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Preliminary Study Using Species-Specific Oligonucleotide Probe for rRNA of *Bilophila wadsworthia*

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Portions of the 16S RNA from a urease-positive *Bilophila wadsworthia* strain were sequenced, and a probe was constructed. The probe was end labeled with [^{32}P]ATP and polynucleotide kinase and hybridized on a nylon filter (by dot blot hybridization) to the immobilized rRNA of 12 *B. wadsworthia* strains and eight other anaerobic isolates. The probe efficiently hybridized only to the *Bilophila* strains. Cross-reactivity at high RNA levels (2,000 ng) was observed with one strain of *Bacteroides thetaiotaomicron* and one strain of *Bacteroides fragilis* (with 10× SET buffer [20× SET buffer is 0.5 M NaCl, 0.03 M Tris, and 2 mM EDTA]) but was not seen at lower RNA levels or with 5× SET buffer. When tested against mixed cultures of aerobic and anaerobic isolates representative of appendiceal abscess flora, the probe did not react with mixed cultures containing no *Bilophila* cells and could detect ≥10^5 *Bilophila* CFU/ml when the mixture was seeded with *Bilophila* cells. This probe is of potential use in the rapid identification of pure isolates and in the direct identification of *B. wadsworthia* in clinical specimens.

Bilophila wadsworthia, a recently described gram-negative anaerobic bacillus, was initially isolated from patients with perforated and gangrenous appendicitis (4). *B. wadsworthia* was isolated from approximately one-half of the clinical specimens studied and was the third most common anaerobe isolated yet is present in the feces of about 50% of humans in counts of only 10^5 to 10^6 CFU/g (1, 2). The lower gastrointestinal tract is the presumed ecological niche for *B. wadsworthia*, although it has been occasionally isolated as part of the indigenous oral and vaginal flora. It has also been isolated recently from a variety of clinical specimens, such as specimens from scrotal abscesses, mandibular osteomyelitis, axillary hidradenitis suppurativa, pleural fluid, joint fluid, and blood (8). There is, therefore, growing evidence that *B. wadsworthia* is a significant clinical pathogen.

B. wadsworthia is stimulated by bile and may be isolated from *Bacteroides* bile-esculin agar and on nonselective anaerobic media containing blood. It is an obligately anaerobic nonmotile gram-negative bacillus that is non-spore forming, is strongly catalase positive, does not hydrolyze esculin, and is asaccharolytic and whose growth is stimulated by pyruvate. Most of the strains are urease positive. *Bilophila* strains may take up to 7 days to grow; in mixed culture, isolation and identification of the microorganism may take even longer. In a previous study from our laboratory (2), the API ZYM system showed very weak positive results for lipase esterase and weak to strong reactions for acid phosphatase. There were no positive reactions with the Rapid ANA system. Therefore, the possibility of developing and utilizing a more rapid method of identification was explored in this study. Analysis of 16S rRNA sequences has been useful for the study of molecular evolution (20), and 16S RNA is also a useful target for specific oligonucleotide probes (19). In this study, the 16S rRNA of a urease-positive strain of *B. wadsworthia* was sequenced, and an oligonucleotide probe complementary to unique segments of the rRNA sequence was constructed. This probe was used in hybridization experiments with several strains of *Bilophila* and with 16 other aerobic and anaerobic isolates (13 species), concentrating on microorganisms frequently coisolated in appendicitis specimens.

MATERIALS AND METHODS

Organisms. The *Bilophila* strains used in the study were obtained from the Wadsworth Anaerobic Laboratory (WAL) collection (strains WAL 7959 [ATCC 49260], WAL 9077, WAL 8594, WAL 8839, WAL 9974, WAL 8571, WAL 8448, WAL 8283, WAL 9798, WAL 8144, WAL 9294, and WAL 9344). Eight of the 12 strains studied were urease producers. Other organisms studied included the following strains: *Bacteroides fragilis* (ATCC 25285, WAL 9076, and WAL 9088), *Bacteroides thetaiotaomicron* (ATCC 29741 and WAL 9039), *Bacteroides distasonis* (WAL 10032), *Fusobacterium nucleatum* (ATCC 10953), a *Desulfovibrio* sp. (ATCC 7757), *Peptostreptococcus micros* (WAL 9248), *Fusobacterium varium* (WAL 8913), *Bacteroides intermedius* (WAL 7784), *Lactobacillus catenaforme* (WAL 9708), *Clostridium clostridioforme* (WAL 8657), *Eubacterium lentum* (WAL 9771), and one clinical isolate each of *Escherichia coli* and a *Streptococcus* sp. (viridans group.)

