Typing of *Staphylococcus aureus* strains by PCR-amplification of variable-length 16S–23S rDNA spacer regions: characterization of spacer sequences

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To develop a rapid and accurate method of typing large numbers of clinical isolates of *Staphylococcus aureus*, the spacer region C of the rRNA operon [1391–507 (16S–23S)] was enzymically amplified from 322 strains. When the products were separated by denaturing PAGE, 15 variable-length *rrn* alleles were demonstrated, ranging in size from 906 to 1223 bp. The variable-length *Hpa*II-digested region C [(region E; 1446–196 (16S–23S)] amplification products were cloned into M13mp18RF to sequence separate variable-length alleles. A total of 17 region E inserts were sequenced, aligned and divided into nine alleles by length (938–1174) and sequence properties. The 16S–23S spacer rDNA varied in length (303–551 bp) and in properties; three alleles contained a *tRNA*^{Leu} gene alone, two alleles contained a *tRNA*^{Leu} and a *tRNA*^{Ala} gene, and four alleles lacked *tRNA* genes. The sequences of two alleles showed less than 1% variation when isolated from two or three *S. aureus* strains. The 48 penicillin- and methicillin-sensitive strains were divided into 26 ribotypes; in contrast, the 274 methicillin-resistant *S. aureus* (MRSA) strains were divided into nine ribotypes (A–I) with 97% typing as either ribotype A or B (*rrnL* was missing in B). The sequence conservation of the *rrn* operons argues for the use of the 16S–23S spacer region as a stable and direct indicator of the evolutionary divergence of *S. aureus* strains.

Keywords: *Staphylococcus aureus*, typing, rDNA, *rrn* operon, spacer regions

INTRODUCTION

Disease caused by *Staphylococcus aureus* is often the result of hospital-acquired (nosocomial) infections. Strains of *S. aureus* that are resistant to the penicillinase-resistant antibiotic methicillin are now common, the first major nosocomial epidemic of a methicillin-resistant strain of *S. aureus* (MRSA) having been described by Stewart & Holt (1963). The determination of whether isolates of *S. aureus* represent a single strain is of considerable epidemiological value in a hospital setting.

Numerous methods for typing MRSA isolates have been described, including bacteriophage typing (Blair &

Williams, 1961), biotyping (Cookson *et al.*, 1986), electrophoretic protein typing (Costas *et al.*, 1989; Stephenson *et al.*, 1986), immunoblots (Mulligan *et al.*, 1988), plasmid DNA analysis (Hartstein *et al.*, 1989), multilocus enzyme electrophoresis (Musser & Kapur, 1992), ribotyping (see below), analysis of the *mec* gene (Kreiswirth *et al.*, 1993) and pulsed-field gel electrophoresis (Prevost *et al.*, 1991; Wei & Grubb, 1992).

The 16S rRNA gene has been used extensively for evolutionary studies of bacterial species (Woese, 1987) and the rRNA operon has been used for evolutionary studies of bacterial strains (Gürtler, 1993; Frothingham & Wilson, 1993). The Southern hybridization of rRNA operons to detect restriction fragment length polymorphisms (RFLPs) between MRSA strains has been reported (Blumberg *et al.*, 1992; De Buyser *et al.*, 1992; Monzon *et al.*, 1991; Preheim *et al.*, 1991). However, Southern hybridization is slow and labour-intensive. The rRNA RFLPs found in *Clostridium difficile* have been

Abbreviations: HRH, Heidelberg Repatriation Hospital; MRSA, methicillin-resistant *Staphylococcus aureus*; RFLP, restriction fragment length polymorphism.

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shown in part to be due to variable-length 16S–23S rDNA spacer regions (Gürtler, 1993). A method was described to type *C. difficile* strains based on the variable-length 16S–23S rDNA spacer regions (Gürtler, 1993). In this report an identical approach is used to type *S. aureus* isolates.

The number of rRNA operons has been completely determined (by Southern hybridization or PCR of the rDNA) in only a few eubacteria, including *Escherichia coli*, where there are seven operons (Morgan *et al.*, 1977); *Bacillus subtilis*, 10 operons (Loughney *et al.*, 1982); *Clostridium perfringens*, 10 operons (Garnier *et al.*, 1991); *C. difficile*, 10 operons (Gürtler, 1993); and *Mycobacterium* spp. (Bercovier *et al.*, 1986) and *Mycoplasma* spp. (Amikam *et al.*, 1984), one and two operons respectively. The reports describing sequence data for the 16S–23S spacer region are so far incomplete: of seven operons in *E. coli* five spacers have been sequenced (Harvey *et al.*, 1988); in *B. subtilis* two of 10 spacers have been sequenced (Loughney *et al.*, 1982); in *Plesiomonas shigelloides* three spacers (East *et al.*, 1992); in *Aeromonas hydrophila* three spacers (East & Collins, 1993) and in *Caulobacter crescentus* (Feingold *et al.*, 1985), *Acholeplasma laidlawii* (Nakagawa *et al.*, 1992) and *Enterococcus hirae* (Sechi & Daneo-Moore, 1993) two spacers have been sequenced. In this report we describe for the first time the 16S–23S spacer sequences for the complete rRNA operon set in *S. aureus*.

METHODS

Bacterial strains and their cultivation. The bacterial strains used are listed in Table 1. The identity of all strains was determined by biochemical tests (Kloos & Schleifer, 1986), and antibiotic sensitivity tests were assessed by the agar dilution method (break points were determined according to NCCLS guidelines, vol. 13, no. 25, 1993). Purified stocks were stored in glycerol broth at -70°C . All strains were grown in trypticase soya broth (TSB, Oxoid). The stability of ribotype patterns was tested by passaging single colonies from sheep blood agar plates five times a week for six weeks.

DNA isolation and amplification. Genomic DNA was isolated by the protocol of Gürtler *et al.* (1991) except that the cell walls of *S. aureus* were disrupted by incubating the strain with 200 μg lysostaphin ml^{-1} at 37°C for 5–10 min. DNA regions (Fig. 1) were amplified by the protocol of Gürtler (1993) using the primers shown in Table 2. For the amplification of M13 clone inserts, 50–100 ng single-stranded M13 clone DNA was added to PCR mixtures using primers M13F and R.

