Improved Method for Polynucleotide Probe-Based Cell Sorting, Using DNA-Coated Microplates

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We developed an improved method for cultivation-independent sorting of bacterial cells. The technique is based on labeling the target cells by in situ hybridization with polynucleotide transcript probes. Due to the probes’ length, part of the probe remains outside the cell and can subsequently be used to capture the cells. Target cells are immobilized during a second hybridization step in microplates that are coated with DNA that is complementary to the probe sequence. The method was applied successfully to artificial mixtures of cells with polynucleotide probes targeting either rRNA, a plasmid-borne beta-lactamase gene, or a chromosome-borne glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. Cells could be separated based on phylogenetic parameters (using rRNA-targeted probes) as well as on other DNA-encoded traits.

A key interest of microbial ecology is the analysis of structure and function of bacterial communities. Given that only a small percentage of prokaryotic cells are culturable (2), the development of culture-independent methods for detection and enrichment of microbial cells is necessary. For detection of bacteria in environmental samples, fluorescence in situ hybridization (FISH) using fluorescently labeled oligonucleotides that target rRNA has become the method of choice (4, 7). Culture-independent enrichment can be achieved by combining FISH with flow cytometry (1). However, while very efficient, this method has the drawback of requiring expensive equipment.

A few years ago, a cost-effective alternative to flow cytometry was introduced (12). Probe-based cell fishing is based on in situ hybridization with biotin-labeled polynucleotide transcript probes targeting a hypervariable region of the rRNA. Due to the probe size (ca. 250 nucleotides [nt]), some probes or partial probes remain outside the cell (13, 19). This allows the target cells to be captured by incubating the sample with streptavidin-coated paramagnetic beads, which bind to the biotin-labeled probes protruding from the target cells. Labeled and unlabeled cells can then be separated in a magnetic field.

The work presented here describes an advancement of probe-based cell fishing. The process of cell separation was changed so that it was performed in microplates coated with DNA that is complementary to the probe sequence. The cell sorting is thus based on a hybridization of the extracellular probes to the DNA in the microplate. This renders the cell sorting independent of a (possibly insufficient) labeling of the probes with biotin and at the same time permits simultaneous handling of multiple samples and possible automation of the process. In addition, it is now possible to use plasmid- or chromosome-borne genes as target sequences for the probe-based cell fishing, which for the first time allows cells to be separated according to DNA-encoded traits such as antibiotic resistance. This sets the method apart from previously published techniques, which are restricted to the use either of antibodies binding to surface antigens (5, 9, 10) or of rRNA-targeted probes (1, 15).

MATERIALS AND METHODS

Organisms. The organisms used in this study were Acinetobacter calcoaceticus ATCC 17978, Nitisera kitsui LMG 8383T, Escherichia coli ATCC 17757T, and E. coli carrying plasmid pCR2.1 TOPO (Invitrogen, Carlsbad, Calif.).

Cell fixation. Cells were fixed with paraformaldehyde (PFA) as described previously (3) and stored in 50% ethanol in phosphate-buffered saline (PBS) (137 mM NaCl, 10 mM Na2HPO4–KH2PO4, 2.7 mM KCl [pH 7.2]) at −20°C.

Isolation of genomic DNA. Genomic DNA was isolated as described previously by Wisotzkey et al. (16).

Generation of polynucleotide transcript probes. Polynucleotide RNA probes were generated via in vitro transcription as described previously (18). The PCR primers that were used to generate the template for the in vitro transcription and the sizes of the resulting polynucleotide probes are listed in Table 1.

Coating of microplates with DNA. Nunc Maxisorb 96-well plates (Nunc, Roskilde, Denmark) were coated with PCR products (using the same primers and PCR conditions as stated above) as described previously (17). For PCR in microplates directly after the cell sorting, Nunc Nucleolink plates were used by applying the same DNA-coating protocol.

