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Flow cytometric sorting of fecal bacteria after *in situ* hybridization with polynucleotide probes

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ABSTRACT

The gut microbiome represents a key contributor to human physiology, metabolism, immune function, and nutrition. Elucidating the composition and genetics of the gut microbiota under various conditions is essential to understand how microbes function individually and as a community. Metagenomic analyses are increasingly used to study intestinal microbiota. However, for certain scientific questions it is sufficient to examine taxon-specific submetagenomes, covering selected bacterial genera in a targeted manner. Here we established a new variant of fluorescence *in situ* hybridization (FISH) combined with fluorescence-activated cell sorting (FACS), providing access to the genomes of specific taxa belonging to the complex community of the intestinal microbiota. In contrast to standard oligonucleotide probes, the RNA polynucleotide probe used here, which targets domain III of the 23S rRNA gene, extends the resolution power in environmental samples by increasing signal intensity. Furthermore, cells hybridized with the polynucleotide probe are not subjected to harsh pretreatments, and their genetic information remains intact. The protocol described here was tested on genus-specifically labeled cells in various samples, including complex fecal samples from different laboratory mouse types that harbor diverse intestinal microbiota. Specifically, as an example for the protocol described here, RNA polynucleotide probes could be used to label *Enterococcus* cells for subsequent sorting by flow cytometry. To detect and quantify enterococci in fecal samples prior to enrichment, taxon-specific PCR and qPCR detection systems have been developed. The accessibility of the genomes from taxon-specifically sorted cells for subsequent molecular analyses was demonstrated by amplification of functional genes.

Introduction

Environmental microbial communities often exhibit a highly diverse composition, and the functional interactions among the community members or between microbes and host organisms, as well as their dynamics, are still not well understood. The analysis of such complex communities is mainly limited by the fact that the majority (~99%) of the microbial diversity on earth is estimated not to grow under lab conditions and therefore, remain inaccessible to further analyses [4]. Nevertheless, understanding the composition and genetics of whole microbial populations under certain conditions to date is a big scientific challenge to gain insights into how microbes function individually as well as within the complex populations they are embedded in.

Recently, research on the human intestinal microbiota increasingly became a focus of attention. The individual human intestinal microbiota contains approximately 10^{13}–10^{14} microorganisms, collectively comprising around 1800 genera and 15,000–36,000 species that carry at least 100 times as many unique genes as are found in the complete human genome [15]. This huge genetic resource provides a wide range of metabolic functions related to human hosts (*e.g.*, carbohydrate metabolism, energy metabolism, and storage) [27]. The composition has been shown to vary greatly between individuals, each preserving their relatively stable unique structure of intestinal microbiota [5,13,33,50], independent of origin and health status, that are defined primarily by species and functional composition [5], and dominated by only two phyla, *Firmicutes* and *Bacteroidetes*, followed by minor proportions of *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *Verrucomicrobia* [13]. Thus, research on the influence of a disruption of commensal microbiota (dysbiosis) on human health is increasingly in the center of interest [15,22,23].

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To address the challenge of understanding in-depth linkages between microbial diversity and their functional features, various culture-independent technologies have been developed over the past years. Most of them commonly use 16S or 23S ribosomal RNA (rRNA) genes as targets for taxonomic characterization which are presently the most widely investigated phylogenetic markers for bacteria. Comprehensive public rRNA databases are available which allow rapid identification and profiling of the gastrointestinal microbiota or any other environment [8,11,12,24,26,32]. While microbiome profiling with phylogenetic markers excludes functional aspects, latest next-generation sequencing techniques, including whole genome shotgun sequencing (WGS), enable the generation of a wide range of data, *e.g.* on the human microbiome [33,44], which provide simultaneous analysis of all genes and their putative functions in the respective organisms, even in complex microbial samples. However, despite various advances in binning technology and promising metagenomic tools, which aid the *de novo* assembly of microbial genomes without the need for reference sequences, the complexity of environmental samples such as the gut microbiome still poses major challenges. The main limitations of the metagenomics approach to retrieving complete genomes are microdiversity and very low abundance of the target population [1,2,30,41].

