11-26-2014

Design and evaluation of peptide nucleic acid probes for specific identification of Candida albicans.

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Abstract
Candida albicans is an important cause of systemic fungal infections, and rapid diagnostics for identifying and differentiating C. albicans from other Candida species are critical for the timely application of appropriate antimicrobial therapy, improved patient outcomes, and pharmaceutical cost savings. In this work, two 28S rRNA-directed peptide nucleic acid-fluorescence in situ hybridization (PNA-FISH) probes, P-Ca726 (targeting a novel region of the ribosome) and P-CalB2208 (targeting a previously reported region), were evaluated. Hybridization conditions were optimized by using both fluorescence microscopy (FM) and flow cytometry (FCM), and probes were screened for specificity and discriminative ability against a panel of C. albicans and various nontarget Candida spp. The performance of these PNA probes was compared quantitatively against that of DNA probes or DNA probe/helper combinations directed against the same target regions. Ratiometric analyses of FCM results indicated that both the hybridization quality and yield of the PNA probes were higher than those of the DNA probes. In FCM-based comparisons of the PNA probes, P-Ca726 was found to be highly specific, showing 2.5- to 5.5-fold-higher discriminatory power for C. albicans than P-CalB2208. The use of formamide further improved the performance of the new probe. Our results reinforce the significant practical and diagnostic advantages of PNA probes over their DNA counterparts for FISH and indicate that P-Ca726 may be used advantageously for the rapid and specific identification of C. albicans in clinical and related applications, especially when combined with FCM.

Disciplines
Food Microbiology | Food Science | Fresh Water Studies | Human and Clinical Nutrition | Molecular, Genetic, and Biochemical Nutrition | Plant Sciences

Comments
This article is published as Kim, H.J. and **Brehm-Stecher, B.F.** Design and evaluation of peptide nucleic acid probes for specific identification of *Candida albicans*. *J. Clin. Microbiol.* 53: 511-21 (2015). Doi: 10.1128/JCM.02417-14. Posted with permission.

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Design and Evaluation of Peptide Nucleic Acid Probes for Specific Identification of *Candida albicans*

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Candida albicans **is an important cause of systemic fungal infections, and rapid diagnostics for identifying and differentiating *C. albicans* from other *Candida* species are critical for the timely application of appropriate antimicrobial therapy, improved patient outcomes, and pharmaceutical cost savings. In this work, two 28S rRNA-directed peptide nucleic acid-fluorescence *in situ* hybridization (PNA-FISH) probes, P-Ca726 (targeting a novel region of the ribosome) and P-CalB2208 (targeting a previously reported region), were evaluated. Hybridization conditions were optimized by using both fluorescence microscopy (FM) and flow cytometry (FCM), and probes were screened for specificity and discriminative ability against a panel of *C. albicans* and various nontarget *Candida* spp. The performance of these PNA probes was compared quantitatively against that of DNA probes or DNA probe/helper combinations directed against the same target regions. Ratiometric analyses of FCM results indicated that both the hybridization quality and yield of the PNA probes were higher than those of the DNA probes. In FCM-based comparisons of the PNA probes, P-Ca726 was found to be highly specific, showing 2.5- to 5.5-fold-higher discriminatory power for *C. albicans* than P-CalB2208. The use of formamide further improved the performance of the new probe. Our results reinforce the significant practical and diagnostic advantages of PNA probes over their DNA counterparts for FISH and indicate that P-Ca726 may be used advantageously for the rapid and specific identification of *C. albicans* in clinical and related applications, especially when combined with FCM.**

The genus *Candida* includes several species that are pathogenic for humans, including *Candida albicans*, *C. dubliniensis*, *C. krusei*, *C. glabrata*, *C. tropicalis*, and others. Early detection and differentiation of pathogenic *Candida* species from patient samples are critical to patient outcomes. *Candida albicans* is the major pathogen in this genus, causing approximately one-half of all infections caused by *Candida* spp. (1, 2). Because *Candida* spp. differ in their patterns of resistance to common antifungals, differentiation of *C. albicans* (fluconazole responsive) from fluconazole-resistant species such as *C. krusei*, *C. glabrata*, and *C. tropicalis* is required for appropriate antimicrobial therapy (1–6). The close phenotypic similarity between *C. dubliniensis* and *C. albicans* further underlines the need for specific identification of *C. albicans* in clinical settings (7, 8).

Peptide nucleic acid (PNA) probes are synthetic DNA mimics with improved performance characteristics compared to DNA probes. Specifically, these characteristics include faster hybridization kinetics, enhanced single-mismatch discrimination, and improved penetration of structures such as the cell wall (9, 10). Fluorescence *in situ* hybridization (FISH) is a rapid method for whole-cell detection of specific microorganisms. The clinical utility of FISH for the detection of *Candida* spp. has been shown by using DNA-based probes targeting the small (18S) ribosomal subunit (11–15). The advent of PNA and other probe technologies and the expansion of public databases containing previously limited large-subunit (28S) rRNA gene sequences have led to further improvements in FISH-based detection of *Candida* spp. (3–6, 9, 10, 16–22; J. J. Hyldig-Nielsen, H. Stender, K. M. Oliveria, and S. Rigby, U.S. patent application US2003/0175727 A1). Commercial probes targeting *C. albicans* include the DNA-based molecular beacon (18S gene) sold by Miacom Diagnostics, Inc. (Research Triangle Park, NC, USA), and the PNA probe (28S gene) available from AdvanDx, Inc. (Woburn, MA, USA). Identification and characterization of previously undescribed variable regions in the yeast 28S rRNA gene may allow the development of new probes or probe sets with advantageous properties, including greater discrimination between *C. albicans* and other *Candida* spp.

The present study builds on our previous work using DNA-based FISH (DNA-FISH) for detection of *C. albicans*, with a key interest being whether further improvements in performance characteristics could be gained through the use of PNA technology. In our previous work, we evaluated CalB2208, a 28S rRNA-targeted DNA probe reported in the patent literature, and found it to be both suitable for FISH and specific for *C. albicans* (23). The purpose of this study was 3-fold: (i) to evaluate the performance of a PNA version of CalB2208 and to compare its performance to that of its DNA counterpart, (ii) to screen for and identify new regions within the 28S rRNA gene that may allow specific detection of *C. albicans* or other clinically important *Candida* spp., and (iii) to evaluate the performance of *C. albicans*-targeted PNA probes resulting from this screen by using both qualitative (fluorescence microscopy [FM]) and quantitative (flow cytometry [FCM]) methods.

Received 20 August 2014 **Returned for modification** 22 September 2014
Accepted 24 November 2014

Accepted manuscript posted online 26 November 2014

Citation Kim H-J, Brehm-Stecher BF. 2015. Design and evaluation of peptide nucleic acid probes for specific identification of *Candida albicans*. J Clin Microbiol 53:511–521. doi:10.1128/JCM.02417-14.

