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Expression profiling of *Helicobacter pylori* based on microarray

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Title (English)

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Abstract

Helicobacter pylori colonizes the gastric mucosa where it will induce chronic gastric inflammation, which can progress to superficial gastritis, peptic ulcer and gastric cancer. *Helicobacter pylori* exists in two different forms, a rod-like shape and a coccoid form. What kind of functions this form has and its significance in infection is still not fully known. To study the virulence of *H. pylori* in the coccoidal form, mRNA-levels in a large quantity of *Helicobacter pylori* genes were detected using the microarray technique. For this purpose 85 different *H. pylori* genes, mostly virulence genes and metabolic genes, of strain 26695 were selected and investigated by array hybridisations. Difficulties appeared as the hybridisations were inhibited by contaminants, presumably from the cell debris or the media, in the RNA preparations. This study demonstrates the great importance of the purity of the RNA and further improvements of the preparation of RNA are required.

Keywords

Helicobacter pylori, rod-like shape, coccoid form, microarray technique, hybridisation, RNA

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Expression profiling of *Helicobacter pylori* based on microarray

Caroline Pérez

Sammanfattning

Magbakterien *Helicobacter pylori* isolerades för första gången 1982 av de australiensiska forskarna Marshall och Warren. Bakterien är orsaken till uppkomsten av magkatarr, magsår och magcancer, och har hittats i magen på människor över hela världen. Infektionen är kronisk och livslång om inte patienten behandlas. *Helicobacter pylori* förekommer i två olika former, stavform och kockform. *H. pylori* i stavform är aktiv och infektiös, men det är idag inte känt om *H. pylori* i kockform, bakteriens viloform, orsakar inflammation. Målet med denna studie var att undersöka om *H. pylori* i kockform är infektiös. Detta gjordes med hjälp av en metod kallad microarraytekniken. Med denna metod kan man undersöka genuttrycket, dvs vilka gener som är ”påslagna” och därför kommer att generera proteiner som sedan orsakar inflammation i magen, hos *Helicobacter pylori* i kockform. Därigenom fås en indikation på om *H. pylori* i kockform kan orsaka inflammation i magen eller om bakterien enbart befinner sig i en icke infektiös viloform.

Examensarbete 20 poäng i Molekylär bioteknikprogrammet

Uppsala universitet, maj 2001

Table of contents

1. Introduction

1.1 The *Helicobacter pylori* bacteria

Helicobacter pylori was first isolated 1982 by B.J. Marshall and J.R. Warren at The Royal Perth Hospital, Australia [1]. It colonizes the gastric mucosa where it will induce chronic gastric inflammation, which can progress to a variety of diseases, ranging in severity from superficial gastritis and peptic ulcer to gastric cancer [2]. *Helicobacter pylori* has been found in the stomachs of humans in all parts of the world. Once acquired, the infection becomes chronic and probably persists for life unless the patient is treated [1] [2]. The number of infected is higher in developing countries than in developed countries (figure 1). In developing countries, 70% to 90% of the population carries *Helicobacter pylori* and almost all of these acquire the infection before the age of 10 years. In developed countries, the prevalence is not as high, ranging from 25% to 50% [3]. The differences in prevalence between developing and developed countries are suggested to be a connection to poor sanitary circumstances. When the socio-economic and sanitary standards are improved, the number of infected children is declining. The incidence of infection is then decreasing gradually over time. This explains the high prevalence of infection among the elderly over the world. They were born in a time when the common hygienic standard was much lower than today [4].

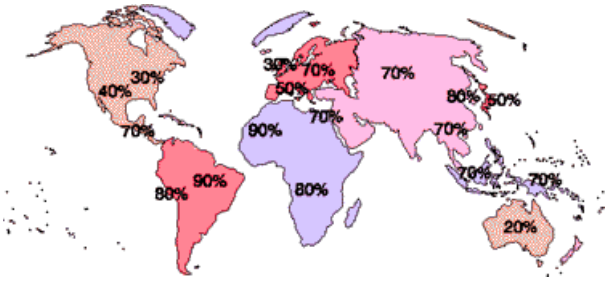


Figure 1. Map showing percentage of population infected with *H. pylori* as determined by epidemiological studies. The rates of infection can be attributed to socio-economic conditions in childhood. As populations achieve better living conditions, the incidence of *H. pylori* decreases. This image is from the *Helicobacter* Foundation website: http://www.helico.com [17].

