

## Sequence Heterogeneities of Genes Encoding 16S rRNAs in *Paenibacillus polymyxa* Detected by Temperature Gradient Gel Electrophoresis

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Sequence heterogeneities in 16S rRNA genes from individual strains of *Paenibacillus polymyxa* were detected by sequence-dependent separation of PCR products by temperature gradient gel electrophoresis (TGGE). A fragment of the 16S rRNA genes, comprising variable regions V6 to V8, was used as a target sequence for amplifications. PCR products from *P. polymyxa* (type strain) emerged as a well-defined pattern of bands in the gradient gel. Six plasmids with different inserts, individually demonstrating the migration characteristics of single bands of the pattern, were obtained by cloning the PCR products. Their sequences were analyzed as a representative sample of the total heterogeneity. An amount of 10 variant nucleotide positions in the fragment of 347 bp was observed, with all substitutions conserving the relevant secondary structures of the V6 and V8 regions in the RNA molecules. Hybridizations with specifically designed probes demonstrated different chromosomal locations of the respective rRNA genes. Amplifications of reverse-transcribed rRNA from ribosome preparations, as well as whole-cell hybridizations, revealed a predominant representation of particular sequences in ribosomes of exponentially growing laboratory cultures. Different strains of *P. polymyxa* showed not only remarkably differing patterns of PCR products in TGGE analysis but also discriminative whole-cell labeling with the designed oligonucleotide probes, indicating the different representation of individual sequences in active ribosomes. Our results demonstrate the usefulness of TGGE for the structural analysis of heterogeneous rRNA genes together with their expression, stress problems of the generation of meaningful data for 16S rRNA sequences and probe designs, and might have consequences for evolutionary concepts.

The characterization of bacterial genes for small-subunit rRNA is widely used in evolutionary, taxonomic, and ecological studies. Sequence information has phylogenetic meaning, can be interpreted in the context of large databases, and can be obtained independently from cultivation techniques (20, 42). By PCR, rRNA sequences can be retrieved from small amounts of genomic DNA extracted from laboratory cultures or natural environments (16, 17, 31, 34).

The separation of PCR-amplified segments of 16S rRNA genes (rDNA) different in sequence by denaturing gradient gel electrophoresis for characterization of bacterial communities was described by Muyzer et al. (23). We have combined PCR and temperature gradient gel electrophoresis (TGGE) to study the complexity of microbial communities in soils and their variation in a similar approach (unpublished data). TGGE can be used to separate DNA molecules identical in length but different in sequence (28). A linear gel temperature gradient results in drastic mobility shifts of DNA molecules at some position during electrophoresis due to sequence-dependent melting of molecule domains. With a GC-rich sequence (GC clamp) attached to one end of the molecules of interest via

PCR, complete strand dissociation during electrophoresis is prevented and the other positions of the sequence, becoming parts of the melting domain(s), may contribute to the extent of mobility shifts. Within a short fragment, almost 100% of all single base pair substitutions could be detected by using this technique (33).

TGGE analysis of PCR-amplified segments of 16S rDNAs from pure cultures of several strains of bacteria resulted in a number of bands each, indicating their heterogeneity. The significance of this observation was studied in some detail because it might interfere not only with analyses of denaturing gel patterns and other methods in microbial ecology but also with concepts in bacterial taxonomy and evolution. For *Paenibacillus polymyxa* (basonym: *Bacillus polymyxa* [3a]) DSM 36<sup>T</sup> (T = type strain), it is shown that the reason for this phenomenon is the existence of several cistrons encoding 16S rRNAs with different sequences within a single genome.

The abundance and extent of such sequence heterogeneities have not been studied systematically. When more than a single 16S rRNA gene from one eubacterial genome has been analyzed, the sequences determined have been identical or differed from each other by less than 1% of their nucleotide positions (8, 12, 13, 26). Bigger insertions are an exception to these microheterogeneities; such “intervening sequences,” thought to be excised from the primary transcripts during rRNA processing, have been reported for rRNA genes from several species (6). However, a higher level of divergence has been described for small-subunit rRNA genes from archaeobacteria (24) and eukaryotes (18). A recent comparative analysis of sequences deposited in GenBank revealed a level of in-

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traspecific and intrastain sequence variation that cannot be explained exclusively by errors in laboratory procedures (9).

Our results demonstrate a surprisingly high degree of sequence divergence among rRNA cistrons within strains of *P. polymyxa* and the suitability of the combination of PCR and TGGE as a method for its detection. Potential consequences for sequencing strategies, the design of taxon-specific probes, functional studies, and the taxonomic and phylogenetic interpretation of rRNA sequence data are discussed.

#### MATERIALS AND METHODS

**Organisms and culture techniques.** Strains of *P. polymyxa* (DSM 36<sup>T</sup>, DSM 292, DSM 356, and DSM 365) and *B. subtilis* (DSM 402) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. *Escherichia coli* NM 522 was obtained from Promega, Heidelberg, Germany. Cells from single colonies, obtained from a second restreaking of a single colony suspension on solid LB medium, were used for inoculation of liquid cultures and were grown aerobically at 30°C (37°C for *E. coli*) in LB medium (32).

**DNA extraction.** Cell cultures (2 ml) in the late exponential growth phase were harvested by centrifugation and lysed by incubation for 15 min in 150 µl of glucose buffer (50 mM glucose, 25 mM Tris-Cl [pH 8.0], 10 mM EDTA) (20) containing lysozyme at 4 mg/ml and subsequent addition of sodium dodecyl sulfate (SDS) to a final concentration of 1% (wt/vol). Chromosomal DNA was extracted as described by Ausubel et al. (5).

