ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1997, p. 2121–2126 Vol. 41, No. 10
0066-4804/97/$04.00+0

Mechanism of Sulfonamide Resistance in Clinical Isolates of *Streptococcus pneumoniae*

JEFFREY P. MASKELL,* ARMINE M. SEFTON, AND LUCINDA M. C. HALL

Department of Medical Microbiology, St. Bartholomew's and the Royal London School of Medicine and Dentistry, London E1 2AD, United Kingdom

Received 21 April 1997/Returned for modification 6 June 1997/Accepted 30 July 1997

The genetic basis of sulfonamide resistance in six clinical isolates of *Streptococcus pneumoniae* was demonstrated to be 3- or 6-bp duplications within *sulA*, the chromosomal gene encoding dihydropteroate synthase. The duplications all result in repetition of one or two amino acids in the region from Arg_{58} to Tyr_{63}, close to but distinct from the *sul*-d mutation, a duplication previously reported in a resistant laboratory strain (P. Lopez, M. Espinosa, B. Greenberg, and S. A. Lacks, J. Bacteriol. 169:4320–4326, 1987). Six sulfonamide-susceptible clinical isolates lacked such duplications. The role of the duplications in conferring sulfonamide resistance was confirmed by transforming 319- or 322-bp PCR fragments into the chromosome of a susceptible recipient. Two members of a clone of serotype 9V, one susceptible and one resistant to sulfonamide, which are highly related by other criteria, were shown to have *sulA* sequences that differ in 7.2% of nucleotides in addition to the duplication responsible for resistance. It is postulated that horizontal gene exchange has been involved in the acquisition (or loss) of resistance within this clone. However, five of the six resistant isolates have distinct duplications and other sequence polymorphisms, suggesting that resistance has arisen independently on many occasions.

Streptococcus pneumoniae remains a major cause of acute respiratory disease and is considered to be responsible for the death of well over a million children per year. For developing countries, the World Health Organization recommends that co-trimoxazole (sulfamethoxazole plus trimethoprim) be used for the therapy of respiratory tract infection, because it is both effective and cheap. However resistance to co-trimoxazole is now prevalent, and high rates of resistant isolates have been reported in South Africa (12), Spain (18), Portugal (23), Hungary (15), and elsewhere (11). Furthermore isolates of *S. pneumoniae* resistant to penicillin and other antimicrobials are very often also resistant to co-trimoxazole (11).

The sulfonamide agents inhibit the growth of bacteria by interfering with the biosynthesis of folic acid, essential for the production of purines, pyrimidines, and amino acids. Within the folic acid pathway, dihydropteroate synthase (DHPS) catalyzes the synthesis of 7,8-dihydropteroate from pterin pyrophosphate and *para*-aminobenzoate; sulfonamides compete with *para*-aminobenzoate for this enzyme and inhibit its activity (2).

Sulfonamide resistance in most gram-negative species involves the acquisition of an additional DHPS gene on a plasmid or transposable element, producing an enzyme that is not inhibited by sulfonamide and that hence bypasses inhibition of the chromosomally encoded enzyme (10). In contrast, resistance in clinical isolates of *Neisseria meningitidis* was shown to be due to a 6-bp insertion in the normal chromosomal DHPS gene, probably acquired by horizontal gene transfer (20). Similarly, a sulfonamide-resistant laboratory mutant of *S. pneumoniae* was found to have acquired a 6-bp insertion (a duplication of an existing sequence) in *sulA*, the chromosomal gene encoding DHPS in *S. pneumoniae* (although in a region of the gene distant from the insertion in *Neisseria meningitidis*) (14). The mechanism of sulfonamide resistance in clinical isolates of *S. pneumoniae* has not previously been determined. In this study, we analyzed the *sulA* genes from six sulfonamide-resistant clinical isolates of *S. pneumoniae* and investigated their role in resistance.

MATERIALS AND METHODS

Bacterial isolates. The sources of the clinical isolates of *S. pneumoniae* used in this study and their serotypes and susceptibilities to sulfamethoxazole and trimethoprim are shown in Table 1.