Restriction enzyme analysis. PCR product M13F-M13R was digested with 10–15 units *DraI* or *HinfI*, as instructed by the manufacturer (Boehringer). Genomic DNA was digested with 20 units *HpaII*. The digested PCR products and genomic DNA were resolved on 2% (w/v) low-gelling-temperature plus 2% (w/v) 'AR' agarose gels.

Southern hybridization. The protocol of Gürtler *et al.* (1991) was followed to hybridize digoxigenin-labelled PCR products I and J (Fig. 1: amplified using V42 and V12, respectively, as templates) to *S. aureus* genomic DNA.

Denaturing PAGE. [^{32}P]dATP-labelled PCR product C was amplified and electrophoretically separated by the protocol of Gürtler (1993).

Table 1. *S. aureus* strains

The numbers of strains isolated from various locations at various times are shown. The strains from Ireland are listed in Townsend *et al.* (1987) as WG1761–3 (plus six other strains) while those from RFH are listed in Townsend *et al.* (1984) as WG2710, 2715, 2720 and 2724 and in Townsend *et al.* (1987) as WG2716 (plus three other strains).

Location*	Year	No. of isolates
Methicillin-resistant		
Guildford, UK, NCTC 10442	1960	1
New York, ATCC 33952	1981	1
Melbourne, RMH	1982	10†
UK, NCTC 11940	1982	1
UK, NCTC 11939	1982	1
Melbourne, HRH	1982	6‡
Ireland	1982–83	9
London, RFH	1983	8
London, NCTC 12232	1986	1
Melbourne, HRH	1988–9	7
Melbourne, HRH	1992–1993	226§
Melbourne, RCH	1993	3
Penicillin-sensitive		
HRH	1992–4	14
Oxford, UK, ATCC 9144	1944	1
Bundaberg, Australia, NCTC 2669	1928	1
UK, NCTC 8532	1953	1
Methicillin-sensitive		
HRH	1992–3	31

* NCTC, National Collection of Type cultures, UK; ATCC, American Type Culture Collection; HRH, Heidelberg Repatriation Hospital; RMH, Royal Melbourne Hospital; RCH, Royal Children's Hospital, Melbourne; RFH, Royal Free Hospital, London, UK.

† Including H21.

‡ Including H11, H12, H14.

§ Including D46.

Cloning. PCR product C (Fig. 1) was amplified (described above) from *S. aureus* genomic DNA from strains D46, H11 and ATCC 33952 using primers R1391F and LR488, R1391FH and LR488H, or R1391F and LR194F (Table 2). For each strain, ten equivalent reactions were pooled, precipitated with 26% (w/v) polyethylene glycol in 20 mM MgCl_2 (Paithankar & Prasad, 1991) and digested with *HpaII*. For D46, 1 μg of the *HpaII*-digested products was end-repaired with 4 units of T4 DNA polymerase (Boehringer), 200 μM dNTPs, 33 mM Tris/acetate (pH 8.0), 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol, 0.1 mg bovine serum albumin ml^{-1} and incubated at 11°C for 30 min. For ATCC 33952 and H11 the *HpaII*-digested products (1–25 ng) were ligated directly into *AccI*-digested M13mp19RF (50 ng: in a total of 10 μl) and the end-repaired *HpaII*-digested products (1–25 ng) from strain D46 were ligated into *SmaI*-digested M13mp19RF (50 ng: in a total of 10 μl) with 1 unit T4 DNA ligase (Boehringer), 66 mM Tris/HCl (pH 7.5), 5 mM MgCl_2 , 1 mM dithiothreitol, 1 mM ATP and incubated at room temperature overnight. Competent JM109 *E. coli* cells (50 μl ; Promega) were transformed with

Fig. 1. Approaches for typing and sequencing 16S–23S rDNA alleles in *S. aureus*. The diagram shows the distribution of rDNA genes and restriction sites in the region of interest. The solid line joining these genes can vary in length in the same strain or in different strains (Fig. 3b). At the bottom of the diagram, the dashed lines show positions of the PCR products C, I and J, which were obtained using the primers R1391F and LR488, SP1F and SP2R, and SP1F and LR20F, respectively (Table 2). The dotted lines show the origins of *Hpa*II fragments (E, F, G and H) obtained from PCR product C. The locations of other primer-binding regions that were used to sequence *Hpa*II fragment E are also shown.

Table 2. Sequencing primers

Code	Region	Position*	direction (sense)	Sequence
R1391FH†	16S	1400–1417 ^a	F(+)	GGCCGGTTGTACACACCGCCGTC
R1391F	16S	1400–1417 ^a	F(+)	TTGTACACACCGCCGTC
SP1F	16S–23S spacer	51–70 ^b	F(+)	ATTGTATTCAGTTTGAATG
SP1R	16S–23S spacer	51–70 ^b	R(–)	TTACTTACTTATCTAGTTT
TRNAF	tRNA ^{Ile}	100–120 ^b	F(+)	ATAGCTCAGCTGGTTAGAGC
TRNAR	tRNA ^{Ile}	100–120 ^b	R(–)	GCTCTAACCAGCTGAGCTAT
SP2F	16S–23S spacer	281–300 ^b	F(+)	AAAAGTAGATAAGTAAGTAA
SP2R	16S–23S spacer	281–300 ^b	R(–)	GTGGATGCCTTGGCACTAG
SP3F	16S–23S spacer	390–410 ^b	F(+)	CACTCACAAGATTAATAACG
SP3R	16S–23S spacer	390–410 ^b	R(–)	CGTTATTAATCTTGTGAGTG
LR20R	23S	24–42 ^c	F(+)	GTGGATGCCTTGGCACTAG
LR20F	23S	24–42 ^c	R(–)	CTAGTGCCAAGGCATCCAC
LR194F	23S	194–214 ^c	R(–)	CTTTCTCTTCTCCGGGTACT‡
LR488	23S	502–520 ^c	R(–)	CCTTCCCTCACGGTACTG
LR488H†	23S	502–520 ^c	R(–)	AACCGGCCTTCCCTCACGGTACTG
M13R	Phage M13	6205–6221 ^d	F(+)	CAGGAAACAGCTATGAC
M13F	Phage M13	6291–6307 ^d	R(–)	GTTTTCCAGTCACGAC

* Numbering according to: *a*, the published 16S rRNA sequence from *S. aureus* (Ludwig *et al.*, 1992); *b*, the aligned spacer sequence for *S. aureus* (Fig. 3); *c*, the published 23S rRNA sequence from *S. aureus* (Ludwig *et al.*, 1992); *d*, the published sequence for bacteriophage M13.

