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Detection of Low-Copy-Number Genomic DNA Sequences in Individual Bacterial Cells by Using Peptide Nucleic Acid-Assisted Rolling-Circle Amplification and Fluorescence In Situ Hybridization▽†

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An approach is proposed for in situ detection of short signature DNA sequences present in single copies per bacterial genome. The site is locally opened by peptide nucleic acids, and a circular oligonucleotide is assembled. The amplicon generated by rolling circle amplification is detected by hybridization with fluorescently labeled decorator probes.

Fluorescence in situ hybridization (FISH) is a convenient method for bacterial identification (23, 25, 27, 31, 34). Due to their high copy numbers (10^4 to 10^5), rRNAs have long been regarded as the most suitable targets for bacterial FISH (2, 3, 8). With the advent of the era of "global" genome sequencing and the parallel development of potent signal amplification techniques (7, 21, 25, 29), it has recently become possible to target other nucleic acid sites (4, 13, 22, 24, 32), including regions of bacterial chromosomal DNA (34, 36). However, these new approaches require long probes, very high probe concentrations, and long hybridization (35, 36). A shorter target sequence would increase assay specificity: shorter probes are less tolerant of base pair mismatches and also can result in better discrimination between otherwise very similar bacteria. Recently, another approach has been described for the in situ detection of low-copy-number genomic sequences by using short (35- to 39-nucleotide [nt]-long) target sequences, rolling-circle amplification (RCA), and FISH. However, this approach cannot be performed directly on slides and involves embedding the cells into membrane filters and heating them to denature genomic DNA (19, 20).

An isothermal amplification method reported in the literature, the multiple displacement amplification reaction, uses phi29 DNA polymerase and random primers to amplify genomic DNA (6, 18). It is applied for DNA microarray-based screening and direct detection of pathogen-specific DNA (1, 14, 28, 30, 33).

We describe here a new approach to using low-copy-number genomic sequences as targets for the detection of specific bacteria. The technique expands both the utility and the resolving power of whole-cell FISH for the detection of microbes and may be useful in food, environmental, clinical diagnostic, and national security-related applications.

Our major tool is peptide nucleic acid (PNA) (Fig. 1). PNA openers have a unique ability to locally open double-stranded DNA (dsDNA) via binding to one of the two DNA strands, leaving the other strand accessible for hybridization with synthetic oligonucleotide probes. Such a complex between dsDNA, a pair of PNAs, and the oligonucleotide probe is called a PD-loop (5). The binding sites for each of the two PNA openers must be short (7- to 10-bp-long) homopurine-homopyrimidine tracts, and they are separated by an arbitrary sequence of nucleobases of up to 10 bp. We call such a site consisting of two PNA-binding sites and the linker sequence the PD-loop site. Statistically, such sites are expected to exist once per a few hundred base pairs of DNA sequence (see reference 9 and the supplemental material).

After the opening of a PD-loop site with a pair of PNA openers, a circular probe is assembled by T4 DNA ligase by using a circularizable oligonucleotide with termini that are complementary to the displaced strand (11, 15, 16) (Fig. 1, step 2). This circular probe assembly is exceedingly sequence specific since only chosen sites are opened by PNA openers and the remaining DNA maintains its duplex form and is inaccessible for mismatch hybridization (10, 11, 16, 26).

Fluorescence labeling is achieved by an RCA reaction on the circular probe (17) in the presence of fluorescently labeled decorators. These decorators consist of linear oligonucleotides with fluorophores at their termini; their hybridization to single-stranded DNA produced by RCA yields the multiply fluorescently labeled product (Fig. 1, step 3). The fluorescent signal is readily detected by standard techniques using fluorescence microscopy (Fig. 1, step 4).

Our approach integrates the highly specific and sensitive recognition of target dsDNA sequences by using PNA openers and circularized probes with efficient signal amplification by RCA. We report here the results of proof-of-principle experiments performed with three bacterial species: *Escherichia coli*, *Bacillus subtilis*, and *Streptococcus mutans*.