Editor: D. W. Warnock

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doi:10.1128/JCM.02417-14

TABLE 1 Yeast strains used in this study

Organism[a]	Strain
Candida albicans	ATCC 10231[b]
C. albicans	ATCC 90028[b]
C. dubliniensis	ATCC MYA-646
C. dubliniensis	ATCC MYA-583
C. dubliniensis	ATCC MYA-577
C. dubliniensis	ATCC MYA-180
C. glabrata	ATCC 15126[b]
C. tropicalis	ATCC 750
C. parapsilosis	ATCC 22019[b]
C. krusei	ATCC 14243[b]
Zygosaccharomyces bailii	ATCC 60483
C. albicans	ATCC 752
C. albicans	ATCC 14053
C. amphixiae	ATCC MYA-4331
C. bohiensis	ATCC MYA-4363
C. buenavistaensis	ATCC MYA-4365
C. catenulata	ATCC 10565
C. chauliodes	ATCC MYA-4356
C. colliculosa	ATCC 2507
C. dubliniensis	ATCC MYA-179
C. famata	ATCC 2560
C. glabrata	ATCC 2001
C. gropengiesseri	ATCC 10669
C. guilliermondii	ATCC 6260
C. holmii	ATCC 10670
C. kefyr	ATCC 2512
C. krusoides	ATCC 7345
C. labiduridarum	ATCC MYA-4368
C. lambica	ATCC 2146
C. melinii	ATCC 10568
C. molischiana	ATCC 2516
C. neerlandica	ATCC MYA-4367
C. parapsilosis	ATCC 96141
C. parapsilosis	ATCC 96143
C. rugosa	ATCC 10571
C. sphaerica	ATCC 2504
C. stellata	ATCC 10667
C. tetrigidarum	ATCC MYA-4369
C. tropicalis	ATCC 66029
C. valida	ATCC 9982
C. zeylanoides	ATCC 4933

[a] Organisms comprising the initial limited test panel are shown in boldface type. The remaining strains represent those added to form the expanded test panel.
[b] Sourced from Microbiologics, Inc. (St. Cloud, MN).

MATERIALS AND METHODS

***Candida* strains and culture conditions.** *Candida* species and other related strains used in this study were collected from the American Type Culture Collection (ATCC) (Manassas, VA, USA) or from Microbiologics, Inc. (St. Cloud, MN, USA), as shown in Table 1. Strains were grown on yeast mold (YM) agar plates (Becton, Dickson and Company, Sparks, MD, USA) with overnight incubation at between 25°C and 30°C. Individual colonies were inoculated into YM broth (10 ml in 50-ml conical tubes) and incubated at 35°C for 15 to 17 h on a rotary shaker (230 rpm) (model SI2/-2; Sheldon Manufacturing, Cornelius, OR, USA).

Alignment of 28S rRNA gene sequences of *Candida albicans* and other *Candida* spp. The following 28S rRNA gene sequences of nine clinically important *Candida* spp. and one *Saccharomyces cerevisiae* strain were obtained from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/): *C. albicans* SC5314 (locus tag Ca.rrn003; GenBank accession number AACQ01000295), *C. albicans* WO-1 chromosome R cont1.18 (region spanning positions 11095 to 14456; accession number AAFO01000018), the *C. dubliniensis* 18S rRNA gene (accession number AF405231), *C. dubliniensis* CD36 chromosome R (region spanning positions 1865607 to 1869583), *C. albicans* WO-1 (chromosome R cont1.18, whole-genome shotgun sequence, region spanning positions 11095 to 14456; accession number NC_012867), *C. glabrata* CBS 138 (chromosome L, region spanning positions C1448197 to 1444790; accession number NC_006035), the *C. orthopsilosis* strain 90-125 25S rRNA gene (region spanning positions 3546 to 6908; accession number FN812686), *C. parapsilosis* strain CDC317 25S rRNA (region spanning positions 322944 to 326306; accession number HE605209), *C. tropicalis* ATCC MYA-3404 (genomic scaffold supercont3.10, region spanning positions 409292 to 412653; accession number GG692404), and *Saccharomyces cerevisiae* YJSH1 (contig.chr12.02, whole-genome shotgun sequence, region spanning positions 80971 to 77577; accession number AGAW01000022). These 28S rRNA gene sequences were aligned by using Vector NTI Advance (version 11.5; Invitrogen) and screened for potentially diagnostic sequences for evaluation as targets for the development of new FISH probes.

Design of DNA and PNA probes. As shown Table 2, a total of three regions were chosen in for targeting with DNA and/or PNA probes. One of these regions (CalB2208 [28S rRNA gene positions 2033 to 2055]) was previously shown by our group to be suitable as a target for DNA-FISH, and the remaining two (Ca2536 [positions 2536 to 2556] and Ca720 [Ca726 for PNA] [positions 720 to 740]) were newly selected in this work. Apart from spanning key differences within each gene sequence, probe composition was also guided by practical considerations such as melting temperature (T_m), with PNA probes being restricted to 15 nucleobases due to the intrinsically higher T_m of PNA.

DNA and PNA probe synthesis and preparation. All DNA and PNA probes were labeled with 6-carboxyfluorescein (FAM) at the 5′ end (DNA probes) or the N terminus (PNA probes), with the exception of one DNA probe labeled at the 3′ end due to the existence of a potential fluorophore-quenching guanine residue at the 5′ end. DNA probes were synthesized by Integrated DNA Technologies (IDT) (Coralville, IA, USA). PNA probes were synthesized by Panagene (Daejeon, South Korea) and obtained from a licensed distributor (PNA Bio, Inc., Thousand Oaks, CA, USA), with the exception of the EuUni-1 probe, which was donated by AdvanDx, Inc. (Woburn, MA, USA). DNA probes were resuspended in "low-TE" buffer (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA) to a concentration of 100 pmol/μl (100 μM) and stored frozen at −20°C. PNA probes were resuspended in dimethyl formamide (DMF) at a concentration of 100 pmol/μl (100 μM), with incubation at 50°C and with periodic agitation to ensure complete probe suspension. Once in suspension, PNA probes in DMF were stored at −20°C.

Cell fixation. For each yeast strain, 1 ml of liquid culture was added to a 1.5-ml microcentrifuge tube and harvested by centrifugation (2,000 × *g* for 5 min). The bulk of the supernatant was removed, and the pellet was vigorously vortexed for 30 s in order to resuspend the pellet in the remaining ~25 to 30 μl of supernatant. One milliliter of fixative solution (a 6:4 mixture of 10% neutral buffered formalin [HT50; Sigma] and absolute ethanol) was added, and the mixture was vortexed thoroughly and held at ambient temperature (~25°C) for 1 h. To prevent clumping due to cell sedimentation, tubes were inverted by hand (~5 times every 10 min) during the fixation process. Fixed cells were harvested as described above, resuspended in 1 ml cell storage buffer (a 1:1 mixture of 1× phosphate-buffered saline [PBS] and absolute ethanol), and then stored at −20°C until needed.

Fluorescence *in situ* hybridization. Previously fixed cells in storage buffer were vortexed thoroughly, 100 μl of this suspension was transferred into new microcentrifuge tubes, and cells were pelleted via centrifugation (2,000 × *g* for 5 min). The bulk of the supernatant was removed, and the pellet was vigorously vortexed for 30 s in order to resuspend the pellet in the remaining ~25 to 30 μl of cell storage solution. One hundred microliters of DNA or PNA hybridization buffer containing each type of

TABLE 2 DNA and PNA probes used in this study

Probe[a]	Systematic name[b]	Probe sequence (5′–3′ for DNA or N terminus to C terminus for PNA)[e]	Size (bp)	T_m (°C)[c]	Probe location (base positions in 28S rRNA gene)	Reference(s) and/or source
D-CalB2208		FAM-CGG CCA TAA AGA CCT ACC AAG CG	23	60	2033–2055	20, 23
DH-CalB2171		TGT CTA CAG CAG CAT CCA CCA GCA GTC CGT CGT G	34	68.8	1999–2032	20, 23
DH-CalB2233		CCA GTT CTA AGT TGA TCG TTA AAC GTG CCC CGG A	34	65.1	2056–2089	20, 23
D-Ca2536		FAM-CGG ATC GCC CAG AGG GCT TAA	21	61.6	2536–2556	Hogan et al., U.S. patent application 20100159530 A1; this study
D-Ca720		GCA CCC TAA TGG GCC CCC AC-FAM[d]	20	63.6	720–739	This study
DH-Ca690		TCG TTC ACT TTC ATT ACG CGT ACG GGT T	28	61.8	690–717	This study
DH-Ca741		TCC GAA CAC ATC AGG ATC GGT CGA TGG	27	63.4	741–767	This study
P-CalB2208	L-S-C.alb-741-b-A-15	FAM-OO-CGG CCA TAA AGA CCT	15	75.5	2041–2055	This study
P-Ca2536	L-S-C.alb-2536-b-A-15	FAM-OO-GCC CAG AGG GCT TAA	15	79.9	2536–2550	Hogan et al., U.S. patent application 20100159530 A1; this study
P-Ca726	L-S-C.alb-726-b-A-15	FAM-OO-TGC ACC CTA ATG GGC	15	77.1	726–740	This study
D-Euk 516		TEX-ACC AGA CTT GCC CTC C	16	75.2		33
P-EuUni-1		FAM-OO-ACC AGA CTT GCC CTC	15	72.8		34

