The ISME Journal (2010) 4, 959–961

www.nature.com/ismej

COMMENTARY

Further limitations of phylogenetic group-specific probes used for detection of bacteria in environmental samples

Jeremy J Barr, Linda L Blackall and Philip Bond

The ISME Journal (2010) **4,** 959–961; doi:10.1038/ismej.2010.37; published online 1 April 2010

Fluorescence *in situ* hybridization (FISH) with fluorochrome-labelled oligonucleotides targeting rRNA is a powerful tool for the identification and quantification of micro-organisms that are important in environmental and industrial processes (Amann, 1995). Phylogenetic group-specific (PGS) oligonucleotide probes, targeting rRNA of many different taxa, are commonly used to screen environmental samples. The use of broad-spectrum PGS FISH probes is quite limited because they might detect micro-organisms outside the PGS group containing the target sequence (false positives), and they frequently miss micro-organisms within the group lacking the target sequence (false negatives) (Loy *et al.*, 2007; Amann and Fuchs, 2008).

The removal of unwanted nutrients, such as carbon, nitrogen and phosphorus from wastewater, is crucial in maintaining our waterways and preventing eutrophication. The enhanced biological phosphorus removal (EBPR) process is a highly applied and globally important biological process for the removal of phosphorus and treatment of wastewater. FISH has been regularly used to assess the bacteria present in EBPR-activated sludge biomass, and to link EBPR process performance with resident microbial communities (Daims *et al.*, 2006). This assessment has substantial implications for EBPR, specifically relating to the identification of two critical, relatively abundant and competing bacterial populations of '*Candidatus* Accumulibacter phosphatis' (henceforth Accumulibacter) (Crocetti *et al.*, 2000) and '*Candidatus* Competibacter phosphatis' (henceforth Competibacter) (Crocetti *et al.*, 2002), members of the *Betaproteobacteria* and *Gammaproteobacteria*, respectively. Accumulibacter is responsible for the majority of phosphorus removal in EBPR wastewater treatment processes, as Competibacter is detrimental for this vital nutrient removal process, through competition for carbon while not removing phosphorus.

The two common PGS probes BET42a and GAM42a, targeting the 23S rRNA in *Betaproteobacteria* and *Gammaproteobacteria,* respectively (Manz *et al.*, (1992)), span positions 1027–1043 and differ by only one nucleotide at position 1033, where BET42a has thymine (T) and GAM42a has adenine (A). Both PGS probes identify false-positive and -negative micro-organisms (Amann and Fuchs, 2008) with important implications in environmental sample assessments.

Competibacter contains polymorphisms at position 1033 (T, targeted by GAM42a, or guanine (G)) in the 23S rRNA (Yeates *et al.*, 2003), leading to inconsistent FISH results with the use of BET42a and GAM42a. This inconsistency was resolved by the addition of probe GAM42_C1033 (Yeates *et al.*, 2003), containing cytosine (C) at position 1033. Consequently, there are single-base mismatches between BET42a, GAM42a and GAM42_C1033 oligonucleotide probes, the use of an unlabelled competitor oligonucleotide is required to limit cross-hybridizations to non-target 23S rRNA when FISH experiments are performed on environmental samples.

It has been shown that a mix of probes targeting Accumulibacter 16S rRNA, called PAOmix (Crocetti *et al.*, 2000), when used simultaneously with BET42a (plus unlabelled GAM42a as a competitor), showed that Accumulibacter was a *Betaproteobacterium*, as the PAOmix-targeted cells also bound to BET42a (Crocetti *et al.*, 2000; He *et al.*, 2007). However, we found in subsequent investigations of EBPR biomass, using BET42a along with both unlabelled oligonucleotide competitors (GAM42a and GAM42_C1033), that Accumulibacter cells (that is, PAOmix targeted cells) failed to bind to labelled BET42a (Figure 1a). By repeating the FISH experiments in the absence of the competitor oligonucleotides, we reconfirmed that Accumulibacter cells bound to BET42a (Figure 1b). We thus posed the question 'Was the nucleotide for Accumulibacter 23S rRNA at position 1033 atypical of *Betaproteobacteria*?'. A metagenomic study of two EBPR biomasses produced a near-complete genome sequence for Accumulibacter (García-Martín *et al.*, 2006), wherein the 23S rRNA gene nucleotide at position 1033 was G, not A, typical of *Betaproteobacteria*.

We addressed these anomalous Accumulibacter FISH results by DNA extraction (Yeates and Gillings,