Susceptibility testing. Growth on brain heart infusion agar (Unipath Ltd., Basingstoke, Hampshire, United Kingdom) supplemented with 5% defibrinated horse blood (Unipath) after 18 h of incubation at 35°C was examined for purity, and a few colonies were inoculated into Iso-Sensitest broth (Unipath) supplemented with 2% horse serum (Unipath) and incubated for 5 h at 35°C. The suspension was diluted in peptone water (Unipath) to provide a final inoculum

TABLE 1. *S. pneumoniae* isolates used in this study

Isolate	Source[a]	Serotype[b]	MIC (mg/liter)	
			Sulfamethoxazole	Trimethoprim
PN93/1802	United Kingdom (BC)	9V	256	256
J93/155	Japan	19F	1,024	32
PN94/720	United Kingdom (CSF)	23F	512	256
P48	United Kingdom	ND	1,024	256
R12	United Kingdom (sputum)	ND	256	512
J94/76	Japan	14	512	4
PN94/258	United Kingdom (BC)	23F	32	4
PN93/908	United Kingdom (CSF)	14	32	4
J93/196	Japan	14	32	4
PN93/1791	United Kingdom (BC)	19F	16	4
PN93/917	United Kingdom (CSF)	9V	32	8
CP1015	Laboratory strain	NC	64	2

[a] BC, blood culture; CSF, cerebrospinal fluid.
[b] ND, not done; NC, noncapsulated.

* Corresponding author. Mailing address: Department of Medical Microbiology, St. Bartholomew's and the Royal London School of Medicine and Dentistry, Turner St., Whitechapel, London E1 2AD, United Kingdom. Phone: (44) 171 377 7259. Fax: (44) 171 375 0518. E-mail: J.Maskell@mds.qmw.ac.uk.

