Shine-Dalgarno [SD] sequence), putative −10 and −35 promoter regions, and the sequence of the PCR primer P1 are also underlined. A potential transcription terminator sequence (inverted arrows) is indicated. Nucleotides 3019 to 3535 (shown in boldface type) encode the C-terminal portion of the *dexB* ORF on the minus strand.

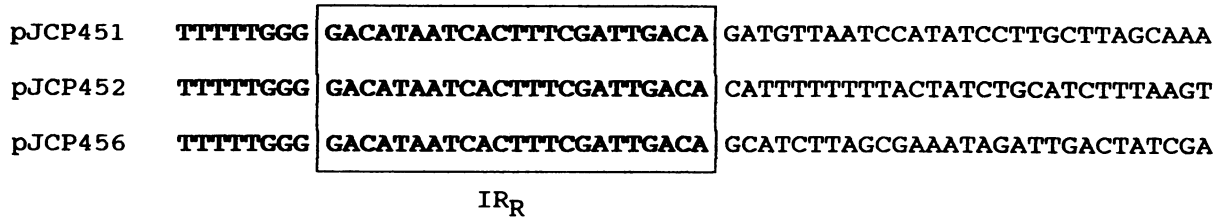

FIG. 4. Alignment of sequences surrounding the right terminal repeats (IR_R) for three of the integration sites of IS*1202* in the *S. pneumoniae* SSZ genome. Sequence data for the three sites were obtained from pJCP451, pJCP452, and a third cloned PCR-walking product of SSZ DNA, designated pJCP456. IS*1202* sequences are shown in boldface.

between the different genomic copies of this sequence in SSZ if the copy of the repetitive element in pJCP451 is different from the copy transformed into Rx1 during construction of Rx1-19F. Screening of a large Rx1-19F cosmid library (2,000 cosmids) with the insert of pJCP451 used as a probe revealed that this region of the pneumococcal chromosome was not represented in the Rx1-19F library, suggesting that this segment of DNA may be unstable in *E. coli* K-12. The complete nucleotide sequence of the 1.8-kb *Bam*HI fragment was determined by using various nested deletion derivatives of pJCP451 as templates (constructed by the method of Henikoff [13] with an Erase-a-base kit [Promega Corporation, Madison, Wis.]). Double-stranded DNA sequencing was carried out by using dye-labelled primers on an API model 373A automated DNA sequencer.

PCR walking. The pJCP451 sequence data were used to design oligonucleotide primers P1 (5′-TCGCTAAATGGG AAGCTTTTCC-3′) (the location of P1 is shown in Fig. 3) and P2, which is the complement of P1, for amplification of flanking sequences in the Rx1-19F chromosome by PCR walking, as described by Willoughby et al. (29). PCR products obtained with Rx1-19F template DNA were electrophoretically purified and subcloned into pBluescript SK^+, to generate plasmids pJCP452 and pJCP453, as shown in Fig. 2. Southern hybridization analysis indicated that the PCR product in pJCP452 hybridized to both Rx1 and Rx1-19F DNAs, whereas the PCR product in pJCP453 hybridized with Rx1-19F DNA only (data not shown).

Sequence analysis. The nucleotide sequences of the pneumococcal DNA inserts of pJCP452 and pJCP453 were determined from both DNA strands. The region of DNA 5′ to the *Bam*HI site of pJCP453 was obtained by *Cla*I digestion of chromosomal DNA from Rx1-19F-B1 (see below) followed by self-ligation and transformation into *E. coli* DH1. A 1.8-kb *Spe*I-*Sal*I fragment spanning and extending 340 bp 5′ to the *Bam*HI site was subcloned from this construct into pBluescript SK^+ to generate pJCP454 (Fig. 2), and both strands of this DNA were also sequenced. The sequence was analyzed by using DNASIS version 7 software (Hitachi Software Engineering, San Bruno, Calif.).

Inspection of the compiled sequence data (Fig. 3) revealed a segment of DNA between nucleotides 264 and 2010 that exhibited all the characteristics of an insertion element. This segment is 1,747 bp long, with imperfect terminal inverted repeats of 23 bp (four mismatches). Insertion elements encode at least one protein which is involved in the transposition process. These proteins, called transposases, are basic and directly bind target DNA (2, 11). The left and right inverted repeats of IS*1202* are separated by a single open reading frame (ORF), which encodes a protein with a predicted M_r of 54,420 Da. A ribosome binding site precedes the ATG start codon, and a potential promoter is located 27 bp upstream of the initiation codon. A stemmed loop structure resembling a rho-independent transcription terminator sequence (Gibbs free energy, −17.3 kcal [ca. −72.4 kJ]/mol) is located 132 bp downstream from the termination codon (nucleotides 1883 to 1908). The repetitive element is flanked by an unusually long duplicated target sequence of 27 bp. Together, these data strongly suggest that this portion of the Rx1-19F genome (originating from strain SSZ) represents a bona fide insertion sequence, the first to be described for *S. pneumoniae*, and it has been designated IS*1202*.

