Establishment of a continuous model system to study *Helicobacter pylori* survival in potable water biofilms

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Abstract Close association of the pathogen *Helicobacter pylori* in drinking water biofilms has been suggested. Using a two-stage water model, the survival and development of the pathogen in potable water biofilms was monitored. Filter-sterilized tap water was used as the growth medium and the inoculum consisted of a naturally occurring consortium of microorganisms. Biofilms were generated on removable stainless steel coupons that were placed in the second vessel. Novel technology peptide nucleic acid (PNA) molecular probes were used to detect and locate the pathogen within the biofilms which were also stained with the vital fluorophore, CTC, as a measure of viability. The PNA-labelled oligonucleotide probes were highly specific, and complementary to the helix 6 region of *H. pylori* 16S rRNA. The pathogen was tracked in the biofilms using epifluorescence microscopy (EF) and episcopic differential interference contrast microscopy (EDIC).
Keywords episcopic differential interference contrast microscopy, *Helicobacter pylori*, peptide nucleic acids.

Introduction

Helicobacter pylori is a gram-negative, microaerophilic, flagellated bacteria, whose relatedness to organisms of the genus *Campylobacter* led scientists to first name it as *Campylobacter pyloridis* (Warren and Marshal, 1983). However, the bacterium revealed important differences at the biochemical, morphological and genetic level to other *Campylobacter* spp. and was eventually renamed (Goodwin *et al*., 1989).

Infection with *H. pylori* leads to several gastrointestinal diseases such as gastritis, peptic and duodenal ulcers (Taylor and Blaser, 1991), gastric carcinomas and mucosa-associated lymphoid tissue tumours (Moran, 1997). In Western countries rates of infection are as high as 60% by age 65, while in developing countries the prevalence of infection can be as high as 90% by 20 years of age (Foreman and Webb, 1993), however, most infections are asymptomatic. Although the mode of transmission remains a subject open to discussion, *H. pylori* has already been detected in Peruvian drinking water (Hulten *et al*.,1996), tap and well water in Japan (Sasaki *et al*., 1999), and, more recently, identified in a water distribution pipe biofilm in Scotland using a nested PCR technique (Park *et al*., 2001). This data points to the necessity of studying *Helicobacter* in water systems, especially in biofilms, where the bacteria can have better chances of survival in the microenvironments generated by the heterogeneous structure and activity of the complex consortia (Keevil, 2001a)

Materials and Methods

Chemostat setup

The two-stage model, which has been described previously (Rogers *et al*., 1994; Keevil, 2001b), consisted of two 1L glass vessels linked in series to simulate conditions found in a water system. In both vessels the top plate and the sample port hood were made of titanium, in order to eliminate extraneous iron from the culture. The first vessel (seed) was fed by filter-sterilized tap water to produce a dilution rate of 0.05 h^{-1} and, to ensure reproducibility, conditions in this vessel remained constant. The heterotrophic consortium of microorganims collected during the first filtration of the tap water was utilised as the inoculum for the seed vessel. A constant 0.05 h^{-1} flow from this vessel was supplied into a second vessel, which was used to grow biofilms. Additional sterile water was added to this biofilm-generating vessel to produce an overall dilution rate of 0.2 h^{-1}. Effluent from the second vessel was collected in a waste container. The temperature in both vessels was maintained at 30 ˚C using an external electrical pad controlled by a proportional integral derivative unit system. The stirrer speed was 300 rpm in both vessels and ensured liquid homogenisation as well as sufficient oxygen concentration in the water.

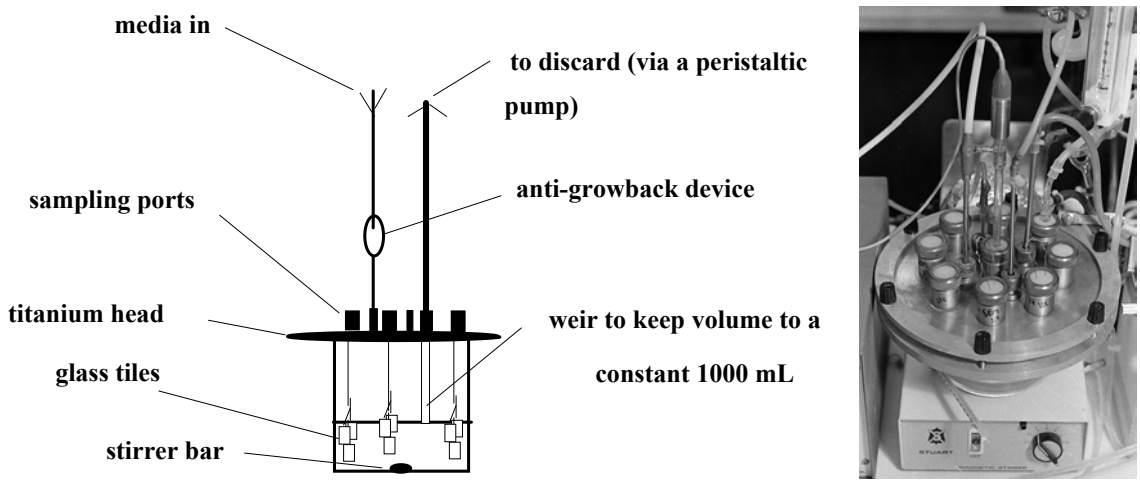

Figure 1 Diagram (left) and picture (right) of the model biofilm system with multiple assemblages of coupons suspended from rigid titanium wire inserted through silicone rubber bungs in the top ports. The weir system was used to maintain the volume at the required level

