Serotype 14 variants of the Spanish penicillin-resistant serotype 9V clone of *Streptococcus pneumoniae* arose by large recombinational replacements of the *cpsA*–*pbp1a* region

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The high prevalence of penicillin resistance among *Streptococcus pneumoniae* isolates from Uruguay has been associated with the emergence of a penicillin-resistant clone of serotype 14. Isolates of this clone were identical by multilocus sequence typing to members of the Spanish penicillin-resistant serotype 9V clone and possessed indistinguishable forms of the penicillin-binding protein 2b and 2x genes. Their *pbp1a* genes were also identical, except at the 3' end. The serotype 14 isolates were shown to be a variant of the Spanish serotype 9V clone which arose by a 22.2 kb recombinational replacement that introduced the capsular biosynthetic locus, and part of the neighbouring *pbp1a* gene, from a serotype 14 isolate. One end of the recombinational replacement was within the first gene of the capsular polysaccharide operon, *cpsA*, as the sequence of the upstream *dexB* gene, through most of *cpsA*, was identical in the penicillin-resistant serotype 9V and 14 isolates, but the sequences differed in the rest of *cpsA* and in *cpsB*. The other recombinational junction was at the end of the divergently transcribed *pbp1a* gene, which is approximately 11.6 kb downstream of the capsular biosynthetic locus. Isolates of this serotype variant were also detected in Spain and Denmark. Penicillin-resistant serotype 14 isolates from Poland were also closely related to the penicillin-resistant serotype 9V clone, but have emerged independently, as one end of the recombinational replacement was upstream of *dexB* and the other was within *pbp1a*, but at a different position from that in the serotype 14 variants from Uruguay, Spain and Denmark. Serotype 14 variants of the Spanish serotype 9V clone have therefore arisen on more than one occasion by large recombinational replacements that extend from the start of the *cps* region into the *pbp1a* gene.

Keywords: recombination, capsular locus, pneumococcus, serotype exchange, penicillin-binding protein 1a

INTRODUCTION

Since the first report of an isolate of *Streptococcus pneumoniae* that was resistant to penicillin in the 1960s, the incidence of both penicillin-resistant and multiple-

Abbreviations: MLST, multilocus sequence typing; MPSp9V, major penicillin-resistant Spanish serotype 9V clone; TMP-SMX, trimethoprim/sulphamethoxazole.

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antibiotic-resistant pneumococci has increased world-wide (Tomasz, 1997; Crook & Spratt, 1998). Molecular characterization of resistant strains from around the world has highlighted the considerable diversity among these isolates, but has also identified a number of successful clones of pneumococci highly resistant to penicillin (MIC > 1 µg penicillin ml⁻¹), some of which have spread globally (Crook & Spratt, 1998).

The best characterized of these clones are those that appear to have emerged in Spain during the 1980s

Table 1. Pneumococcal strains

Isolate	Isolation			Susceptibility to:†					Fingerprint pattern			Serotype
	Date	Site	Country	Pen	Cml	Tet	T/S	Ery	<i>pbp2b</i>	<i>pbp2x</i>	<i>pbp1a</i>	
SP-665	1988	Ear	Spain	R	S	S	R	S	1	1	1	9V
PO-273	1994	Ear	Poland	R	S	R	R	S	1	1	1'	14
PO-341	1994	Nose	Poland	R	S	R	R	S	1	1	1'	14
PO-342	1994	Nose	Poland	R	S	S	R	S	1	1	1'	14
M134	1997	Blood	Denmark	R	S	S	R	S	1	1	1'	14
URU-E135	1994	Blood	Uruguay	R	S	S	R	S	1	1	1'	14
URU-E157	1994	CSF*	Uruguay	R	S	S	R	S	1	1	1'	14
URU-E159	1994	Ear	Uruguay	R	S	S	R	S	1	1	1'	14
URU-E206	1994	Blood	Uruguay	R	S	S	R	S	1	1	1'	14
URU-E228	1994	Blood	Uruguay	R	S	S	R	S	1	1	1'	14
URU-E238	1994	Blood	Uruguay	R	S	S	R	S	1	1	1'	14
URU-94203	1994	Throat	Uruguay	R	S	S	R	S	1	1	1'	14
URU-94204	1994	Throat	Uruguay	R	S	S	R	S	1	1	1'	14
URU-E513	1996	CSF*	Uruguay	R	S	S	R	S	1	1	1'	14
M291	1996	Blood	Uruguay	R	S	S	R	S	1	1	1'	14
M322	1997	CSF*	Spain	R	S	S	R	S	1	1	1'	14
M339	1997	CSF*	Spain	R	S	S	R	S	1	1	1'	14
M359	1997	CSF*	Spain	R	S	S	R	S	1	1	1'	14

* Cerebrospinal fluid.

