**REVIEW
ARTICLE****New approaches to typing and identification of bacteria using the 16S–23S rDNA spacer region**Volker Gürtler¹ and Vilma A. Stanisich²

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Identification of bacteria using the rRNA operon

Medical microbiology is extremely reliant on the culture of bacteria from clinical specimens and their subsequent identification by biochemical and phenotypic characteristics for the diagnosis of disease. Following determination of the structure of DNA by Watson & Crick (1953), studies in bacteriology have seen a major shift from functional to molecular techniques for identifying bacteria (Towner & Cockayne, 1993). This review will deal with the 16S–23S spacer region of the rRNA operon (Fig. 1) and its use in the identification of micro-organisms at the species and strain (typing) levels.

The rRNA genes are essential for the survival of all organisms and are highly conserved in the bacterial and other kingdoms. Consequently, characterization of the 16S rRNA gene is now well-established as a standard method for the identification of species, genera and families of bacteria (Amann *et al.*, 1995; Woese, 1987). This is reflected in the large body of information that has been accumulated on the phylogenetic analysis of micro-organisms using this gene (Olsen *et al.*, 1991). In contrast, relatively few 23S gene sequences have been reported, and neither this gene nor the 5S gene have been widely used for phylogenetic purposes. Sequence information has also become available on the 16S–23S spacer region and suggests that considerable variation can occur between species in both the length and the sequence of this region. The fact that many bacteria have multiple copies (alleles) per genome of the rRNA operon therefore raises the possibility that spacer variations between strains, species and genera may be used for identification and typing purposes. Systematic studies of spacer region variation would require amplification of the regions by PCR (Mullis & Faloona, 1987) and hence the availability of conserved regions in the flanking 16S and 23S rRNA genes to serve in primer recognition. A variety of primers have already been used for such studies (see Table 3), but the appropriateness of the choices, particularly for those

based on the 23S rRNA gene, has been limited by the paucity of sequenced genes. One purpose of this review is to take account of increased knowledge of such sequences to better identify conserved regions that may be used for spacer amplification; the second is to evaluate the potential use of spacer region variation as a rapid approach for bacterial typing and identification.

Conserved regions in the 16S and 23S rRNA genes flanking the 16S–23S rDNA spacer region

The rRNA operon is transcribed into one pre-rRNA transcript that contains the following components in the order (5' to 3'): 16S, spacer, tRNA, spacer, 23S, spacer and 5S rRNA sequences (Watson *et al.*, 1987; Fig. 1). Transcription of the operon results in the formation of a pre-rRNA transcript that is cleaved into separate rRNA and tRNA molecules. In the remaining discussion, therefore, reference to rDNA will include both the gene and the intergenic sequences, whereas reference to tRNA or rRNA will specifically exclude the spacer sequences.

Any studies directed towards the PCR-mediated amplification of *all* of the copies of the 16S–23S rDNA spacer regions of one or more bacteria are totally dependent on the occurrence of suitable, highly conserved, regions in the flanking termini of the 16S and 23S rRNA genes. Such regions can only be reliably identified if a sufficient body of sequence information is available for these rRNA genes. This is the case for the 16S rRNA gene as full-length sequences for this gene have been reported for about 2500 different bacterial species (Ribosomal RNA Database Project; Olsen *et al.*, 1991). These genes are similar in length (about 1.5 kb) throughout the bacterial kingdom and contain highly conserved regions as well as others that vary according to species and family; they also yield RNA products with a similar secondary structure (Neefs *et al.*, 1990; Woese, 1987). Fig. 1 shows the location of sequences within the conserved regions at each end of the 16S rRNA gene that are suitable for amplification of the adjacent DNAs; of sequences 2, 3 and 4 that could be used to amplify the spacer DNA, region 2 is the

Fig. 1. Position of conserved regions within the rRNA operon. The boxed areas represent the various genes of the bacterial rRNA operon. Transcription of the operon is from left to right. Some bacteria have two tRNA genes as shown; others have either one (either tRNA^{glu}, tRNA^{ala} or tRNA^{ile}) or none. The dark lines represent the spacer regions that separate the various rRNA genes and the dark jagged lines represent breaks in the 16S and 23S genes which have not been shown fully. The 5S rRNA gene that occurs to the right of the 23S rRNA gene is not shown. The thin numbered line shows the nucleotide numbering of the 16S and 23S rRNA genes of *E. coli* with breaks shown as dashed lines. Multiple copies of the rRNA operon occur in a single copy of many bacterial chromosomes. The numbers 1–10 represent the following nucleotide sequence blocks. 16S rRNA gene: 1, 8–27; 2, 1390–1407; 3, 1491–1506; 4, 1525–1541. 23S rRNA gene: 5, 21–38; 6, 115–132; 7, 188–208; 8, 422–437; 9, 441–460; 10, 456–474. The sequences (5'–3') of regions 1–4 are AGAGTTTGATCCTGGCTCAG, TTGTACACACCGCCGTC, AAGTCGTAACAAGGTA and GGCTTGGATCACCTCCTT, respectively.

most highly conserved sequence that is present in eubacteria, archaea and eukaryotes (Lane *et al.*, 1985), while regions 3 and 4 are present in most or many eubacteria, respectively (Weisburg *et al.*, 1991).

The number of full-length 23S rRNA gene sequences now known is far fewer than for the 16S rRNA gene and is due, in part, to the larger size of the 23S rRNA gene (about 3 kb). The relative paucity of 23S rRNA gene sequences means that phylogenetic analyses based on this gene remain incomplete (Ludwig *et al.*, 1992). However, the analysis of full-length sequences from 21 species that represent 18 genera, reveals six conserved regions within the first 520 bp of the gene (Fig. 1). Overall sequence conservation is important in deciding which region is most likely to be successful for primer construction. However, sequence conservation at the start of regions 5–10 is more important because mismatches at this end may result in the failure of *Taq* polymerase to bind (see below). Using these criteria, region 10 (nt 456–474) is the most highly conserved, with sequence identity in 14 of the species and variations at only one nucleotide location in six of the seven remaining species (Table 1). Next, in order of identity, are regions 6, 9, 8, 7 and 5. At least from a theoretical standpoint, these observations suggest that amplification of all of the 16S–23S rDNA spacer regions from a strain is most likely to be successful using region 2 of the 16S rRNA gene and region 10 of the 23S rRNA gene for primer construction.

Characterization of 16S–23S rDNA spacer sequences

16S–23S rDNA copy number and length

Southern hybridization studies between 16S rDNA sequences and genomic DNA digested with restriction enzymes that cut relatively infrequently (e.g. *Hind*III or *Eco*RI), have shown that several bands may hybridize to the probe and, moreover, that the number of these often corresponds to the number of rRNA operons (or *rrn* alleles) in the genome [seven copies in *E. coli* (Morgan *et al.*, 1977), 10 in *Bacillus subtilis* (Loughney *et al.*, 1982), ten in

Clostridium perfringens (Garnier *et al.*, 1991), one in *Mycobacterium* sp. (Bercovier *et al.*, 1986), and one to two in *Mycoplasma* sp. (Amikam *et al.*, 1984)]. Such bands range in size from approximately 0.5 to 20 kb, suggesting differences both in the locations of restriction sites within the operons and in the DNA that flanks them. Among the *intra*-operon differences that could lead to fragment-length variation are differences in the lengths (and hence in the cut sites) of the 16S–23S spacer DNAs. This specific source of variation has been shown to occur in strains of *Clostridium difficile* (Gürtler, 1993). Additional studies that have provided information on the variable length of the 16S–23S rDNA spacer regions, and which are based on DNA cloning and sequencing, are listed in Table 2. At first glance, this body of information appears substantial but, in fact, it deals only with a small number of the approximately 3500 bacterial species that comprise the *Approved Lists of Bacterial Names*. Moreover, the 16S–23S rDNA sequences that have been determined are, in most cases, limited to a fraction of the total number of operons per genome in that species.