It is still not proved how the infection of *H. pylori* is transmitted from the stomach of one person to that of another. Three routes have been described: 1. The least common is iatrogenic, in which tubes, endoscopes, or specimens in contact with the gastric mucosa from one person are introduced to another person. Improved disinfections of endoscopes have reduced the incidence of transmission. 2. The most important transmission is the fecal-oral transmission. *H. pylori* has been isolated from the feces of young children infected with the organism but the isolation is not common. This could indicate that detaching of the bacteria is irregular. Fecally contaminated water could also be a source of infection, but the organism has still not been isolated from water, which is also the case for food-borne transmission. 3. Finally, oral-oral transmission has been identified at African women who premasticate foods given to their infants. But there is no identified association of infection with sexual transmission or transmission via aspiration from vomitus, though *H. pylori* bacteria have been showed in small amounts in dental layers. The transmission of *H. pylori* is not yet clearly understood [3] [4].

The *H. pylori* is a spiral-shaped and gram-negative bacterium, which is movable and have flagella (figure 2) at one end [4]. Many factors contribute to the virulence of *H. pylori*. Expressed by all isolates are factors required for colonization and survival in the human stomach. Among these factors are the urease and flagella the most notable. Urease metabolises urea to carbon dioxide and ammonia to buffer the gastric acid. This is important when the bacterium reaches the acid environment in the human stomach. Meanwhile the bacterium neutralizes the gastric acid, it has to flee away from it, which it does with the help of its flagella. The flagella allow the bacterium to swim across the viscous gastric mucus and reach the more neutral pH below the mucus [4] [11]. Below the mucus, *H. pylori* adheres tightly to the underlying epithelial cells with the help of adhesins that bind to specific receptors on the membrane of the epithelial cells. Among the molecules that act directly on the surrounding tissues, the most important is the vacuolating cytotoxin (VacA), which is a secreted protein toxin that is responsible for the gastric epithelial erosion observed in infected hosts. It causes vacuolar degeneration of target cells by interfering with intracellular membrane fusion [4] [11].

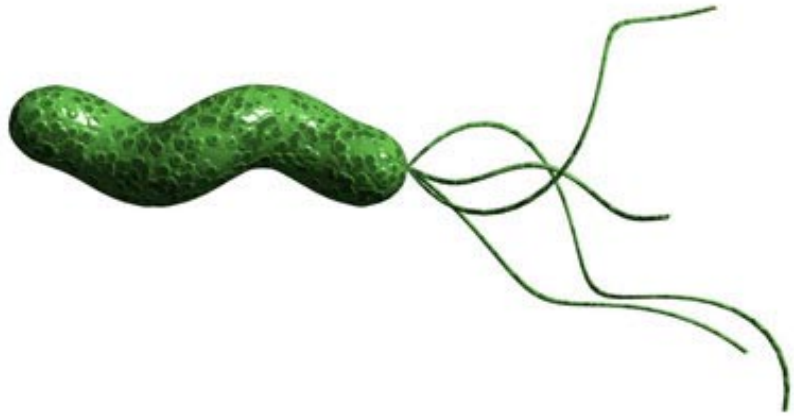


Figure 2. A 10,000x computer-aided design image of *H. pylori* showing spiral shape and flagella that enables the bacteria to propel themselves into the mucus layer of the stomach. The image by Luke Marshall is from the article by Lynch N.A.: http://www.faseb.org/opar/pylori/pylori.html [18].

The infection of *H. pylori* is chronic and life-long, as described above. This indicates that some kind of symbiosis probably exists where a certain degree of inflammation is maintained to support the bacteria with nutrition, but without being harmful to the human host organism. The body's immune system is not capable to exterminate the bacteria at this kind of infection, and the body requires help from the outside (e.g. antibiotics) to be able to overcome the infection [4].