[a] Initial capital letters indicate the type and use of each probe, as follows: D, DNA probe; P, PNA probe; DH, DNA helper probe. The same convention is also used here for previously reported DNA and PNA probes.
[b] Systematic names according to Alm et al. (35). Systematic names are noted here only for newly reported probes. Systematic names are based on absolute 28S rRNA gene base numbering, determined by using NCBI GenBank accession number AACQ01000295, as described in Materials and Methods.
[c] The T_m of DNA was calculated by using the default settings of OLIGOAnalyzer software (Integrated DNA Technologies); the T_m of PNA was calculated by using PNA tool software (PNA Bio).
[d] 3′ labeling was used to avoid potential fluorescence quenching by the 5′ G residue.
[e] FAM, 6-carboxyfluorescein; TEX, Texas Red; O, 8-amino-3,6-dioxaoctanoic acid linker.

probe at concentrations ranging from 0.2 to 2.0 μM (or up to 2.5 μM for DNA helper probes) was added to the concentrated cell suspension. DNA hybridization buffer consisted of 100 mM Tris (pH 8.0), 10 mM EDTA, 0.1% sodium dodecyl sulfate, and 0.9 M NaCl with or without 10% formamide, depending on the experiment; PNA hybridization buffer contained 20 mM Tris-HCl (pH 9.0), 2 mM EDTA, 100 mM NaCl, and 0.5% SDS with or without 10% formamide, depending on the experiment. Upon the addition of probes, cell suspensions were incubated at 55°C (Thermomixer R; Eppendorf North America, Hauppauge, NY, USA) and hybridized for 1 h, with shaking every 10 min. Next, 1 ml of preheated and probe-free DNA hybridization buffer or PNA wash solution (10 mM Tris [pH 8.0], 1 mM EDTA) was added to remove any nonspecifically bound probe. This washing step was carried out for 30 min at 55°C with continuous shaking, cells were harvested via centrifugation (2,000 × *g* for 5 min), the supernatant was removed, and hybridized cells were resuspended in 200 μl PBS for FM or diluted further for FCM.

Fluorescence microscopy. Ten microliters of each hybridized cell suspension was added to the surface of a poly-L-lysine-coated slide (Polysciences, Warrington, PA, USA), smeared with a pipette tip for even distribution, and dried on a hot plate. Ten microliters of antifade solution (Vectashield mounting medium with 4′,6-diamidino-2-phenylindole [DAPI]; Vector Laboratories, Burlingame, CA, USA) was added to the slide and distributed with gentle compression after the addition of a coverslip. Samples were viewed on a Leitz Laborlux S fluorescence microscope equipped with a 63× 1.4-numerical-aperture (NA) oil immersion objective, and photographs (not shown) were taken by using a Canon PowerShot A640 consumer-grade digital camera (Canon Canada, Inc., Mississauga, Ontario, Canada) controlled with Axiovision software (v. 4.6; Carl Zeiss Microimaging, Inc., Thornwood, NY, USA).

Flow cytometry. FCM was performed on a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) with 488-nm excitation. Samples were examined by using the "fast" setting, and green fluorescence was collected in the FL1 channel (533/30-nm-band-pass filter) by using yeast side scatter to trigger event detection. FCM data were analyzed by using the BD Accuri C6 software package. As is typical of FCM analysis, absolute fluorescence values varied for experiments conducted on different days, but trends between treatments and across different experiments remained consistent, and representative data are shown.

RESULTS

Target-specific 28S rRNA gene regions and DNA/PNA probe design. A total of three unique 28S rRNA gene regions where sequences for *C. albicans* and other *Candida* spp. differed were identified by sequence alignment and used to design DNA and PNA probes for the specific identification of *C. albicans*. One region (28S ribosomal DNA [rDNA] positions 2033 to 2055, targeted by the D-CalB2208 and P-CalB2208 probes) was previously identified in the patent literature (20) and later shown to be suitable for use in DNA-based FISH (23). An additional region (28S rDNA positions 2536 to 2556, targeted by the D-Ca2536 and P-Ca2536 probes), also from the patent literature (J. J. Hogan, I. Andruszkiewicz, J. J. Bungo, and S. K. Kaplan, U.S. patent application 20100159530 A1), was evaluated for use in FISH. Finally, the region spanning 28S rDNA positions 720 to 739 and targeted by the D-Ca720 and P-Ca726 probes was identified and is first reported here. Sequence alignments, probe positions, mismatch locations,

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D-CalB2208 probe                3′-GCGAACCATCC.AGAAATAC.CGGC-5′
P-CalB2208 probe                C′-........TCC.AGAAATAC.CGGC-N′
C. albicans SC5314              5′-CGCTTGGTAGG.TCTTTATG.GCCG-3′
C. albicans WO-1                   CGCTTGGTAGG.TCTTTATG.GCCG
C. dubliniensis AF405231           CGCTTGGTAGG.CTCTTGTA.GCCG
C. dubliniensis CD36               CGCTTGGTAGG.CTCTTGTA.GCCG
C. glabrata CBS 138                GTCTTGGTAGGTCTCTTGTAGGCCG
S. cerevisiae YJSH1                GCCTTGGTAGGTCTCTTGTAGACCG
C. orthopsilosis                   CACTTGGTAGG.CATTTAT..GCCG
C. parapsilosis CDC317             CACTTGGTAGG.CATTTAT..GTCG
C. tropicalis MYA-3404 cont3.10    CGCTTGGTAGG.TTCTTGTA.ACCG

D-Ca720 probe                   3′-CACCCCCG..GGTAA....TCCCACG.-5′
P-Ca726 probe                   C′-......CG..GGTAA....TCCCACGT-N′
C. albicans SC5314              5′-GTGGGGGC..CCATT....AGGGTGCA-3′
C. albicans WO-1                   GTGGGGGC..CCATT....AGGGTGCA
C. dubliniensis AF405231           ATGGGGGC..CTGTA....TGGGTGCA
C. dubliniensis CD36               ATGGGGGC..CTGTA....TGGGTGCA
C. glabrata CBS 138                GTTGGGGCCCTCCACCTGGGGGGTGCA
S. cerevisiae YJSH1                GTTGGGGCCTCGCAA....GAGGTGCA
C. orthopsilosis                   GTAGGACCTCCTTTA....GGAGTGCA
C. parapsilosis CDC317             GTAGGACCTCCTTTA....GGAGTGCA
C. tropicalis MYA-3404 cont3.10    GTGGGGGC..CCGTA....TGGGTGCA
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FIG 1 Sequence variation in the 28S rRNA genes of *C. albicans* and nontarget *Candida* spp. and antiparallel alignment of DNA- and PNA-FISH probes targeting the two probe regions examined in this work. The following sequences were aligned: *C. albicans* SC5314 (locus tag Ca.rrn003; GenBank accession number AACQ01000295), *C. albicans* WO-1 chromosome R cont1.18 (region spanning positions 11095 to 14456; accession number AAFO01000018), the *C. dubliniensis* 18S rRNA gene (accession number AF405231), *C. dubliniensis* CD36 chromosome R (region spanning positions 1865607 to 1869583), *C. albicans* WO-1 (chromosome R cont1.18, whole-genome shotgun sequence, region spanning positions 11095 to 14456; accession number NC_012867), *C. glabrata* CBS 138 (chromosome L, region spanning positions C1448197 to 1444790; accession number NC_006035), the *C. orthopsilosis* strain 90-125 25S rRNA gene (region spanning positions 3546 to 6908; accession number FN812686), *C. parapsilosis* strain CDC317 25S rRNA (region spanning positions 322944 to 326306; accession number HE605209), *C. tropicalis* ATCC MYA-3404 (genomic scaffold supercont3.10, region spanning positions 409292 to 412653; accession number GG692404), and *Saccharomyces cerevisiae* YJSH1 (contig.chr12.02, whole-genome shotgun sequence, region spanning positions 80971 to 77577; accession number AGAW01000022). Residues differing from those present in the *C. albicans* sequence are shown in boldface type.

