***Clostridium paradoxum* DSM 7308^T contains multiple 16S rRNA genes with heterogeneous intervening sequences**

Fred A. Rainey,¹ Naomi L. Ward-Rainey,¹ Peter H. Janssen,² Hans Hippe¹ and Erko Stackebrandt¹

Author for correspondence: Fred A. Rainey. Tel: +49 531 2616 101. Fax: +49 531 2616 418.
e-mail: rainey@gbf-braunschweig.de

¹ DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, D-38124 Braunschweig, Germany

² Max-Planck-Institut für Terrestrische Mikrobiologie, D-35043 Marburg, Germany

Sequence analysis of the cloned 16S rRNA genes of *Clostridium paradoxum* DSM 7308^T revealed the presence of 15 different sequences in variable region I (*Escherichia coli* positions 73–97) of the 16S rDNA. The majority of the cloned genes contained intervening sequences (IVSs), which varied in length from 120–131 nt, and were present in the DNA obtained from single colonies of *C. paradoxum*. The absence of IVSs in the mature rRNA was demonstrated by Northern hybridization and sequence analysis of the 16S rRNA reverse transcriptase (RT)-PCR product. This finding was supported by the failure of oligonucleotide probes specific for certain IVSs to hybridize to the RT-PCR product obtained from *C. paradoxum*. Alterations in culture conditions (temperature, pH, salt) or culture age did not lead to expression of RNA containing IVSs, as indicated by the size of RT-PCR products. Hybridization of the restriction-enzyme-digested genomic DNA of *C. paradoxum* with probes derived from the IVSs demonstrated that the 16S rRNA genes containing different IVSs are located at different sites on the chromosome.

Keywords: *Clostridium paradoxum*, 16S rRNA/rDNA, multiple operons, operon heterogeneity, intervening sequence

INTRODUCTION

In recent years, thermophilic bacteria have been the subject of intense investigation due to the potential industrial application of their thermostable enzymes (Kristjansson, 1992). A thermophilic alkaliphilic bacterium was recently described and assigned to the genus *Clostridium* as *Clostridium paradoxum* (Li *et al.*, 1993). On the basis of 16S rRNA sequence analysis, *C. paradoxum* was shown to belong to the *Bacillus/Clostridium* subphylum of the Gram-positive line of descent.

The nucleotide sequence of the genes coding for the small and large rRNA subunits are now widely used for the identification and phylogenetic placement of new isolates within the domains Bacteria and Archaea (Olsen *et al.*, 1994; Ludwig & Schleifer, 1994). In addition, the primary structures of the 16S and 23S rRNAs have been shown to have enormous potential in the detection of uncultured

micro-organisms in environmental samples (Ward *et al.*, 1995). The usefulness of these molecules has stimulated interest in variation in their structure (Gutell *et al.*, 1994). The presence of intervening sequences (IVSs) in rRNA genes could have potential as species- or strain-specific markers. Patel *et al.* (1992) were first in reporting the presence of an extended stem at variable position I [*Escherichia coli* positions 73–97 (Brosius *et al.*, 1978)] in the 16S rRNA gene of the thermophilic bacterium *Desulfotomaculum australicum*; a similar phenomenon was found later in the species *Desulfotomaculum thermobenzoicum* (Redburn & Patel, 1993). Subsequent studies demonstrated the presence of large insertions in the 16S rRNA genes of thermophilic clostridia (Rainey *et al.*, 1993), *Rhizobium tropici* (Willems & Collins, 1993), *Caedibacter caryophila* (Springer *et al.*, 1993), *Campylobacter sputorum* (van Camp *et al.*, 1993), *Campylobacter helveticus* (Linton *et al.*, 1994b) and *Helicobacter canis* (Linton *et al.*, 1994a). Similar IVSs in the 23S rRNA gene have been described for *Leptospira* (Hsu *et al.*, 1990; Ralph & McClelland, 1993), *Salmonella* (Winkler, 1979; Smith *et al.*, 1988; Burgin *et al.*, 1990; Hsu *et al.*, 1992), *Yersinia* (Skurnick & Toivanen, 1991) and *Campylobacter* (Konkel *et al.*, 1994;

Abbreviations: IVS, intervening sequence; RT, reverse transcriptase.

The EMBL accession numbers for the sequences reported in this paper are Z69927–Z69942.

Trust *et al.*, 1994) species, and for *Coxiella burnetii* (Afseth *et al.*, 1995).