† Addition of *Hpa*II sites (underlined) at 5' ends of R1391F and LR488.

‡ Sequence obtained from this study.

Fig. 2. *S. aureus* ribotypes. Denaturing PAGE of PCR product C amplified from strains of *S. aureus*. (a) MRSA ribotypes A (lanes 1–3) and B (lanes 4–8). (b) All MRSA ribotypes A–I (excluding C) and penicillin-sensitive ribotype Pa (ATCC 9144). (c) Penicillin- and methicillin-sensitive *S. aureus* strains: lanes 1–6, ribotypes Pi, Pj, Pj, Pf, A (strain H11) and Mg; lanes 9–16, ribotypes B, Mi, Mh, Mi, Pi, Mh, Mj and Mj. The size markers shown in (c) are λ DNA digested with HindIII and EcoRI (lane 8; 947 bp band only) and SPP1 DNA digested with EcoRI (lane 7; 1150 and 1000 bp bands only). The sizes (bp) of the respective alleles are shown on the right [mean ± SEM (number of determinations)].

2–3 µl of the ligation mixtures according to the protocol described in Sambrook *et al.* (1989). After plates had been incubated overnight at 37 °C, bacteriophage M13 plaques were either picked off and grown in Luria Broth (LB), or colony hybridizations (Sambrook *et al.*, 1989) to HpaII-digested PCR product C labelled with digoxigenin were performed. Positive plaques were then picked off and grown in LB (Sambrook *et al.*, 1989). Single-stranded bacteriophage M13 DNA was then prepared from all the positive clones (Sambrook *et al.*, 1989). To determine the presence and size of the inserts, the single-stranded DNA from the M13 clones was used as a template in the PCR using M13F and R primers which flank the *Sma*I and *Acl*I restriction sites (Yanisch-Perron *et al.*, 1985).

DNA sequencing. Sequencing was done by the dideoxynucleotide method of Sanger *et al.* (1977) using the Bst DNA sequencing kit (Bio-Rad). 7-Deaza-2'-deoxyguanosine triphosphate was used to minimize band compression due to GC-rich regions. The primers used for sequencing are listed in Table 2 and Fig. 1.

Data analysis. DNA sequences were processed and analysed by the following methods. The DNASIS program (version 6; Pharmacia) was used to orient, join and edit DNA sequences. The orientation of inserts was deduced by alignment with the 16S or 23S rDNA sequences from *Bacillus subtilis* (Green *et al.*, 1985) or *S. aureus* (Ludwig *et al.*, 1992). The 17 sequences

(fragment E, Fig. 1) were aligned using CLUSTAL V (Higgins *et al.*, 1992) and were aligned to the 16S and 23S rDNA sequences from *S. aureus* (Ludwig *et al.*, 1992). Further modifications to the alignment were done using MACCLADE software (Maddison & Maddison, 1992). Phylogenetic analysis was done with the software package PHYLIP using DNAPENNY (Felsenstein, 1993). The resulting treefile was then imported into MACCLADE for further analysis and presentation.

The presence or absence of PCR product C bands was analysed by the protocol of Gürtler (1993) with intense bands reported as positive; when the faint bands were also reported as positive the results did not change.

RESULTS

Characterization of 16S–23S rDNA spacers

Using the DNA typing approach used previously (Gürtler, 1993) and the primers LR1391F and LR488, PCR product C (Fig. 1) was amplified from the *S. aureus* strains listed in Table 1. These included 274 MRSA from three geographically distinct clinical sources (261 from the HRH) and 45 penicillin- or methicillin-sensitive strains

Table 3. Presence or absence of sequences within the nine (*rrnA*, *C*, *E*, *F*, *G*, *H*, *J*, *K* and *L*) 16S–23S spacer alleles sequenced

The sequence information presented in Fig. 3 is tabulated to show from which *S. aureus* strains the original PCR product was isolated, which clones were characterized, the GenBank accession number, the sizes of the 16S–23S spacer, the *HpaII* insert (fragment E, see Fig. 1) and fragment C, the presence or absence of sequences (CS, conserved sequence; VS, variable sequence; positions and lengths are shown according to numbering in Fig. 3b), and the base-pair differences between clones for the 13 regions.

Allele	Clone	Strain from which isolated	GenBank no.	Size (bp) of:			CS1	tRNA ^{Ile}	VS1	VS2	tRNA ^{Ala}	VS3	VS4	CS2	VS5	VS6	VS7	CS3	VS8	Base- pair changes																
				16S–23S spacer	Fragment E	Fragment C*															Position:															
																					1–73	93–172	74–119	120–228	191–266	232–270	248–270	271–415	416–449	450–527	530–537	538–546	547–550			
<i>rrnL</i>	V13	H11	U11775	303	603	981	+	–	+	–	–	–	+	+	–	–	–	+	–	NA																
<i>rrnK</i>	V41	33952	U11785	319	616	994	+	–	+	–	–	+	–	+	–	–	–	+	–	NA																
<i>rrnJ</i>	4,	D46	U11773	335	634	1012	+	+	–	–	–	–	+	–	–	–	–	+	–	5																
	V4,	H11	U11783																																	
	V17,	H11	U11776																																	
	V8,	H11	U11789																																	
	V32,	33952	U11780																																	
V43	33952	U11787																																		
<i>rrnH</i>	V27	33952	U11778	362	661	1039	+	–	+	–	–	+	–	+	+	–	+	+	+	2																
	V7	H11	U11788		663	1041																														
<i>rrnG</i>	V2	H11	U11777	382	672	1050	+	+	–	–	–	–	–	+	+	–	+	+	+	NA																
<i>rrnF</i>	V30	33952	U11779	460	756	1134	+	+	–	–	–	–	–	+	+	+	+	+	+	4																
	V38	33952	U11782																																	
<i>rrnE</i>	V12	H11	U11774	469	764	1142	+	–	+	+	–	+	–	+	+	–	+	+	+	NA																
<i>rrnC</i>	V34	33952	U11781	473	774	1152	+	+	–	–	+	–	–	+	+	–	+	+	+	5																
	V42	33952	U11786																																	
<i>rrnA</i>	V40	33952	U11784	551	849	1227	+	+	–	–	+	–	–	+	+	+	+	+	+	NA																
Length (bp)							73	79	33	109	75	38	23	145	33	77	8	8	4																	

* The size of fragment C (see Fig. 1) can be obtained by adding 331 bp (*HpaII*₂ to LR520) and 47 bp (R1391 to *HpaII*₁) to fragment E.

from a single Melbourne source; three MRSA and three sensitive strains from type culture collections were used. These strains yielded various amplified products: in total, 15 alleles were recognized (named *rrnA*–*rrnO*), 14 of which are shown in Fig. 2, varying in length from 935 to 1223 bp (*rrnO* was 906 bp in length; not shown).

