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Identification of Bacterial Cells by Chromosomal Painting

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Chromosomal painting is a technique for the microscopic localization of genetic material. It has been applied at the subcellular level to identify regions of eukaryotic chromosomes. Here we describe the development of bacterial chromosomal painting (BCP), a related technology for the identification of bacterial cells. Purified genomic DNAs from six bacterial strains were labeled by nick translation with the fluorochrome Fluor-X, Cy3, or Cy5. The average size of the labeled fragments was ca. 50 to 200 bp. The probes were hybridized to formaldehyde-fixed microbial cells attached to slides and visualized by fluorescence microscopy. In reciprocal comparisons, distantly related members of the class *Proteobacteria* (*Escherichia coli* and *Oceanospirillum linum*), different species of the genus *Bacillus* (*B. subtilis* and *B. megaterium*), and different serotypes of the subspecies *Salmonella choleraesuis* subsp. *choleraesuis* (serotype typhimurium LT2 and serotype typhi Ty2) could easily be distinguished. A combination of two probes, each labeled with a different fluorochrome, was used successfully to simultaneously identify two cell types in a mixture. Lysozyme treatment was required for the identification of *Bacillus* spp., and RNase digestion and pepsin digestion were found to enhance signal strength and specificity for all cell types tested. Chromosome in situ suppression, a technique that removes cross-hybridizing fragments from the probe, was necessary for the differentiation of the *Salmonella* serotypes but was not required to distinguish the more distantly related taxa. BCP may have applications in diverse branches of microbiology where the objective is the identification of bacterial cells.

Chromosomal paints are fluorescent DNA probes synthesized enzymatically from DNA templates. Templates can range in size from single genes to entire chromosomes. Probes are prepared by randomly digesting templates to small fragments (50 to 200 bp is optimal) (15) and labeling the fragments by nick translation with fluorescently derivatized nucleotides. In situ hybridization of the probes to target molecules paints the target DNA, with each labeled fragment serving as a single brushstroke. Results are observed by fluorescence microscopy or flow cytometry.

Chromosomal painting has been used by eukaryotic cell biologists to microscopically identify specific chromosomes or regions of chromosomes (14, 15, 20). Metaphase spreads, interphase nucleii, and whole eukaryotic cells have been investigated. This method has been shown to be highly specific for individual chromosomes or chromosomal regions in several eukaryotic systems, including *Saccharomyces cerevisiae* (17, 22), mammals (15, 20), and plants (28). Chromosomal painting has been used for cytogenetic analyses (2, 9, 27), gene mapping (12, 13), analyses of aberrant chromosomes (3, 16), and determining changes in the copy number of chromosomal regions (5), as well as studies of interspecies evolutionary divergence (8, 24), chromosome condensation (22), radiation biology (19), and, most recently, complete karyotyping (23, 24).

No applications of chromosomal painting to bacteria have been described. However, in principle, this method offers a versatile approach for the microscopic detection of bacteria and the localization of specific genes in bacterial cells. One potential application is the identification of cells in nature. Fluorescence in situ hybridization with oligonucleotide probes for rRNAs is now widely used for this purpose (reviewed in reference 1). Fluorescent probes for ribosomes often fail to label a significant fraction of the bacterial cells in natural ecosystems, possibly because some cells are dormant and therefore have few ribosomes or because the probes fail to enter some cells. Chromosomes are present in living cells regardless of their growth rate; therefore, chromosomal painting should be useful for detecting dormant cells. Moreover, as targets, chromosomes have the advantages that they are not subject to digestion by the proteases used to permeabilize cells and are very slow to diffuse because of their large size.

In theory, chromosomal painting has the potential to be as specific as solution-based DNA-DNA reassociation. Genomic DNA-DNA hybridization is an important measure of similarity between bacterial strains and is regarded as crucial for establishing relatedness among strains within a species for taxonomic purposes (reviewed in reference 6). In solution-based tests, conspecific strains exhibit greater than 70% DNA-DNA reassociation. DNAs from cells more distantly related than the genus level do not reassociate significantly due to the divergence of gene sequences at synonymous sites. Thus, genomic DNA-DNA hybridization is particularly useful for resolving close phylogenetic relationships and therefore complements the sequence information provided by conserved genes such as rRNAs.