The number of rRNA operons in bacterial genomes has been shown to range from 1 to 14 (Young & Cole, 1993). In most cases, the rRNA genes are linked as a transcription unit in the order 5'-16S-23S-5S-3' (Nomura *et al.*, 1984). Although sequence heterogeneity between 16S rDNA operons within the same organism has been shown (Carbon *et al.*, 1979; Mylvaganam & Dennis, 1992), only a single type of IVS has been described for a given strain.

In the course of an attempt to determine the sequence of the variable region I of *C. paradoxum*, with the view to designing a specific oligonucleotide probe, we observed unusually large PCR amplicons of the 16S rRNA gene of *C. paradoxum* DSM 7308^T. Here we present evidence of both multiple 16S rRNA genes, the presence of different sequence composition in insertions at variable region I, and 16S rRNA operon heterogeneity.

METHODS

Bacterial strains and growth conditions. *C. paradoxum* strain JW-YL-7 (= DSM 7308^T) (Li *et al.*, 1993) was grown under a variety of culture conditions for DNA and RNA preparation: temperature (37, 55 or 60 °C), pH (7.7, 9.4 or 10.2), culture time (5, 18 or 48 h) and NaCl concentration (1 or 45 g l⁻¹). Except where noted, cultures were grown at pH 9.4, at 55 °C and with 1 g NaCl l⁻¹, and harvested after 18 h. One culture was supplemented with 50 ml soil extract l⁻¹ (Cote & Gherna, 1994). A bicarbonate-containing, sulfide-reduced mineral medium containing trace elements, selenite/tungstate and vitamins

(Dörner & Schink, 1990), was prepared in 50 or 200 ml volumes, under a headspace of N₂ plus CO₂ (4:1, v/v) in 125 or 250 ml bottles, respectively. Just prior to inoculation with a stationary-phase culture (1% inoculum, v/v), the medium was supplemented with the appropriate buffer, 10.5 mM nitrilotriacetic acid, 1 g yeast extract l⁻¹ and 10 mM glucose. The buffers (Sigma) were added from concentrated stock solutions to the following final concentrations: 50 mM HEPPS/NaOH for pH 7.7, 25 mM CHES/NaOH plus 25 mM CAPS/NaOH for pH 9.4, and 50 mM CAPS/NaOH for pH 10.2. The final pH of the medium was checked before and after growth, correcting for the growth temperature.

Preparation of single colonies. The agar medium used for the isolation of single colonies of *C. paradoxum* had the following composition (per l): 5.3 g Na₂CO₃, 0.7 g Na₂HPO₄·12H₂O, 0.075 g KCl, 20 ml trace element solution (Balch *et al.*, 1979), 5 g yeast extract, 10 g tryptone, 0.5 mg resazurin, 15 g agar (Difco), 4 g glucose, 0.8 g L-cysteine. The pH of the agar medium was adjusted to 10 by adding 10 ml 2 M NaOH (autoclaved under nitrogen atmosphere). For colony isolation, a sample taken from a well-sporulated culture was streaked on agar. After anaerobic incubation (Anaerocult bag; Merck) at 55 °C overnight, well-separated colonies developed. Streaking of single colonies was repeated twice and finally a single colony was transferred into a liquid medium of the same composition.

Preparation of genomic DNA and amplification of the 16S rRNA genes. The cells from 10 ml culture were recovered by centrifugation and resuspended in 200 µl saline/EDTA buffer [150 mM NaCl, 10 mM EDTA (pH 8.0)]. After the addition of 5 µl lysozyme (10 mg ml⁻¹), the solution was incubated at 37 °C for 30 min. Proteinase K (5 µl of 15 mg ml⁻¹) and 10 µl SDS (25%, w/v) were added, followed by incubation at 55 °C for 30 min. DNA was recovered using the Prep-A-Gene kit (Bio-

Table 1. Oligonucleotide primers and probes used in this study

Primer/probe	Sequence (5'-3')	Target region*
16S rDNA/RNA primers		
27f	GAGTTTGATCCTGGCTCAG	9-27
124f	CACGTGTTACTCACCGTCC	105-124
343r	CTGCTGCCTCCCGTA	343-357
357f	TACGGGAGGCAGCAG	343-357
519r	G(T/A)ATTACCGCGGC(T/G)GCTG	519-536
530f	CAGC(C/A)GCCGCGGTAAT(T/A)C	519-536
803f	ATTAGATACCCTGGTAG	787-803
907r	CCGTCAATTCATTTGAGTTT	907-926
1114f	GCAACGAGCGCAACCC	1099-1114
1241f	TACACACGTGCTACAATG	1224-1241
1385r	CGGTGTGT(A/G)CAAGGCCC	1385-1401
1406f	G(A/G)GCCTTG(C/T)ACACACCG	1385-1401
1525r	AGAAAGGAGGTGATCCAGCC	1525-1545
M13 primers		
M13 (-20)	GTAAAACGACGGCCAGT	Cloning vector
M13 reverse	GGAAACAGCTATGACCATG	Cloning vector
IVS-specific probes		
para1	ATGTGTTATGCTTTATTTTGC	Within IVS
para32	TTATTCATAATTTGCGAAAAG	Within IVS
para82	TCAAAGGCTATCAAATTAGC	Within IVS

