Miniprimer PCR, a New Lens for Viewing the Microbial World

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Molecular methods based on the 16S rRNA gene sequence are used widely in microbial ecology to reveal the
diversity of microbial populations in environmental samples. Here we show that a new PCR method using an
engineered polymerase and 10-nucleotide “miniprimers” expands the scope of detectable sequences beyond
those detected by standard methods using longer primers and Taq polymerase. After testing the method in
silico to identify divergent ribosomal genes in previously cloned environmental sequences, we applied the
method to soil and microbial mat samples, which revealed novel 16S rRNA gene sequences that would not have
been detected with standard primers. Deeply divergent sequences were discovered with high frequency and
included representatives that define two new division-level taxa, designated CR1 and CR2, suggesting that
miniprimer PCR may reveal new dimensions of microbial diversity.

Characterization of 16S rRNA gene sequences has become a central feature of microbial ecology. Frequently, these analyses are initiated by using PCR to amplify 16S rRNA genes directly from environmental samples without culturing (4). Indeed, these types of studies have transformed our view of the microbial world. However, as the 16S rRNA sequence database has grown, it has become evident that many sequences deviate within the most conserved regions targeted by “universal” 16S rRNA gene PCR primers (8, 9, 38). To accommodate these deviations, commonly used primers have been modified with degenerate positions to enable the primers to target a wider range of 16S rRNA gene sequences. However, because polymerases used for PCR require primers of ~20 to 30 nucleotides (nt), 16S rRNA gene primer design has been constrained to target conserved regions of those lengths. New thermostable polymerases have recently become available (40), opening the possibility of changes in primer design. Prethermostable polymerases have recently become available
strains to target conserved regions of those lengths. New
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MATERIALS AND METHODS

PCR conditions. The S-Tbr (DyNamo II; Finnzymes Oy, Espoo, Finland) and
Taq (Roche, Indianapolis, IN) DNA polymerases were used for PCR. S-Tbr is an
engineered polymerase where the N-terminal 5’-3’ exonuclease domain of Ther-

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PCRs were combined for cloning and subsequent sequencing. All sample handling was performed in a clean, sterile laminar flow hood.

Cloning and sequencing. PCR products were extracted from ~1% agarose gels and cloned using the TOPO cloning kit for sequencing (Invitrogen, Carlsbad, CA). Escherichia coli genomic DNA was prepared using the QIAamp DNA mini kit (Qiagen, Valencia, CA) or purchased from USB Corporation (Cleveland, OH); to prepare Halobacterium salinarum genomic DNA, H. salinarum strain MPK1 (24) was grown in culture as previously described (12), followed by genomic DNA preparation using the QIAamp DNA mini kit. Automated DNA sequencing of clones was performed by the Massachusetts General Hospital DNA Sequencing Core Facility (Cambridge, MA) or by the University of Wisconsin Biotechnology Center (Madison, WI) using sequencing primers located outside the bounds of the cloned 16S rRNA gene inserts. Sequence reads were assembled using Phred and Phrap as implemented in XplorSeq (D. Frank, unpublished data) or the Staden package, release 1.6.0. Soil study clones A01 and A04 were fully sequenced with two- to fivefold coverage by independent sequence reads and had at least threefold coverage of all highly conserved regions. Mallard was used as the primary tool to identify and remove anomalous or chimeric 16S rRNA gene sequences (7); for some ambiguous cases, Bellerophon (20), PinTal (6), BLAST, and manual checking were used to supplement results from Mallard analysis.

Informatics and computation. Sequences were aligned using the NAST aligner (16) and imported into Arb version 05.10.26 (27), using the full greengenes database (see below) as a 16S rRNA reference database (15). The Arb database of Cabo Rojo and reference sequences is available at plantpath.wisc.edu/database (see below) as a 16S rRNA reference database (15). The Arb database was submitted to GenBank under accession numbers EU245047 to EU246330. 