and sequences of DNA and PNA probes (including helper probes) used in this study are provided in Fig. 1 and Table 2. As noted in Materials and Methods, choices regarding DNA or PNA probe length included considerations of both intrinsic differences in T_m values between DNA and PNA and, for the PNA probes especially, the type and position of mismatches between *C. albicans* and other yeasts within the target region. DNA probes ranged between 20 and 23 nucleotides in length, and PNA probes contained 15 nucleobases. The *in silico* specificities of probes and their helpers were verified individually by using the probeCheck database (http://131.130.66.200/cgi-bin/probecheck/content.pl?id=home) (24). Because initial testing of PNA probes against *C. albicans* and other *Candida* spp. (at a 0.2 μM concentration) resulted in only weak hybridizations with P-Ca2536, this probe was not evaluated further.

Evaluation and optimization of PNA-FISH parameters. In order to optimize PNA-FISH for probes P-CalB2208 and P-Ca726, various factors, including probe concentration, hybridization time, and salt concentration, were examined. Additionally, we sought to evaluate the abilities of DNA helper probes to enhance PNA-FISH in a hybrid DNA/PNA system. Results for these experiments are summarized in Fig. 2. Due to the number of factors examined, these experiments were limited to two *Candida* spp., *C. albicans* (ATCC 90028 or ATCC 10231, depending on the experiment) and the closely related *C. dubliniensis* (ATCC MYA-180 or ATCC MYA-646, depending on the experiment). Responses of target and nontarget cell types to hybridization parameters were determined by using FCM, and ratiometric analyses were used to investigate probe discriminatory power. Not surprisingly, higher probe concentrations and longer hybridization times led to brighter hybridizations with *C. albicans*, as shown in Fig. 2A and B. Also, as demonstrated in other studies of PNA, increasing salt concentrations led to lower hybridization intensities for both PNA probes (Fig. 2C). Finally, DNA helper probes were examined for their abilities to enhance PNA probe signals in a DNA/PNA hybrid system. As illustrated in Fig. 2D, the presence of DNA helper probes had a positive, concentration-dependent impact on PNA-FISH results, although this effect was not dramatic. Based on our evaluation of these PNA-FISH hybridization parameters, subsequent experiments were carried out by using 0.5 μM PNA probe, 1 h of hybridization at 55°C, and 100 mM NaCl, without DNA helper probes. In order to conserve these reagents, the lowest effective concentrations of PNA probes were used for this exploratory research. However, higher probe concentrations may be used in future applications.

Comparative performances of DNA and PNA probes. The original motivation for this study was to examine the impact of the use of PNA technology on the quality of hybridizations with CalB2208, which we had demonstrated in a previous report to be both suitable for DNA-FISH and specific for *C. albicans* (23). Although we had achieved satisfactory results with DNA-FISH, we hypothesized that the use of PNA would result in the brighter, faster, and more uniform hybridizations typical of PNA chemistry (25). After optimization of hybridization conditions for the two PNA probes, the performances of DNA-FISH (with and without DNA helper probes) and PNA-FISH probes were compared directly. The results shown in Fig. 3A and B clearly demonstrate the superior performance of the PNA probes over DNA probes or DNA probe/helper combinations targeting the same region. Although DNA-FISH with and without helpers enabled the differentiation of positive and negative samples for cells grown under optimal conditions, PNA probe hybridizations were characterized by dramatic improvements over DNA probes in staining intensity and uniformity, with the absolute fluorescence of the P-CalB2208 probe showing a 7.2-fold increase in hybridization yield over the D-CalB2208 probe and with that of P-Ca726 showing a 2.3-fold increase in hybridization yield over D-Ca720 (Fig. 3).

Analysis of PNA probe specificity and discriminative power. Although sequence alignment and *in silico* (probeCheck) vetting of P-CalB2208 and P-Ca726 probe sequences indicated their uniqueness for *C. albicans*, probe performance must ultimately be confirmed by testing against a panel of target and nontarget organisms. In an initial expanded test of the specificity of P-CalB2208 and P-Ca726, we used the same core panel of organisms examined in our previous report on the suitability of the CalB2208 probe for DNA-FISH (23), modified to contain an additional *C. albicans* strain (ATCC 90028) and replacing the basidiomycete *Rhodotorula mucilaginosa* with the ascomycete *Zygosaccharomyces bailii* as the negative control. Figure 4A shows hybridization results for P-CalB2208 and P-Ca726. While the overall fluorescence intensity of P-CalB2208 was higher, so was the background signal. Assuming that the higher levels of background for P-CalB2208 resulted from nonspecific fluorescence instead of actual cross-hybridization, our data suggested that both probes were specific for