Despite these deficiencies, several general observations can be made. First, among the Gram-negative and Gram-positive species studied, at least half carry multiple copies of the rRNA operon (13/22 and 11/20, respectively); this feature is likely to extend to a proportion of the remaining species where the actual number of copies is currently unknown. In many of the species the copy number can be as high as five to ten (e.g. *Haemophilus* and *Bacillus*), but in other groups the number is typically only one or two (thermophiles, *Mycobacteria*, *Mycoplasma* and archaea). Second, the length of the 16S–23S rDNA spacer region varies both between species (e.g. 60 bp in *Thermoproteus tenax* to 1529 bp in *Bartonella elizabethae*) and between alleles of a given species. The complete set of rRNA alleles has been sequenced only for *Staphylococcus aureus* and *Haemophilus influenzae* in which the 16S–23S spacer region varies from 303 to 551 bp and from 478 to 723 bp, respectively. Third, variations in the length of the 16S–23S rDNA spacer regions are due, in part, to the number and type of tRNA genes that it may contain (Fig.

Table 1. Conserved regions in the first 520 bp of the bacterial 23S rRNA gene

The positions of the conserved sequence regions 5–10 are shown in Fig. 1. The numbering of the nucleotides is based on the sequence of the *E. coli* 23S rRNA gene (Brosius *et al.*, 1980). Dots represent nucleotides corresponding to the *E. coli* 23S rRNA sequence; dashes represent absence of a nucleotide.

Bacterium	Region 5 (nt 21–38)	Region 6 (nt 115–132)	Region 7 (nt 188–208)
Gram-negative			
<i>Escherichia coli</i>	A-CGGTGGATGCCCTGGCA	CCGAATGGGGAAACCCAG	GAAGTGAACATCTAAGTACC
<i>Aeromonas hydrophila</i>	.-T.....T....	T...A.....T.-.C	TTT
<i>Frankia</i> sp.	.-.....T....	G.	...G.....C.T....
<i>Listeria monocytogenes</i>	.-.....T....	G.....C	
<i>Neisseria gonorrhoeae</i>	-CA..C.....T...G	C	C.....
<i>Rhodobacter sphaeroides</i>	--T.....TA....	C	
<i>Rhodopseudomonas capsulata</i>	T-T.....T....	C	
<i>Plesiomonas shigelloides</i>	.-.....T....	C	
<i>Pseudomonas aeruginosa</i>	.-.....T....	.T.....G.....C	
<i>Burkholderia cepacia</i>	G-T.....T...G	C	A.
Gram-positive			
<i>Bacillus stearothermophilus</i>	.-.....T....	G.....C	T.....
<i>Bacillus subtilis</i>	.-.....T....	C	
<i>Clostridium botulinum</i>	.-T...A.....T....	A.....T..C	
<i>Enterococcus hirae</i>	T-.....T....	G.....	
<i>Staphylococcus aureus</i>	.-.....T....		T.....
<i>Staphylococcus carnosus</i>	.-.....T....	G.....	T.....
<i>Streptomyces griseus</i> subsp. <i>griseus</i>	.-.....T....	G.	...G.....C.....
<i>Streptococcus thermophilus</i>	.-.....T....	G.....G.	G.
Other groups of bacteria			
<i>Mycoplasma hyopneumoniae</i>	T-T.....T...GT	G.....A	...T.....T...G.
<i>Mycobacterium leprae</i>	.-T.....T....	A	...G.....C.....
<i>Ruminobacter amylophilus</i>	.-T.....TA....	G.....	...G.....C.....
Bacterium	Region 8 (nt 422–437)	Region 9 (nt 441–460)	Region 10 (nt 456–474)
Gram-negative			
<i>Escherichia coli</i>	AAGGCTAAATACTCC	TGACCGATAGTGAACCAGTA	CAGTACCGTGA-GGGAAAGG
<i>Aeromonas hydrophila</i>		C.....	C.....
<i>Frankia</i> sp.	...C.....	C.G..T....	T.....
<i>Listeria monocytogenes</i>			
<i>Neisseria gonorrhoeae</i>	A	C.....	
<i>Rhodobacter sphaeroides</i>	G.....	C.....	
<i>Rhodopseudomonas capsulata</i>	G.....	C.....	
<i>Plesiomonas shigelloides</i>		C.....	A.....
<i>Pseudomonas aeruginosa</i>	A.		
<i>Burkholderia cepacia</i>	G	C.....	
Gram-positive			
<i>Bacillus stearothermophilus</i>		C.....	
<i>Bacillus subtilis</i>			
<i>Clostridium botulinum</i>	A.	G.....	
<i>Enterococcus hirae</i>			
<i>Staphylococcus aureus</i>	T		
<i>Staphylococcus carnosus</i>	T		
<i>Streptomyces griseus</i> subsp. <i>griseus</i>	...C.....T..	C.G..T-...	T.....-.....T..
<i>Streptococcus thermophilus</i>	..CC.....T		
Other groups of bacteria			
<i>Mycoplasma hyopneumoniae</i>	AA	AC.....T...	T.....-.....
<i>Mycobacterium leprae</i>	...C.....T.	C.G..T....	-.....T..
<i>Ruminobacter amylophilus</i>	A.	A....	A.....-.....

1; Table 2, column 4). Most of the spacers in Gram-negative bacteria contain both a tRNA^{ala} and tRNA^{ile} gene, but others (*Aeromonas hydrophila* and *H. influenzae*) contain only a tRNA^{glu} gene. By contrast, the spacers of Gram-positive bacteria contain either a tRNA^{ala} or a tRNA^{ile} gene or both of these. However the majority of the Gram-positive species (15/19) have no tRNA genes

present at all, and this is also the case for 10 of the 11 species of *Mycobacterium*, *Mycoplasma* and archaea. These various findings show considerable heterogeneity in the number, length and composition of the 16S–23S rDNA spacer regions of a small but diverse range of eubacteria and some archaea. Similar heterogeneity would be expected to extend to other species and, where it occurs,

Table 2. 16S–23S rDNA spacer sequences isolated from eubacteria and archaeobacteria

