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Potential Risks of Gene Amplification by PCR as Determined by 16S rDNA Analysis of a Mixed-Culture of Strict Barophilic Bacteria

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Abstract. The 16S rDNA genes of an apparently pure culture of a psychrophilic and strict barophilic bacterium (WHB 46) were studied by PCR-mediated amplification and cloning into phage M13 mp18. Sequence analysis of five individual clones revealed the presence of two different 16S rDNA types. The homology value of 90% indicates that culture WHB 46 is actually composed of two closely related species (WHB 46-1 and 46-2). Both strains are members of the γ-subdivision of proteobacteria. Analysis of a sixth clone (WHB 46-½) leads to the conclusion that it represents a 16S rDNA hybrid molecule assembled during the PCR reaction. This hypothesis was confirmed by secondary structure analysis of the chimeric rDNA. The appearance of such hybrid molecules point to a potential risk in studies on the diversity of bacterial populations by analysis of rDNA pattern via PCR-mediated amplification because they suggest the existence of organisms that do not actually exist in the sample investigated.

Introduction

Within a few years, application of the PCR technique to amplify small amounts of DNA has become a routine method. The technique was even further improved when combined with sequence analysis of amplified fragments, either after cloning [8] or directly by analyzing single- [7] or double-stranded DNA [1]. For molecular taxonomists, PCR is in particular suited for the analysis of evolutionarily conserved genes, e.g., rRNAs, where PCR primers are available for a broad range of even distantly related organisms [10]. The method is especially powerful for those organisms that cannot be grown as pure cultures and for the detection of the molecular diversity of microbial populations. Advantages of the molecular approach over classical enrichment and selection procedures have recently been demonstrated in the analysis of natural samples [4, 21].

Here we describe the PCR-mediated amplification of 16S rRNA genes from

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a psychrophilic and strict barophilic culture collected from a depth of 4,995 meters. Sequence analysis of the cloned rDNA fragments shows that in the presence of genomic DNA from different origins, amplification by PCR supports the formation of hybrid molecules.

Materials and Methods

Isolation and Cultivation

The strict barophilic bacteria (WHB 46) were isolated from a near-bottom seawater sample from a depth of 4,995 m collected during the ANT V/2 cruise of RV "Polarstern" at a station located in the eastern part of the Weddell Sea (63°17.54'S, 03°03.99'E). The water sample was retrieved by means of a rosette water sampler with conventional Niskin bottles. The sample underwent decompression during lifting. Upon retrieval aboard, undiluted, diluted, and concentrated seawater subsamples were added to nutrient-poor medium (Bacto yeast extract, 0.02% (w/v); KNO_3, 0.1% (w/v); and $FePO_4 \cdot 4H_2O$, 0.001% (w/v) in 75% (v/v) natural seawater) resulting in cultures containing 0.013% added organic compounds. During cultivation, aerobic conditions were obtained by using gas permeable polypropylene bags instead of culture tubes. The bags were heat-sealed, placed into stainless steel pressure vessels, and pressurized to 500 bars using FC 77 (3M) as hydraulic fluid [6]. The enrichment cultures were decompressed after an incubation period of 8 weeks at 2°C, then serially diluted, and a duplicate set was subcultivated in ZoBell medium 2216 (Difco) with natural seawater at atmospheric and deep sea pressure. Growth developed under pressurized conditions only [23].

Culture WHB 46 was isolated by means of an agarose medium (Bacto peptone, 0.25% (w/v); Bacto yeast extract, 0.05% (w/v); KNO_3, 0.025% (w/v); low gelling point agarose (Serva), 1.5% (w/v); and $FePO_4 \cdot 4H_2O$, 0.001% (w/v) in 75% (v/v) natural seawater). Appropriate dilutions from the subcultures were mixed with liquid agarose at 15°C. After 3 weeks at 3°C and 500 bars, visible colonies were picked, transferred into sterile seawater, and an appropriate dilution of the suspension again introduced into fresh agarose medium. Three colony-transfers were accomplished before the culture was considered to be pure. Standard taxonomic tests concerning degradation of organic substrates, utilization of organic compounds as sole carbon sources, production of acid from different carbohydrates, and sensitivity to antibiotics were performed on polypropylene bags or syringes under increased pressure at 400 bars. To obtain quantities of cells necessary for the isolation of genomic DNA multiple small volume cultures (100 ml) were grown at 3°C and 500 bars in ZoBell medium 2216 (Difco) until late logarithmic phase. The cells were harvested by centrifugation at 8,000 × *g* for 15 min at 4°C, washed twice with sterile artificial seawater, and stored at −80°C.