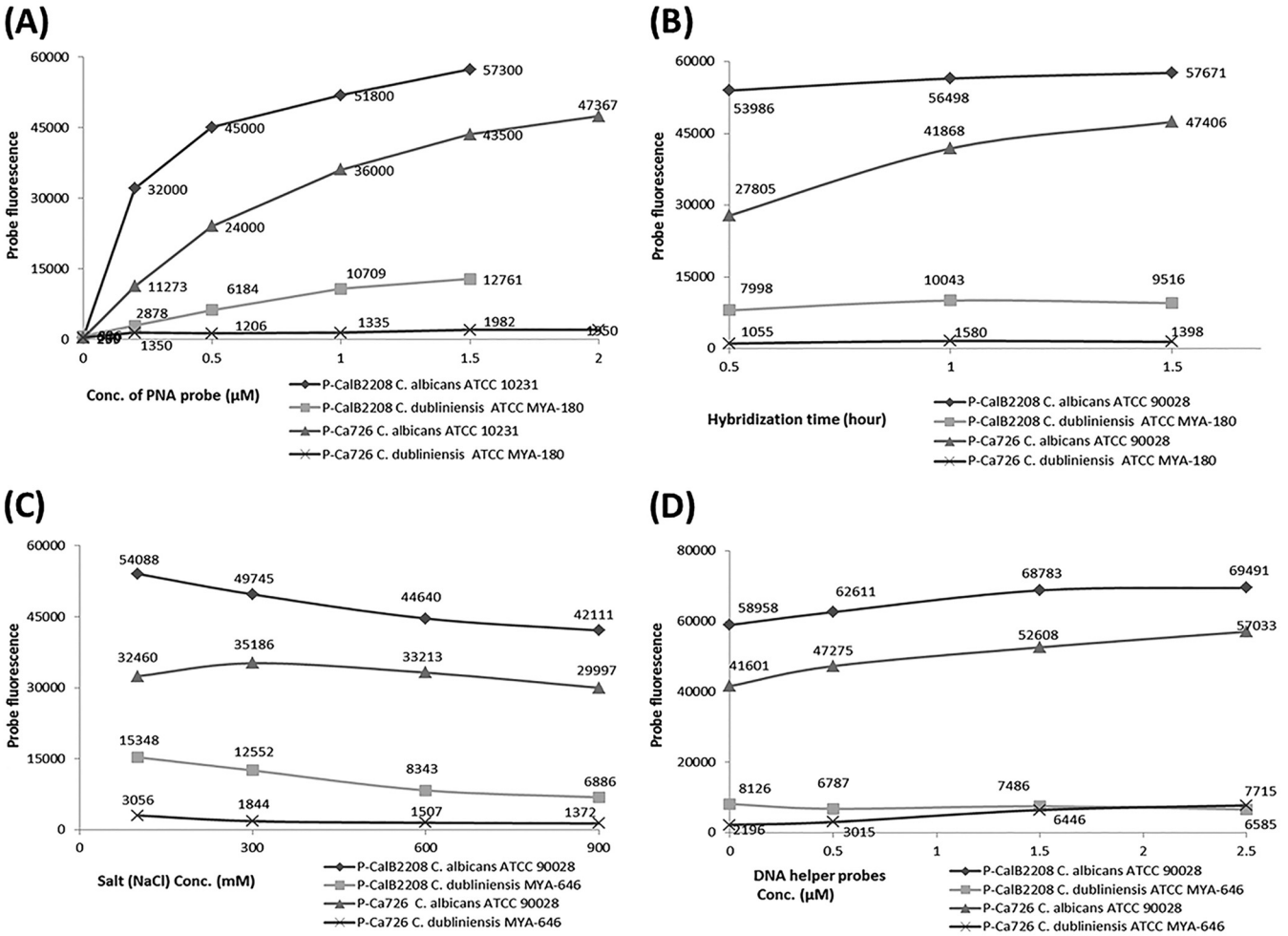

FIG 2 Effect of hybridization variables on the yield of *Candida albicans*-specific PNA-FISH using probes P-CalB2208 and P-Ca726, quantified via FCM. Variables examined included PNA probe concentration (A), hybridization time (B), salt (NaCl) concentration (C), and effect of a DNA helper probe (D). All hybridizations were carried out at 55°C. In addition to the variable addressed in each panel, hybridization conditions for individual experiments include a hybridization time of 1 h with an NaCl concentration of 100 mM (A); a probe concentration of 1 μM and an NaCl concentration of 100 mM (B); a probe concentration of 0.5 μM and a hybridization time of 1 h (C); and a probe concentration of 0.5 μM, a hybridization time of 1 h, and an NaCl concentration of 300 mM (D). Representative data from a single experiment are shown.

C. albicans. Next, we applied a ratiometric approach to these data to compare the discriminative powers of each probe. Using *C. albicans* ATCC 10231 as an "anchor point," we calculated the ratio of this strain's probe-conferred fluorescence to that of each other strain within the panel. Higher values for this ratio indicate each probe's capacity to discriminate between target and nontarget organisms. From these results (Fig. 4B), it is clear that while the overall fluorescence intensity of P-Ca726 was not as high as that of P-CalB2208, it showed a greater capacity to discriminate between *C. albicans* and the other yeasts in this panel. In order to more thoroughly explore the specificity of these two probes for *C. albicans*, we expanded our test panel to encompass an additional 30 *Candida* spp. Surprisingly, P-CalB2208 showed high cross-reactivity to several strains in the expanded panel, including *C. kefyr* ATCC 2512, *C. famata* ATCC 2560, and *C. tropicalis* ATCC 66029 (Fig. 5A). These strains also showed increased fluorescence with P-Ca726 but not to the extent that they might present as false positives, as was the case for P-CalB2208. Combined, these results indicate that P-Ca726 has both higher specificity for *C. albicans* and higher discriminatory power for target versus nontarget organisms than does P-CalB2208.

Impact of formamide on the discriminative ability of P-Ca726. In an effort to further enhance the discriminative ability of the P-Ca726 probe, we examined the impact of 10% formamide, using a test panel that included three *C. albicans* strains and four of the nontarget *Candida* spp. shown previously to have higher baseline signals (high-level nonspecific hybridization or high background level). Interestingly, while formamide had only a modest effect on reducing background staining of the nontarget *Candida* spp., substantial increases in probe-conferred fluorescence were seen for all three *C. albicans* strains tested. These results are shown in Fig. 6. These data highlight the capacity of formamide to further increase the staining intensity and discriminative ability of P-Ca726, enhancing its diagnostic utility.

DISCUSSION

Methods for the identification and differentiation of pathogenic *Candida* spp. are needed to direct appropriate health care inter-

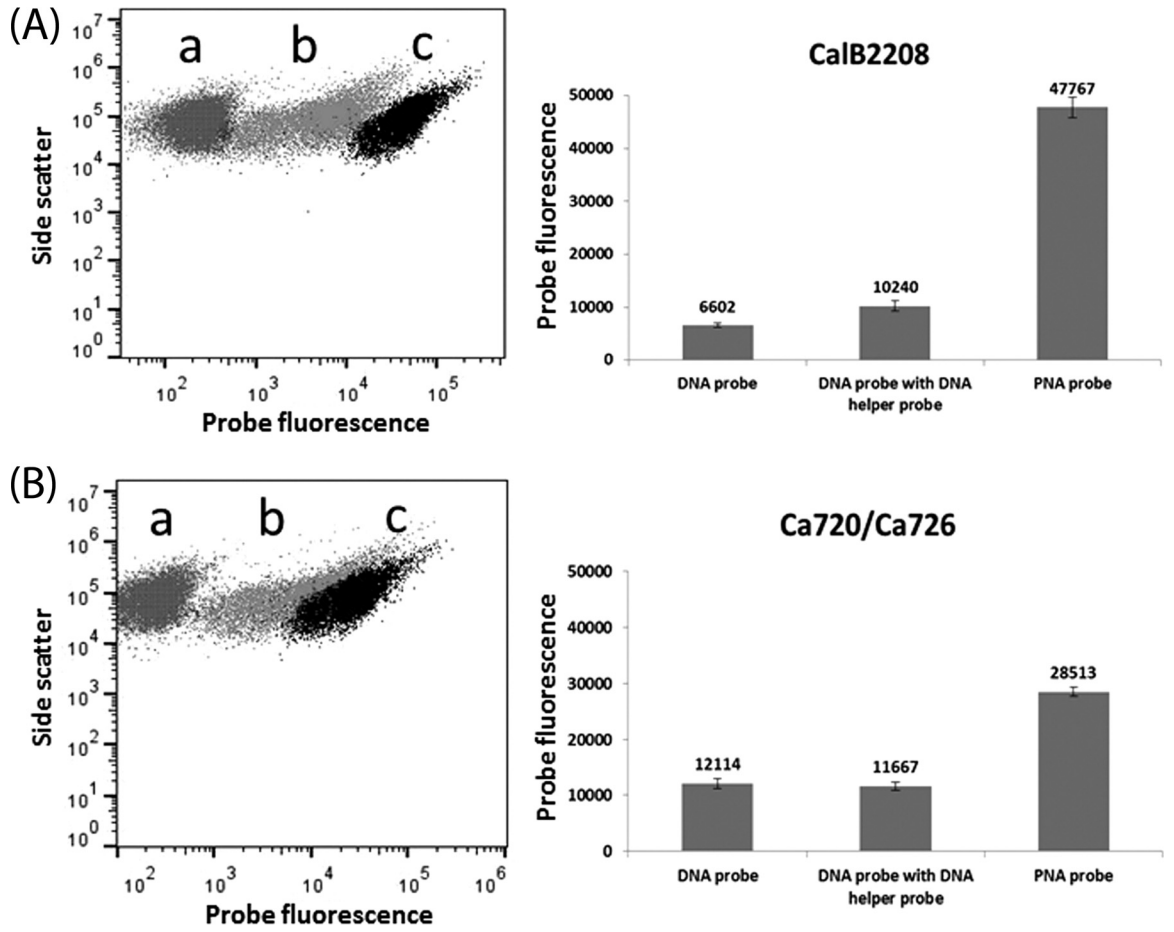

FIG 3 Comparative staining of *C. albicans*-directed DNA- and PNA-FISH probes analyzed via flow cytometry. Probes D-CalB2208 and D-Ca720, associated DNA helpers, and probes P-CalB2208 and P-Ca726 were used. (A) Scatter plot data for treatments with the D-CalB2208 probe with *C. dubliniensis* ATCC MYA-180 (negative control) (a), the D-CalB2208 probe plus DNA helpers with *C. albicans* ATCC 90028 (b), and the P-CalB2208 probe with *C. albicans* ATCC 90028 (c). (B) Corresponding results for the D-Ca720 and P-Ca726 probes. Bar graphs to the right of each scatter plot provide quantitative comparisons of results for the hybridization of DNA probes, DNA probes plus helpers, or PNA probes against *C. albicans* ATCC 90028.