Table 1 presents the PD-loop sites chosen for the three bacterial species along with corresponding PNA openers, cir-

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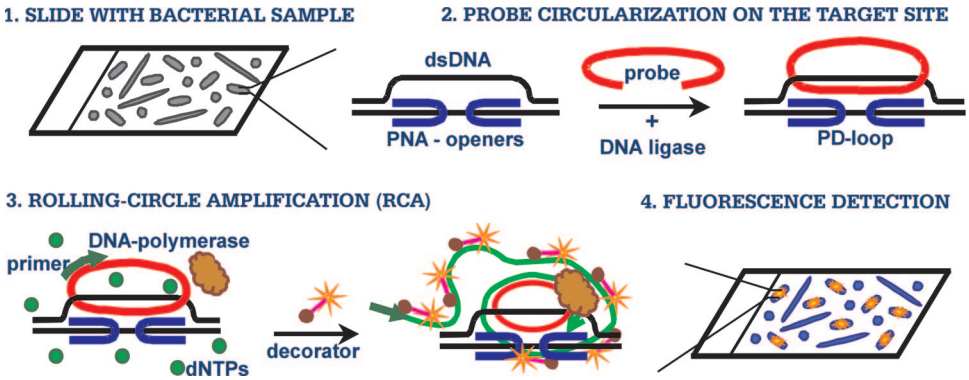

FIG. 1. Major steps of a DNA-based assay for fluorescence in situ detection of short DNA sequences in a single bacterial cell. dNTPs, deoxynucleoside triphosphates.

cularizable probes, decorators, and primer sequences (see methods in the supplemental material). The chosen target sites (Table 1) correspond to various genomic regions and to various DNA strands: coding and noncoding regions and sense and antisense strands in coding regions. All these sites yielded similar signals (see below), which excluded the possibility that the signal was due to the targeting of mRNA transcripts. To additionally exclude RNA targeting by our probes, RNA was degraded by subjecting all samples to RNase A treatment (see methods in the supplemental material).

We first validated our approach using *E. coli* and *B. subtilis* as model organisms. All steps were sequentially performed (Fig. 1) with cells fixed on polylysine slides (see methods in the supplemental material). Cells were counterstained by DAPI (4′,6′-diamidino-2-phenylindole), and slides were evaluated by fluorescence microscopy using standard FISH equipment and software. Figure 2 shows typical results of experiments with *E. coli* and *B. subtilis*. Both bacteria gave clear signals (red for *E. coli* and green for *B. subtilis*) when a set of site-specific genomic probes was applied (Fig. 2A and D). Figure 1 presents the data for only one site for each species (see the legend to Fig. 1). Very similar data (not shown) were obtained for three other sites (two for *E. coli* and one for *B. subtilis*).

TABLE 1. Signature sites, PNAs, circularizable oligonucleotides, decorator probes, and primer used in this work