Bacterium	Properties of alleles* (<i>rrn</i>)			GenBank accession no.		Reference
	No. known†	Designation	Length of spacer‡	23S rRNA gene	16S–23S spacer	
Gram-negative						
<i>Aeromonas hydrophila</i>	?	<i>rrn</i> II	511 ^G	X67946		East & Collins (1993)
		<i>rrn</i> I	472 ^{IA}			East & Collins (1993)
		<i>rrn</i> III	544 ^G			East & Collins (1993)
<i>Bartonella bacilliformis</i>	?	<i>rrnA</i>	906 ^{IA}		L26364	Minnick <i>et al.</i> (1994)
<i>Bartonella elizabethae</i>	?	<i>rrnA</i>	1529 ^{IA}		L35103	Roux & Raoult (1995b)
<i>Bartonella henselae</i>	?	<i>rrnA</i>	1262 ^{IA}		L35101	Roux & Raoult (1995b)
<i>Bartonella quintana</i>	?	<i>rrnA</i>	1211 ^{IA}		L35100	Roux & Raoult (1995b)
<i>Bartonella vinsonii</i>	?	<i>rrnA</i>	1258 ^{IA}		L35102	Roux & Raoult (1995b)
<i>Caulobacter crescentus</i>	2	<i>rrnA</i>	653 ^{IA}			Feingold <i>et al.</i> (1985)
<i>Escherichia coli</i>	7§	<i>rrnB</i>	441 ^G	J01695	J01695	Brosius <i>et al.</i> (1981)
		<i>rrnC</i>	355 ^G	V00331	X12420	Harvey <i>et al.</i> (1988)
		<i>rrnD</i> , X	437 ^{IA}		K00763	Young <i>et al.</i> (1979)
		<i>rrnE</i>	355 ^G	V00331	M20399	Harvey <i>et al.</i> (1988)
		<i>rrnG</i>	431 ^G		M20397	Harvey <i>et al.</i> (1988)
<i>Haemophilus influenzae</i>	6	<i>rrnB</i> , E, F	478 ^G	L42023	L42023	Fleischmann <i>et al.</i> (1995)
	6	<i>rrnA</i> , C, D	723 ^{IA}	L42023	L42023	Fleischmann <i>et al.</i> (1995)
<i>Plesiomonas shigelloides</i>		<i>rrn</i> II	264 ^G	X65487	X65486	East <i>et al.</i> (1992)
		<i>rrn</i> I	191			East <i>et al.</i> (1992)
		<i>rrn</i> III	191			East <i>et al.</i> (1992)
<i>Pseudomonas aeruginosa</i>	?	<i>rrnA</i>	470 ^{IA}	Y00432	L28150	§
		<i>rrnB</i>	469 ^{IA}		L28149	§
		<i>rrnC</i>	469 ^{IA}		L28148	§
<i>Burkholderia cepacia</i>	?	<i>rrnA</i>	394	<i>Burk.</i> <i>cepacia</i>	L28155	§
		<i>rrnB</i>	530 ^{IA}		L28152	§
		<i>rrnC</i>	588 ^{IA}		L28151	§
		<i>rrnD</i>	530 ^{IA}		L28153	§
		<i>rrnE</i>	531 ^{IA}		L28154	§
<i>Pseudomonas fluorescens</i>	?	<i>rrnA</i>	518 ^{IA}		L06306	Gill <i>et al.</i> (1994)
		<i>rrnB</i>	513 ^{IA}		L06307	Gill <i>et al.</i> (1994)
<i>Pseudomonas gladioli</i>	?	<i>rrnA</i>	580 ^{IA}		L28156	§
		<i>rrnB</i>	547 ^{IA}		L28157	§
<i>Pseudomonas mallei</i>	?	<i>rrnA</i>	604 ^{IA}		L28158	§
<i>Pseudomonas mendocina</i>	?	<i>rrnA</i>	512 ^{IA}		L28159	§
		<i>rrnB</i>	515 ^{IA}		L28160	§
		<i>rrnC</i>	517 ^{IA}		L28161	§
<i>Pseudomonas pickettii</i>	?	<i>rrnA</i>	514 ^{IA}		L28163	§
<i>Pseudomonas pseudomallei</i>	?	<i>rrnA</i>	642 ^{IA}		L28165	§
		<i>rrnB</i>	604 ^{IA}		L28164	§
<i>Rhodobacter sphaeroides</i>	3	<i>rrnA</i> –C	667 ^{IA}	X53853	X53853	Dryden & Kaplan (1990)
<i>Rhodopseudomonas capsulata</i>	?	<i>rrnA</i>	910?			Yu <i>et al.</i> (1982)
<i>Rhodothermus marinus</i>	1	<i>rrnA</i>	429 ^{IA}		X80994	Andrésson & Fridjónsson (1994)
<i>Thiobacillus ferrooxidans</i>	2	<i>rrnA</i> , B	440 ^{IA}			Sagredo <i>et al.</i> (1992)
Gram-positive						
<i>Bacillus subtilis</i>	10	<i>rrnA</i>	164	K00637	K00637	Green <i>et al.</i> (1985)
		<i>rrnB</i>	344 ^{IA}	K00637	J01551	Loughney <i>et al.</i> (1982)
<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i>	?	<i>rrnA</i>	485		U09381	§
<i>Clostridium botulinum</i> p4088.8	?	<i>rrnA</i>	365		M97699	Campbell <i>et al.</i> (1993)
<i>Clostridium botulinum</i> B1	?	<i>rrnA</i>	366	M94260	M97696	Campbell <i>et al.</i> (1993)
<i>Clostridium botulinum</i> E18	?	<i>rrnA</i>	367		M97697	Campbell <i>et al.</i> (1993)

Table 2 (cont.)

Bacterium	Properties of alleles* (<i>rrn</i>)			GenBank accession no.		Reference
	No. known†	Designation	Length of spacer‡	23S rRNA gene	16S–23S spacer	
<i>Clostridium botulinum</i> F3	?	<i>rrnA</i>	437		M97698	Campbell <i>et al.</i> (1993)
<i>Enterococcus faecalis</i>	?	<i>rrnA</i>	327 ^A		L16515	Hall (1994)
	?	<i>rrnB</i>	225		L16515	Hall (1994)
<i>Enterococcus hirae</i>	6	<i>rrnA</i>	319 ^A	L00925	L00925	Sechi & Daneo-Moore (1993)
		<i>rrnB</i>	138	L00924	L00924	Sechi & Daneo-Moore (1993)
<i>Frankia</i> strain ORS020606	2	<i>rrnA</i>	411	M55343	M55343	Normand <i>et al.</i> (1992)
<i>Lactobacillus brevis</i>	?	<i>rrnA</i>	216		X74221	Nakagawa <i>et al.</i> (1994)
<i>Lactobacillus casei</i>	?	<i>rrnA</i>	219		X74220	Nakagawa <i>et al.</i> (1994)
<i>Lactobacillus heterobiochii</i> (1198)	?	<i>rrnA</i>	218		X74223	Nakagawa <i>et al.</i> (1994)
<i>Lactobacillus heterobiochii</i> (13118)	?	<i>rrnA</i>	214		X74224	Nakagawa <i>et al.</i> (1994)
<i>Lactobacillus japonicus</i>	?	<i>rrnA</i>	205		X74222	Nakagawa <i>et al.</i> (1994)
<i>Listeria monocytogenes</i>	?	<i>rrnA</i>	243	X64533	L05172	Emond <i>et al.</i> (1993)
<i>Staphylococcus aureus</i>	9	<i>rrnA</i>	551 ^{1A}	X68425	U11784	Gürtler & Barrie (1995)
		<i>rrnC</i>	473 ^{1A}		U11781	Gürtler & Barrie (1995)
		<i>rrnE</i>	469		U11774	Gürtler & Barrie (1995)
		<i>rrnF</i>	460 ^I		U11782	Gürtler & Barrie (1995)
		<i>rrnG</i>	382 ^I		U11777	Gürtler & Barrie (1995)
		<i>rrnH</i>	362		U11788	Gürtler & Barrie (1995)
		<i>rrnJ</i>	335 ^I		U11773	Gürtler & Barrie (1995)
		<i>rrnK</i>	319		U11785	Gürtler & Barrie (1995)
		<i>rrnL</i>	303		U11775	Gürtler & Barrie (1995)
<i>Streptococcus anginosus</i>	?	<i>S.ang</i> ₂₄₅₀	388/391 ^{1A}		L36931	Whiley <i>et al.</i> (1995)
<i>Streptococcus anginosus</i>	?	<i>S.ang</i> ₃₃₅₀	295 ^{1A}		L36932	Whiley <i>et al.</i> (1995)
<i>Streptococcus constellatus</i>	?	<i>S.con</i> ₃₈₀	319 ^{1A}		L36933	Whiley <i>et al.</i> (1995)
<i>Streptococcus intermedius</i>	?	<i>S.int</i> ₃₃₀	270 ^{1A}		L36934	Whiley <i>et al.</i> (1995)
<i>Streptococcus intermedius</i>	?	<i>S.int</i> ₄₅₀	391/392 ^{1A}		L36935	Whiley <i>et al.</i> (1995)
<i>Streptococcus pneumoniae</i>	6	<i>rrnA</i>	249 ^A		M60763	Bacot & Reeves (1991)
<i>Streptomyces ambofaciens</i>	4	<i>rrnD</i>	303			Pernodet <i>et al.</i> (1989)
<i>Streptomyces griseus</i>	6	<i>rrnE</i>	304	M76388	M76388	Kim <i>et al.</i> (1993)
subsp. <i>griseus</i>						
<i>Streptomyces lividans</i> TK21	6	<i>rrnA</i>	278		M20148	Suzuki <i>et al.</i> (1988)
Mycobacterium and Mycoplasma spp.						
<i>Acholeplasma laidlawii</i>	2	<i>rrnA</i>	167		D13260	Nakagawa <i>et al.</i> (1992)
		<i>rrnB</i>	374 ^{1A}		D13259	Nakagawa <i>et al.</i> (1992)
<i>Mycobacterium paratuberculosis</i>	1	<i>rrnA</i>	278	X74495	X74495	van der Giessen <i>et al.</i> (1994)
<i>Mycobacterium phlei</i>	1	<i>rrnA</i>	363	X74493	X74493	van der Giessen <i>et al.</i> (1994)
<i>Mycobacterium tuberculosis</i>	1	<i>rrnA</i>	276		X58890	Frothingham & Wilson (1994)
<i>Mycoplasma capricolum</i>	2	<i>rrnA</i>	228			Iwami <i>et al.</i> (1984)
		<i>rrnB</i>	226			Sawada <i>et al.</i> (1984)
<i>Mycoplasma hyopneumoniae</i>	1	<i>rrnA</i>	500	X68421	X58551	Taschke & Herrmann (1986)
<i>Mycoplasma pneumoniae</i>	1	<i>rrnA</i>	253		D14528	Harasawa <i>et al.</i> (1993)
SAY-MLO**	2	<i>rrnA</i>	243 ^I		M86340	Kuske & Kirkpatrick (1992)
Archaeobacteria						
<i>Methanococcus vannielii</i>	2	<i>rrnA</i>	156	X02729		Jarsch & Böck (1983)
		<i>rrnB</i>	242 ^A		X00083	Jarsch & Böck (1983)
<i>Sulfolobus solfataricus</i>	1	<i>rrnA</i>	237	M67495	M67495	Olsen <i>et al.</i> (1985)
<i>Thermoproteus tenax</i>	1	<i>rrnA</i>	60	Y00346	Y00346	Kjems <i>et al.</i> (1987)