ventions, including effective antimicrobial therapy. A variety of "classical" identification methods are available, including culture, phenotypic methods, biochemical tests, and immunoassays, but these methods may not be timely enough to contribute to positive patient outcomes. Rapid molecular biological methods, such as PCR, are available, but they require extensive sample preparation and do not report on the presence of intact or viable cells (26–30). In a clinical setting, whole-cell methods for pathogen identification have several advantages: they preserve information about cell morphology and number and are amenable to simple qualitative analyses such as FM or quantitative approaches such as FCM. Fluorescence *in situ* hybridization (FISH) is a rapid whole-cell method that has promising applications for the identification of *Candida* and other fungal or bacterial pathogens. FISH uses fluorescently labeled RNA, DNA, or other probes capable of recognizing and binding to pathogen-specific rRNA sequences inside whole cells. The ribosome is a naturally amplified target, thousands of which are present in actively growing cells. Ideally, cells whose ribosomes have been targeted with FISH probes are brightly labeled and are easily differentiated from nontarget cells. A variety of groups have reported the use of DNA-based FISH for the identification of *C. albicans* using probes targeting the 18S rRNA subunit; however, either the specificities of some of these probes have not been rigorously examined or the probes have been subsequently shown to react with nontarget yeasts (11–15, 23). More recently, probes based on alternative chemistries, such as peptide nucleic acid (PNA), have been used for the identification of microbial pathogens. PNA probes have several advantages over DNA probes and typically result in brighter, more homogeneous labeling of target cells. PNA-FISH assays targeting 28S rRNA for *C. albicans* and other pathogenic *Candida* spp. have been described and evaluated clinically and are commercially available (3–5, 16–19, 21, 22; Hyldig-Nielsen et al., U.S. patent application US2003/0175727 A1).

Previously, we demonstrated the utility of a probe sequence reported in the patent literature, CalB2208, for the specific detection of *C. albicans* via DNA-FISH (23). While we were able to increase the hybridization intensity using DNA helper probes, this probe was intrinsically dimmer than other probes evaluated in that study, including the positive control, EUK-516. We hypothesized that the use of PNA chemistry might improve the performance characteristics of a probe targeted to the CalB2208 region. An examination of the literature indicated that existing probes for *C. albicans* either targeted the small ribosomal subunit (18S) or

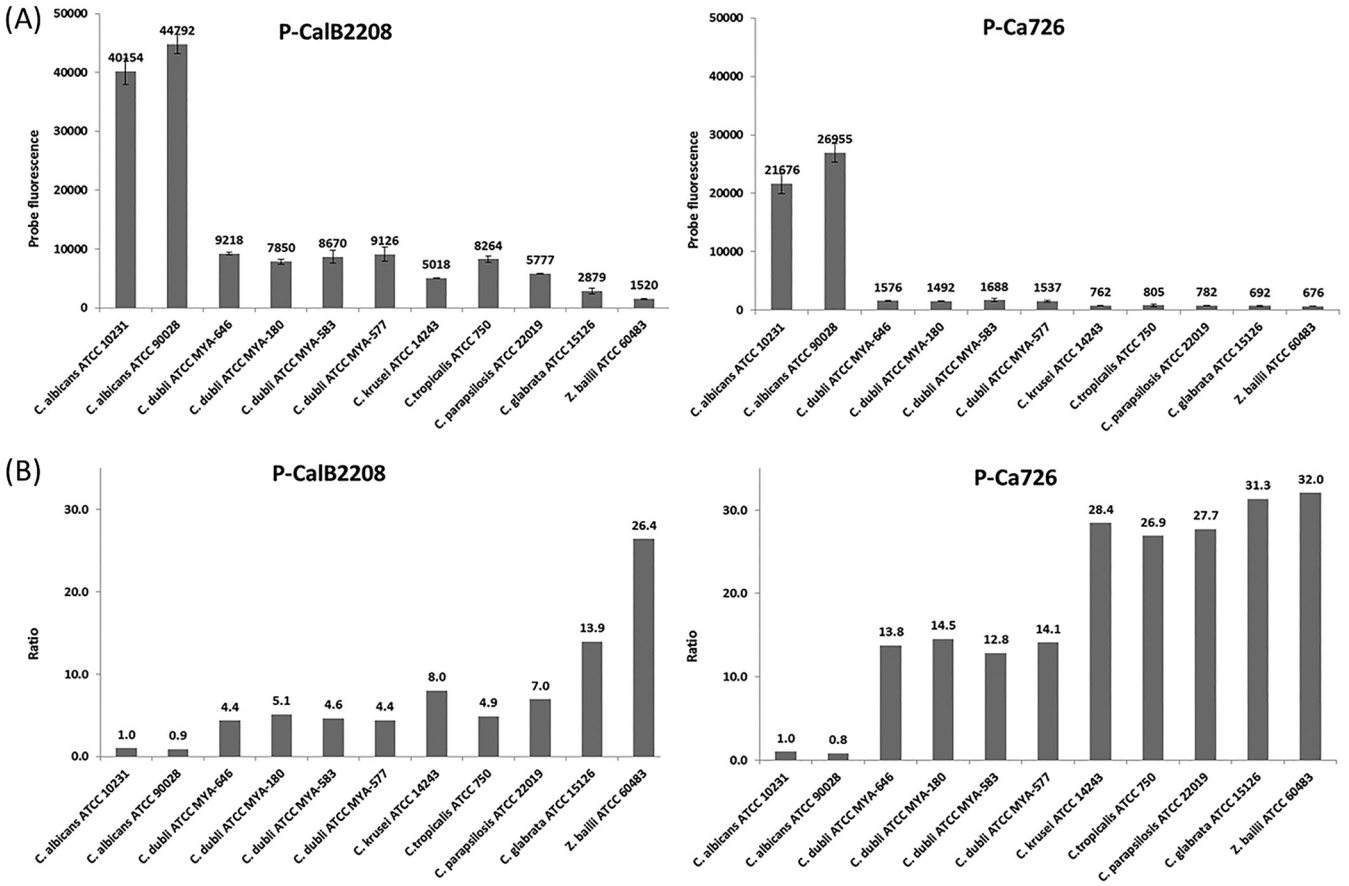

FIG 4 Specificity and discriminatory power of the P-CalB2208 and P-Ca726 probes examined against a panel comprised of *C. albicans* and nontarget yeasts. (A) Intensity of FISH results for each test strain, quantified by FCM. (B) Results for analysis of probe discriminatory power, expressed as the ratio of *C. albicans* ATCC 10231 fluorescence to that of each test strain. Higher values for this ratio indicate each probe's capacity to discriminate between target and nontarget organisms.

had been developed when only a portion of the large ribosomal subunit (28S) sequence was publicly available. The possibility that there may be additional regions on the 28S subunit that are diagnostic for *Candida* spp., including *C. albicans*, led to our screening of publicly available sequence data and identification of the regions targeted by Ca2536 and Ca726 (Ca720), which we subsequently examined using both DNA and PNA probes.

In early work, we noted that the DNA probe D-Ca2536 yielded bright hybridizations, but a PNA probe targeting this region yielded only dim results (data not shown). These results probably stem from the presence of six contiguous purines, which can result in PNA aggregation and precipitation (10). This probe also had a strong potential for self-hybridization. These results highlight the limitation that base composition within a variable and potentially diagnostic target sequence may preclude the effective use of PNA chemistry.

