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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 of biofilms found 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.

Keywords:

Biofilms; DNA; Microbial Growth; RNA; LNA/2'OMe-FISH; confocal laser scanning microscopy.

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; ΔGº, Gibbs' Free Energy; bp, base pair.

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 little attention as most studies have assessed single-species adhesion and biofilm formation [8-10], due to the lack of adequate methodologies 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 biofilms, 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 application 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 biofilms. 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 bio-stability (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 analogue 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 application 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 *E. 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 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 17061, 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.

2.1. 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].

2.2. 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 sulphur atom at each internucleotide linkage [44].The choice of this type of modifications was also based on a previous work of our group [20], which demonstrated that LNA/2'OMe oligonucleotide probes with a PS linkage are good candidate probes to be used in FISH experiments. Oligonucleotide synthesis was carried out on an automated nucleic acid synthesizer (PerSpective Biosystems Expedite 8909 instrument) under anhydrous conditions in 1.0 µmol scale, according to Fontenete *et al.* [20].

2.3. Melting temperature analysis

In order to predict optimal hybridization temperatures for each oligonucleotide probe, the Kierzek website (http://rnachemlab.ibch.poznan.pl/calculator2.php) was used. Here, the thermodynamic parameters are calculated for a 10^{-4}M oligonucleotide probe concentration. A lower concentration, 200 nM, was used in our FISH experiments. Thus, as the probe concentration affects the melting temperature, as it is also the case for the denaturant agent, the data provided by this software was only used as an indicative value. To address this problem, the melting temperatures were also experimentally determined as described by Fontenete *et al*. [20].

2.4. FISH protocol development

Standard hybridization procedures on glass slides were performed in order to determine the optimal hybridization temperature of each oligonucleotide probe on pure cultures. Nonetheless, since flow cytometry is the easiest way to quantify the bacterial populations, FISH signals at selected hybridization temperatures were confirmed by performing the hybridizations in suspension.

Hybridization on slides and in suspension was performed as previously described [19, 43, 45], with a few modifications (see supplemental material 1 for details on the optimization of the hybridization procedures).

The specificity of each oligonucleotide probe was evaluated in a culture smear of the remaining species selected for this study, at the optimal hybridization temperature. In addition, since probes are intended to work in multiplex experiments, it is important to confirm the ability of the oligonucleotide probes to discriminate between the species. A mix of two probes was applied simultaneously in a mixed smear of the two corresponding species. For this, 10 µL of the final suspension from each species were mixed, spread on glass slides and hybridization was performed as described in the supplemental material.

2.5. Microscopic visualization and image quantification

For image acquisition a Leica DM LB2 epifluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with Leica DFC300 FX camera (Leica Microsystems GmbHy, Germany) and filters capable of detecting the LNA/2'OMe oligonucleotide probes (BP 450-490, FT 510, LP 515 for FAM- labelling and BP 570/20, FT 590, LP 640/40 for Cy3- labelling) was used. For image capture, Leica IM50 Image Manager, was used and the fluorescence intensity of each oligonucleotide probe (at different temperatures) was quantified in the microscopy images using the ImageJ program (National Institutes of Health Software, http://rsbweb.nih.gov/ij/index.html). The quantification by ImageJ software was performed in order to determine the average fluorescence intensity of each image obtained by epifluorescence microscopy. Data was plotted as mean of arbitrary fluorescence units (AFU) which represented the mean fluorescence intensity.

2.6. LNA/2'OMe-FISH correlation with cultivability and PI staining

In order to determine whether the LNA/2'OMe-FISH method is able to detect bacteria in different physiological states, the correlation between LNA/2'OMe-FISH counts, colony forming units (CFUs) counts (for cultivable counts assessment) and propidium iodide (PI) staining (for total cells counts assessment) was performed at selected time points of the bacterial growth (lag phase, exponential phase, early stationary phase and late stationary phase; see the supplemental material - Figure S1). Bacterial growth curves were previously assessed as described below. Cells were harvested at different growth stages for cultivability, LNA/2'OMe-FISH and total cells assessments. The quantification of cells by LNA/2'OMe-FISH was performed in suspension as described above. The number of cultivable cells was determined by standard CFU counts. The plates were incubated at 37 °C for 16 h (*E. coli*), 24 h (*D. tsuruhatensis*) and 48 h (*A. xylosoxidans* and *B. fungorum*). For PI staining, 100 µL of fixed cells (prepared as described above) were centrifuged at 10.000 x *g* for 5 min, and resuspended in 100 µL of PI (Invitrogen; 50 ug/mL). After 10 min in the dark at room temperature, the cell suspension was centrifuged and the cells were resuspended in saline solution. The counting of the stained cells (by PI or LNA/2'OMe-FISH) was performed by flow cytometry as described in the supplemental material (see supplemental material 2). All the data from CFU, LNA/2'OMe-FISH and PI counts values were log transformed and used to calculate the Pearson correlation coefficient for each species (LNA/2'OMe-FISH *vs.* CFU counts; and LNA/2'OMe-FISH *vs.* PI counts); a linear regression model was also used to adjust the data. The experiment was performed in duplicate.

2.6.1. Bacterial growth curve: The growth curve for each species was determined on Tryptic Soy Broth (TSB) (Merk, Germany) at 37 °C. For this, each species was grown overnight (16-18 hours) at 37 °C, 150 rpm. Subsequently, cells were diluted in order to obtain a final optical density at 620 nm ($O.D._{620\ nm}$) of 0.1. Cellular suspensions were then incubated at 37 °C, 150 rpm. The growth was monitored by measuring the $O.D._{620\ nm}$ every 30 min until the stationary stage. The experiment was performed in duplicate.

2.6.2. Resazurin assay: Resazurin is a blue fluorescent dye which is reduced by viable cell bacteria to a pink colored resofurin. The amount of conversion from blue to pink is proportional to the number of viable cells [46]. The resazurin assay was performed in order to check the viability of *B. fungorum* during the growth curve in TSB medium. At selected time points of the growth of the bacterium (lag phase, exponential phase, early stationary phase and late stationary phase), 190 µl of *B. fungorum* suspension was dispensed into wells (8 wells) of a 96-well microtiter plate (Orange Scientific, Belgium) followed by the addition of 10 µl of 0.1 g/L resazurin (Sigma-Aldrich, USA) solution [47]. Plates were incubated during 90 min in darkness at 37 ºC [48]. Afterwards, the fluorescence was measured ($\lambda_{excitation}$=570 nm and $\lambda_{emission}$=590 nm) using a microtiter plate reader (SpectraMax M2E, Molecular Devices, UK)[47]. Wells containing sterile TSB were used as a control. The experiment was performed in duplicate.