Extraction of rRNA. Organisms were grown to stationary phase in 50 ml of *Brucella* broth (Difco Laboratories, Detroit, Mich.) and then centrifuged at 2,000 × *g* at 4°C for 20 min. Pellets were resuspended in 0.5 ml of LETS buffer (100 mM

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LiCl, 10 mM EDTA, 10 mM Tris [pH 7.8], 1% sodium dodecyl sulfate). An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and 0.05 volume of glass beads (25- to 50-μm diameter) were added, and the mixtures were agitated in a 1.5-ml Eppendorf tube with a minibead beater (Biospec Products, Bartlesville, Ind.) for 80 s at room temperature. The samples were then centrifuged for 1 min at 13,000 × *g* in an Eppendorf centrifuge (model 235B; Fisher Scientific, Pittsburgh, Pa.). The aqueous phases were reextracted with phenol-chloroform-isoamyl alcohol (25:24:1) and then with chloroform-isoamyl alcohol (24:1). The aqueous phases were transferred to a clean Eppendorf tube, and the nucleic acids were precipitated with 0.1 volume of 2 M lithium chloride and 2.5 volumes of cold absolute ethanol and allowed to stand for 15 min on ice. The tubes were then centrifuged for 20 min at 16,000 × *g* at 4°C in an Eppendorf centrifuge, and the resulting pellets were washed with cold 95% ethanol. The alcohol was decanted, and the pellets were dried in vacuo. The dried pellets were redissolved in 50 μl of diethyl pyrocarbonate-treated water, precipitated again with 0.1 volume of 2 M sodium acetate and 3 volumes of 95% ethanol, and allowed to stand for another 15 min on ice. The mixtures were again centrifuged for 15 min, and the pellets were washed with cold 95% ethanol. The mixture was then centrifuged for 15 min, the supernatant was decanted, and the pellets were dried in vacuo. The dry pellets were then resuspended in 50 μl of diethyl pyrocarbonate-treated water. The concentration of nucleic acids was determined spectrophotometrically (i.e., by A_{260} measurements).

Sequencing reaction and synthesis of the *Bilophila* oligonucleotide probe. RNA was purified, and the 16S rRNA fragment was sequenced with reverse transcriptase as previously described (10, 11, 17). 16S ribosomal DNA (rDNA) was amplified in a PCR using as a template a small sample from one colony of each strain. Primers were directed at conserved segments corresponding to *E. coli* positions 787 to 806 and 1507 to 1492. The amplicon was sequenced directly as described previously (9). The sequence was aligned with 16S rDNA sequences from other gram-negative bacteria, and a probe site was chosen. A DNA synthesizer (model 380; Applied Biosystems, Foster City, Calif.) was used to synthesize oligonucleotides by the phosphoamidite method (3), as described by the manufacturer.

16S rRNA analysis. Whole DNA extracted from the type strain of *B. wadsworthia* was amplified with primers directed at conserved regions of the 16S rDNA as previously described (9). The PCR product was reamplified by the method of Zintz and Beebe (22). The product from the amplification was sequenced with the Dye Deoxy Terminator Cycle Sequencing Kit in conjunction with the 373A ABI automated DNA sequencer (Applied Biosystems). Sequences from both strands of the DNA were generated to decrease the number of errors and ambiguous bases read.

Phylogenetic analysis. The 1,448 nucleotides of inferred sequence from *B. wadsworthia* were aligned on the basis of conserved primary and secondary structure (21) with a selection of sequences from the Ribosomal Database Project (15) by using the Genetic Data Environment, version 2.2 (16), on a Sun Sparcstation II. Regions of the sequence of *B. wadsworthia* that were not determined (because they were not between the positions of the first and last primers) or that displayed substantial length variation were excluded from the analyses because of uncertain homology; 1,355 nucleotides were retained for the analyses. The sequence of *E. coli* was included in the alignment as an out-group. The data were subjected to analysis by the least-squares distance method with the distance correction of Jukes and Cantor (12), by the parsimony method, and by the maximum likelihood method of PHYLIP, version 3.5 (7). The results from the analyses were compared, and the confidence limits in the trees generated were estimated by the bootstrap method (6).