* *E. coli* numbering (Brosius *et al.*, 1978).

Fig. 1. Fragment size analysis of PCR products. Unless indicated otherwise, PCR products were generated from *C. paradoxum* DNA or RNA. Unfilled peaks represent bands of size standard; filled peaks represent sample bands. PCR template was: (a) genomic DNA (fragment sizes 114, 214, 223 nt); (b) isolated colony 1 DNA (114, 214, 223 nt); (c) isolated colony 2 DNA (114, 214, 223 nt); (d) insert of clone para23 DNA (114 nt); (e) insert of clone para27 DNA (214 nt); (f) insert of clone para82 DNA (223 nt); (g) RNA (114 nt); (h) *E. coli* DNA (118 nt).

Rad). The 16S rDNA was amplified by PCR, using the reagents supplied in the TA cloning kit (Invitrogen), 50–100 ng genomic DNA and the primer pair (0.5 µg each) 27f and 1525r (Table 1). Thermal cycling was performed in a model 480 apparatus (Perkin Elmer). The samples were subjected to an initial denaturing step of 2 min at 94 °C, after which 2 U *Taq* polymerase was added to each sample at 90 °C. The thermal profile consisted of 28 cycles of 1 min at 94 °C, 2 min at 57 °C, and 3 min at 72 °C. A final extension step of 7 min at 72 °C was performed.

Direct cloning of 16S rDNA PCR product. The 16S rDNA amplicon was cloned using the TA cloning kit according to the manufacturer's instructions, and transformants containing a complete 16S rDNA gene were detected by a rapid disruption method and agarose gel electrophoresis (Sambrook *et al.*, 1989). Cloned inserts were amplified by PCR using the conditions described for 16S rDNA above, with the exception that the primers (0.5 µg each) used were M13 (–20) and M13 reverse (Table 1).

Preparation of RNA and amplification of 16S rRNA by reverse transcriptase (RT)-PCR. The total cellular RNA was extracted from the cell pellet obtained from 50 ml culture, using the RNA isolation kit (Stratagene) according to manufacturer's instructions. DNA was completely removed by digestion with 10 U RNase-free DNase (Boehringer Mannheim) at 37 °C for 1 h. RNA was recovered by extraction with an equal volume of phenol, followed by extraction with an equal volume of

chloroform, and precipitation in 0.4 M lithium chloride and 3 vols absolute ethanol. 16S rRNA was amplified by RT-PCR using the RT-PCR kit (Perkin Elmer), according to the manufacturer's instructions, using combinations of the primers (0.5 µg each) 27f, 124r, 343r, 519r, 357f, 530f, 907r, 803f and 1385r (Table 1).

Direct sequencing of PCR products. PCR products were purified using the Prep-A-Gene kit. Sequencing reactions were performed with the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) according to the protocol and thermal profile recommended by Applied Biosystems. The sequencing primers used are listed in Table 1. Purified sequence reactions were electrophoresed on a 6% (w/v) polyacrylamide sequencing gel for 12 h using an Applied Biosystems model 373A Automated DNA Sequencer.

Dot blot hybridization of RT-PCR products. Dot blots of RT-PCR products were prepared using a BioDot microfiltration apparatus (Bio-Rad), and Hybond-N nylon membrane (Amersham). Oligonucleotide probes were 3' end-labelled using the end-labelling kit of Boehringer Mannheim, and PCR products were random prime-labelled using the DNA labelling kit of Boehringer Mannheim. Hybridization and chemiluminescent detection was performed as recommended by Boehringer Mannheim.

Southern hybridization of genomic DNA, and Northern hybridization of RNA. Genomic DNA (5 µg) was digested with

Table 3. Signature nucleotides for the different *C. paradoxum* 16S rDNA clones, outside of variable region I

Variant bases are given in bold.