2.7. Spatial discrimination of biofilm populations using LNA/2'OMe-FISH

In other to evaluate whether LNA/2'OMe-FISH would be useful to discriminate and elucidate the spatial organization of the biofilm populations without disturbing the biofilm structure, CLSM was used to visualize *in situ* hybridized single- and dual-species biofilm samples. Single- (*B. fungorum*) and dual-species biofilms (*E. coli*/ *B. fungorum*) were formed as previously described [11, 25]. Briefly, the strains were grown overnight in artificial urine medium (AUM) and then the inoculum was diluted in AUM in order to obtain a final concentration of 1 x 10^5 CFUs/ml AUM was prepared as previously described[49]. For single-species biofilms, 6 ml of each inoculum were transferred to six-well tissue culture plate (Orange Scientific, Belgium) containing coupons of silicone, prepared as previously described [50]. For dual-species biofilms, equal volumes of two-fold concentrated solutions were mixed. Plates were incubated (FOC 225I - VELP Scientifica, Italy) at 37 °C, under static conditions, during 192 hours. Every 48 h the medium was carefully replaced by fresh AUM. FISH protocol was applied to biofilms formed on silicone coupons at 192 h. Before hybridization, coupons were washed in 0.85% (v/v) sterile saline, dried at ~60 °C for 15 minutes and fixed with 100% methanol for 20 min to prevent the detachment of biofilm during hybridization. After this, the FISH procedure was similar to the one applied for slides (see above). After hybridization, the silicone coupons were allowed to air dry, mounted with 1 drop of mounting oil and covered with a coverslip. The experiment was performed in triplicate.

2.8. Confocal laser scanning microscopy

The biofilm CSLM images were acquired in a FluoView FV1000 microscope (Olympus). Biofilms were observed using a 60x water-immersion objective (60x/1.2 W). Multichannel simulated fluorescence projection images and vertical cross sections through the biofilm were generated by using the FluoView application Software package (Olympus). *E. coli* cells were identified as green fluorescent rods and the atypical bacteria as bright red fluorescent rods.

3. Results and discussion

Previous studies have demonstrated that atypical bacteria might enhance the pathogenicity of virulent bacteria, increase the overall resistance of the biofilm to antibiotics, and influence the clinical outcome of the infection [51-54]. Thus, it is crucial to have a better knowledge about the formation of biofilms and the contribution of each population in polymicrobial infections.

The FISH methodology has been shown to be a valuable technique to discriminate and to analyze the spatial distribution of the individual species *in situ* without disturbing the biofilm structure [11, 18, 25]. The characterization of biofilms has been limited to PNA molecules, despite the well-known advantages of other molecules, such as LNA and 2'OMe. As such, was developed a procedure, based on a multiplex LNA/2'OMe FISH and CLSM, for the analysis of the spatial organization of *in vitro* biofilms.

3.1. Analysis of LNA/2'OMe oligonucleotide probes

3.1.1. Probe design and theoretical evaluation: For the selection of useful oligonucleotides, conserved regions for the 23S rRNA (*E. coli*) and 16S rRNA (*D. tsusruhatensis*, *A. xylosoxidans* and *B. fungorum*) sequences were identified using ClustalW. The sequences selected for *E. coli* hybridize between the position 1505 and 1520 (Ec1505_LNA/2'OMe_13 and Ec1505_LNA/2'OMe_16 probes) of the 23S rRNA gene sequence (accession number: X80724). The other probes target the 16S rRNA sequences with target positions between 404 and 419 (Dt404_LNA/2'OMe_13 and Dt404_LNA/2'OMe_16) for *D. tsuruhatensis* (accession number EU779949); 411 and 426 (Bf411_LNA/2'OMe_13 and Bf411_LNA/2'OMe_16) for *B. fungorum* (accession number AF215705); and 590 to 605 (Ax590_LNA/2'OMe_13 and Ax590_LNA/2'OMe_16) on *A. xylosoxidans* (accession number AF225979).

The theoretical specificity and sensitivity of each selected oligonucleotide probe was evaluated using the probeCheck program coupled to the large subunit (LSU) database for *E. coli* probes, or using ProbeMatch coupled with the RDP II database for the other three microorganisms (Table 2). According to the LSU database, the *E. coli* probes (Ec1505_LNA/2'OMe_13 and Ec1505_LNA/2'OMe_16) detected 3260 out of 4619 *E. coli* sequences present in the database which corresponds to a sensitivity of 70.6% (last accession, September 2014). The sensitivity value shows that the *E. coli* probes are not able to detect all *E. coli* strains; however, these oligonucleotides are suitable to be used in this study since both detected the *E. coli* strain CECT 434. According to the ProbeMatch from RDP II database (isolates with good quality and sequence size >1200 bp), theoretical sensitivities of 97.82%, 90.47% and 97.80% were obtained for *D. tsuruhatensis*, *B. fungorum*, and *A. xylosoxidans*, respectively. In addition, as shown in Table 2, all oligonucleotide probes display high specificity. While the specificity and sensitivity values are good indications of probe performance, especially for species identification purposes, this is in this particular case not the most important feature. As the intention is to form dual-species biofilms (*E. coli* co-cultured with atypical species), the absence of cross-hybridization with the other species under study is the most important criterion. In fact, none of the non-target sequences detected by the probes belong to the other species under study. As such, the *in silico* analyses indicated that the oligonucleotide probes are able to detect specifically each target species, with no cross-complementarity observed with the other species to be used in the biofilm experiments. These are actually the first LNA/2'OMe oligonucleotide probes specifically designed for *E. coli*, *D. tsusurhatensis*, *A. xylosoxidans* and *B. fungorum* detection.