Preparation of Genomic DNA

Cells (50 mg wet weight) were suspended in 0.5 ml of saline EDTA (0.15 M NaCl, 0.01 M EDTA, pH 8.0) and 4% (w/v) sodium dodecylsulfate. Lysis of cells was obtained by incubation for 30 min at 60°C. The lysate was adjusted to 1 M NaCl, and the suspension subsequently extracted with 1 volume of phenol and 1 volume of phenol-chloroform (1:1, w/v). After two additional extractions with chloroform the upper phase was ethanol-precipitated. Because only fragmented genomic DNA could be extracted, nucleic acids were separated on an 0.7% agarose gel. A 4–6 kb fraction was recovered by means of NA-45 DEAE paper (Schleicher and Schuell, Dassel, FRG) according to Sambrook et al. [17]. This fraction was used for PCR-mediated amplification of 16S rDNA.

PCR Reaction

PCR was performed according to Saiki et al. [16], using 20 nmol of each dNTP and 100 pmol of each primer. The 5' and 3' primers, generated on an automated DNA synthesizer (Applied Bio-

systems, model 381A) were GCGGGATCCGAGTTTGATCCTGGCTCAG (*E. coli* positions 9 through 27) and CGCGGATCCAGAAAGGAGGTGATCCAGCC (*E. coli* positions 1,525 through 1,542), respectively. The underlined region of the primers represents overhangs with a BamHI restriction site. These primers, which under nonstringent conditions are applicable to most eubacterial 16S rDNA genes, allow the Taq polymerase to synthesize a stretch of 1,531 bp between positions 9 and 1,542 (IUB nomenclature of *E. coli*). Thirty cycles (annealing for 2 min at 43°C, elongation for 2.5 min at 72°C, denaturation for 1 min at 93°C) were carried out in a Biozym DNA Incubator II. The PCR products were extracted with chloroform to remove the light mineral oil and precipitated with ethanol [8].

Cloning and Sequence Analysis

After digestion with Bam HI, 16S rDNA fragments were cloned into phage M13 mp18. Identification of recombinants was done according to the M13 Cloning/Dideoxy Sequencing Manual (1980, Bethesda Research Laboratories, Inc., Gaithersburg, MD). DNA sequencing was performed with Sequenase according to the manual of the manufacturer (USB) using both universal M13 and internal 16S rDNA (RNA) oligonucleotide primer.

Data Analysis

16s rDNA sequences were aligned to those of several reference proteobacteria, the sequences of which have been published. Phylogenetic analyses were carried out using the neighborliness method [3, 18]. The algorithm was implemented as part of the program package "Sage" (Technoma GmbH, Heidelberg, FRG) designed for the IBM XT/AT and compatibles.

Results and Discussion

Sequence Analysis and the Phylogenetic Position of Culture WHB 46

The Gram-negative bacteria of culture WHB 46 showed shortest generation times at 5°C and 400 bars. Even after cultivation of cells under these conditions it was only possible to isolate genomic DNA of a molecular weight ranging from approximately 1 to 6 kb. The genomic DNA was therefore separated on an 0.7% agarose gel and a fraction isolated that ranged between 4 and 6 kb.