With the aim of isolating and comparing the variable-length sequences of the 16S–23S rRNA alleles, PCR product C from strains D46 (ribotype B), H11 and ATCC 33952 (both ribotype A) were cleaved with *HpaII* and the resulting fragments cloned into M13 vectors. Four clones were isolated from D46, one containing insert E (Table 3), one containing insert F, one containing insert G and one containing insert H. Sixteen clones (V2–V17) were isolated from H11: seven contained insert E (Table 3), four contained insert H, four contained insert G, and one contained insert F. With the sequence information of insert H, primer LR194F was designed, which contained a *HpaII* site. For strain ATCC 33952, primers LR194F and R1391F were used to obtain a mixture of PCR products, which were digested with *HpaII* to yield predominantly product E and cloned into M13mp19: clones V18–V47 were isolated and nine of these contained insert E (Table 3).

It was confirmed that inserts were of variable length when they were amplified by PCR (not shown) and then digested

with *DraI* or *HinfI* (not shown; see Fig. 1 for restriction enzyme cleavage sites). It was determined from the first insert sequenced (clone 4) that *HinfI* digested at the end of the tRNA^{Ile} gene (Figs 1 and 3b). All the PCR products which digested with *HinfI* (not shown) contained a tRNA^{Ile} gene, which was subsequently confirmed by DNA sequence data (Fig. 3b).

The designation of alleles (Table 3) was made by direct correlation with fragment C molecular sizes (Fig. 2a). The length of the spacer varied from 303 bp to 551 bp (Table 3). The fragment E insert sequences were aligned to the 16S (Fig. 3a), 23S (Fig. 3c) and 16S–23S spacer (Fig. 3b) rDNA sequences. There were only four base-pair differences in the 16S rDNA sequences (Fig. 3a), in contrast to 71 base-pair differences in the 23S rDNA sequences (Fig. 3c). In the 16S–23S spacer rDNA (Fig. 3b), Table 3) there were no differences in CS1 and CS2; however, there were striking gaps between alleles in regions VS2, tRNA^{Ala}, VS3, VS4, VS5, VS6, VS7 and VS8. The tRNA^{Ile} gene was present in *rrnJ*, *G*, *F*, *C* and *A*, and the tRNA^{Ala} gene was only present in *rrnA* and *C*. The number of base-pair differences between clones judged to be the same allele was five for *rrnJ* (from strains H11, D46 and ATCC 33952, isolated in 1982, 1992 and 1981 respectively), two for *rrnH* (from strains ATCC 33952 and H11), four for *rrnF* (strain ATCC 33952) and five for *rrnC* (ATCC 33952) (Table 3). Using phylogenetic analysis, the *rrn* alleles were divided

Table 4. Distribution of alleles in *S. aureus* ribotypes

PCR product C was amplified from various *S. aureus* strains and separated by denaturing PAGE (Fig. 2). The presence of variable-length alleles (*rrnA–O*) is shown. The size of each allele is shown in Fig. 2. The data were collated (using BioImage Software) from Fig. 2 and four other denaturing polyacrylamide gels.

Ribotype	Allele (<i>rrn</i>):															No. of isolates
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	
Methicillin sensitive																
Ma	+	−	+	−	−	−	+	−	+	−	−	−	−	−	+	2
Mb	−	−	+	−	−	−	−	+	−	+	−	+	−	−	−	1
Mc	−	−	+	−	−	+	+	+	+	−	−	−	−	−	−	1
Md	+	−	−	−	−	+	−	−	−	+	+	+	−	−	−	1
Me	−	−	+	+	+	+	−	+	−	+	+	−	−	−	−	1
Mf	−	−	+	+	+	+	−	+	−	−	−	−	−	−	−	1
Mg	−	−	+	+	+	−	+	+	−	−	−	−	−	−	−	2
Mh	−	+	+	+	+	+	−	+	−	+	+	−	−	−	−	2
Mi	+	−	+	+	−	−	+	+	−	−	−	−	−	−	−	2
Mj	−	−	+	−	−	+	+	+	+	+	+	+	−	−	−	2
Mk	−	−	+	−	−	+	−	+	−	−	−	−	−	−	−	2
MI	+	−	−	−	−	+	−	−	−	+	−	+	−	−	−	1
Mm	−	−	+	−	+	+	−	+	−	−	−	−	−	−	−	1
Mn	+	+	−	−	−	+	−	−	+	+	−	+	−	−	−	1
Mo	+	−	+	−	−	+	−	−	+	+	−	+	−	−	−	1
Mp	+	−	+	−	−	−	−	+	−	−	−	+	−	−	−	2
Mq	+	−	+	+	−	+	+	−	+	−	−	−	−	−	−	1
Penicillin sensitive																
Pa	−	+	+	+	−	+	−	+	−	−	−	−	−	−	−	2
Pc	+	−	+	+	+	+	−	−	−	−	−	−	−	−	−	6
Pd	+	−	+	+	+	−	+	+	−	+	−	−	−	−	−	1
Pe	−	−	+	−	−	+	−	−	−	+	−	+	−	−	−	1
Pf	−	−	+	−	−	+	−	+	−	+	−	+	−	−	−	1
Pg	−	−	+	−	−	+	+	+	−	−	−	−	−	−	−	1
Ph	−	−	+	−	−	+	+	+	−	+	−	+	−	−	−	1
Pi	+	−	−	−	+	−	+	+	+	−	−	−	−	−	−	2
Pj	+	−	+	−	−	−	+	−	+	−	−	−	−	−	−	1
MRSA																
A*	+	−	+	−	−	+	−	+	−	+	−	+	−	−	−	94
B†	+	−	+	−	−	+	−	+	−	+	−	−	−	−	−	178
C‡	−	−	+	−	−	+	−	+	−	+	−	−	−	−	−	3
D§	+	−	+	−	−	+	−	+	−	+	−	+	+	−	−	1
E	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	1
F	−	−	−	+	−	−	−	−	+	+	+	−	−	−	−	1
G	+	−	+	−	−	−	−	+	−	+	−	−	−	−	−	1
H	+	−	+	−	−	+	−	+	−	+	−	+	−	+	−	1
I	+	−	+	−	−	−	+	−	+	−	−	−	−	−	−	6

* Including H11 and ATCC 33952; one isolate was penicillin-sensitive and another was methicillin-sensitive.