† Pen, penicillin; Cml, chloramphenicol; Tet, tetracycline; T/S, TPM-SMX; Ery, erythromycin; S, susceptible; R, resistant.

(Fenoll *et al.*, 1991). These include the major multi-resistant Spanish serotype 6B, 14 and 23F clones, and the penicillin-resistant serotype 9V clone (Muñoz *et al.*, 1991; Soares *et al.*, 1993; Coffey *et al.*, 1991, 1996). Isolates of the Spanish resistant clones have been recovered in many countries in the 1990s. For example, the major multiresistant Spanish serotype 23F clone (MMSp23F) has been identified throughout Europe, the USA, South-East Asia and South Africa (Muñoz *et al.*, 1991; Klugman *et al.*, 1994; McGee *et al.*, 1997; Tarasi *et al.*, 1997; Shi *et al.*, 1998). Similarly, the major penicillin-resistant Spanish serotype 9V clone (MPSp9V) was first identified in Spain (Coffey *et al.*, 1991), has been prevalent in France during the last 10 years (Gasc *et al.*, 1995, 1997), and has been recovered in many other countries including Germany, Greece, the Netherlands, Canada, Uruguay, Thailand, South Africa and Taiwan (Sibold *et al.*, 1992; Reichman *et al.*, 1995; Hermans *et al.*, 1997; Enright & Spratt, 1998; Shi *et al.*, 1998).

Isolates have been reported that are identical (or very closely related) in overall genotype to the major penicillin-resistant and multiresistant clones, and which possess the same altered forms of the *pbp1a*, *pbp2b* and *pbp2x* genes, but which differ in serotype (Coffey *et al.*, 1991, 1998a; Sibold *et al.*, 1992; Barnes *et al.*, 1995; Hermans *et al.*, 1997; Nesin *et al.*, 1998). It has been proposed that these serotype variants of the penicillin-resistant clones have arisen by recombinational exchanges at the capsular biosynthetic locus (*cps*); molecular evidence in support of this suggestion has been

reported for serotype 19F variants of the MMSp23F clone (Coffey *et al.*, 1998b).

In recent years, more than 40% of disease isolates of *S. pneumoniae* from Uruguay have been resistant to penicillin ($\text{MIC} \geq 0.12 \mu\text{g ml}^{-1}$) (Hortal *et al.*, 1997). The rise in prevalence of resistance has been linked to the emergence of isolates of serotype 14 that are resistant to both penicillin and trimethoprim/sulphamethoxazole (TMP-SMX), but are susceptible to tetracycline and chloramphenicol (Hortal *et al.*, 1997; Camou *et al.*, 1998). The relationship of the penicillin-resistant serotype 14 isolates to other penicillin-resistant pneumococci was determined using multilocus sequence typing (MLST), and they were shown to be serotype variants of the MPSp9V clone. The molecular basis of the emergence of these variants was elucidated by analysis of their capsular locus and flanking regions.

METHODS

Bacterial strains. The properties of the penicillin-resistant serotype 14 pneumococcal isolates are shown in Table 1. Those with the URU prefix were randomly selected examples of the penicillin- and TMP-SMX-resistant serotype 14 clone that is prevalent in Uruguay. Strains with the M prefix were penicillin-resistant serotype 14 isolates identified during a study of the genetic relatedness of isolates from serious invasive disease (Enright & Spratt, 1998 and unpublished), and were chosen for further study as they were indistinguishable (or very closely related) in genotype to the penicillin-resistant serotype 14 Uruguayan clone. PO-273, PO-341 and

PO-342 are penicillin-resistant serotype 14 isolates from Poland that were also found to be closely related to the Uruguayan serotype 14 clone. SP-665 is a well characterized member of the MPSP9V clone (Coffey *et al.*, 1991).