**PCR.** PCR primers F-968-GC (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CCG GGG GAA CGC GAA GAA CCT TAC-3') and R-1346 (5'-TAG CGA TTC CGA CTT CA-3') or R-1401 (5'-CGG TGT GTA CAA GAC CC-3') were combined to amplify the segment of eubacterial 16S rDNA from nucleotide 968 to nucleotide 1346 or 1401, respectively (*E. coli* numbering [7]). The 40-nucleotide GC-rich sequence at the 5' end of primer F-968-GC improves the detection of sequence variations of amplified DNA fragments by subsequent TGGE (33). The GC clamp of Muzer et al. (23) was modified at positions 7 and 8 and positions 15 and 16 to avoid some complementarity to other primers in use. PCR amplification was performed as follows. Twenty picomoles of each primer, 5 nmol of each deoxyribonucleoside triphosphate, 50 nmol of MgCl<sub>2</sub>, 2.5 µl of dimethyl sulfoxide, 5 µl of 10× PCR buffer (100 mM Tris-HCl [pH 8.3], 100 mM KCl), and 0.5 U of *Taq* DNA polymerase (Hofstadter; Perkin Elmer Cetus, Norwalk, Conn.) were combined with H<sub>2</sub>O to a volume of 50 µl in a 0.5-ml test tube and overlaid with 50 µl of mineral oil. After addition of 10 ng of template DNA, the samples were incubated in a Hybaid OmniGene Temperature Cycler (Hybaid, Teddington, United Kingdom) programmed as follows: initial denaturation of double-stranded DNA for 5 min at 94°C; 35 cycles each consisting of 1 min at 94°C, 1 min at 63°C, and 1 min at 72°C; and extension of incomplete products for 5 min at 72°C. Amplification products were analyzed by electrophoresis in 1.5% (wt/vol) agarose gels and stored at -20°C until they were used.

**rRNA extraction.** A protocol employing ribosome harvesting by differential centrifugation steps and subsequent RNA isolation was used. Cells from a 40-ml culture in the late exponential growth phase were harvested by centrifugation, resuspended in 4 ml of TM buffer (10 mM Tris-Cl [pH 7.5], 10 mM MgCl<sub>2</sub>), and transferred to a cell homogenizer vial containing 4 g of glass beads (diameter, 170 to 180 µm; Braun, Melsungen, Germany). Cell lysis was performed by treatment in a cell homogenizer (Braun) for 90 s at 4,000 rpm. The suspension was centrifuged for 15 min at 15,000 × g (Sorvall RC-5B, SM 24 rotor). Following transfer of the supernatant, centrifugation was repeated for 30 min at 30,000 × g. Ribosomes were then pelleted by centrifugation for 2 h at 100,000 × g in a Centrikon K-2080 ultracentrifuge using a Konton TFT 80.13 rotor and subsequently resuspended in 500 µl of TN150 buffer (10 mM Tris-Cl [pH 8.0], 150 mM NaCl, 2 mM vanadyl ribonucleoside complexes [Fluka GmbH, Neu-Ulm, Germany]) by agitation with a small stirring bar at 0°C. Following extraction with an equal volume of water-saturated phenol-chloroform-isoamyl alcohol (25:24:1), rRNA was precipitated by addition of 50 µl of 3 M sodium acetate (pH 5.0) and 2 volumes of ethanol (-20°C) to the aqueous phase. Following sedimentation in a microcentrifuge for 10 min at 12,000 × g, the RNA pellet was rinsed with 1 ml of 70% ethanol, dried, and redissolved in 100 µl of TMC buffer (10 mM Tris-Cl [pH 7.5], 5 mM MgCl<sub>2</sub>, 0.1 mM CsCl) containing 5 U of RNase-free DNase (Promega) to remove traces of DNA. After incubation for 15 min at 37°C, the extraction and precipitation procedures were repeated as described above and the RNA isolated was redissolved in 100 µl of TE buffer (10 mM Tris-Cl [pH 8.0], 1 mM EDTA).

**RT-PCR.** Segments of 16S rRNA were reverse transcribed and subsequently amplified by applying the *rTth* DNA polymerase (Perkin Elmer Cetus) and the primers described for PCR. For reverse transcription (RT), 15 pmol of reverse primer R-1346, 10 nmol of each deoxyribonucleoside triphosphate, 1 µl of dimethyl sulfoxide, 2 µl of 10× RT buffer (100 mM Tris-Cl [pH 8.3], 900 mM KCl), 20 nmol of MnCl<sub>2</sub>, and 5 U of *rTth* DNA polymerase were combined with H<sub>2</sub>O to a volume of 20 µl in a 0.5-ml test tube and overlaid with 50 µl of mineral oil. Template RNA (200 ng) was added, and the samples were incubated at 70°C

TABLE 1. Sequences of oligonucleotide probes used in this study

Probe	Sequence <sup>a</sup>						Complementary sequence(s)
1114	CAA	CGA	GCG	CAA	CCC		16S rDNA
1/2	CAG	ATC	GCT	CCT	TCG	CT	1, 2; D16276 <sup>b</sup>
3/4	CAC	CTC	GCG	ATT	TCG	CT	3, 4
5	CAC	CTC	GCG	GCT	TCG	CT	5
Pp	CAC	CTC	GCT	CCT	TCG	CT	X60632 <sup>b</sup> X57308 <sup>b</sup>

<sup>a</sup> Variant positions in *P. polymyxa* sequences are boxed.

<sup>b</sup> Accession number of sequence in the EMBL database.

for 15 min. Afterwards, 80 µl of a solution containing 1× chelating buffer [10 mM Tris-Cl (pH 8.3), 100 mM KCl, 0.75 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.05% (wt/vol) Tween 20, 5% (vol/vol) glycerol], 3 mM MgCl<sub>2</sub>, each deoxyribonucleoside triphosphate at 50 µM, and 15 pmol of primer F-968-GC was added and the samples were incubated in a Hybaid OmniGene Temperature Cycler programmed as follows: initial denaturation for 1 min at 94°C; 35 cycles each consisting of 10 s at 94°C, 1 min at 60°C, and 1 min at 68°C; and extension of incomplete products for 7 min at 68°C. Amplification products were analyzed by electrophoresis in 1.5% (wt/vol) agarose gels and stored at -20°C until they were used. A PCR without an RT step was performed to verify the absence of DNA.

