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Detection and discrimination of biofilm populations using locked nucleic acid/2′-*O*-methyl-RNA fluorescence *in situ* hybridization (LNA/2′OMe-FISH)

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ABSTRACT

Multispecies biofilms are the dominant form in nature. The application of fluorescence *in situ* hybridization (FISH)-based techniques to the discrimination of biofilm populations might contribute to the understanding of microorganism interactions in these structures, and might allow the development of efficient strategies to prevent or minimize biofilm-associated diseases. This work presents the first study that develops, optimizes and validates a multiplex FISH procedure using locked nucleic acid (LNA) and 2′-*O*-methyl RNA (2′OMe) oligonucleotides probes for the *in vitro* discrimination within mixed populations. As a case study, *Escherichia coli*, the major cause of urinary tract infections (UTIs), and three other atypical colonizers of urinary catheters (*Delftia tsuruhatensis*, *Achromobacter xylosoxidans* and *Burkholderia fungorum*) with unproven pathogenic potential, were selected. Specific probes for these species were designed and optimized for specific hybridization in multiplex experiments. Results showed that the LNA/2′OMe-FISH method performed well in multiplex experiments and presented a good correlation with total and cultivability counts, regardless of the cells physiological state. In fact, the method was also able to report variations of viable but non-cultivable populations. Further analysis of mixed biofilm structures by confocal laser scanning microscopy provided a clear discrimination in three dimensions between the location of the different populations.

1. Introduction

In nature, it is well established that the microorganisms form biofilm structures in response to hostile environmental conditions. In this mode of life, bacteria live predominantly adhered to abiotic or biotic surfaces embedded within a self-produced matrix of extracellular polymeric substances (EPS) [1,2]. Typically, the bacterial biofilms are mostly polymicrobial and are responsible for most public health (*e.g.*, device-related infections, persistent and recurrent infections) [3–5], industrial (*e.g.*, food processing) [6] and environmental (*e.g.*, drinking water distribution) [7] problems.

Biofilm dynamics and interactions have received a little attention as most studies have assessed single-species adhesion and biofilm formation [8–10], due to the lack of adequate methodolo-

Abbreviations: EPS, extracellular polymeric substances; FISH, fluorescence *in situ* hybridization; rRNA, ribosomal RNA; CLSM, confocal laser scanning microscopy; PNA, peptide nucleic acid; LNA, locked nucleic acid; 2′OMe, 2′-*O*-methyl-RNA; CAUTIs, catheter-associated urinary tract infections; TSA, tryptic soy agar; NCBI, National Centre for Biotechnology Information; EBI, European Bioinformatics Institute; Tm, melting temperature; PS, phosphorothioate; PO, phosphodiester; FAM, fluorescein; CY3, Cyanine 3; AFU, arbitrary fluorescence units; CFUs, colony forming units; PI, propidium iodide; PBS, phosphate buffered saline; O.D., optical density; LSU, large subunit; ΔG°, Gibb's free energy; bp, base pair.

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Table 1
Sequence of LNA/2′OMe oligonucleotide probes synthesized in this study.

Target organism	Name	Sequence (5′Label-3′)	Lenght (bp)
E. coli	Ec1505_LNA/2′OMe_13	5′-FAM-lGmCmClTmCmAlGmCmClTmUmGlA-3′	13
	Ec1505_LNA/2′OMe_16	5′-FAM-lCmAmClGmCmClTmCmAlGmCmClTmUmGlA-3′	16
D. tsuruhatensis	Dt404_LNA/2′OMe_13	5′-CY3-lGmAmGlCmUmUlTmUmUlCmGmUlT-3′	13
	Dt404_LNA/2′OMe_16	5′-CY3-lGmAmGlCmUmUlTmUmUlCmGmUlTmCmClG-3′	16
B. fungorum	Bf411_LNA/2′OMe_13	5′-CY3-lTmAmUlTmAmAlCmCmAlCmGmGlC-3′	13
	Bf411_LNA/2′OMe_16	5′-CY3-lGmGmUlAmUmUlAmAmClCmAmClGmGmClG-3′	16
A. xylosoxidans	Ax590_LNA/2′OMe_13	5′-CY3-lAmAmAlTmGmClAmGmUlTmCmClA-3′	13
	Ax590_LNA/2′OMe_16	5′-CY3-lAmAmAlTmGmClAmGmUlTmCmClAmAmAlG-3′	16

LNA nucleotide monomers are represented with "l"; 2′-OMe-RNA monomers are represented with "m". Labels: FAM – Fluorescein; CY3 – Cyanine 3.

gies to discriminate the populations *in situ* [11,12]. Recent advances in fluorescence-based techniques and in molecular biology allow *in situ* studies of the spatial organization and the species interactions in bacterial biofilms [13–15]. For instance, the green fluorescence protein (GFP) has been applied to monitor the development of multispecies biofilms *in situ* [15,16]. However, this technique requires the construction of strains that express GFP and is thus not applicable to natural biofilm samples. Alternatively, researchers have been using a different molecular biology approach, namely fluorescence *in situ* hybridization (FISH) [17]. FISH is used for the identification/detection of microorganisms based on its phylogenetic markers at 16S or 23S ribosomal RNA (rRNA), particularly abundant in viable cells [17]. It is based on the hybridization of a fluorescent oligonucleotide probe with a conserved rRNA sequences, and subsequent detection by epifluorescence microscopy, confocal laser scanning microscopy (CLSM) or flow cytometry. FISH in combination with CLSM is being increasingly used to visualize/study the co-localization of each species in biofilm, and can be useful to quantify the microbial populations without disturbing biofilm structure (see examples [11,18]).