When cultured on solid medium, the bacteria assume a rod-like shape and the spiral shape is infrequent or absent. After prolonged culture on solid or in liquid medium, coccoid forms typically predominate [3]. What kind of functions this form has and its significance in infection is still not fully known. Such forms may represent an environmentally resistant or dormant phase that can be induced to revert to the virulent bacillary form *in vivo* [3], and also may they play a role in the survival of the bacterium in a hostile environment [5]. The coccoid form has also been found in the human stomach [6]. Since *H. pylori* has been recovered only occasionally from sources outside the human

stomach, this indicates that every environment outside the human stomach is unfavourable to *H. pylori* and hence stimulates its conversion to the coccoid, nonculturable form [5]. Early studies have shown that the aging coccoid forms of *H. pylori*, although reportedly non-culturable *in vitro* remains genetically unchanged indicating that it is likely to be viable [7]. But Kusters *et al* [5] conclude that conversion of *H. pylori* from the bacillary to the coccoid form is a passive process that does not require protein synthesis. Their data suggest that the coccoid form is the morphologic manifestation of bacterial cell death. But their assumption that coccoids are dead does not rule out the existence of metabolic activity: bacterial enzymes can have extremely long half-lives, and hence enzymatic activity is no proof of viability [5]. Other investigators have suggested that the coccoidal form of *H. pylori* represents a degenerative form with no infectious capability [8]. So whether the coccoid form is in the process of degeneration or in the dormant stage prior to subsequent bacterial transmission remains an open question.

Successful infection with coccoid forms of *H. pylori* in animal models has been reported [9] and these findings have highlighted the possible role of the coccoid forms in transmission of infection and morphological conversion of coccoids to the spiral form. The acidity of gastric secretions is considered to be harmful to most organisms in the gastric environment. But it is suggested that acidity is essential for the establishment of colonization of the stomach by *H. pylori*. Mizoguchi *et al* [10] investigated whether coccoid forms had the capacity to synthesize proteins and, if so, whether coccoids produced by various procedures exhibited diversity in protein synthesis levels or viability. They also examined the potential morphological conversion of the coccoid to the spiral form following acid exposure. Their data suggests that the protein synthesis response of coccoid *H. pylori* to acid stress plays an important role in triggering conversion from the coccoid to the spiral form, and also that diversity in the viability following exposure to different stresses of coccoids correlates with their ability to cause infection [10]. In conclusion, there are two types of coccoids, “reversible” coccoids, capable of transition to the spiral form, and “irreversible” coccoids, which can progress to cell death [10].

1.2 The microarray technique

Various methods are available for detecting and quantitating gene expression levels, including northern blots, S1 nuclease protection, differential display, sequencing of cDNA libraries and serial analysis of gene expression (SAGE). To this collection, two array-based technologies as cDNA and oligonucleotide arrays can be included. These allow the study of expression levels in parallel [12], which will give static information about gene expression (in which tissue the gene is expressed) and dynamic information (how the expression pattern of one gene relates to those of others) [13]. There exist differences between the cDNA and oligonucleotide arrays, although both methods are capable of analysing patterns of gene expression [13]. In this study only the cDNA arrays were used, therefore merely the cDNA microarray technique will be explained (figure 3, adapted from Duggan *et al* [13]).