For footnotes see p. 6.

to provide a basis for identification and typing of the organisms see below).

16S–23S rDNA spacer sequence alignment

To determine the extent of sequence similarity between the 16S–23S rDNA spacer regions of various bacteria, the sequence alignment shown in Fig. 2 was constructed from the DNA database using 33 spacer sequences from 13 of the species listed in Table 2. The criterion used to select these species was the availability of the corresponding 23S rDNA sequences since these served as the reference points for the alignment process. In some cases only one or a few spacer sequences have been included either because the organism contains only a single rRNA operon (e.g. *Mycoplasma*, *Methanococcus* and species of the archaea) or because only some of the spacer sequences have been determined [e.g. *Pseudomonas aeruginosa* and *Burkholderia* (*Pseudomonas*) *cepacia*]. In the two cases in which the full complement of spacers have been identified (seven in *E. coli* and nine in *S. aureus*), all but two of the *E. coli* and four of the *S. aureus* sequences have been included. Despite the limited scope of the analysis and the occurrence of gaps in the DNA alignments due to the variable length of the spacers and the relative positions of the tRNA genes, it is evident that no highly conserved regions are present. The greatest homology that does occur is in the region of the tRNA genes (from positions 221 to 298, 302 to 379 and 364 to 440), but, as noted earlier, these genes are not present in all species nor in all of the rRNA operons of a single strain. Using the DNASIS (Pharmacia) program, the only homology that was detected outside the tRNA regions was a sequence of 19 bp that was common to both *S. aureus* and *Enterococcus hirae*. It is possible that homology searches based on very short regions (20 bp or less) might uncover inter-species homologies additional to those shown in Fig. 2; such regions would need to be avoided when designing species-specific primers or, alternatively, could be of great use in the design of genera-specific primers. The findings of this alignment analysis are the basis of the contention to be raised in the next section, that bacteria with multiple copies of the rRNA operon can be identified by the variations they display in both the length and the number of the 16S–23S spacer regions.

Typing and identification of bacteria by PCR analysis of 16S–23S rDNA spacer regions

General observations

The advent of PCR technology has opened the way for the

rapid and more complete analysis of multiple-copy 16S–23S rDNA spacer regions and undoubtedly will be used in the future for detailed study and sequence determinations of these regions. However, PCR technology has also been used in several recent studies as a rapid means of amplifying the spacer regions for the specific purpose of detecting heterogeneity between and within species (Barry *et al.*, 1991; Jensen *et al.*, 1993; Kostman *et al.*, 1995). Such heterogeneity has been found in terms of both the number and the length of the spacers and raises the possibility that this methodology has broad applicability as a rapid, possibly automatable, general method for bacterial identification and typing. The likely success of this approach can be gauged from a consideration of the studies carried out to date that have used PCR-based amplification of the 16S–23S rDNA spacer regions. These, and some of the associated findings, are listed in Table 3.

In total, some 27 genera and 44 species of bacteria have been studied and in at least half of these spacer-length variations were detected at the species level (Table 3, column 4) and also between strains of the same species (Table 3, column 6); in some cases the strain differences were identified by RFLPs or by single-strand conformation polymorphisms (SSCP) (Table 3, column 7). Moreover, in the five instances where the same species of bacteria have been studied by independent groups of workers, spacer length variations were confirmed for *C. difficile*, *Burk. cepacia* and *S. aureus*, but were not found in the *Rochalimaea* species; both studies on *Acinetobacter* sp. found strain differences by RFLP analysis and only limited spacer-length variations between strains. When balanced against the methodological differences used in these studies, particularly with respect to the choice of primers (see below), both the level of detectability of spacer variation and the reproducibility of the finding is very good and suggests the broad applicability of the approach.

Choice of PCR primers and conditions

One issue that is critical for the successful detection of spacer variation is the choice of the PCR primers. As discussed earlier, conserved regions in the 16S rRNA gene can be identified accurately and all four of those shown in Fig. 1, as well as several that are organism-specific (e.g. for *Neisseria* or *Rochalimaea* species) have been used for primer construction in the studies listed in Table 3; the most frequently used are regions 2 and 3. Similarly, primers based on regions 5–10 of the less well-

* The term allele is interchangeable with the term used in the text (operon).

†?, Total number of *rrn* operons is not known.

‡ Superscripts denote the presence of tRNA^{glu} (G), tRNA^{ala} (A) and tRNA^{ile} (I) genes; ?, unknown.

§ Of the seven operons in *E. coli*, two (which contain tRNA^{ile} and tRNA^{ala}) have not had their 16S–23S spacer sequences determined (Morgan *et al.*, 1977).

|| Also available from the Ribosomal RNA Database Project (RDP; Olsen *et al.*, 1991), except for *Burk. cepacia* (RDP code) which is only available from the RDP.

¶ The only available data are the GenBank entries.

** SAY-MLO, western aster yellows mycoplasma-like organism.

characterized 23S rRNA gene have been used in various combinations with the 16S-primers, but not all have been successful in detecting spacer variation. More often than not, studies involving the region 5 primer have either failed to detect spacer variation at the species or strain levels (e.g. Gill *et al.*, 1994; McLaughlin *et al.*, 1993), or have had only limited success (e.g. Jensen *et al.*, 1993; Nowak *et al.*, 1995). The reason for this may be the considerable species variation that occurs in sequences at the start of region 5 (Fig. 1). The complement of these sequences forms the 3' end of the primer sequences and hence is the region at which *Taq* polymerase binds to commence amplification in the PCR procedure. Mismatches in this region could therefore decrease the efficiency of priming, or abolish priming altogether, resulting either in failure to detect spacer variation or detection of only some of the spacers that are present. Pertinent to the latter outcome are our findings of significant variability in region 5 of the *S. aureus* rRNA operons and the likelihood that not all operons would be detected by a region 5 primer (Gürtler & Barrie, 1995). In contrast, primers based on regions 7 and 10 of the 23S rRNA gene have been used successfully with various 16S-primers to detect strain differences *solely* on the basis of spacer-length variation [e.g. 2, 7 (Kostman *et al.*, 1995) and 2, 10 (Gürtler, 1993)]. These 23S-primers are therefore the ones recommended as the most likely to detect all copies of the spacer region (ranging from two to ten copies) and hence to detect spacer variation should it be present. A primer to regions 6, 8 and 9 should also be appropriate given the level of sequence conservation of the region (Table 1), but these have not been successful in detecting spacer variation in the instances in which they have been used (e.g. Leys *et al.*, 1994; Matar *et al.*, 1993; Nour *et al.*, 1994).