Novel taxa identified with colloquial names and for which references are not available at plantpath.wisc.edu/isen/CR. For each primer search, pertinent BLAST parameters were as follows: no gaps, $M = 1$, $N = -3$, $V = 1,000,000$, and $B = 1,000,000$, and both the word size ($W$) and score threshold ($S$) were set to the length of the primer sequence used for the search. Putative 16S rRNA gene amplifications found in the environmental sequence database were confirmed by a BLAST score ratio (33) of greater than 0.80, computed for each sequence by dividing the BLAST score for its best match to the reverse 16S rRNA gene database by the BLAST score of the sequence compared to itself; default BLAST parameters were used for these searches. All BLAST searches were implemented using WU-BLAST (blastn 2.0MP-WashU [10 May 2005]; W. Gish, personal communication).

**Final note on primer sequences, nomenclature, and citation.** In reviewing the literature to prepare this work, we found many instances of ambiguity regarding 16S rRNA gene PCR primers. These ambiguities resulted primarily from using the same name (e.g., “27F”) to refer to primers having different sequences. Because of this, we feel it is important to be cautious in reporting 16S rRNA gene primer sequences, especially as more variations of commonly used primers are developed and published. Though there may eventually be a need to agree on a more systematic method of naming primers, most ambiguities can easily be minimized in publications by reporting the sequences of primers instead of designating them by a common name and reference.

**Nucleotide sequence accession numbers.** These sequence data have been submitted to GenBank under accession numbers EU245047 to EU246330.

### RESULTS

**Minimum primer length for Taq and S-Tbr polymerases.** We conducted studies to determine the minimum primer length required for PCR amplification by the S-Tbr and Taq polymerases. For initial testing, primers were designed based on the nondegenerate versions of the 27F (sometimes called 8F) and 1492R primers used to amplify bacterial 16S rRNA genes (designated 27F-P and 1492R-P in this work) (18). Beginning with a 20-nt forward primer sequence (Fig. 1A), we designed a series of progressively shorter primers with lengths from 20 to 8 nt (Fig. 1A). To determine the lower limit for primer length, PCRs were performed using a forward primer from the series of decreasing lengths paired with a 19-nt reverse primer; *E. coli* genomic DNA was used as the template (Fig. 1B). With S-Tbr polymerase, successful amplification resulted with a primer as short as 10 nt. In a parallel experiment, *Taq* required the primer to be longer than 14 nt for detectable amplification (Fig. 1B). PCR products were produced with forward and reverse primers of 10 nt at annealing temperatures as high as ~49°C (Fig. 1C). To distinguish this new PCR method from traditional *Taq* PCR, we refer to the shortened primers as “miniprimers” and to PCR using them as “minipimer PCR.”

**Minipimer design and specificity.** Several more 16S rRNA gene minipimer candidates of 9 to 11 nt were designed (Fig. 2A) and tested to assess their performance and specificity (Fig. 2A).
2B). Miniprimers were designed by consulting 16S rRNA gene sequence alignments and published primer sequences to choose the most conserved 10-nt regions within several long 16S rRNA gene primers. We based our designs on variants most often reported in the literature (Table 1). The 788F-10 and 797R-10 primers (Fig. 2A) were designed to target the 10-nt region found to be the longest string of totally conserved bases in an alignment of 500 bacterial 16S rRNA gene sequences (8). By use of several combinations of miniprimers, PCRs were performed to amplify 16S rRNA genes from Escherichia coli or Halobacterium salinarum genomic DNA (from the domains Bacteria and Archaea, respectively). First, we selected miniprimer pairs that produced amplicons of the expected sizes with a minimum of spurious products. Then, amplicons from several PCRs were sequenced to verify that they were regions of 16S rRNA genes. All sequences from amplicons of the expected sizes were from the correct regions of the 16S rRNA gene targeted by each particular miniprimer pair. This analysis yielded several pairs of miniprimers that performed well for the amplification of bacterial and archaeal 16S rRNA genes (Fig. 2); in particular, 27F-10, 524F-10, and 2F-10 paired with 1505R-10 have reproducibly performed well in our analyses.