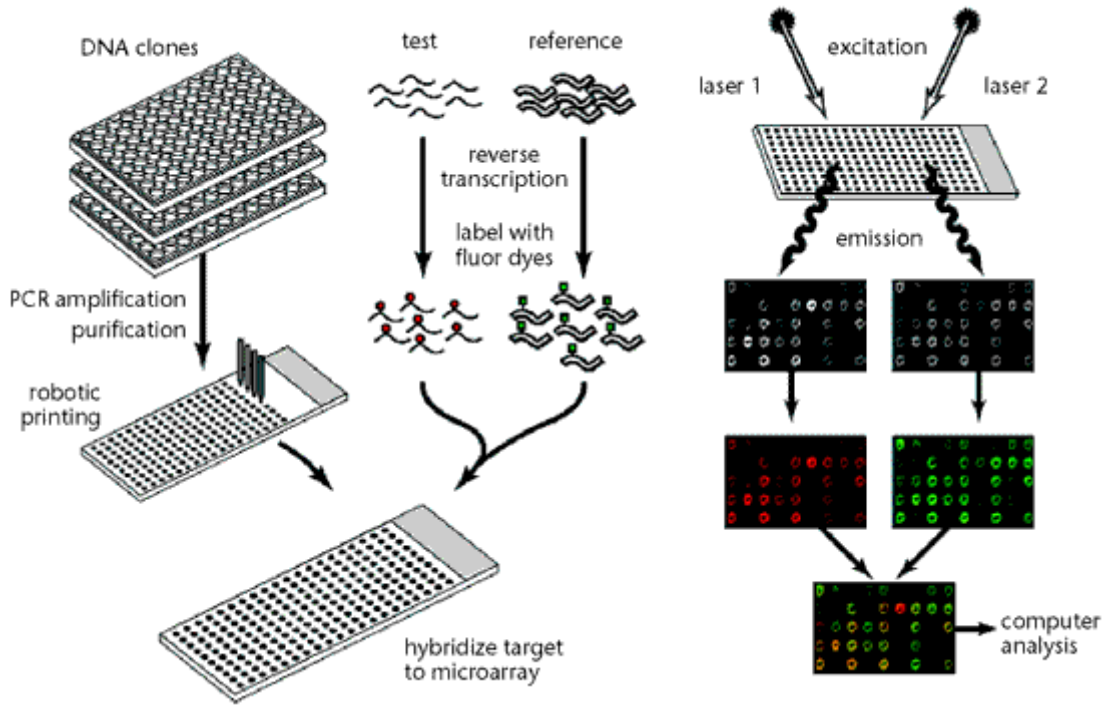


Figure 3. A schematic illustration of the cDNA microarray procedure. This image is from Duggan *et al* [13].

cDNA microarrays are capable of profiling gene expression patterns of tens of thousands of genes in a single experiment. cDNA arrays are produced by spotting PCR (polymerase chain reaction) products, representing specific genes with known sequences, onto a matrix. The PCR products are prepared in 96-well [14] or 384-well plates and are thereafter spotted in a known order onto the matrix. There are two types of matrices, glass and membrane matrices. The types of membranes commonly used are nitrocellulose and charged nylon. Arrays based on glass are made on microscope slides, which are coated with poly-lysine, amino silanes or amino-reactive silanes. These different coats enhance both the hydrophobicity of the slide and the adherence of the spotted DNA droplet on the slide. The PCR products are usually purified by precipitation or gel filtration to remove unwanted salts, detergents, PCR primers and proteins that can contaminate the arrays. Printing is carried out by a computer-controlled, high-speed robot that spots a sample of each gene product onto a number of matrices in a serial operation. There are many variations on the printing device, but the most common ones are the fountain pen and the capillary tube, to which a low but constant pressure is applied [13].

After DNA samples are arrayed onto slides, they are often immobilized by ultraviolet irradiation [14]. After fixation, residual amines on the slide surface are reacted with succinic anhydride to reduce the positive charge at the surface. In the final step, the DNA deposited will be rendered to single-stranded by heat or alkali [13].

The targets for the arrays are labelled representations of cellular mRNA pools. A reverse transcription of the mRNA is made, producing a labelled product directly complementary to the immobilized probes. The RNA must be purified as cellular protein, lipid and carbohydrate can mediate non-specific binding of fluorescently labelled cDNAs to slide surfaces. For fluorescent detection, Cy3-dUTP and Cy5-dUTP are frequently paired as they are widely separated in their excitation and emission spectra and have high incorporation efficiencies with reverse transcriptase. The matrix is then probed with the fluorescently tagged

cDNA representations of total RNA pools from test and reference cells, allowing one to determine the relative amount of transcript present in the pool by the type of fluorescent signal generated. Above the test and reference genes there are also control cells from another species, which genes function as controls as they are not complementary to the probes except to their own complementary counterparts on the matrix. The fluorescent targets are hybridised under stringent conditions to the clones on the array. The incorporated targets yield by laser excitation an emission with a characteristic spectrum, which is measured using a scanning laser microscope. The images from the scanner are sent into a computer program where the images are pseudo-coloured and merged. Information about the clones is associated to each target and data from each hybridisation is analysed in the computer program. Data from a single hybridisation experiment is viewed as a normalized ratio, that is Cy3/Cy5, in which significant deviations from 1, no change, are indicative of increased, >1, or decreased, <1, levels of gene expression relative to the reference sample [13].