S. pneumoniae isolates were routinely grown at 37 °C in an atmosphere of 95% air/5% CO₂ on tryptic soy agar (TSA) containing 5% (v/v) defibrinated sheep's blood, or TSA agar containing catalase (4000 units ml⁻¹). MICs for TMP-SMX were kindly determined by D. Griffiths at the Department of Microbiology, John Radcliffe Hospital, Oxford, UK. MICs of other antibiotics were determined using the E-test on TSA containing 5% defibrinated sheep's blood.

Analysis of genetic relatedness. MLST was carried out as previously described (Enright & Spratt, 1998). Briefly, ~450 bp internal fragments of the *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt* and *ddl* genes were amplified by the PCR and were sequenced on both strands with an ABI 377 Prism automated sequencer with dRhodamine-labelled terminators (PE Applied Biosystems) using the same primers. The sequences at each locus were compared with those in our pneumococcal MLST database (<http://mlst.zoo.ox.ac.uk/>) and sequences were assigned as known alleles if they were identical to alleles in our database, or as new alleles if they differed in sequence from any of the known alleles. The combination of alleles at each locus defined the allelic profile, and the relatedness of isolates was visualized by constructing a dendrogram by the unweighted pair group method with arithmetic means from the matrix of pairwise differences between the allelic profiles (Enright & Spratt, 1998). The allelic profiles of isolates (e.g. 7-11-10-1-6-8-1) are shown with the alleles at the seven loci in the order *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt* and *ddl*.

Analysis of *pbp* genes. *pbp1a*, *pbp2b* and *pbp2x* gene fingerprinting was carried out as previously described (Coffey *et al.*, 1991). The *pbp1a* gene was amplified by PCR and sequenced using an ABI 377 sequencer as described previously (Coffey *et al.*, 1995a).

Analysis of capsular genes. Amplification of the *cps14K* and *cps14L* genes by PCR was carried out using the primers 14Kup (5'-d-GTGACTATATAGAYATHCC-3') and 14Kdown (5'-d-TACTATAAACTTTRTARAA-3'), and 14Lup (5'-d-TTGTGATAACTTCTCCNTA-3') and 14Ldown (5'-d-TGTTTCGTGGTAACATNAC-3'), respectively. The *dexB-cpsB* region and the *aliA* gene were amplified using the primers *cpsUP1* (5'-d-GGCTTTCTCCGTTTATG-3') and *cpsC3rev* (5'-d-CCAGTGTGTGCATCAGAAA-3'), and *aliAup* (5'-d-GTGACATTATTGGCGGAC-3') and *aliAdown* (5'-d-GGATTTCAGCATTCAAGAGAAATAC-3'), respectively. The *aliA-pbp1a* and *cpsL-aliA* regions were amplified using the primers *aliA-A1* (5'-GAATACGCGGCAGTCAA-3') and *pbp1A-1rev* (5'-ATGATGACCGACATGATGAA-3'), and 14Lup and *aliAdown*, respectively.

RESULTS

Genetic relatedness of penicillin-resistant serotype 14 isolates from Uruguay, Spain, Denmark and Poland

The overall genetic relatedness of the penicillin-resistant serotype 14 isolates was assessed using MLST. The allelic profiles of the isolates URU-E135, URU-E157, URU-E159, URU-E206, URU-E228, URU-E238, URU-94203, URU-94204 and M291 from Uruguay, M134 from Denmark, and M322, M339 and M359 from Spain were all identical (allelic profile 7-11-10-1-6-8-1). Com-

parison of this allelic profile with those in our pneumococcal MLST database showed that it was identical to the allelic profile of members of the MPSP9V clone (e.g. strain SP-665).

One penicillin-resistant serotype 14 isolate from Uruguay (M288) differed from the MPSP9V clone at a single locus (allelic profile 7-11-10-1-6-34-1). The three penicillin-resistant serotype 14 isolates from Poland had an identical allelic profile (7-5-10-18-6-8-1), but this profile differed at 2/7 loci from that of the MPSP9V clone.

The serotype 14 strains from Uruguay, Spain and Denmark were resistant to penicillin (MIC 0.5–2 µg ml⁻¹) and TMP-SMX, but susceptible to chloramphenicol and tetracycline, which is the profile typical of the MPSP9V clone. One of the serotype 14 strains from Poland had the same antibiotic profile but the other two were additionally resistant to tetracycline (Table 1). The sequences of the alleles at all seven loci, and the properties of the pneumococci, are available from the MLST Web site (<http://mlst.zoo.ox.ac.uk/>).