Posn*	para															RT†	DNA‡
	1	11	24	25	27	39	83	89	32	78	82	99	85	23	2		
119	A	A	A	A	A	A	A	A	A	A	A	A	A	G	A	A	A
232	G	G	G	G	G	G	G	G	C	G	G	G	G	G	G	G	G
365	T	T	T	T	T	T	C	T	T	T	T	T	T	T	T	U	T
595	A	A	A	A	A	A	A	A	A	A	G	A	A	A	A	A	A
682	G	G	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A
699	C	C	C	C	C	C	C	C	T	T	C	C	C	C	C	C	N
721	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	A	A
748	A	A	A	G	A	A	A	A	A	A	A	A	A	A	A	A	A
811	C	C	C	C	T	C	C	C	C	C	C	C	C	C	C	C	C
815	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
1002	T	T	T	T	T	T	T	C	T	T	T	T	T	T	T	U	N
1003	A	A	A	A	A	A	A	G	A	A	A	A	A	A	A	A	N
1005	G	G	G	G	G	G	G	A	A	A	A	G	G	G	A	N	N
1022	T	T	T	C	C	C	C	C	C	C	C	C	C	C	C	C	N
1028	C	C	C	C	C	C	C	C	C	C	T	C	C	C	C	C	C
1062	T	T	C	T	T	T	T	T	T	T	T	T	T	T	T	U	T
1124	G	G	G	G	G	G	G	A	G	G	G	G	G	G	G	G	G
1180	A	A	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A
1265	C	C	C	C	C	C	C	C	C	C	C	C	T	C	C	C	C
1346	A	A	A	A	A	A	G	A	A	A	A	A	A	A	A	A	A
1443	C	C	G	C	C	C	C	C	C	C	C	C	C	C	C	C	C

* *E. coli* numbering (Brosius *et al.*, 1978).

† 16S rRNA sequence determined from the RT-PCR product.

‡ 16S rDNA sequence of PCR product prior to cloning.

contain an extra sequence block of 98–109 bp which is not present in the first-described rRNA sequence (Li *et al.*, 1993) (Table 2). Sequence differences in other parts of the cloned genes are listed in Table 3.

Sequence determination and fragment size analysis of the 16S rRNA of *C. paradoxum*

Sequence determination of the RT-PCR product generated from the RNA of *C. paradoxum* showed variable region I to consist of 22 bp (Table 2). Fragment size analysis of the RT-PCR product using GENESCAN showed only one fragment of 114 nt (Fig. 1g), in contrast to the rDNA PCR products with three fragments (Fig. 1a–c). The sequence data obtained for the RT-PCR product had two ambiguities at positions 89 and 1005 (*E. coli* positions), and subsequent cloning and sequencing of the RT-PCR product revealed the presence of two rRNA copies which differed at these positions. Sequence analysis of the RT-PCR products generated from RNA of cells grown under various conditions (extremes of pH, temperature and salinity) or harvested in different growth phases showed the same sequence as that obtained from cells grown under standard conditions (including the

Fig. 2. Agarose gel electrophoresis of RNA (lanes 1 and 2), and Northern hybridization with a DIG-labelled *C. paradoxum* 16S rDNA probe (lanes 3 and 4). Lanes 1 and 3, *E. coli*; lanes 2 and 4, *C. paradoxum*.

Fig. 3. Hybridization of Southern blots of digested *C. paradoxum* DNA against DIG-labelled probes. (a) Lanes: 1, Boehringer Mannheim DNA molecular mass marker II, DIG-labelled; 2, *EcoRV* digest hybridized with 16S rDNA probe; 3, *BclI* digest hybridized with 16S rDNA probe; 4, *EcoRV* digest hybridized with 23S rDNA probe; 5, *BclI* digest hybridized with 23S rDNA probe. (b) Lanes: 1, Boehringer Mannheim DNA molecular mass marker II, DIG-labelled; 2–5, *AflIII* digest hybridized with the following probes: 2, 16S rDNA probe; 3, clone para1 group-specific oligonucleotide probe (representing para1, 11, 24, 25, 27, 39, 83, 89); 4, clone para32-specific oligonucleotide probe; 5, clone para 82-specific oligonucleotide probe.

ambiguity at position 1005). Fragment size analysis of partial RT-PCR products generated from these RNAs showed only one fragment, of the same size as that obtained when the PCR template was RNA from cells grown under standard conditions (data not shown). No signal was obtained after hybridization of dot-blotted RT-PCR products with probes specific for the different IVSs (data not shown). Agarose gel electrophoresis of *C. paradoxum* RNA showed three bands corresponding in size to the 16S and 23S rRNAs as compared to *E. coli* (Fig. 2). Northern blot analysis using the complete *C. paradoxum* 16S rDNA gene as a hybridization probe indicated that only one of the three bands represented a single 16S rRNA species.