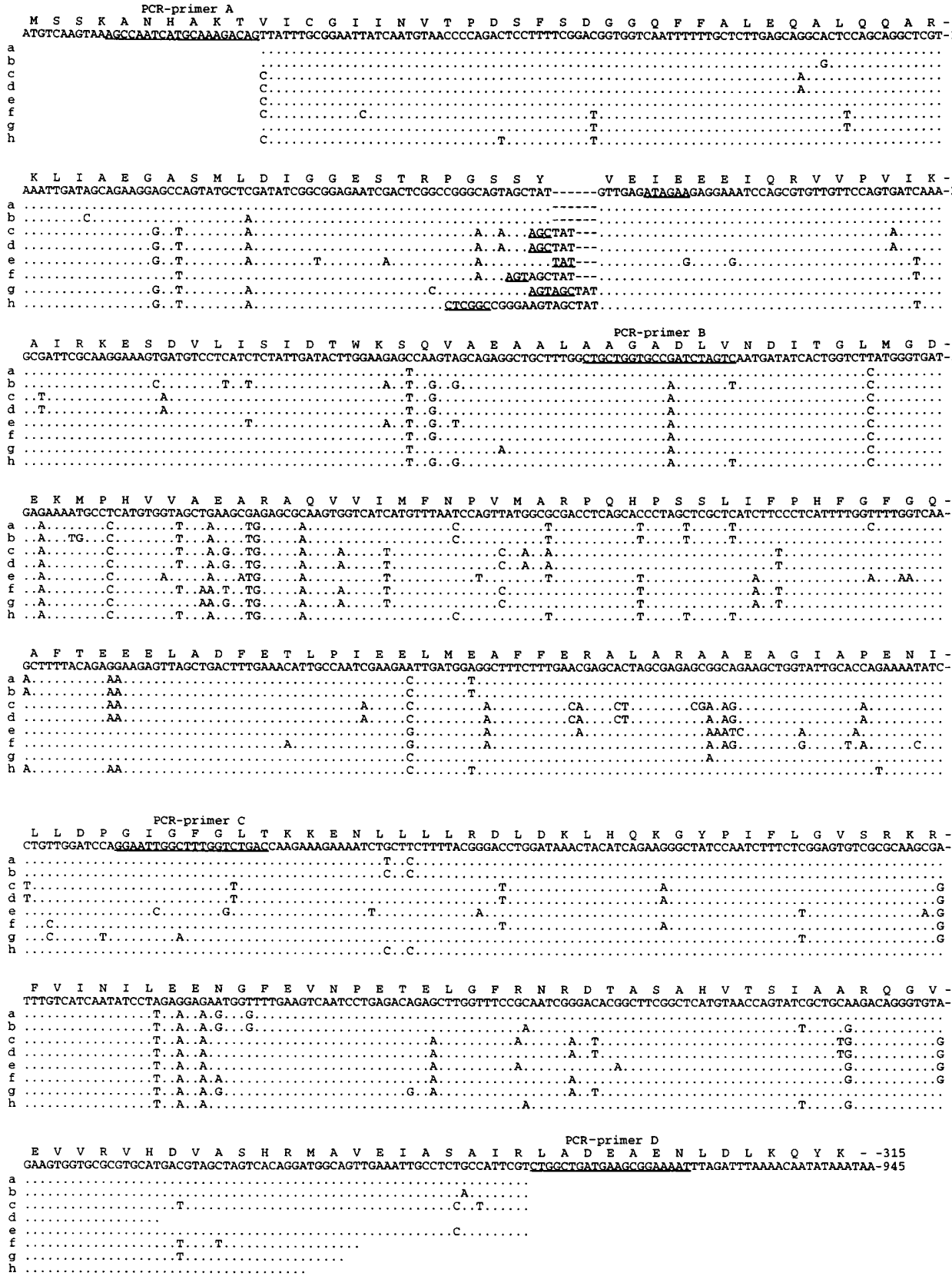

FIG. 1. Sequences of *sulA* from clinical isolates of *S. pneumoniae* compared to the sequences of R6 and CP1015 (top line). Identical residues are indicated by a dot, and spaces inserted to give alignment in the region of the duplications are indicated by dashes. Underlining indicates the positions of the primers, duplications in clinical isolates, and the position of the sequence duplicated in the *sul*-d mutation of R6. The clinical isolates represented are J93/196 (a), PN93/917 (b), PN93/1802 (c), R12 (d), J93/155 (e), PN94/720 (f), J94/76 (g), and P48 (h).

of 10^3 CFU when applied to Iso-Sensitest agar (Unipath) plates supplemented with 5% lysed defibrinated horse blood (Unipath) and containing doubling dilutions of sulfamethoxazole or trimethoprim (Sigma Chemical Co., Poole, Dorset, United Kingdom) with a multipoint inoculator (Denley-Tech. Ltd.) delivering approximately 1 μl/spot. Plates were incubated at 35°C for 18 h in an atmosphere of 7% carbon dioxide in air. The MIC was defined as the lowest antibiotic concentration showing a 90% reduction in growth in comparison to that on the control plate containing no antibiotic (17). Each batch of medium was tested with *Enterococcus faecalis* NCIB 12756 (ATCC 33186) to ensure that the thymidine content was suitable for sulfamethoxazole-trimethoprim susceptibility testing (1).

DNA extraction. DNA was extracted from 18-h cultures on brain heart infusion agar (Unipath) supplemented with 5% horse blood. Suspensions were centrifuged, washed once in phosphate buffer (pH 7), and then resuspended in 0.1 ml of TE (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). Lysozyme (10 μl at 50 mg/ml) was added, and the mixture was then incubated for 30 min at 37°C, followed by the addition of 0.5 ml of GES (5 M guanidine thiocyanate, 0.1 M EDTA [pH 8.0], 0.5% Sarkosyl) and incubation at room temperature for 10 min. Following the addition of ammonium acetate (0.25 ml at 7.5 M), the cells were mixed and placed on ice for 10 min. Chloroform-isoamyl alcohol (24:1) was added, and the components were mixed and then separated by centrifugation at 13,000 × *g* for 10 min, after which, 0.7 ml of the aqueous phase was recovered. DNA was precipitated with 0.54 volumes of cold isopropanol and then redissolved and reprecipitated in 70% ethanol. The DNA was finally recovered in 50 to 100 μl of TE and stored at −20°C.