In silico evaluation of miniprimer targets in environmental sequences. To determine whether miniprimer PCR might expand the phylogenetic breadth of 16S rRNA gene sequences detected in environmental samples, the environmental sequence database was searched for putative 16S rRNA gene amplicons. It is important to note that this database compiles sequences obtained not by PCR but by random shotgun cloning of DNA directly extracted from environmental samples. A modeled PCR search identified putative 16S rRNA gene amplicons delimited by forward and reverse primer sequences separated by an appropriate distance (Fig. 3). Our goal was not to determine the fraction of 16S rRNA sequences in the database identified by the primers but to compare experimental methods for 16S rRNA gene discovery. Each database sequence and its reverse complement were searched using several of the best-performing primer pairs (Fig. 2); additional primer pairs were also designed to identify smaller amplicons.

![Figure 1](image1.png)  
**FIG. 1.** Establishing conditions for miniprimer PCR. (A) Minimum primer length. Beginning with a 20-nt primer, a series of shortened primers having a common 5’ end was designed. These primers were used in combination with a 19-nt reverse primer (B) and a 10-nt primer (C) to demonstrate that 10-nt primers can be used for PCR. (B) Amplification using the forward primer series. When electrophoresed on an agarose gel, PCR with S-Tbr produces detectable amplification using primers as short as 10 nt (F10/R19). (C) Miniprimer PCR. Agarose gel electrophoresis of PCR products with S-Tbr and a range of annealing temperatures demonstrates robust amplification for a pair of 10-nt primers (F10/R10). In panels B and C, agarose gels are stained with ethidium bromide and arrows mark the expected size of PCR products.

![Figure 2](image2.png)  
**FIG. 2.** Miniprimers designed and tested. (A) Sequences of miniprimers tested. (B) Performance of miniprimer pairs. Forward and reverse miniprimers were used to PCR amplify 16S rRNA genes from bacterial (Escherichia coli [Ec]) or archaeal (Halobacterium salinarum[Hs]) genomic DNA. PCR was determined successful if a band of the correct size was detected with minimum background. Success or failure with each template is indicated with + or −, respectively, in the appropriate column; a mark of +/− indicates that the product was in low abundance or contained a moderate amount of background but may be clonable. In several cases, amplicons were sequenced to confirm them to be the expected 16S rRNA sequences; confirmation is indicated by a + in the column labeled “16S.” nd, not determined.
because the average sequence length of the environmental sequence database entries is \( \sim 1,000 \) nt, lowering the probability of identifying long amplicons. To compare the abundances of putative amplicons defined by pairs of long primers and miniprimers, matched primer pairs were used to target the same regions of the 16S rRNA gene (Fig. 3). Miniprimers identified 1,648 putative amplicons and the standard primers identified 448; surprisingly, of the 1,648 miniprimer sequences, 971 were not identified by any long-primer pair tested. By BLAST comparison against a database of 30,312 nonredundant, nonchimeric bacterial and archaeal 16S rRNA gene sequences of at least 1,350 nt, 1,068 miniprimer and 301 long-primer sequences were verified to be regions of 16S rRNA genes (Fig. 3). Importantly, each miniprimer pair identified 2- to 10-fold more putative 16S rRNA gene amplicons (Fig. 3). This analysis predicted that miniprimer PCR can amplify more 16S rRNA gene sequences in environmental samples than can standard long primers.

Next, the phylogenetic breadth represented by the putative 16S rRNA gene amplicons was determined. In sum, the miniprimer and long-primer pairs identified 1,648 and 301 total putative 16S rRNA gene sequences, respectively. To build the best phylogenies and remove redundant sequences, the longest sequence identified from each database entry was used in instances when the same database entry was identified by multiple primer pairs. After removing replicated sequences, the remaining 68S miniprimer and 20S long-primer sequences were aligned to a comprehensive 16S rRNA gene sequence database (16). One long-primer sequence and 14 miniprimer sequences could not be satisfactorily aligned to the database. A phylogenetic tree was constructed using the remaining sequences, and the resulting taxonomic distribution indicated that almost all the sequences originated from *Bacteria*, with *Proteobacteria* sequences in the majority (see S1 in the supplemental material). For almost every taxonomic class, miniprimers identified more sequences than long primers. Also, miniprimers identified sequences in some classes for which long primers identified no sequences (see S1 in the supplemental material); this generally occurred for classes in which few members were identified but also occurred for Sargasso Sea group 11 (SAR11), the most abundant class of marine bacteria currently known (29). In addition, miniprimers identified nine sequences from *Archaea*, a domain for which long primers identified no sequences. This suggests that miniprimer PCR can amplify more sequences in more phylogenetic groups than can standard long primers.