Coupons of stainless steel were made with an area of 1 cm^2, with a 1 mm hole allowing them to be suspended from titanium wire (three coupons per set). These coupons were inserted in the second vessel, and after the formation of the biofilm, were either used for microscopy observations or for plate counts on R2A agar.

The model was inoculated with *H. pylori* (NCTC 11637) at 10^6 $cfu.ml^{-1}$ that had been cultured in Brucella broth at 37 ˚C to late exponential phase. Survival of the pathogen was monitored by plating planktonic and resuspended biofilm phases on to Columbia Blood Agar. It was also tracked using peptid nucleic acid fluorescence *in situ* hybridisation (FISH).

Peptide Nucleic Acids (PNA)

PNA is a DNA analogue with the same interbase spacing and an aminoethyl glycine charge-neutral backbone, which allows it to bind to complementary nucleic acid sequences (Figure 2). Peptide nucleic acids were originally developed as a gene-targeting drug, but soon it was found that they could also play a role in improving existing or developing new techniques within DNA hybridisation-based methods in genetic diagnostics and molecular biology. The hybridisation strength, expressed as thermal stability (Tm), is usually higher for PNA/DNA duplexes. However, this is highly influenced by the purine/pyrimidine content of the oligonucleotide, with purine–rich oligonucleotides being much more stable than pyrimidine-rich ones (Giesen *et al*., 1998). 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 desestabilize the secondary structures of the rRNA, resulting in an improved access to the target sequences (Stefano and Hyldig-Nielsen, 1997). PNA has already been successfully used for in situ detection of *Escherichia coli* in water (Prescott and Fricker, 1999).

A species-specific PNA oligonucleotide probe directed against the 16S rDNA molecule was synthesized for the detection of *H. pylori* in water. The oligonucleotide used was complementary to the 19 bp primer ACT-1 (Thoreson *et al*., 1994) which was tested against related and non-related bacteria and was found to have insignificant homology to them. Because PNA achieve optimal combination of specificity and binding strength between 12 and 18 bases long, one base was taken from the ACT-1 primer.

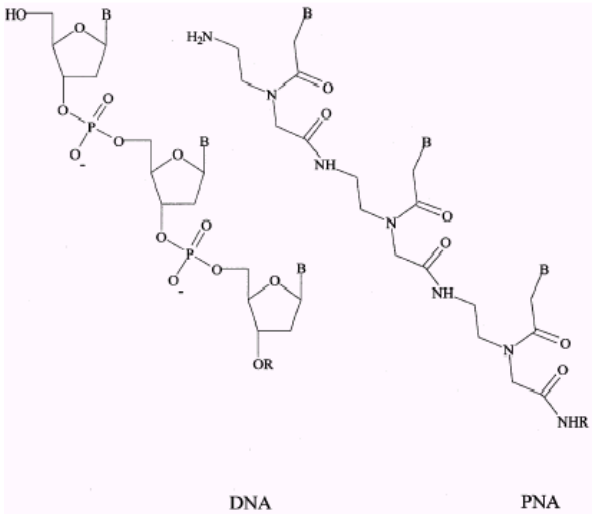

Figure 2 Chemical structure of DNA and PNA.

The sequence used was therefore 5'-(TAATCAGCACTCTAGCAA)-3'. An Advanced BLAST search of the Genbank nr-database (www.ncbi.nlm.nih.govlast) showed that this new oligonucleotide differed by at least 2 bases from the sequences of other bacteria species.

Microscopy techniques

Microscopy examination of entire biofilms has significantly increased our understanding of the spatial organisation that occurs within them and on the surfaces supporting their environment (Lawrence *et al*., 1991). The information that may be gained includes measurement of the depth of biofilm (Bakke and Olson, 1986) and identification of specific species within the biofilm consortia using labels (Rogers and Keevil, 1992).

Biofilms were visualized using episcopic differential interference contrast (EDIC) and fluorescence (EF) techniques. EDIC/EF has been described previously (Keevil and Walker, 1992) and consists of a conventional light microscope with UV fluorescence and DIC powered through a mercury lamp. Adaptations include the siting of the polariser above the specimen, which allows opaque specimens to be viewed, addition of a larger housing to accommodate appropriate filter blocks and the presence of mirror plates in the mercury light housing to increase the light intensity. DIC can be used to examine dense biofilms without prior preparation of the biofilm. The objectives used were non-coverslip corrected, so that a coverslip was not required. This technique therefore gives excellent topographical information because there is no compression of the biofilm.

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