

16S–23S rDNA spacer of *Pectinatus*, *Selenomonas* and *Zymophilus* reveal new phylogenetic relationships between these genera

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The 16S–23S rDNA spacer regions of two *Pectinatus* species, two *Zymophilus* species and one *Selenomonas* species were cloned after PCR amplification. The results of PCR amplification showed that these species had two types of spacer regions which differ in molecular size (long and short). Only the long spacer regions in these bacteria contained one or two tRNA genes (alanine and/or isoleucine). The spacer regions in these bacteria had a relatively high level of homology. Homology was particularly high for bacteria belonging to the same genus. Interestingly, the order of the two tRNA genes present in the long spacer regions of *Pectinatus* and *Selenomonas* was the reverse of that which had been previously reported for other bacteria. The results of spacer homology analysis and the order of the tRNA genes suggest that the taxonomic classification of anaerobic bacteria isolated from the brewing process should be re-examined.

Keywords: *Pectinatus*, *Selenomonas*, *Zymophilus*, 16S–23S rDNA spacer, phylogeny

INTRODUCTION

Certain new bacteria, *Pectinatus cerevisiiphilus*, *Pectinatus frisingensis*, *Selenomonas lactificifex*, *Zymophilus paucivorans* and *Zymophilus raffinovorans*, have been isolated from breweries (Haikara, 1989, 1991; Lee *et al.*, 1978; Schleifer *et al.*, 1990). As these bacteria have a similar morphology, they were initially classified as *Pectinatus* (Haikara, 1989). However, the results of taxonomic studies, such as G + C content, DNA–DNA hybridization, utilization of various carbohydrates and 16S rRNA sequences, have indicated that these bacteria should be divided into three genera: *Pectinatus*, *Selenomonas* and *Zymophilus* (Schleifer *et al.*, 1990). It has previously been reported that the ribotypes of *P. cerevisiiphilus*, *P. frisingensis*, *S. lactificifex*, *Z. raffinovorans* and *Z. paucivorans* were completely different (Motoyama *et al.*, 1998). Moreover, *Pectinatus* strains differ from other bacteria in

terms of their beer spoilage ability. When *P. frisingensis* or *P. cerevisiiphilus* infect beer, serious contamination will occur (Haikara, 1989, 1991; Lee *et al.*, 1978). However, *Selenomonas* and *Zymophilus* grow more poorly in beer than *Pectinatus* (Haikara, 1989).

Comparative analysis of 16S rRNA sequences is a suitable technique for studies on taxonomy of bacteria, but these sequences are highly conserved among eubacteria, so more information is needed, especially for the taxonomic study of closely related bacteria (Woese, 1987). Recently, the 16S–23S rDNA spacer sequence has become of interest due to its application in the differentiation of several closely related bacterial species. This is because sequences of this region are less conserved than those of the 16S rRNA gene (Graham *et al.*, 1997; Gürtler & Stanisich, 1996). In this study, the spacer regions of these bacteria were cloned and DNA sequences were determined for phylogenetic analyses.

METHODS

Bacterial strains, culture conditions and DNA extraction. Bacterial strains used were *P. cerevisiiphilus* (DSM 20467^T), *P. frisingensis* (DSM 6306^T), *S. lactificifex* (DSM 20757^T), *Z. paucivorans* (DSM 20756^T) and *Z. raffinovorans* (DSM 20765^T). Each strain was cultivated at 30 °C in thioglycollate

The DDBJ accession numbers for the long 16S–23S rDNA spacer sequences of *Pectinatus cerevisiiphilus* DSM 20467^T, *Pectinatus frisingensis* DSM 6306^T, *Selenomonas lactificifex* DSM 20757^T, *Zymophilus paucivorans* DSM 20756^T and *Zymophilus raffinovorans* DSM 20765^T are AB022061, AB022063, AB022094, AB022066 and AB022068, respectively. Corresponding DDBJ accession numbers for the short 16S–23S rDNA spacer sequences are AB022062, AB022064, AB022065, AB022067 and AB022093, respectively.

medium (Nissui) under anaerobic conditions. DNA was extracted using the method of Olsen *et al.* (1991).