PCR. DHPS gene sequences were amplified with primers (PE-Applied Biosystems) produced according to the published sequence for the DHPS gene of *S. pneumoniae* R6 (14). The forward primer (A) was biotin labelled and had the sequence 5′-AGCCAATCATGCAAAGACAG (bp 12 to 31), and the sequences of the reverse primers were 5′-GACTAGATCGGCACCAGCAG (bp 308 to 327) (B), 5′-GTCAGACCAAAGCCAATTCC (bp 607 to 626) (C), and 5′-ATTTTCCGCTTCATCAGCCAG (bp 901 to 921) (D). Amplification reactions were performed in reaction mixtures containing 100 μl of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphates, 100 ng of each primer, and 1.2 U of Amplitaq polymerase (Perkin-Elmer) in a Hybaid thermal reactor. The program involved 1 cycle of denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min. PCR products were run on 0.9% agarose gels in Tris-borate-EDTA buffer (TBE) in parallel with a 1-kb ladder (Gibco-BRL, Paisley, United Kingdom) and located by staining with ethidium bromide. For sequencing, pure single-stranded DNA was prepared from PCR products with streptavidin paramagnetic beads (M-280; Dynal A.S., Norway).

Nucleotide sequence determination. The DNA sequences of purified PCR amplification products were determined by the dideoxy chain termination method, with the primers described above in conjunction with the Sequenase version 2.0 kit (U.S. Biochemicals), and labelled with ^{35}S-dATP (Amersham, Buckinghamshire, United Kingdom). Additional primers were used where necessary to resolve ambiguous sequences. Most sequences were determined from the products of more than one PCR to ensure that differences were not the result of PCR artifacts.

Transformation. The sulfamethoxazole-susceptible recipient strain of *S. pneumoniae*, CP1015, was transformed with whole DNA and PCR products of the amplified DHPS gene of resistant isolates according to a method based on that of Morrison et al. (16). The recipient strain was grown in CAT medium (19), which contained (per liter) 10 g of Casitone (Difco), 5 g of tryptone (Difco), 4 g of yeast extract (Unipath), 5 g of NaCl (Sigma), 2 g of glucose (Sigma), and 17 mM K_2HPO_4 (Sigma) (pH 7.5), at 37°C to an A_{550} of 0.4. Glycerol was added to a final concentration of 15%, and the suspensions were stored at −70°C. For the production of competent cells, the suspensions were thawed and diluted 1:100 in transformation medium, which consisted of CAT medium supplemented with final concentrations of 1 mM $CaCl_2$ and 0.4% bovine serum albumin fraction V (Sigma) (pH 7). After aerobic incubation at 37°C for 2 h, the pH was raised to 7.8 by the addition of 1 M NaOH, and the mixture was incubated for a further 20 min. Ten microliters of DNA or PCR product was added to 1 ml of medium, and this mixture was then incubated at 30°C for 30 min, followed by a further 2 h of incubation at 37°C. DNA-free controls were tested in parallel in order to confirm that any growth was a result of transformation and not mutation. After incubation, 50-μl volumes of 10-fold dilutions of suspensions were plated onto Iso-Sensitest agar supplemented with 5% lysed horse blood and sulfamethoxazole at 256 and 512 mg/liter and onto antibiotic-free medium for the determination of viable counts. Plates were incubated for 24 to 48 h at 37°C in 7% CO_2. The susceptibility of transformants to sulfamethoxazole was determined as described above.

RESULTS

Sequence of *sulA* in clinical isolates. Primers deduced from the published *sulA* sequence of *S. pneumoniae* R6 were used to amplify the *sulA* gene from six sulfonamide-resistant and six susceptible clinical isolates (Table 1). Resistant and susceptible