**TGGE.** TGGE was performed on the Diagen TGGE system (Diagen, Düsseldorf, Germany) by using horizontal polyacrylamide gels (6% [wt/vol] acrylamide, 0.1% [wt/vol] bisacrylamide, 8 M urea, 20% [vol/vol] formamide, 2% [vol/vol] glycerol) prepared in electrophoresis buffer (20 mM morpholinepropanesulfonic acid, 1 mM EDTA, pH 8.0). After 4 h of electrophoresis at 350 V with a temperature gradient of 35 to 50°C parallel to the electric field, DNA bands were visualized by silver staining (22).

**Cloning of PCR products.** The PCR product was purified by using the QIAquick PCR Purification Kit (Diagen) and ligated into a pGEM-T plasmid vector (Promega) in accordance with the manufacturers' instructions. Transformation of *E. coli* NM 522 and small-scale preparations of plasmid DNA were performed as described by Sambrook et al. (32).

**Sequence analysis of plasmid inserts.** Extracted plasmid DNA was purified by using the QIAGEN Plasmid Kit (Diagen) and used as a template in sequencing reactions by applying the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). Sequences of both strands of plasmid inserts were determined by using primers complementary to the T7 and SP6 promoters, flanking the multiple cloning site of the vector. Products of sequencing reactions were analyzed by using an Applied Biosystems 373A DNA Sequencer.

**Oligonucleotide probes.** The sequences of the probes used for hybridization experiments are shown in Table 1. Oligonucleotides were synthesized with a C6-TFA aminolinker [6-(trifluoroacetyl amino)-hexyl-(2-cyanoethyl)-(N,N-diisopropyl)phosphoramidite] at the 5' end (MWG Biotech, Ebersberg, Germany). For hybridizations to nucleic acids fixed on nylon membranes, they were labeled with digoxigenin (43). Labeling with the fluorescent dye tetramethylrhodamine isothiocyanate (Molecular Probes, Eugene, Oreg.) for whole-cell hybridizations was performed as described previously (2).

**Southern blot hybridization.** A 5-µg sample of genomic DNA from *P. polymyxa* DSM 36<sup>T</sup> was digested with 50 U of restriction endonucleases (Promega) for 2 h at 37°C. Separation of restriction fragments by agarose gel electrophoresis (0.8% [wt/vol] in 0.5× TBE) and capillary transfer to nylon membranes (Amersham Buchler, Braunschweig, Germany) were performed as described by Sambrook et al. (32). Nucleic acids were immobilized by baking for 2 h at 80°C. The membranes were prehybridized with 10 ml of hybridization solution (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 2% [wt/vol] blocking reagent [Boehringer, Mannheim, Germany], 0.1% [wt/vol] N-lauroylsarcosine, 0.02% [wt/vol] SDS) for 3 h at a hybridization temperature which depended on the sequence of the respective digoxigenin-labeled oligonucleotide subsequently used as a probe (50, 54, 56, and 58°C for probes 1114, 1/2 and 3/4, Pp, and 5). Hybridizations were performed at the hybridization temperature for at least 12 h with 1 ml of a hybridization solution containing 10 pmol of a digoxigenin-labeled probe. After washing the membranes twice for 5 min at the hybridization temperature in washing buffer (2× SSC, 0.1% [wt/vol] SDS) hybridized probes were detected by using antidigoxigenin antibodies coupled with alkaline phosphatase and the chemiluminescent substrate disodium 3-(4-methoxyphosphoryl)-1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1<sup>3,7</sup>]decan-4-yl]phenyl phosphate in accordance with the manufacturer's (Boehringer) instructions. After removal of the bound probe by incubation in 50 ml of 0.1× SSC plus 0.5% (wt/vol) SDS for 10 min at 80°C, filters could be hybridized with another probe.

**Whole-cell hybridization.** Cell fixation with a paraformaldehyde solution, hybridization of fixed cells with tetramethylrhodamine-isothiocyanate-labeled oligonucleotides, and epifluorescence microscopy have been described previously (1).

**Nucleotide sequence accession numbers.** The GenBank accession numbers of sequences 1 to 6 determined in this study are U60654 to U60659, respectively.

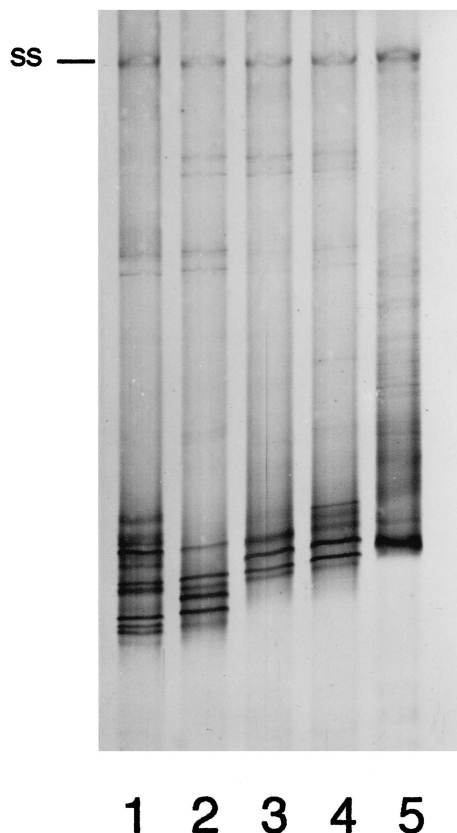


FIG. 1. TGGE separation patterns of PCR-amplified segments of 16S rRNA genes derived from *P. polymyxa* DSM 36<sup>T</sup> (lane 1), DSM 365 (lane 2), DSM 356 (lane 3), DSM 292 (lane 4), and *B. subtilis* DSM 402 (lane 5). Primers F-968-GC and R-1401 were used for amplification. ss, single-stranded DNA, characterized by its reddish color after silver staining.

