RT-PCR and PNA-FISH as Novel Methods for the Detection of Specific Microorganisms in Heterotrophic Biofilm Communities

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Biofilms occur in a large number of environments and medical devices relevant for public health. In natural environments they are usually multispecies microbial communities. Two recent advances of fluorescence in situ hybridisation (FISH) and polymerase chain reaction (PCR), peptide nucleic acid FISH and real time PCR, have been used successfully in the detection and quantification of specific microorganisms. Advantages and pitfalls for each of the techniques when applied to biofilms will be highlighted in this review.

Introduction

Elder and colleagues (1995) define a biofilm as "a functional consortium of microorganisms organized within an extensive exopolymer matrix". The formation of biofilms is now a well-know strategy used by microorganisms to survive within hostile environments, such as those encountered in host tissues (antibodies, phagocytes, etc.) or on inert surfaces exposed to inhospitable conditions (UV light, desiccation, heat, cold, shear forces). Organisms within a biofilm are far more resistant to antimicrobial agents than those in suspension (Mah and O'Toole 2001). In natural environments, they usually subsist as multispecies microbial communities, which imply the existence of complex interactions and cell-to-cell signaling pathways. To decipher these relationships, it is important to identify and quantify the organisms present and also understand their genetic expression changes. Two recent advances of fluorescence *in situ* hybridisation (FISH) and polymerase chain reaction (PCR), peptide nucleic acid FISH (PNA-FISH) and real time PCR (RT-PCR), have been used successfully in the detection and quantification of specific microorganisms. Nevertheless, PNA-FISH and RT-PCR have, so far, been applied only in a few

biofilm studies. However, there is a disadvantage of using these techniques which is the incapacity to resolve cell viability. Whether bacterial cells are dead or alive is an important question with many implications for monitoring biofilms. It is clinically and industrially relevant to understand the antimicrobial effect against biofilms and identify which specific cells within a biofilm are susceptible. The most common used strategies to overcome this difficulty are the detection of RNA, the use of dyes for microscopic differentiation and culture-based techniques. Although these methods are well established, they present some disadvantages. Working with RNA is technically demanding, and RNA is prone to contamination with RNA-degrading enzymes, resulting in problems of reproducibility (Alifano *et al.* 1994). For the use of dyes, due to the lack of an amplification step, the sensitivity of microscope- based techniques is a limiting factor, as is the inability to specify individual species (Virta *et al.* 1998). Culture-based techniques, on the other hand, are slow, providing results only after a few days, are dependent on the medium and the incubation temperature and do not support the growth of potentially viable but non-cultivable bacteria (Kell *et al.* 1998). A few ways to overcome this difficulty will be presented, by taking advantage of PNA-FISH and RT-PCR. This review will focus, briefly, on the advantages and pitfalls of these two molecular techniques in biofilm research and give an overview of FISH and RT-PCR procedures and applications in recent publications.

The Application of PNA-FISH for Biofilm Studies

FISH is based on the specific binding of nucleic acid probes to specific regions on RNA or DNA (Amann *et al.* 1997). Traditionally, FISH methods are based on the use of conventional DNA oligonucleotide probes, containing around 20 bases. Over the past years, this method has become a crucial biotechnological tool in environmental and clinical diagnosis.

In the early 90s, Nielsen *et al.* (1991) reported the development of a synthetic DNA analogue, named peptide nucleic acid (PNA). This molecule proved to be capable of forming PNA/DNA and PNA/RNA hybrids of complementary nucleic acid sequences. The PNA properties made PNA-FISH procedures easier and more efficient than DNA FISH (Stender *et al.* 2002; Wilks *et al.* 2006). PNA molecules have the negatively charged sugar-phosphate backbone replaced by an achiral, neutral polyamide backbone formed by repetitive units of *N*-(2-aminoethyl) glycine. They can hybridize to complementary nucleic acid targets obeying the Watson-Crick base-pairing rules (Egholm *et al.* 1993). PNA molecules present a quicker and stronger binding, attributed to the lack of charge repulsion between the neutral PNA strand and the complementary DNA or RNA strand. Consequently, the probes used for PNA-FISH are shorter, usually consisting of 15 bases, than conventional DNA probes, that allow an easy penetration in the cell (Stender *et al.* 2002). In fact, PNA probes are better for targeting highly structured nucleic acids, like rRNAs, since

hybridisation with PNA probes can be performed efficiently under low salt concentrations. These conditions destabilize the secondary structures of the rRNA, resulting in an improved access to the target sequences (Stefano and Hyldig-Nielsen, 1997). Other advantages of the use of PNA oligonucleotides include a faster hybridisation time, resistance to nuclease and protease attack, and higher specificity and sensitivity. As general rule, the melting temperature (*Tm*) of a PNA/DNA or PNA/RNA duplex is 1°C higher per base pair than *Tm* of the corresponding DNA/DNA duplex (Nielsen 1998).