TABLE 2. Amino acid differences in predicted SulA product between isolates

Isolate	Duplication(s) in 58–63 region (position)	Difference in amino acid no. from isolate CP1015[a]																														
		S-61	D-85	W-93	D-107	M-121	P-122	V-125	E-127	A-128	R-129	Q-131	M-140	G-157	A-159	E-163	T-170	E-175	E-178	A-179	E-182	P-202	N-247	R-260	A-274	A-287	A-298
PN93/917					N	W					G	K			T	K		D	V				S				T
J93/196							A				G	K			T	K		D	V				S					
PN93/1802	S (62)	R	N		N		A	I	D	D	G	K	I			K		D		T	D			H	V			
J93/155	Y (63)				N		A				G	K		N						T				H				
P48	R, P (58, 59)			R	N		A				G	K			T	K		D	V				S				ND[b]	
PN94/720	S (61)				N		A				G	K					K			T					V		ND	
J94/76	S, S (61, 62)				N		A		V	V	G	K						D			L	S					ND	
R12	S (62)	R	N		N		A				G	K	I			K		D		T	D				V	ND	ND	

[a] Only polymorphic positions are given.
[b] ND, not done.

TABLE 3. Sequence divergence for *sulA* and its deduced product

Isolate	% Divergence for isolate[a]:								
	CP1015	J94/76	J93/155	PN93/1802	PN94/720	R12	P48	PN93/917	J93/196
CP1015		4.8	6.8	6.7	5.5	6.5	5.1	4.9	3.1
J94/76	3.2		6.3	5.3	3.9	5.1	5.3	6.1	4.7
J93/155	4.5	3.9		6.8	5.9	6.8	6.7	7.1	6.7
PN93/1802	5.9	4.9	4.8		4.5	0.5	6.4	7.2	6.3
PN94/720	3.5	3.2	3.2	4.2		4.0	5.9	6.7	5.6
R12	5.5	4.4	5.1	0.7	3.3		6.3	7.0	6.1
P48	3.9	2.1	5.0	4.6	4.3	4.0		2.0	2.0
PN93/917	3.5	2.5	5.2	5.5	4.6	4.4	1.4		2.1
J93/196	3.1	2.1	4.8	5.2	3.9	3.6	0.7	1.4	

[a] Nucleotide differences are given above and amino acid differences are given below the diagonal. Duplications in resistant isolates were excluded from the calculation of divergence.

isolates covered a similar range of serotypes and countries of origin and included in particular two serotype 9V United Kingdom isolates that had been previously shown to be highly related by multilocus enzyme electrophoresis and pulsed-field gel electrophoresis pattern, but to differ in antibiotic resistance profile (PN93/1802 and PN93/917). The sequence of *sulA*, excluding the first 11 and last 14 codons (from which the PCR primers were designed), was determined for the six resistant and two susceptible isolates (Fig. 1). Partial sequences, between the A and B primers, were determined for the remaining susceptible isolates (not shown).

All resistant isolates contained a duplication of either 3 or 6 bp, resulting in the addition of one or two amino acids in the region between amino acids Arg_{58} and Tyr_{63}. None of the six susceptible isolates contained duplications in this region. In addition, there was considerable sequence diversity elsewhere in the gene, among both resistant and susceptible isolates (Fig. 1 and Table 2). Most mutations were synonymous, but some involved amino acid changes (Tables 2 and 3). No amino acid change was present in all resistant isolates and absent in all susceptible isolates. The highest level of sequence difference was between PN93/1802 and PN93/917, the resistant and susceptible serotype 9V isolates, with 7.2% divergence in DNA sequence in addition to the duplication (Table 3).

TABLE 4. Transformation to sulfonamide resistance

Donor	Donor DNA (no. of transformations)	Frequency of transformation	Sulfamethoxazole MIC (mg/liter)	
			Donor	CP1015 transformants[a]
PN93/1802	Total DNA (1)	3×10^{-3}	256	512
	PCR A–B (3)	3.2×10^{-4}–2×10^{-6}		
	PCR A–D (2)	2.9×10^{-2}–2.1×10^{-3}		
J94/76	Total DNA (1)	1.8×10^{-3}	512	1,024
	PCR A–B (1)	5.1×10^{-7}–7.7×10^{-7}		
J93/155	Total DNA (1)	2.5×10^{-3}	512	1,024
	PCR A–B (2)	1.3×10^{-7}–7.7×10^{-7}		
PN93/720	PCR A–B (1)	2.3×10^{-4}	256	512 or 1,024
	PCR A–D (1)	4.3×10^{-2}		
P48	PCR A–B (1)	4.8×10^{-5}	1,024	1,024
	PCR A–D (1)	2.2×10^{-2}		
None	No DNA	None detected		

[a] MICs were determined independently for three to six transformants for all donor DNA types.