**Evaluation of miniprimer PCR with a soil sample.** To determine whether the computational analysis reflected experimental results, miniprimer PCR was used in a study to amplify 16S rRNA genes from soil by use of the primer pairs 27F-10/1505R-10, 12F-10/1505R-10, and 524F-10/1505R-10 (Table 1 and Fig. 2). In this study, we did not attempt a comprehensive analysis of a soil sample but only an assessment of the miniprimers and whether they could detect sequences with mismatches to the standard primers. After PCR and cloning, 32 clones from each primer pair were partially sequenced from one end to examine the primer binding regions. Products from the 27F-10/1505R-10 PCR were chosen for further study because 27F-10 comprises the first 10 nt from the 5’ half of the 27F suite of primers (Table 1) and thus enabled the identifi-
culation of amplicons whose sequences had mismatches to the 3′ half of 27F (see S2 in the supplemental material). In addition, the 27F-10/1505R-10 miniprimer pair amplifies nearly the entire 16S rRNA gene, and it performed well in pilot experiments. Very-high-quality sequences were obtained for 10 of the 32 27F-10/1505R-10 sequences; of these 10 end sequences, 5 began with the 27F-10 sequence and 5 began with the 1505R-10 sequence. Two of the sequences anchored by the miniprimer 27F-10, A01 and A04, contained mismatches to the 27F-P binding sequence and also to the sequence of 27F-HT, a degenerate primer designed to be more general (Table 1; also see S2 in the supplemental material).

After A01 and A04 were fully sequenced, it was noted that the A01 sequence also had a mismatch within the 4 nt at the 3′ end of the 1492R binding site that are not shared by 1505R-10 (see S2 in the supplemental material). The A01 and A04 sequences were aligned to the comprehensive 16S rRNA gene sequence database to assess the sequences within conserved regions (see S2 in the supplemental material). The alignment revealed that A01 and A04 contained mismatches within the 27F-P and 1492R-P primer binding regions and within other regions of the 16S rRNA gene that are very highly conserved across the Bacteria domain (see S2 in the supplemental material). Importantly, predictions of rRNA secondary structure based on the Arv alignments supported the presence of the divergent bases in the A01 and A04 sequences—of the nine divergent bases observed (see S2 in the supplemental material), seven are located in base-paired regions, and for five of these bases, the expected complementary changes are present at the proper locations to preserve Watson-Crick pairing for all 20 sites chosen (data not shown). Thus, given the covariation present in the sequences, it is exceedingly unlikely that the divergent bases arose from PCR errors or chimeric sequences. 

**Miniprimer PCR characterization of the Candelaria microbial mat community.** We applied the method to a mature microbial mat community from an extreme environment, the hypersaline Candelaria lagoon of the Cabo Rojo saltlens. This mat community was chosen for study because it was expected to have intermediate diversity and interesting phylotypes. To identify members of the mat community for this study, we constructed six 16S rRNA gene sequence libraries with either the miniprimer or standard long-primer techniques.

We constructed libraries with the miniprimer pair 27F-10/1505R-10 and two different long-primer pairs: 27F-P/1492R-P and 27F-HT/1492R-HT (Table 1). The primers 27F-P and 1492R-P are “first-generation” nondegenerate universal bacterial primers that remain in widespread use today (1, 5, 18, 19, 39), and 27F-HT and 1492R-HT are more recently published primers that are based on the original first-generation primers and include several degenerate positions to broaden their scope of targets (37). These primers produce nearly full-length 16S rRNA gene sequences and thus provide a fair assessment of the miniprimer method relative to standard methods in common use; duplicate libraries were constructed with each of the primer pairs (Table 2).

The Candelaria microbial mat sequence libraries comprised over 40 bacterial divisions, with the Chloroflexi, Bacteroidetes, Halanaerobiales, and Planctomycetes divisions having the greatest representation (Fig. 4). All of the most highly populated divisions identified were represented in each of the libraries, though in different proportions (Fig. 4). Notably, miniprimers amplified a greater proportion of sequences that could not be classified at or below the division level (see below).