## RESULTS

### Analysis of PCR-amplified segments of 16S rDNA by TGGE.

The segment of bacterial 16S rDNA containing rapidly evolving regions V6, V7, and V8 (25) was amplified and attached to a GC clamp via PCR. Figure 1 shows the patterns of bands obtained by TGGE of PCR products derived from various *P. polymyxa* strains and *B. subtilis* 168 (DSM 402). The *P. polymyxa* patterns are reproducible and characteristic for the respective strains under study. The clonality of each strain was ensured by conventional microbiological techniques as described in Materials and Methods. It was independently supported by microscopic analysis during the whole-cell hybridizations described below.

To separate DNA molecules with differences in electrophoretic migration behavior in TGGE gels, the PCR product(s) derived from *P. polymyxa* DSM 36<sup>T</sup> (type strain) were cloned into a pGEM-T plasmid vector. Six different plasmid inserts were identified by TGGE analysis of PCR products obtained by amplification with the same primers as before. They can be tentatively assigned to bands of the complex pattern (Fig. 2). PCR products derived from templates with single sequences as inserts of plasmids frequently emerge as two or even more bands in the TGGE (lanes 2, 3, 5, and 6). This observation indicates their composition of DNA molecules with slightly different migration behavior. In this case, it is very likely due to abortion of the elongation reaction during PCR caused by the GC clamp (hairpin formation). Sequencing re-

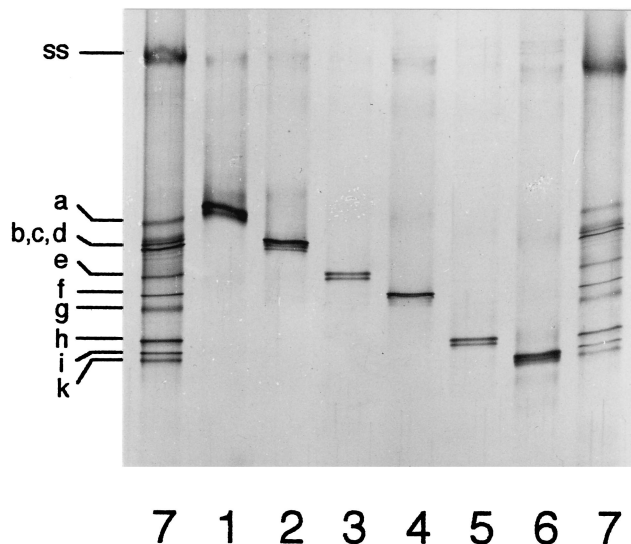


FIG. 2. TGGE separation patterns of PCR-amplified segments of 16S rRNA genes derived from *P. polymyxa* DSM 36<sup>T</sup>. PCR products derived from total genomic DNA emerged as a complex pattern (lane 7). Bands were singularized by cloning of PCR products. Subsequent PCR amplification of cloned inserts and analysis of products demonstrated their migration as shown in lanes 1 to 6 in the text. They can tentatively be assigned to the following (individual) bands of the pattern: lane 1, a; lane 2, b, c, and d; lane 3, e; lane 4, f; lane 5, h; lane 6, i and k. ss, single-stranded DNA. Primers F-968-GC and R-1346 were used for amplification.

vealed incomplete extension and the position of elongation termination within the GC clamp in some of the clones. The apparent missing of respective double or triple bands in the pattern of bacterial strains might be explained by the divergence of PCR conditions, e.g., with respect to target sequence abundance (lane 7 in Fig. 2). In a TGGE analysis of plasmid inserts (obtained by restriction endonuclease digestion), the fragments appeared as single bands (results not shown).

**Sequence analysis of cloned PCR products.** Ten positions of variable nucleotides were detected by comparing the sequences of the plasmid inserts. In pairwise comparisons, one to eight transitions and transversions are equivalent to sequence differences of 0.3 to 2.3% within the DNA segment analyzed, which is 347 bp long (Table 2). A theoretical calculation of the relative gel mobilities of these fragment sequences with the computer program POLAND (36) was done to check for the consistency of the results of sequencing with respect to migration behavior (data not shown).

The variable nucleotide positions detected correspond to nucleotides in rapidly evolving regions V6 and V8 of the respective 16S rRNA molecules. The potential secondary struc-

TABLE 2. Binary comparisons of sequences determined for a segment<sup>a</sup> of 16S rRNA genes from *P. polymyxa*

Sequence	No. of nucleotides different from sequence:				
	1	2	3	4	5
2	2				
3	8	8			
4	8	6	2		
5	7	5	4	2	
6	8	6	5	3	1

<sup>a</sup> Nucleotides 984 to 1330.