Unexpectedly, sequence analysis of 5 individual clones revealed the presence of basically two different types of 16S rDNA sequences (WHB 46-1 and WHB 46-2). The three clones corresponding to WHB 46-1 showed not more than 12 differences in total, with a maximum of 10 differences between either two sequences (0.7% of total). Four of these differences, occurring in one of the three clones (WHB 46-1*), can be considered microheterogeneity of rrn genes because they are involved in coordinated base exchanges (*E. coli* positions 837/838 and 848/849). Two other differences can be interpreted as PCR-mediated errors because they distort the secondary structure (*E. coli* positions 391 and 1488). The other six differences occurred in loop regions or they were involved in substitutions without disrupting the secondary structure (*E. coli* positions 285[C-G → T-G], 338[A → G], 607[A → G], 727[A → G], 926[G-T → A-T], and 1243[T-G → C-G]). The two clones corresponding to WHB 46-2 had a sequence similarity of 99.9%. The only differences were found at *E. coli* position

Table 1. Equally weighted evolutionary distances[a] between 16S rRNA pairs of members of the gamma subclass of Proteobacteria. Several representatives of the alpha and beta subclasses were used as outgroup reference organisms (data not shown)

Organisms	Evolutionary distances								
	1	2	3	4	5	6	7	8	9
1. Barophilic organism WHB 46-1	—	30	105	107	118	111	118	131	140
2. Chimeric structure WHB 46-½		—	81	109	126	128	130	132	153
3. Barophilic organism WHB 46-2			—	108	114	109	113	144	151
4. *Listonella anguillarum*				—	104	96	102	140	164
5. *Xenorhabdus nematophilus*					—	57	48	128	144
6. *Escherichia coli*						—	51	117	164
7. *Proteus vulgaris*							—	132	163
8. *Ruminobacter amylophilus*								—	151
9. *Pseudomonas aeruginosa*									—

[a] The values were calculated from an alignment with the total length of 1,130 positions (determined by the shortest sequence in this alignment [*X. nematophilus*])

1303[T-A → C-A]. A consensus sequence of the three clones of WHB 46-1, excluding those differences that occurred in only one of the three sequences, were derived for deposition in EMBL data library. The accession numbers for the sequences of WHB 46-1 and WHB 46-2 are X54744 and X54745, respectively.

The occurrence of 154 to 155 nucleotide differences (depending on the clone investigated) between clones WHB 46-1 and WHB 46-2 (homology of around 90%) is too high to indicate strain-specific microheterogeneities. It can rather be assumed that culture WHB 46 consists of two strains belonging to two moderately related species exhibiting similar morphological and physiological characteristics (see below). Cells of both strains may have intensive surface interactions (e.g., by their flagella, see "phenotypic characterization" below) that prevents their separation under the isolation conditions used. Both strains are members of the gamma subclass of Proteobacteria as derived from evolutionary distance values (Table 1) and the phylogenetic tree derived therefrom (Fig. 1).

PCR and Phylogenetic Chimera

In addition to the five clones, a sixth 16S rDNA clone was analyzed (WHB 46-½) which showed between 97 and 97.2% and 92.1% sequence similarity to the three clones of WHB 46-1 and the two clones of 46-2, respectively. Almost complete homology (99.5%) existed in the first 1,095 nucleotides of clones WHB 46-1* and 46-½ (five differences, three of which were PCR errors in the WHB 46-½ sequence), while no differences were found in the last 505 bases of clones WHB46-2 and 46-½; the overlapping region of 101 bases is totally conserved in all of the three sequences.

The unusual distribution of stretches of homology allows the conclusion that clone WHB 46-½ does not represent a naturally occurring 16S rDNA, but a