Bacterium	Signature site[a] (description)	PNA(s)[b]	Decorator probe, ODNs, and primer[c]
E. coli	GGAGAGAGACTCAAAAG AAGG (major cold shock protein gene [*csp*] region; 1.050 Mb; antisense strand; mRNA for *cspG*)	PNA1, H-Lys$_2$-CTCTCTCC-(eg1)$_3$-JJTJTJTJ-Lys-NH$_2$; PNA2, H-Lys$_2$-TTTJTTJJ-(eg1)$_3$-CCTTCTTT-Lys-NH$_2$	decR, 5′-Cy3-TCACGGAATGGTTACTTGCAC AGC-biotin-3′; ODNcspG, 5′-p-tcaAAAGAAG G(tcacggaatggttacttgcCAGC)CAGCAGCC(TC ACggaatggttacttgccagc)GGAGAGAGac-3′; ODNrpoN, 5′-p-gctGAAAGAAG(tcacggaatggtt acttgcCAGC)CAGCAGCC(TCACggaatggttactt gccagc)GAAAGAAGatg-3′; ODNrnr, 5′-p-ccG GAAGAAG(tcacggaatggttacttgcCAGC)CAGC AGCC(TCACggaatggttacttgccagc)GAAAGAA Gaagtg-3′
	GAAAGAAGATGTGCTGAAA GAAG (RNA polymerase sigma N factor gene *rpoN*; 3.343 Mb; sense strand)	PNA3, H-Lys$_2$-JTTTJTTJ-(eg1)$_3$-CTTCTTTC-Lys-NH$_2$	
	GAAAGAAGAAGTGCCGGAA GAAG (exoribonuclease R gene *rnr*; 4.405 Mb; sense strand)	PNA3 and PNA4, H-Lys$_2$-JJTTJTTJ-(eg1)$_3$-CTTCTTCC-Lys-NH$_2$	
B. subtilis	GAAAAGAAACCCTTCAGAGGA AG(*serA* region; 2.391 Mb; noncoding region)	PNA5, H-Lys$_3$-JTTTTJTT-(eg1)$_3$-TTCTTTTC-Lys-NH$_2$; PNA6, H-Lys$_2$-TJTJJTTJ-(eg1)$_3$-CTTCCTCT-Lys-NH$_2$	decG, 5′-FITC-CCTCAATCGTCGTCGTGTAC TAC-FITC-3′; ODNserA, 5′-p-ttcAGAGGAA GttatCAGCCAGCAGCCTCA(Cctcaatcgtcgtcgt gtactac)tattGAAAAGAAaccc-3′; ODNyxjA, 5′-p-cactAAAGAAAAagtCAGCCAGCAGCC TCA(Cctcaatcgtcgtcgtgtactac)taattGGAAGAA Gcg-3′
	GGAAGAAGCGCACTAAAG AAAA (*yxjA* gene; 4.005 Mb; antisense strand)	PNA4 and PNA7, H-Lys$_2$-TTTJTTTT-(eg1)$_3$-TTTTCTTT-Lys-NH$_2$	
S. mutans	AAAGAAAAATATTTAAAGA GGAA (*dnaK* region; 0.085 Mb; sense strand)	PNA7 and PNA8, H-TTJTJJTT-(eg1)$_3$-TTCCTCTT-Lys-NH$_2$	decG, 5′-FITC-CCTCAATCGTCGTCGTGTAC TAC-FITC-3′; ODNdnaK, 5′-p-ttaAAGAGGA AttatCAGCCAGCAGCCTCA(Cctcaatcgtcgtcgt gtactac)tattAAAGAAAAatat-3′; ODNwapA, 5′-p-tttAAGAGGAAtattCAGCCAGCAGCCT CA(Cctcaatcgtcgtgtactac)tattAAAAGAGGta t-3′; ODNhypP, 5′-p-ggtgAGAGGAAGttatCA GCCAGCAGCCTCA(Cctcaatcgtcgtgtactac) tattGGAAGAAGttcg-3′; primer, 5′-GTGAGG CTGCTGGCTG-3′
	AAAAGAGGTATTTTAAGAGG AA (*wapA* region; 0.934 Mb; sense strand)	PNA9, H-Lys$_2$-TTTTJTJJ-(eg1)$_3$-CCTCTTTT-Lys-NH$_2$; PNA8	
	GGAAGAAGTTCGGGTGAGAG GAAG (hypothetical protein gene [*hyp*] region; 1.329 Mb; noncoding region)	PNA4 and PNA6	

[a] PNA binding sites are underlined.
[b] eg1, Lys, and J denote the bis-PNA linker segment, the amino acid lysine, and the nucleobase pseudoisocytosine, respectively (12).
[c] In circularizable probe sequences, letters both capitalized and underlined represent primer annealing sites, letters capitalized but not underlined represent sequences identical to those of PNA binding sites, and letters in parentheses represent sites for decorator hybridization. ODNs, circularizable oligodeoxynucleotides; FITC, fluorescein isothiocyanate.

