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Selective isolation of bacterial cells within a microfluidic device using magnetic probe-based cell fishing

Jérémy Pivetal[a,*], Sylvain Toru[a], Marie Frenea-Robin[a], Naoufel Haddour[a], Sébastien Cecillon[a], Nora M. Dempsey[b], Frédéric Dumas-Bouchiat[c], Pascal Simonet[a]

[a] Université de Lyon, Lyon, F-69622, France ; université Lyon 1, Lyon, F-69622, France ; CNRS, UMR5005, Laboratoire AMPERE, Villeurbanne, F-69622, France
[b] Univ. Grenoble Alpes, Inst NEEL, F-38042 Grenoble, France and CNRS, Inst NEEL, F-38042 Grenoble, France
[c] SPCTS, CNRS - University of Limoges, 12 Rue Atlantis, 87068 Limoges, France

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ABSTRACT

This paper demonstrates the selective isolation of bacterial cells from a mixture by combining magnetic probe-based cell fishing with the use of microfluidics. A microfluidic cell sorter was obtained by integrating permanent micromagnets in a PDMS microchannel. While non-target cells pass through the device, the magnetically labeled target cells can be efficiently captured thanks to the high magnetic field gradients generated by the micro-flux sources. The sorting device capabilities were evaluated by performing enrichment of *Escherichia coli* DH10β from a bacterial cell mixture containing an initial concentration of 0.04% *E. coli* DH10β and 99.96% *Acinetobacter* sp. ADP1. The probes used to specifically target *E. coli* cells were RNA transcripts from PCR amplicons of 23S rRNA genes from *E. coli* DH10β DNA. Those probes were biotinylated to enable magnetic labeling with 50 nm streptavidin-coated superparamagnetic particles after *in situ* hybridization. qPCR analysis of the sorted fractions revealed that the target cells were enriched from an initial concentration of 0.04% to a final concentration of 98.46%, with a 50% recovery rate, at a throughput of 10^9 cells per hour. This approach offers great potential to address issues linked to exploration of bacterial diversity in complex ecosystems, where labeling strategies applicable to unculturable bacteria are required.

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1. Introduction

Exploration of bacterial genetic and functional diversity in environmental samples not only provides better understanding of ecological processes but can also enable key advancements in different application areas. For example, analysis of functional processes can be required in biomedicine to identify potential novel therapeutic targets [1–3] as well as in bioengineering to find new strains usable for bioremediation of contaminated soils [4,5].

Since only a small percentage of bacteria (≈1%) are cultivable *in vitro* [6], much effort has been directed over the last decade toward the development of culture-independent methods to study microbial communities [7]. In particular, thanks to the development of high-throughput sequencing, metagenomics has become a fast growing discipline, which has considerably improved our knowledge of the uncultured microbial majority [8–10]. This approach involves analyzing the combined genetic material of bacteria recovered from an environmental sample rather than studying single bacterial species. However, in environments such as soils and sediments, the exhaustive deciphering of the microbial diversity is precluded by the complexity of the microbiome, and by methodological limitations (DNA extraction bias, sequencing errors, etc.) [11,12].

Consequently, genome reconstructions are simplified descriptions of reality, which is even truer when biodiversity is hidden in lysis-recalcitrant bacteria or rare taxa [13].

To circumvent some of the limitations of culture and metagenomic approaches and reduce cell diversity, one possible route is to perform upstream selection of bacterial cells based on taxonomic or functional criteria, thus physically separating targeted bacteria from other microorganisms and environmental contaminants prior to DNA extraction and sequencing [14]. This approach offers the advantage of preserving individual genomes in cells – and therefore solving the problem of assembly inherent to metagenomic approaches – while enabling the genomic analysis of isolates regardless of their culturability.

* Corresponding author. Tel.: +33 609246123.
E-mail addresses: jeremy.piv@voila.fr (J. Pivetal), sylvain.toru@ens-cachan.fr (S. Toru), marie.robin@univ-lyon1.fr (M. Frenea-Robin), naoufel.haddour@ec-lyon.fr (N. Haddour), sebastien.cecillon@ec-lyon.fr (S. Cecillon), nora.dempsey@neel.cnrs.fr (N.M. Dempsey), frederic.dumasbouchiat@gmail.com (F. Dumas-Bouchiat), pascal.simonet@ec-lyon.fr (P. Simonet).

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Specific isolation of uncultured bacterial cells may be performed using fluorescence *in situ* hybridization (FISH). In the FISH technique, the sample is first fixed to stabilize the cells and permeabilize their membranes. Fluorescently labeled oligonucleotide probes are then added and allowed to hybridize complementary rRNA targets located within the permeabilized cells [15,16]. Efficient separation of target cells can then be performed using fluorescent-activated cell sorting, based upon the specific fluorescent characteristics acquired by cells [17,18]. However, flow cytometers are expensive instruments requiring maintenance from skilled operators, and their miniaturization constitutes a huge challenge [19]. Moreover, the effectiveness of this technique in complex environments such as soil remains limited by the natural autofluorescence of certain mineral constituents and the presence of contaminants in the matrix that hinder the process of cell sorting [20–23].

Magnetic probe-based cell fishing provides an alternative to fluorescence-based methods [24]. The principle consists in performing *in situ* hybridization with probes tagged with superparamagnetic particles. As demonstrated by Stoffels et al., hybridized target cells can therefore be subsequently sorted using a magnetic column (such as a MACS® column) specifically retaining labeled cells [24]. However, the recovery rate of target cells can be rather low (between 20 and 30% in the study performed by Stoffels and coworkers) and this low recovery rate may be notably due to the magnetic physical property of the column. Such a limitation may be compromising when the goal is to capture bacteria present at very low abundance in a given sample.