Transformation of sulfonamide resistance. The determinant of sulfonamide resistance was located by transformation of DNA from resistant isolates into the highly transformable recipient CP1015 (16). (Sequence determination of *sulA* from CP1015 revealed it to be identical to the published sequence from R6.) Either total DNA, the *sulA* PCR fragment amplified with primers A and D (913 or 916 bp, almost the complete gene), or the *sulA* PCR fragment amplified with primers A and B (a 319 or 322-bp fragment that includes the duplications) was used as donor DNA (Table 4). Rates of transformation decreased with decreasing length of donor DNA, so that typically 10^2- to 10^3-fold less transformants were obtained with fragments A-B than with fragments A-D (Table 4). However, in control experiments without donor DNA, no spontaneous resistant mutants were detected.

For all five of the resistant isolates tested, fragments A-B (as well as larger fragments) were capable of transforming CP1015 to sulfonamide resistance. The sixth resistant isolate, R12, was indistinguishable from PN93/1802 in the A-B fragments, and so it was not independently tested. The MIC of sulfamethoxazole in the transformants was always as high as or higher than that in the original donor. The higher MIC for many transformants may reflect the genetic background of the recipient, for which the MIC of sulfamethoxazole is 64 mg/liter, compared to 32 mg/liter for R6 and typical susceptible clinical isolates. The sequence of the A-B segment of *sulA* was determined in two transformants from each donor. All transformants had acquired the duplication of their respective donors. For four donors (J93/155, P48, PN94/720, and J94/76) at least one of the two transformants sequenced had acquired no other coding difference, confirming that resistance was due to the duplication. In the case of PN93/1802, both sequenced transformants had acquired the Ser_{61}-to-Arg_{61} mutation of the parent as well as the duplication. This would be expected, because the two sites are so close together that recombination between them would be very infrequent. However, the Ser_{61}-to-Arg_{61} mutation cannot be required for resistance, because isolate PN94/720 has a duplication which results in an amino acid sequence equivalent to that of PN93/1802, but lacks the mutation.

DISCUSSION

The results described above demonstrate that 3- or 6-bp duplications in *sulA*, the DHPS gene, confer resistance to sulfonamide in clinical isolates of *S. pneumoniae*. The duplica-

tions characterized all occur within the region of amino acids Arg_{58} to Tyr_{63} (Fig. 1). Previously, Lopez et al. had demonstrated that the laboratory mutation *sul*-d comprises a 6-bp duplication in the DHPS gene, resulting in duplication of amino acids 66 and 67 (14). Secondary structure prediction by the method of Garnier (7) suggests that the *sul*-d duplication lies in an alpha helix, as also reported by Lopez et al. However the duplications in clinical isolates are predicted to fall outside the alpha helix, in a region of extended or coil conformation.

Sulfonamide resistance in meningococci is thought to have arisen through the acquisition of segments of a modified DHPS gene by horizontal gene transfer (20). The same mechanism is understood to be responsible for penicillin resistance in pneumococci (6). Our results suggest that recombination is also involved in sulfonamide resistance in pneumococci. The two serotype 9V isolates studied here have been described previously to share the same multilocus enzyme electrophoresis profile and highly related pulsed-field gel electrophoresis patterns, but differ in antibiotic resistance: PN93/1802 is resistant to sulfamethoxazole, trimethoprim, and penicillin (intermediate resistance), while PN93/917 is susceptible to all of these agents (9). The DHPS genes of these two isolates differ by 7.2% (Table 3), too great a difference to be accounted for by point mutation between isolates that by other criteria are believed to have a recent common ancestor. We suggest that resistance probably arose by transformation with a DHPS gene from another source; it is possible that resistance to other agents was transferred at the same time.