3.1.2. Thermodynamic parameters: The Tm for each oligonucleotide probe was predicted using the LNA-2'OMeRNA/RNA calculator (http://rnachemlab.ibch.poznan.pl/calculator2.php) and was furthermore experimentally determined (Table 3). These experiments showed Tm-values in the range of 76-88 °C for all oligonucleotide probes. The theoretical Tm-values are higher (between 100 and 129 ºC) since this estimation does not consider the presence of the denaturing agent. Nonetheless, the correlation coefficient observed between the theoretical and the experimental Tm values presented an acceptable value of 0.85 (p<0.05) (see supplemental material - Figure S2).

The affinity to the target is also affected by several other factors like target accessibility and probe size [55]. The oligonucleotide probe affinity is defined as ΔGº, and a threshold ΔGº of -13 kcal/mol has been recommended for the design of DNA probes to guarantee a good hybridization efficiency [55]. However, for LNA/2'OMe oligonucleotide probes there is no information about the recommended threshold ΔGº. It is known that the introduction of LNA monomers increases the target affinity by having a positive and additive effect on the Tm (one LNA substitution increases Tm between 1 and 10 ºC against RNA) [56-58].

Also the Na^+ concentration has an important effect on the ΔGº values. Positive ions promote rRNA folding by reducing the repulsion between phosphates groups which could reduce accessibility [59-62]. However, positive ions are also essential to stabilize the hybrid duplex[63]. Thus, for the ΔGº and theoretical Tm calculations a [Na+] of 0.9 M was used, which corresponds to the NaCl concentration used in the hybridization solution. This high concentration has a strong impact on the ΔGº values that can be reduced in average -10 kcal/mol (±1.1), when compared to values obtained with no NaCl (data no shown). In general, analysis of the data in Table 3 shows the lower ΔGº values (between -32 and -42 kcal/mol) for the 16 nucleotide probes. The shorter ones display ΔGº values between -26 and -33 kcal/mol, which suggest a lower affinity. Nonetheless, too much affinity might be undesirable for those situations where a one mismatch distinction is required, which means that hybridization might occur with sequences that are not 100% complementary.

3.2. Optimization of hybridization conditions

Different hybridization temperatures, between 51 ºC and 59 ºC, were tested using the FISH method in glass slides for each oligonucleotide probe to achieve the best FISH signals. The results demonstrated that the strongest fluorescence intensity was obtained at 55 ºC (Figure 1) for most of the oligonucleotide probes. The exception was for the *A. xylosoxidans* probes (Ax590_LNA/2'OMe_13 and Ax590_LNA/2'OMe_16) that presented a peak of fluorescence at 53 ºC. Concerning the specificity of each probe against the non-target strains, a slight cross-hybridization was observed for some of the oligonucleotide probes at 55 ºC, especially for the *E. coli/ D. tsuruhatensis* and *E. coli/ A. xylosoxidans* combinations (Figure 2). As observed in columns for 57 °C, this problem was solved by increasing the hybridization temperature 2 ºC. No cross-hybridization between the two LNA/2'OMe oligonucleotide probes was observed and, thus, an accurate discrimination between the two species involved was obtained. In addition, the signal-to-noise ratio was also optimal at 57 ºC. Therefore, to achieve an appropriate specificity, 57 ºC was used for all subsequent experiments. As such, for each microorganism, the LNA/2'OMe oligonucleotide probes that presented the best signal at 57 ºC was selected for further experiments. For *E. coli*, *D. tsusruhatensis* and *A. xylosoxidans*, the 16 nucleotide probes (Ec1505_LNA/2'OMe_16, Ax590_LNA/2'OMe_16 and Dt404_LNA/2'OMe_16, respectively) were selected, while for *B. fungorum* the 13 nucleotide probe (Bf411_LNA/2'OMe_13) was chosen.

As described above, hybridization in suspension is the easiest way to quantify the bacterial population by subsequent flow cytometry or epifluorescence microscopy analysis, and it is important to ensure that the signal obtained on a standard glass slide test is maintained in suspension. For these, the FISH procedure in suspension was also performed for the probes selected at 57 °C. Comparing the values of fluorescence signal intensity, the results showed that the signals obtained in hybridizations performed in suspension were similar or higher than those obtained in standard smears (hybridization procedures performed in glass slides) (Figure 3).

Once the hybridization temperature had been optimized and the probes for subsequent experiments were selected, multiplex FISH was tested against a smear of two species mixed together (*E. coli* in combination with the atypical microorganism). The results showed that both hybridization protocols (on slides and in suspension) provided an accurate discrimination between the two species at 57 ºC. We have shown that the LNA/2'OMe oligonucleotide probes selected for subsequent experiments are able to successfully hybridize with the target microorganisms at 57 ºC providing a species-specific hybridization signal. The results thus confirmed the potential applicability of the selected oligonucleotide probes to a multiplex LNA/2'OMe-FISH experiment.

3.3. LNA/2’OMe-FISH validation

With the intended application of the FISH method here developed in mind, and considering that LNA/2’OMe-FISH application to biofilms are inexistent so far, it is important to establish the scope and limits of this methodology. As the rRNA is usually the target for the oligonucleotide probes used in FISH, the fluorescence signal is expected to be affected by the cell rRNA content. Microbial cells with a high metabolic activity have a rRNA content sufficient to generate a strong FISH signal [62, 64]. However, it is well established that the physiological state of biofilm cells vary spatially and temporally [1]. Therefore, changes in the number of rRNA molecules occur, and consequently, a low hybridization rate can be observed. In an early biofilm, the cells are metabolically active and have all requisites to divide and grow, due to the presence and rapid diffusion of the nutrients. In a mature biofilm, different physiological states are observed, namely active, dormant or dead cells [1].

3.3.1. LNA/2’OMe-FISH correlation with cultivability and PI staining: For each species under study, cells at different physiological states (lag phase, exponential phase, early stationary phase and late stationary phase; Figure S1) were collected and evaluated using CFU counts; PI staining and LNA/2’OMe-FISH staining, for further correlation purposes. CFU is a conventional method for viability assessment, since only actively growing cells (cultivable cells) will be measured, while a general fluorescent nucleic acid dye is usually used for total cell quantification s[65, 66]. The PI binds to DNA and is commonly used in combination with SYTO9 to discriminate live and dead cells, but it can also be used to count the total cells in previously fixed samples. The LNA/2’OMe-FISH allows the identification and quantification of cells with intact or significant rRNA content; which might eventually correlates with viability since RNA content is rapidly degraded after cell dead [67].