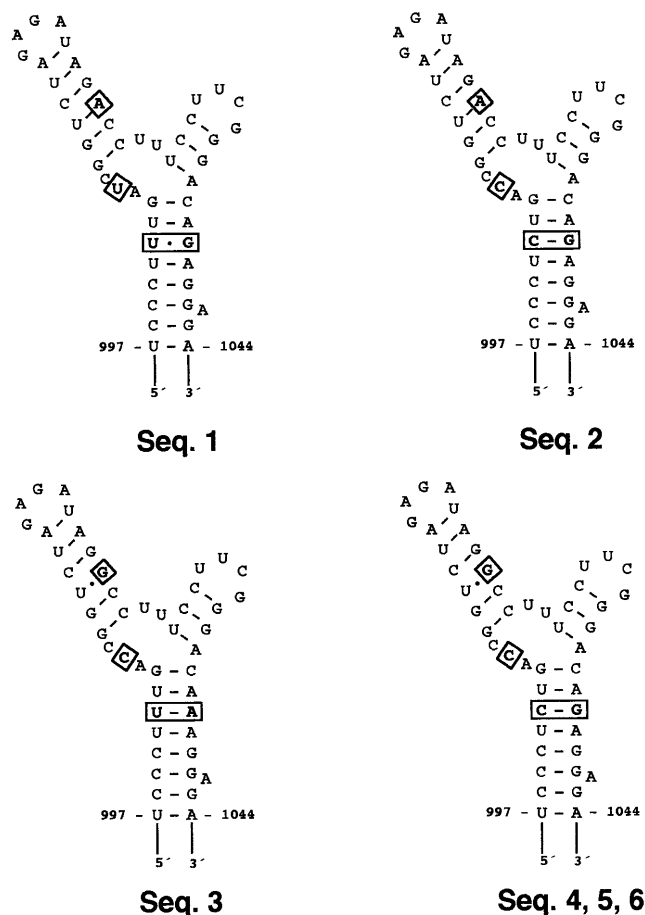


FIG. 3. Secondary structures of highly variable region V6 of 16S rRNA molecules from *P. polymyxa*. Seq., sequence.

tures of these regions in biologically active rRNAs (19), as inferred from alignment with database sequences, are shown in Fig. 3 and 4. One variable nucleotide in region V6 is thought not to be involved in helix formation; other base differences are either due to covariation of pairing ribonucleotides (transitions and transversions) or of the transition type  $U:G \leftrightarrow U:A/C:G$ . Thus, the exchanges do not significantly interfere with secondary structure formation.

**Southern blot hybridizations.** Hybridization experiments with discriminating oligonucleotide probes were designed to verify the presence of the different 16S rDNA sequences in the genome of *P. polymyxa* DSM 36<sup>T</sup>. The probe sequences are shown in Table 1. Probe 1114, a general bacterial probe, is complementary to the stretch of bacterial 16S rDNA between nucleotides 1099 and 1114 (*E. coli* numbering [7]). Probes 1/2, 3/4, and 5 are complementary to positions 1257 to 1273 in the amplified region of the respective RNA cistrons. Figure 4 demonstrates the position of the complementary sequence within the V8 region. Sequences 5 and 6 differ at only one nucleotide position ( $T \leftrightarrow C$ ); they are difficult to discriminate by specific hybridization. Therefore, no probe complementary to sequence 6 was used.

The EMBL database contains three sequences for 16S rRNA from *P. polymyxa*. Probe 1/2 is complementary to the sequence (accession no. D16276) determined by direct sequencing of PCR-amplified 16S rDNA (37). Probe Pp was designed to be complementary to the sequences (accession no. X60632 and X57308) published by Ash et al. (4) and Rössler et al. (29), which were determined by reverse transcriptase sequencing of rRNA. Their agreement in the stretch analyzed here suggested the use of this probe as an additional test for the heterogeneity of this region, even though they did not correspond to any of our sequence determinations and were not consistent with their secondary structure prediction.

Genomic DNA of *P. polymyxa* DSM 36<sup>T</sup> was digested with restriction enzymes *Nco*I and *Pst*I, for which no recognition site was present in the published 16S rRNA sequences for this species. Following the separation of restriction fragments and Southern transfer onto nylon membranes, rDNA sequences complementary to the digoxigenin-labeled oligonucleotide probes used were detected by specific hybridization (Fig. 5 and 6).

Probe 1114, a general eubacterial probe, detected at least 12 fragments, taking the various intensities of the hybridization signals and the bands detected by the other probes into account. No hybridization signal was obtained with probe Pp, even at low hybridization stringency. Probes 1/2, 3/4, and 5 showed hybridization signals at different positions on the nylon membrane. Thus, they hybridized with nonoverlapping specificity to different DNA fragments with complementary sequences. However, the possibility of hybridization to sequences of nonperfectly matching complementarity (one mismatch) cannot be excluded. All of the signal positions observed can be assigned to signals generated by probe 1114. Hybridization with probe 1/2 generated five hybridization signals, probe 3/4

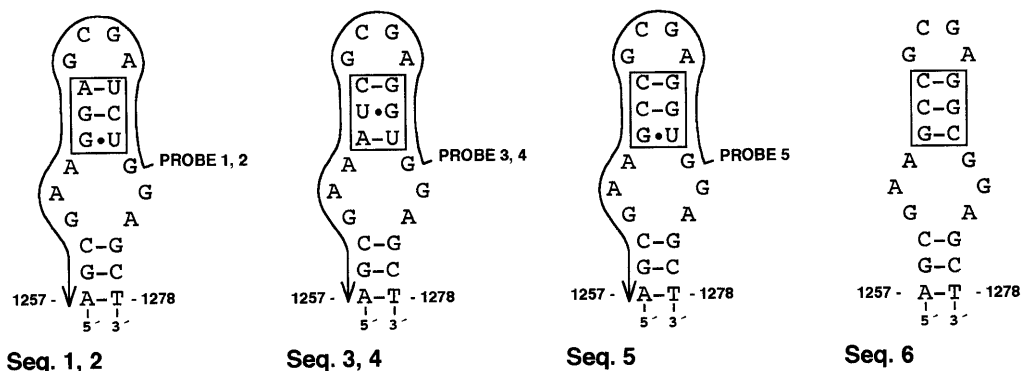


FIG. 4. Secondary structures of highly variable region V8 of 16S rRNA molecules from *P. polymyxa*. The positions of oligonucleotide probes are indicated. Seq., sequence.