Relatedness of the *pbp1a*, *pbp2b* and *pbp2x* genes of the serotype 14 isolates and the MPSP9V clone

The DNA fragments of the *pbp2b* and *pbp2x* genes, obtained by digestion with multiple frequently cutting restriction endonucleases, were identical for the penicillin-resistant serotype 14 isolates from Uruguay, Spain, Denmark and Poland, and the reference isolate of the MPSP9V clone (SP-665). The identity of the gene fingerprints implied that these strains all possessed the same mosaic forms of the *pbp2b* and *pbp2x* genes.

The DNA fragments obtained by *HinfI* digestion of the *pbp1a* genes of the penicillin-resistant serotype 14 isolates were also identical to those of the MPSP9V clone. However, while *DdeI*/*MseI* digestion produced identical fragment patterns from all of the serotype 14 isolates, this pattern differed slightly from that of the MPSP9V clone. Comparison of the *DdeI*/*MseI* gene fingerprint pattern of the serotype 14 strains with that expected from the sequence of the *pbp1a* gene of the MPSP9V clone suggested that the genes differed slightly at the 3' end.

The *pbp1a* gene (2160 bp) was therefore sequenced from each of the penicillin-resistant serotype 14 isolates and compared to the sequence from the MPSP9V clone (SP-665) and the penicillin-susceptible strain, R6. The gene from the MPSP9V clone is identical to that in the MMSp23F clone (Coffey *et al.*, 1991) and has the typical mosaic structure found in penicillin-resistant strains, which is believed to have arisen by the introduction of highly divergent sequences from the *pbp1a* genes of related streptococcal species (Coffey *et al.*, 1995b). The genes from SP-665 and R6 were identical in sequence between nucleotides 1–306 and 2000–2160, but the resistant isolate contained a highly divergent block (13.5% divergence) between nucleotides 307 and 1999. The *pbp1a* genes from the serotype 14 strains from Uruguay, Spain and Denmark were found to be identical

[illegible]

Fig. 1. Polymorphic sites within the *pbp1a* gene of the serotype 14 variants of the MP5p9V clone. The nucleotides present at each polymorphic site are shown for the penicillin-susceptible laboratory strain R6, but only those sites that differ from R6 are shown for the other strains; nucleotides identical to R6 are shown by dots. The polymorphic sites are numbered above, in vertical format, with the numbering starting at the initiation codon. The *pbp1a* sequence of the MP5p9V clone was identical to that of the serotype 14 isolates from Uruguay, Spain and Denmark up to nucleotide 1928, and was identical to the serotype 14 isolates from Poland up to nucleotide 1859. The arrows show the putative recombinational cross-over points.

to each other, and were identical to the gene from the MPSp9V clone, except that the divergent block was slightly truncated, and extended only to nucleotide 1922 (Fig. 1). Downstream of this position the *pbp1a* sequence of these serotype 14 isolates was identical (except at one site) to that of the penicillin-susceptible strain R6.

The sequences of the *pbp1a* genes from the three Polish resistant serotype 14 strains were also identical to each other, and to the MPSp9V clone, except that the divergent block was further truncated, and extended only to nucleotide 1854 (Fig. 1). Downstream of this position, the gene was identical to that of strain R6.

Analysis of the capsular biosynthetic locus and flanking regions

The genetic organization around the serotype 14 capsular biosynthetic locus (*cps*) is shown in Fig. 2. The *cps14K* and *cps14L* genes hybridize only to serotype 14 and serogroup 15 pneumococci, which have similar capsular polysaccharide structures (Kolkman *et al.*, 1998). As expected, internal fragments of these genes could be amplified by the PCR from DNA of each of the penicillin-resistant serotype 14 isolates, but not from DNA of isolates of the MPSP9V clone (data not shown).

A 500 bp internal fragment of the *cps14L* gene was sequenced from the penicillin-resistant serotype 14 variants. The sequences were identical and differed at only one position from the published sequence of the *cps14L* gene (Kolkman *et al.*, 1997). The serotype 14 variants of the MPSP9V clone therefore contained *cps* genes characteristic of serotype 14 pneumococci.