Here we report the development, optimization, and testing of bacterial chromosomal painting (BCP), a procedure for in situ identification of bacteria based on the method of chromosomal painting. The chromosomal paints were constructed from bacterial genomic DNAs and hybridized to aldehyde-fixed, permeabilized bacterial cells. The results demonstrate that BCP can be used to extend the principle of genomic DNA-DNA hybridization to the detection of single cells.

MATERIALS AND METHODS

Strains. *Escherichia coli* AB1157 was a gift from L. Walter Ream, Oregon State University. *Oceanospirillum linum* ATCC 11336, *Bacillus subtilis* ATCC 6633, *Bacillus megaterium* ATCC 14581, *Salmonella choleraesuis* subsp. *choleraesuis* serotype typhimurium LT2 ATCC 29946, and *Salmonella choleraesuis* subsp. *choleraesuis* serotype typhi Ty2 ATCC 19430 were obtained from the American Type Culture Collection, Rockville, Md.

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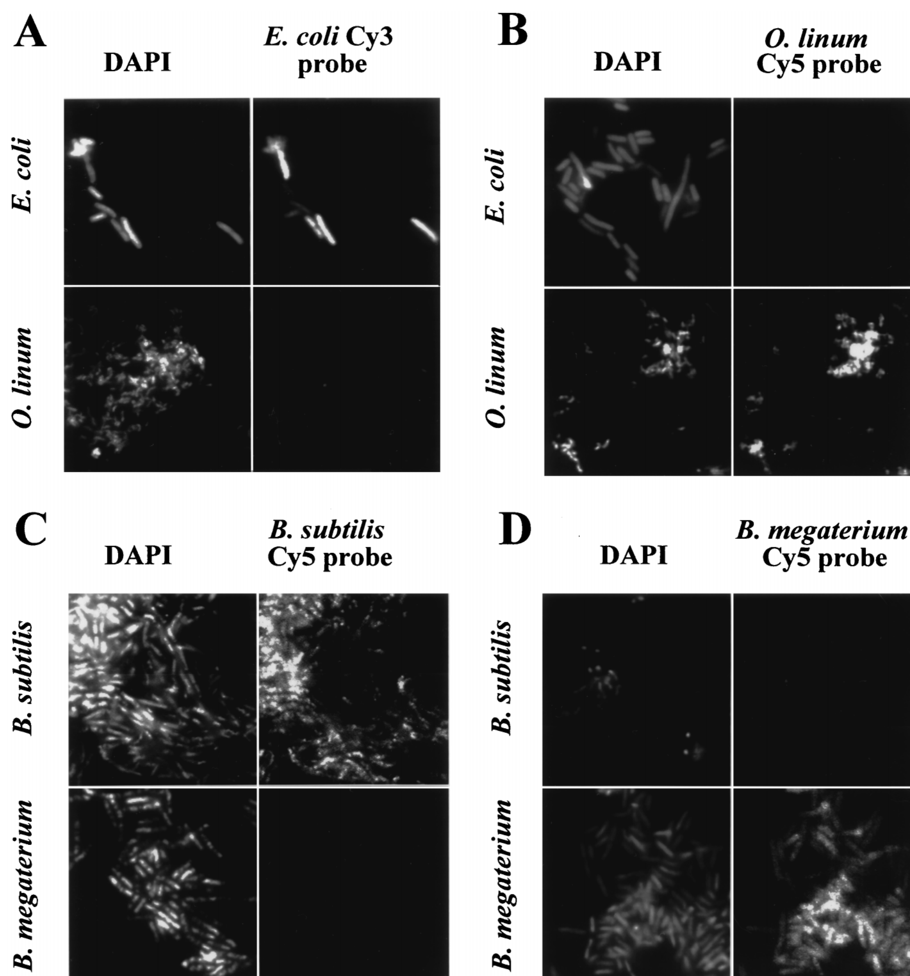