† Including D46 and H12; one isolate was methicillin-sensitive.

‡ Including H14.

§ Including H21.

|| Five isolates were methicillin-sensitive.

into three distinct groups (Fig. 4) containing tRNA^{Ile} and tRNA^{Ala}, tRNA^{Ile} only, or no tRNA genes.

To confirm the presence of variable-length 16S–23S spacer regions in genomic DNA, PCR products I and J (Fig. 1) were hybridized to genomic DNA isolated from *S. aureus* isolates (not shown). Between four and seven bands, ranging in size from ~ 600 bp to ~ 850 bp, were obtained for all strains, with variation between strains. The results showed that variable-length 16S–23S spacer regions occur in genomic DNA whose size range is similar to the results obtained with PCR-ribotyping (Fig. 2) and DNA sequencing (Fig. 3b, Table 3).

Typing of *S. aureus* strains

The majority of strains presented in this study were from the HRH (274 strains of a total of 322). Among the MRSA strains, two ribotypes (A and B) that were highly reproducible in individual isolates were obtained (Fig. 2a) with 101 and 180 strains respectively (including five type strains). An additional seven ribotypes were found among the remaining nine MRSA strains (Fig. 2b shows eight MRSA ribotypes). Ribotype A was the major ribotype found (ribotype A/total no. of strains in location) between 1960 and 1989 in Melbourne (19/22), Ireland (9/9), New York (1/1) and the UK (12/12). After 1989, ribotype B was the major ribotype found at the HRH (176 were ribotype B and 57 were ribotype A).

In contrast to the MRSA strains, the sensitive strains showed considerably more variation in the presence or absence of bands, yielding an additional 26 ribotypes from the 48 strains studied (Fig. 2c shows some of these strains). The MRSA ribotypes A, B and I included some of the penicillin- or methicillin-sensitive strains (Table 4). The occurrence of the alleles in the various ribotype classes is summarized in Table 4.

The stability of ribotypes A, B, C, D and Pa was investigated by 30 serial passages of strains ATCC 9144, H11, H12, H14 and H21 over a six-week period. The ribotype was assessed after every fifth passage by visual comparison with reference patterns and was found to be stable except for strains H12 and H21. Strain H12 was identified as ribotype B at all passages except number 5, where *rrnL* appeared, making it ribotype A. Strain H21 was originally found to be ribotype D; however, during the stability experiment it was found to be ribotype A at all passages subsequently investigated (the colonies from which the DNA was prepared were used completely). After plating out colonies from the original frozen stock, genomic DNA was prepared from 10 separate colonies of strain H21: in all cases the ribotype was found to be A. The instability of strains H12 and H21 could be explained by a contaminant or by the rearrangement, duplication or deletion of an *rrn* operon to yield *rrnL* or *rrnM*, respectively. This instability did not affect the typing of *S. aureus* strains significantly since they were infrequent events.

DISCUSSION

The main finding of the present study was that the presence or absence of specific variable-length rDNA spacer regions varied between *S. aureus* strains. The patterns obtained were mostly stable within strains upon repeated testing, allowed the designation of strains to specific types, discriminated within the species *S. aureus*, and allowed for the easy testing of large numbers of strains. With these criteria met, the molecular typing method described here may be applied to epidemiological studies of *S. aureus*.

The 16S–23S rDNA spacer sequences of nine *rrn* operons were determined from three MRSA strains. The variation in 16S–23S spacer length (303 bp to 551 bp) was accounted for by the type (tRNA^{Ala} or tRNA^{Ile}) and number (one, both or none) of tRNA genes and by the presence or absence of other sequences of unknown function.

The type of tRNA gene found in the 16S–23S spacer varies in number (0, 1 or 2) and combination between operon and between species: *Aeromonas hydrophila* (East & Collins, 1993) and *E. coli* (Morgan *et al.*, 1977) have tRNA^{Ala}, tRNA^{Ile} and tRNA^{Glu}; *Bacillus subtilis* (Loughney *et al.*, 1982) and *Caulobacter crescentus* (Feingold *et al.*, 1985) have tRNA^{Ile} and tRNA^{Ala}; *Plesiomonas shigelloides* (East *et al.*, 1992) has tRNA^{Glu}; *Methanococcus vannielii* (Jarsch & Böck, 1983) and *Enterococcus hirae* (Sechi & Daneo-Moore, 1993) have tRNA^{Ala}; and *Mycobacterium bovis* has no tRNA genes (Suzuki *et al.*, 1987). The length of the spacer varies from 156 bp for *M. vannielii* (Jarsch & Böck, 1983) to 551 bp (this report). There is intraspacer and interspacer variation of other sequences besides tRNA genes (this report).

The variation in length and sequence of the 16S–23S spacer makes it an ideal candidate for typing of strains [(*C. difficile* (Gürtler, 1993), *Mycobacterium avium* (Frothingham & Wilson, 1993, 1994)] and species identification (Jensen *et al.*, 1993) which can potentially be comprehensively applied to the bacterial kingdom.

The instability of *rrn* operons has been reported in *B. subtilis* (Widom *et al.*, 1988) and *E. coli* (Hill & Harnish, 1982). These reports show evidence for the loss of an *rrn* operon (Loughney *et al.*, 1983; Widom *et al.*, 1988) and recombination leading to chromosomal rearrangement of *rrn* operons (Hill & Harnish, 1982). The instability of ribotype D (strain H21) in the present study could be explained by the loss of *rrnM* giving rise to the stable ribotype A. The relative instability of ribotype B in this study was due to the loss of *rrnL*. The frequency of such events is low [$\sim 10^{-4}$; Hill & Harnish (1982)] and thus will have little practical effect on this method.

