Quantitative Measure of Small-Subunit rRNA Gene Sequences of the Kingdom *Korarchaeota*

CLIFFORD F. BRUNK^{1,2*} AND NICOLE EIS¹

Lehrstuhl für Mikrobiologie und Archaeenzentrum der Universität Regensburg, Regensburg, Germany,¹ and Biology Department and Molecular Biology Institute, University of California, Los Angeles, California²

Received 22 June 1998/Accepted 22 September 1998

Comparative PCR amplification of small-subunit (SSU) rRNA gene (rDNA) sequences indicates substantial preferential PCR amplification of pJP27 sequences with korarchaeote-specific PCR primers. The coamplification of a modified SSU rDNA sequence can be used as an internal standard to determine the amount of a specific SSU rDNA sequence.

Recently a group of deep-branching archaea of considerable phylogenetic interest have been identified on the basis of their small-subunit (SSU) rRNA gene (rDNA) sequence from the Obsidian Pool, Yellowstone National Park, Wyo. (1a, 2, 11). A mixed culture taken from the Obsidian Pool, which contains pJP27 as well as other archaea, has been established in a chemostat in the laboratory (6, 12).

The presence of pJP27 in the laboratory culture has been determined by phylogenetic staining (8) and PCR amplification of the SSU rDNA sequences by using korarchaeote-specific probes and primers (6, 12). The initial sequences of the PCR-amplified SSU rDNAs identified pJP27, but not pJP78, among the PCR products; however, specific PCR amplification reported here indicates pJP78 is also present in the chemostat culture. Although both phylogenetic staining and PCR amplification indicate the presence of korarchaeotes in the chemostat culture, they give dramatically different estimates of the relative amounts of these organisms in the chemostat.

In this study we establish that the pJP27 SSU rDNA sequence is preferentially amplified relative to other SSU rDNA sequences found in the chemostat culture derived from the Obsidian Pool. We also are able to determine the abundance of the korarchaeote SSU rDNA sequences by using a modified SSU rDNA sequence as an internal standard. This approach also allows the determination of the abundance of specific SSU rDNA sequences from environmental or laboratory samples.

Cells from the chemostat culture were harvested and DNA was prepared as previously described (1). PCR amplification of the SSU rDNA sequences in the DNA preparation was performed with various primer sets (Table 1) and a standard PCR protocol: 90 s at 96°C; 10 cycles of 30 s at 96°C, 30 s at 60°C, and 60 s at 72°C; 25 cycles of 20 s at 94°C, 30 s at 60°C, and 60 s (increased by 2 s each cycle) at 72°C; and 600 s at 72°C. All enzyme digestions and cloning (pAMP1; Gibco-BRL, Eggenstein, Germany) were performed according to the manufacturer's protocols. The *Sac*II restriction sites of pJP27 and pJP78 SSU rDNA sequences were converted to *Pst*II or *Eco*RI, respectively, with modified PCR primers (Pst-F, 5'-GCC AGC CGC TGC AGT AAA ACC-3'; Pst-R, 5'-GGT TTT ACT GCA GCG GCT GGC-3'; *Eco*R-F, 5'-GCC AGC CGC CGA

ATT CAA ACC AGC-3'; and *Eco*R-R, 5'-GCT GGT TTG AAT TCG GCG GCT GGC-3').

The amount of pJP27 or pJP78 SSU rDNA sequence present in a DNA sample was determined by coamplifying a known amount of the appropriate modified SSU rDNA sequence along with the SSU rDNA sequences in the DNA sample and analyzed following endonuclease restriction enzyme digestion by agarose gel electrophoresis. The gels were documented and the files were analyzed with NIH Image 1.62b7 software.

In an initial PCR characterization of the organisms present in the chemostat established from the Obsidian Pool, roughly one-third of the sequences were identical to the pJP27 sequence (12), but pJP78 was not detected, although a few pJP78 sequences were detected in the Barns et al. characterization of the Obsidian Pool (1a, 2). These results create a conundrum, as phylogenetic staining suggests that pJP27 is a minor component, possibly as low as 1 in 10⁴ cells (6). This disparity between the microscopic observations and the PCR analysis suggests that some of the SSU rDNA sequences may be pref-

TABLE 1. PCR primer sets^a

Primer set	Forward primer ^b	Reverse primer ^c
L	23F	1390R
T	236F	1146R
C	384F	890aR
F	624F	1390R
R	23F	604R
A	236F	890aR
B	384F	1146R
Q	533F	1146R
K	236F	1390R
J	23F	1146R
W	624F	1146R
X	797aF	1146R

^a The primer numbers refer to the position in the *Escherichia coli* SSU rRNA sequence (4). For cloning, linkers appropriate for the pAMP1 vector system (Gibco-BRL) were added to the primers.

^b Forward primers and their sequences are as follows (references are given parenthetically): 23F, 5'-TCY GGT TGA TCC TGC C-3' (9); 236F, 5'-GAG GCC CCA GGR TGG GAC CG-3' (8); 384F, 5'-CTC CGC AAT RCG CGM AAG-3' (5); 533F, 5'-TGB CAG CMG CCG CGG TAA-3' (7); 624F, 5'-GTT AAA TCC GCC TGA AGA CA-3' (5); and 797aF, 5'-GCR AAS SGG ATT AGA TAC CC-3' (7).

^c Reverse primers and their sequences are as follows: 604R, 5'-TGT CTT CAG CGG GAT TTA AC-3' (8); 890aR, 5'-TTT CAG YCT TGC GRC CGT AC-3' (7); 1146R, 5'-CCG GGC AAA CTA GGG GGA C-3' (this work); and 1390R, 5'-ACG GGC GGT GTG TRC AA-3' (15).