In situ hybridization with polynucleotide transcript probes. Hybridization was carried out in 0.5-ml reaction tubes. A quantity of 5 to 10 µl of PFA-fixed cells was washed with 200 µl of PBS (137 mM NaCl, 10 mM Na2HPO4–KH2PO4, 2.7 mM KCl [pH 7.2]), centrifuged for 3 min at 10,000 × g, and resuspended in 30 µl of hybridization buffer (75 mM NaCl, 20 mM Tris-HCl [pH 8.0], 0.001% sodium dodecyl sulfate, 5% formamide). For rRNA-targeted probes, a formamide concentration of 80% (vol/vol) was used, and plasmid and chromosomal DNA-targeted probes were hybridized with formamide concentrations of 10 and 5%, respectively. Five microliters of transcript probe (~4 to 5 µg) was added, and the solution was incubated at 80°C for 20 min. The subsequent hybridization was carried out at 53°C for 5 to 10 h (for rRNA-targeted probes), 18 to 24 h (for plasmid-targeted probes), or 24 to 30 h (for chromosomal DNA-targeted probes).

Cell sorting in microplates. After in situ hybridization in 0.5-ml reaction tubes, 1 ml of PBS was added; cells were centrifuged for 5 min at 12,000 × g and resuspended in microplate buffer (MP; 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.02% sodium dodecyl sulfate, 2% blocking reagent [10% (wt/vol) stock solution in maleic acid buffer; Roche, Mannheim, Germany], 0.1% N-lauroylsarcosine, 33% formamide). One hundred microliters of MP buffer was used per microliter of PFA-fixed cells for the hybridization. DNA-coated and noncoated microplates were washed with 100 µl of PBS per microwell to remove unbound DNA and were then prehybridized with 50 µl of MP buffer for 5 min at room temperature. One-half of the hybridized cells was distributed in a DNA-coated microplate (fraction B, 50 µl per well); the other...
half was distributed in a microplate not coated with DNA (fraction A [negative control], also 50 μl per well). Microplates were covered with adhesive tape to prevent evaporation and incubated for 90 min at 53°C (for RNA-targeted probes) or 37°C (plasmid- and DNA-targeted probes), respectively. The supernatants were removed (if several microwells were used, the supernatants of each fraction were pooled), centrifuged, and resuspended in 10 to 20 μl of PBS. Target cells immobilized in the microplate were either used directly for PCR analysis or recovered from the microplate by adding 100 μl of H2O2, incubating them at 94°C for 5 min, and thoroughly rinsing them to wash off the cells from the microplate (fraction C). Fraction A represents the original distribution of target and nontarget cells, fraction B should be depleted in target cells, and fraction C should be enriched in target cells.

Quantification of cell sorting efficiency. For quantification of the cell sorting efficiency, fractions A, B, and C were hybridized with fluorescently labeled oligonucleotide probes EUB338 (5’-GCT GCC TCC GTG AGG AGT-3’ [1], which stained all cells used in this study; the fractions were also hybridized with target cell-specific probe Aca23a (5’-ATCCTCTCCCATACTCTA-3′ [H11032]) for Acinetobacter spp. and with Eco444 (5’-CTT TAC TCC TCC CTT CCC-3’) for E. coli as described previously (11). The fluorescently labeled cells were analyzed with a confocal laser scanning microscope (CLSM 510; Zeiss, Ober-Kochen, Germany). The percentage of targeted cells in each fraction was determined as described previously (6).

The cell sorting efficiency was expressed as percent depletion of target cells and calculated as follows: \[ \frac{\text{percent of target cells before cell sorting} - \text{percent of target cells after cell sorting}}{\text{percent of target cells before cell sorting}} \times 100 \]

PCR after cell sorting. To confirm the availability of genetic material for further analysis, a PCR was performed directly with the fixed cells after the cell sorting. Universal primer 1019V (5’-TGG TTC TCC TCT (CG) CCG AAA-3’), which binds to 23S rDNA, E. coli positions 807 to 821 and E. coli-specific primer 91R-E (5’-GCT TAA ACC GGG ACA ACC-3’, which binds to 23S rDNA, E. coli positions 1459 to 1477) were used to specifically amplify E. coli DNA and primer 1019V in combination with N. cani-specific primer 91R-N (5’-CGG CTT AAA CAA GCT ATT-3’, which binds to 23S rDNA, E. coli positions 1461 to 1479), which was used for amplification of N. cani DNA. The following PCR parameters were used: initial denaturation, 10 min for 94°C; 30 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C; followed by a final elongation step of 3 min at 72°C.