Finally several technical considerations are noteworthy. First, in several of the studies listed in Table 3 only a single PCR product was obtained so that species-specific differences were usually detected by digestion of the PCR product by a restriction endonuclease prior to electrophoresis (Liveris *et al.*, 1995; Matar *et al.*, 1993; McLaughlin *et al.*, 1993; Navarro *et al.*, 1992; Nowak *et al.*, 1995; Ponsonnet & Nesme, 1994; Salzano *et al.*, 1994; Vaneechoutte *et al.*, 1992; de Wit & Klatser, 1994). There may be three reasons for the recovery of only a single PCR product: (a) as mentioned above, the use of primer 5 may result in only one of the multiple 16S–23S rDNA spacer regions being amplified; (b) only one copy of the 16S–23S rDNA spacer region is actually present per genome of the organism being studied; (c) when primer 1 is used (which is at the beginning of the 16S rRNA gene) a large fragment of at least 2000 bp is obtained after PCR which may be difficult to resolve into multiple bands. Second, it may be possible to distinguish between fragments of the same size by the application of techniques such as denaturing-gradient gel electrophoresis (Sheffield *et al.*, 1989), temperature-gradient gel electrophoresis (Wartell *et al.*, 1990) or SSCP (de Wit & Klatser, 1994). Third, issues such as the method of DNA extraction prior to PCR (Waterhouse & Glover, 1993), and the possibility of

anomalous hybridization during the reaction (Jensen & Straus, 1993), need to be noted. In the latter case, this can be overcome by the use of denaturing PAGE and it is noteworthy that good separation of bands was achieved in the only two studies in which this technique was used (Gürtler, 1993; Gürtler & Barrie, 1995).

Bacteria with one or two copies of the rRNA operon

Certain bacteria and archaea have only one or two rRNA operons per genome (Table 2), making it unlikely that spacer length and number variation could be used as a general method of strain differentiation. In fact, no such variation has been seen among strains of *Mycobacterium* or *Mycoplasma* that fall into this category. However, some variation has been detected at the species level. In the case of *Mycoplasma*, a two-step PCR protocol was used to detect the spacer-length variation, followed by nucleotide sequencing to locate the differences (Nakagawa *et al.*, 1992; Uemori *et al.*, 1992). Direct nucleotide sequencing was also used by van der Giessen *et al.* (1994) to demonstrate that the spacer region of fast-growing species of *Mycobacterium* (*M. chelonae*, *M. fortuitum* and *M. phlei*) was 85 bp longer than the 278 bp spacer in slow-growing forms (*M. paratuberculosis* and *M. avium*). An alternative approach that is available for the differentiation of strains with few rRNA operons is the direct approach of nucleotide sequencing (Frothingham & Wilson, 1993, 1994).

Summary and future perspectives

The focus of this review has been the proposal that a rapid and universal bacterial identification and typing scheme is possible based on amplification of the variable length 16S–23S rDNA spacer regions. This is based on the observation that many bacteria carry multiple rRNA operons (Table 2) and that considerable length and sequence heterogeneity is apparent when the 16S–23S rDNA spacer sequences of such strains are aligned (Fig. 2). We suggest that region 2 of the 16S rRNA gene (nt 1390–1407) and region 10 of the 23S rRNA gene (nt 456–474) are the regions of choice for the construction of primers, the former because of its proximity to the spacer region and the latter because of the high level of sequence conservation among the species analysed (Fig. 1 and Table 1). Thus far, these primers have been used successfully to discriminate between clinical strains of *C. difficile* and of *S. aureus* (Table 3). Other primer combinations are also possible, that of regions 2 and 7 being noteworthy in respect to use by various workers and success in detecting spacer variation in the species studied (Table 3). By contrast, primer combinations involving region 5 of the 23S rRNA gene (nt 21–38) are not recommended since the sequence conservation of this region is relatively poor (Table 1) and only limited success has been found in strain discrimination (Table 3).

In the clinical situation, rapid and cost-effective identification of strains is an imperative. Up until now, the methods used for the phylogenetic analysis of bacteria based on the 16S rRNA gene invariably involve the

Fig. 2. Alignment of 16S–23S rDNA spacer sequences from various bacterial species starting from the end of the 16S rRNA gene to the beginning of the 23S rRNA gene. Alignment was obtained using the CLUSTAL W program (Thompson *et al.*, 1994) and DNA sequences that commenced at region 2 of the 16S rRNA gene, proceeded through the 16S–23S rDNA spacer region, and ended at region 10 of the 23S rRNA gene (see Fig. 1). Alignment across the 16S and 23S rRNA sequences was very good (sequences not shown), but gaps were present within the spacer region. Some of these gaps were manually removed