1.3 The aim of the study

The aim of the study was to detect mRNA-levels in a large quantity of *Helicobacter pylori* genes based on the microarray technique, and to investigate if the coccoid form is virulent. For these purpose 85 different *H. pylori* genes of strain 26695 (provided by Lars Engstrand, Karolinska Institute, Stockholm, Sweden) were selected and then spotted on poly-L-lysine coated glass-slides (table 2, appendix). The genes we chose to investigate were mainly virulence genes and metabolic genes. The virulence genes contribute to the pathogenic effects of the bacterium in the rod-shaped form [3] and we wanted to investigate if there was an expression of these genes in the bacteria with coccoid form. Since the metabolic genes contribute to the metabolism of the bacterium, we assumed that the expression of these genes would decrease among the coccoidal-formed bacteria as the metabolic activity decreases in the dormant phase. We also selected some maintenance genes, which allow the bacterium to colonize and remain within the host [3] and hence always will be expressed among both the rod-shaped and the coccoidal-shaped bacteria. The 85 *H. pylori* genes were amplified with each gene's specific primer pair[1], wearing the same name as the gene in this study.

As negative controls, five different human genes were randomly selected. It is not important what kind of genes that are used as negative controls as long as they are from another species than the test- and reference genes and hence not complementary to the probes. The human genes were amplified with each gene's specific primer pair (table 1), wearing the same name as the gene in this study.

[1] The sequences of each *H. pylori* gene's specific primer pair will be provided by request: Maria.Lagerstrom-Fermer@Astrazeneca.com.

Table 1. The sequences of each human gene's specific primer pair and the lengths of each amplified human gene.

Primer pair	Sequence	Gene length
AGTRI fo	5'-C CCC AAA AGC CAA ATC CCA C-3'	305 nt
AGTRI re	5'-AGA AAA GTC GGT TCA GTC CAC-3'	
ACE14521 fo	5'-A ATT CTC TGA GCT CCC CTT AC-3'	175 nt
ACE14521 re	5'-GCC CTC CCA TGC CCA TAA C-3'	
REN204 fo	5'-G AAT GCC CTC AAT CCG AGA AA-3'	192 nt
REN204 re	5'-AGC CAA GCA CTC ACG TCC A-3'	
AGT fo	5'-G CAC AAG GTC CTG TCT GCC-3'	344 nt
AGT re	5'-AAA GCC AGG GTG CTG TCC A-3'	
B-AMEL-1	5'-CTA TGG TTA CGA ACC CAT GGG T-3'	110 nt
B-AMEL-5	5'-CTG GCA CCA TGG GGA TGT GGT G-3'	

The primer pairs AGTRI, ACE14521, REN204 and AGT were provided by Ulrika Liljedahl, Department of Medical Sciences, Molecular Medicine, Uppsala university, Sweden, and the primer pair AMEL was provided by Maria Lagerström-Fermér, Department of Medical Sciences, Molecular Medicine, Uppsala university, Sweden.

The project was divided into five parts (figure 4). The first part involved building the microarray glass-slide including the amplification of five negative control genes (human genes) and 85 *H. pylori* genes and then the printing on poly-L-lysine coated glass-slides, which were then post processed. The second part involved preparation of human RNA from the amplified human genes (negative control), and the next step was to prepare and isolate RNA from *H. pylori* cells. The fourth part included cDNA synthesis of *H. pylori* RNA (test- and reference genes) and human RNA (negative control), labelling of *H. pylori* cDNA and human cDNA and array hybridisation. The last step was to scan the microarrays and then analysing the data.