The serotype 9V isolates described here belong to the same clone of penicillin-resistant serotype 9V that has been described in France, Spain, Portugal, and Germany as well as the United Kingdom (3, 4, 8, 9, 13, 21, 22). Only in France and the United Kingdom were susceptible isolates of serotype 9V investigated, and in both cases, they were also found to belong to the clone (9, 13). Several models are possible to explain these findings. (i) A clone of susceptible 9V was prevalent in Europe, and resistance was acquired independently in different areas. (ii) A resistant clone spread through Europe, and resistance genes were subsequently lost by some members of the clone. (iii) Resistance arose in a single event (or series of events), and resistant and susceptible variants of the clone spread independently. Further analysis of DHPS and penicillin-binding protein (PBP) genes in both susceptible and resistant members of the clone should help to distinguish between these possibilities. Interestingly, it has already been shown that resistant Spanish and United Kingdom isolates belonging to the 9V clone share identical (by restriction pattern) PBP1A, -2B, and -2X genes with a widespread Spanish 23F multiply-resistant clone (4).

Given the apparent role of transformation in the acquisition of sulfonamide resistance in isolates of serotype 9V, it is tempting to speculate that this is likely to be the principal mechanism of the spread of resistance in pneumococci. However, it should be noted that in five of the six resistant isolates, the resistance-mediating duplications were distinct. Furthermore, whereas in both the DHPS genes of sulfonamide-resistant *N. meningitidis* (20) and the PBP2B genes of penicillin-resistant pneumococci (5, 6), there was evidence that sequences very different from the susceptible isolate had derived from one or more common ancestor, no comparable relatedness was apparent in the *sulA* sequences of sulfonamide-resistant pneumococci. The gene encoding DHPS is relatively diverse in pneumococci, in sulfonamide-susceptible as well as -resistant isolates, particularly when compared to the PBP2B gene from penicillin-susceptible isolates and to the amylomaltase gene *malM* (incorrectly referred to by Dowson et al. as *malP*) (6). Horizontal gene transfer may have had a role in generating such diversity and appears to have been involved in the spread of sulfonamide resistance, but it must also be concluded that resistant alleles have arisen independently, probably on multiple occasions.

ACKNOWLEDGMENTS

We are especially grateful to D. Morrison, M. C. Trombe, and J. P. Claverys for advice, assistance, and provision of the recipient strain and control DNA for pneumococcal transformation and N. Rikitomi for provision of Japanese isolates.