	430	440	450	460	470	480	490	500	510	520	530	540	550	560
<i>E. coli</i> rrnG	ACAGACGTTAAAGTCTGGTTG	ATATATTTGCTCTTTAA	AAATCTGGAT											
<i>E. coli</i> rrnC	ACTCATCTTCGGGTGATGTTG	AGATATTTGCTCTTTAA	AAATCTGGAT											
<i>E. coli</i> rrnB	ACTCATCTTCGGGTGATGTTG	AGATATTTGCTCTTTAA	AAATCTGGAT											
<i>E. coli</i> rrnE	ACTCATCTTCGGGTGATGTTG	AGATATTTGCTCTTTAA	AAATCTGGAT											
<i>A. hydrophila</i> II														CTCTCTTCGTAACAGAA
<i>A. hydrophila</i> III														CTCTCTTCGTAACAGAA
<i>A. hydrophila</i> I	atcccgatagctccacata													ATCCGTAAGA-ATTTCG
<i>E. coli</i> rrnD & X	atcccgatagctccacata	CTCTCTTCGTAACAGAA												
<i>P. aeruginosa</i> rrnA	atccctcttggctccacccc	AT-AAA-CAATCG-TCGAAG	CTCAGAAATGAATGTTCTGTAATGAACATGATTTCTGGCTTTG											GTCTTTTAAAAATTCGG
<i>P. aeruginosa</i> rrnB	atccctcttggctccacccc	AT-AAA-CAATCG-TCGAAG	CTCAGAAATGAATGTTCTGTAATGAACATGATTTCTGGCTTTG											GTCTTTTAAAAATTCGG
<i>P. aeruginosa</i> rrnC	atccctcttggctccacccc	AT-AAA-CAATCG-TCGAAG	CTCAGAAATGAATGTTCTGTAATGAACATGATTTCTGGCTTTG											GTCTTTTAAAAATTCGG
<i>B. cepacia</i> rrnB	atcccgcttgcctccaccaa	TCTCAATGCAAAACATTCGAATGA	CGATGTTGCGAGTCTTTCATTTGGCGATTGAG	CCAGTCAGAGGATATCAACAGATATCGCGTCTGTTCTTTAAACATCTGGAAAGATGA										
<i>B. cepacia</i> rrnC	atcccgcttgcctccaccaa	TCTCAATGCAAAACATTCGAATGA	CGATGTTGCGAGTCTTTCATTTGGCGATTGAG	CCAGTCAGAGGATATCAACAGATATCGCGTCTGTTCTTTAAACATCTGGAAAGATGA										
<i>B. cepacia</i> rrnD	atcccgcttgcctccaccaa	TCTCAATGCAAAACATTCGAATGA	CGATGTTGCGAGTCTTTCATTTGGCGATTGAG	CCAGTCAGAGGATATCAACAGATATCGCGTCTGTTCTTTAAACATCTGGAAAGATGA										
<i>B. cepacia</i> rrnE	atcccgcttgcctccaccaa	TCTCAATGCAAAACATTCGAATGA	CGATGTTGCGAGTCTTTCATTTGGCGATTGAG	CCAGTCAGAGGATATCAACAGATATCGCGTCTGTTCTTTAAACATCTGGAAAGATGA										
<i>B. cepacia</i> rrnA	atcccgcttgcctccaccaa	TCTCAATGCAAAACATTCGAATGA	CGATGTTGCGAGTCTTTCATTTGGCGATTGAG	CCAGTCAGAGGATATCAACAGATATCGCGTCTGTTCTTTAAACATCTGGAAAGATGA										
<i>S. aureus</i> rrnG														
<i>S. aureus</i> rrnF														
<i>S. aureus</i> rrnJ														
<i>S. aureus</i> rrnA	atcccgcttgcctccaccaa	TCTCAATGCAAAACATTCGAATGA	CGATGTTGCGAGTCTTTCATTTGGCGATTGAG	CCAGTCAGAGGATATCAACAGATATCGCGTCTGTTCTTTAAACATCTGGAAAGATGA										
<i>S. aureus</i> rrnC	atcccgcttgcctccaccaa	TCTCAATGCAAAACATTCGAATGA	CGATGTTGCGAGTCTTTCATTTGGCGATTGAG	CCAGTCAGAGGATATCAACAGATATCGCGTCTGTTCTTTAAACATCTGGAAAGATGA										
<i>E. hirae</i> rrnA	atcccgcttgcctccaccaa	TCTCAATGCAAAACATTCGAATGA	CGATGTTGCGAGTCTTTCATTTGGCGATTGAG	CCAGTCAGAGGATATCAACAGATATCGCGTCTGTTCTTTAAACATCTGGAAAGATGA										
<i>E. hirae</i> rrnB	atcccgcttgcctccaccaa	TCTCAATGCAAAACATTCGAATGA	CGATGTTGCGAGTCTTTCATTTGGCGATTGAG	CCAGTCAGAGGATATCAACAGATATCGCGTCTGTTCTTTAAACATCTGGAAAGATGA										
<i>R. sphaeroides</i> rrnA	atcccgcttgcctccaccaa	TCTCAATGCAAAACATTCGAATGA	CGATGTTGCGAGTCTTTCATTTGGCGATTGAG	CCAGTCAGAGGATATCAACAGATATCGCGTCTGTTCTTTAAACATCTGGAAAGATGA										
<i>B. subtilis</i> rrnB	atcccgcttgcctccaccaa	TCTCAATGCAAAACATTCGAATGA	CGATGTTGCGAGTCTTTCATTTGGCGATTGAG	CCAGTCAGAGGATATCAACAGATATCGCGTCTGTTCTTTAAACATCTGGAAAGATGA										
<i>B. subtilis</i> rrnA	atcccgcttgcctccaccaa	TCTCAATGCAAAACATTCGAATGA	CGATGTTGCGAGTCTTTCATTTGGCGATTGAG	CCAGTCAGAGGATATCAACAGATATCGCGTCTGTTCTTTAAACATCTGGAAAGATGA										
<i>C. botulinum</i> B														
<i>C. botulinum</i> C														
<i>C. botulinum</i> D														
<i>M. vanielii</i> rrnB														
<i>S. griseus</i>														
<i>L. monocytogenes</i>														
<i>M. hyopneumoniae</i>														
	570	580	590	600	610	620	630	640	650	660	670	680	690	700
<i>E. coli</i> rrnG														
<i>E. coli</i> rrnC														
<i>E. coli</i> rrnB														
<i>E. coli</i> rrnE														
<i>A. hydrophila</i> II	TGCAGAGTTAAGACTGATCTTAACTCTGTTTCTTACGCTTGTGCTGTTGCAACATGCTCTTTAAACATCTGGAAAGCTGATTTAAAGTAGTCTTCAACATTTGTTACAAAGTCTTGGAACTCTTGGCG													
<i>A. hydrophila</i> III	TGCAGAGTTAAGACTGATCTTAACTCTGTTTCTTACGCTTGTGCTGTTGCAACATGCTCTTTAAACATCTGGAAAGCTGATTTAAAGTAGTCTTCAACATTTGTTACAAAGTCTTGGAACTCTTGGCG													
<i>A. hydrophila</i> I	AGAACGAAAGCTGATGTTGACGATATGTTGAGTCTTATGCTTCTTGAAGAGTGTGCTCTTAAACATCTGGAAAGCTGATTTAAAGTAGTCTTCAACATTTGTTACAAAGTCTTGGAACTCTTGGCG													
<i>E. coli</i> rrnD & X														
<i>P. aeruginosa</i> rrnA	TGT-----GATA-----GAAG-----TAAGACTGA-ATG-ATCTCTTTCACCTGGTGATCAT-TCAGTCAAGGTAAATTT-TGGAGTTC-----													
<i>P. aeruginosa</i> rrnB	TGT-----GATA-----GAAG-----TAAGACTGA-ATG-ATCTCTTTCACCTGGTGATCAT-TCAGTCAAGGTAAATTT-TGGAGTTC-----													
<i>P. aeruginosa</i> rrnC	TGT-----GATA-----GAAG-----TAAGACTGA-ATG-ATCTCTTTCACCTGGTGATCAT-TCAGTCAAGGTAAATTT-TGGAGTTC-----													
<i>B. cepacia</i> rrnB	AGTAATTTGGATAGCGAAGCGTCTTGAGATGGAAGCTGGAACATATCCGGTGTGATTTGATGATGATGATCTCAAGATGATTCGAATC-----													
<i>B. cepacia</i> rrnC	AGTAATTTGGATAGCGAAGCGTCTTGAGATGGAAGCTGGAACATATCCGGTGTGATTTGATGATGATGATCTCAAGATGATTCGAATC-----													
<i>B. cepacia</i> rrnD	AGTAATTTGGATAGCGAAGCGTCTTGAGATGGAAGCTGGAACATATCCGGTGTGATTTGATGATGATGATCTCAAGATGATTCGAATC-----													