Once the P-CalB2208 and P-Ca726 probes were synthesized, our first step was to optimize FISH conditions for these probes. These determinations were fairly straightforward, and the general trends regarding the effects of probe concentration, hybridization time, and salt concentration were not surprising. However, we also noted that the ratios of hybridization intensities for the positive samples (*C. albicans*) to those for the negative samples (*C. dubliniensis*) reported in Fig. 2C increased as a function of increasing salt concentrations. These results highlight the competing influences of higher salt concentrations on reaction completion versus their lesser effect on nonspecific probe binding. Importantly, this effect suggests the possibility of improved wash steps using higher salt concentrations, once the hybridization step is complete. This also prompted us to examine the utility of additional ratiometric analyses for the evaluation of probe performance. The bulk of FISH analyses reported in the literature are qualitative. Decisions regarding the quality of hybridization results are made on the basis of apparent brightness or dimness, as viewed microscopically. Quantitative means of comparing FISH outcomes, such as FCM, open the potential for exploring phenomena that are not readily apparent by visual means.

Next, we examined the potential for the use of DNA-based helper probes in a hybrid DNA/PNA system. PNA synthesis is expensive, and while it may be possible to use unlabeled PNA probes as helpers to increase the accessibility of the target region, this would be cost-prohibitive. Although the low-salt conditions for PNA hybridization are not optimal for DNA-FISH, they are similar to those used for PCR, a DNA-based, hybridization-dependent process that typically uses 50 mM KCl. We therefore hypothesized that DNA helper probes added to a hybrid DNA/PNA system might still perform some level of helper function, with higher concentrations of helper probe being expected to drive the reaction toward completion, compensating for nonoptimal reaction conditions. Additionally, a higher NaCl concentration of 300

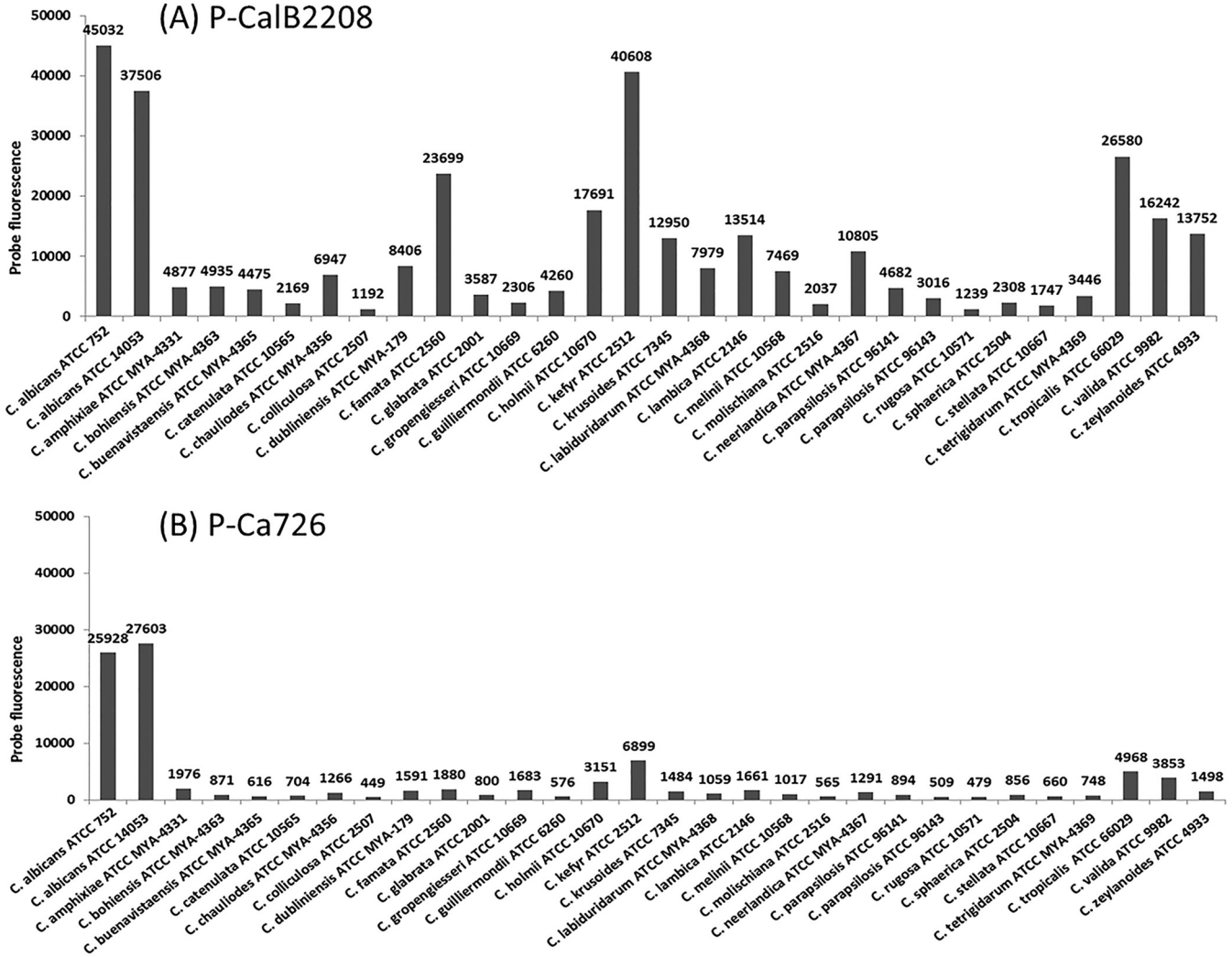

FIG 5 Specificity of P-CalB2208 and P-Ca726 for *C. albicans*, determined by using an expanded test panel of target and nontarget organisms. (A) Results for P-Calb2008. (B) Results for P-Ca726.

mM was used in this system (versus the 100 mM typically used for PNA), in an effort to improve DNA hybridization. As shown in Fig. 2D, the DNA helper probes did help open the local structure of the ribosome, leading to brighter PNA-FISH results, although it was not a dramatic effect. The use of DNA helper probes as an adjunct to PNA-FISH suggests an additional and inexpensive means of denaturation of higher-order ribosome structures that may be used in concert with chemical approaches, such as the use of formamide. Alternatively, modified DNA probes with higher binding affinities, such as DNA helper probes containing locked nucleic acid (LNA) monomers, might provide another means of enhancing PNA-FISH signals.

Once the optimal conditions for FISH with P-CalB2208 and P-Ca726 were determined, we performed a direct comparison between these probes and their DNA counterparts, using the DNA hybridization conditions reported in our previous work (23). DNA probes were examined with and without helpers. The PNA probes outperformed the DNA-based treatments, yielding brighter hybridizations and more uniform staining, as shown in Fig. 3. Although this is not surprising, we are not aware of published studies comparing the performance of DNA-FISH and PNA-FISH probes directed toward the same target region. Apart from their abilities to penetrate recalcitrant cellular structures, such as thick cell walls, and to bind avidly to their targets, the improved results for PNA may in part be due to its capacity to target sequences located within higher-order structures of the ribosome (25). The contribution of this effect to the observed results is not known, as the previously reported ribosomal accessibility map for DNA-FISH probes for yeast 28S rRNA did not include the regions targeted in our work (31).