The miniprimers amplified more sequences with poor matches to previously isolated 16S rRNA gene sequences (Fig. 5). Miniprimer libraries contained a larger fraction than did long-primer libraries of sequences that matched the database at distances greater than 0.10 (Fig. 5); conversely, the miniprimer libraries matched many fewer sequences at distances less than or equal to 0.05. The distributions of database matches for the two long-primer libraries were very similar and

<table>
<thead>
<tr>
<th>Library</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Polymerase</th>
<th>No. of sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>27F-10</td>
<td>1505R-10</td>
<td>S-Tbr</td>
<td>255</td>
</tr>
<tr>
<td>P1</td>
<td>27F-P</td>
<td>1492R-P</td>
<td>Taq</td>
<td>147</td>
</tr>
<tr>
<td>H1</td>
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<td>1492R-HT</td>
<td>Taq</td>
<td>150</td>
</tr>
<tr>
<td>M2</td>
<td>27F-10</td>
<td>1505R-10</td>
<td>S-Tbr</td>
<td>343</td>
</tr>
<tr>
<td>P2</td>
<td>27F-P</td>
<td>1492R-P</td>
<td>S-Tbr</td>
<td>202</td>
</tr>
<tr>
<td>H2</td>
<td>27F-HT</td>
<td>1492R-HT</td>
<td>S-Tbr</td>
<td>184</td>
</tr>
</tbody>
</table>

**Fig. 4.** Distribution of microbial mat library sequences in major bacterial divisions identified. The 10 taxonomic divisions with the highest representation in the libraries are shown as their relative proportions within the miniprimer (M) and two types of long-primer (P, 27F-P/1492R-P; H, 27F-HT/1492R-HT) libraries. The aggregate distribution of all libraries is also shown (all). Phylogenetic assignments were performed in Arv; the “other divisions” category comprises 31 divisions whose representation is less than ~3 to 5% in each library. *Proteobacteria* class names with standing have been shortened using Greek letters.

