Design and Evaluation of 16S rRNA-Targeted Peptide Nucleic Acid Probes for Whole-Cell Detection of Members of the Genus *Listeria*

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Six fluorescein-labeled peptide nucleic acid oligomers targeting *Listeria*-specific sequences on the 16S ribosomal subunit were evaluated for their abilities to hybridize to whole cells by fluorescence in situ hybridization (FISH). Four of these probes yielded weak or no fluorescent signals after hybridization and were not investigated further. The remaining two FISH-compatible probes, Lis-Un-3 and Lis-Un-11, were evaluated for their reactivities against 22 *Listeria* strains and 17 other bacterial strains belonging to 10 closely related genera. Hybridization with Bac-Un-1, a domain-specific eubacterial probe, was used as a positive control for target accessibility in both *Listeria* spp. and nontarget cells. RNase T1 treatment of select cell types was used to confirm that positive fluorescence responses were rRNA dependent and to examine the extent of nonspecific staining of nontarget cells. Both Lis-Un-3 and Lis-Un-11 yielded rapid, bright, and genus-specific hybridizations at probe concentrations of approximately 100 pmol ml⁻¹. Lis-Un-11 was the brightest probe and stained all six *Listeria* species. Lis-Un-3 hybridized with all *Listeria* spp. except for *L. grayi*, for which it had two mismatched bases. A simple ethanolic fixation yielded superior results with *Listeria* spp. compared to fixation in 10% buffered formalin and was applicable to all cell types studied. This study highlights the advantages of peptide nucleic acid probes for FISH-based detection of gram-positive bacteria and provides new tools for the rapid detection of *Listeria* spp. These probes may be useful for the routine monitoring of food production environments in support of efforts to control *L. monocytogenes*.

The genus *Listeria* is comprised of six species, *L. monocytogenes*, *L. grayi*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, and *L. welshimeri* (17). Of these species, only one, *L. monocytogenes*, is pathogenic to humans. The high mortality (~25 to 30%) associated with food-borne cases of listeriosis underscores the need for control of this pathogen. It has been suggested that the presence of generic *Listeria* in a food production facility can serve as an indicator for conditions that may support the growth of *L. monocytogenes* (5). New rapid genotypic methods for the detection of generic *Listeria* may therefore allow more efficient monitoring of the plant sanitation practices aimed at reducing the incidence of listeriosis.

Fluorescence in situ hybridization (FISH) is a rapid and highly specific nucleic acid-based method for the whole-cell identification of bacteria (3, 13). In the FISH technique, fluorescently labeled nucleic acid probes complementary to genus or species-specific rRNA sequences are hybridized to whole bacterial cells, resulting in the selective staining of target cells (13). As a whole-cell method, FISH allows the simultaneous collection of information on both cell morphology and molecular identity. Recently, two DNA-based FISH probes have been developed for the detection of *Listeria* spp. (20, 21). The first, Lis-1255 (*Escherichia coli* nucleotide positions 1255 through 1272), was originally reported for use as a PCR primer (26) but has been adapted for use as a FISH probe (20, 21). This probe is complementary to the 16S rRNA of all six species of *Listeria* but also reacts with *Brochothrix* spp. (20, 21, 25). The other probe, Lis-637 (*E. coli* nucleotide positions 637 through 658) (20, 21), reacts with all members of the genus *Listeria* except *L. grayi*. An ideal probe for the detection of generic *Listeria* would both be restricted to the genus and react with all six species.

Gram-positive bacteria present unique challenges to the use of DNA-based FISH probes due to the permeability barrier posed by their thick and highly anionic cell walls (8, 15). As a result, DNA-based FISH analysis of gram-positive cells often requires extensive preparatory steps, including lysozyme and proteinase K digestions (15, 25). Because an unknown sample may contain cells that differ markedly in their requirements for permeabilization, these steps may result in overdigestion and cell loss (25). Extensive processing may also result in the alteration of cellular light-scatter properties, which could interfere with analyses by fluorescence microscopy or flow cytometry that are often used in conjunction with FISH.