At an early stage of the probe screening process, we also evaluated specificity with DNA versions of both probes (D-CalB2208 and D-Ca720) against a representative panel of *Candida* spp. Although the results for D-CalB2208 were in agreement with data from our previous report (23), D-Ca720 reacted strongly with *C. tropicalis* ATCC 750 (data not shown). This can be explained by fewer mismatches in the *C. tropicalis* sequence and contiguous matches with this probe on both ends of the target sequence (Fig. 1). However, the P-Ca726 probe did not cross-react with *C. tropicalis* to any great degree, highlighting an instance where the

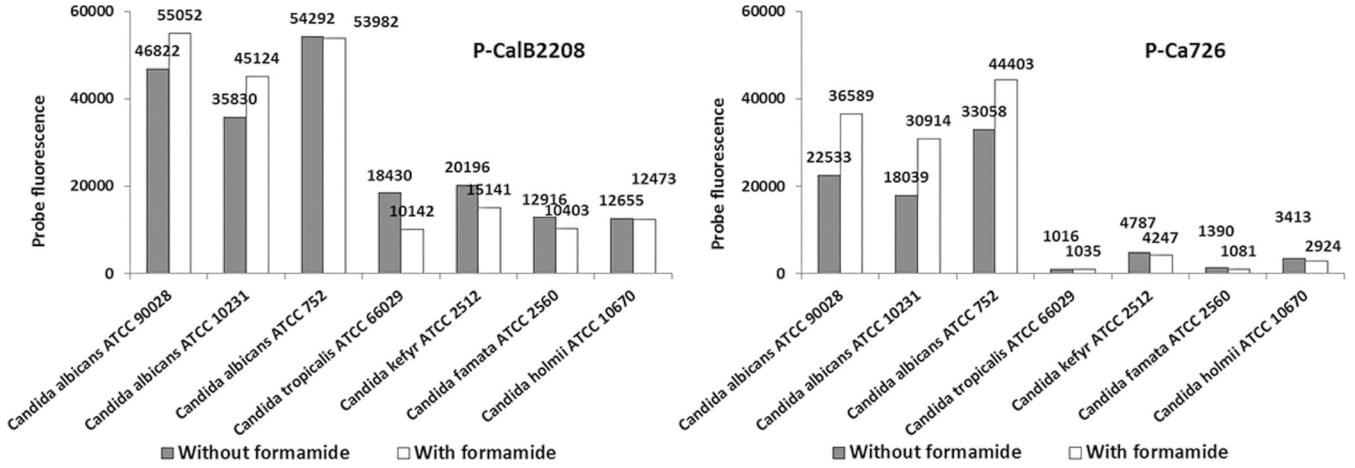

FIG 6 Effects of formamide (10%) on PNA-FISH probe specificity and intensity. The panel used includes an additional *C. albicans* strain and select nontarget strains that showed high cross-reactivity in the experiments summarized in Fig. 5.

shorter length of PNA probes can be an advantage, rather than a liability, as the destabilizing impact of the short central mismatch is greater in the resulting PNA/RNA duplex.

The substantial cross-reaction of P-CalB2208 with additional *Candida* spp. in our expanded test panel was unexpected, but it may be possible to modify the specificity of PNA probes targeting this region by shifting the binding site of the probe to a different segment within the mismatch cluster. However, as the synthesis of multiple PNA probe variants targeting slightly different regions was cost-prohibitive for the present study, this strategy was not evaluated.

Reagent performance is central to the success of any assay, and for PNA probes, this is defined by factors such as probe brightness, specificity, uniformity of staining, and discriminatory power. Comparison of histograms for DNA-FISH and PNA-FISH, shown in Fig. 3, indicated that cultures of *C. albicans* grown overnight are comprised of populations with differing permeabilities. The presence of a left-hand "shoulder" of less fluorescent cells in the DNA-FISH histograms indicates that the DNA probes are unable to fully permeate and stain these cells, which presumably have a thicker cell wall. This shoulder is much less pronounced in the PNA-labeled cells, indicating that the PNA probes are better able to penetrate and stain these cells. While both P-CalB2208 and P-Ca726 yielded bright and uniform staining of target cells, the data presented in Fig. 4B and 5 highlight the greater discriminatory power and higher specificity of P-Ca726 versus P-CalB2208 for *C. albicans*.

Most previous PNA-FISH studies on the identification of *C. albicans* have used FM, which, for some samples, could be inconclusive, as the method is qualitative and the decision between positive and negative results may be subjective (3–5, 16). Although expert microscopists are more likely to make a correct call, operator fatigue or artifacts caused by nonspecific staining, drying of the slide, or close cell grouping could also impact the outcome. The use of more "impartial" single-cell analysis methods such as FCM may enhance sample throughput and tie positive/negative outcomes to more quantitative measures (17, 23). Trnovsky et al. (17) suggested the use of FCM for rapid screening of clinical isolates from liquid or solid media for the presence of *C. albicans*. This entailed the use of positive (*C. albicans*) and negative (*C. tropicalis*) cell types hybridized with a *C. albicans*-specific PNA probe to set a gate delineating positive and negative results, followed by examination of FISH-labeled samples. Samples with ≥60% of events above this linear gate were considered positive (*C. albicans*), and those with ≤60% of events below this gate were considered negative (non-*C. albicans*). In the hands of those investigators, this approach yielded the same results as those determined via FM but in a higher-throughput format that did not require the same level of subjective interpretation as might be needed for FM. The use of a highly specific and discriminative probe such as P-Ca726 may enable this type of approach to be applied on more complex samples, such as blood cultures, sputum samples, or vaginal swabs, for early, high-throughput detection of *C. albicans* in various sample types. The presence of nontarget organisms or particulate matter in these samples would undoubtedly present challenges, but the use of a bright, robust, and discriminative probe such as P-Ca726 may enable clear distinction of positive and negative events, as we have shown for FISH-based detection of *Salmonella* in similarly complex food samples such as alfalfa sprouts and peanut butter enrichments (32).

Results from our formamide experiments (Fig. 6) show that 10% formamide improved the performance of P-Ca726. With formamide, nonspecific binding was reduced, and the intensity of positive hybridizations with *C. albicans* was increased. These data suggest that the P-Ca726 binding site is located within an area of higher-order structure and that the use of a denaturant such as formamide can provide enhanced access of the probe to its binding site.

In summary, we have designed and evaluated a new *C. albicans*-specific PNA-FISH probe, P-Ca726, which targets a region of the 28S ribosomal gene that has not been used previously for *Candida* diagnostics. The new probe yields bright, homogeneous, and specific hybridizations with *C. albicans*. Ratiometric analyses of FCM data for hybridization against both positive and negative cell types indicate that P-Ca726 shows very low nonspecific binding, highlighting the discriminatory power of this probe. Although a DNA version of this probe, D-Ca720, was found to be suitable as a FISH probe, the staining intensity of D-Ca720 was much lower than that of P-Ca726, even when DNA helper probes were used. The enhanced signals observed in both the DNA/PNA and formamide experiments suggest that it may be possible to further en-

hance the signal of P-Ca726 by using alternative chemical denaturants; helper probe chemistries with higher T_m values, such as LNA; or combinations of these approaches. Clinical applications of the P-Ca726 probe may include high-throughput FCM-based screening of yeast isolates for confirmation as *C. albicans* or FCM/PNA-FISH for the rapid detection of *C. albicans* in complex clinical samples. The ability to rapidly identify *C. albicans* in these samples could enable the administration of appropriate antifungal therapy and improved patient outcomes.

The sequence variability among *Candida* spp. in the region targeted by probes D-Ca720 and P-Ca726 suggests the utility of this region for the development of improved diagnostics for various *Candida* spp. of emerging disease concern. Additional assay formats based on this region may include multiplex PNA-FISH assays capable of differentiating key *Candida* spp. present in the same sample or other molecular diagnostics such as PCR.

ACKNOWLEDGMENTS

We thank AdvanDx, Inc. (Woburn, MA, USA), for providing the EuUni-1 probe used as a control in this work and Advanced Analytical Technologies, Inc. (Ames, IA, USA), for providing yeast strains used in the expanded test panel.

This work was supported in part by the Midwest Dairy Association, by Iowa State University's Grow Iowa Values Fund, and by an Accuri Creativity Award to B.F.B.-S.

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