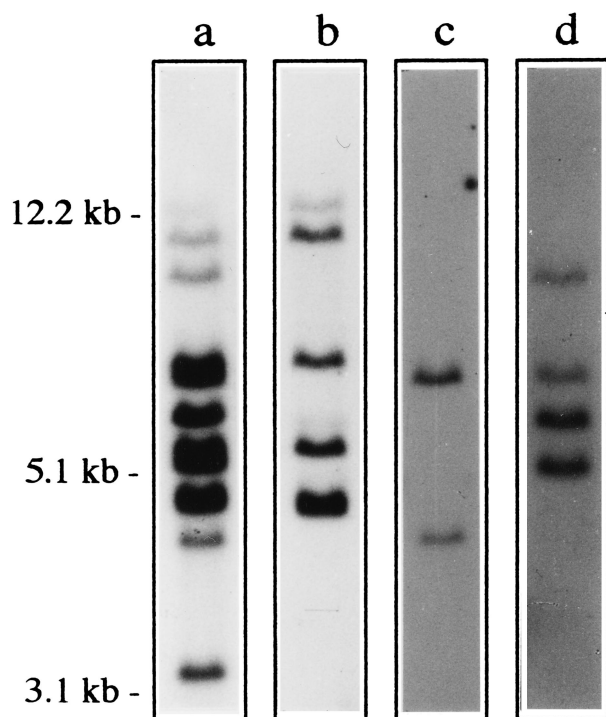


FIG. 5. Chromosomal DNA from *P. polymyxa* DSM 36<sup>T</sup> was digested with *Nco*I. Fragments were separated by agarose gel electrophoresis. Southern blotted, and hybridized to oligonucleotide probes 1114 (lane a), 1/2 (lane b), 3/4 (lane c), 5 (lane d), and Pp (no signals obtained).

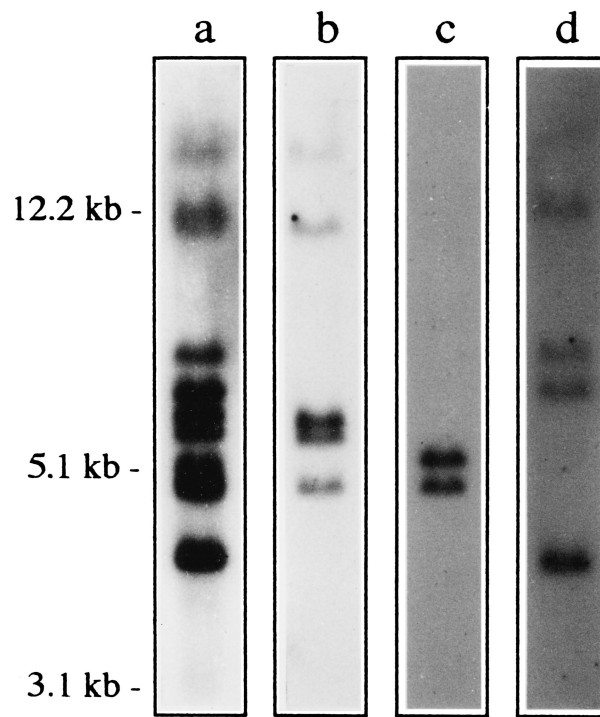


FIG. 6. Chromosomal DNA from *P. polymyxa* DSM 36<sup>T</sup> was digested with *Pst*I. Fragments were separated by agarose gel electrophoresis. Southern blotted, and hybridized to oligonucleotide probes 1114 (lane a), 1/2 (lane b), 3/4 (lane c), 5 (lane d), and Pp (no signals obtained).

generated two, and probe 5 generated four, totalling a minimum of 11 fragments hybridizing to these probes in each of the digests. The sequences determined and used for probe design do not represent the heterogeneity of the region completely. This is most significantly apparent in the *Nco*I digest, where the smallest fragment detected by general probe 1114 did not hybridize to any of the other probes (Fig. 5).

An unequivocal counting of fragment numbers in the different lanes is confronted with several factors contributing to signal strength distributions: (i) the efficiency of DNA transfer by blotting, (ii) the degree of matching of probes with target sequences, and (iii) the potential presence of minor bands resulting from incomplete restriction digestion. However, the interpretation of Fig. 5 and 6 can be summarized by the statement of clearly separable specificity of the probes to target sequences of different operons and the existence of at least 12 operons with 16S rRNA sequences in this strain. A more precise description of the organization of the rRNA operons, which was not intended here, would require much more detailed restriction analysis and hybridizations.

**RT-PCR of 16S rRNA.** rRNA was extracted from cultures of *P. polymyxa* DSM 36<sup>T</sup> and *E. coli* in the late exponential growth phase (optical density at 600 nm of 0.55 after incubation for 69 h). Following RT, segments of small-subunit rRNA from nucleotide 968 to nucleotide 1346 (*E. coli* numbering) were amplified and attached to a GC clamp via PCR. Compared with the respective PCR product obtained by amplification of rDNA, the RT-PCR product derived from the type strain of *P. polymyxa* emerged as a less complex pattern of bands after TGGE (Fig. 7). No band corresponding to sequence 5 (band h of the strain pattern in lane 1) was detected in the RT-PCR

products from these ribosomes. In contrast, respective amplification products derived from *E. coli* RNA and rDNA looked fairly similar, with a slight difference in density distribution.