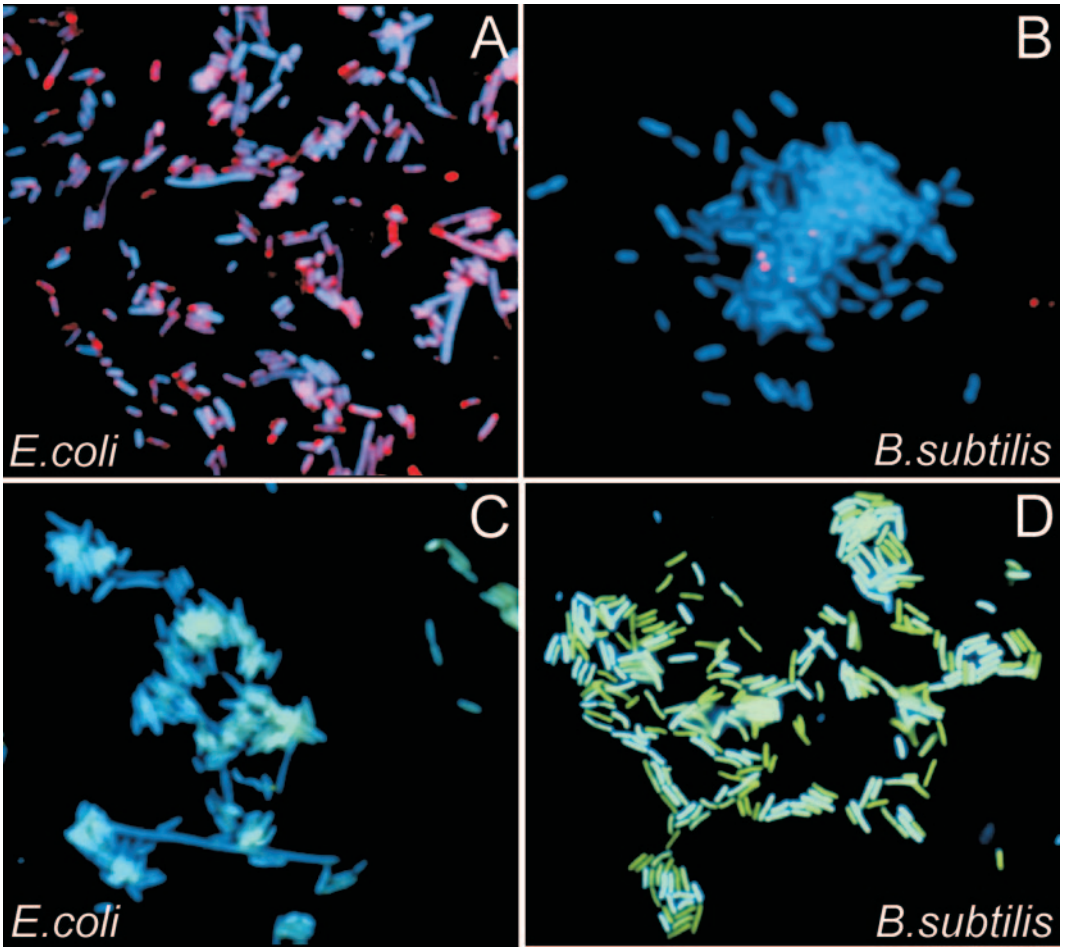

FIG. 2. Images of bacterial cells observed by using a fluorescence microscope in experiments performed according to the scheme presented in Fig. 1. The fluorescence signals were acquired separately using three filter sets (DAPI for DNA and Cy3 or fluorescein for the labeled RCA product). Each image is a superposition of two separate images, with DAPI and Cy3 or DAPI and fluorescein signals pseudocolored in blue and red or blue and green, respectively. (A) *E. coli* cells to which the probes corresponding to the 21-nt target site in the *E. coli* cold shock protein gene (*csp*) region, PNA1, PNA2, ODNcspG, and decR, were applied (Table 1). Virtually all cells displayed very bright spots. No such spots were observed in numerous negative control experiments in which any of the steps of the protocol given in Fig. 1 were omitted (see the supplemental material). (B) The same procedure as that described in the legend to panel A was carried out with a combination of all probes specific to *E. coli* (PNA1, PNA2, PNA3, PNA4, ODNcspG, ODNrpoN, ODNrnr, and decR) (Table 1) applied to *B. subtilis* cells. No signal was detected. (C) A combination of probes specific to *B. subtilis* (PNA4, PNA5, PNA6, PNA7, ODNserA, ODNyxjA, and decG) was applied to *E. coli* cells. No signal was detected. (D) *B. subtilis* cells to which the probes corresponding to the 23-nt target site in the *B. subtilis* phosphoglycerate dehydrogenase gene (*serA*) region (PNA5, PNA6, ODNserA, and decG) were applied.

No signal was observed in various negative control experiments, which are described in the supplemental material. To confirm the specificity of detection and to exclude the possibility of nonspecific probe binding to cell structures, a mixture of all *E. coli*-specific probes was applied to *B. subtilis* cells and vice versa. No cross signals were observed (Fig. 2B and C). The data shown in Fig. 2 demonstrate that our approach can specifically target single-copy, short DNA sequences in bacteria and can generate a detection signal readily seen by fluorescence microscopy.

We then performed our protocol with a mixture of *E. coli* and *B. subtilis* cells, combining the corresponding probes (Table 1). Nearly all cells on mixed slides displayed a red or green signal only (Fig. 3A). Although on these slides *E. coli* and *B. subtilis* cells can appear morphologically similar, the results of the control experiments (Fig. 2) support the premise that red fluorescence is specific to *E. coli* and green fluorescence is specific to *B. subtilis*.

We also investigated a mixture of bacillary and coccal bacteria. Figure 3C shows some resulting data for the mixture of *E. coli* and *S. mutans*, in which round cocci and rod-shaped bacilli show green and red signals, respectively.