**Table 2.** Candelaria microbial mat 16S rRNA gene libraries constructed

<table>
<thead>
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<td>255</td>
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<td>P1</td>
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<td>1492R-P</td>
<td>Taq</td>
<td>147</td>
</tr>
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<td>1492R-HT</td>
<td>Taq</td>
<td>150</td>
</tr>
<tr>
<td>M2</td>
<td>27F-10</td>
<td>1505R-10</td>
<td>S-Tbr</td>
<td>343</td>
</tr>
<tr>
<td>P2</td>
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</tr>
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<td>27F-HT</td>
<td>1492R-HT</td>
<td>S-Tbr</td>
<td>184</td>
</tr>
</tbody>
</table>
subtaxa were largely populated by long-primer sequences, sug-
which miniprimer sequences contributed; the other
1492R-HT libraries, respectively). From the 27F-P/1492R-P and 1.2% from the 27F-HT/
5.9% of the miniprimer sequences contributing and 1.5% of
more similar to each other than either distribution was to the
A small fraction of sequences matched the database at dis-
sequences were distributed over many
are likely to be representatives of
The coverage and richness of the 1,281 Candelaria se-
were almost entirely composed of sequences cloned from microbial mats. Several miniprimer
sequences were distributed over many Deltaproteobacteria groups. Five
sequences contributed to the Deltaproteobacteria group GN04, which was
was first defined as a group of Guerrero Negro sequences and is
currently exclusive to sequences cloned from microbial mats. Very few miniprimer sequences
were from the Bacteroidetes
Firmicutes
divisions, though large fractions of long-primer sequences were classified in these divisions (Fig. 4). The
remaining divisions into which library sequences were placed each contributed less than 5% to their respective libraries.
To evaluate the reproducibility of the three library construction
methods and to compare the methods, the 16S rRNA gene sequence libraries were compared to each other by calculating similarity indices (34) using 97% sequence similarity to define
Here, we focus on the classic incidence-based Sørenson similarity index, a measure of membership, and the Clayton
similarity indices (34) using 97% sequence similarity to define
sequence libraries were compared to each other by calculating
similarity methods and to compare the methods, the 16S rRNA gene
sequences were estimated by calculating rarefaction curves and
nonparametric diversity estimators Chao1 and Ace1 (see S4 in the supplemental material). Rarefaction analysis sug-
gested that differences in compositions of the libraries constructed with minprimers and long-primer libraries are not
due to the enzyme used for amplification.
The coverage and richness of the 1,281 Candelaria se-
sequences were estimated by calculating rarefaction curves and
the nonparametric diversity estimators Chaol and Ace1 (see
sequences comprised monophyletic groups at distances of at least 0.17, with
6.9% of the miniprimer sequences contributing and 1.5% of
the two long-primer libraries combined contributing (1.7% from the 27F-P/1492R-P and 1.2% from the 27F-HT/1492R-HT libraries, respectively).
Other Candelaria sequences expanded the membership of
previously defined taxonomic groups. The Chloroflexi
group Eub6 is almost entirely composed of sequences cloned in this
study and from the Guerrero Negro microbial mats (26) and
was the largest single taxonomic group from any division to which miniprimer sequences contributed; the other
Chloroflexi
subtaxa were largely populated by long-primer sequences, sug-
miniprimer method appears to amplify more novel sequences than the long-primer
methods.
A small fraction of sequences matched the database at dis-
tances greater than 0.20 (Fig. 5). Most of these sequences were not
placed into defined taxa or were placed into recently de-
defined taxa, many of which include sequences isolated from
similar environments (Fig. 6). A few groups contain clusters of
miniprimer sequences, in particular a subgroup within WS6
and the CR1 and CR2 groups (Fig. 6). The clusters CR1 and
CR2 (Fig. 6; also see S3 in the supplemental material) formed
at distances of 0.26 and 0.24, respectively, from previously
isolated sequences and thus are likely to be representatives of
new division-level taxa. Another four clusters formed at dis-
tances slightly below the division level: CR3 to CR5 at 0.18 and
CR6 at 0.17 (see S3 in the supplemental material). Interesting-
ly, seven of the eight sequences defining CR1 and CR2, and
25 of the 32 sequences defining CR3 to CR6, are miniprimer
sequences. Many other sequences also branched deeply at dis-
tances of 0.29 to 0.16 to their nearest neighbors but did not
meet all the criteria for defining new division-level taxa. A
small fraction of library sequences accounted for these putative
novel groups: approximately 3.5% of library sequences
met the Guerrero Negro microbial mat. Several miniprimer
sequence and was the largest single taxonomic group from any division to which miniprimer sequences contributed; the other
Chloroflexi
subtaxa were largely populated by long-primer sequences, sug-
gest the existence of a primer bias for particular groups of
Chloroflexi. Similar subdivision bias is demonstrated by the
Halanaerobiales
sequences: most
sequences were from miniprimer libraries, whereas most
Halanaerobiales
family sequences were from long-primer libraries. A large fraction of miniprimer sequences was clustered
within the Planctomycetes
group and sequences from all libraries were divided over many
Deltaproteobacteria
groups. Five
sequences contributed to the Deltaproteobacteria group GN04, which was
recently defined based on sequences isolated from the
Guerrero Negro microbial mat. Several miniprimer
sequences populated the
Spirochaetes
group GN05-1, which was
first defined as a group of Guerrero Negro sequences and is
currently exclusive to sequences cloned from microbial mats. Very few miniprimer sequences were from the
Bacteroidetes
and
Firmicutes
divisions, though large fractions of long-primer sequences were classified in these divisions (Fig. 4). The
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### Table 3. Estimation of library similarities

