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The feasibility of improved live-dead distinction in qPCR-based microbial source tracking

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ABSTRACT

PCR-based microbial source tracking (MST) has become a useful tool to identify dominant sources of fecal pollution in water. The method has previously been successfully combined with viability PCR (using propidium monoazide) allowing the preferential detection of membrane-intact bacteria. This study aimed at further improving the selectivity for intact cells when targeting host-specific markers in *Bacteroidales* bacteria. One approach was to increase amplicon sizes that had been shown to be useful for other applications of viability PCR. For this purpose, two different amplicon sizes were compared when targeting either the genus of *Bacteroidales* or subgroups thereof specifically associated with human and ruminant fecal material. When applied to different environmental samples, the proposed proportion of intact cells could drop by up to 38% (for sewage treatment effluent from 64 to 26%) when targeting longer sequences. Furthermore co-incubation of the viability dye with dimethylsulfoxide (DMSO) was found to be beneficial, although this observation is currently still empirical. When examining signal decay of artificially contaminated unfiltered river water over six weeks, the PMA treatment effect was observed from the beginning, but the ratio of intact and damaged cells remained constant over time with signals disappearing at the same rate independent of PMA treatment. In this instance the contribution of other factors to overall signal decay seemed more important than loss of membrane integrity.

1. Introduction

The origin of fecal contamination in natural water bodies is in many cases unknown. Apart from bathing waters for recreational activities, this lack of knowledge is especially problematic for raw waters that serve for drinking water production and that do not undergo extensive treatment to lower the microbial load. Minimization of contamination loads in impaired water bodies is however also an important component of the multi-barrier approach to safe drinking water spanning from source to the consumer's tap (Damikouka et al., 2007; Schill and Mathes, 2008; Marti et al., 2011). Problem remediation critically relies on knowledge about the sources of contamination. Microbial source tracking (MST) has shown to be a useful tool to fill this gap and to identify potential sources (Harwood et al., 2014).

Among a number of MST methods, quantitative PCR based on detection of host-specific bacterial groups has a prominent role in laboratories. *Bacteroidales* are the most important bacterial group with 16S rRNA genes serving as genetic markers. As for any PCR-based method, however, the approach per se cannot distinguish between recent and past contamination due to its inability to discriminate between DNA from live or dead organisms (Bae and Wuertz, 2009a). In addition to dead bacteria, free DNA that can persist in water and sediments for elongated times can distort results. Detection of free DNA over up to 21 days (freshwater), 40 days (sediments) and 55 days (marine water) have been reported (Nielsen et al., 2007). The combination of sample treatment with propidium monoazide (PMA) prior to analysis has been suggested to be a promising approach to overcome this problem (Bae and Wuertz, 2012). PMA both binds to free DNA and selectively only enters membrane-damaged cells followed by intercalation into their DNA (Nocker et al., 2006). Subsequent light exposure leads to irreversible modification of the DNA and suppresses its amplification. As a result the treatment allows preferential detection of DNA from membrane-intact cells whose DNA does not undergo modification. Applying this method, it could be shown that the signals from membrane-intact *Bacteroidales* undergo a continuous decline in water as shown in microcosm studies (Bae and Wuertz, 2012). Determination of genetic markers from *Bacteroidales* with and without prior PMA treatment was suggested to be a means to provide more relevant information and an estimate time of the contamination event (Bae and Wuertz, 2012). Signals derived without treatment with a viability dye on the other

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hand only provide information about the overall contamination, but not when it occurred.

Despite successful applications of viability quantitative PCR (qPCR) to MST, a current research focus still lies on improving the efficiency of suppression of signals from membrane-compromised bacteria. Approaches so far included higher PMA concentrations and light exposure times (Bae and Wuertz, 2009a). Optimization of these parameters improved the efficiency of dead cell exclusion in samples with high organic content. In this study we addressed the usefulness of increased amplicon lengths that have been shown beneficial in other applications of viability PCR (Contreras et al., 2011; Li and Chen, 2013; Schnetzinger et al., 2013; Soejima et al., 2011). Furthermore the effect of incubating the viability dye together with DMSO was assessed. DMSO is well known to affect permeability of cell membranes (Gurtovenko and Anwar, 2007; Notman et al., 2006). The idea was to test whether DMSO might facilitate dye penetration into cells with possibly moderate membrane damage, comparable to the effect of PMA incubation at elevated temperatures or in the presence of deoxycholate (Nkuipou-Kenfack et al., 2013). *Bacteroidales*-based assays with specificity for fecal material of human and ruminant origin were applied for this purpose together with a universal *Bacteroidales* test. Primers producing different amplicon lengths and the optimized DMSO concentration were finally applied to samples from a wastewater treatment plant (WWTP), a river and creek samples.