In this study, every site arbitrarily chosen (eight sites total) from the genome database worked as a specific signature site for the corresponding bacterium. The data support the idea that the proposed approach could be used as a universal tool for specific bacterial detection.

We also verified that bacterial detection by our method is possible with clinical and environmental samples (see the supplemental material).

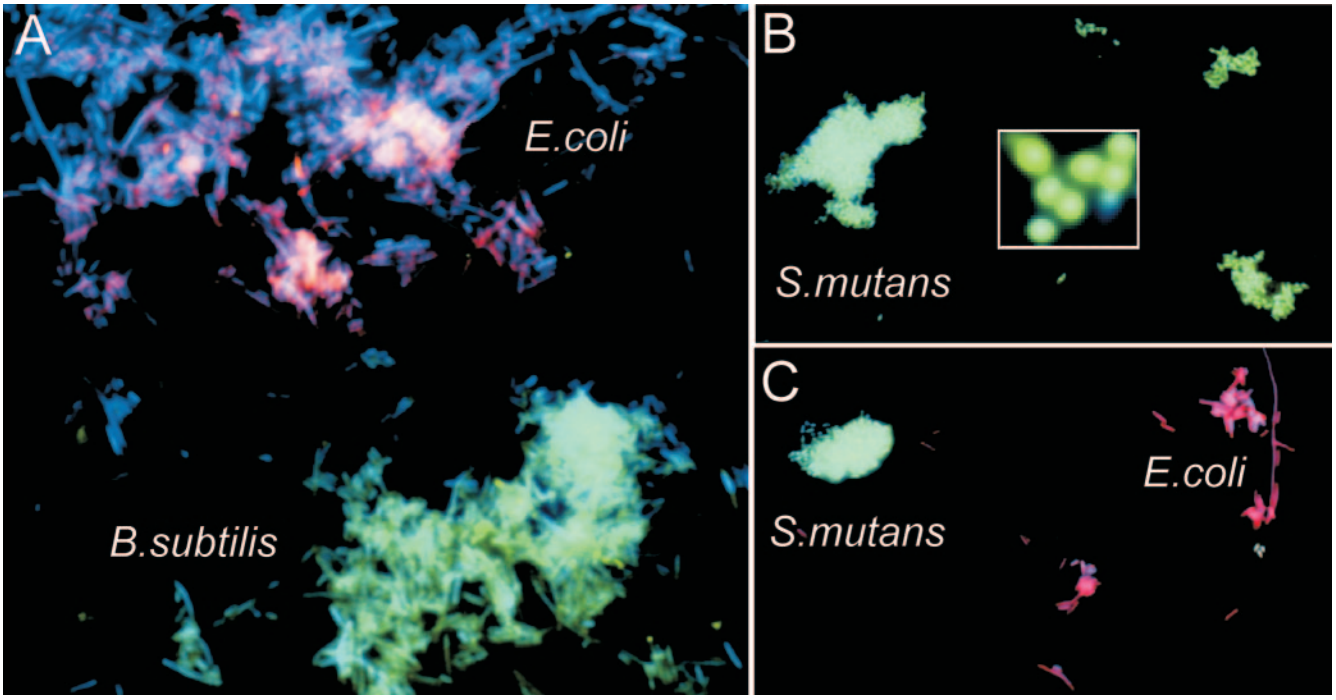

FIG. 3. (A) Images of bacterial cells observed by using a fluorescence microscope in experiments performed with a mixture of *E. coli* and *B. subtilis* cells to which a combination of probes specific to *E. coli* and *B. subtilis*, PNA3, PNA4, PNA7, ODNrnr, ODNyxjA, decR, and decG, were applied. *E. coli* and *B. subtilis* bacteria can be distinguished from each other in the mixture of both types of cells. (B) *S. mutans* cells to which the probes corresponding to the 22-nt target site in the *S. mutans* wall-associated protein gene (*wapA*), PNA8, PNA9, ODNwapA, and decG, were applied. Virtually all cells are colored green. No such spots were observed in various negative control experiments in which any of the steps were omitted (data not shown). The insert shows an enlarged image of several *S. mutans* cells. (C) Images of bacterial cells observed by using a fluorescence microscope in experiments performed with *E. coli* and *S. mutans* cells with the combination of probes specific to *E. coli* (*rpoN* and *rnr*) and *S. mutans* (*hypP*): PNA3, PNA4, PNA6, ODNrpoN, ODNrnr, ODNhypP, decR, and decG. The fluorescent signals were acquired separately using three filter sets with DAPI, Cy3, and fluorescein.

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