<i>B. cepacia</i> rrnE	AGTAATTTGGATAGCGAAGCGTCTTGAGATGGAAGCTGGAACATATCCGGTGTGATTTGATGATGATGATCTCAAGATGATTCGAATC-----													
<i>B. cepacia</i> rrnA	CGGTACGAAAGTATCGCTGCTGCTTTTAAACATCTGGAAGAGTAAATTTGGATAGCGAAGCGTCTTGAGATGGAAGCTGGAACATATCCGGTGTGATTTGATGATGATGATCTCAAGATGATTCGAATC-----													
<i>S. aureus</i> rrnG	AAACCTCACAAGATTAATAACCGGTTT-----													
<i>S. aureus</i> rrnF	AAACCTCACAAGATTAATAACCGGTTT-----													
<i>S. aureus</i> rrnJ	AAACCTCACAAGATTAATAACCGGTTT-----													
<i>S. aureus</i> rrnA	AAACCTCACAAGATTAATAACCGGTTT-----													
<i>S. aureus</i> rrnC	AAACCTCACAAGATTAATAACCGGTTT-----													
<i>E. hirae</i> rrnA	AAACCTCACAAGATTAATAACCGGTTT-----													
<i>E. hirae</i> rrnB	AAACCTCACAAGATTAATAACCGGTTT-----													
<i>R. sphaeroides</i> rrnA	GGGTTCGACCCCGCAGCGATATTGTT-----													
<i>B. subtilis</i> rrnB														
<i>B. subtilis</i> rrnA														
<i>C. botulinum</i> B	TTCAATTTTGAAGACTAAGTCTTTCAAAATGTTCTTTGAAAATTCGCATAG-----													
<i>C. botulinum</i> C	CAATTTTGAAGACTAAGTCTTTCAAAATGTTCTTTGAAAATTCGCATAG-----													
<i>C. botulinum</i> D	TTCAATTTTGAAGACTAAGTCTTTCAAAATGTTCTTTGAAAATTCGCATAG-----													
<i>M. vanielii</i> rrnB														
<i>S. griseus</i>														
<i>L. monocytogenes</i>														
<i>M. hyopneumoniae</i>														
	710	720	730	740	750	760	770							
<i>E. coli</i> rrnG	ACAATGAAAGTGTCTGAGTCTCTCAAAATTTTCGCAACTC-----TGAAGTG-----AAACATCTTCGGGTGTGA													[432]
<i>E. coli</i> rrnC	ACAACGAGAGTGTCTGAGTCTCTCAAAATTTTCGCAACTC-----TGAAGTG-----AAACATCTTCGGGTGTGA													[355]
<i>E. coli</i> rrnB	ACAACGAGAGTGTCTGAGTCTCTCAAAATTTTCGCAACTC-----TGAAGTG-----AAACATCTTCGGGTGTGA													[441]
<i>E. coli</i> rrnE	ACAACGAGAGTGTCTGAGTCTCTCAAAATTTTCGCAACTC-----TGAAGTG-----AAACATCTTCGGGTGTGA													[355]
<i>A. hydrophila</i> II	AAACCAAAATTTTATTTGGTCTTGTGATGACCAACAGCTAGCTGGCTTTTCCAGGACATCTTCGGGTGTGAT													[511]
<i>A. hydrophila</i> III	AAACCAAAATTTTATTTGGTCTTGTGATGACCAACAGCTAGCTGGCTTTTCCAGGACATCTTCGGGTGTGAT													[544]
<i>A. hydrophila</i> I	AAACCAAAATTTTATTTGGTCTTGTGATGACCAACAGCTAGCTGGCTTTTCCAGGACATCTTCGGGTGTGAT													[472]
<i>E. coli</i> rrnD & X	ACAATGAAAGTGTCTGAGTCTCTCAAAATTTTCGCAACTC-----TGAAGTG-----AAACATCTTCGGGTGTGA													[437]
<i>P. aeruginosa</i> rrnA	-----GAATTT-----TTCGGCG-ATGTC-CTCTTCACAGTATAACCA-----GATTGCTTGGGGTTATA-													[467]
<i>P. aeruginosa</i> rrnB	-----GAATTT-----TTCGGCG-ATGTC-CTCTTCACAGTATAACCA-----GATTGCTTGGGGTTATA-													[467]
<i>P. aeruginosa</i> rrnC	-----GAATTT-----TTCGGCG-ATGTC-CTCTTCACAGTATAACCA-----GATTGCTTGGGGTTATA-													[468]
<i>B. cepacia</i> rrnB	-----TCAATGGAATACCGCACAACCGGAGACTCAACCTGTAGCA-----GACAGCTCTTTATA-													[528]
<i>B. cepacia</i> rrnC	CCGACATCAATTTGGAATACCGCACAACCGGAGACTCAACCTGTAGCA-----GACAGCTCTTTATA-													[586]
<i>B. cepacia</i> rrnD	-----TCAATGGAATACCGCACAACCGGAGACTCAACCTGTAGCA-----GACAGCTCTTTATA-													[528]
<i>B. cepacia</i> rrnE	-----TCAATGGAATACCGCACAACCGGAGACTCAACCTGTAGCA-----GACAGCTCTTTATA-													[529]
<i>B. cepacia</i> rrnA	-----GTTTGCATCAATGGAATACCGCACAACCGGAGACTCAACCTGTAGCA-----GACAGCTCTTTATA-													[392]
<i>S. aureus</i> rrnG	-----CGTAAGTTCATGTTTAACTGTTGACTTTATAAAATGTTGGAACATA-													[382]
<i>S. aureus</i> rrnF	ATGTAATGAGCATTTAAATAATGAAAGCAAGCGGATGTTGAGCGTTT-GACTTTATAAAATGTTGGAACATA-													[457]
<i>S. aureus</i> rrnJ	-----CCTGTAGGA-----TGGAAACATA-													[335]
<i>S. aureus</i> rrnA	ATGTAATGAGCATTTAAATAATGAAAGCAAGCGGATGTTGAGCGTTT-GACTTTATAAAATGTTGGAACATA-													[551]
<i>S. aureus</i> rrnC	-----CGTAAGTTCATGTTTAACTGTTGACTTTATAAAATGTTGGAACATA-													[476]
<i>E. hirae</i> rrnA	AGTTTCATTTGCTTATTTCTTGTATCGACTTCTATCGCTAGAGAAAGATCAAAACCCCAACCGGTAGGGTTGATTA													[330]
<i>E. hirae</i> rrnB	AGTTTCATTTGCTTATTTCTTGTATCGACTTCTATCGCTAGAGAAAGATCAAAACCCCAACCGGTAGGGTTGATTA													[330]
<i>R. sphaeroides</i> rrnA	CGCAGGACCTTTGGGTCTATCGATGGAATGATACATGCTTCTGACATGGAAGAGCTTGTCTTTTCGGATCAG-													[664]
<i>B. subtilis</i> rrnB	-----AGTAACCAAGATATCACATAGTGATTTTAAAC													[344]
<i>B. subtilis</i> rrnA	-----AGTAACCAAGATATCACATAGTGATTTTAAAC													[167]
<i>C. botulinum</i> B	ATATACCAACAGCCAGGAATATATTTCTTGTAT- AAATAAGAACTATTCATTAATGTTTAAATTTAAATTAATA													[366]
<i>C. botulinum</i> C	ATATACCAACAGCCAGGAATATATTTCTTGTAT- AAATAAGAACTATTCATTAATGTTTAAATTTAAATTAATA													[365]
<i>C. botulinum</i> D	ATATACCAACAGCCAGGAATATATTTCTTGTAT- AAATAAGAACTATTCATTAATGTTTAAATTTAAATTAATA													[437]
<i>M. vanielii</i> rrnB	-----GATTAATTAATGATGTTGCTGACAAAGCTTTTATATCTGGTGTATTCAGATGTTTAAATTT													[232]
<i>S. griseus</i>	GTGAATCTGGCTTTCAGGCGGGGTGATGGTGGCTGGCTGGCTTGGAGAACTGCACAGTGGCGGAGCATCTGT													[304]
<i>L. monocytogenes</i>	ATCTTTCATCTGATGGAAGTATCATGCTGATACGGAAGAACTCAGAAACCAACTTTTACTTCGTGAAGATTAAT													[243]
<i>M. hyopneumoniae</i>	AGATTATGCTTAAATGTTTAAATATCTTAAGATATATTCTCTAAAATAGCTATCTACCTAAAATAGTTTAACT-													[491]