Linear regression and Pearson correlation analysis for each species was performed to compare the CFU and PI counts against LNA/2’OMe-FISH counts (Table 4 and see supplemental material - Figure S3). As shown in Table 4, the Pearson correlations were significant for all pairs of methods (LNA/2’OMe-FISH counts *vs.* CFU counts and LNA/2’OMe-FISH counts *vs.* PI counts) with $p<0.05$. *E. coli*, *A. xylosoxidans*, and *D. tsuruhatensis* presented strong and similar correlation values between the LNA/2’OMe-FISH *vs.* CFU and LNA/2’OMe-FISH *vs.* PI counts. The comparison between LNA/2’OMe-FISH and CFU counts showed a correlation coefficient of 0.95, 0.88, and 0.83 for *E. coli*, *D. tsuruhatensis*, and *A. xylosoxidans*, respectively. Correlation coefficient values of 0.93 (for *E. coli*), 0.84 (for *D. tsuruhatensis*), and 0.99 (for *A. xylosoxidans*) were found for the comparison of LNA/2’OMe-FISH *vs.* PI counts. For *B. fungorum*, as expected, a good correlation was observed between the LNA/2’OMe-FISH and PI counts ($r=0.83$). However, a negative correlation was obtained LNA/2’OMe-FISH *vs.* CFU counts ($r=-0.92$). In order to explain this result, the *B. fungorum* growth curve was analyzed in terms of CFU and LNA/2'OMe-FISH counts. The CFU assessment showed a decrease in the cultivable population, which was inconsistent with the LNA/2'OMe-FISH counts that increased over time (Figure 4a). Therefore, the negative correlation obtained in LNA/2'OMe-FISH *vs.* CFU counts was due to the decrease of CFU counts during the *B. fungorum* growth. However, the OD measurement (Figure 4b) seemed to indicate that the cells are viable and metabolically active, since the OD is increasing as a result of active cellular division. This result corroborated the data obtained by LNA/2'OMe-FISH counts. To further investigate this issue, the resazurin assay was performed at selected time points during the growth of *B. fungorum*. The resazurin assay is commonly used for the assessment of bacterial viability with the fluorescence output being proportional to the number of viable cells. Despite the decrease in CFUs counts, resazurin and OD data showed that cellular viability increased until the stationary phase (Figure 4b). The decrease observed in the cultivable population might be the result of a transition to a non-cultivable/dormant state of the bacteria, which is activated when growth condition are not optimal.

Overall, the values produced by LNA/2'OMe-FISH correlated well with viable and total cells counts for all species under study. The only exception was noticed for *B. fungorum*, which seems to present a non-cultivable state that renders CFU unable to correctly estimate changes in the population. In fact, this particular result showed the potential of FISH techniques for species with known dormant stages or unknown physiological behavior. To our knowledge no study has so far analyzed the correlation between LNA/2'OMe-FISH and CFU and total cell counts. The results provided evidences of the utility of this technique for studies of population dynamics, especially for biofilms which present a variety of cellular fractions at different metabolic stages.

3.3.2. Characterization of biofilm 3D structures using multiplex LNA/2'OMe-FISH: While our results have provided evidence of the suitability of LNA/2'OMe-FISH for characterization of heterogeneous populations, analysis of biofilms requires that probes are able to penetrate over the complex matrix that surrounds the cells. A biofilm matrix is rich in extracellular nucleic acid, proteins, and expolissacharydes [68, 69], which might hinder the LNA/2'OMe oligonucleotide probe diffusion (also negatively charged). To evaluate the ability of the method to provide an *in situ* 3D characterization of biofilm populations, biofilms were formed in artificial urine for 8 days. Samples from single- and dual-species biofilms were taken, fixed, *in situ* hybridized and then evaluated by CLSM. As an example, Figure 5 showed that the LNA/2'OMe oligonucleotide probes were able to penetrate over the biofilm matrix, providing a complete staining of the biofilm surface and a strong signal, even in a thicker biofilm with 40 μm (Figure 5a). This clearly demonstrated that the probes, despite their negative charge, did not face any diffusion barrier within the biofilm structure. Unlike their DNA counterparts, for which diffusion problems have been attributed to the negative charge and size of the probes [11, 17], the LNA/2'OMe probes apparently present a different behavior. It might be possible that the size of the oligonucleotides might be more determinant than the charge with respect to assuring an efficient diffusion thought the biofilm matrix. Regarding the multiplex assay, it was also possible to analyze an 8-days dual-species biofilm of *E. coli*/ *B. fungorum* (Figure 5b), where the discrimination and localization of the two populations was evident. In this case, the transversal biofilm image showed both species mixed together, which corresponds to a typical coaggregation organization of the bacteria in polymicrobial biofilms. This distribution is commonly associated with cooperation or synergetic interaction within biofilms [70]. This might suggest that, in this particular case, these species might benefit from the mixed consortium; or, at least, they are not negatively affected by each other presence, as antagonistic iterations typically involve the formation of separate microcolonies.

4. Conclusions

Multiplex FISH methodology in combination with CLSM is becoming common in biofilm experiments providing a simple way to analyze *in situ* the spatial distribution of natural biofilm populations. While the potential of DNA or PNA probes have already been proven for biofilm studies, the potential application of LNA-modified probes have been studied for the first time with this report. We have herein developed and validated a multiplex LNA/2'OMe-FISH procedure which, in combination with CLSM, is capable of discriminating among bacterial species providing spatial localization data of complex biofilm populations, in conditions mimicking the CAUTIs.