* Corresponding author. Mailing address: Biology Department, University of California, Los Angeles, CA 90095-1606. Phone: (310) 825-3114. Fax: (310) 206-3987. E-mail: cbrunk@ucla.edu.

FIG. 1. Dilution series in which the ratio of pJP78 target increases relative to the pJP27 target by twofold in each lane. The lower panel displays the target DNAs (PCR product of a previous amplification), and the upper panel displays the PCR product following amplification using T primers. Stars indicate the lanes in which the sequences are in equal amounts in the target, while squares indicate the lanes in which the sequences are in equal amounts in the PCR product. Scanning analysis of these gels indicates that the PCR products are equal in amounts when the pJP78 target exceeds the pJP27 target by 65-fold.

entially PCR amplified. Thus, the relative PCR amplification of pJP27 and pJP78 using korarchaeote-specific primers (set T) was examined.

The T primers are virtually a perfect match to both the pJP27 and pJP78 sequences (pJP78 has a single mismatch in the 5' end of the reverse primer). As shown in Fig. 1, when the pJP78 plasmid exceeds the pJP27 plasmid by 65-fold in the target DNA, equal amounts of pJP27 and pJP78 PCR products are produced. This preferential amplification is clearly associated with the sequence between the primer binding sites rather than simply with the primer binding sites themselves, as a similar preferential PCR amplification is observed with plasmid or PCR product. The addition of 5% acetamide to the PCR has previously been observed to alleviate the differential ampli-

cation (17); however, acetamide did not significantly affect the preferential amplification of pJP27 relative to pJP78.

When other primer sets are examined, the differential PCR amplification varies widely. Table 2 lists the ratio of pJP78 to pJP27 target required to produce equal amounts of PCR products for each primer set. Different portions of the pJP27 and pJP78 SSU rDNA sequences are PCR amplified with significantly different efficiencies. Comparative PCR amplification of pJP27 with other SSU rDNA sequences obtained from the chemostat in an earlier analysis (12), using L primers, are also shown in Table 2. Substantial differential amplification is observed among these SSU rDNA sequences as well (Table 3).

Knowing that there is a significant preferential PCR amplification of pJP27, it is possible that pJP78 is present in the chemostat but is not detected. Within the region spanned by the T primers, pJP27 has a *Bgl*I restriction site but pJP78 does not; thus, cleavage with *Bgl*I prior to PCR amplification should eliminate intact pJP27. When the DNA was digested with *Bgl*I prior to PCR amplification, the pJP78 sequence could be readily detected.

In order to determine the exact amount of korarchaeotes

TABLE 2. Differential PCR amplification of various regions of the SSU rRNA sequences and SSU rRNAs from different organisms^a

Primer set	Ratio of pJP78/pJP27 target required to produce equal amounts of PCR product
W	>300
Q	200
B	150
X	125
J	100
T	65
R	50
L	4
A	2
K	2
F	1
C	0.2

^a The targets for all comparisons were PCR products from a PCR amplification using L primers. PCR products of amplifications were analyzed by restriction endonuclease digestion. The pJP78 sequence was provided by the N. Pace laboratory.

TABLE 3. Relative PCR amplification of the pJP27 SSU rDNA sequence compared with SSU rDNA sequences of different organisms from a previous analysis (12)^a

Sequence compared to pJP27	Ratio of target/pJP27 required to produce equal amounts of PCR product
I-3	6
I-9	10
II-22	3
III-4	3
M11TL	5
pJP78	4

^a See the footnote to Table 2 for additional information.

present in a sample, a modified pJP27 SSU rDNA sequence can be used as an internal standard during PCR (3, 10, 13, 14, 16). A single *SacII* restriction site in pJP27 was modified to a *PstI* site. When the PCR amplifications of the original and modified pJP27 sequences were compared by using various primers, their amplifications were identical, and the relative amounts of each sequence in the PCR product were easily determined by cleavage with *SacII* or *PstI* following the PCR amplification. The reciprocal nature of these analyses significantly increases the accuracy with which the relative abundance of each sequence can be determined. By this assay, DNA prepared from the chemostat contains about 4.7 fg of pJP27 SSU rDNA sequence per ng of total DNA.

A similarly modified pJP78 SSU rDNA sequence was constructed in which the *SacII* restriction site was converted to an *EcoRI* site. By using this modified pJP78 sequence and chemostat DNA, extensively digested with *BglI* to eliminate pJP27 sequences, 0.9 fg of pJP78 SSU rDNA sequence per ng of total DNA was detected.

A direct comparison of the PCR amplification of the SSU rDNA from pJP27 and that of SSU rDNA sequences from other organisms indicates that there is a significant preferential PCR amplification of the pJP27 SSU rDNA sequences. This preferential PCR amplification is a property of the SSU rDNA sequences themselves, not simply the primer binding region. Including a modified sequence as an internal standard which PCR amplifies identically to the unmodified sequence but can be distinguished from the unmodified sequence after amplification by a unique restriction site allows the amount of unmodified sequence in the original sample to be determined.

We thank K. O. Stetter for providing laboratory and technical assistance while C.F.B. was a visiting scientist at his laboratory at the University of Regensburg. A clone containing the pJP78 SSU rDNA sequence was kindly provided by the Pace laboratory. We thank Gary Fogel, Jinliang Li, and Erik Avaniss-Aghajani for critical reviews of the manuscript.

This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie to K. O. Stetter.

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