The fact that ribotypes common to MRSA strains (A, B and I) were also found amongst the sensitive strains suggests that the transfer of *Mec^R* to a methicillin- or penicillin-sensitive strain may have occurred; the initial *Mec^R* transfer event may have been to a penicillin-

(a)		1456	1466	1476	1486	1496	1506	1516	1526
SA16S		CGGTGGAGTA	ACCTTTTAGG	AGCTAGCCGT	CGAAGGTGGG	ACAAATGATT	GGGGTGAA-G	TCGTAAACAAG	GTAGCCGTAT
rrnG		.....	.....	.....	.....	.....	.....	.....	.....
rrnC	V34	.....	.....	C.....	.....	.....	.....	.....	.....
rrnC	V42	.....	.....	.....	.....	.....	.....	.....	.....
rrnI		.....	.....	.....	.....	.....	.....	.....	.....
rrnH	v7	.....	.....	.....	.....	.....	T.....	.....	.....
rrnH	V27	.....	.....	.....	.....	.....	.....	.....	.....
rrnK		.....	.....	.....	.....	.....	.....	.....	.....
		1536		1546	1556				
SA16S		CGGAAGGTGC	GGCTGGATCA	CCTCCTTTCT					
rrnJ,F,C,A,H,K,L		.....	.....	.....					
rrnE		.....	.....	.....					
(b)		10	20	30	40	50	60	70	80
rrn J		AAGGATATAT	TCGGAACATC	TTCTTCAGAA	GATCGCGAAT	AACGTGACAT	ATTGTATTCA	GTTTGAATG	TTTATTAAAC
rrn G		AAGGATATAT	TCGGAACATC	TTCTTCAGAA	GATCGCGAAT	AACGTGACAT	ATTGTATTCA	GTTTGAATG	TTTATTAAAC
rrn C		AAGGATATAT	TCGGAACATC	TTCTTCAGAA	GATCGCGAAT	AACGTGACAT	ATTGTATTCA	GTTTGAATG	TTTATTAAAC
rrn A		AAGGATATAT	TCGGAACATC	TTCTTCAGAA	GATCGCGAAT	AACGTGACAT	ATTGTATTCA	GTTTGAATG	TTTATTAAAC
rrn F		AAGGATATAT	TCGGAACATC	TTCTTCAGAA	GATCGCGAAT	AACGTGACAT	ATTGTATTCA	GTTTGAATG	TTTATTAAAC
rrn E		AAGGATATAT	TCGGAACATC	TTCTTCAGAA	GATCGCGAAT	AACGTGACAT	ATTGTATTCA	GTTTGAATG	TTTATTAAAC
rrn H		AAGGATATAT	TCGGAACATC	TTCTTCAGAA	GATCGCGAAT	AACGTGACAT	ATTGTATTCA	GTTTGAATG	TTTATTAAAC
rrn K		AAGGATATAT	TCGGAACATC	TTCTTCAGAA	GATCGCGAAT	AACGTGACAT	ATTGTATTCA	GTTTGAATG	TTTATTAAAC
rrn L		AAGGATATAT	TCGGAACATC	TTCTTCAGAA	GATCGCGAAT	AACGTGACAT	ATTGTATTCA	GTTTGAATG	TTTATTAAAC
		90	100	110	120	130	140	150	160
rrn J		ATTCAAAAAA	ATGG-GCCTA	TA-GCTCAGC	TGGTTAGAGC	GCACGCCCTGA	TAAGCGTGAG	GTCGGTGGTT	CGAGTCCAC-
rrn G		ATTCAAAA-AA	ATGG-GCCTA	TA-GCTCAGC	TGGTTAGAGC	GCACGCCCTGA	TAAGCGTGAG	GTCGGTGGTT	CGAGTCCAC-
rrn C		ATTCAAAATTA	ATGG-GCCTA	TA-GCTCAGC	TGGTTAGAGC	GCACGCCCTGA	TAAGCGTGAG	GTCGGTGGTT	CGAGTCCAC-
rrn A		ATTCAAAATTA	ATGG-GCCTA	TA-GCTCAGC	TGGTTAGAGC	GCACGCCCTGA	TAAGCGTGAG	GTCGGTGGTT	CGAGTCCAC-
rrn F		ATTCAAAAAA	ATGG-GCCTA	TA-GCTCAGC	TGGTTAGAGC	GCACGCCCTGA	TAAGCGTGAG	GTCGGTGGTT	CGAGTCCAC-
rrn E		ATTCAAAATAT	TTTTTGGTTA	AA-GTGATAT	TGCTTAT-GC	GAGCGCTTGA	CAATC-TATT	CTTTTAAAG	AAAGCGGTG
rrn H		ATTCAAAATAT	TTTTTGGTTA	AA-GTGATAT	TGCTTAT-G-	-----	-----	-----	-----
rrn K		ATTCAAAATAT	TTTTTGGTTA	AACGTGATAT	TGCTTAT-G-	-----	-----	-----	-----
rrn L		ATTCAAAATAT	TTTTTGGTTA	AAG-TGATAT	TGCTTAT-G-	-----	-----	-----	-----
		170	180	190	200	210	220	230	240
rrn J		TTAGGCCCCAC	CATTA-----	-----	-----	-----	-----	-----	-----
rrn G		TTAGGCCCCAC	CATTA-----	-----	-----	-----	-----	-----	-----
rrn C		TTAGGCCCCAC	CATTAATTTA	ATACCTATTT	GGGGGCTTAG	CTCAGCTGGG	AGAGCGCCTG	CTTTGCACGC	AGGAGGTCAG
rrn A		TTAGGCCCCAC	CATTAATTTA	ATACCTATTT	GGGGGCTTAG	CTCAGCTGGG	AGAGCGCCTG	CTTTGCACGC	AGGAGGTCAG
rrn F		TTAGGCCCCAC	CATTA-----	-----	-----	-----	-----	-----	-----
rrn E		TTAGACAATG	CATTAA--GA	AAAAATTAAG	CGGAGTTTAC	TTTTGTAAAT	-GAGCATTTG	ATTTTTTG--	-AAAAATAAG
rrn H		-----	-----	-----	-----	-----	-----	-----	-AAAAATAAG