FIG. 1. Reciprocal hybridizations between *E. coli* and *O. linum* (gram negative) (A and B) and *B. subtilis* and *B. megaterium* (gram positive) (C and D). BCP and DAPI staining were as described in Materials and Methods. (A) *E. coli* and *O. linum* cells probed with *E. coli* genomic DNA labeled with Cy3; (B) *E. coli* and *O. linum* cells probed with *O. linum* genomic DNA labeled with Cy5; (C) *B. subtilis* and *B. megaterium* cells probed with *B. subtilis* genomic DNA labeled with Cy3; (D) *B. subtilis* and *B. megaterium* cells probed with *B. megaterium* genomic DNA labeled with Cy5. Both the DAPI and the specific fluorochrome emission images of the same field are depicted. Images at the same wavelength within each panel were normalized to the same maximum and minimum pixel intensities to allow direct comparison of signal intensities.

Conditions. All solutions were filtered through a 0.2-μm-pore-size filter, either Acrodisc membrane filters (used for growth media; Gelman Sciences, Ann Arbor, Mich.) or Tuffryn (used for BCP solutions; Gelman Sciences) membrane filters, prior to contact with cells. All incubations and washes were performed at room temperature unless otherwise indicated. For the effect of varying specific aspects of this protocol, see Table 1.

Cell collection and fixation. All strains were grown in LB broth except *O. linum*, which was grown in marine broth (Difco Laboratories, Detroit, Mich.). Approximately 1.5×10^9 to 3.0×10^9 cells were collected by centrifugation at $6,000 \times g$ in a JA-20 Sorvall centrifuge rotor. Cells were washed once in 1× phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4 [pH 7.0]) and then resuspended in 5 ml of 1× PBS–4% (vol/vol) formalin. Cells were fixed for 1 h, washed once in 1× PBS, and resuspended in 5 ml of 50% ethanol with 0.5× PBS. Fixed cells in this buffer could be stored at −20°C for at least 3 months with no effect on signal strength.

Preparation of fixed cells for hybridization. Fixed cells were spotted onto Superfrost Plus slides (Fisher) and air dried for at least 1 h. The slides were then dehydrated through an ethanol series (70, 90, and 100%; 3 min each) and air dried. Slides containing *Bacillus* sp. cells were treated with lysozyme (1 mg/ml; Sigma) in 1× PBS for 30 min at 37°C and then washed twice for 5 min in 1× PBS and once for 5 min in 1× SSC (150 mM NaCl, 15 mM Na citrate). All slides were treated with RNase A (100 μg/ml; Sigma) in 2× SSC for 30 min at 37°C and washed three times in 2× SSC for 5 min. The slides were then treated with pepsin in 0.01 M HCl (0.01%, vol/vol) for 10 min at 37°C and washed twice for 5 min in 1× PBS and once for 5 min in 1× PBS plus 50 mM $MgCl_2$. The slides were treated for 10 min in 1× PBS–50 mM $MgCl_2$ with 1% (vol/vol) formalin, washed once in 1× PBS for 5 min, dehydrated through an ethanol series, and air dried. Intracellular DNA was denatured by incubation in 70% formamide–2× SSC at 80°C. The slides were then dehydrated through an ice-cold ethanol series and air dried.

Genomic DNA isolation. DNAs were isolated by the guanidine thiocyanate method of Pitcher et al. (21), followed by RNase A treatment (100 μg/ml; Sigma) at 37°C for 30 min. *Bacillus* spp. were treated with lysozyme (50 mg/ml; Sigma) at 37°C for 30 min prior to extraction. DNA concentrations and purities were determined by measuring the absorbances at 260 and 280 nm.

Probe labeling. Probes were labeled by a variation of the nick translation method (10) in which digestion and labeling steps were separated to better control the probe fragment size. For each DNA preparation, a digestion time series was performed in which 1 μg of DNA was incubated in nick translation buffer (50 mM Tris-HCl, 10 mM $MgSO_4$, 0.1 mM dithiothreitol [pH 7.2]) and 2×10^{-4} U of DNase I (Sigma) at 37°C for 0 to 60 min. Incubation times yielding a majority of fragments in the 50- to 200-bp size range were then used to digest 10- to 40-μg DNA aliquots to be labeled for BCP probes. Fragment sizes were determined by electrophoresis on a 3% NuSieve GTG agarose gel.