Peptide nucleic acid (PNA) is a pseudopeptide DNA mimic with an uncharged, achiral backbone (24). The unique chemical makeup of PNA probes confers a number of beneficial properties, including rapid hybridization kinetics, resistance to nucleases, and the ability to hybridize to positions on the ribosome that are inaccessible to DNA probes (24). PNA probes are also able to penetrate recalcitrant biological structures such as mycobacterial and gram-positive cell walls (22). In the present study, six *Listeria*-targeted PNA probes were evaluated for their suitability as FISH probes. Two probes were found to be FISH compatible and were evaluated for their specificities against a number of target and nontarget cells. This work adds two new probes to the set of tools available for the rapid molecular detection of *Listeria* spp. and clearly demonstrates...
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Listeria

previously (22). Briefly, 16S rRNA sequences representing all six species of

synthesized at Boston Probes, Inc. (Bedford, MA), and supplied by Applied

probes used in this study are given in Table 1. Probes were designed and

sequences of the peptide nucleic acid oligomers used in this study

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Systematic namea</th>
<th>Probe location</th>
<th>Sequence</th>
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<td>LisUn-2-1</td>
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<td>85–99, H61</td>
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<td>134–148, H122 and H144</td>
<td>CCC CAA CCT ACA GGC</td>
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<tr>
<td>LisUn-11</td>
<td>S-G-Lis-0466-a-A-14</td>
<td>466–480, H441</td>
<td>AAG GGA CAA GCA GT</td>
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<tr>
<td>LisUn-19</td>
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<td>1433–1449, H1399</td>
<td>GGT TAC CCT ACC GAC TT</td>
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<tr>
<td>LisUn-20</td>
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<td>1437–1453, H1399</td>
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</tr>
</tbody>
</table>

a Systematic names according to Alm et al. (1).
b E. coli base and helix numbering according to the rRNA secondary structure maps of Gutell et al. (9).
c Analogous to 5’-3’ convention used for DNA.
d Sequence reported previously by Perry-O’Keefe et al. (16).

tentially diagnostic target sequences were identified and checked against the GenBank database for significant similarities to nontarget sequences using BLAST (National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov]) and GeneMan softwares (version 3.3; DNASTAR, Madison, WI).

Bacterial strains. A total of 39 bacterial strains from both the genus Listeria and 10 closely related genera were examined. Nontarget strains were chosen in light of previous studies on the 16S rRNA-based phylogeny of the listeriae (4, 11, 19). From these alignments, potentially diagnostic target sequences were identified and checked against the GenBank database for significant similarities to nontarget sequences using BLAST (National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov]) and GeneMan softwares (version 3.3; DNASTAR, Madison, WI). Candidate sequences were also screened for secondary structure using Primer-Select software (version 4.03; DNASTAR, Madison, WI). Probes were synthesized according to the method previously described by Stender et al. (23), with the notable exception that solubility-enhancing groups (7) were not used, as these have been implicated in reduced hybridization efficiency against gram-positive bacteria (24). PNA probes (50 μl) were received suspended in 50% N,N-dimethylformamide (DMF) at an approximate concentration of 250 to 350 μM. Probes were diluted to a working concentration of approximately 100 μM in 50% DMF-water and stored in polypropylene microcentrifuge tubes at −20°C until needed.

the advantages of PNA probes for FISH-based detection of this genus.

MATERIALS AND METHODS

Chemicals. RNase T1 (EC 3.1.27.3, 90 Kunitz units mg−1) was from Sigma-Aldrich (St. Louis, MO); Unless otherwise stated, all chemicals were from Sigma-Aldrich or from Fisher Scientific (Itasca, IL). Microbiological media were from Difco Laboratories (Detroit, MI).