Dot blot hybridizations. Probes were labeled at the 5′ ends with [^{32}P]ATP by using polynucleotide kinase (13). rRNA was denatured in formaldehyde at 65°C for 15 min and diluted in formaldehyde. rRNA (2 to 2,000 ng) was applied to a nylon membrane (GeneScreen; Dupont, NEN Research Products, Boston, Mass.). The rRNA was bonded to the membrane by exposure to UV light in a Stratalinker (model 1800; Stratagene, La Jolla, Calif.) as recommended by the manufacturer. Dot blots were performed as previously described (5, 18). Various dilutions of 20× SET buffer (0.5 M NaCl, 0.03 M Tris [pH 7.4], and 2 mM EDTA) were used in order to determine the optimal concentration. The membrane was prehybridized for 4 h at 38°C in 15 ml of SET buffer at the optimal concentration with 0.1% sodium PP_i, 0.2% sodium dodecyl sulfate, and 2 mg of heparin per ml added. Labeled probe (5×10^6 to 10×10^6 cpm) was then added to the reaction mixture, and the membrane was incubated overnight at 38°C with slow agitation. The next day, the membrane was washed three times (38°C, 45 min each time) in the same concentration of buffer used for hybridization. The blot was air dried and then autoradiographed with SB5 film (Eastman Kodak Co., Rochester, N.Y.) after exposure at −70°C for 48 h.

The probe was tested against 12 strains of *Bilophila* (including the strain used to make the probe). Other anaerobes tested included three strains of *B. fragilis*, two *B. thetaiotaomicron* strains, one *B. distasonis* strain, one *F. nucleatum* strain, and one *Desulfovibrio* sp. strain. Also, mixed cultures were prepared by adding *Bilophila* cells to a mixture of these organisms, which are representative of those commonly isolated in appendiceal abscess specimens (4). Pure cultures of each organism were diluted to 0.45 to 0.6 optical density unit (595 nm; model 35 spectrophotometer; Beckman Instruments, Irvine, Calif.). The organisms used included viridans streptococcus, *P. micros*, *B. thetaiotaomicron*, *C. clostridioforme*, *L. catenaforme*, *F. varium*, *B. intermedius*, *E. coli*, *B. fragilis*, and *E. lentum* (the strains of *B. thetaiotaomicron* and *B. fragilis* were different from the strains used in the specificity experiments described above). Organisms were mixed together in equal volumes and seeded with serial dilutions of the *Bilophila* culture. A mixture with no *Bilophila* added served as a negative control. Colony counts were performed with a spiral plater (10) (Spiral Biotech, Bethesda, Md.).

RESULTS AND DISCUSSION

Sequence data. By using the program PCGENE (Intelligenetics Corp., Mountain View, Calif.), we showed that the chosen probe site differed from all rDNA sequences in GenBank release 77 and the Ribosomal Database Project (1 August 1993 version) by 3 or more bases. Comparison of the 16S rDNA sequence from *B. wadsworthia* with other sequences available from the Ribosomal Database Project revealed that the organisms most closely related to *B. wadsworthia* belonged to the genus *Desulfovibrio*. All the analyses retained the grouping of *B. wadsworthia* with *Desulfovibrio longus* and *Desulfovibrio desulfuricans*. The degree of similarity between *B. wadsworthia* and *D. desulfuricans* (90.45%) is greater than the degree of similarity between *D. desulfuricans* and *D. longus* (86.9%). Figure 1 shows a constructed phylogenetic tree showing these relationships.