In each labeling reaction, 10 μg of digested DNA, 1× nick translation buffer, 50 μM each dGTP, dATP, and dTTP, 0.1 μM dCTP, 0.1 mM fluorochrome-labeled dCTP (either Fluor-X, Cy3, or Cy5; Amersham, Arlington Heights, Ill.), and 83 U of *E. coli* DNA polymerase I (New England Biolabs, Beverly, Mass.) were mixed and incubated overnight at 15°C. Reactions were stopped by boiling for 5 min. Labeled products were separated from contaminants in a Microcon 10 microconcentrator (Amicon, Inc., Beverly, Mass.) as described in the manufacturer's instructions. Labeled probes were stored for up to 1 year at −20°C with no detectable loss of activity.

Probe preparation (for slides not requiring chromosome in situ suppression [CISS]). For each cell spot, 200 ng of probe and 5 μg of sheared calf thymus DNA (Sigma) were dried in a Speed-Vac concentrator (Beckman). The dried probe mix was resuspended in 100% deionized formamide (5 μl/cell spot) by vortexing for 30 min. An equal volume of 20% dextran sulfate (Sigma)–2× SSC (5 μl/cell spot) was added to the resuspended probe. These solutions were treated for 5 min at 80°C and chilled on ice to denature the probes. Denatured probes were spotted directly onto slides of prepared, fixed cells.

Probe preparation (with CISS). To differentiate conspecific bacteria, regions of highly conserved DNA held in common by probes and heterologous genomic DNA were removed by CISS (15). Specifically, suppressor DNA (unlabeled DNA from the negative control organism) was added in excess to the probe, denatured, and allowed to preanneal for an empirically determined optimum duration prior to application to the slide.

For combinations of serotype typhimurium LT2 and serotype typhi Ty2, which require CISS, probes were prepared as described above except that 2 μg of calf thymus DNA was replaced by an equivalent amount of suppressor DNA. After denaturation as described above, the probe was immediately cooled on ice and then placed at 37°C for 1 h prior to application to the slides.

Hybridization. Ten microliters of probe was added to each cell spot, covered with a coverslip, sealed with rubber cement, and incubated for 2 days at 37°C in the dark.

Washes. After removing the dried rubber cement, the coverslips were shaken off in wash 1 (50% deionized formamide with 2× SSC, preheated to 50°C; for other stringencies tested, see Table 1) in a Coplin jar. The slides were washed three times in succession in wash 1, at 50°C for 10 min, at 50°C for 5 min, at room temperature for 5 min, and finally at room temperature for 10 min. The wash buffer was changed to wash 2 (0.2× SSC, preheated to 65°C; for other stringencies tested, see Table 1). The slides were then washed three times in succession in wash 2, at 65°C for 10 min, at 65°C for 5 min, at room temperature for 5 min, and finally at room temperature for 10 min.

Counterstaining and mounting. Cells were counterstained with 1 μg of DAPI (4′,6-diamidino-2-phenylindole) per ml (Sigma) in 2× SSC for 5 min and washed once in 2× SSC for 5 min. Slides were rinsed in 0.1× PBS and air dried briefly. Twelve microliters of DABCO solution (1,4-diazabicyclo[2,2,2]octane [Sigma]; 2.3% (wt/vol) in a 9:1 mixture of glycerin–Tris-HCl [pH 8.0]) per cell spot was added to the slides, covered with a coverslip, and sealed with clear nail polish. The slides were stored at −20°C for several months without detectable loss of signal.

Image capture and processing. The slides were examined with a Leica DMRB microscope equipped with a 75-W xenon vapor arc lamp. Images were captured with a Photometrics (Tucson, Ariz.) Star I cooled-charge-coupled device camera head with an attached Photometrics Star I camera controller. Integration times were the same for all images captured for a specific fluorochrome within a single experiment. Images were normalized with IP Labs Spectrum versions 3.0 and 3.1 software (Signal Analytics Corporation, Vienna, Va.) to the same maximum and minimum pixel intensities within each figure to allow direct comparison between the cell types. Figure 3 was subjected to a single round of sharpening with a 5-by-5 sharpen nat algorithm. Images were converted from a 16-bit to an 8-bit format and then imported into Adobe Photoshop version 3.0.2, where figures were composed.