Here, we demonstrate that the magnetic separation of bacterial cells labeled using magnetic probe-based cell fishing can be performed efficiently within a microfluidic device integrating hard magnetic micro-flux sources. Indeed, the magnetic force exerted on targeted cells are greatly enhanced thanks to the huge magnetic field gradients (10^6 T/m) obtained by miniaturizing the magnetic sources [25]. Moreover, micro-patterned hard magnetic structures have the advantage of requiring neither an external magnetic field nor a power supply, and have been used to fabricate compact autonomous devices for the trapping of magnetic particles and cells functionalized with such particles [25–27]. In the present study, we demonstrate the selective isolation of targeted *Escherichia coli* from a mixture of two bacterial species using magnetic probe-based cell fishing, followed by quantitative evaluation of separation efficiency using qPCR.

2. Materials and methods

2.1. Organisms and growth conditions

The organisms used in this study were *E. coli* DH10β and *Acinetobacter* sp. ADP1. All these strains were cultured in Luria Bertani (LB) broth at 37 °C.

2.2. Cell fixation

Cell fixation was performed from exponentially growing cultures (optical density = 0.8–1) using paraformaldehyde, a chemically fixative agent. For this purpose, cells from 2 ml of culture were collected by centrifugation for 10 min at 2500 × *g*. The pellet was then resuspended in 500 μl of a 3% paraformaldehyde solution (PFA) diluted in phosphate-buffered saline (130 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2) and incubated 12 h at 4 °C. PFA-fixed bacteria were then washed three times by centrifugation (2500 × *g* for 10 min) with PBS and finally resuspended in 500 μl of the same buffer. For storage at −20 °C, 50% (v/v) absolute ethanol was added to samples.

2.3. Preparation of rDNA templates for in vitro transcription

PCR amplification of 23S RNA gene fragments encoding the variable region of domain III was performed on *E. coli* DH10β genomic DNA extracted using the NucleoSpin® Tissue kit (Macherey-Nagel), following the manufacturer's instructions. The nucleotide sequences of the primers were 5′-MADGCGTAGNCGAWGG-3′ (1900V [28]) and 5′-**TAATACGACTCACTATAGGG**GGACCWGTGTCSGTTTHBGTAC-3′ (317RT [24]). The latter contained the T7-RNA polymerase promoter sequence (underlined) needed for the *in vitro* transcription. PCR was performed using Illustra Hot Start Mix RTG PCR beads (GE Healthcare), according to the supplied protocol. The cycling parameters were 95 °C for 10 min, followed by 35 cycles at 95 °C for 30 s, 53 °C for 60 s and 72 °C for 90 s, followed by 72 °C for 10 min. The resulting rDNA fragments had an expected size of about 236 nucleotides. They were analyzed by electrophoretic separation in 1% (w/v) agarose gel stained with ethidium bromide. Purified PCR products (GFX PCR DNA and Gel Band purification Kit, GE Healthcare) were used as templates for *in vitro* transcription.

2.4. Preparation and labeling of transcript probes

The transcription of the 23S rDNA fragments was performed using an RNA transcription kit (Invitrogen). The RNA probe products were purified with the NucleoSpin® RNA purification kit (Macherey-Nagel) and subsequently labeled with a biotin labeling kit (Mirus), offering a labeling density of about 1 labeled nucleotide every 50 nt.

2.5. Magnetic labeling of bacterial cells

Targeted cells were specifically labeled in liquid samples by performing magnetic *in situ* hybridization. Ten microliters of PFA-fixed cells were washed with 200 μl of PBS (130 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2), centrifuged for 3 min at 10,000 × *g*, and resuspended in 30 μl of a hybridization buffer containing 100 mM NaCl, 0.01% SDS, 20 mM Tris–HCl (pH 8.0), 5 mM EDTA (pH 8.0), 80% formamide and 1 μg of labeled transcript probes. The solution was incubated at 80 °C for 20 min and the subsequent hybridization was carried out at 53 °C for 4 h. After cell hybridization with the biotinylated transcript probes, 60 μl of PBS (130 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4, 0.5 M EDTA, pH 7.2) and 10 μl of streptavidin-coated paramagnetic beads (Miltenyi Biotec MicroBeads, diameter 50 nm) were added to 40 μl samples. An incubation step was then performed overnight at 4 °C. Two reference control samples were also prepared. One contained a bacterial cell suspension with no probe but subjected to hybridization conditions and incubated with nanoparticles. The other was obtained similarly, but did not contain nanoparticles.

2.6. Micro-magnet design and fabrication

High rate triode sputtering was used to deposit 5 μm thick hard magnetic neodymium iron boron (NdFeB) films on Si wafers [29]. Ta (100 nm) was used as both a buffer and capping layer to prevent diffusion into the Si and oxidation, respectively. NdFeB films present a coercivity of around 2 T and a remanence up to 1.3 T. Micro-magnet arrays were obtained by Thermo-Magnetic Patterning (TMP), an approach which consists in exploiting the temperature dependence of coercivity to locally switch magnetization [30]. Using a KrF (248 nm) nanosecond pulsed excimer laser, the magnetized hard magnetic films were irradiated through a mask with 100 μm × 100 μm square patterns. At the same time, an external magnetic field was applied opposite to the initial magnetization direction, which induced magnetization reversal in the

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