Localization of different sequence insertions in the genome of *C. paradoxum*

Hybridization of Southern blots of digested genomic DNA of *C. paradoxum* with oligonucleotide probes specific for some of the insertion types showed that the different insertions are located on different DNA fragments (Fig. 3). Subsequent hybridization with a PCR-generated 16S rDNA probe, that would hybridize to all clone types, revealed the location of the 16S rRNA genes. The pattern obtained from the restriction digest shown in

Fig. 3 suggests that there were at least nine genes. Hybridization with a probe generated from a 23S rDNA PCR product of *C. paradoxum* showed that the 23S rRNA genes are located on the same restriction fragments as the 16S rRNA genes (Fig. 3).

DISCUSSION

Here we present the first report of heterogeneous IVSs within the 16S rRNA genes of a single strain of a bacterial species. The results obtained from sequencing the cloned 16S rDNA of *C. paradoxum* suggest that the number of 16S rRNA genes is at least 15, but may be greater, given the possibility that there is more than one copy of each of the 15 sequence types. The differences between some of the operons is only 1 bp, e.g. para11 differs from the other clone sequences in having an A rather than a T at a given position. It must therefore be considered that such small differences could be the result of PCR-introduced errors. However, the presence of a pairing nucleotide at a distant position in the primary structure of the sequence lends credibility to such differences. Some of these one-base differences are in regions not involved in direct secondary structure pairing and so cannot be verified in this respect.

The presence of additional undetected copies with IVSs of a different size to those reported here is unlikely, given the fact that only three different-sized fragments were detected in the 16S rDNA PCR products that were analysed by using GENESCAN software (Fig. 1a–c). It was not possible to determine the exact number of genes by hybridization experiments, as the fragments carrying the 16S rRNA genes are in some cases very close together and difficult to resolve, and some signals obtained after hybridization may represent doublet or even triplet bands (Fig. 3). However, it would appear that there are at least nine different chromosomal locations of the 16S rRNA genes. Hybridization with oligonucleotide probes specific for certain IVSs demonstrates that the different IVSs are located at different positions in the chromosome (Fig. 3), although the proximity of the copies has not been determined.

The possibility that the multiple IVSs arise from a culture containing multiple strains was shown to be unlikely by the results of GENESCAN fragment size analysis of single colonies of *C. paradoxum*, in which fragments of the same size as those found in the analysis of the original culture were detected (Fig. 1).

The reported IVSs in 23S rRNA genes are transcribed, and subsequently excised, producing a fragmented mature 23S rRNA molecule (Trust *et al.*, 1994; Konkel *et al.*, 1994; Ralph & McClelland, 1993; Skurnik & Toivanen, 1991). The 23S rDNAs of the archaea *Desulfurococcus mobilis*, *Staphylothermus marinus* and *Pyrobaculum organotrophum* have been shown to contain true introns, which are spliced off from the 23S rRNA (Kjems & Garrett, 1985, 1991; Kjems *et al.*, 1992). The phenomenon has also been reported in the 16S rRNA gene of *Pyrobaculum aerophilum* (Burggraf *et al.*, 1993). Sequence analysis of 16S rRNA RT-PCR products generated from the RNA of *C.*

paradoxum revealed that no IVSs are present in the mature 16S rRNA which can be amplified by RT-PCR. The 16S rRNA sequence determined by Li *et al.* (1993), using RT sequencing, also contained no IVSs. The RT sequence data alone does not prove that the operons carrying IVSs were not transcribed, since it should not be possible to amplify the rRNA if the IVS-bearing genes were transcribed and the IVSs excised without subsequent religation of the mature fragmented rRNA. The Northern hybridization clearly shows that only one 16S rRNA band hybridizes with the 16S rDNA probe. This result indicates that the IVSs are not excised from IVS-bearing transcripts resulting in fragmented 16S rRNA. Although not the subject of this study, it is interesting to note that the 23S rRNA appears to be fragmented.