rrn K		-----	-----	-----	-----	-----	-----	-----	-AAAAATAAG
rrn L		-----	-----	-----	-----	-----	-----	-----	-AAAAATAAG
		250	260	270	280	290	300	310	320
rrn J		-----	-----	-----T	TTGTACATTG	AAAACTAGAT	AAGTAAGTAA	AATATAGATT	TTACCAAGCA
rrn G		-----	-----	-----T	TTGTACATTG	AAAACTAGAT	AAGTAAGTAA	AATATAGATT	TTACCAAGCA
rrn C		CGGTTGATC	CCGCTAGTCT	CCACCATTAT	TTGTACATTG	AAAACTAGAT	AAGTAAGTAA	AATATAGATT	TTACCAAGCA
rrn A		CGGTTGATC	CCGCTAGTCT	CCACCATTAT	TTGTACATTG	AAAACTAGAT	AAGTAAGTAA	AATATAGATT	TTACCAAGCA
rrn F		-----	-----	-----T	TTGTACATTG	AAAACTAGAT	AAGTAAGTAA	AATATAGATT	TTACCAAGCA
rrn E		CAGTATGCGA	GCGCTTGACT	AAAAAG-AAA	TTGTACATTG	AAAACTAGAT	AAGTAAGTAA	AATATAGATT	TTACCAAGCA
rrn H		CAGTATGCGA	GCGCTTGACT	AAAAAG-AAA	TTGTACATTG	AAAACTAGAT	AAGTAAGTAA	AATATAGATT	TTACCAAGCA
rrn K		CAGTATGCGA	GCGCTTGACT	AAAAAG-AAA	TTGTACATTG	AAAACTAGAT	AAGTAAGTAA	AATATAGATT	TTACCAAGCA
rrn L		-----CGA	GCGCTTGACT	AAAAAG-AAA	TTGTACATTG	AAAACTAGAT	AAGTAAGTAA	AATATAGATT	TTACCAAGCA
		330	340	350	360	370	380	390	400
rrn J		AAACCGAGTG	AATAAAGAGT	TTTAAATAAG	CTTGAATTCA	TAAGAAATAA	TCGCTAGTGT	TCGAAAGAAC	ACTCACAAGA
rrn G		AAACCGAGTG	AATAAAGAGT	TTTAAATAAG	CTTGAATTCA	TAAGAAATAA	TCGCTAGTGT	TCGAAAGAAC	ACTCACAAGA
rrn C		AAACCGAGTG	AATAAAGAGT	TTTAAATAAG	CTTGAATTCA	TAAGAAATAA	TCGCTAGTGT	TCGAAAGAAC	ACTCACAAGA
rrn A		AAACCGAGTG	AATAAAGAGT	TTTAAATAAG	CTTGAATTCA	TAAGAAATAA	TCGCTAGTGT	TCGAAAGAAC	ACTCACAAGA
rrn F		AAACCGAGTG	AATAAAGAGT	TTTAAATAAG	CTTGAATTCA	TAAGAAATAA	TCGCTAGTGT	TCGAAAGAAC	ACTCACAAGA
rrn E		AAACCGAGTG	AATAAAGAGT	TTTAAATAAG	CTTGAATTCA	TAAGAAATAA	TCGCTAGTGT	TCGAAAGAAC	ACTCACAAGA
rrn H		AAACCGAGTG	AATAAAGAGT	TTTAAATAAG	CTTGAATTCA	TAAGAAATAA	TCGCTAGTGT	TCGAAAGAAC	ACTCACAAGA
rrn K		AAACCGAGTG	AATAAAGAGT	TTTAAATAAG	CTTGAATTCA	TAAGAAATAA	TCGCTAGTGT	TCGAAAGAAC	ACTCACAAGA
rrn L		AAACCGAGTG	AATAAAGAGT	TTTAAATAAG	CTTGAATTCA	TAAGAAATAA	TCGCTAGTGT	TCGAAAGAAC	ACTCACAAGA
		410	420	430	440	450	460	470	480
rrn J		TTAATAACGC	GTTT-----	-----	GAACGTAAC	TCATGTTAA-	-----	-----	-----
rrn G		TTAATAACGC	GTTTAAATCT	TTTTATAAAA	GAACGTAAC	TCATGTTAA-	-----	-----	-----
rrn C		TTAATAACGC	GTTTAAATCT	TTTTATAAAA	GAACGTAAC	TCATGTTAA-	-----	-----	-----
rrn A		TTAATAACGC	GTTTAAATCT	TTTTATAAAA	GAACGTAAC	TCATGTTAA-	GA-GTTAAAT	TATTTTAAAG	CAGGAGTTTA
rrn F		TTAATAACGC	GTTTAAATCT	TTTTATAAAA	GAACGTAAC	TCATGTTAA-	GAAGTTAAAT	TATTTTAAAG	CAG-AGTTTA
rrn E		TTAATAACGC	GTTTAAATCT	TTTTATAAAA	GAACGTAAC	TCATGTTAA-	-----	-----	-----
rrn H		TTAATAACGC	GTTTAAATCT	TTTTATAAAA	GAACGTAAC	TCATGTTAA-	-----	-----	-----
rrn K		TTAATAACGC	GTTT-----	TTTTA-AAA	GAACGTAAC	TCATGTTAA-	-----	-----	-----
rrn L		TTAATAACGC	GTTT-----	TTTTA-AAA	GAACGTAAC	TCATGTTAA-	-----	-----	-----
		490	500	510	520	530	540	550	559
rrn J		-----	-----	-----	-----	-----	-----CCTG	TAGGA----	T
rrn G		-----	-----	-----	-----	-----	CG	TTTGACCTTA	TAAAAATGGT
rrn C		-----	-----	-----	-----	-----	CG	TTT-GACTTA	TAAAAATGGT
rrn A		CTTATGTAAA	TGAGCATTTA	AAATAATGAA	AACGAAGCCG	TATGTGAGCG	TTT-GACTTA	TAAAAATGGT	GGAAACATA
rrn F		CTTATGTAAA	TGAGCATTTA	AAATAATGAA	AACGAAGCCG	TATGTGAGCG	TTT-GACTTA	TAAAAATGGT	GGAAACATA
rrn E		-----	-----	-----	-----	-----	CG	TTT-GACTTA	TAAAAATGGT
rrn H		-----	-----	-----	-----	-----	CG	TTT-GACTTA	TAAAAATGGT
rrn K		-----	-----	-----	-----	-----	CG	TTT-GACTTA	TAAAAATGGT
rrn L		-----	-----	-----	-----	-----	CG	TTT-GACTTA	TAAAAATGGT