The nucleotide base sequence of the 16S rRNA probe

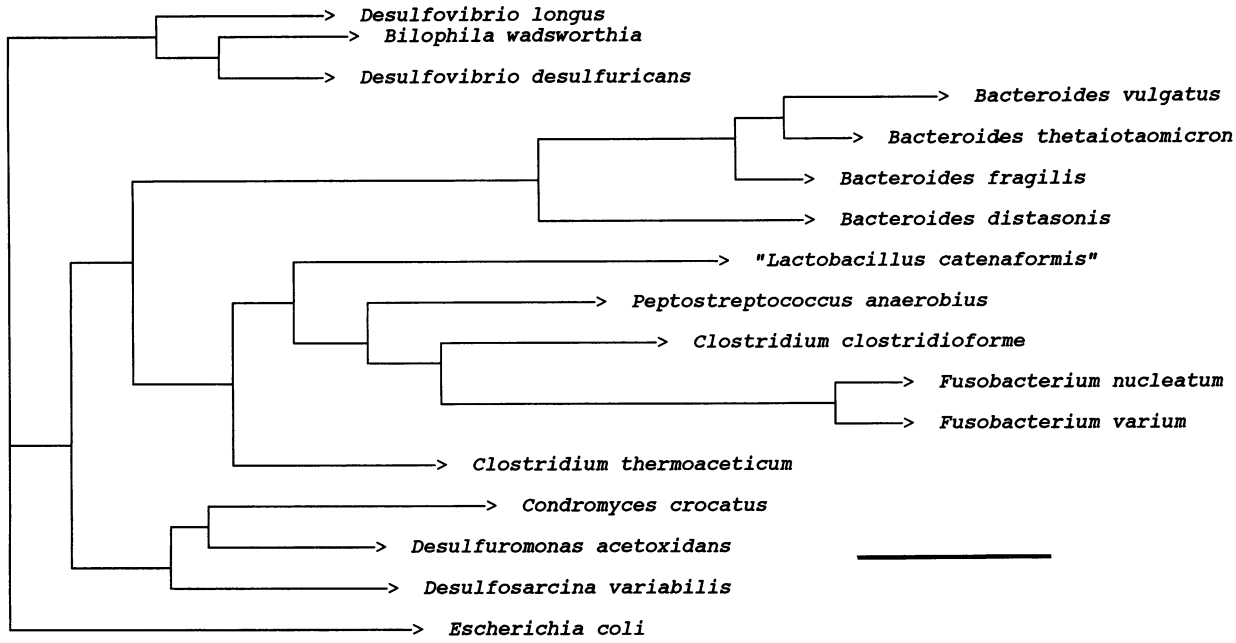

FIG. 1. Maximum-likelihood phylogenetic tree of small-subunit rRNA sequences showing the relationship of *B. wadsworthia* to a selection of sequences from the Ribosomal Database Project. The sequence from *E. coli* was used to root the tree. The lengths of the horizontal branches indicate the estimated number of substitutions per sequence position. Bar, 0.10 substitution per sequence position.

obtained from *B. wadsworthia* WAL 7959 (ATCC 49260) (urease positive) was CGGCTGCTCCTCCTGC.

The *Bilophila* probe used in this study hybridized with all of the 12 *Bilophila* strains tested. The reactions with the four urease-negative strains were equivalent to those with the urease-positive strains (the probe strain was urease positive). From 2 to 2,000 ng of RNA was applied to a nylon membrane. Reactions were seen easily at the 20-ng level and sometimes faintly at the 2-ng level. Two of the eight other strains tested (*B. fragilis* and *B. thetaiotaomicron*) showed very faint reactivity at 2,000 ng (*B. fragilis*) and at 2,000 and 200 ng (*B. thetaiotaomicron*). This very faint reactivity was seen only with 10× SET buffer and not with 5× SET buffer. The probe reaction clearly distinguished between the *Bilophila* and the non-*Bilophila* strains. A representative blot is shown in Fig. 2.

Mixed cultures without *Bilophila* strains were not reactive with the *Bilophila* probe (Fig. 3). If a *Bilophila* strain was added to mixed cultures, gradually decreasing reactivity was seen as the dilution factor increased. The highest dilutions showing a faint trace of reactivity were 1:16 (at 20 ng of RNA) and 1:64 (at 200 ng of RNA).