To identify the end points of the recombinational exchange that occurred during the emergence of the serotype 14 variants of the MPSp9V clone, the sequence from *dexB*, which flanks the *cps* locus, to the end of *cpsB*, was determined for the penicillin-resistant serotype 14 strains and for SP-665. The sequences from SP-665 and the serotype 14 strains from Uruguay, Spain and Denmark were identical (except at one nucleotide) throughout *dexB*, the *dexB*–*cpsA* intergenic region, and the first 1067 bp of *cpsA* (a total of 3926 bp). The single difference was within a poly-A tract in the *dexB*–*cpsA* intergenic region, where there were eight consecutive A residues in the serotype 14 strains from Uruguay, Spain and Denmark, but only seven A residues in strain SP-665. Sequencing of this region from four other members of the MPSp9V clone showed the presence of eight A residues, as observed in the serotype 14 variants. The sequences of the rest of *cpsA* (378 bp) and of *cpsB*

Fig. 2. Genetic organization of the *dexB*–*cps*–*pbp1a* region. The region from a serotype 14 strain is shown in black and is based on the sequence of the serotype 14 *dexB*–*cps* locus (Kolkman *et al.*, 1997) and the results presented in this paper. The *dexB*–*cps*–*pbp1a* region from the MPSp9V clone is shown in white. The large ORF (labelled *orf*) located between *aliA* and *pbp1a* (or its translation product) was homologous to an incomplete ORF downstream of *aliA* in the *cps* region of a serotype 3 pneumococcus (García *et al.*, 1997), but showed no homology to any gene of known function in the databases. The shaded regions indicate *cps* genes that are not shown (the entire serotype 9V *cps* locus has not been described). The two distinct *cps* regions found among the serotype 14 variants are shown. Regions that are identical to, or are assumed to be identical to, those of the MPSp9V clone are shown in white, whereas regions that are believed to have been introduced by recombination from a serotype 14 donor are shown in black. The arrows at the bottom show the regions that were sequenced for all strains. The hatched region in the Polish serotype 14 variants represents the position of the unidentified recombinational cross-over upstream of *dexB*. The *dexB*–*cpsA* intergenic region from the MPSp9V clone and the serotype 14 variants from Uruguay, Spain and Denmark contained a 353 bp insertion/deletion (indicated by the inverted triangles) that was 98% identical to a sequence in the *dexB*–*cpsA* intergenic region of the *cps* locus from a serotype 1 pneumococcus, and which encodes a putative 115 amino acid product that is 52% identical to a transposase from *Synechocystis* sp. Similar insertion/deletions (98% identity) have also been observed in the *cps* locus of some serotype 19F isolates (Coffey *et al.*, 1998b).

(732 bp), from the serotype 14 strains from Uruguay, Spain and Denmark, differed from the homologous regions from SP-665 at 7/378 (1.9%) and 123/732 (16.8%) nucleotide sites, respectively (Fig. 3). In these strains, one end of the recombinational exchange that replaced the serotype 9V *cps* genes with those from a serotype 14 isolate therefore occurred towards the end of *cpsA* (Fig. 2).

The amplification of the *cps14K* and *cps14L* genes by PCR from the serotype 14 strains, but not from the MPSp9V clone, implied that the recombinational replacement that introduced the *cps* region from a serotype 14 isolate included the most distal *cps* genes, and located the other recombinational cross-over downstream of the *cps* locus. The *aliA* gene is downstream of the *cps* locus in all of the serotypes that have been examined (García *et al.*, 1997; Arrecubieta *et al.*, 1994; Dillard *et al.*, 1995; Morona *et al.*, 1997; Muñoz *et al.*, 1997; Coffey *et al.*, 1998b; Llull *et al.*, 1998; Ramirez & Tomasz, 1998; Morona *et al.*, 1999). PCR with primers based on the sequences of the *cps14L* and *aliA* genes showed that these genes were separated by about 3.8 kb in the penicillin-resistant serotype 14 strains from Uruguay. The *aliA* gene was amplified and sequenced from each of the serotype 14 strains from Uruguay, Spain and Denmark. The *aliA* sequences of these serotype 14 strains were all identical, but differed from that of the

MPSp9V clone at 21/1983 (1.1%) nucleotide sites. The differences between the *aliA* genes of the serotype 14 isolates and the MPSp9V clone suggested that this gene was introduced along with the serotype 14 *cps* genes and implied that the recombinational junction was downstream of *aliA*.