In recent years, studies have been carried out describing the development and application of novel PNA probes for the identification of several clinically and industrially relevant microorganisms, including *Escherichia coli* (Prescott and Fricker, 1999), *Staphylococcus aureus* (Oliveira *et al.* 2003), *Salmonella* spp. (Perry-O'Keefe *et al.* 2001), Listeria spp. (Brehm-Stecher, 2005) and *Campylobacter* species such as *C. coli*, *C. jejuni* and *C. lari* (Lehtola *et al.* 2005). Other studies have been applying PNA-FISH to differentiate closely related species. For example, Hongmanee *et al.* (2001) used PNA-FISH to distinguish between tuberculous and non-tuberculous *Mycobacterium* species. All the PNA probes presented in these studies have high sensitivity and specificity, and recent work has demonstrated the clinical potential of this technique. Chaping and Musgnug (2003) evaluated three rapid methods for the direct identification of *S. aureus* from blood cultures, API RAPIDEC (API), tube coagulase test (TCT), and PNA-FISH which exhibited the highest sensitivity (98.6%) and a specificity of 100%. More recently, Forrest *et al.* (2006) demonstrated that rapid candidemias identification, by PNA-FISH, resulted in a significant reduction of caspofungin usage, with great cost savings. Others authors have also demonstrated that FISH may become a very useful diagnostic tool for bloodstream infections (Peters *et al.* 2006). This technology has also been applied to yeasts, for the identification of *C. albicans* from blood culture bottles (Rigby *et al.* 2002); *Dekkera bruxellensis* from wine - a spoilage yeast that causes an undesirable flavour (Stender *et al.* 2001); and even to detect extracellular protozoan parasites from the subgenus *Trypanozoon* (Radwanska *et al.* 2002).

The PNA-FISH method is a promising technique for the *in situ* visualization of microorganisms in biofilms, especially because the hydrophobic nature of the PNA molecule allows a better diffusion through the biofilm matrix and thus, improved discrimination of microbes in their naturally occurring three dimensional states (Azevedo *et al.* 2003). Despite of the potential of PNA molecules, there are few studies regarding their application to biofilms. Azevedo *et al.* (2003) reported the first application of a PNA oligonucleotide probe in heterogeneous biofilms, to detect *Helicobacter pylori*, having concluded that this new technology provides an easy and quick way of performing FISH assays in this type of structure. In 2006, PNA-FISH was applied successfully to detect *Mycobacterium avium* subsp. *avium* (Lehtola *et al.* 2006), *Legionella* spp.

and *L. pneumophila* (Wilks and Keevil 2006) in drinking-water biofilms. These studies indicated that this technology is advantageous for complex environmental samples, as it allows locating specific bacteria directly in intact biofilms (when combined with an episcopic differential interference contrast/ epifluorescence microscope) thus providing valuable information about structure-function relationships and physiological niches.

BacLight™ and PNA-FISH Combination to Assess Viability

One limitation of the PNA-FISH procedure is it's incapacity to assess cellular viability. To overcome this limitation PNA-FISH could be coupled, for instance, with the two-colour fluorescence LIVE/DEAD® BacLight™ assay. This kit is used to assess viability of microorganisms for microscopy and consists of two dyes: SYTO 9 (which emits in the green channel) and propidium iodide (PI- which emits in the red channel). Intact cells can take up SYTO9 which then becomes interconnected with their genomic DNA, while the larger red fluorescent PI molecule is excluded. PI can, however, freely flow through compromised cell membranes and stain the DNA; hence, green labelled cells are considered alive and red cells are considered dead (Azevedo *et al.* 2007). PNA probe could be applied with one or both dyes as long as the PNA probe is coupled with a fluorochrome emitting in a different wavelength. As BacLight™ dyes stain DNA and PNA probes bind specifically to RNA, a simultaneous application should theoretically be possible. If this suggested technique works it could identify organisms, count and determine the percentage of viable/non-viable cells in the sample.