The putative translation product of the ORF was designated ORF1202. Data base searches with IS*1202* or ORF1202 did not reveal any significant homology at the DNA or protein level with other known insertion sequences or transposable elements, or indeed with any other sequence. However, ORF1202 does contain the consensus motifs common to many unrelated transposases, described recently by Rezsöhazy et al. (23), as shown in Fig. 3. Moreover, it shares the general characteristic of transposases of being highly basic in nature (the predicted pI is 9.84).

Comparisons of the complete IS*1202* sequence and portions of two other copies either contained in pJCP451 or obtained by PCR walking with SSZ DNA (which contains four copies) as a template were also made. Within IS*1202*, a number of DNA polymorphisms were found, one of which accounts for the presence of a second *Hin*dIII site in the pJCP451 copy, as previously predicted by genomic restriction mapping.

Analysis of the sequence surrounding the 3′ ends of three copies of IS*1202* (Fig. 4) indicated that the repetitive element always diverged at exactly the same position, that is, after the terminal inverted repeat sequence. This provided additional evidence that IS*1202* is indeed a mobile genetic element.

Distribution of IS*1202*. The distribution of IS*1202* in four other type 19F clinical isolates of *S. pneumoniae*, single type 19A, 19B, and 19C isolates, and a further 14 clinical isolates belonging to serotypes 2, 3, 4, 6, 14, 18C, and 23F was investigated by Southern hybridization (Fig. 5). Of the eight group 19 strains tested (including SSZ), all but one type 19F strain hybridized with the IS*1202* probe. The copy number in those strains containing IS*1202* varied from one to five. One of three group 6 strains tested contained a single IS*1202*-related sequence, and one of three type 14 strains tested reacted weakly with the probe. The remaining 12 strains, belonging to serotypes 2, 3, 4, 6, 14, 18C, and 23F, did not contain IS*1202*-related DNA sequences.

Chromosomal location of IS*1202* in Rx1-19F. BLASTX analysis (1) of the data presented in Fig. 3 indicated that nucleotides 3019 to 3535 encode the distal portion of an ORF on the minus strand, which has 65% homology to the terminal 180 amino acids of the *dexB* gene product (glucan-1,6-α-glucosidase) from *Streptococcus mutans* (25). The translated product of this partial ORF is also homologous to other

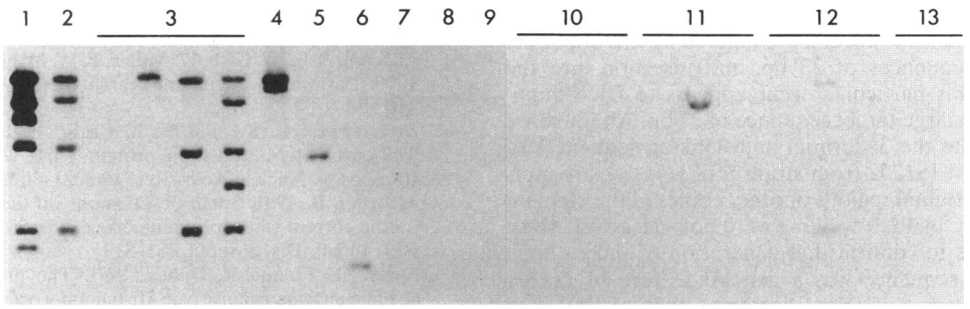

FIG. 5. Distribution of IS*1202* among clinical isolates of *S. pneumoniae* belonging to various serotypes or groups. Chromosomal DNA from the indicated strains was digested with *Cla*I, electrophoresed, blotted, and hybridized with the insert of pJCP451. Lanes: 1, DNA size markers (fragment sizes are the same as for Fig. 1); 2, type 19F strain SSZ; 3, four different type 19F clinical isolates; 4, type 19A; 5, type 19B; 6, type 19C; 7, type 2 strain D39; 8, type 3; 9, type 23F; 10, three different type 4 isolates; 11, three different group 6 isolates; 12, three different type 14 isolates; 13, two different group 18 isolates.

glucosidases from the same family as *dexB* (Fig. 6) and therefore is likely to represent part of the equivalent gene of *S. pneumoniae*; in Rx1-19F, IS*1202* has inserted 1 kb downstream from this gene. In other streptococci, *dexB* genes form part of a multiple-sugar metabolism (*msm*) operon (27).