RESULTS AND DISCUSSION

The procedure consists of two discrete hybridization steps. During the first step, target cells are labeled with polynucleotide probes, followed by the immobilization of the target cells in DNA-coated microplates during the second step. Depending on whether a depletion or enrichment is desired, either the supernatant (depleted in target cells) or the cells in the microplates (highly enriched in target cells) are used for further analysis. The cells in the microplates can be either recovered by heat denaturation of the hybrids or employed directly for PCR in the plates.

The concept of probe-based cell fishing is based on the assumption that probes or partial probes are located outside the cell (12, 13). When fluorescently labeled polynucleotide probes for in situ hybridization are used, the appearance of a halo, i.e., a concentration of fluorescence signal around the cell, supports this idea. We have good evidence that the probes form a network on the basis of their secondary structure. Due to the limited permeability of the cell envelope, only part of the probe is linked to its intracellular target site, while the remaining part is located outside the cell and can form a network by hybridizing with single-stranded probes (18). According to this hypothesis, the number of probe molecules associated with a target cell can easily exceed the copy number of intracellular target sequences and therefore allow the detection of low-copy-number targets such as plasmin and even chromosome-borne genes (19). To ensure the formation of such a network, a large amount of probe has to be used (3 to 5 μg). This is especially important for plasmid- and DNA-targeted probes, where only few intracellular target sequences are available. A high percentage of target cells in the sample was found to decrease the cell sorting efficiency, possibly due to competition of the cells for the limited amount of probe.

The cell sorting method was tested with probes targeting rRNA (probe DIII, targeting a ~250–nt region of 23S rRNA domain III), a plasmid-encoded antibiotic resistance gene (probe betaLact, targeting an 840-nt fragment of the beta-lactamase gene), and a chromosome-borne housekeeping gene (probe GAPDH-E, targeting a 238-nt fragment of the E. coli glyceraldehyde-3-phosphate dehydrogenase [GAPDH]).

The probes were generated via in vitro transcription by using T3 RNA polymerase as described previously (18). In the course of transcription, the probes can be labeled with biotin, digoxigenin, or a fluorescent dye by incorporation of labeled UTP. Although labeling is not necessary for the cell sorting procedure, the procedure may be desirable for subsequent analysis of the cells by epifluorescence microscopy. Prior to the cell sorting experiments, the specificity of all probes was confirmed by FISH (19).

With regard to the very different copy numbers of the target molecules (up to 10^7 for rRNA, ~10^2 for the [high-copy-number] plasmid, and less than 10 for the chromosomal DNA) and

| Primer     | Sequence (5’-3’) | Size of polynucleotide probe (nt) | Probe target                  |
|------------|-----------------|----------------------------------|-------------------------------|
| 317RT^3    | ATA GGT ATT AAC CAC TAA AGG G ACC (A/T)GT GTC (C/G)GT | 236 | 23S rRNA, domain III |
| 1900V°     | (A/C)(A/G/T) GCG TAG (G/C/T)CCA(A/T)G | 840 | Plasmid-borne beta-lactamase gene |
| BetaLact-RT3 | ATA GGT ATT AAC CAC TAA AGG G ACC AAT GCT TAA TCA GTG | 258 | Chromosome-borne GAPDH gene |
| BetaLact-V  | GAG TAT TCA ACA TTT CCG | | |
| GAPDH-E-RT3 | ATA GGT ATT AAC CAC TAA AGG G CA GTT TCG TCA GTG A | | |
| GAPDH-E-V  | GCT GCT CAG AAA CGT TCT | | |

^a Shown are the primers that were used to generate the template for the in vitro transcription; the resulting polynucleotide probe sizes are also indicated.
^b The T3 promoter sequence is indicated by underlined and boldface type.
^c Ludwig et al. (8).
the different stability of the hybrids (RNA-RNA for rRNA-targeted probes and RNA-DNA for the plasmid- and chromosomal DNA-targeted probes), the hybridization conditions for the first hybridization (stringency, incubation time, and amount of probe) had to be adapted (19). For the second hybridization, the temperature was reduced to 37°C (instead of 53°C) when plasmid- or DNA-targeted probes were used.