RT-PCR Applications in Biofilm Research

Real time PCR (RT-PCR) refers to the detection of PCR products while the reaction is occurring, opposed to the traditional PCR, where detection only occurs at the end-point of the reaction. The signal is readily followed on a computer screen, allowing each point to be automatically plotted and the extent of amplification followed as an continual graphical plot (Levin 2004). RT-PCR presents some additional advantages in comparison to PCR. For instance, the later technology does not allow the detection by electrophoretic separation, of small fragments, being the minimum size ca. 200pb. In addition, conventional PCR requires visualization of amplified products by agarose gel electrophoresis, which obviously requires additional time. RT- PCR completely eliminates this step through the use of a fluorometer built into the RT-PCR thermal cycler that is able to measure the fluorescence intensity after each amplification cycle. Agarose gel electrophoresis is now usually replaced with real-time PCR systems by programmed generation of a thermal denaturation curve of the amplified product after the PCR, allowing automatic calculation of the Tm value.

RT-PCR depends on the emission of a UV-induced fluorescent signal propor-

tional to the quantity of DNA that has been synthesized. Several RT-PCR-based techniques have been developed for this purpose. Among the fluorescent reporter systems employed, the most common are SYBR green, a class of molecular probes designated by TaqMan, fluorescent resonance energy transfer (FRET) and molecular beacons.

The simplest, least expensive, and most direct fluorescent system for RT-PCR involves the incorporation of the dye SYBR green whose fluorescence under UV greatly increases when bound to the minor groove of the DNA double helix (Hein *et al.* 2001). SYBR green lacks the specificity of fluorescent DNA probes but has the advantage of allowing the construction of a DNA melting curve. A more specific procedure is the TaqMan-PCR, also known by 5' nuclease fluorogenic TaqMan assay, which explores the property of *Taq* polymerase to act as a 5'-3' exonuclease. Briefly, an oligonucleotide probe that has a reporter fluorescent dye attached to its 5' end, and a quencher dye attached to its 3' end, are used in this assay. Initially, the unbound probe is unable to emit a fluorescent signal. When the probe hybridizes to its target template, the reporter dye is cleaved by the 5' nuclease activity of *Taq* polymerase and becomes capable of emitting a fluorescent signal without the suppression activity of the quencher dye (Livak *et al.* 1995). FRET-PCR is a technique that involves the incorporation of two different probes into the PCR. One probe (light donor) harbors a fluorescein label at its 3'-end and the other probe (light acceptor) is labeled with LightCycler Red 640 at its 5'-end. Probes are selected so that they hybridize to sequences in the same strand standing close to each other (no more than 1 to 5 nucleotides interval). Under UV, the fluorescein emits a green light which then excites the Red 640 dye because of their close proximity which in turn emits a red light which is proportional to the amount of amplicon present (Loh and Yap 2002). Molecular Beacons probes are used to perform other RT-PCR assays. These probes have GC-rich complementary terminal nucleotides that form a hairpin configuration with a hybrid stem. The probes harbor a reporter dye at one end and a quencher dye at the other end. The reporter dye is non-fluorescent in the hairpin configuration. When the probe hybridizes to its target, the reporter dye is separate from the quencher resulting in fluorescence emission (Fortin *et al.* 2001). Based on the methods described above, several RT-PCR assays were developed for the rapid screening of microorganisms (Table 1).

Despite the ability to use RT-PCR assays to study specific populations in biofilms, most researchers have applied this technique to identify and quantify changes in genes expression, usually comparing biofilm and planktonic cultures. Gilmore el al (2003) used RT-PCR to quantify changes in expression of *Streptococcus gordonii* genes known or thought to be involved in dental plaque biofilm formation. Beloid *et al.* (2006) investigated the influence of regulatory and pathogenicity island-associated factors on biofilm formation by *E. coli*. Ledeboer

et al. (2006) investigate how biofilm growth of tissue culture cells affects genes expression in *Salmonella*. They compared global gene expression during planktonic and biofilm growth by applying microarray technology. RT-PCR was then used to quantify significant transcription differences, associated with the growth as a biofilm. A quantitative analysis by RT-PCR confirmed results obtained by microarray platforms. Olioggi *et al.* (2006) characterized genes expression of *Streptococcus pneumoniae* and concluded that sessile cells were more effective in inducing meningitis and pneumonia, while planktonic cells were more effective in inducing sepsis. Lin Xu *et al.* (2006) used RT-PCR to try and understand how cellular intercommunication or Quorum sensing, is processed in biofilms.