RESULTS

Initial optimization of BCP. *E. coli* AB1157 and *O. linum* cells were easily distinguished with BCP (Fig. 1A and B). After the initial positive result, cell growth phase, lysozyme digestion, RNase digestion, pepsin digestion duration, chromosome in situ suppression, hybridization duration, and wash stringency were all tested for their effects on specificity and signal intensity (Table 1).

TABLE 1. Effects of various cell and probe treatments on the specificity and signal strength of BCP

Treatment or condition	Effect[a] on:			
	Selected gram-negative spp.		*Bacillus* spp.	
	Specificity[b]	Signal[c]	Specificity	Signal
Growth state				
Log phase	+++	+++	+++	+++
Stationary phase	+++	+++	+++	+++
Lysozyme digestion				
0 min	+++	+++	−	−
5 min	+++	+++	+	+
10 min	+++	+++	+	++
15 min	+++	+++	+	+++
20 min	+++	+++	++	+++
30 min	+++	+++	++	+++
45 min	+++	+++	++	++
60 min	+++	+++	++	+
RNase A digestion[d]				
+	+++	++	ND	ND
−	+	+++	ND	ND
Hybridization				
1 h	No signal[e]	−	ND	ND
4.5 h	++	+	ND	ND
Overnight	+++	++	ND	ND
2 days	+++	+++	ND	ND
3 days	+++	+++	ND	ND
Pepsin digestion				
0 min	No signal	−	No signal	−
2 min	++	+	ND	ND
5 min	+++	++	ND	ND
10 min	+++	+++	+++	+++
20 min	+++	+++	ND	ND
30 min	+++	++	ND	ND
Wash stringency[f]				
Low	+	+++	+	+++
Medium	++	+++	++	+++
High	+++	++	+++	++
Very high	+++	+	+++	+

[a] Symbols: −, no or extremely low specificity or signal; +, low specificity or signal; ++, moderate specificity or signal; +++, strong specificity or signal; ND, no data.

[b] Specificity, ability to differentiate between closely related species.

[c] Signal, signal intensity.

[d] +, RNase A treatment; −, no RNase A treatment.

[e] No signal, specificity could not be determined because no signal was detectable.

[f] Low-stringency conditions: wash 1, 50% formamide with 2× SSC preheated to 45°C; wash 2, 0.2× SSC preheated to 60°C. Medium-stringency conditions: wash 1, 50% formamide with 2× SSC preheated to 50°C; wash 2, 0.2× SSC preheated to 65°C. High-stringency conditions: wash 1, 35% formamide with 2× SSC preheated to 50°C; wash 2, 10% formamide with 0.2× SSC preheated to 60°C. Very high stringency conditions: wash 1, 50% formamide with 2× SSC preheated to 60°C; wash 2, 25% formamide with 0.2× SSC preheated to 60°C.

The growth state of the cells, i.e., logarithmic or stationary phase, had no discernible effect on signal strength or specificity. The duration of the lysozyme treatment, which is required for *Bacillus* spp. but not for the gram-negative species, was optimized for signal intensity and specificity. There was a peak in signal strength at 15 to 30 min of digestion with lysozyme, after which the signal strength decreased. RNase A digestion was performed to reduce nonspecific background hybridization due to cross-hybridization with rRNA gene fragments in the probe and was found to increase the signal-to-noise ratio. An alternate approach, CISS with ribosomal DNA (rDNA) genes to block this nonspecific signal, did not work as well (data not shown). The time course of digestion with pepsin was optimized for maximum permeabilization with minimum loss of signal due to leakage of target from the cell. Pepsin digestion, similar to lysozyme digestion, had a peak in signal strength, after which the signal decreased. CISS had no effect on hybridizations among distantly related organisms. However, in tests with *S. choleraesuis* subsp. *choleraesuis* serotypes, CISS was optimized to provide the greatest signal difference between the two organisms. At least 2 days of hybridization were required to reach equilibrium, at which point signal intensity reached a plateau. Wash conditions of medium stringency were chosen for maximum signal with minimum background.