As an alternative to analyzing whole communities by metagenomics, there have also been attempts to enrich specific populations or even single cells from complex specimens, prior to sequence analysis [35,47]. In this context, flow cytometry (FCM) is one of the most commonly used tools. FCM can be applied to enrich specific bacterial cells by fluorescence-activated cell sorting (FACS) based on light scattering properties and detection of fluorescently labeled cells. In combination with fluorescence *in situ* hybridization (FISH) analysis, a widely used technique for monitoring and identifying microorganisms in complex ecosystems, FACS has the potential to not only count but also to sort specific microbial communities in a high-throughput manner. This allows for the parallel characterization of microorganisms with respect to both physical and biochemical properties [3,16,34,40,46]. The mono-labeled oligonucleotide probes commonly used for this purpose are however known to produce high background signals specifically in environmental samples [19,47]. Furthermore, the signal intensity of the detected cells is directly related to the number of target molecules in the cell, naturally slow growing or starving bacteria might be below the detection limit [19,28]. Finally, to date, no technique has been introduced to reliably enrich a target population from feces for subsequent subjection to molecular analyses such as metagenomics. The molecular process is mainly restricted by the existing rigid signal amplification protocols which at the same time limit the access to the cells' genomic information [3,6,17,29,37,47].

Facing the challenge to specifically sort populations from a complex microbial community while simultaneously being able to recover DNA in high quality, we focused on a technology that uses specific polynucleotide probes for *in situ* hybridization. Because of their reduced specificity and more complex *in vitro* synthesis compared with oligonucleotides, less attention was paid to these probes over the past years [38].

We focused on polynucleotide probes targeting domain III of the 23S rRNA, because Trebesius et al. [45] discovered special features of these multiple fluorescently labeled ssRNA probes. First, these approximately 250–300 nucleotide probes were characterized by a relatively high variability in contrast to the general conservation of the 23S rRNA gene, resulting in a specificity roughly on genus-level. Still, it was shown that the specificity could be adjusted by changing the stringency of the hybridization. Second, the reduced specificity was meant to be partly compensated by the strong halo-shaped hybridization signals that these polynucleotide probes could bring about under certain hybridization conditions, even when detecting microbes with a low ribosome content. In addition, these probes were already shown to detect a larger percentage of cells in environmental samples in contrast to oligonucleotide probes [14,31,45]. The natural signal amplification induced by one single probe was formerly explained by the "network hypothesis" [51] and was already successfully utilized for certain applications [43,51–54].

The purpose of this work was to enable the cultivation-free, targeted enrichment, and finally the recovery of DNA, from key gut microorganisms. *Enterococcus* spp. was chosen as a representative genus to introduce the novel technique by use of specific polynucleotide probes. To this end, we established and tested a protocol combining FISH and FACS for taxon-specific cell enrichment of enterococci from fecal samples. For certain scientific questions such as in-depth sequence analysis of a specific genus out of a complex sample, this methodology might be an alternative to other existing techniques. Further, it can probably be adopted to other microbial communities than the gut microbiome, for example samples related to clinical or food microbiology. Since only a specifically targeted part of a complex microbial consortium is extracted, the novel methodology developed and evaluated here could be used in combination with metagenomic characterization of the extracted population to (i) study certain taxonomic groups in greater depth than is possible with conventional metagenomics, including possible rare species and strains, (ii) study functional gene catalogs of taxa under different conditions (*e.g.*, in response to diet or inflammation and competing strains), and (iii) compare intestinal taxon-specific metagenomes between different individuals.

Materials and methods

Reference strains and culture conditions

The 19 intestinal or clinical relevant reference strains used in this study were obtained from various sources, including the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany and the CCUG, Culture Collection, University of Göteborg, Sweden (Table A.1). The strains were cultivated as described in the catalogs of these organizations. Exponentially grown cells were harvested by centrifugation at 14,000 rpm and 4 °C for 10 min, washed with sterile-filtered (0.2 μm) phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4).

Polynucleotide probe generation

The ssRNA polynucleotide probe (polyDIII) with a size of approximately 250 nucleotides was generated *via in vitro* transcription of PCR-amplified rDNA based on the protocol by Zwirglmaier et al. [51]. PCR was performed using the TaKaRa Ex-Taq PCR system (TaKaRa Bio Inc., Kusatsu, Shiga, Japan), according to the manufacturer's protocol, using genomic DNA of selected *Enterococcus* reference strains as a template. Genomic DNA was isolated using a protocol according to Wisotzkey et al. based on a chloroform/isoamyl alcohol extraction [49]. Modified universal primers used for PCR amplification of the DNA target region (23S rDNA domain III) of bacteria were 1900V (HAG GCG TAG GCG ATG) and the reverse primer 317RT3 (ATA GGA ATT AAC CCT CAC TAA AGG GAC CTG TGT CGG TTT RCG GTA), which included the promoter sequence for T3 polymerase (underlined) [25]. Initial denaturation for 10 min at 94 °C was followed by 32 cycles of 10 s denaturation at 98 °C, 30 s annealing at 54 °C, 1 min primer extension at 72 °C, and a final elongation step of 10 min at 72 °C. PCR products were verified by agarose gel electrophoresis, applying the GeneRuler 1 kb Plus DNA Ladder (Thermo