Taking advantage of progress within nucleic acid mimics development, notably peptide nucleic acid (PNA), locked nucleic acid (LNA), and 2′-*O*-methyl-RNA (2′OMe), we were aiming at improving FISH efficiency [19–22]. Despite the potential of the different nucleic acid mimics, studies regarding the applications of FISH to assess the spatial species organization in biofilm samples have been limited to rather few PNA probes (some examples [11,18,23–25]). These studies have shown that the PNA-FISH method is a robust technique able to discriminate and locate the species within biofilm. Despite the absence of studies applying other mimics to biofilm characterization, the properties of other molecules are promising. For instance, the use of LNA probes offers several advantages compared to DNA probes, including a greater affinity toward DNA/RNA targets, a higher biostability (resistance to nuclease degradation), a better signal-to-noise ratio, and a better sensitivity and specificity [26–28]. In addition, LNA probes are highly soluble in water and were found to hybridize with RNA (and DNA) more efficiently than PNA probes. Thus, for at least some FISH applications, the use of LNA would be advantageous comparing to PNA [29–31].

LNA is a RNA analog which contains a ribose ring locked by an O2′-C4′-methylene linkage resulting in a *N*-type (C3′-*endo*) furanose ring conformation [32,33]. The 2′OMe is another RNA mimic which – though not locked – preferentially displays a C3′-*endo* furanose ring conformation, enhancing its affinity for RNA targets [34]. It was reported that the introduction of LNA nucleotides at every third position in a 2′OMe probe increases the target affinity with a concomitant increase in sensitivity [22]. In fact, the remarkable hybridization properties of LNA-modified probes enable the use of these molecules in FISH experiments [20]. For example, LNA-modified probes might be used for therapeutic (*e.g.*, *via* inhibition of gene expression) [35,36] and for diagnostic purposes (*e.g.*, for the detection of microRNAs and for SNP genotyping) [37,38]. However, there are no available studies regarding their applications for the detection/identification of bacterial populations within a biofilm. As such, this article describes the first development and validation of FISH method to assess the biofilm spatial organization and the species distribution/discrimination without disturbing the biofilm structure, using the LNA technology (LNA/2′OMe probes) in combination with CLSM.

As a case study, we selected *Escherichia coli*, the major cause of urinary tract infections [39–41], and three other atypical colonizers of urinary catheters (*Delftia tsuruhatensis*, *Achromobacter xylosoxidans* and *Burkholderia fungorum*). Despite their unproven pathogenic potential, it was reported that these microorganisms can coexist on the urinary catheter surface with pathogenic bacteria (*e.g.*, *E. coli*) [42]. In fact, catheter-associated urinary tract infections (CAUTIs) are the most common nosocomial infection, but there is a lack of knowledge about the impact that multispecies biofilms have on CAUTIs outcome and, particularly, on the role that these atypical microorganisms have on outcome of this type of infection. The ability of LNA/2′OMe oligonucleotide probes to discriminate the biofilm populations would give insights on the type of interactions (*e.g.*, symbiotic, antagonistic, and synergistic) that might occur between different species, and thus, could provide valuable knowledge on how to prevent, minimize or treat these infections.

2. Material and methods

2.1. Culture of bacterial strains

The bacterial strains, *E. coli* CECT 434, *A. xylosoxidans* B3, *D. tsuruhatensis* BM90 and *B. fungorum* DSM 17,061 were maintained on Tryptic Soy Agar (TSA) (Merk, Germany) and incubated at 37 °C. Single colonies were streaked onto fresh plates at 37 °C for 20–24 h (for *E. coli*, *A. xylosoxidans*, *D. tsuruhatensis*) or 48 h (for *B. fungorum*) prior to the experiments.

2.2. Design and theoretical evaluation of oligonucleotide probes

Oligonucleotide probes with different sizes (13 bp and 16 bp) were designed and synthesized to increase the chances of finding oligonucleotide probes that work at the same temperature (Table 1). The oligonucleotide probe design and the theoretical specificity and sensitivity assessment were performed as described in Almeida et al. [43].

2.3. Synthesis and purification of LNA/2′OMe oligonucleotide probes

Based on previous studies [20,22], LNAs were incorporated at every third 2′OMe monomer. The LNA/2′OMe oligonucleotide probes included a phosphorothioate (PS) backbone instead of a phosphodiester (PO). The PS monomers include replacement of one of the two non-bridging oxygen atoms by a sulfur atom at each internucleotide linkage [44]. The choice of this type of modifica-