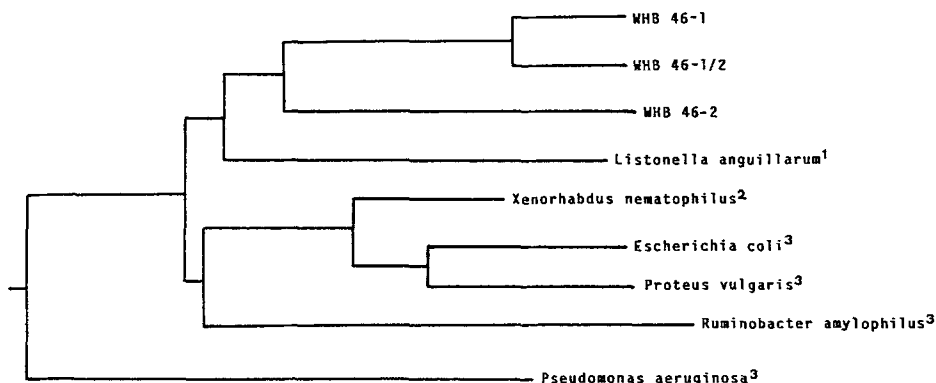

Fig. 1. Phylogenetic tree of members of the gamma subclass of proteobacteria, indicating the position of the two cultures of strict barophilic organisms WHB 46-1 and 46-2 and the intermediately branching, nonexisting chimeric "organism" WHB 46-½. Sequences were obtained from the following sources: [1]Valle et al. [20], [2]Puetz et al. [15], [3]Neefs et al.[13]

hybrid assembled under the conditions used in the PCR reaction. This hypothesis is supported by a feature of the secondary structure of the chimeric rDNA. While for clones WHB 46-1* and WHB 46-2 regions 984 to 990 formed a perfect helix with regions 1215 to 1221, the hybrid sequence showed two mismatches and a new U-G base pair (Fig. 2), which originated from the primary structure of the two "pure" sequences.

The two main factors that cause chimera to be formed from DNA from mixed cultures during amplification are (a) the presence of a large number of highly conserved stretches along the primary structure of rDNAs (the length of these regions is expanded with increasing degree of relationship between the molecules investigated) and (b) the availability of low molecular weight genomic DNA (here 4–6 kb, see above) used in this amplification assay. Fragmented, single-stranded rDNA will be synthesized and under the reassociation conditions used, which in most cases can be considered nonstringent, even molecules of different origin may form duplexes in overlapping and conserved regions leading to the amplification of hybrid molecules (Fig. 3). The chimeric nature of a composite molecule may even be increased by the presence of a higher proportion of low molecular DNA fragments, the occurrence of which is mainly caused by physical shearing during the isolation of DNA from environmental samples [14, 19]. The finding that in our example only one of six clones was a chimeric form allows the conclusion that a high proportion of DNA molecules isolated contained an intact 16S rDNA gene. In order to reduce the probability of chimeric structures to be formed it is recommended to isolate a small amount of the highest molecular weight DNA obtainable rather than to use a mixture of all available size fragments. The phenomenon described here should not be confused with PCR-mediated DNA recombination, which is probably caused by pausing and unspecific termination of Taq polymerase during the elongation reaction [12].

The formation and subsequent sequence analysis of hybrid molecules (and this is true for all conserved phylogenetic markers) points toward potential

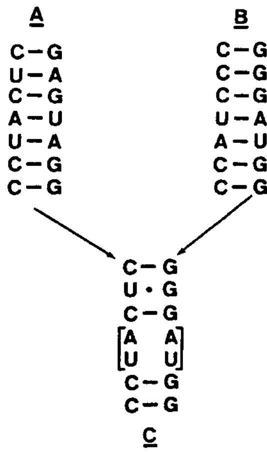

Fig. 2. 16S rRNA helix 984–990/1215–1221 (IUB nomenclature of *E. coli*). A WHB 46-1*, B WHB 46-2, C WHB 46-½; diagnostic mismatches are marked by brackets.