The microorganisms used in the study of mixed cultures yielded 10^8 to 10^9 CFU/ml at the optical density used (with the exception of the *Lactobacillus* sp., which yielded 3.7×10^7 CFU/ml). We calculated that 3.45×10^8 *Bilophila* CFU yielded approximately 2,000 ng of RNA. On the basis of these data, it appears that the probe used in this study was able to detect $\geq 10^5$ CFU of *B. wadsworthia* per ml in artificially seeded mixed cultures that mimicked those seen in intestinal infections.

The oligonucleotide *B. wadsworthia* probe used in this limited study demonstrated good specificity and sensitivity. Both urease-positive and urease-negative strains were reactive, even though the probe was derived from a urease-positive strain. It appears that this probe is of potential use in the rapid identification of *B. wadsworthia* isolates. This probe will especially be useful since this organism is asaccharolytic and since the results obtained from the rapid diagnostic systems tested were not very specific (2). The observation that *B. wadsworthia* is usually β-lactamase positive and tends to be resistant to the bactericidal activity of some of the more commonly used antimicrobial agents could make this probe of a special utility. The ability to detect a *Bilophila* sp. at $\geq 10^5$ CFU/ml suggests that this probe has potential in identifying *Bilophila* strains in mixed infections.

Previously published reports suggest that this microorganism is an important pathogen, especially in intra-abdominal infections (1, 2, 4). Future studies may determine whether *B. wadsworthia* in counts of $<10^5$ CFU/ml in mixed cultures implies significant pathogenicity as well. If lower counts are

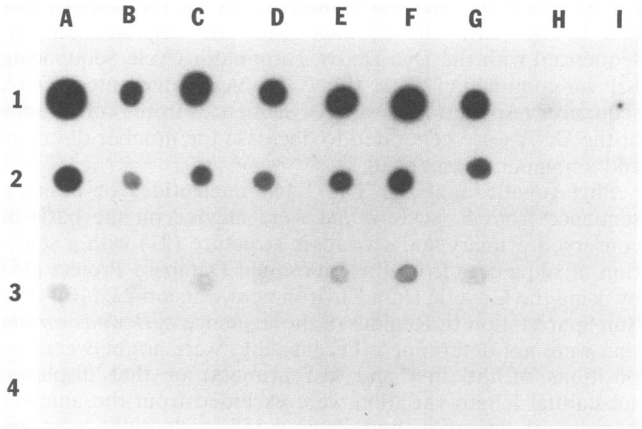

FIG. 2. Dot blot hybridization of *Bilophila* probe to RNA of individual strains of *B. wadsworthia* (A to G), *B. thetaiotaomicron* (H), and *B. fragilis* (I). Rows 1 to 4 are 2,000, 200, 20, and 2 ng of RNA, respectively.

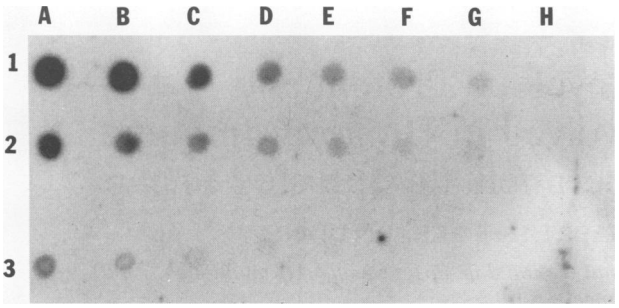

FIG. 3. Dot blot hybridization of *Bilophila* probe with a mixed culture of intestinal organisms seeded with 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64 dilutions of *B. wadsworthia* (A to G, respectively) or no *B. wadsworthia* (H). Rows 1 to 3 are 2,000, 200, and 20 ng of RNA, respectively.

shown to have clinical significance, PCR amplification may be used to augment the sensitivity of this probe. The utility of this probe in the direct detection of *B. wadsworthia* in clinical specimens merits further investigation.