The incomplete pneumococcal genome sequence (<http://www.tigr.org>) indicated that the *pbp1a* gene is 5.8 kb downstream of *aliA* and is transcribed in the opposite direction. PCR using primers based on the sequences of *aliA* and *pbp1a* confirmed that the two genes were separated by about 5.8 kb in strain URU-E238, indicating that the *pbp1a* gene was located about 11.6 kb downstream of the end of the *cps* locus in the Uruguayan penicillin-resistant serotype 14 clone. The sequences of an 838 bp region from *aliA* towards *pbp1a*, and a 798 bp region from *pbp1a* towards *aliA*, were identical in URU-E238 and M134, but the sequence differed from that of the MPSp9V clone at 8/838 (1%) and 24/798 (3%) of nucleotide sites, respectively. These differences between the serotype 14 isolates and the MPSp9V suggested that the region between *aliA* and *pbp1a* was also introduced with the *cps* and *aliA* genes. The identification of a clear recombinational junction, near the end of *pbp1a* where the sequence of the serotype 14 variants becomes identical to that in the MPSp9V clone (Fig. 1), suggested that this marked the other end

[illegible]

Fig. 3. Polymorphic sites within the *dexB*–*cpsB* region. The positions of the *dexB*, *cpsA* and *cpsB* genes are shown. The sequence is numbered from the initiation codon of the *dexB* gene. The nucleotides present at each polymorphic site are shown in full for a member of the MPSP9V clone (SP-665), but only those sites that differ are shown for the serotype 14 variants from Uruguay, Spain, Denmark and Poland; identical nucleotides are shown by dots. Nucleotides that are not present are shown as dashes. The # symbol denotes the position of a 353 bp insertion/deletion which was present in the *dexB*–*cpsA* intergenic region of the serotype 14 variants from Uruguay, Spain and Denmark, and the MPSP9V clone, but was not found in the serotype 14 variants from Poland. The difference at position 2445 corresponds to the presence of an extra A residue within a polyA tract in the serotype 14 strains from Uruguay, Spain and Denmark (8 A residues), compared to a run of 7 A residues in SP-665. Other isolates of the MPSP9V clone possessed 8 A residues. The serotype 14 isolates from Poland possessed either 7 A residues (as shown; strains PO-341 and PO-342) or 8 A residues (strain PO-273). The position of the putative recombinational cross-over in the serotype 14 isolates from Uruguay, Spain and Denmark is shown by the arrow. The sequences of all of the serotype 14 isolates were identical from nucleotide 3574 to the end of *cpsB*.

of the recombinational replacement that introduced the serotype 14 capsular biosynthetic genes.

The size of the proposed replacement in the serotype 14 isolates from Uruguay, Spain and Denmark can be calculated to be approximately 22.2 kb from the distance between the *cpsA* recombinational junction to the end of the *cps* region (obtained from the published sequence of the serotype 14 *cps* locus), plus the size of the region from the end of *cpsL* to the *pbp1a* recombinational junction (estimated by the PCR). A schematic representation of the *cps* locus and flanking regions of the MPSP9V clone and the penicillin-resistant serotype 14 variants from Uruguay, Spain and Denmark is shown in Fig. 2.

The three penicillin-resistant serotype 14 isolates from Poland were identical to each other within the *dexB*-*pbp1a* region, except that PO-273 possessed 8 A residues in the polyA tract within the *dexB*-*cpsA* intergenic region, whereas the other two strains had 7 A residues. In these serotype 14 variants, one recombinational cross-over point appeared to be upstream of

the *cps* locus, as their *cpsA* and *cpsB* genes differed at 1.4% and 16.8% of sites, respectively, from those of the MPSP9V clone. The cross-over point was not identified in the Polish strains but it appeared to be upstream of *dexB* as this gene differed at 2.2% of sites from that of the MPSP9V clone (Fig. 2). The *aliA* genes from the three Polish isolates were identical to each other but differed from that of the other serotype 14 isolates, and from that of the MPSP9V clone, at 26 (1.3%) and 17 (0.9%) nucleotide sites. The other cross-over point in the Polish strains was therefore also downstream of *aliA* and presumably corresponded to the recombinational junction identified at the end of the *pbp1a* gene. The recombinational replacement that appears to have introduced the serotype 14 capsular genes into the Polish strains therefore appears to extend for >25 kb (Fig. 2).