**Whole-cell hybridizations with different strains of *P. polymyxa*.** Cells from *P. polymyxa* cultures were fixed with paraformaldehyde solution and hybridized with fluorescent-dye-labeled oligonucleotide probes with the sequences described in Table 1. Results obtained with cells from exponentially growing cultures (optical density at 600 nm of 0.06 to 0.15 after incubation for 20 h) are presented in Table 3. Ribosomes of the type strain simultaneously contained 16S rRNA with sequences 1/2 and 5. The apparent conflict with the result of the rRNA analysis presented in Fig. 7 was (partly) resolved by analyzing cells from older cultures of DSM 36 (optical densities at 600 nm of 0.45 and 0.55 after incubation for 48 and 69 h). These demonstrated a less intense hybridization signal with probe 5, whereas the signal intensity remained unchanged with probe 1/2 (data not shown in Table 3). No rRNA with the sequences designated 3 and 4 was detected in the type strain. However, all of the different sequences of region V8 represented in the complementary probes were proven to be present in biologically active ribosomes, but in different strains. Probe 3/4 yielded strong hybridization signals with strains 292 and 356 only. In ribosomes of strain 365, 16S rRNA complementary to probe 5 seemed to be predominant. Probe Pp, complementary to previously published sequences, gave no hybridization signal with any of the strains. As mentioned for Southern hybridization, binding of probes to RNA with single mismatches cannot be excluded with certainty. However, probes 3/4 and 5, differing at two nucleotide positions, discriminate their target sequences in strains DSM 36, DSM 292, and DSM 356.

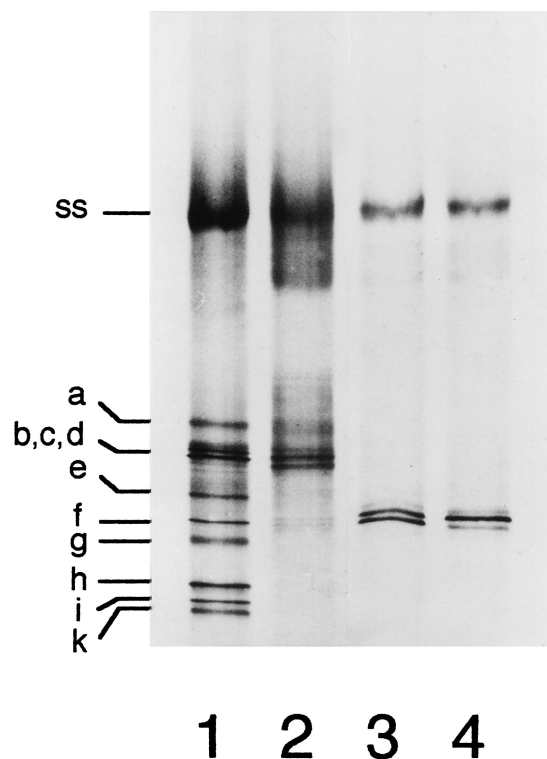


FIG. 7. TGGE separation patterns of PCR products obtained by amplification of segments of 16S rRNA genes (lanes 1 and 3) and reverse-transcribed rRNA (lanes 2 and 4) from cells in the late exponential growth phase (see text). Amplification products derived from *P. polymyxa* DSM 36<sup>T</sup> (lanes 1 and 2) and *E. coli* NM 522 (lanes 3 and 4) are compared. For designations of individual bands, see the legend to Fig. 2. Primers F-968-GC and R-1346 were used for amplification. ss, single-stranded DNA.

## DISCUSSION

**Sequence heterogeneity of 16S rRNA genes from *P. polymyxa*.** Sequences of cloned PCR-amplified segments of 16S rDNA (347 bp corresponding to *E. coli* positions 984 to 1330) from the type strain of *P. polymyxa* differ from each other by one to eight nucleotides at 10 sites in total. The analyzed sequences of region V8 were demonstrated to be present in the genome of the organism under study by Southern blot hybridization. Most likely, the sequences in total, including the V6 regions, represent segments of single 16S rRNA genes, although the possibility of errors introduced by PCR cannot be excluded.

So far, such a degree of heterogeneity in the small-subunit rRNA genes of one strain has not been described for bacteria. Although most bacteria have multiple *rrn* operons, only a few

studies have addressed questions of their identity in structure and function (10). Most investigations so far support the notion of a high degree of sequence identity of *rrn* genes within one organism. In the seven genes encoding 16S rRNA in the genome of *E. coli*, 16 variable sites have been detected (8). Nine substitutions and seven deletions-insertions are scattered over the rapidly evolving sections. The segment analyzed in this work contains only three of them (nucleotides 1071 and 1074, 1280, and 1321), at positions different from the 10 variant positions in *P. polymyxa*. The 16S rRNA genes in four adjacent operons from *B. subtilis* 168 (DSM 402) differ at less than 0.2% of their nucleotide positions (26). The three 16S rRNA sequences of *Rhodobacter sphaeroides* are identical (12), as are the six sequences in *Haemophilus influenzae* Rd, which are organized in two kinds of operons differing only with respect to the spacer between the 16S and 23S rRNA genes (13).

In contrast, two *rrn* operons with an overall sequence divergence of 5% and a pronounced uneven distribution of variant positions were described to exist in the archaeobacterium *Haloarcula marismortui* (24). In a systematic comparison of published 16S rRNA sequences of prokaryotic species, a rather high level of sequence variation was observed recently (9). In addition to our experimental data, these observations may support the notion of more interoperon variability in single species than previously acknowledged.

In addition, the distinct band patterns derived from different *P. polymyxa* strains indicate interstrain (intraspecific) variability in the abundance of heterogeneous sequences. (Note that identical band positions do not unequivocally demonstrate their sequence identity. In a segment of some 400 bp, such as that analyzed here, compensating effects of exchanges in different melting domains may account for similar mobility shifts of nonidentical sequences.) Some bands clearly demonstrate the existence of sequences not found in the type strain. Conversely, missing bands could be due to the absence of respective variants or to deletions of *rrn* operons. Such deletions caused by intrachromosomal recombination events have been reported for *B. subtilis* (41).