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REFERENCES

1. **Baron, E. J., M. Curren, G. Henderson, H. Jausimies-Somer, K. Lee, C. A. Strong, P. Summanen, K. Tuner, and S. M. Finegold.** 1992. *Bilophila wadsworthia* isolates from clinical specimens. J. Clin. Microbiol. **30:**1882–1884.
2. **Baron, E. J., P. Summanen, J. Downes, M. C. Roberts, H. Wexler, and S. M. Finegold.** 1989. *Bilophila wadsworthia*, gen. nov. and sp. nov., a unique gram-negative anaerobic rod recovered from appendicitis specimens and human faeces. J. Gen. Microbiol. **135:** 3405–3411.
3. **Beaucage, S. L., and M. H. Caruthers.** 1981. Dideoxynucleotide phosphoramidites—a new class of key intermediates for deoxypolynucleotide synthesis. Tetrahedron Lett. **22:**1859–1862.
4. **Bennion, R. S., E. J. Baron, J. E. Thompson, J. Downes, P. Summanen, D. A. Talan, and S. M. Finegold.** 1990. The bacteriology of gangrenous and perforated appendicitis—revisited. Ann. Surg. **2:**165–171.
5. **Berent, S. L., M. Mahmoudi, R. M. Torczynski, P. W. Bragg, and A. P. Bollon.** 1985. Comparison of oligonucleotide and long DNA fragments as probes in DNA and RNA dot, Southern, Northern, colony and dot hybridizations. BioTechniques **3:**208–220.
6. **Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39:**783–791.
7. **Felsenstein, J.** 1989. PHYLIP—Phylogeny Inference Package (version 3.2). Cladistics **5:**164–166.
8. **Finegold, S., P. Summanen, S. Hunt-Gerardo, and E. Baron.** 1992. Clinical importance of *Bilophila wadsworthia*. Eur. J. Clin. Microbiol. Infect. Dis. **11:**1058–1063.
9. **Frothingham, R., R. L. Allen, and K. H. Wilson.** 1991. Rapid 16S ribosomal DNA sequencing from a single colony without DNA extraction or purification. BioTechniques **11:**40–44.
10. **Hill, G. B., and S. Schalkowsky.** 1990. Development and evaluation of the spiral gradient end point method for susceptibility testing of anaerobic gram-negative bacilli. Rev. Infect. Dis. **12** (Suppl. 2):S200–S209.
11. **Huibregtse, J. M., and D. R. Engelke.** 1986. Direct identification of small sequence changes in chromosomal DNA. Gene **44:**151–158.
12. **Jukes, T. H., and C. R. Cantor.** 1969. Evolution of protein molecules, p. 21–132. *In* H. N. Munro (ed.), Mammalian protein metabolism. Academic Press, Inc., New York.
13. **Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin, and N. R. Pace.** 1985. Rapid determinations of 16S ribosomal RNA sequences for phylogenetic analysis. Proc. Natl. Acad. Sci. USA **82:**6955–6959.
14. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 122–123. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Olsen, G. J., R. Overbeek, N. Larsen, T. L. Marsh, M. J. McCaughey, M. A. Maciukenas, W.-M. Kuan, T. J. Macke, Y. Xing, and C. R. Woese.** 1992. The Ribosomal Database Project. Nucleic Acids Res. **20:**2199–2200.
16. **Smith, S. W., C. Wang, P. M. Gillevet, and W. Gilbert.** 1992. Genetic Data Environment and the Harvard Genome Database. Genome mapping and sequencing. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Wexler, H. M., E. Molitoris, F. Jashnian, and S. M. Finegold.** 1991. Comparison of spiral gradient and conventional agar dilution for susceptibility testing of anaerobic bacteria. Antimicrob. Agents Chemother. **35:**1196–1202.
18. **Wilson, K. H., R. Blitchington, B. Hindenach, and R. C. Greene.** 1988. Species-specific oligonucleotide probes for rRNA of *Clostridium difficile* and related species. J. Clin. Microbiol. **26:**2484–2488.
19. **Wilson, K. H., R. B. Blitchington, and R. C. Greene.** 1990. Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. J. Clin. Microbiol. **28:**1942–1946.
20. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.
21. **Woese, C. R., R. Gutell, R. Gupta, and H. F. Noller.** 1983. Detailed analysis of the higher-order structure of 16S-like ribosomal ribonucleic acids. Microbiol. Rev. **47:**621–669.
22. **Zintz, C. B., and D. C. Beebe.** 1991. Rapid reamplification of PCR products purified in low melting point agarose gels. BioTechniques **11:**158–162.