<table>
<thead>
<tr>
<th>Library</th>
<th>M1</th>
<th>M2</th>
<th>P1</th>
<th>P2</th>
<th>H1</th>
<th>H2</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>0.60 ± 0.09</td>
<td>0.64 ± 0.07</td>
<td>0.07 ± 0.02</td>
<td>0.08 ± 0.03</td>
<td>0.06 ± 0.02</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>M2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>0.58 ± 0.16</td>
<td>0.49 ± 0.14</td>
<td>0.06 ± 0.02</td>
<td>0.08 ± 0.02</td>
<td>0.03 ± 0.01</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>P2</td>
<td>0.50 ± 0.12</td>
<td>0.37 ± 0.10</td>
<td>0.76 ± 0.15</td>
<td>0.52 ± 0.08</td>
<td>0.51 ± 0.11</td>
<td>0.56 ± 0.10</td>
</tr>
<tr>
<td>H1</td>
<td>0.42 ± 0.15</td>
<td>0.49 ± 0.16</td>
<td>0.88 ± 0.13</td>
<td>0.74 ± 0.15</td>
<td>0.73 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>H2</td>
<td>0.51 ± 0.12</td>
<td>0.55 ± 0.13</td>
<td>0.72 ± 0.10</td>
<td>0.60 ± 0.09</td>
<td>0.65 ± 0.11</td>
<td></td>
</tr>
</tbody>
</table>

*The Sørenson (S, lower left) and Clayton (C, upper right) indices were computed for each pair of libraries using an OTU definition of 97% sequence identity. For both indices, values can range from 0 to 1, with 1 indicating identity. Values are reported with their standard errors. See Table 2 for details regarding the six libraries.*

sity at a greater taxonomic resolution and that many organisms have yet to be identified.

Lastly, we examined the miniprimer clone sequences to quantify mismatches within the regions that would be targeted by the long primers 27F-P, 27F-HT, 1492R-P, and 1492R-HT (Fig. 7). For the 27F binding region, 309 and 266 of the 598 miniprimer sequences deviated from the 27F-P and 27F-HT primer sequences, respectively, at one or more nucleotides; sequences with two mismatches to 27F-P and 27F-HT numbered 20 and 10, respectively, and 1 sequence had three mismatches to the 27F-P sequence. Though the miniprimer sequence consensus largely agreed with the sequences of the long 27F primers, the nucleotide frequency at position 12 did not agree with the degeneracy present at this position in 27F-HT. Position 12 of 27F-HT is designed to match A or C; however, while in the cloned miniprimer sequence libraries C was present in the majority of sequences, G was present more than four times as often as was A, and T was observed twice. Similar analysis of the 1492R binding region identified one sequence from the miniprimer clone libraries having a single mismatch to 1492R-P and 1492R-HT (data not shown).

### DISCUSSION

In this work, we show that 10-nt miniprimers can be used to identify divergent 16S rRNA gene sequences from environmental samples. Miniprimer PCR amplified a greater proportion than did standard primers of sequences that were novel or that poorly matched a database of previously isolated 16S rRNA genes; a similar calculation for long primers yields a similar false-positive rate (19/320 ≈ 5.9%). In addition, the rates of identifying mitochondrial and chloroplast sequences (see S1 in the supplemental material) were also the same (8/685 ≈ 3/205 ≈ 1%) for both methods. In support of this, the microbial mat libraries contained no eukaryote, chloroplast, or mitochondrial sequences, though eukaryotes are known to associate with the Candelaria mats (11). Overall, the results suggest that miniprimers can identify more 16S rRNA gene sequences without an increased rate of amplifying false positives. However, much additional testing and sequencing of amplicons isolated from environmental samples should be completed to test this prediction.

**Benefits of miniprimer PCR.** Adding miniprimer PCR to the tools used for analyzing microbial communities may enable a more accurate measure of 16S rRNA gene sequences in environmental samples by expanding the sequences detectable by PCR. For example, in the 1 Gbp of randomly cloned sequence in the environmental sequence database, if one assumes a microbial genome size of 2 to 3 Mbp and an average of 2.5 16S rRNA genes per genome (2), it is expected that ~800 to 1,200 16S rRNA genes are present in the sample. When totaled, the miniprimer computational searches identified 1,068 putative 16S rRNA gene amplicons (Fig. 3), in close agreement with the estimate, while the searches with the longer primers totaled 301 (Fig. 3). If one assumes that decreasing the length of a PCR primer by 1 nt can increase by fourfold the number of perfectly matched sequence targets, then reducing the length of a primer from 20 nt to 10 nt could theoretically increase the possible targets by a factor of a million (~ 4^10). Sequence variation within the regions targeted by these particular miniprimers may render the theoretical limit meaningless in this