Initial experiments on transformation into Rx1 indicated that IS*1202* cotransformed with the type 19F *cps* locus to form Rx1-19F. To investigate the influence of the product of ORF1202 on capsule biosynthesis in Rx1-19F, this gene was interrupted by insertion-duplication mutagenesis. This was achieved first by subcloning an internal fragment of ORF1202 (nucleotides 493 to 1569) into pVA891, a vector previously employed for interruption of the pneumolysin and autolysin genes of *S. pneumoniae* (5, 7, 8). The recombinant plasmid, designated pJCP455, was then used to transform Rx1-19F. Homologous recombination between the internal ORF1202 fragment and the Rx1-19F chromosome generated an erythromycin-resistant transformant, designated Rx1-19F-B1. Southern hybridization analysis indicated that Rx1-19F-B1 contained a single chromosomal copy of pJCP455 integrated within ORF1202. Phenotypic examination revealed that Rx1-19F-B1 continued to produce apparently normal levels of type 19F capsule.

The erythromycin-resistant marker disrupting ORF1202 in Rx1-19F-B1 was then exploited to study the linkage between IS*1202* and the type 19F *cps* locus. Chromosomal Rx1-19F-B1 DNA was used to transform the nonencapsulated strain Rx1, and erythromycin-resistant transformants were phenotypically screened for cotransformation of the capacity to produce type 19F capsule. In three independent transformation experiments, 5.5, 4.7, and 9.5% (mean, 6.5%) of the erythromycin-resistant transformants expressed the type 19F capsule. Moreover, during the initial experiments involving transformation of strain Rx1 with SSZ DNA, all encapsulated primary and secondary transformants generated also contained a single copy of IS*1202* located on a 4.2-kb *Cla*I fragment (Fig. 1). This degree of cotransformation confirms the close linkage between the 19F capsule locus and the copy of IS*1202* present in Rx1-19F.

Conclusions and discussion. This paper describes the characterization of a region of DNA which is unique to the encapsulated derivative of Rx1, Rx1-19F. However, this segment was not involved in capsule production, but rather contained IS*1202*, the first insertion sequence to be described for *S. pneumoniae*. IS*1202* has all the general characteristics of a transposable insertion element; it is a short segment of DNA

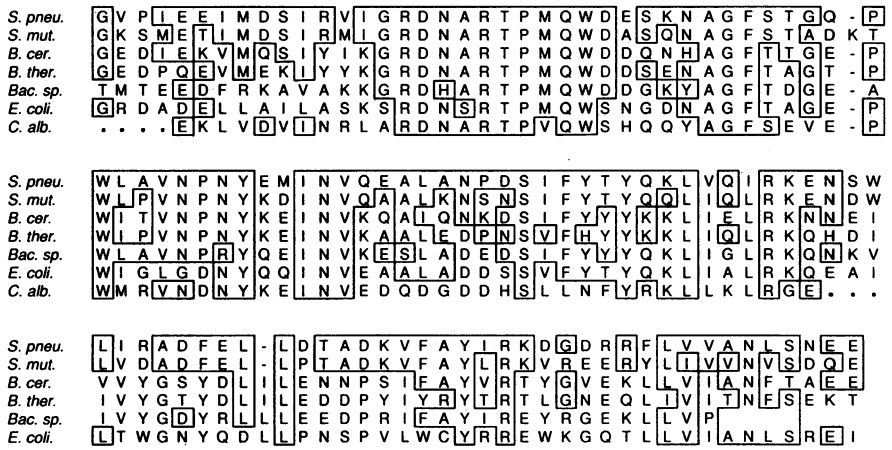

FIG. 6. Amino acid sequence alignment of the C-terminal portions of the glucan-1,6-α-glucosidases from *S. pneumoniae* (*S. pneu.*), *S. mutans* (*S. mut.*), *Bacillus cereus* (*B. cer.*), *B. thermoglucosidasius* (*B. ther.*), *Bacillus* sp. (*Bac. sp.*), *E. coli*, and *Candida albicans* (*C. alb.*). Data for *S. mutans* are from Russell and Ferretti (25), while those for the other species are deposited in GenBank under accession numbers X53507, D10487, D00638, L06097, and M94674, respectively. Alignments were performed with the program CLUSTAL (14). Dashes indicate absence of a residue, while dots indicate that sequence data are not available.