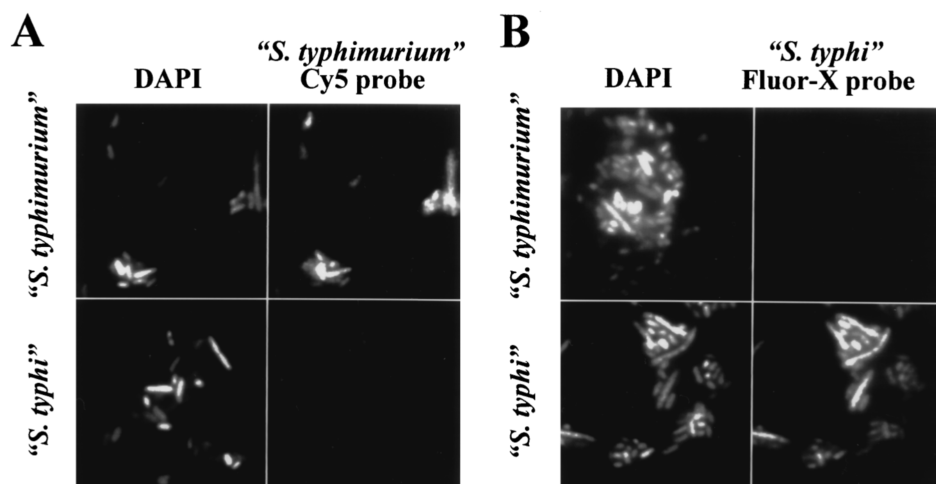

FIG. 2. Reciprocal hybridizations of bacteria related at the strain level. BCP and DAPI staining were as described in Materials and Methods. (A) *S. choleraesuis* subsp. *choleraesuis* serotype typhimurium LT2 ("*S. typhimurium*") and *S. choleraesuis* subsp. *choleraesuis* serotype typhi Ty2 ("*S. typhi*") cells probed with serotype typhimurium LT2 genomic DNA labeled with Cy5; (B) serotype typhimurium LT2 ("*S. typhimurium*") and serotype typhi Ty2 ("*S. typhi*") cells probed with serotype typhi Ty2 genomic DNA labeled with Fluor-X. Images were captured and analyzed as described in the legend to Fig. 1. A 1-h CISS step was added for enhanced specificity as described in Materials and Methods.

Effect of cell wall composition. To determine the effect of cell wall composition on signal strength, reciprocal BCP hybridizations were performed on two members of the gram-positive genus *Bacillus* (*B. subtilis* and *B. megaterium*) as well as the gram-negative bacteria *E. coli* and *O. linum*. BCP worked well with both sets of organisms (Fig. 1), although the *Bacillus* spp. required a lysozyme treatment not required by the gram-negative species to obtain a strong and specific signal. In each case, the signal for the target organism was much stronger than that for the negative control organism. Quantitation of the signal-to-noise ratio was difficult because of variability in signal and background and differences due to the use of different fluorochrome labels.

Differentiation of closely related organisms. Figure 2 demonstrates that BCP can differentiate between very closely related organisms. The two *Salmonella* strains used to test the specificity of BCP have 88% DNA-DNA solution reassociation at 60°C, indicating that they are members of the same species (4). CISS for 1 h at 37°C was required to obtain sufficient specificity in the probe to distinguish between these two organisms. In these experiments, the signal-to-noise ratio was lower than that in other experiments, most likely due to the removal of many cross-hybridizing fragments from the probe mix by CISS. In this case also, variability between experiments and between replicate samples made quantitation difficult.

Differentiation of multiple cell types in a mixed sample. To determine whether multiple species could be detected simultaneously with more than one differentially labeled probe, a mixture of approximately 50% *E. coli* AB1157 and 50% *O. linum* was probed with an equal mix of *E. coli* Cy3 and *O. linum* Cy5 probes (Fig. 3). The two cell types could be easily distinguished. There was some background hybridization of the *E. coli* Cy3 probe to the *O. linum* cells, but this was considerably lower than the true hybridization signal.