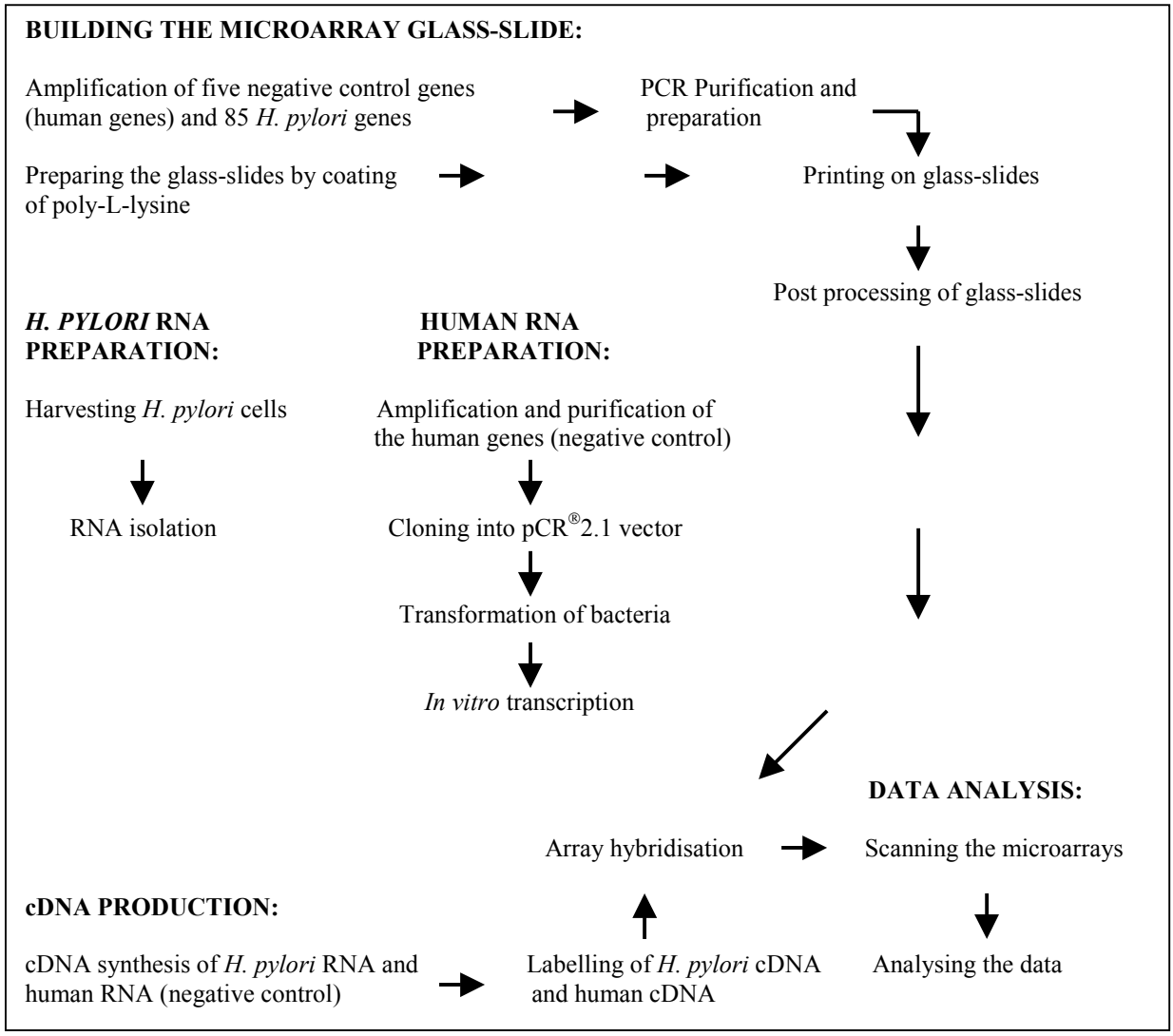


Figure 4. Project overview.

2. Materials and methods

2.1 Preparing negative control genes (human DNA)

2.1.1 PCR amplification and purification of PCR product

In each reaction 1 µl 0,295 µg/µl of human DNA was used as template and five different primer pairs (AGTRI, ACE14521, REN204, AGT, AMEL) with the concentration of 1 µM of each primer. Each reaction also contained 0.5 µl Taq-polymerase (AmpliTaq Gold, Perkin Elmer), 0.2 mM dNTP (Gibco BRL), 10 µl 10x PCR buffer containing 15 mM $MgCl_2$ (Perkin Elmer), and ddH_2O was added to a final volume of 100 µl. The program (95 °C 10 min) x 1, (95 °C 1 min, 56 °C 1 min, 72 °C 1 min) x 35, (72 °C 7 min) x 1 was performed on a PTC-225 Peltier Thermal Cycler (SDS). The PCR products were analysed on a 2% agarose gel (5 g agarose, 250 ml 0.5x TEB) stained with 8 µl ethidium bromide and visualised with UV-light. The products were then purified using QIAquick™ PCR Purification Kit (Merck Eurolab).

