DEVELOPMENT OF PEPTIDE NUCLEIC ACID PROBES TO DETECT *HELICOBACTER PYLORI* IN DIVERSE SPECIES POTABLE WATER BIOFILMS

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The application of a novel technology which utilises peptide nucleic acid (PNA) molecular probes for the visualization of ***Helicobacter pylori*** *in biofilms was assessed. The probes selected were highly specific for the pathogen and did not hybridize with any of the autochthonous bacteria isolated or with any other component from the water biofilm. The use of this technique made pathogen detection quick and reliable. However, the presence of autofluorescent microorganisms and stacks in the biofilm samples implied that care had to be taken not to misidentify the bacteria.* ***H. pylori*** *can successfully incorporate within biofilms and the three characteristic* ***H. pylori*** *morphotypes were visualised. PNA probes appear to be a promising new technique for the in situ visualization of microorganisms in biofilms and a further development of this technique may allow the simultaneous detection of different species.*

Introduction

In order to understand the structure/function relationships of a bacterial biofilm, it is important to identify the bacteria that compose it and where they are predominantly located in the biofilm microenvironment. Fluorescence *in situ* hybridization (FISH) is one of the techniques available for that purpose. It is now widely established in diagnostic and other fields of microbiology as a reliable way to detect and visualize microbial species, and has been successfully adapted for the study of bacteria in biofilms in several works (Jang *et al.* 2003, Manz *et al.* 1999, Nogueira *et al.* 2002, Okabe *et al.* 1999).

In the early 1990s 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, and its neutrally charged polyamide backbone made PNA FISH procedures easier and more efficient for different reasons:

- Hybridization can be performed efficiently under low salt concentrations, a condition that promotes the destabilization of rRNA secondary structures and results in an improved access to target sequences that would be elusive using conventional FISH.
- Hybridization does not suffer from the electrostatic repulsions that occur when a DNA oligonucleotide is used. Because of the lower free energy of the hybrid a better specificity and sensitivity of the probe is achieved.

- Diffusion through the membrane might be easier due to the hydrophobic character of PNA as compared to DNA.
- It is not a substrate for the attack of proteases or endonucleases (Demidov *et al.* 1994).

Taking advantage of these features, a new procedure was developed for the simultaneous detection of both Gram-negative and Gram-positive species; something never accomplished using DNA probes (Perry-O'Keefe *et al.* 2001). This highlights PNA FISH as a very desirable technique to use for heterotrophic biofilms, where inter-species interactions determining the development and process of the biofilms are conditioned by their location and activity levels. A more extensive review of PNA applications in FISH technology can be found in Stender *et al.* (2002).

Helicobacter pylori is a Gram-negative, flagellated bacterium closely related to *Campylobacter* spp. Most people who are infected with *H. pylori* are asymptomatic, however, some may develop gastric or duodenal ulcers, and the occurrence of certain stomach cancers is two to six times higher in infected individuals. Contaminated food or water has been suggested as one of the sources of infection, but the evidence is not conclusive. To clarify this debate it is consequentely necessary to study *H. pylori* in water systems, especially in biofilms, where the bacteria have a better chance of survival in the microenvironments generated by the heterogeneous structure and activity of the complex consortia (Keevil 2003). The public concern about the survival of this microorganism in water was acknowledged by the United Sates Environmental Protection Agency by including it in their Contaminant Candidate List in their 1998 report (United States Environmental Protection Agency 1998).

Methods

Several sequences for *H. pylori* NCTC 11637 16S rRNA described in the literature were evaluated (Russmann *et al.* 2001, Thoreson *et al.* 1995, Trebesius *et al.* 2000) for its suitability for PNA FISH technology. The main adaptation performed at an average DNA oligonucleotide probe is the shortening of the sequence to comply with the optimum of between 12 and 18 bases necessary to achieve an optimal combination of specificity and binding strength in PNA hybridization. For the purpose of checking the impact of this modification, both an advanced BLAST search of the Genbank nr-database and a 16S rRNA sequence match analysis (http://rdp.cme.msu.edu/html/) were carried out. Based on the primer ACT-1 described in Thoreson *et al.* (1995), the sequence 5'-(TAATCAGCACTCTAGCAA)-3' was found to fulfil the necessary requirements. The advantage of this sequence is that there are at least two base mismatches for every other microbial species, which will theoretically minimize the chances of non-specific binding of the PNA probe to the very diverse microflora in the biofilm. However, the option to choose this more specific sequence meant that some other *H. pylori* strains would also not hybridize with the probe (Table 1).

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Table 1 Alignment of partial 16S rRNA of *H. pylori* and related microorganisms sequences. Base differences between the target sequence and other sequences are in bold and underlined.