DISCUSSION

Our initial experiments were designed to demonstrate the principle of BCP with cultured organisms in controlled conditions as well as to optimize and simplify the protocol (Table 1). A model system consisting of *E. coli* and *O. linum* (Fig. 1A and B) was used to test the effect of varying several BCP protocol parameters. These organisms are distantly related members of the same bacterial class (*Proteobacteria*). They have rDNA sequence similarities of 85% (18) and are easily distinguished by differences in cell size and shape. Once a strong and specific signal was obtained, the method was tested under more challenging conditions, including with target organisms with less permeable cell walls (Fig. 1C and D), those with closer evolutionary relationships (Fig. 2), and those in simple cell mixtures (Fig. 3). BCP was effective under all conditions tested.

Although BCP consistently allowed the identification of bacterial cells, there was some variability in fluorescence among slides and among cells on the same slide. For example, in reciprocal hybridizations between serotype typhimurium LT2 and serotype typhi Ty2, higher background was observed with the serotype typhimurium LT2 probe than with the serotype typhi Ty2 probe (Fig. 2). Variability in background, specificity, and signal strength was also evident in comparisons of replicate experiments (data not shown). Possible explanations for this include variable permeabilization of individual cells and variation in the completeness of hybridization and washing caused by boundary layer effects. In future applications of BCP to natural systems, signal variability may pose problems for accurate discrimination between populations of cells that are closely related. However, appropriate positive and negative controls can demonstrate the expected range of signal strengths for positive cells as well as any overlap between positive cells and background. These controls will allow researchers to identify threshold values for the discrimination of positive signals.

A variety of approaches have been described for the specific microscopic detection of microbial cells. These include general approaches based on differential staining or autofluorescence (30) as well as techniques based on highly specific chemistry, such as fluorescent antibodies for cell surface antigens (29) or oligonucleotide probes for rRNAs (reviewed in reference 1). A major advantage of rRNA probes is that they are applicable to the specific detection of uncultured species. In theory, two other approaches, in situ PCR and chromosomal painting, could be used for similar purposes.