(1,747 bp) which is found in one or more copies in the bacterial chromosome. The ends of IS*1202* are defined by two imperfect inverted repeat sequences of 23 bp, and insertion into the chromosome in this particular event appears to have duplicated an unusually large target sequence of 27 bp. Although we were able to isolate the 3′ termini and flanking sequences of two other copies of IS*1202* from strain SSZ (Fig. 4), attempts to isolate the 5′-terminal regions of other copies of the element by direct cloning or by PCR walking were not successful. Thus, it was not possible to confirm that generation of such a large duplicated target sequence was a general feature of IS*1202* transposition. It was possible, therefore, that IS*1202* had integrated into a preexisting directly repeated sequence. To examine this, we PCR amplified and sequenced a segment of DNA, corresponding to the IS*1202* integration site of strain Rx1-19F, from the type 19F strain that did not contain the element (Fig. 5). No directly repeated sequence was found at the integration site (data not presented). Thus, integration of IS*1202* was indeed responsible for generation of the 27-bp sequence duplication.

IS*1202* appears to be unrelated to any previously characterized insertion element and may therefore have originated in *S. pneumoniae*. However, given the capacity of pneumococci to undergo natural transformation, the possibility that IS*1202* was acquired from another bacterial species cannot be eliminated. The G+C content of IS*1202* (39.5%) is within the range reported for members of the genus *Streptococcus* (9).

Analysis of the DNA sequence data has shown that IS*1202* in Rx1-19F and one of the copies of IS*1202* in SSZ have integrated into a large (approximately 1-kb) intergenic region immediately downstream from what is believed to be the pneumococcal *dexB* gene. Transformation studies also indicate close linkage to the type 19F *cps* locus. In experiments using donor DNA from Rx1-19F-B1 (a derivative of Rx1-19F in which ORF1202 had been interrupted by insertion-duplication mutagenesis with pJCP455), erythromycin resistance (encoded by the pJCP455 sequences) and type 19F capsule production showed an average of 6.5% cotransformation. This linkage is likely to be highly significant, because the minimum amount of DNA required to encode the type 19F capsule is probably in the range of 10 to 15 kb and pJCP455 is approximately 6 kb in length. Thus, even if the loci are very close together, a double recombination event involving at least 20 kb of DNA would be required for cotransformation. The observed close linkage between the markers is also supported by the finding that when Rx1 was transformed with SSZ DNA, all encapsulated transformants also contained IS*1202*.

Interestingly, the group B streptococcus type III *cps* locus is located approximately 2 kb downstream from an unrelated insertion sequence, IS*861* (24), which, like IS*1202*, does not appear to interfere with capsule synthesis. Also, the *Haemophilus influenzae* type b encapsulation locus is located on a large transposon flanked by two copies of IS*1016* (17). It is unlikely that the type 19F capsule locus is located on a similar mobile element flanked by IS*1202*, as some type 19F strains contain only one copy and at least one strain lacks the element altogether.

Nucleotide sequence accession number. The nucleotide sequence described in this paper has been deposited with GenBank under accession number U04047.

We thank Andrew Lawrence and Anne Berry for assistance with the Quellung reaction and pneumococcal transformation, respectively.