![FIG. 6. Microbial mat sequences with low-scoring database matches. Miniprimer (red), long-primer (green and blue), and reference (black) sequences were assembled into a phylogenetic tree by use of maximum likelihood (35). The best-scoring tree of 20 independent calculations is shown; branches supported by bootstrap values of greater than 50 (100 iterations) are labeled. Some bootstrap values near closely spaced terminal clusters have been removed for clarity; none of the removed values were less than 50 and most were greater than 80. Uncultured isolates are identified by their accession numbers and by the environment from which the sequences were cloned; cultured isolates are in italics. Taxonomic groups are indicated for clusters of three or more sequences that could be classified. Seven archaeal 16S rRNA gene sequences were used to root the tree.](image-url)
instance; nonetheless, this upper limit could inform other applications of the method.

In addition to expanding the range of detectable targets, combining miniprimer PCR with standard techniques might increase the accuracy of environmental sampling by enabling estimates of microbial diversity to reflect sampled communities more closely. When PCR is used to analyze samples containing multiple heterogeneous templates, many mechanisms can result in a biased distribution of amplicons that does not accurately represent the distribution of templates in the sample (3, 22, 30, 36). Miniprimers may reduce these kinds of biases in two ways. First, because miniprimers are shorter and can target the most conserved regions of the 16S rRNA gene, the potential for mismatch is reduced compared with long primers. The degeneracy of 27F-HT was not appropriate for the nucleotide frequencies of the miniprimer sequences (Fig. 7), suggesting that particular communities may be better analyzed with a miniprimer such as 27F-10. Second, because miniprimers target smaller regions, the requirement for degenerate positions in primers can be eliminated or minimized, thus removing or decreasing preferential binding of particular primer species within a degenerate primer mixture. Obviously, the use of shortened primers may introduce different biases into PCR-based analyses of microbial communities, and more study is required to understand that potential, perhaps using known mixtures of bacterial DNAs. However, despite the biases of any PCR method, miniprimer PCR may be useful to supplement traditional methods either to reduce or make apparent the biases that occur in analysis of environmental samples and allow a more accurate description of microbial communities.

Miniprimer design. Though general rules have been established for Taq PCR primer design, it is not yet evident what parameters require optimization when primers are substantially shortened. Successful amplification was obtained using miniprimers whose G+C content covered a broad range from 30 to 90% (Fig. 2). Thus, product formation appeared unrelated to the G+C content of miniprimers. In our tests, a high rate of success has been obtained with 1505R-10 paired with many different forward primers (Fig. 2). Of the putative primer binding sequences identified in the environmental sequence database, the fewest sites were found for 1505R-10, suggesting that a productive miniprimer maximizes specificity for the target sequence in the context of the total template sequence present in the PCR. This may be a good rule with which to begin miniprimer design. It should be noted that the miniprimers used here are initial versions and that they should be optimized further. For instance, neither 27F-10 nor 27F perfectly matches Chlamydia, and 788F-10 does not match Dehalococcoides. Thus, some problems associated with long primers remain concerns in miniprimer design, and regularly checking, curating, and updating miniprimer sequences are as required for miniprimers as for the longer primers.

Future prospects for miniprimer PCR. The use of miniprimers in PCR can benefit many areas of biological research in several ways. PCR with miniprimers may enable PCR strategies that are not possible when primer design is restricted to a minimum length of \( \sim 20 \) nt. Some strategies might benefit from using even shorter primers—preliminary data indicate that miniprimers can be as short as 8 nt when paired with a primer of 15 to 20 nt (data not shown). Also, miniprimer sequences provide a small amount of additional sequence data that may be helpful for making fine discriminations among closely related sequences. Finally, the combination of highly processive enzymes and shorter primers may decrease the cost of PCR. The increased processivity enables PCR to be performed with smaller amounts of enzyme (40), and shorter primers are less costly to synthesize. Although cost savings may be minimal for small projects, these considerations become important for projects requiring very large numbers of PCRs.

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