Fluorescent in situ hybridization with oligonucleotide probes

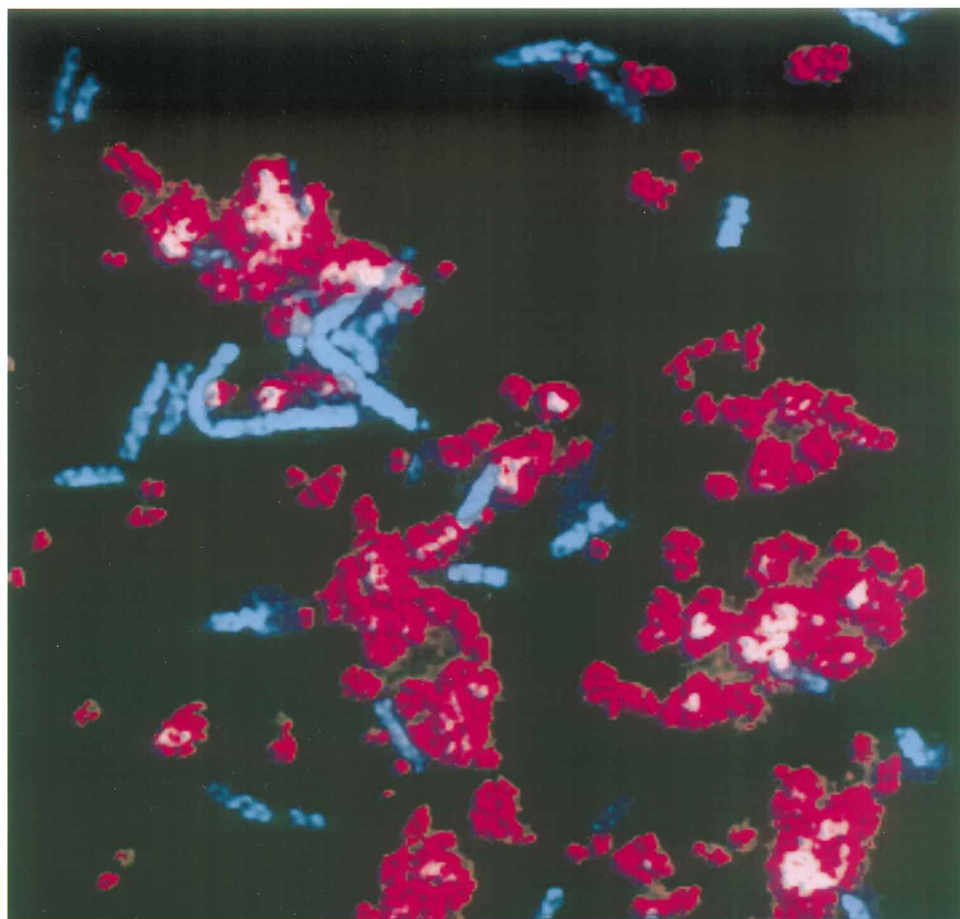

FIG. 3. Differentiation of two cell types in mixed culture by BCP. *E. coli* and *O. linum* cells (mixed ca. 1:1) were probed simultaneously with *E. coli* genomic DNA labeled with Cy3 (blue) and *O. linum* genomic DNA labeled with Cy5 (red). Separate images were captured, pseudocolored, and combined as described in Materials and Methods. *O. linum* cells clumped together in the process of fixation.

for rRNAs has been applied to a wide variety of ecosystems (reviewed in reference 1). rRNA-fluorescence in situ hybridization provides information on the ribosome content of cells and therefore can be used to assess cellular growth rates in some cases. However, the detection of fluorescent signals from these probes requires large numbers of ribosomes (10^3 to 10^4 per cell) (4); thus, dormant cells may be undetectable by this method. In applications to natural systems, a substantial percentage of direct cell counts cannot be detected with universal rRNA probes (11). Although untested in natural systems, BCP may be useful for identifying cells that are either too small or growing too slowly to be observed with rRNA probes. Additionally, rRNA sequences are most useful for examining phylogenetic relationships at the genus level and above, due to the high sequence conservation of the rRNA genes (25). BCP offers a complementary approach that may be more useful for discriminating among closely related populations of cells.

In situ PCR, another method from eukaryotic cell biology, has recently been adapted by Hodson and coworkers for the detection of multicopy plasmids (7). In situ PCR may offer some advantages for the engineering of phylogenetic and functional probes with broad specificities. However, this technique requires a difficult balance between high cell permeability, to allow the polymerase access to its target, and low permeability, to prevent the diffusive loss of the product. Chromosomal painting approaches are less sensitive to the diffusive loss of the signal because the probes are bound to chromosomes, which are large and diffuse slowly.

For BCP to be of use in natural systems, it will be necessary to obtain large genomic DNA fragments from numerically significant uncultured bacterial species. One approach to obtaining such probes is the use of large-insert vectors such as the bacterial artificial chromosome, fosmid, or P1 phage systems to clone large genomic DNA fragments from total environmental DNA. In one example of this approach, Stein and coworkers recently used a fosmid library to retrieve a 38.5-kb marine archaeal genomic DNA fragment from a marine water sample (26). Whether a genomic fragment of this size will be sufficient for BCP has not been determined; however, regions as small as 6 kb have been detected by eukaryotic chromosomal painting (16).

Chromosomal painting has the potential to augment rRNA and rDNA-based methods to provide information about specific bacterial biomass and cell counts, cell fate, bacterial succession, and other issues in microbial ecology. In addition, BCP-like methods could determine the metabolic and genetic potential of uncultured and numerically important bacterial community members by use of conserved structural genes or operons as probes. Many metabolically and ecologically important functions, such as nitrogen fixation, bacterial photosynthesis, and carbon fixation, are encoded by operons that are approximately the same size as the minimum fragment size

detectable in eukaryotic systems. Future research may explore the potential of BCP for the determination of phylogenetic distance and almost certainly will explore its potential as a means of linking functional genes to other markers, such as rDNAs, in uncultured microbes.

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