The results of this study suggest that only two copies of the 16S rDNA (without large IVSs) are transcribed, and the other copies are silent or redundant. Supporting evidence for this theory is the fact that the sequence of the 16S rRNA RT-PCR product contains only two ambiguous base positions corresponding to the heterogeneity of two positions in the transcribed 16S rRNA genes without inserts (para2 and para23 clone types, Table 2). If more than these two 16S rDNA genes were transcribed, then the variable positions found outside variable region I (Table 3) should result in sequence ambiguity in those positions. In contrast, the sequence data obtained by direct sequencing of the 16S rDNA PCR product includes five ambiguous bases (Table 3), corresponding to positions where the different genes have been shown to contain different bases.

Having concluded that the 16S rRNA genes bearing IVSs are redundant (not expressed) during normal growth conditions, we attempted to determine whether these genes might be reserved for use under certain environmental conditions. GENSCAN fragment size analysis and sequence analysis of 16S rRNA RT-PCR products generated from RNA from cells of *C. paradoxum* grown under simulated 'extreme' conditions (extremes of temperature, pH, NaCl concentration and culture age) clearly demonstrated that these stresses had no influence on the expression of the 16S rRNA genes. This contrasts with the results of similar studies with *E. coli*, in which the levels of expression of certain operons was reported to be affected by culture conditions (Condon *et al.*, 1992).

The apparent dormancy of IVS-bearing 16S rRNA genes in *C. paradoxum* invites speculation as to the possible origin of these sequences. Comparison of IVS sequences with the sequence information available in databases such as GenBank has been previously used to determine whether the IVSs are derived from the genomic DNA of other bacteria (Ralph & McClelland, 1993; Linton *et al.*, 1994a). A similar comparison of the *C. paradoxum* IVSs was performed, but no similarity was found to any sequence contained in GenBank. Another possible IVS source is the genomic DNA of *C. paradoxum* itself, with the DNA base composition of the IVSs ranging from 33.3 to 38.5 mol% G + C, which is near that of the genomic DNA of *C. paradoxum* (30 mol% G + C, Li *et al.*, 1993).

This contrasts with the findings of Konkel *et al.* (1994) who noted a 22.2 mol% G + C in the 23S rDNA IVS of *Campylobacter jejuni*, much lower than that reported for the *C. jejuni* genome (32.5 mol% G + C; Owen & Leaper, 1981). In this case, the authors suggested an exogenous source for the IVS.

The results of this study demonstrate a large degree of sequence heterogeneity within the 16S rRNA genes of *C. paradoxum*. This finding has important implications for the interpretation of data obtained from 16S-rDNA-based taxonomy and microbial ecology studies. These results highlight the need to sequence more than one clone, where cloning is required to obtain readable sequence data. If the sequence of only one cloned gene is analysed and deposited in the database, subsequent comparisons of 16S rDNA sequence data from similar strains may lead to erroneous taxonomic conclusions, especially if the highly variable regions were used for the design of oligonucleotide taxon-specific probes.

In microbial ecology studies, the amplified 16S rRNA genes under investigation are often size-selected to avoid cloning nonspecific amplification products. It is possible that larger 16S rRNA genes such as those described here may be mistaken for nonspecific products and removed prior to cloning, resulting in loss of this intra-strain heterogeneity and thus some of the microbial diversity. It should be considered that a group of highly related environmental 16S rDNA clone sequences such as those that have been found in many environments may represent not a group of separate, phylogenetically highly related strains, but rather the sequence heterogeneity of the 16S rDNA contained within one strain. Similarly, the apparent complexity of communities analysed by newly developed methods such as TGGE/DGGE (Muyzer *et al.*, 1993; Muyzer & de Waal, 1994) could in some situations be attributable to this phenomenon.

The findings of this study, demonstrating the presence of heterogeneity within the 16S rRNA genes of a single organism, raise some concerns regarding the use of 16S rRNA gene sequences for taxonomic and microbial ecology studies. However, such heterogeneity needs to be further investigated within groups of highly related strains to assess the potential use of this phenomenon as a strain-specific characteristic for strain identification and differentiation.

REFERENCES

- Afseth, G., Mo, Y.-Y. & Mallavia, L. P. (1995). Characterization of the 23S and 5S rRNA genes of *Coxiella burnetii* and identification of an intervening sequence within the 23S rRNA gene. *J Bacteriol* **177**, 2946–2949.
- Balch, W. E., Fox, G. E., Magrum, R. J. & Wolfe, R. S. (1979). Methanogens: reevaluation of a unique biological group. *Microbiol Rev* **43**, 260–296.
- Brosius, J., Palmer, M. L., Kennedy, J. P. & Noller, H. P. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci USA* **75**, 4801–4805.
- Burggraf, S., Larsen, N., Woese, C. R. & Stetter, K. O. (1993). An