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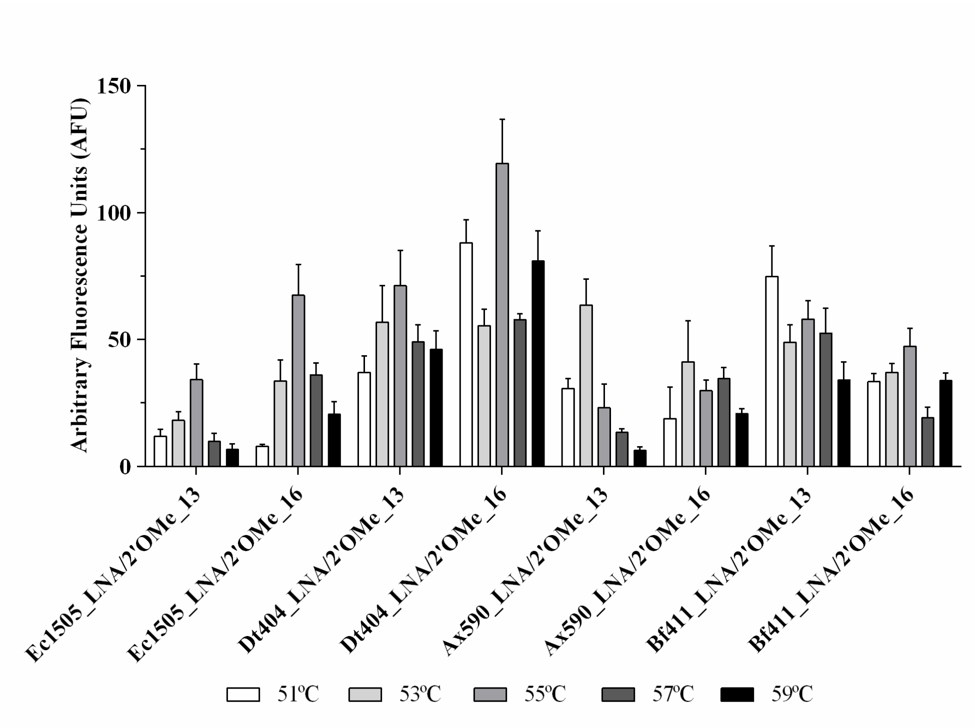


Figure 1. Fluorescence intensity of *E. coli* (Ec1505_LNA/2'OMe_13; Ec1505_LNA/2'OMe_16), *D. tsuruhatensis* (Dt404_LNA/2'OMe_13; Dt404_LNA/2'OMe_16), *A. xylosoxidans* (Ax590_LNA/2'OMe_13; Ax590_LNA/2'OMe_16), and *B. fungorum* (Bf411_LNA/2'OMe_13; Bf411_LNA/2'OMe_16) LNA/2'OMe oligonucleotide probes at different hybridization temperatures (between 51ºC and 55ºC). Hybridizations were performed in pure culture smears on glass slides. Fluorescence signal intensity, determined using ImageJ software, was expressed in arbitrary fluorescence units (AFU). All images were acquired at equal exposure conditions. Error bars represent standard deviation.

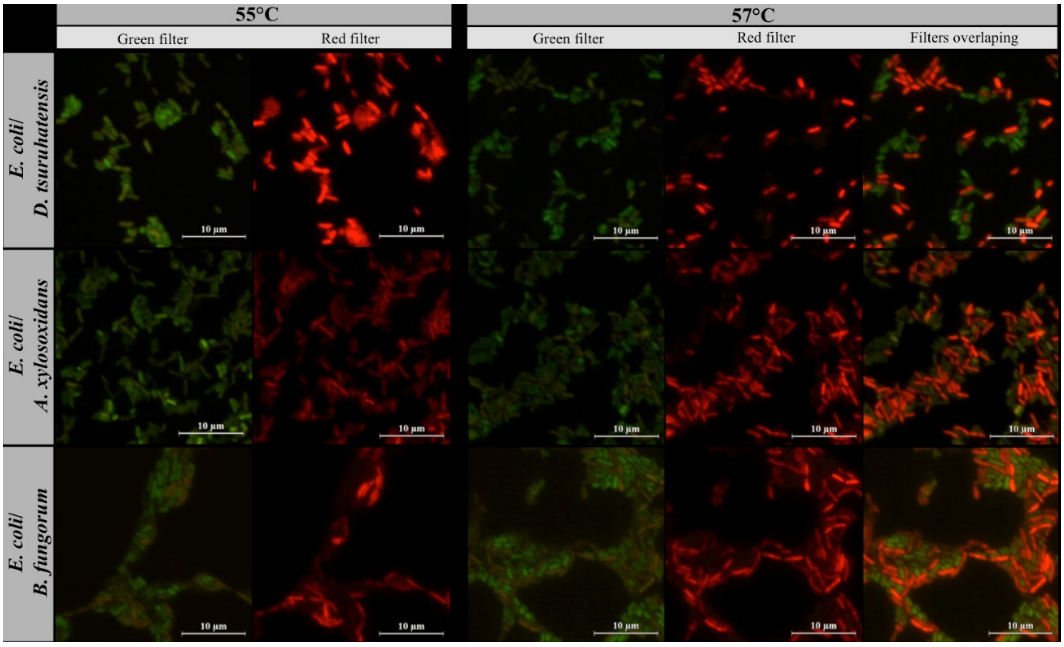


Figure 2. Epifluorescence microscopy images of a multiplex LNA/2'OMe-FISH assay for dual-species smears. In the first two columns is possible observe some degree of cross-hybridization between the two LNA/2'OMe oligonucleotide probes at 55 °C, especially for the *E. coli/ D. tsuruhatensis* and *E. coli/ A. xylosoxidans* combinations. Columns for 57 °C of hybridization show no cross-hybridization and, thus, an accurate discrimination between the two species involved.

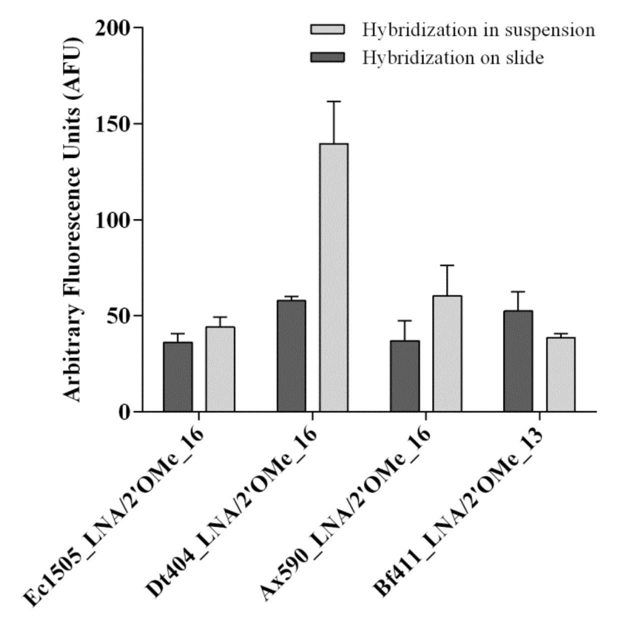


Figure 3. Comparison of the average fluorescence intensity of the selected LNA/2'OMe oligonucleotide probes obtained in standard smears (hybridization procedures performed in glass slides) and in suspension at 57 °C. Fluorescence signal intensity is expressed in arbitrary fluorescence units (AFU) and was quantified using ImageJ software. All images were acquired at equal exposure conditions. Data are means of three independent experiments and error bars represent standard deviation.