2.1.2 Cloning into pCR®2.1 vector

Cloning of PCR products into pCR®2.1 vector (figure 5) was performed using Original TA Cloning® Kit (Invitrogen) and the ligation reaction included 2 µl fresh PCR product, 1 µl 10x ligation buffer, 1.5 µl pCR®2.1 vector (25 ng/µl), 1 µl T4 DNA ligase (4.0 Weiss units). ddH_2O was then added to a final volume of 10 µl. The ligation reaction was incubated overnight at 14 °C.

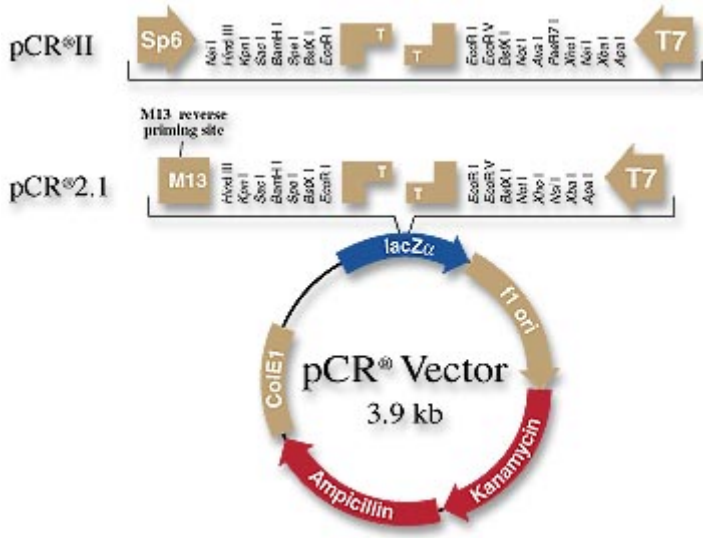


Figure 5. Map over the linearized vector pCR®2.1. This image is from the website of Invitrogen: http://www.invitrogen.com/vectordata/index.html [16].

2.1.3 Transformation of bacteria

The ampicillin resistant plasmids were used to transform competent *E. coli* cells of strain INVαF' (provided with the cloning kit, Invitrogen). The transformation was performed using Original TA Cloning® Kit (Invitrogen). The protocol from Invitrogen was followed with one exception: the LB plates only contained ampicillin (500 ml LB medium, 500 µl 100 mg/ml

ampicillin), as no blue/white selection would be performed. 50 µl respectively 200 µl of the cells from each sample were plated on the ampicillin plates and incubated overnight at 37 °C.

2.1.4 Colony PCR

20 colonies from each sample (20 colonies totally from the plate with 50 µl respectively 200 µl of the cells) were picked with a toothpick and replated on a master plate containing ampicillin and the same toothpick was then put in eppendorf tubes containing 100 µl of ddH_2O. The master plate was incubated overnight at 37 °C. The tubes containing H_2O and bacterial cells were incubated at 100 °C for 10 min to lyse the cells, and then centrifuged at 2,000 rpm for 1 min. 2 µl of the cell-mix (containing DNA) was used as template in a colony PCR reaction of 25 µl, which also contained 0.125 µl Taq-polymerase (AmpliTaq Gold, Perkin Elmer), 2.5 µl 10x PCR buffer containing 15 mM $MgCl_2$ (Perkin Elmer), 1 µM respectively of primer JS1 (5'-GAG CGA ATA ACA ATT TCA CAC AGG-3') and JS2 (5'-GCC AGG GTT TTC CCA GTC ACG A-3'), 0.2 mM dNTP (Gibco BRL) and ddH_2O was added to a final volume of 25 µl. The program as follows was used for the reaction: (94 °C 5 min) x 1, (94 °C 1 min, 55 °C 1 min, 72 °C 1 min) x 25, (72 °C 7 min) x 1. The PCR products were analysed on a 2% agarose gel (5 g agarose, 250 ml 0.5x TEB) stained with 8 µl ethidium bromide and visualised with UV-light. Plasmids with inserts equivalent to the genes AGTRI, ACE14521, REN204, AGT and AMEL (colonies taken from the master plate) were prepared using QIAGEN Plasmid Midi Kit (Merck Eurolab) and the DNA concentrations were quantified in a DU 530 Life Science UV/Vis Spectrophotometer (Beckman). A plasmid PCR was performed to verify the presence of inserts. The PCR reaction included 0.5 µl plasmid-DNA as template, 0.25 µl Taq-polymerase (AmpliTaq Gold, Perkin Elmer), 5 µl 10x PCR buffer containing 15 mM $MgCl_2$ (Perkin Elmer), 0.2 mM dNTP (Gibco BRL), 1 µM of each primer (AGTRI, ACE14521, REN204, AGT, AMEL) and ddH_2O to a final volume of 50 µl. The same program as described in section 2.1.1 was used. The PCR products were analysed on a 2% agarose gel (1 g agarose, 50 ml 0.5x TEB) stained with 3 µl ethidium bromide and visualised with UV-light.