- intron within the 16S ribosomal RNA gene of the archaeon *Pyrobaculum aerophilum*. *Proc Natl Acad Sci USA* **90**, 2547–2550.
- Burgin, A. B., Parodos, K., Lane, D. J. & Pace, N. R. (1990). The excision of intervening sequences from *Salmonella* 23S ribosomal RNA. *Cell* **60**, 405–414.
- van Camp, G., van de Peer, Y., Nicolai, S., Neefs, J.-M., Vandamme, P. & de Wachter, R. (1993). Structure of 16S and 23S ribosomal RNA genes in *Campylobacter* species: phylogenetic analysis of the genus *Campylobacter* and presence of internal transcribed spacers. *Syst Appl Microbiol* **16**, 361–368.
- Carbon, P., Ehresmann, C., Ehresmann, B. & Ebel, J.-P. (1979). The complete nucleotide sequence of the ribosomal 16S RNA from *Escherichia coli*. *Eur J Biochem* **100**, 399–410.
- Condon, C., Philips, J., Fu, Z.-Y., Squires, C. & Squires, C. L. (1992). Comparison of the expression of the seven ribosomal RNA operons in *Escherichia coli*. *EMBO J* **11**, 4175–4185.
- Cote, R. J. & Gherna, R. L. (1994). Nutrition and media. In *Methods for General and Molecular Microbiology*, pp. 155–178. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Dörner, C. & Schink, B. (1990). *Clostridium homopropionicum* sp. nov., a new strict anaerobe growing with 2-, 3-, or 4-hydroxybutyrate. *Arch Microbiol* **154**, 342–348.
- Gutell, R. R., Larsen, N. & Woese, C. R. (1994). Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. *Microbiol Rev* **58**, 10–26.
- Hsu, D., Pan, M.-J., Zee, Y. C. & LeFebvre, R. B. (1990). Unique ribosome structure of *Leptospira interrogans* is composed of four rRNA components. *J Bacteriol* **172**, 3478–3480.
- Hsu, D., Zee, Y. C., Ingraham, J. & Shih, L.-M. (1992). Diversity of cleavage patterns of *Salmonella* 23S rRNA. *J Gen Microbiol* **138**, 199–203.
- Kjems, J. & Garrett, R. A. (1985). An intron in the 23S rRNA gene of the archaeobacterium *Desulfurococcus mobilis*. *Nature* **318**, 675–677.
- Kjems, J. & Garrett, R. A. (1991). Ribosomal RNA introns in archaea and evidence for RNA conformational changes associated with splicing. *Proc Natl Acad Sci USA* **88**, 439–443.
- Kjems, J., Larsen, N., Dalgaard, J. Z. & Garrett, R. A. (1992). Phylogenetic relationships amongst the hyperthermophilic archaea determined from partial 23S rRNA gene sequences. *Syst Appl Microbiol* **15**, 203–208.
- Konkel, M. E., Marconi, R. T., Mead, D. J. & Cieplak, W., Jr (1994). Identification and characterization of an intervening sequence within the 23S ribosomal RNA genes of *Campylobacter jejuni*. *Mol Microbiol* **14**, 235–241.
- Kristjansson, J. K. (1992). *Thermophilic Bacteria*. Boca Raton, FL: CRC Press.
- Li, Y., Mandelco, L. & Wiegel, J. (1993). Isolation and characterization of a moderately thermophilic anaerobic alkaliphile, *Clostridium paradoxum* sp. nov. *Int J Syst Bacteriol* **43**, 450–460.
- Linton, D., Clewley, J. P., Burnens, A., Owen, R. J. & Stanley, J. (1994a). An intervening sequence (IVS) in the 16S rRNA gene of the eubacterium *Helicobacter canis*. *Nucleic Acids Res* **22**, 1954–1958.
- Linton, D., Dewhirst, F. E., Clewley, J. P., Owen, R. J., Burnens, A. P. & Stanley, J. (1994b). Two types of 16S rRNA gene are found in *Campylobacter helveticus*: analysis, applications and characterization of the intervening sequence found in some strains. *Microbiology* **140**, 847–855.
- Ludwig, W. & Schleifer, K.-H. (1994). Bacterial phylogeny based on 16S and 23S rRNA sequence analysis. *FEMS Microbiol Rev* **15**, 155–173.
- Muyzer, G. & de Waal, E. C. (1994). Determination of the genetic diversity of microbial communities using DGGE analysis of PCR-amplified 16S rDNA. In *Structure, Development and Environmental Significance of Microbial Mats*, pp. 207–214. NATO ASI Series, vol. G35. Edited by L. J. Stal & P. Caumette. Berlin: Springer-Verlag.