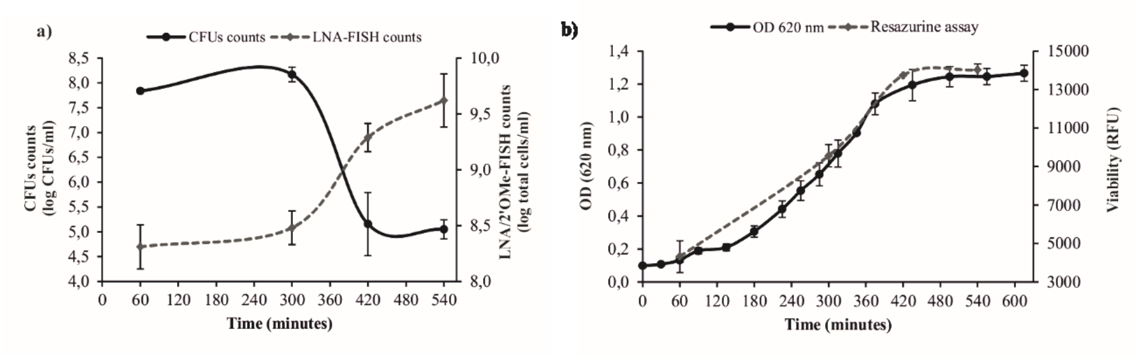


Figure 4. Evaluation of the *B. fungorum* growth curve by CFU and LNA/2'OMe-FISH counts (a) and by OD and the resazurine assay (b). The CFU assessment shows a decrease in the cultivable population, which is inconsistent with the LNA/2'OMe-FISH counts that increase over time. OD measurements and viability assessment using resazurine assay, show active growth over time, which corroborate the results obtained with the LNA/2'OMe-FISH counts. Data are means of two independent experiments and error bars represent standard deviation.

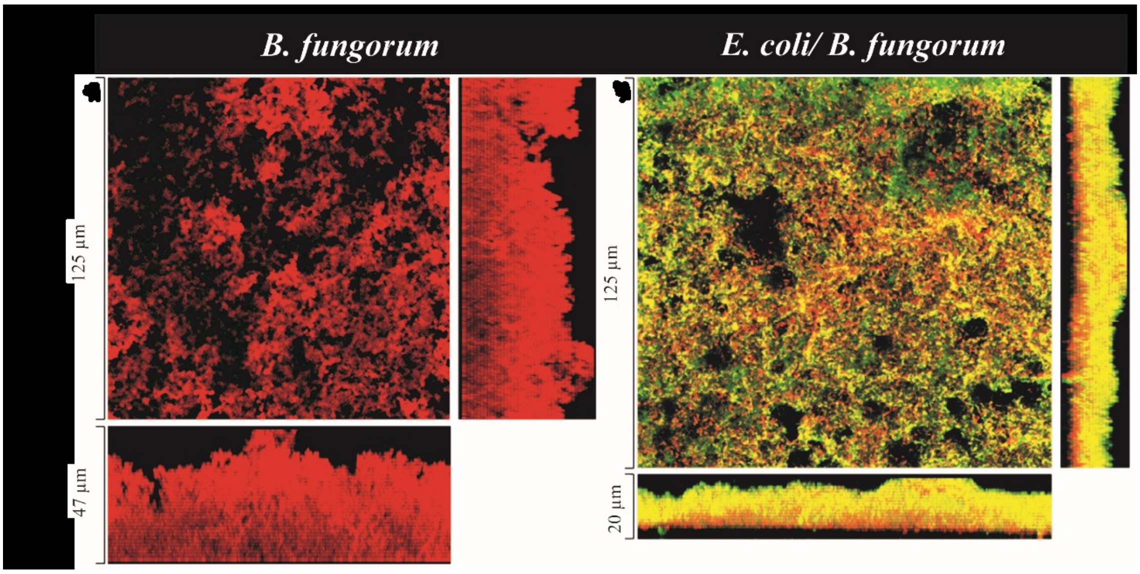
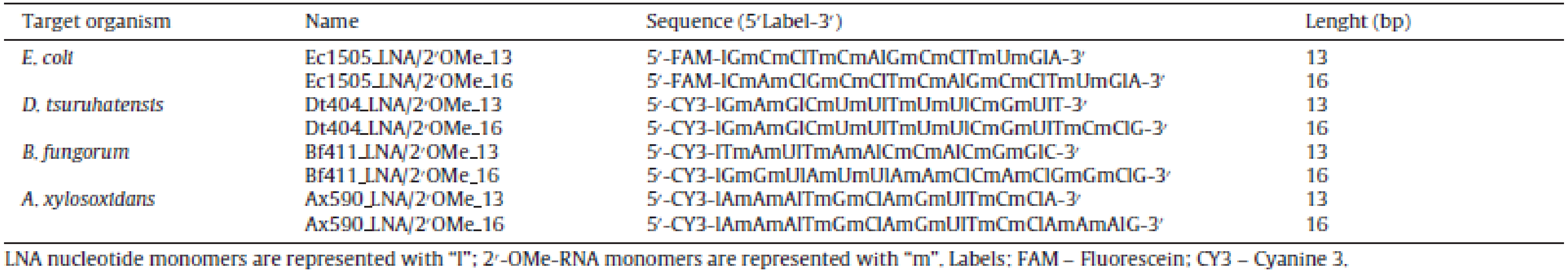


Figure 5. An example of LNA/2’OMe-FISH combined with CLSM analysis. CLSM images for *B. fungorum* single-species biofilm (a), and for *E. coli*/ *B. fungorum* dual-species biofilm showing the localization of species in the biofilm formed in conditions mimicking the CAUTIS for 8 days on silicone coupons. For dual-species biofilms, the transverse biofilm image shows both species mixed together in the direction of the z-axis. Green fluorescent cells represent *E. coli*; red fluorescent cells represent *B. fungorum*.