Probe design and synthesis. The common and systematic names, ribosomal locations (Escherichia coli base and helix numbering), and sequences of the PNA probes used in this study are given in Table 1. Probes were designed and synthesized at Boston Probes, Inc. (Bedford, MA), and supplied by Applied Biosystems, Inc. (Foster City, CA). Probe design was carried out as described previously (22). Briefly, 16S rRNA sequences representing all six species of Listeria and several closely related genera were aligned using MegAlign software (version 4.0; DNASTAR, Madison, WI). Choices regarding which genera should be represented in these alignments were informed by previous studies on the 16S rRNA-based phylogeny of the listeriae (4, 11, 19). From these alignments, potentially diagnostic target sequences were identified and checked against the GenBank database for significant similarities to nontarget sequences using BLAST (National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov]) and GeneMan softwares (version 3.3; DNASTAR, Madison, WI). Candidate sequences were also screened for secondary structure using Primer-Select software (version 4.03; DNASTAR, Madison, WI). Probes were synthesized according to the method previously described by Stender et al. (23), with the notable exception that solubility-enhancing groups (7) were not used, as these have been implicated in reduced hybridization efficiency against gram-positive bacteria (24). PNA probes (50 μl) were received suspended in 50% N,N-dimethylformamide (DMF) at an approximate concentration of 250 to 350 μM. Probes were diluted to a working concentration of approximately 100 μM in 50% DMF-water and stored in polypropylene microcentrifuge tubes at −20°C until needed.

Bacterial strains. A total of 39 bacterial strains from both the genus Listeria and 10 closely related genera were examined. Nontarget strains were chosen in light of previous studies on the 16S rRNA phylogeny of Listeria (4, 11, 19). The strains and their sources are listed in Tables 2 and 3. Each of the six Listeria

<table>
<thead>
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<th>Organism</th>
<th>Strain</th>
<th>Comment</th>
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<td>Type strain</td>
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<td>FSL-J2-066b</td>
<td>Serotype 1/2a</td>
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<td>Serotype 1/2b</td>
<td>+</td>
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<td>Listeria monocytogenes</td>
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<td>+</td>
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<td>DD6821b</td>
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<td>ATCC 19118c</td>
<td>Serotype 4e</td>
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<td>Listeria grayi</td>
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<td>Listeria seeligeri</td>
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<td>Listeria welshimeri</td>
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<td>Type strain</td>
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<tr>
<td>Listeria welshimeri</td>
<td>JLI-20d</td>
<td>+</td>
<td>+</td>
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</table>

a From Microbiologics, Inc., St. Cloud, MN.
b From Martin Wiedmann, Cornell University, Ithaca, NY.
c From the American Type Culture Collection, Manassas, VA.
d From CartoonGram, Inc., St. Paul, MN.
e From the American Type Culture Collection, Manassas, VA.
 f Type strain for L. murrayi.
g From Kathy Glass, University of Wisconsin Food Research Institute, Madison, WI.
h —, no strain designation.

TABLE 1. Common names, systematic names, locations, and sequences of the peptide nucleic acid oligomers used in this study

TABLE 2. Inclusivity data for LisUn-3 and LisUn-11 PNA probes/
Cells were centrifuged (2,000 rpm for 5 min), resuspended in fresh wash solution, and incubated for another 7 min. RNase treatment. In order to verify that PNA probes were targeting rRNA and to investigate the potential for nonspecific binding due to probe interactions with cell surfaces, suspensions of selected cells were treated with RNase T1 prior to hybridization. Ethanol-fixed cells (100 μl) of L. monocytogenes ATCC 15313, G. haemolymphus ATCC 10379, and Staphylococcus aureus ATCC 29123 were pelleted (2,000 × g, 7 min) and resuspended in 1 volume (100 μl) RNase T1 solution (4 mg ml⁻¹ in 0.1 M Tris [pH 8.0], 1 mM EDTA; final enzyme concentration, 36 Kunitz units). Cell suspensions were digested for 1 h at room temperature, pelleted (2,000 × g, 7 min), resuspended in probe-containing hybridization buffer, and hybridized as described above. To facilitate comparison between treatments, RNase-digested cells were hybridized in parallel with untreated cell suspensions.

**Flow cytometry.** Flow cytometry was used to examine the impact of different fixation methods on hybridization quality and to selectively identify *Listeria* subpopulations in mixed cultures after FISH. Samples were hybridized and washed as described above and then resuspended and diluted further (1:10) in 0.5 ml 10× TE buffer (0.1 M Tris [pH 8.0], 1 mM EDTA; final enzyme concentration, 36 Kunitz units). Cells were digested for 1 h at room temperature, pelleted (2,000 × g, 7 min), resuspended in probe-containing hybridization buffer, and hybridized as described above. To facilitate comparison between treatments, RNase-digested cells were hybridized in parallel with untreated cell suspensions.