- Muyzer, G., de Waal, E. & Uitterlinde, A. (1993). Profiling of complex microbial populations using denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59**, 695–700.
- Mylvaganam, S. & Dennis, P. P. (1992). Sequence heterogeneity between the two genes encoding 16S rRNA from the halophilic archaeobacterium *Haloarcula marismortui*. *Gen Soc Am* **130**, 399–410.
- Nomura, M., Gourse, R. & Baughman, G. (1984). Regulation of the synthesis of ribosomes and ribosomal components. *Annu Rev Biochem* **53**, 75–117.
- Olsen, G. J., Woese, C. R. & Overbeek, R. (1994). The winds of (evolutionary) change: breathing new life into microbiology. *J Bacteriol* **176**, 1–6.
- Owen, R. J. & Leaper, S. (1981). Base composition, size and nucleotide sequence similarities of genome deoxyribonucleic acids from species of the genus *Campylobacter*. *FEMS Microbiol Lett* **12**, 395–400.
- Patel, B. K. C., Love, C. A. & Stackebrandt, E. (1992). Helix 6 of the 16S rRNA of the bacterium *Desulfotomaculum australicum* exhibits an unusual structure idiosyncrasy. *Nucleic Acids Res* **20**, 5483.
- Rainey, F. A., Ward, N. L., Morgan, H. W., Toalster, R. & Stackebrandt, E. (1993). Phylogenetic analysis of anaerobic thermophilic bacteria: aid for their reclassification. *J Bacteriol* **175**, 4772–4779.
- Ralph, D. & McClelland, M. (1993). Intervening sequence with conserved open reading frame in eubacterial 23S rRNA genes. *Proc Natl Acad Sci USA* **90**, 6864–6868.
- Redburn, A. C. & Patel, B. K. C. (1993). Phylogenetic analysis of *Desulfotomaculum thermobenzoicum* using polymerase chain reaction-amplified 16S rRNA-specific DNA. *FEMS Microbiol Lett* **113**, 81–86.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Skurnik, M. & Toivanen, P. (1991). Intervening sequences (IVSs) in the 23S ribosomal RNA genes of pathogenic *Yersinia enterocolitica* strains. The IVSs in *Y. enterocolitica* and *Salmonella typhimurium* have a common origin. *Mol Microbiol* **5**, 585–593.
- Smith, N. H., Crichton, P. B., Old, D. C. & Higgins, C. F. (1988). Ribosomal-RNA patterns of *Escherichia coli*, *Salmonella typhimurium* and related Enterobacteriaceae. *J Med Microbiol* **25**, 223–228.
- Springer, N., Ludwig, W., Amann, R., Schmidt, H. J., Goertz, H.-D. & Schleifer, K.-H. (1993). Occurrence of fragmented 16S rRNA in an obligate bacterial symbiont of *Paramecium caudatum*. *Proc Natl Acad Sci USA* **90**, 9892–9895.
- Trust, T. J., Logan, S. M., Gustafson, C. E., Romaniuk, P. J., Kim, N. W., Chan, V. L., Ragan, M. A., Guerry, P. & Gutell, R. R. (1994). Phylogenetic and molecular characterization of a 23S rRNA gene positions the genus *Campylobacter* in the epsilon subdivision of the Proteobacteria and shows that the presence of transcribed spacers is common in *Campylobacter* spp. *J Bacteriol* **176**, 4597–4609.
- Ward, N., Rainey, F. A., Goebel, B. & Stackebrandt, E. (1995). Identifying and culturing the 'unculturable': a challenge for microbiologists. In *Microbial Diversity and Ecosystem Function*, pp. 89–110. Edited by D. Allsopp, D. L. Hawksworth & R. R. Colwell. Wallingford: CAB International.
- Willems, A. & Collins, M. D. (1993). Phylogenetic analysis of

rhizobia and agrobacteria based on 16S ribosomal RNA gene sequences. *Int J Syst Bacteriol* **43**, 305–313.

Winkler, M. E. (1979). Ribosomal ribonucleic acid isolated from *Salmonella typhimurium*: absence of the intact 23S species. *J Bacteriol* **139**, 842–849.

Young, M. & Cole, S. T. (1993). *Clostridium*. In *Bacillus subtilis and*

Other Gram-Positive Organisms, pp. 35–52. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.

Received 23 October 1995; revised 18 March 1996; accepted 28 March 1996.