2. Material and methods

2.1. Environmental samples

Samples used in this study were collected at different sites in North Rhine-Westphalia and Lower Saxony between August 2015 and June 2016. Water samples were collected from the river Ruhr at a site next to a field with a herd of grazing cows, two different creeks and a WWTP (influent and effluent). The WWTP operated on the principle of conventional mechanical (solids removal), biological (activated sludge) and chemical (phosphorous precipitation) treatment without final disinfection. Samples were cooled (4 °C), transported to the laboratory and processed within 24 h. Volumes of 500 mL were filtered using a vacuum manifold (Sartorius, Göttingen, Germany) and mixed cellulose filters (0.45 μm pore size, 45 mm diameter; Millipore, Molsheim, France). Filtered material was subjected to PMA treatment (or not) followed by extraction of genomic DNA directly from the filters. Fecal material for experiments to study the effect of DMSO or that served to test specificity was collected from humans, cows, badger, deer, dogs and pigs. Human samples were given by anonymous donors. Cow and pig samples were collected from different farms, dog samples were collected from public parks frequently used by dogs. Deer and badger fecal material were collected from wild living animals in forests. For the assessment of signal specificity, at least three independent samples from each source were pooled with the exception of badgers and deer where only one and two samples were available, respectively. Aliquots of 0.1 g of fecal material (wet weight) were suspended in 10 ml filtered (0.2 μm) mineral water (Evian, France) to a final concentration of 10 g/L. Dead cell aliquots of fecal material of human and cow origin were prepared by transferring 1 mL volumes into 1.5 mL microcentrifuge tubes and exposing them to 60 °C for 20 min using a standard laboratory heat block. Higher temperatures resulted in substantial signal losses even without PMA treatment, potentially through thermal damage of the DNA or partial cell lysis.

2.2. Preparation of standards

For every primer-probe combination and targeted fecal source a standard curve was made. Detected fecal concentrations were expressed in mg fecal material (wet weight) per liter water instead of genome units. The approach was chosen to become independent of the ambiguities associated with the amplification of the cloned sequence of a single *Bacteroidales* strain, which we considered little representative of the large diversity contained in fecal samples. To obtain material for a standard, at least three different fecal samples from each animal target were collected and three identical aliquots of every sample were processed (translating to at least 9 data points). Fecal samples were suspended in filtered (0.2 μm) mineral water (Evian, France) to a final concentration of 0.1 g per 10 mL. Genomic DNA was extracted from 1 mL aliquots and subsequently serially diluted. The highest concentration corresponded to 10 mg fecal material (wet weight) per 10 mL water. Each dilution series was amplified using the matching primers and probes. When using universal *Bacteroidales* primers, mixed samples with fecal material of human and cow origin were used. Standard curves together with standard deviations were made using Excel (Microsoft Office).

2.3. PMA treatment

PMA (0.7 mg aliquots; QIAGEN, Hilden, Germany) was dissolved in 550 μL water (DNA and nuclease-free, provided by QIAGEN) to give a concentration of 2.5 mM. This stock was either used directly or stored at − 20 °C in the dark. In experiments with suspended fecal material aiming at studying the effect of amplicon length and DMSO, 10 μL PMA were added to 500 μL of suspended fecal samples (concentration of 10 g wet weight per L; untreated and heat-treated) to obtain a final dye concentration of 50 μM as commonly used in other studies (Fittipaldi et al., 2012). Samples were supplemented before with different concentrations (v/v) of DMSO. Samples without DMSO served as controls. Incubation with PMA was performed for 10 min at 37 °C in the dark with occasional mixing by inverting tubes multiple times. Tubes were subsequently light-exposed for 15 min using a PhAST Blue photoactivation system (GenIUL, Barcelona, Spain). Biomass was collected by centrifugation at 5000*g* for 5 min followed by removal of the supernatant. Resulting pellets were processed directly or were stored at − 20 °C until DNA isolation.

In case of filtered water samples from a sewage treatment plant, the river Ruhr and two creeks, 40 μL of the PMA stock were diluted in 2 mL filtered (0.2 μm) mineral water (Evian, France) to a final concentration of 50 μM. Filters on the vacuum manifold were overlaid with this solution and incubated for 10 min in the dark with occasional mixing (by swinging the filter multiple times in different directions). After incubation, PMA solution was removed by applying vacuum. Filters were placed in Petri dishes (without lid) and light exposed for 15 min using the PAUL (Photo Activation Universal Light) photoactivation system (GenIUL, Barcelona, Spain).

2.4. DNA isolation

Pellets with fecal material were collected from 500 μL fecal suspensions (untreated or heat-treated, with and without PMA) or in the case of DNA standards from 1 mL suspensions with a concentration of 0.01 g per mL. Genomic DNA was extracted using the PowerSoil® DNA Isolation Kit (cat.nr. 12,888; Mobio Laboratories, Carlsbad, USA) following the instructions by the manufacturer. Genomic DNA from filtered environmental samples was extracted directly from filters using the PowerWater® DNA Isolation Kit (cat.nr. 14900; Mobio Laboratories, Carlsbad, USA). For this purpose, filters were loosely rolled using sterile forceps and inserted into 5 mL PowerWater® Bead Tube with the top side of the filter facing the inner side of the tube. DNA was subsequently extracted following the instructions by the manufacturer. DNA was finally eluted from the spin columns with a volume of 100 μL and stored at − 20 °C until qPCR amplification.

2.5. End point PCR

For all endpoint PCR reactions, 2 μL of extracted genomic DNA were