Fig. 3. For legend see opposite.

sensitive strain since the ATCC 8532 strain (from 1953) was ribotype A. The transfer of *Mec^R* to *S. aureus* may have occurred by genetic transfer since it has been shown that the *mec* gene is encoded on a transposon (Trees & Iandolo, 1988). In support of this are the findings that the *mec* gene from *S. aureus* and *S. epidermidis* is highly conserved (Ryffel *et al.* 1990) and that there is significant homology of the *mec* gene from *S. aureus* and methicillin-resistant strains of *S. haemolyticus* and *S. simulans* (Ubukata *et al.*, 1990).

The potential utility of the *S. aureus* typing method described in this study is shown by its application to the typing of MRSA strains, the majority of which were from the HRH. Most of the MRSA strains from the HRH were ribotype B; however, ribotype A predominated from other parts of the world – significantly the first MRSA isolated in 1960 (Jevons, 1961; Stewart & Holt, 1963) was ribotype A. The sample size from parts of the world other than Australia in this study is too small to draw firm conclusions; however, the results are consistent with the origin of a single MRSA clone in the early 1960s (Lacey & Grinstead, 1973). The analysis of strains by multilocus enzyme electrophoresis has demonstrated the occurrence

of significant levels of genetic polymorphism among extant MRSA isolates (which also occur in methicillin-sensitive strains), leading to the hypothesis that many MRSA organisms have arisen independently (Musser & Kapur, 1992). Further studies on isolates from continents other than Australia using the method described in this paper may help to confirm this hypothesis.

Whereas the bacteriophage typing system was the standard method for *S. aureus* (Williams *et al.*, 1953), many current MRSA strains are not typable by the International Set of Phages (Richardson *et al.*, 1988), requiring the addition of further experimental phages (Vickery *et al.*, 1993). RFLP analysis by pulsed-field gel electrophoresis has been shown to be more discriminating than phage typing (Schlichting *et al.*, 1993). However, RFLP analysis relies on the stability of restriction enzyme recognition sites such that a point mutation within a site will result in a different RFLP. The sequence conservation of the *rrn* operons (Woese, 1987) argues for the use of the 16S–23S spacer region as a more stable and direct indicator of the evolutionary divergence of *S. aureus* strains and will be a valuable addition to the large number of typing methods available.

(c)

	10	20	30	40	50	60	70	80
SA238	GATTAAGTTA	TTAAGGCCGC	ACGG--TGGA	TOCCTTGGCA	CTAGAAGCCG	ATGAAGGACG	TTACTAACGA	CGATATGCTT
rrnJ†	.....	.....	.....	.....	.....	.....	.....	.....
rrnJ-V4	.....	.....	..A.GA...	.....	.....	.....	.....	.....
rrnJ-V8	.....	.....	.....	.....	.....	..TT...	.....	.....
rrnJ-V43	.....	.....	.....	.....	.....	.....	.....	.....
rrnE	.....	.....	.....	.....	.....	.....	.....	.....
rrnF	.....	.....	.....	.....	.....	.....	.....	.....
rrnF	.....	.....	.....	.....	.....	.....	.....	.....
rrnA,C,K,H	.....	.....	.....	.....	.....	.....	.....	.....
rrnE	.....	.....	..A....	.....	.....	.....	.....	.....
rrnL	.....	.....	.....	.....	.....T	.....TT	.....	.....
	90	100	110	120	130	140	150	160
SA238	TGGGGAGCTG	T-AAGT-AAG	CTTTGATCCA	GAGATTTCGG	AATGGGGAAA	CCC-AGCATG	AGTTATGTCA	TGTTATCGAT
rrnJ§	.....	.....	.....	.....	.....	.....	.....	.....
rrnJ-V17	.....	.....	.....	.....	.....	...C....	.....	.....
rrnE-V12	.....	.....	.....	.....	.....	.....	.....	.....
rrnE-V2	.....	.....	.....	.....	.....	.....	.....	.....
rrnF-V30	.....	.....	.....	.....	.....	.....	.....	.....
rrnF-V38	.....	..T...T...	.....	.....	...T...G...	A..C....	.....	.....
rrnA	.....	.....	.....	.....	.....	.....	.....	.....
rrnC	.....	.....	.....	.....	.....	.....	.....	.....
rrnH	.....	.....	.....	.....	.....	.....	.....	.....
rrnK	.....	.....	.....	.....	.....	.....	.....	.....
rrnL	.....	.....	.....	.....	.....	.....	...C....	.....
	170	180	190					
SA238	ATGTG-AAT-	ACATAGCATA	TCAGAAGGCA	CACCCG				
rrnJ-4	.....	.....T	.....	.....				
rrnJ-V4, V17, V32	.....	.....	.....	.....				
rrnJ-V8	.....	.....	.....G.	.....				
rrnJ-V43F	.....	.....T	.....	.....				
rrnE-V12	.....	.....-AT	.....	.....				
rrnE-V2	.....	.....-AT	.....	.....				
rrnF-V30	.....	.....-AT	.....	.....				
rrnF-V38	.....	..A...TG.AT	.....	.....				
rrnA	.....	.....-AT	.....	.....				
rrnC	.....	.....	.....	.....				
rrnH-V7	.....	.....-AT	.....	.....				
rrnH-V27	.....	.....TAT	.....	.....				
rrnK-V41	.....	.....TG.AT	.....	.....				
rrnL-V1	.....	...C...TG.AT	.....	.....				

Fig. 3. Alignments of 16S–23S spacer sequences from *S. aureus*. Fragment E from *S. aureus* strains (Table 3) was cloned into M13mp18RF and sequenced with the primers listed in Table 2 and Fig. 1. The sequences were derived from the clones and isolates listed in Table 3. The sequences SA16S and SA23S were taken from Ludwig *et al.* (1992). Shown is the alignment of *rrn* alleles with SA16S (a), of *rrnA* with *rrnC*, *E*, *F*, *G*, *H*, *J*, *K*, and *L* (b), and of *rrn* alleles with SA23S (c). The symbols refer to an identical base (.) and an absent base (–). † = *rrnA*, *E*, *J*, *L*, *F*; ‡ = clones 4, V17, V32; § = clones 3, V4, V8, V32, V43.

Fig. 4. Dendrogram showing the relationships of the 16S-23S alleles. The same tree was obtained using the data from either Fig. 3(b) or Table 3. One parsimonious tree was obtained with DNAPENNY from the PHYLIP package. Then, using MACCLADE, of the 16 possible rerootings, the tree shown was used because it was drawn using the longest allele *rrnA* as the root. The numbers show the number of nucleotide changes per branch.

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