PCR amplification, cloning and DNA sequence analysis. The 16S rRNA sequences of *P. frisingensis*, *P. cerevisiiphilus*, *S. lactificifex* and *Z. paucivorans* have been reported (Schleifer *et al.*, 1990), but only the 23S rRNA sequence of *P. frisingensis* has been reported (Ludwig *et al.*, 1992). The sequence lying between nt 557–576 of the 16S rRNA of *P. cerevisiiphilus*, *P. frisingensis*, *S. lactificifex* and *Z. paucivorans* (Schleifer *et al.*, 1990) was used for the 16S primer, whereas the sequence between nt 21–38 of 23S rRNA of *P. frisingensis* (Ludwig *et al.*, 1992) was used for the 23S primer. The 16S primer was highly conserved among these bacteria (Schleifer *et al.*, 1990) and the 23S primer was highly conserved among eubacteria (Gürtler & Stanisich, 1996). The DNA sequence of the 16S primer was 5'-GGCAAGCGTTGTCCGGAATT-3' and that of the 23S primer was 5'-CGCCAAGGCATCCACCAT-3'. PCR was carried out in a 2400 Gene Amplification PCR thermal cycler (Perkin Elmer) with *rTaq* DNA polymerase (TOYOBO) or Ready-To-Go PCR Beads (Pharmacia Biotech). The amplification program was 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s in a total volume of 50 µl (TOYOBO) or 25 µl (Pharmacia Biotech) for 30 cycles. The PCR products were purified with the High Pure PCR Product Purification kit (Boehringer Mannheim) and cloned into a pCR2.1 DNA plasmid vector using the TA cloning system 2.2 (Invitrogen) according to the manufacturer's instructions. Ligation mixtures were used to transform INVαF' *Escherichia coli* One Shot competent cells (Invitrogen). Plasmids containing insert DNA were extracted from the bacterial cells using a Plasmid Mini kit (Qiagen). Each DNA insert was sequenced using a SequiTherm Long-Read Cycle Sequencing kit for LI-COR (Epicenter Technology) with an IRD-41 Infrared Dye-labelled M13 primer (Aloka). The nucleotide sequence of insert DNA was determined using LI-COR according to the manufacturer's instructions. For sequence data analysis, creation of the dendrogram and scanning of tRNA genes, DNASIS was used (Hitachi Software Engineering).

RESULTS

Cloning of the 16S–23S rDNA spacer of *Pectinatus*, *Selenomonas* and *Zymophilus*

Using the PCR conditions described in Methods, two PCR-amplified products were obtained from these bacteria which differed in molecular size (long and short) (Fig. 1). The long fragments were about 1.6 kb, whereas the short fragments were about 1.4 kb. Each PCR-amplified product was thought to contain about 1000 bp fragments derived from 16S rRNA genes. However, a few fragments, smaller than the long and short PCR products, were amplified when using *P. frisingensis* and *Z. paucivorans* DNAs as templates (Fig. 1, lanes 2 and 4). Two major PCR products (the long, about 1.6 kb, and the short, 1.4 kb) were cloned into the plasmid pCR2.1.

The long 16S–23S rDNA spacer region of *Pectinatus*, *Selenomonas* and *Zymophilus*

The long PCR fragments of *P. cerevisiiphilus*, *P. frisingensis*, *S. lactificifex*, *Z. paucivorans* and *Z. raffinosivorans* contained 724, 624, 579, 553 and 591 bp of the

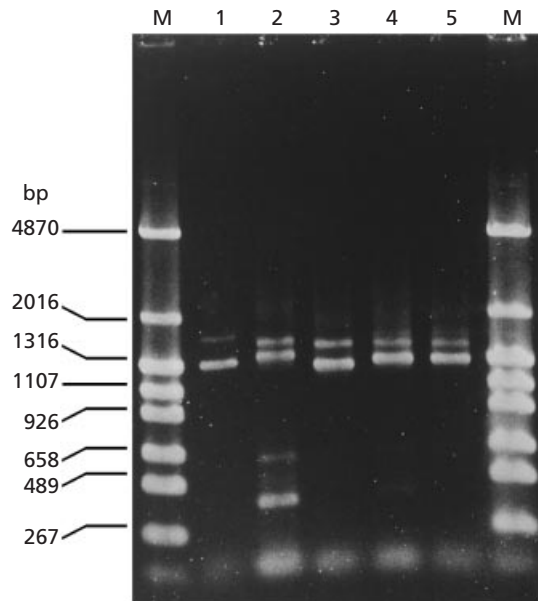


Fig. 1. Agarose gel electrophoresis of PCR-amplified 16S–23S rDNA spacer regions from *Pectinatus*, *Selenomonas* and *Zymophilus*. Lanes: M, molecular size marker; 1, *P. cerevisiiphilus* DSM 20467^T; 2, *P. frisingensis* DSM 6306^T; 3, *S. lactificifex* DSM 20757^T; 4, *Z. paucivorans* DSM 20756^T; 5, *Z. raffinosivorans* DSM 20765^T.