using the program MACCLADE (Maddison & Maddison, 1992) so that regions with the greatest homology (the tRNA genes) were aligned. The sequences in lower-case letters correspond to tRNA^{le} (220–440), tRNA^{glu} (300–378) and tRNA^{ala} (364–439). The short (17 bp) sequence in lower-case letters (52–68) highlights sequence homology between *S. aureus* and *Ent. hirae* which is not a tRNA sequence.

Table 3. Studies on bacterial 16S–23S rDNA spacer regions

When a region not shown in Fig. 1 has been chosen, it has been on the basis that the region is specific to the organism being sought.

Bacterium	Location of:		Variable length rDNA spacers:			Strain differences by RFLP or SSCP	Reference
	16S primer*	23S primer*	Presence of:	Sequence data available	Strain differences†		
Gram-negative							
<i>Acinetobacter</i> sp.	3	5	Yes	No	Limited	Yes	Nowak <i>et al.</i> (1995)
<i>Acinetobacter</i> sp.	2	7	Yes	No	Limited	Yes	Dolzani <i>et al.</i> (1995)
<i>Actinobacillus actinomycetemcomitans</i>	1,4	8	Yes	Yes	No‡	NA	Leys <i>et al.</i> (1994)
<i>Actinobacillus actinomycetemcomitans</i>	4	8	Yes	No	No	Yes	Griffen <i>et al.</i> (1992)
<i>Agrobacterium</i> sp.	1	6	No	No	No	Yes	Ponsonnet & Nesme (1994)
<i>Bordetella pertussis</i>	3	§	No	No	No	No	Rossau <i>et al.</i> (1992)
<i>Citrobacter diversus</i>	3	5	Yes	No	Limited	NA	Jensen <i>et al.</i> (1993)
<i>Citrobacter freundii</i>	3	5	Yes	No	Limited	NA	Jensen <i>et al.</i> (1993)
<i>Comamonadaceae</i>	1	10	No	No	Limited	Yes	Vaneechoutte <i>et al.</i> (1992)
<i>Enterobacter</i> sp.	3	5	Yes	No	Limited	NA	Jensen <i>et al.</i> (1993)
<i>Enterobacter</i> sp.	2	7	Yes	No¶	Yes	NA	Kostman <i>et al.</i> (1995)
<i>Escherichia coli</i>	2	7	Yes	No¶	Yes	NA	Kostman <i>et al.</i> (1995)
<i>Haemophilus influenzae</i>	**	**	?	No	No	Yes	Smith-Vaughan <i>et al.</i> (1995)
<i>Legionellaceae</i>	††	6	Yes	No	No	NA	Hookey <i>et al.</i> (1995)
<i>Moraxella canis</i>	3	§	No	No	No	Yes	Jannes <i>et al.</i> (1993)
<i>Neisseria meningitidis</i>	††	5	No	No	No	Yes	McLaughlin <i>et al.</i> (1993)
<i>Neisseria meningitidis</i>	††	5	Yes	No	Yes	NA	Auriol <i>et al.</i> (1995)
<i>Nitrobacter</i> sp.	4	5	No	No	No	Yes	Navarro <i>et al.</i> (1992)
<i>Burkholderia cepacia</i>	2	7	Yes	No	Yes	NA	Dasen <i>et al.</i> (1994)
<i>Burkholderia cepacia</i>	2	7	Yes	No	Yes	NA	Kostman <i>et al.</i> (1992)
<i>Pseudomonas fluorescens</i>	3	5	No	Yes	No	NA	Gill <i>et al.</i> (1994)
<i>Pseudomonas putida</i>	3	5	No	Yes	No	NA	Gill <i>et al.</i> (1994)
<i>Rochalimaea</i> sp.	3	9	No	No	No	Yes	Matar <i>et al.</i> (1993)
<i>Rochalimaea</i> sp.	§§	5	No	Yes	No	Yes	Roux & Raoult (1995a)
<i>Rhizobium</i> sp.	1	6	No	No	No	Yes	Nour <i>et al.</i> (1994)
<i>Salmonella enterica</i>	2	7	Yes	No	Yes	NA	Nastasi & Mammina (1995)
<i>Salmonella</i> sp.	3	5	Yes	No	Limited	NA	Jensen <i>et al.</i> (1993)
Gram-positive							
<i>Bacillus thuringiensis</i>	3	5	No	Yes	No	No	Bourque <i>et al.</i> (1995)
<i>Clostridium difficile</i>	2	10	Yes	No	Yes	NA	Gürtler (1993)
<i>Clostridium difficile</i>	2	7	Yes	No	Yes	NA	Cartwright <i>et al.</i> (1995)
<i>Clostridium difficile</i>	2	7	Yes	No	Yes	NA	Martirosian <i>et al.</i> (1995)
<i>Clostridium</i> sp.	3	5¶¶	No	Yes	No	No	Barry <i>et al.</i> (1991)
<i>Enterococcus faecalis</i>	3	5	Limited	Yes	No	NA	Hall (1994)
<i>Enterococcus faecium</i>	2	7	Yes	No¶	Yes	NA	Kostman <i>et al.</i> (1995)
<i>Listeria</i> sp.	3	5	Yes	No	Limited	NA	Jensen <i>et al.</i> (1993)
<i>Staphylococcus aureus</i>	2	10	Yes	Yes	Yes	NA	Gürtler & Barrie (1995)
<i>Staphylococcus aureus</i>	2	7	Yes	No¶	Yes	NA	Kostman <i>et al.</i> (1995)
<i>Staphylococcus aureus</i>	2	7	Yes	No	Yes	NA	Dolzani <i>et al.</i> (1994)
<i>Staphylococcus</i> sp.	3	5	Yes	No	Limited	NA	Jensen <i>et al.</i> (1993)
<i>Streptococcus milleri</i> group	3	5	Limited	Yes	No	NA	Whiley <i>et al.</i> (1995)
<i>Streptococcus thermophilus</i>	1	10	No	No	No	Yes	Salzano <i>et al.</i> (1994)

Table 3 (cont.)

Bacterium	Location of:		Variable length rDNA spacers:			Strain differences by RFLP or SSCP	Reference
	16S primer*	23S primer*	Presence of:	Sequence data available	Strain differences†		
Other bacterial groups							
<i>Borrelia burgdorferi</i> ***	NA	NA	No	No	No	Yes	Liveris <i>et al.</i> (1995)
<i>Borrelia andersonii</i>	NA	NA	No	No	No	Yes	Marconi <i>et al.</i> (1995)
<i>Borrelia japonica</i>	NA	NA	No	No	No	Yes	Marconi <i>et al.</i> (1995)
<i>Coxiella burnettii</i>	3	5	No	Yes	No	No	Thiele <i>et al.</i> (1994)
<i>Mycobacterium abscessus</i>	3	5	Yes	No	No	NA	Abed <i>et al.</i> (1995a)
<i>Mycobacterium avium</i>	3	5	Yes	No	No	NA	Abed <i>et al.</i> (1995a)
<i>Mycobacterium fortuitum</i>	3	5	Yes	No	No	NA	Abed <i>et al.</i> (1995a)
<i>Mycobacterium gordonae</i>	3	5	Yes	No	No	NA	Abed <i>et al.</i> (1995a)
<i>Mycobacterium kansasii</i>	3	5	Yes	No	Yes†††	NA	Abed <i>et al.</i> (1995a)
<i>Mycobacterium leprae</i> ‡‡‡	NA	NA	NA	No	No	Yes	de Wit & Klatser (1994)
<i>Mycobacterium tuberculosis</i>	3§§§	5§§§	NA	No	No	No	Abed <i>et al.</i> (1995b)
<i>Mycobacterium tuberculosis</i>	3	5	No	Yes	No	NA	Glennon <i>et al.</i> (1994)
<i>Mycobacterium tuberculosis</i>	3	5	Yes	No	No	NA	Abed <i>et al.</i> (1995b)
<i>Mycoplasma</i>			No	No	No	Yes	Harasawa <i>et al.</i> (1993); Nakagawa <i>et al.</i> (1992); Uemori <i>et al.</i> (1992)

NA, Not applicable to this study.

* The primers used were constructed from the regions numbered 1–10 in Fig. 1. In some cases the primers were either longer or shorter than the regions described in Table 1: refer to the respective references to obtain the exact sequences used.

† Variable length 16S–23S rDNA spacer regions the presence of which is variable between strains.

‡ Strain differences demonstrated by DNA sequence variability only and not by spacer length or number variations.

§ The 23S primer used was only 'quasi-conserved', the sequence of which is located near the spacer and does not match any of regions 5–10.

|| The *Comamonadaceae* are comprised of the following genera: *Comamonas*, *Xylophilus*, *Hydrogenophaga*, *Aquaspirillum* and *Acidovorax*. This study primarily showed genus and species differences with limited sub-species differentiation.

¶ Partial only.

** The sequence of the forward primer was at the beginning of the 16S rRNA gene and the reverse primer was within the 5S rRNA gene (located after the 23S rRNA gene). Variable length 16S–23S spacer regions (?) were inferred from RFLPs.

†† Nucleotide position for the 16S primer is 1113–1130.

‡‡ 1424–1441 (coordinates based on the 16S rRNA gene of *N. gonorrhoeae*).

§§ 1464–1484 (coordinates based on the aligned 16S rRNA gene of *Bartonella quintana* with *E. coli*).

||| Single PCR products were obtained for ten *Clostridium* species (and six other eubacteria) and 16S–23S spacer DNA sequences were reported for *C. perfringens*, *C. pasteurianum* and *C. difficile*.

¶¶ The 23S primer sequence used was different at three nucleotide positions to the sequence shown in Table 1.

*** *B. burgdorferi* has only one 16S rRNA gene and two 23S rRNA genes arranged in tandem.

††† Different types may be subspecies.

‡‡‡ This study used SSCP analysis in a nested PCR reaction using primers specific to *M. leprae* rRNA genes demonstrating strain-specific sequence differences.

§§§ The 16S–23S spacer region amplicon was subjected to a further round of randomly amplified polymorphic DNA PCR to produce variations between strains.

||| Primers specific to *Mycoplasma* species were used in a nested PCR to obtain a single band which varied in size between species.

sequence determination of PCR products; this is both costly and time consuming. The alternative favoured here, that of bacterial typing based on spacer-length variation is rapid, possibly automatable, and cost-effective. Reliable identification and typing of strains will be greatest in

species with multiple rRNA operons (at least three or four) and in those in which the full genomic complement of spacer regions can be amplified. The pattern of products obtained can then be matched to a database of band patterns from standard strains representing the

various species listed in the *Approved Lists of Bacterial Names*. To date, the 16S–23S spacer length and sequence database is far from complete, but increasing interest in this area, as shown by Table 3, is sure to redress this deficiency.

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