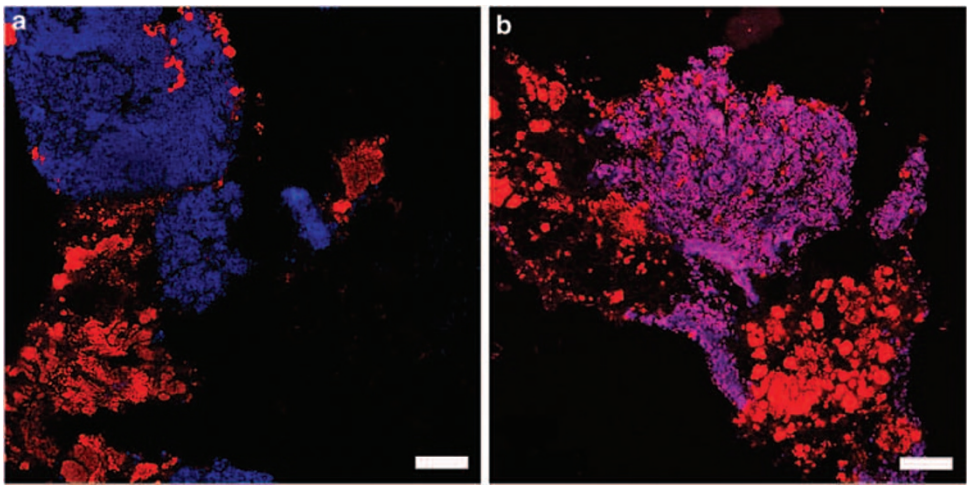

Figure 1 Confocal laser-scanning micrographs of sequentially cryo-sectioned granules from EBPR biomass, showing the anomalous FISH results. All FISH experiments used fluorescently labelled BET42a targeting *Betaproteobacteria* (red cells) and PAOmix targeting Accumulibacter (blue cells). Cells targeted by both probes are shown in magenta. When the labelled BET42a probe was used with competitors GAM42a and GAM42_C1033, no Accumulibacter cells bound to BET42a, resulting in mutually exclusive FISH probe targeting (**a**). When labelled BET42a was used with no competitors, cells binding to Accumulibacter probes (PAOmix) also bound to BET42a (for *Betaproteobacteria*) (**b**), resulting in dual-labelled Accumulibacter cells. The scale bar in both images is 20 μm.

1998) from a laboratory-scale EBPR reactor biomass followed by polymerase chain reaction amplification with an Accumulibacter-specific 16S rRNA gene forward primer (PAO651; 5′-CCCTCTGCCAAA CTCCAG-3′) (Crocetti *et al.*, 2000) and a general bacteria-specific 23S rRNA gene reverse primer (1091r; 5′-RGTGAGCTRTTACGC-3′) (Lane, 1991). Amplicons were cloned, sequenced (Bond *et al.*, 1995; Yeates *et al.*, 2003) and the 16S rRNA gene portions of clones (ca. 60% of the 16S rRNA gene) showed 98–100% identity to the previously reported Accumulibacter sequences (He *et al.*, 2007), confirming that the source cells for clones were Accumulibacter. The 23S rRNA gene portion clarified that Accumulibacter in our bioreactor has a G at position 1033, thus explaining the irregular FISH results.

As the target region is not perfectly matched by either BET42a (T at 1033) or GAM42a (A at 1033), G–T and G–A hybrids can exist, and therefore BET42a or GAM42a can bind to Accumulibacter cells. G–T and G–A hybrids are stronger than many other mismatches such as A–A, T–T, C–T or C–A (Lathe, 1990). When the unlabelled competitor probe was added to the probe mix, it likely hybridized to the Accumulibacter 23S rRNA because of the strong G–C hybridization, precluding labelled BET42a to bind and thus fluoresce (Figure 1a). When labelled BET42a was used with no unlabelled competitors, Accumulibacter 23S rRNA bound to BET42a owing to the G–T hybridization (Figure 1b). Other examples of anomalous FISH with *Gammaproteobacteria* have been reported previously (Siyambalapitiya and Blackall, 2005).

This commentary cautions against the trusting use of PGS probes in environmental samples, specifically the BET42a, GAM42a and GAM42_C1033 probes, without the user being fully aware of their limitations. The BET42a and GAM42a PGS probes were designed before extensive 23S rRNA sequence databases were available, but nonetheless have proven to be extremely useful in many instances for rapid pre-screening of environmental samples. However, owing to the increasing frequency of inconsistencies and complications when using PGS probes, we highlight that caution is required when PGS probes are used for microbial community structure analysis. Contrary to the review by Amann and Fuchs (2008), the use of unlabelled competitor probes does not always improve PGS probe specificity, and in this case can actually cause further probing inconsistencies. It was of particular importance on this occasion, as the trusting use of PGS probes would confuse the monitoring of key bacteria that have competing roles in EBPR, which is a widely used and assessed wastewater treatment process.

Acknowledgements

This work was funded by the Environmental Biotechnology Cooperative Research Centre (EBCRC), which was established and funded by the Australian Government together with industry and university partners. Barr acknowledges the EBCRC for funding of a PhD Scholarship. Bond acknowledges the EBCRC for part-time funding of a postdoctoral fellowship.

JJ Barr is at The University of Queensland (UQ), Advanced Water Management Centre (AWMC), Brisbane, Queensland, Australia and JJ Barr is at Environmental Biotechnology Cooperative Research Centre, Sydney, New South Wales, Australia;

LL Blackall is at Australian Institute of Marine Science (AIMS), Cape Ferguson, Australia
P Bond is at The University of Queensland (UQ), Advanced Water Management Centre (AWMC), Brisbane, Queensland, Australia
and P Bond is at Environmental Biotechnology Cooperative Research Centre, Sydney, New South Wales, Australia
E-mail: phil.bond@uq.edu.au

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