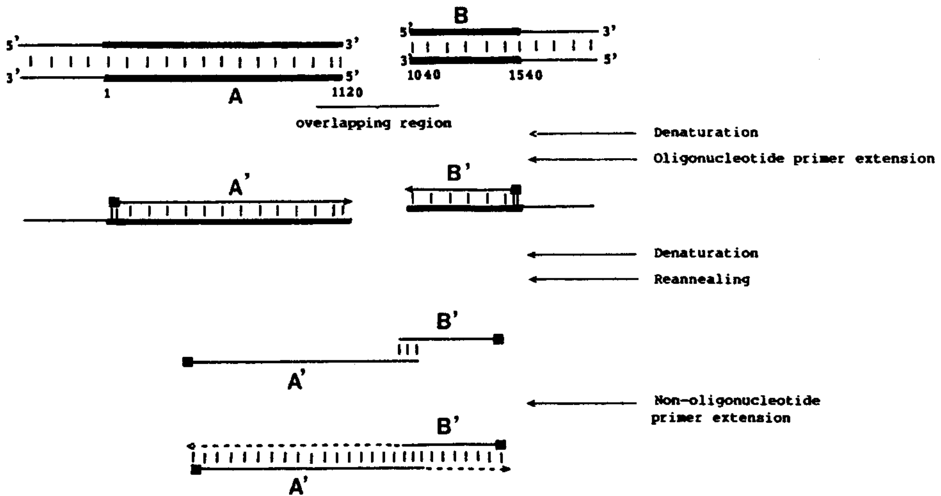

Fig. 3. Schematic illustration of the formation of chimeric 16S rDNA during PCR-mediated gene amplification of a mixture of two fragmented rDNA genes.

risks in the use of PCR for unravelling community structures. Without critical analysis of the sequences, either by checking the secondary structure of highly conserved helices or by calculation of separate phylogenetic trees for individual domains of the sequences, the existence of a chimeric sequence will escape attention. Since homology values calculated for the parent and the composite molecules will always be lower than 100% (with the homology depending on the degree of the chimeric nature of the artifact) the existence of chimerae may lead to the misleading interpretation of these molecules as representing new organisms (Fig. 1).

An alternative strategy to direct PCR-mediated amplification of DNA in-

cludes the extraction of rRNA from natural habitats, followed by the synthesis of rcDNA and creation of a rDNA library in pBR322 [9, 21, 22]. On the other hand, this strategy has the disadvantage that it may not completely cover the diversity of natural communities. The 16S rRNA of members of certain genera (e.g., *Streptomyces* [9]) exhibits specific post-transcriptional modifications that cause the reverse transcriptase to terminate rcDNA synthesis. In the case of *Streptomyces,* termination occurs at position 966/967 of 16S rRNA [5]. Using 3′ neighboring primers, this strategy would fail to detect streptomycetes in environmental samples when the available genus-specific probe that is used targets a region in the 5′ adjacent part of the posttranscriptional modification (positions 955–967) [9].

Phenotypic Characterization of the Culture

Although culture WHB 46 is a mixture of two strains, information on phenotypic characters may be helpful to others working with similar organisms. While negative reactions indicate that both strains share the same properties, a positive reaction in only one strain will mask a negative reaction in the other. Positive reactions will therefore not be listed. The characteristics are as follows: Gram-negative; straight or curved rods, monopolar monotrichous sheathed flagellum; optimal growth temperature about 5°C, maximum growth temperature 10°C; strictly barophilic, optimal growth at about 400 bars; no growth at atmospheric pressure; shortest generation time (11 hours) at 5°C and 400 bars in Zobell medium 2216; negative reactions: catalase; indol formation; Voges-Proskauer test; growth without seawater; degradation of starch, gelatin, and precipitated chitin; acetate, L-alanine, L-asparagine, L-serine, L-proline as sole carbon source; acid formation from L-rhamnose, D-xylose, D-fructose, cellobiose, lactose, sucrose, salicine, D-mannitol, and D-sorbitole; growth at 8 μg/ml penicillin, at 8 μg/ml chloramphenicol, at 4 μg/ml tetracycline and at 32 μg/ml rifampicin.

The two strains are phylogenetic neighbors of *Listonella* (*Vibrio*) *anguillarum* and other members of *Vibrio* (M. Dorsch, D. Lane, E. Stackebrandt, manuscript submitted). Further assignment has to await 16S rRNA sequence information from other known barophilic isolates especially from members of the genera *Shewanella* [11] and *Colwellia* [2], containing to our knowledge the only barophilic bacteria taxonomically described by rRNA/rDNA analysis so far.

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