**RESULTS**

Identification of FISH-suitable probes and effect of fixation method on hybridization quality. All bacterial strains hybridized with BacUni-1, indicating that they were fully permeable to peptide nucleic acid probes. In an initial screen against the formalin-fixed cells of all six *Listeria* type strains, hybridization with either LisUn-2-1 or LisUn-2-2 did not result in a detectable signal and signals from LisUn-19 and LisUn-20 were below the limit of detection. Flow cytometry was used to examine the impact of different fixation methods on hybridization quality and to selectively identify *Listeria* subpopulations in mixed cultures after FISH. Samples were hybridized and washed as described above and then resuspended and diluted further (1:10) in 0.5 ml 10× TE buffer (0.1 M Tris [pH 8.0], 1 mM EDTA; final enzyme concentration, 36 Kunitz units). Cells were digested for 1 h at room temperature, pelleted (2,000 × g, 7 min), resuspended in probe-containing hybridization buffer, and hybridized as described above. To facilitate comparison between treatments, RNase-digested cells were hybridized in parallel with untreated cell suspensions.

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sistently lower staining with FISH probes than did the other formalin-fixed Listeria spp. These results suggested that either these cells had lower rRNA content or they were poorly permeabilized by formalin fixation. In an effort to obtain higher quality hybridizations, an alternative, ethanol-based fixation step was investigated. This involved simply resuspending freshly harvested, washed cells in a 50:50 mixture of ethanol and PBS, followed by storage at −20°C prior to use. This method yielded stronger, more vivid hybridizations for Listeria spp. and was found to be suitable for all cell types investigated. This fixation protocol was used throughout the rest of this work.

Relative probe hybridization intensities. Although all cells yielded positive hybridizations with BacUni-1, the brightness of these hybridizations varied with each strain. Bacillus cereus, Kurthia spp., Enterococcus faecalis, C. divergens, and some Listeria spp. yielded exceptional results. The dimmest positive hybridization of BacUni-1 occurred with E. rhusiopathiae. Both LisUn-3 and LisUn-11 led to bright staining of stationary-phase target cells. However, LisUn-11 yielded uniformly brighter results than did LisUn-3, despite being present at a lower concentration (80 pmol ml⁻¹ versus 95 pmol ml⁻¹). For most nontarget cells, background staining was either undetectable or very low. Nonspecific staining was highest for G. haemolytics but was still too low to be mistaken for a positive reaction. A photograph depicting typical positive hybridization results for BacUni-1 is shown in Fig. 1A. A photograph depicting typical positive hybridization results for LisUn-11 is shown in Fig. 1B.

Probe inclusivity. Inclusivity is a measure of how comprehensively a probe reacts within its target group. A fully inclusive probe will react with all members of its target group (e.g., the genus Listeria). In order to determine the properties of inclusivity for each probe, LisUn-3 and LisUn-11 were screened against 22 Listeria strains, including the type strains of all six species and representatives of the L. monocytogenes serotypes most often implicated in human disease (1/2a, 1/2b, and 4b). LisUn-3 hybridized with all Listeria spp. except L. grayi. LisUn-11 hybridized with all Listeria spp., including L. grayi. Table 2 summarizes these data. Alignments for the region targeted by LisUn-3 (E. coli positions 134 through 148, Fig. 2a) show that nontarget strains have at least three mismatches to the probe sequence, L. grayi has two mismatches, and the other Listeria spp. are fully complementary to the probe. Alignments for the region targeted by LisUn-11 (E. coli positions 466 through 480) (Fig. 2b) show that most nontarget strains have at least four mismatches to the probe sequence and several strains have base insertions, deletions, or both. All Listeria spp. contain target sequences that are fully complementary to LisUn-11.

Probe exclusivity. Exclusivity is a measure of a probe’s restriction to its target group. An exclusive probe will not react with cells outside its target group. In order to determine the properties of exclusivity for each probe, LisUn-3 and LisUn-11 were screened against 17 nontarget organisms from 10 closely related genera. As expected from alignments of probe target regions (Fig. 2), neither probe hybridized to any cell type outside the genus Listeria. Table 3 summarizes these data.