This work was supported by grants from the World Health Organization Programme for Vaccine Development and the National Health and Medical Research Council of Australia.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403–410.
2. **Arciszewska, L. K., and N. L. Craig.** 1991. Interaction of the Tn7-encoded transposition protein TnsB with the ends of the transposon. Nucleic Acids Res. **19**:5021–5029.
3. **Austrian, R.** 1981. Some observations on the pneumococcus and on the current status of pneumococcal disease and its prevention. Rev. Infect. Dis. **3**(Suppl.):S1–S17.
4. **Avery, O. T., and R. Dubos.** 1931. The protective action of a specific enzyme against type III pneumococcus infections in mice. J. Exp. Med. **54**:73–89.
5. **Berry, A. M., R. A. Lock, D. Hansman, and J. C. Paton.** 1989. Contribution of autolysin to the virulence of *Streptococcus pneumoniae*. Infect. Immun. **57**:2324–2330.
6. **Berry, A. M., R. A. Lock, S. M. Thomas, D. P. Rajan, D. Hansman, and J. C. Paton.** 1994. Cloning and nucleotide sequence of the *Streptococcus pneumoniae* hyaluronidase gene and purification of the enzyme from recombinant *Escherichia coli*. Infect. Immun. **62**:1101–1108.
7. **Berry, A. M., J. C. Paton, and D. Hansman.** 1992. Effect of insertional inactivation of the genes encoding pneumolysin and autolysin on the virulence of *Streptococcus pneumoniae* type 3. Microb. Pathog. **12**:87–93.
8. **Berry, A. M., J. Yother, D. E. Briles, D. Hansman, and J. C. Paton.** 1989. Reduced virulence of a defined pneumolysin-negative mutant of *Streptococcus pneumoniae*. Infect. Immun. **57**:2037–2042.
9. **Bridge, P. D., and P. H. A. Sneath.** 1983. Numerical taxonomy of *Streptococcus*. J. Gen. Microbiol. **129**:565–596.
10. **Brown, M. C. M., A. Weston, J. R. Saunders, and G. O. Humphreys.** 1979. Transformation of *E. coli* C600 by plasmid DNA at different phases of growth. FEMS Microbiol. Lett. **5**:219–222.
11. **Derbyshire, K. M., and N. D. F. Grindley.** 1992. Binding of the IS*903* transposase to its inverted repeat *in vitro*. EMBO J. **11**: 3449–3455.
12. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
13. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28**:351–359.
14. **Higgins, D. G., and P. M. Sharp.** 1988. CLUSTAL: a package for performing multiple sequence alignments on a microcomputer. Gene **73**:237–244.
15. **Jobling, M. G., and R. K. Holmes.** 1990. Construction of vectors with the p15a replicon, kanamycin resistance, inducible *lacZ*α and pUC18 or pUC19 multiple cloning sites. Nucleic Acids Res. **18**:5315–5316.
16. **Knott, V., D. J. G. Rees, Z. Cheng, and G. G. Brownlee.** 1988. Randomly picked cosmid clones overlap the *pyr*B and *ori*C gap in the physical map of the *E. coli* chromosome. Nucleic Acids Res. **16**:2601–2612.
17. **Kroll, J. S., B. M. Loynds, and E. R. Moxon.** 1991. The *Haemophilus influenzae* capsulation gene cluster: a compound transposon. Mol. Microbiol. **5**:1549–1560.
18. **Macrina, F. L., R. P. Evans, J. A. Tobian, D. L. Hartley, D. B. Clewell, and K. R. Jones.** 1983. Novel shuttle plasmid vehicles for *Escherichia-Streptococcus* transgeneric cloning. Gene **25**:145–150.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Morelle, G.** 1989. A plasmid extraction procedure on a miniprep scale. Focus **11**:1:7–8.
21. **Musher, D. M.** 1992. Infections caused by *Streptococcus pneumoniae*: clinical spectrum, pathogenesis, immunity and treatment. Clin. Infect. Dis. **14**:801–807.
22. **Paton, J. C., A. M. Berry, R. A. Lock, D. Hansman, and P. A. Manning.** 1986. Cloning and expression in *Escherichia coli* of the *Streptococcus pneumoniae* gene encoding pneumolysin. Infect. Immun. **54**:50–55.
23. **Rezsöhazy, R., B. Hallet, J. Delcour, and J. Mahillon.** 1993. The IS*4* family of insertion sequences: evidence for a conserved

transposase motif. Mol. Microbiol. **9:**1283–1295.

24. **Rubens, C. E., L. M. Heggen, and J. M. Kuypers.** 1989. IS*861*, a group B streptococcal insertion sequence related to IS*150* and IS*3* of *Escherichia coli*. J. Bacteriol. **171:**5531–5535.
25. **Russell, R. R. B., and J. J. Ferretti.** 1990. Nucleotide sequence of the dextran glucosidase (*dexB*) gene of *Streptococcus mutans*. J. Gen. Microbiol. **136:**803–810.
26. **Shoemaker, N. B., and W. R. Guild.** 1974. Destruction of low efficacy markers is a slow process occurring at a heteroduplex stage of transformation. Mol. Gen. Genet. **128:**283–290.
27. **Tao, L., I. C. Sutcliffe, R. R. B. Russell, and J. J. Ferretti.** 1993. Cloning and expression of the multiple sugar metabolism (*msm*) operon of *Streptococcus mutans* in heterologous streptococcal hosts. Infect. Immun. **61:**1121–1125.
28. **Watson, D. A., and D. M. Musher.** 1990. Interruption of capsule production in *Streptococcus pneumoniae* serotype 3 by insertion of transposon Tn*916*. Infect. Immun. **58:**3135–3138.
29. **Willoughby, J. J., W. C. Russell, D. Thirkell, and M. G. Burdon.** 1991. Isolation and detection of urease genes in *Ureaplasma urealyticum*. Infect Immun. **59:**2463–2469.