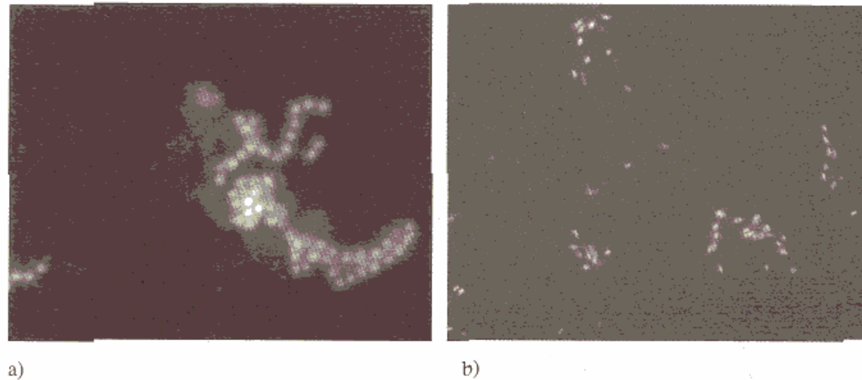
16S rRNA sequence (5'-3')	Species	Strain	Mismatches
U U G C U A G A G U G C U G A U U A	*H. pylori*	NCTC 11637	0
U U G C U A G A G U G C U G A U U A	*H. pylori*	26695	0
U U G C U A G A **A** U G C U G A U U A	*H. pylori*	U00679	1
U U G C U A G A G **A** U G **- -** G A U U A	*H. cinadei*	ATCC 35683	3
U U G C U A G A G **A** U G **- -** G A U U A	*H. fennelliae*	NCTC 11612	3

The hybridization procedure was done according to Stender *et al.* (1999) with slight modifications to ensure an optimal detection. Prior to hybridization, stainless steel coupons with biofilm attached were immersed in 90% ethanol for 10 minutes, and left to air-dry. The coupons were then covered with the hybridization solution, which consisted of 10% (w/v) of dextran sulfate, 10 mM NaCl, 30% (v/v) formamide, 0.1% (w/v) sodium pyrophosphate, 0.2% (w/v) polyvinylpirrolidone, 0.2% (w/v) FICOLL, 5 mM disodium EDTA, 0.1% (v/v) Triton X-100, 50 mM of Tris HCl (pH 7.5) and 200 nM of the PNA probe, for 90 minutes at 55°C. Following hybridization, coupons were washed in a pre-warmed solution containing 5 mM Tris Base, 15 mM NaCl and 1% (v/v) of Triton X (pH 10) at 55°C for 30 min. The coupon was then allowed to air dry after which a drop of mounting oil was added and the coupon covered with a coverslip. The hybridization procedure was then assessed by using a fluorescence microscope equipped with a Green Fluorescent Protein (GFP) filter that was sensitive to the fluorescent carboxyfluorescein molecule that was attached to the 5' terminal of the probe.

A two-stage chemostat system that has been already described (Keevil 2001), was used to grow biofilms, using tap water as the nutrient medium. The system was inoculated with 10^6 cfu.ml^{-1} of *H. pylori* and the coupons used to perform the hybridization technique as described above.

Results

Bacteria cultured from the system using R2A medium were found not to hybridize with the probe. There was also no evidence for non-specific binding of the PNA probe to the general heterotrophic microflora prior to the introduction of *H. pylori*. However, autofluorescence of diverse microorganisms was detected in different filter channels of the fluorescence microscope, including the GFP channel (Figure 1). Care has to be taken not to mistake these microorganisms with *H. pylori*. This can be accomplished by analysing both the morphology of the microorganism and the emission in different filter channels, because generally it was found that the fluorescence emission in these cases is still strong in other channels as well. For instance, in the microorganisms shown in Figure 1a, a strong signal was also emitted when viewed through the Rhodamine filter.



a) b)

Figure 1 Epifluorescence microscopy image showing autofluorescence of microorganisms in the GFP channel. Rods 2-4 μm long (a) and a cluster of cocci about 8 μm in diameter each (b).

Furthermore, coupons visualized before the hybridization procedure exhibited fluorescence in areas where stacks or fronds have formed. Autofluorescence in biofilms had not been observed before, therefore, the tap water was analysed for chemical compounds that could provoke this anomaly. A concentration of 8 ng/l of fluoroanthrene, a non-carcinogenic polyaromatic hydrocarbon (PAH), was detected. Although within the limits of safe drinking water parameters, it might be possible that this compound is able to attach and concentrate within the exopolymeric substances of the biofilm and therefore be able to cause the autofluorescence observed. This autofluorescence could also be identified after the inoculation of *H. pylori* and made detection of the pathogen more difficult since it was detected by the GFP channel of the microscope, as well as by the Rhodamine and DAPI channels (Figure 2a).