RNase treatment. Positive hybridizations with the universal bacterial probe were interpreted as proof that nontarget cells were sufficiently permeabilized to allow access of PNA probes to target rRNA. However, because BacUni-1 is expected to hybridize with nearly all bacteria, it was recognized that false hybridizations due to nonspecific binding would not be immediately obvious. To address this potential pitfall and to verify the dependence on rRNA of the LisUn-3- and LisUn-11-con-
Fig. 2. Sequence variation in the 16S rRNA genes of *Listeria* and related genera. Alignments of partial sequences corresponding to the regions targeted by LisUn-3 (a) and LisUn-11 (b) are shown. The sequence of each probe is provided above the corresponding alignment. Residues differing from those found in the sequence of *L. monocytogenes* are boxed. For LisUn-3, the following GenBank accession numbers were aligned: X56153 (*L. monocytogenes*), X56151 (*L. ivanovii*), X56152 (*L. innocua*), X56148 (*L. welshimeri*), X56149 (*L. grayi*), X56150 (*B. thermosphacta*), X56151 (*S. aureus*), X68417 (*G. haemolysans*), L14326 (*K. zopfii*), X70321 (*E. faecalis*), M58798 (*L. fermentum*), X56157 (*S. aureus*), X56148 (*L. seeligeri*), X56149 (*L. ivanovii*), X56152 (*L. innocua*), X56153 (*L. monocytogenes*), X56150 (*B. thermosphacta*), and X84150 (*S. thermophilus*), X70321 (*G. haemolysans*), X68417 (*S. aureus*), L14326 (*G. haemolysans*), L14326 (*K. zopfii*), X70321 (*E. faecalis*), Z73313 (*Carnobacterium sp.*), and AF302116 (*G. haemolysans*).

Fig. 3. Flow cytometric comparison of cell fixation protocols. Cells from the same overnight culture of *L. monocytogenes* strain Scott A were fixed using either formalin or ethanol, as described in the text. In these histograms, cell number is plotted against probe-conferred fluorescence. The rightward shift and narrower distribution for the ethanol-fixed population (solid histogram) shows that this method resulted in brighter and more homogeneous hybridizations than did formalin-based fixation (dashed histogram).

**Flow cytometry.** Flow cytometry was used to study the effects of fixative choice on hybridization quality. Figure 3 shows that ethanol-fixed cells (solid histogram) were brighter and more uniformly hybridized than formalin-fixed cells (dashed histogram), confirming microscopic observations. The geometric mean fluorescence was used to provide a measure of hybridization intensity. Here, the log of the fluorescence was averaged and reported as a scaled value in fluorescence units. The geometric mean fluorescence was 35.9 for ethanol-fixed cell populations and 27.8 for formalin-fixed cell populations. The coefficient of variation was used to provide a measure of hybridization spread, or uniformity. Coefficients of variation for the ethanol-fixed and formalin-fixed populations were 59.5 and 68.3, respectively.

Flow cytometry was also used to demonstrate the ability of these probes to clearly differentiate *Listeria* spp. from nontarget flora in cell mixtures. The dot plot shown in Fig. 4 illustrates the probe-based detection of a subpopulation of *L. monocytogenes* (16% of the total population) against a high background of nontarget bacteria (*L. fermentum*, 84% of the total population).

**DISCUSSION**

Traditional media-based methods for the detection of *Listeria* spp. are time and labor intensive. Positive detection of *Listeria* in foods or environmental samples can take as long as 5 to 7 days using cultural approaches (14). New tools for the rapid and direct detection of *Listeria* are needed to help ensure the safety of foods or aid efforts to identify and control *Listeria* in food production environments. In this work, two rRNA-targeted peptide nucleic acid probes were used to achieve bright whole-cell hybridizations against stationary-phase *Listeria* spp. Neither of these probes showed any cross-hybridization with nontarget cells from 10 closely related genera. Probe
heat or alcohol-based methods (13, 18, 25). Because these methods yield improved results with DNA probes, it was reasonable to believe that they might also enhance cell wall permeability to PNA probes. In the present study, 10% buffered formalin was used initially, but fixation in 50% ethanol was found to be simpler, lessen concerns regarding fixative toxicity, and produce brighter, more uniform hybridizations.