DISCUSSION

Penicillin resistance in *S. pneumoniae* in Uruguay, and in some neighbouring countries (e.g. Argentina), is predominantly associated with isolates of serotype 14

(Hortal *et al.*, 1997; Camou *et al.*, 1998; Rossi *et al.*, 1998; Tomasz *et al.*, 1998). The majority of these isolates have been shown to be members of a single penicillin-resistant clone of serotype 14 (Camou *et al.*, 1998; Tomasz *et al.*, 1998). In this paper we provide very strong evidence that the penicillin-resistant serotype 14 isolates from Uruguay (and the isolates from Spain and Denmark) are serotype variants of the MPSP9V clone. Putative serotype 14 variants of the MPSP9V clone have been identified in five other Latin American countries (Tomasz *et al.*, 1998) and in the United States (Corso *et al.*, 1998). MLST has extremely high resolving power and the expected frequency of any allelic profile can be estimated from the product of the frequencies of each of the seven alleles within the pneumococcal population. Using allele frequencies obtained from a study of 295 pneumococci from invasive disease (Enright & Spratt, 1998), the allelic profile of the above serotype 14 isolates and the MPSP9V clone was expected to occur by chance at a frequency of 4.5×10^{-7} . The probability that the serotype 14 isolates and the MPSP9V clone had the same allelic profile by chance is therefore close to zero, and these isolates are unambiguously members of the same penicillin-resistant clone that differ in serotype.

The serotype 14 isolates and the MPSP9V clone were also shown by gene fingerprinting to possess indistinguishable, altered forms of their *pbp2b* and *pbp2x* genes, and sequence analysis showed that they possess *pbp1a* genes that are identical, except at the end of the gene closest to the *cps* locus. In addition, the serotype 14 isolates had the characteristic antibiotic-resistance profile of the MPSP9V clone. The penicillin-resistant serotype 14 isolates from Uruguay, Spain and Denmark, and the MPSP9V clone, are therefore essentially identical, and differ only in their serotype and at the end of their *pbp1a* gene. The most likely scenario is that these penicillin-resistant serotype 14 strains are members of the same clonal variant that arose from the MPSP9V clone by a recombinational exchange which replaced the serotype 9V *cps* region with that from a serotype 14 strain, and which has now spread intercontinentally.

This direction of transfer of the *cps* genes is strongly favoured by the fact that all penicillin-susceptible and -resistant serotype 9V isolates that have been examined by MLST possess highly related allelic profiles, suggesting that they are derived from a single, relatively recent common ancestor of serotype 9V (Enright & Spratt, 1998). The presence of a minority of isolates of serotype 14 within this cluster of related genotypes suggests that these have arisen from the serotype 9V isolates, rather than *vice versa*. The apparently earlier emergence of the MPSP9V clone, compared to the serotype 14 variants, also supports the proposed direction of the serotype change.

The upstream cross-over point for the proposed recombinational replacement within the serotype 14 variants from Uruguay, Spain and Denmark could be identified in the *cpsA* gene. As expected, the sequence from *dexB* to the cross-over point in *cpsA* was identical in these

serotype 14 isolates and the MPSP9V clone. The only difference was in the *dexB-cpsA* intergenic region of one isolate of the MPSP9V clone (SP-665) where an additional A residue was present within a polyA tract. This difference in the number of A residues was also found among the three penicillin-resistant serotype 14 variants from Poland (which otherwise were identical throughout the *dexB-pbp1a* region), and was probably due to expansion and contraction of the number of A residues by slipped-strand mispairing.