Nevertheless, *H. pylori* could still be easily detected in the basal layer of the biofilm, and adjacent to the stacks, where the effects of the autofluorescence could not be seen. Spiral forms of the bacteria, ranging from 2 to 4 μm long were still identified after five days of the experiment (Figure 2b). Coccal and U-shaped cell morphologies were also detected throughout the experiment. A cell was considered to be *H. pylori* if it exhibited typical morphology and if the fluorescence emission was intense in the GFP channel and faint or non-existent in all other channels.

Discussion

This study, in the sequence of a work already published (Azevedo *et al.* 2003), represents the first reported use of PNA oligonucleotides for *in situ* detection of microorganisms in biofilms, and offers an efficient alternative to conventional DNA approaches. The PNA technology seems to be easily adapted to the study

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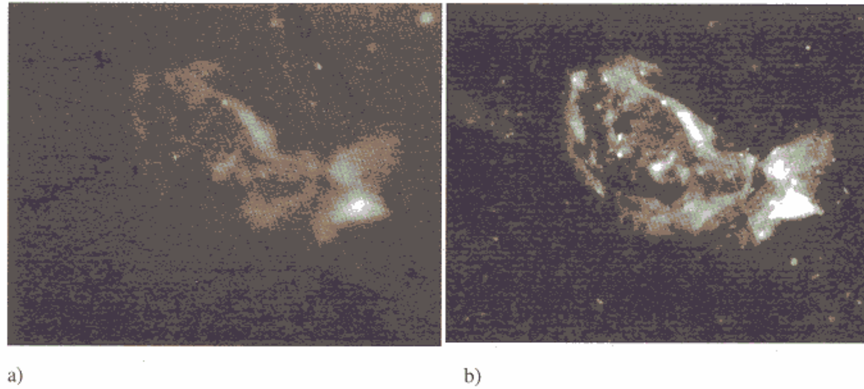

Figure 2 Episcopic fluorescence images of a mature biofilm section formed in a stainless steel coupon two days after inoculation with *H. pylori* using: Rhodamine filter (a); Green Fluorescent Protein filter (b).

of pathogens in biofilms. In the future, a further exploration of the PNA special features, might allow the simultaneous detection of different microorganisms in the same sample.

The existence of autofluorescing stacks and microorganisms means that practice in microscopy visualization is essential for the correct detection of the PNA-labelled pathogen. Autofluorescence of microorganisms was found not to be very problematic mainly because their morphology was significantly different from the *H. pylori*, and, if questions persisted, they could still be dissipated by observing other emission wavelengths. Autofluorescence of stacks implied, however, that detection within these structures was difficult and in some cases even impossible. As this has not been previously reported, it is unlikely to cause significant problems in the future.

All different types of *H. pylori* morphology were identified by the PNA probe and exhibited a strong signal in the GFP channel. It has been suggested that, contrary to most eubacterial mesophiles, *H. pylori* exhibits a relaxed phenotype with respect to accumulation of 16S rRNA under adverse conditions (Scoarughi *et al.* 1999). Therefore, it is not clear if this strong signal implies a metabolically active form of the cell or simply the accumulation of ribosomal RNA in inactive cells.

The observation during these studies of autofluorescing biofilms is intriguing. Autofluorescence of microorganisms and chemical compounds has been previously reported, but we have been unable to find any reference in the literature to natural autofluorescence in biofilms. We believe that it is related to the presence of very low concentrations of some compound present in the local

municipal water supply that is able to attach to and concentrate within the exopolymeric substances (EPS) of the biofilm. An obvious example of this is PAH, and the water analyses confirmed that fluoroanthrene, a non-carcinogenic PAH, was present in low concentrations in the supply to the tap. It has been postulated that PAH appears in water due to coal tar linings, widely used for many years as corrosion protection of cast iron and steel water pipes, being degraded by biofilms (Maier *et al.* 1997). Little PAH is detected in the water in the absence of disinfectant. However, in the presence of disinfectant, PAH and coal tar particles are released from the biofilm. After the disinfection process has ceased, the PAH slowly deceases as a result of the reformation of biofilm which then takes some time to develop its protective characteristics. Maier *et al.* (1997) concluded that the biofilm then begins to break down the PAH. The consorted metabolic activity of high species diversity biofilms to attack normally refractory molecules may support this conclusion. An alternative hypothesis is that the biofilm EPS merely concentrate PAH and that oxidation of EPS by disinfectants such as chlorine or ozone leads to the release of the bound PAH. Further work with non-oxidising disinfectants will be required to investigate this latter possibility.

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