In their work with the DNA-FISH probe Lis-1255, Wagner et al. (25) also used ethanol as a fixative. However, these authors reported that cultures of *L. monocytogenes* grown beyond 9 h in brain heart infusion broth were undetectable using ethanol-based fixation alone. Lysozyme and proteinase K digestions were required to fully permeabilize these cells to the probe. In contrast, the PNA probes studied here yielded rapid (10 to 30 min) and bright hybridizations with stationary-phase cells (18 to 24 h growth) without any need for permeabilization beyond the initial ethanol fixation. It is also worth noting that all of the PNA work done here was accomplished in solution, whereas most literature reports of FISH-based detection of gram-positive bacteria are performed with cells adhered to microscope slides or membrane filters (10, 12, 13, 25). The effects of multiple enzyme digestions on the solution-phase behavior of *Listeria* cells are unknown, but cell clumping could be problematic. In the present study, PNA-hybridized *Listeria* spp. formed even suspensions of individual cells and results of PNA-FISH experiments were readily analyzed using flow cytometry.

The second major advantage to the use of PNA for FISH-based detection of bacteria is that these probes are able to bind areas of the ribosome that are inaccessible to DNA probes. PNA-FISH probes are hybridized under substantially different conditions than are their DNA counterparts. Typically, these hybridizations are carried out under low salt (100 mM NaCl), high-temperature (55°C), and high-pH (pH 9.0) conditions. These conditions contrast with those commonly used in DNA-based FISH protocols (900 mM NaCl, 46°C, and a pH of 7.0 to 8.0) (3, 6, 18). Nucleic acid secondary structures are stabilized at high salt conditions through charge-shielding effects. Low salt conditions are therefore destabilizing for these structures. Alkaline or high-temperature conditions are also common means for nucleic acid denaturation (2). The combined effects of low salt, high pH, and high temperature suggest that target nucleic acids (rRNA) are likely to be in nonnative form (e.g., denatured) under the conditions of PNA hybridization. These ribosome-denaturing conditions are thought to alleviate the influence of a higher-order ribosomal structure on probe accessibility, rendering highly structured regions of the ribosome accessible for detection by FISH (23). The bright hybridization for LisUn-11 (*E. coli* nucleotide positions 466 through 480) highlights this effect, as previous work using DNA-FISH probes has characterized the region spanning *E. coli* nucleotide positions 468 through 486 as the least accessible on the entire 16S subunit (6). Although LisUn-3 targets a supposedly more accessible region of the ribosome (6), hybridizations with this probe were not as bright as those with LisUn-11. This result likely reflects differences in melting temperature between the two probes. The estimated melting temperature of LisUn3 is 70°C, while that of LisUn-11 is 82°C. These data suggest that results with LisUn-3 could be improved by adjusting the hybridization temperature to a lower value (e.g., <55°C).
Sequence comparisons of both 16S and 23S rRNA have demonstrated that the genus *Listeria* can be divided into two relatedness groups: one containing both *L. grayi* subsp. *grayi* and *L. grayi* subsp. *murrayi* and the other containing the rest of the genus (4, 19). This split in sequence homology is reflected in the difficulty of identifying rRNA-targeted probes that both selectively hybridize to the genus *Listeria* and encompass all six *Listeria* species. However, for applications such as environmental monitoring of *Listeria* in food-processing plants, such probes are required. From all appearances, LisUn-11 is an ideal probe for the FISH-based detection of generic *Listeria*. This probe was very bright and hybridized to all *Listeria* spp., including *Listeria* *murrayi*, *Listeria* *monocytogenes*, *Listeria* *spp.*, *Dekkera bruxellensis*, and the other containing the rest of *Listeria* species.

The pace and volume of today’s food production and distribution networks places new emphasis on the need for rapid detection methods for organisms such as *Listeria*. The PNA probes described here provide the food safety community with a powerful and versatile tool for rapid detection, identification and enumeration of specific microorganisms. The development of LisUn-11 is especially timely in light of recently proposed environmental testing requirements for generic *Listeria* (5). Apart from the use described here, these probes may also be adapted for additional assay formats (membrane-based detection of microcolonies, RNA dot blots, as reporter probes for real-time PCR, etc.), although their use will need to be optimized for each application. As part of a *Listeria* testing program, use of these probes may help prevent costly product recalls and reduce the incidence of disease.

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REFERENCES