In serotype 19F variants of the MMSP23F clone, downstream recombinational cross-over points have been identified in the *cpsM* and *cpsN* genes which are close to the end of the *cps* locus (Coffey *et al.*, 1998b). The *cpsL-cpsO* genes of serotype 19F and 23F pneumococci are relatively conserved (Morona *et al.*, 1997; Coffey *et al.*, 1998b), providing sufficient homology for homologous recombination between these regions in serotype 19F and 23F isolates. As homologues of the *cps14I* to *cps14L* genes at the end of the serotype 14 capsular locus have not been detected in serotype 9V isolates (Kolkman *et al.*, 1998), the downstream recombinational cross-over must be in homologous sequences downstream of the *cps* locus. A clear recombinational cross-over was found about 11.6 kb downstream of the *cps* locus and it is very likely that a single recombinational exchange, extending from *cpsA* to *pbp1a* has occurred, from a serotype 14 donor isolate, to alter both the serotype and the *pbp1a* gene of the MPSP9V clone (see below).

The recombinational cross-over points in the three serotype 14 variants from Poland were located upstream of *dexB*, and at the end of *pbp1a*, although at a slightly different position from that in the strains from Uruguay, Spain and Denmark. The different recombinational cross-over points in the isolates from Uruguay, Spain and Denmark, compared to those from Poland, and their different *aliA* sequences, indicates that serotype 14 variants of the MPSP9V clone have arisen on more than one occasion. However, the allelic profile of the isolates from Poland differed from that of the MPSP9V clone at 2/7 loci, whereas the serotype 14 isolates from Uruguay (except strain M288 which differed at 1/7 loci), Spain and Denmark were identical by MLST to the MPSP9V clone. The MPSP9V isolate and the Polish serotype 14 isolates did, however, have indistinguishable *pbp2b* and *pbp2x* genes, and their *pbp1a* genes were also identical, except at the end of the gene where the putative recombinational cross-over occurred.

The origin of the Polish isolates is therefore less clear than that of the serotype 14 isolates from Uruguay, Spain and Denmark. Two scenarios can be considered. Either the same altered *pbp1a*, *pbp2b* and *pbp2x* genes have been introduced on separate occasions into two closely related serotype 9V isolates, and serotype 14 variants of each of the resulting penicillin-resistant serotype 9V lineages arose by different recombinational exchanges in the *dexB-pbp1a* region. Alternatively, the altered *pbp* genes could have entered a serotype 9V

isolate only once, to produce the MPSP9V clone, and serotype 14 variants of this clone arose on two occasions, one within an isolate with the typical allelic profile, and the other within a member of the MPSP9V clone that differed by MLST at 2/7 loci as a result of clonal diversification.

We assume that the cross-over points in *cpsA* (or upstream of *dexB* in the Polish strains), and in *pbp1a*, are the junctions of single recombinational events that resulted in the simultaneous change of both serotype and PBP1a. It is difficult to completely rule out the possibility that two recombination events were involved, one that replaced the *cps* region and another that resulted in an alteration of *pbp1a*. The sequence differences between the serotype 14 variants and the MPSP9V clone at *aliA*, and in the region between *aliA* and *pbp1a*, indicates that these regions of the MPSP9V clone have been replaced and supports a single recombinational replacement event. Probably the strongest argument for a single genetic event is that the serotype 14 strains from Uruguay, Spain and Denmark, and those from Poland, have clearly emerged on separate occasions, but they both have replaced the *cps* region, *aliA* and part of the *pbp1a* gene. The independent occurrence, in both classes of serotype 14 variants, of one recombinational event that replaced the *cps* region, and another that replaced the end of the *pbp1a* gene, is highly unlikely. The recombinational events resulted in the replacements of the last 79 or 102 amino acids of PBP1A with those characteristic of a penicillin-susceptible pneumococcus. These amino acids are outside the region between the active site serine and the KTG motif (Martin *et al.*, 1992). The recombinational replacements were therefore 82 or 59 amino acids downstream of the conserved KTG motif, in a region not known to contribute to the reduced affinity of PBP1A in penicillin-resistant clinical isolates of *S. pneumoniae*.

The recombinational exchanges at the *cps* locus are assumed to have occurred by natural transformation, although the sizes of the replacements, and those in serotype 19F variants of the MMSP23F clone (Coffey *et al.*, 1998b), are large for this genetic exchange mechanism. It is possible that another mechanism of genetic exchange, such as phage-mediated transduction, was involved, although this has not been reported in the pneumococcus. It might be expected that such large recombinational exchanges would be very rare, and the increasing number of reports of serotype changes within pneumococcal lineages, which will typically involve replacement of most of the large *cps* region, argues that occasional changes of serotype may be favoured, presumably by the human immune system.

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