The two hybridization steps vary distinctly in their stringency. While the first hybridization requires stringent conditions, the second hybridization should be carried out under more relaxed conditions to allow all labeled cells to be immobilized in the microplate. Since the second hybridization can differentiate only between labeled and unlabeled cells, it is vital that a successful cell sorting ensure specific and comprehensive binding of the probes during the first hybridization.

To avoid saturation of the surface of the microplate with target cells, the target cell-containing sample was dispersed over 5 to 10 DNA-coated microplate wells. This is especially important if the target cells constitute a high percentage of the sample.

Two types of (96-well) microplates differing in their surface and accordingly in their mode of binding DNA were compared with respect to their usefulness for the cell sorting process. In Maxisorp plates (Nunc), the binding of DNA is based on hydrophobic-hydrophilic interactions, whereas in Nucleolink plates (Nunc), DNA is bound covalently via carbodiimide condensation. Both types of microplates proved to be equally suitable for immobilizing labeled target cells. Nucleolink plates, however, have the advantage of being available in a format suitable for PCR, thus permitting a direct amplification of the genetic material of the target cells after the cell sorting.

Cell sorting efficiency was determined by comparing the number of target cells before and after the cell sorting procedure. FISH was performed with rRNA-targeted oligonucleotide probes, and target cells were quantified by using confocal laser scanning microscopy in conjunction with appropriate software.

The efficiency of the cell sorting process depends mainly on the number of target cells in the sample. Figure 1 summarizes the results of all cell sorting experiments with the three types of probes (rRNA, plasmid, and DNA targeted), showing the initial concentration of target cells in the sample and the depletion rate achieved by the cell sorting. A clear correlation between the concentration of target cells and the depletion rate can be seen, with the best results (up to 70% depletion) being produced at target cell concentrations of <10%. This effect was observed with all three types of probes, although there seemed to be a greater variation between individual experiments for the DNA-targeted probes. Saturation of the microplate surface may account for the observed low depletion rates at high concentrations of target cells. If too many cells compete for a limited amount of probe, a weaker binding to the plate due to an incomplete network of probes around the target cells may also contribute to the effect. The enrichment, however, is not impaired by high concentrations of target cells, since the cells recovered from the surface of the microplates generally contained 90 to 98% target cells. In control experiments with microplates not coated with DNA, no depletion or enrichment of target cells could be observed.

However, even when we used DNA-coated microplates, it
was not possible to completely remove all target cells from a sample. This limitation is mostly due to the fact that not all cells are able to take up the probe, a problem that is more pronounced when DNA-targeted probes (where the target accessibility might be reduced when the DNA is in supercoiled state during the stationary phase of the cell) than when rRNA-targeted ones are used.

The availability of the genetic material for further analysis after cell sorting was confirmed by PCR using primers specific for target cells to amplify sequences from cells immobilized in the microplate and using primers specific for nontarget cells for amplification of the genetic material of the cells in the supernatant.

Cell sorting in microplates offers a low-cost alternative to flow cytometry-based methods (1, 15), requiring only standard equipment (PCR cycler).

Compared to the magnetic cell sorting described by Stoffels et al. (12), the microplate-based method requires less hands-on time and allows the simultaneous processing of multiple samples.

The concept of using DNA-coated microplates also offers the possibility of a simultaneous hybridization with different probes and the subsequent cell separation in several microplates coated with DNA that corresponds to the different probes and the subsequent cell separation in several microplates. This option opens the door for isolating cells according to any DNA-encoded trait, e.g., plasmid- or DNA-targeted probes. This option opens the door for isolating cells according to any DNA-encoded trait, e.g., photosynthesis, nitrate oxidation, antibiotic resistance, etc., thus presenting a valuable new tool for microbial ecologists.

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