Table I - Examples of microorganisms detected by specific RT-PCR-based methods.

Microorganism	Type of sample	Reference
Yersinia enterocolitica	Blood	Sen 2000
Listeria monocytogenes	Pure cultures, water, skimmed milk, and unpasteurized whole milk	Novga *et al.* 2000
Escherichia coli	Human stools and beef	Bellin *et al.* 2001
Legionellae spp.	Hospital water	Wellinghausen *et al.* 2001
Vibrio cholerae	Pure cultures, raw oysters and synthetic seawater	Lyon 2001
Neisseria meningitidis *Haemophilus influenzae* *Streptococcus pneumoniae*	Cerebrospinal fluid, plasma, serum and whole blood	Corless *et al.* 2001
Pseudomonas aeruginosa	Samples from patients with cystic fibrosis	Qin *et al.* 2003
Salmonella spp.	Chicken, fish and raw milk	Malorny *et al.* 2004
E. sakazakii	Infant formula	Seo and Brackett 2005

The knowledge of microbial gene expression in biofilms is crucial for exploring ways of controlling them. Nevertheless, in environmental or clinical samples of already established biofilms it is also important to identify which microorganisms constitute them. For instance, Guilbaud *et al.* (2005) developed a RT-PCR based method to evaluate the population of *Listeria monocytogenes* in artificially made biofilms. That work proves the effective value of this technique on biofilm research that is quite fast and easy to use.

EMA-PCR to Assess Cell Viability

For RT-PCR, the lack of differentiation between DNA from viable and dead bacterial cells has been a major obstacle to broad-range applications of DNA-based molecular diagnostics (Lemarchand *et al.* 2004). A promising alternative has recently been described by Nogva *et al.* (2003) and Rudi *et al.* (2005), who have introduced ethidium monoazide bromide (EMA)-PCR as a diagnostic DNA-based method combining the use of a live-dead staining dye with the speed and sensitivity of RT-PCR. EMA has been used as a dye for microscopic

differentiation between viable and dead cells (Breeuwer *et al.* 2000). Like ethidium bromide, the structurally similar EMA is a phenanthridinium DNA/RNA-intercalating agent (Waring 1965) that can only enter bacterial cells with compromised cell walls and membranes (Rudi *et al.* 2005). When EMA is added to a test sample containing both viable and dead cells, it penetrates the dead cells and reversibly binds to the DNA. Light exposure leads to covalent binding of intracellular EMA to the DNA and inactivation of free EMA. After purification the DNA population from viable cells is unstained, while the DNA from the dead cells is covalently bound to EMA. The unstained DNA from viable cells is PCR amplified, while the DNA from dead cells with bound EMA cannot be amplified (Rudi *et al.* 2005). Nocker and Camper (2006) showed that non-amplification of dead cells DNA was also due to this DNA loss, during the DNA extraction procedure, together with cell debris as part of the pellet fraction. The same authors reported the application of EMA treatment to a drinking-water biofilm. Electrophoresis analyses resulted in community fingerprints dramatically different from those for an untreated biofilm. This work showed that EMA treatment of a mixed population is a valuable tool for the selective removal of DNA of non-viable cells by using conventional extraction protocols, providing a molecular analysis of the viable cells fraction from the population. This technique combines the specific detection and quantification of RT-PCR with the capacity to assess only the DNA from viable cells, showing a great potential in the study of mixed communities such as those present in biofilms.

Conclusions

In conclusion, PNA-FISH and RT-PCR are promising molecular techniques in biofilm studies although the inability to give information about cellular viability is a disadvantage. This limitation could be overcome by coupling these methods with viability tests; LIVE/DEAD for PNA, and ethidium monoazide for PCR. It may be exciting, in future work, to take advantage of both techniques, PCR and FISH, in order to understand microbial dynamics on biofilms. Coupling PCR and FISH brings complementary information about the presence and quantification of a microorganism whilst providing spatial resolution.

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