REFERENCES

1. **Barry, A. L., and C. Thornsberry.** 1985. Susceptibility tests: diffusion test procedures, p. 978–990. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C.
2. **Brown, G. M.** 1962. The biosynthesis of folic acid. II. Inhibition by sulfonamides. J. Biol. Chem. **237:**536–540.
3. **Carvalho, C., P. Geslin, and M. V. Vaz Pato.** 1996. Pulsed field gel electrophoresis in *Streptococcus pneumoniae* isolated in France and Portugal. Pathol. Biol. **44:**430–434.
4. **Coffey, T. J., C. G. Dowson, M. Daniels, J. Zhou, C. Martin, B. G. Spratt, and J. M. Musser.** 1991. Horizontal transfer of multiple penicillin-binding protein genes, and capsular biosynthetic genes, in natural populations of *Streptococcus pneumoniae*. Mol. Microbiol. **5:**2255–2260.
5. **Dowson, C. G., T. J. Coffey, C. Kell, and R. A. Whiley.** 1993. Evolution of penicillin resistance in *Streptococcus pneumoniae*: the role of *Streptococcus mitis* in the formation of a low affinity PBP2B in *S. pneumoniae*. Mol. Microbiol. **9:**635–643.
6. **Dowson, C. G., A. Hutchison, J. A. Brannigan, R. C. George, D. Hansman, J. Linares, A. Tomasz, J. M. Smith, and B. G. Spratt.** 1989. Horizontal transfer of penicillin binding protein genes in penicillin resistant clinical isolates of *Streptococcus pneumoniae*. Proc. Natl. Acad. Sci. USA **86:**8842–8846.
7. **Garnier, J., D. J. Osguthorpe, and B. Robson.** 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J. Mol. Biol. **120:**97–120.
8. **Gasc, A. M., P. Geslin, and A. M. Sicard.** 1995. Relatedness of penicillin-resistant *Streptococcus pneumoniae* serotype 9 strains from France and Spain. Microbiology **141:**623–627.
9. **Hall, L. M. C., R. A. Whiley, B. Duke, R. C. George, and A. Efstratiou.** 1996. Genetic relatedness within and between serotypes of *Streptococcus pneumoniae* from the United Kingdom: analysis of multilocus enzyme electrophoresis, pulsed-field gel electrophoresis, and antimicrobial resistance patterns. J. Clin. Microbiol. **34:**853–859.
10. **Huovinen, P., L. Sundstrom, G. Swedberg, and O. Sköld.** 1995. Trimethoprim and sulfonamide resistance. Antimicrob. Agents Chemother. **39:**279–289.
11. **Klugman, K. P.** 1990. Pneumococcal resistance to antibiotics. Clin. Microbiol. Rev. **3:**171–196.
12. **Koornhof, H. J., A. Wasas, and K. Klugman.** 1992. Antimicrobial resistance in *Streptococcus pneumoniae*: a South African perspective. Clin. Infect. Dis. **15:**84–94.
13. **Lefevre, J. C., M. A. Bertrand, and G. Faucon.** 1995. Molecular analysis by pulsed-field gel electrophoresis of penicillin-resistant *Streptococcus pneumoniae* from Toulouse, France. Eur. J. Clin. Microbiol. Infect. Dis. **14:**491–497.
14. **Lopez, P., M. Espinosa, B. Greenberg, and S. A. Lacks.** 1987. Sulfonamide resistance in *Streptococcus pneumoniae*: DNA sequence of the gene encoding dihydropteroate synthase and characterization of the enzyme. J. Bacteriol. **169:**4320–4326.
15. **Marton, A., M. Gulyas, R. Munoz, and A. Tomasz.** 1991. Extremely high incidence of antibiotic resistance in clinical isolates of *Streptococcus pneumoniae* in Hungary. J. Infect. Dis. **163:**542–548.
16. **Morrison, D. A., S. A. Lacks, W. R. Guild, and J. M. Hageman.** 1983. Isolation and characterization of three new classes of transformation-deficient mutants of *Streptococcus pneumoniae* that are defective in DNA transport and genetic recombination. J. Bacteriol. **156:**281–290.
17. **National Committee for Clinical Laboratory Standards.** 1997. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. National Committee for Clinical Laboratory Standards Villanova, Pa.
18. **Perez, J. L., J. Linares, J. Bosch, M. J. Lopez de Goicoechea, and R. Martin.** 1987. Antibiotic resistance of *Streptococcus pneumoniae* in childhood carriers. J. Antimicrob. Chemother. **19:**278–280.
19. **Porter, R. D., and W. R. Guild.** 1976. Characterization of some pneumococcal bacteriophages. J. Virol. **19:**659–667.
20. **Rådström, P., C. Fermér, B.-E. Kristiansen, A. Jenkins, O. Sköld, and G. Swedberg.** 1992. Transformational exchanges in the dihydropteroate synthase gene of *Neisseria meningitidis*: a novel mechanism for acquisition of

sulfonamide resistance. J. Bacteriol. **174:**6386–6393.
21. **Reichmann, P., E. Varon, E. Gunther, R. R. Reinert, R. Luttiken, A. Marton, P. Geslin, J. Wagner, and R. Hakenbeck.** 1995. Penicillin-resistant *Streptococcus pneumoniae* in Germany: genetic relationship to clones from other European countries. J. Med. Microbiol. **43:**377–385.
22. **Sibold, C., J. Wang, J. Henrichsen, and R. Hakenbeck.** 1992. Genetic relationships of penicillin-susceptible and -resistant *Streptococcus pneumoniae* strains isolated on different continents. Infect. Immun. **60:**4119–4126.
23. **Vaz Pato, M. V., C. Belo de Carvalho, A. Tomasz, and the Multicenter Study Group.** 1995. Antibiotic susceptibility of *Streptococcus pneumoniae* isolates in Portugal. A multicenter study between 1989 and 1993. Microb. Drug Res. **1:**59–69.