**Heterogeneity of rRNA.** TGGE analysis of reverse-transcribed and amplified rRNA and whole-cell hybridizations with oligonucleotide probes complementary to sequenced rRNA cistrons indicates that their transcripts are not evenly represented in ribosomes of *P. polymyxa* cells growing in the laboratory (Fig. 7 and Table 3). In addition, some results indicate a variation of the composition of ribosomes, depending on growth conditions (culture age). With cells from older cultures, a decreased signal intensity with probe 5 was observed, which corresponds to the absence of the respective band in the RT-PCR product analyzed by TGGE (Fig. 7).

The results suggest a distinct pattern of use of 16S *rrn* operon sequences for the assembly of ribosomes and their specific regulation. In *B. subtilis*, some observations indicate growth rate-dependent differential expression of *rrn* operons (30). In some cases, a different pattern of regulation of *rrn* operons may also be inferred from differences in upstream sequence regions (12, 15, 21, 38). Assays of *rrn* gene transcription studies rely on more or less indirect methods, and it is difficult to meet the requirements of a detailed analysis of individual operon regulation beyond the role of gene dosage effects also influenced by the location with respect to the origin of replication (11). It may be considered to be meaningful, however, to investigate whether differential regulation of individual operons, inducing the possibility of uneven selective forces acting on them, does in some cases play the biological role which could reasonably be assigned to it (39). The differentiation of heterogeneous transcripts by RT-PCR and TGGE

TABLE 3. Results of whole-cell hybridizations with various strains of *P. polymyxa*

Strain	Hybridization <sup>a</sup> with probe:			Pp
	1/2	3/4	5	
DSM 36 <sup>T</sup>	+	—	+	—
DSM 292	—	+	—	—
DSM 356	—	+	—	—
DSM 365	—	(+)	+	—

<sup>a</sup> +, strong hybridization signal; (+), weak hybridization signal; —, no hybridization signal.

or specific probes in *P. polymyxa* offers convenient methods to further investigate the regulation of individual operons.

**Evolutionary aspects.** The degree of identity of most of the bacterial operons analyzed so far is thought to be maintained by some "concerted evolution," the mechanism of which is unclear in detail. Selective forces on RNA function contribute to a fixation and spread of sequence variants by some "biased gene conversion" as demonstrated by a sequence conferring antibiotic tolerance (34a). To reconcile the particular degree of diversity analyzed in *P. polymyxa* with such a model, one has to assume that gene conversion and/or selection may act in different manners or frequencies in different species. Also, horizontal gene transfer has been implicated as a potential contribution to diversities in ribosomal operons of bacteria (24, 40). A differential regulation of transcription of rRNA operons with respect to life cycle or growth conditions would then also allow selection and functional adaptations to act on individual operons.

The highly divergent ribosomal sequences in the archaeobacterium *H. marismortui* apparently are both represented in active ribosome structures of laboratory cultures (24). In eukaryotes, however, differential use of ribosomal components has been reported. For example, in *Plasmodium* spp., correlation of the use of heterogeneous 16S rRNAs in ribosomes with the life cycle in its different hosts has been described (18). The distinct abundance of 5S rRNA species in oocytes and somatic cells of *Xenopus laevis* may be considered to be an analogous observation (27). It is conceivable that the coding for tRNAs in many rRNA operons may contribute to some modulation of translation activities, but it is a matter of speculation whether this may be true for the particular structure of rRNAs. Thus, it remains unclear which structural differences of rRNAs might reflect a modulation of ribosome function. The nature of analyzed exchanges (Fig. 3 and 4) and the presence of the different sequence variants in active ribosomes of laboratory-grown cultures of different strains of *P. polymyxa* indicate that all versions of the sequences analyzed are derived from functional operons. Also, these observations do not support a functional significance of the sequence variations observed.

**Consequences for prokaryotic systematics.** The sequence analysis of small-subunit rRNA is of great importance for modern bacterial taxonomy. The phylogenetic classification of prokaryotes is based on the assumption that sequences of macromolecules such as 16S rRNA reflect the evolution of the organisms they have been extracted from. The interpretation of these sequence data might be complicated by the presence of several equivalent molecules with some degree of individually different evolution within a single organism.

If single clones of (amplified) rDNA are sequenced, sequence heterogeneities of gene copies remain undetected. This is also valid for the direct sequencing of rRNA if products of genes different in sequence are not evenly represented in ribosomes. The direct sequencing of PCR-amplified DNA may result in ambiguities due to interoperon variabilities whose extent is unknown. TGGE offers a convenient tool for detection of these heterogeneities and could thus be used routinely to support sequence determinations for phylogenetic analyses.

The determination of bacterial genera, which often is the first step of identification procedures, probably is not confused by the sequence heterogeneities observed here. 16S rRNA sequences are of limited value for measurement of closer phylogenetic relationships of bacteria. Available data suggest that there is no correlation between the results of DNA-DNA reassociation experiments and comparisons of small-subunit rRNA sequences at a sequence similarity exceeding 97% (35). Almost identical 16S rRNA sequences have been reported for

phenotypically divergent bacteria (14). Our results lead to the converse argument that a difference of 2.3% observed in partial sequences is not sufficient to prove the presence of different clones. This stresses problems especially for biodiversity estimates if rRNA sequences are retrieved from uncharacterized mixtures of microorganisms. The genetic diversity of rRNA sequences in a microbial population of an environmental sample may exceed its organismic diversity. Problems also arise for the design of rRNA-specific oligonucleotide probes for detection and identification of prokaryotes. New probes often fail in practical applications (3). In addition to higher-order structures of rRNA molecules and associated proteins as potential reasons, the observed sequence heterogeneity might contribute to this phenomenon with a frequency which cannot be estimated with any confidence. However, it is evident from our observations that sequences determined for rRNA genes do not necessarily match the primary structure of biologically active rRNA.

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