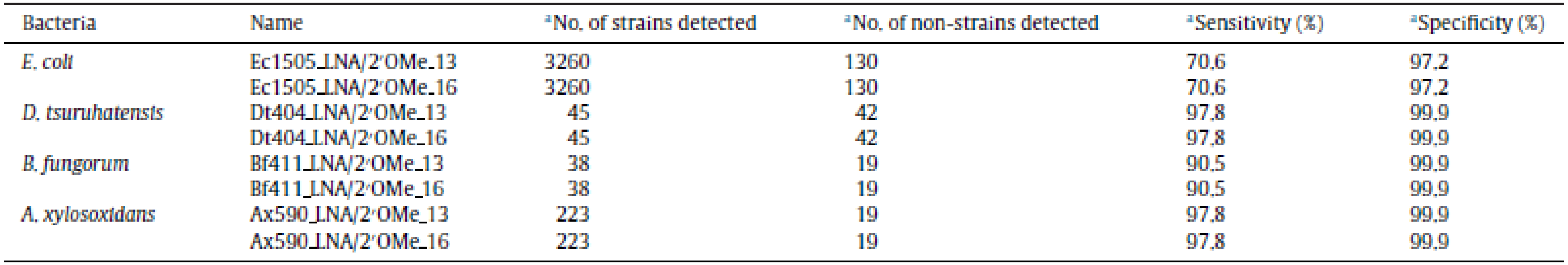
Table 1
Sequence of LNA/2′OMe oligonucleotide probes synthesized in this study.



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.

Table 2
Theoretical sensitivity and specificity of each LNA/2′OMe oligonucleotide probe tested in this study.



Bacteria	Name	[a]No. of strains detected	[a]No. of non-strains detected	[a]Sensitivity (%)	[a]Specificity (%)
E. coli	Ec1505_LNA/2′OMe_13	3260	130	70.6	97.2
	Ec1505_LNA/2′OMe_16	3260	130	70.6	97.2
D. tsuruhatensis	Dt404_LNA/2′OMe_13	45	42	97.8	99.9
	Dt404_LNA/2′OMe_16	45	42	97.8	99.9
B. fungorum	Bf411_LNA/2′OMe_13	38	19	90.5	99.9
	Bf411_LNA/2′OMe_16	38	19	90.5	99.9
A. xylosoxidans	Ax590_LNA/2′OMe_13	223	19	97.8	99.9
	Ax590_LNA/2′OMe_16	223	19	97.8	99.9

[a] Calculated by the TestProbe program (for *Escherichia coli* oligonucleotide probes) coupled to the LSU database with the following data set options: sequence length> 1900 bp; sequence quality> 90% (last accession, September 2014); or ProbeMatch from RDP II database (for the other three microorganisms) with the following data set options: strain – both; source – both; size – >1200 bp; quality – good (last accession, May 2014).

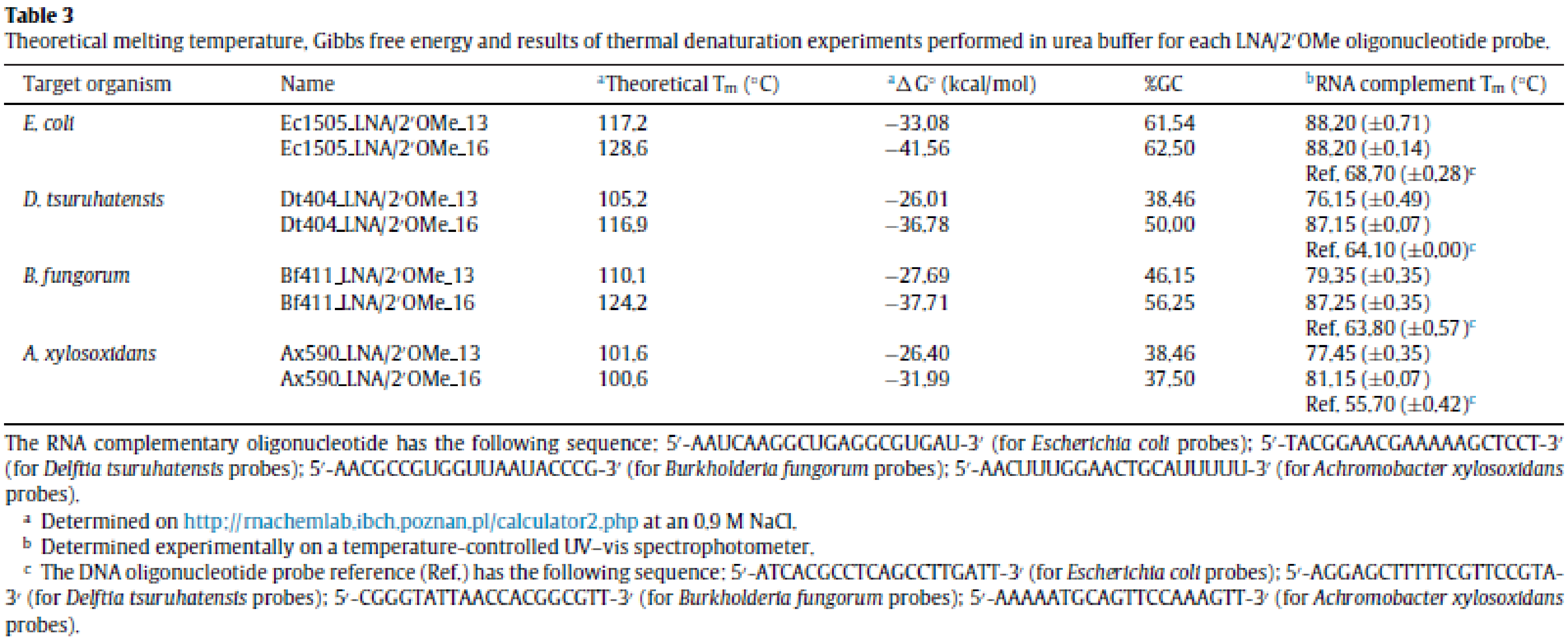


Table 3

Theoretical melting temperature, Gibbs free energy and results of thermal denaturation experiments performed in urea buffer for each LNA/2′OMe oligonucleotide probe.