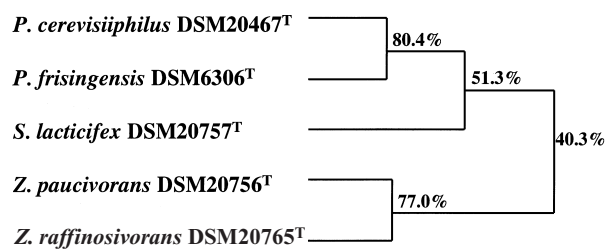
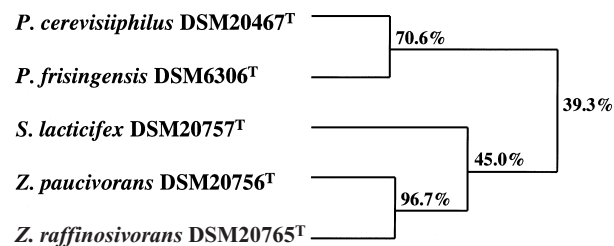
16S–23S rDNA spacer region, respectively (Table 1). The long spacer regions of these bacteria contained one or two tRNA genes. *Pectinatus* spp., *S. lactificifex* and *Z. raffinosivorans* contained two tRNA genes (alanine tRNA gene and isoleucine tRNA gene), whereas *Z. paucivorans* contained only one tRNA gene (alanine tRNA gene). Each of the tRNA gene sequences had very high homologies among these bacteria: homologies of the alanine and isoleucine tRNA gene sequences among these bacteria were 94.7–97.4% and 97.4–100%, respectively. However, sequences of the alanine tRNA genes were not identical with those of the isoleucine tRNA genes.

The 16S–23S rDNA spacer regions of many bacteria contain one or two tRNA genes and these are the alanine and/or isoleucine tRNA gene or the glutamine tRNA gene (Gürtler & Stanisich, 1996). For those containing two tRNA genes (alanine tRNA gene and isoleucine tRNA gene) in the spacer region, the isoleucine tRNA gene was always the first and the alanine tRNA gene was the second gene in 16S to 23S rRNA gene orientation (Gürtler & Stanisich, 1996). In *Pectinatus* and *Selenomonas*, however, the order of these two tRNA genes was the reverse of that previously reported (Table 1; Gürtler & Stanisich, 1996). This is the first report showing the reverse order of the two tRNA genes in the spacer regions.

A dendrogram comparing nucleotide sequences for the long spacer regions is shown in Fig. 2. The long spacer regions of *P. cerevisiiphilus* and *P. frisingensis* were

Table 1. 16S–23S rDNA spacer sequences of bacteria isolated from breweries

Species	Designation	Length of spacer (bp)	tRNA (position)
<i>P. cerevisiophilus</i>	Long	724	Ala (245–320), Ile (330–406)
	Short	399	
<i>P. frisingensis</i>	Long	624	Ala (143–218), Ile (229–305)
	Short	442	
<i>S. lactificifex</i>	Long	579	Ala (245–321), Ile (325–402)
	Short	321	
<i>Z. paucivorans</i>	Long	553	Ala (263–338)
	Short	375	
<i>Z. raffinosivorans</i>	Long	591	Ile (183–259), Ala (301–376)
	Short	376	

**Fig. 2.** Dendrogram showing the genetic relationships among five species of anaerobic bacteria based on the DNA sequences of their long 16S–23S rDNA spacer regions. The phylogenetic tree was created as described in Methods.**Fig. 3.** Dendrogram showing the genetic relationships among five species of anaerobic bacteria based on the DNA sequence of their short 16S–23S rDNA spacer regions. The phylogenetic tree was created as described in Methods.

closely related, with a matching percentage of 80.4%. The long spacer regions of *Z. paucivorans* and *Z. raffinosivorans* were closely related, with a matching percentage of 77.0%. The long spacer region of *S. lactificifex* had a matching percentage of 51.3% with the long spacer regions of *Pectinatus* spp., whereas the long spacer region of *S. lactificifex* had a matching percentage of 40.3% with the long spacer regions of *Zymophilus* spp. The DNA sequence homologies of the long spacer regions indicate that *Pectinatus* spp. are more closely related to *S. lactificifex* than to *Zymophilus* spp. (Fig. 2).