Target organism	Name	[a]Theoretical T_m (°C)	[a]ΔG° (kcal/mol)	%GC	[b]RNA complement T_m (°C)
E. coli	Ec1505_LNA/2′OMe_13	117.2	−33.08	61.54	88.20 (±0.71)
	Ec1505_LNA/2′OMe_16	128.6	−41.56	62.50	88.20 (±0.14)
					Ref. 68.70 (±0.28)[c]
D. tsuruhatensis	Dt404_LNA/2′OMe_13	105.2	−26.01	38.46	76.15 (±0.49)
	Dt404_LNA/2′OMe_16	116.9	−36.78	50.00	87.15 (±0.07)
					Ref. 64.10 (±0.00)[c]
B. fungorum	Bf411_LNA/2′OMe_13	110.1	−27.69	46.15	79.35 (±0.35)
	Bf411_LNA/2′OMe_16	124.2	−37.71	56.25	87.25 (±0.35)
					Ref. 63.80 (±0.57)[c]
A. xylosoxidans	Ax590_LNA/2′OMe_13	101.6	−26.40	38.46	77.45 (±0.35)
	Ax590_LNA/2′OMe_16	100.6	−31.99	37.50	81.15 (±0.07)
					Ref. 55.70 (±0.42)[c]

The RNA complementary oligonucleotide has the following sequence: 5′-AAUCAAGGCUGAGGCGUGAU-3′ (for *Escherichia coli* probes); 5′-TACGGAACGAAAAAGCTCCT-3′ (for *Delftia tsuruhatensis* probes); 5′-AACGCCGUGGUUAAUACCCG-3′ (for *Burkholderia fungorum* probes); 5′-AACUUUGGAACTGCAUUUUU-3′ (for *Achromobacter xylosoxidans* probes).

[a] Determined on http://rnachemlab.ibch.poznan.pl/calculator2.php at an 0.9 M NaCl.

[b] Determined experimentally on a temperature-controlled UV–vis spectrophotometer.

[c] The DNA oligonucleotide probe reference (Ref.) has the following sequence: 5′-ATCACGCCTCAGCCTTGATT-3′ (for *Escherichia coli* probes); 5′-AGGAGCTTTTTCGTTCCGTA-3′ (for *Delftia tsuruhatensis* probes); 5′-CGGGTATTAACCACGGCGTT-3′ (for *Burkholderia fungorum* probes); 5′-AAAAATGCAGTTCCAAAGTT-3′ (for *Achromobacter xylosoxidans* probes).

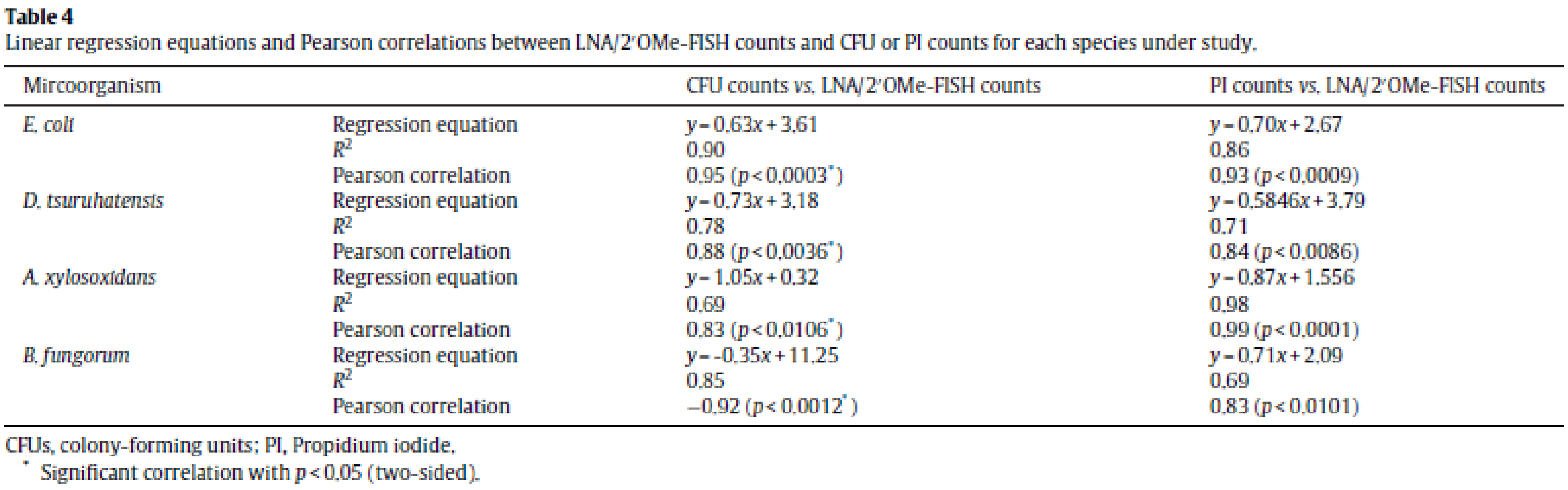


Table 4
Linear regression equations and Pearson correlations between LNA/2′OMe-FISH counts and CFU or PI counts for each species under study.

Mircoorganism		CFU counts *vs.* LNA/2′OMe-FISH counts	PI counts *vs.* LNA/2′OMe-FISH counts
E. coli	Regression equation	$y = 0.63x + 3.61$	$y = 0.70x + 2.67$
	R^2	0.90	0.86
	Pearson correlation	0.95 ($p < 0.0003^*$)	0.93 ($p < 0.0009$)
D. tsuruhatensis	Regression equation	$y = 0.73x + 3.18$	$y = 0.5846x + 3.79$
	R^2	0.78	0.71
	Pearson correlation	0.88 ($p < 0.0036^*$)	0.84 ($p < 0.0086$)
A. xylosoxidans	Regression equation	$y = 1.05x + 0.32$	$y = 0.87x + 1.556$
	R^2	0.69	0.98
	Pearson correlation	0.83 ($p < 0.0106^*$)	0.99 ($p < 0.0001$)
B. fungorum	Regression equation	$y = -0.35x + 11.25$	$y = 0.71x + 2.09$
	R^2	0.85	0.69
	Pearson correlation	−0.92 ($p < 0.0012^*$)	0.83 ($p < 0.0101$)

CFUs, colony-forming units; PI, Propidium iodide.

[*] Significant correlation with $p < 0.05$ (two-sided).