The short 16S–23S rDNA spacer regions of *Pectinatus*, *Selenomonas* and *Zymophilus*

The short PCR fragments of *P. cerevisiophilus*, *P. frisingensis*, *S. lactificifex*, *Z. paucivorans* and *Z. raffinosivorans* contained 399, 442, 321, 375 and 376 bp of the 16S–23S rDNA spacer region, respectively (Table 1). No tRNA genes were found. The short spacer sequences were almost identical within species to the long spacer sequences except that they contained tRNA gene(s).

A dendrogram comparing the nucleotide sequences for

the short spacer regions is shown in Fig. 3. The short spacer regions of *P. cerevisiophilus* and *P. frisingensis* were closely related, with a matching percentage of 70.6%. The short spacer regions of *Z. paucivorans* and *Z. raffinosivorans* were also closely related, with a matching percentage of 96.7%. The short spacer region of *S. lactificifex* had a matching percentage of 45.0% with the short spacer regions of *Zymophilus* spp., whereas it had a matching percentage of 39.3% with the short spacer regions of *Pectinatus* spp. From these homologies, the phylogenetic relationship between *Pectinatus* and *Selenomonas* was almost equal to that between *Pectinatus* and *Zymophilus*. The dendrogram from the short spacer was not identical to that from the long spacer, especially for the phylogenetic relationship of *S. lactificifex*.

DISCUSSION

PCR amplification showed that *Pectinatus*, *Selenomonas* and *Zymophilus* had two types of 16S–23S rDNA spacer (Fig. 1). This result could be expected from previous studies (Graham *et al.*, 1997; Gürtler & Stanisich, 1996). For example, *E. coli* has seven *rrn* operons (rRNA operons). However, the length of the 16S–23S rDNA spacer regions could be divided into

two types. In *P. frisingensis* and *Z. paucivorans*, extra PCR products were amplified (Fig. 1, lanes 2 and 4). These had molecular sizes of about 400–600 bp. PCR primers for amplifying the 16S–23S rDNA spacer were designed to contain about 1000 bp of 16S rRNA gene in their PCR products. Therefore, it is concluded that these 400–600 bp PCR products were laboratory artefacts.

Sequence analysis of the long spacer region showed that these bacteria contained one or two tRNA genes (Table 1). Interestingly, the order of the tRNA genes present in the long spacers of *Pectinatus* spp. and *S. lactificifex* was the reverse of that which has been reported previously for other bacteria (Gürtler & Stanisich, 1996). This observed alanine tRNA/isoleucine tRNA gene order has not been previously reported (Gürtler & Stanisich, 1996). It would be interesting to investigate whether other anaerobic bacteria apart from *Pectinatus* spp. and *S. lactificifex* could also have tRNA genes in this order. *Z. paucivorans* contained only the alanine tRNA gene in the long spacer region and no isoleucine tRNA gene was found, but *Z. raffinivorans* contained both tRNA genes. In other bacteria, only the alanine tRNA gene was found in the spacer regions [e.g. *Enterococcus hirae* (Sechi & Daneo-Moore, 1993)]. Therefore, it was thought that the case of *Z. paucivorans* was not unusual. The short spacer regions of these bacteria were also sequenced and analysed (Table 1). No tRNA genes were present in the short spacer regions of these bacteria. Other bacteria also have spacer regions which contain no tRNA gene (Gürtler & Stanisich, 1996).

The dendrogram from the long spacer regions of these bacteria showed that *S. lactificifex* was more closely related to *Pectinatus* spp. than to *Zymophilus* spp. (Fig. 2). However, the dendrogram of the short spacer regions did not agree with that of the long spacer region. Analysis of the short spacer regions did correspond with that of 16S rRNA sequence (Schleifer *et al.*, 1990): *S. lactificifex* was more closely related to *Zymophilus* spp. than to *Pectinatus* spp. In this study, it was revealed that *Pectinatus* spp. and *S. lactificifex* had the reverse order of the two tRNA genes in the long 16S–23S rDNA spacer regions. Therefore, this study shows that the order of the alanine tRNA/isoleucine tRNA genes and the DNA sequences of the long 16S–23S rDNA spacer regions reflect the phylogenetic positions of these bacteria more than the DNA

sequences of the short 16S–23S rDNA spacer regions and 16S rRNA sequences.

ACKNOWLEDGEMENTS

We thank Drs K. Sakai and M. Ikeda for their continuing support.

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