2.1.5 Linearizing of plasmid (I)

To linearize the recombinant pCR®2.1 vector it was prepared with restriction enzyme *Hind* III (Gibco BRL). The reaction included 20 µg DNA, 10 µl 10x React3 buffer (provided with the enzyme), 10 µl enzyme *Hind* III, RNase-free H_2O to a final volume of 100 µl. The reaction was incubated for at least 3 hours at 37 °C. After the incubation the products were analysed on a 0.9% agarose gel (0.45 g agarose, 50 ml 0.5x TEB) stained with 3 µl ethidium bromide and visualised with UV-light. The procedure of purification of the products was performed as follows: 100 µl RNase-free H_2O and 200 µl phenol (pH~7.5) were added to the product and the whole mixture was centrifuged at 13,000 rpm for 5 min. The upper layer of the two phases (the H_2O-phase) was transferred to a new tube and an equivalent amount of chloroform was added. The mixture was centrifuged again at 13,000 rpm for 5 min and the upper layer was transferred to a new tube. 1/10 volume of 3 M, pH5.4 NaAc and 2.5 volumes of 99% ethanol were added and the DNA was incubated at −20 °C for 30 min. After precipitation the DNA was collected by centrifugation at 13,000 rpm for 20 min, the supernatant was discarded and the pellet was washed carefully with 200 µl 70% ethanol and centrifuged at 13,000 rpm for 20 min. The supernatant was discarded and the pellet was dried at room temperature (RT) and then resuspended in 20 µl RNase-free H_2O. The DNA concentration was quantified in a DU 530 Life Science UV/Vis Spectrophotometer (Beckman).

2.1.6 Linearizing of plasmid (II)

A PCR was performed on the prepared plasmids with the primer pair JS1/JS2 (for sequence, see section 2.1.4): 50 ng plasmid-DNA as template, 1 µl Taq-polymerase (AmpliTaq Gold, Perkin Elmer), 20 µl 10x PCR buffer containing 15 mM $MgCl_2$ (Perkin Elmer), 0.2 mM dNTP (Gibco BRL), 1µM respectively of primer JS1 and JS2, RNase-free H_2O to a final volume of 200 µl. The same program as described in section 2.1.4 was used. To blunt the ends of the fragments, 5 µl 5 u/µl T4 DNA polymerase (Gibco BRL) was added to 200 µl PCR product. After incubations at 11 °C for 15 min, at 37 °C for 45 min and at 65 °C for 20 min the same procedure of purification as described in section 2.1.5 was performed with one exception: double amount of H_2O, phenol, chloroform, ethanol and NaAc was used. The DNA-pellet was resuspended in 20 µl RNase-free H_2O and the DNA concentration was quantified in a DU 530 Life Science UV/Vis Spectrophotometer (Beckman).

2.1.7 *In vitro* transcription

The *in vitro* transcription was performed using *In Vitro* Transcription Kit (Promega) and the reaction was prepared as follows: 8 µl 5x transcription buffer, 4 µl 100 mM DTT, 2 µl T7-polymerase, 2 µg linearized plasmid-DNA, 8 µl 2.5 mM rNTP, 1 µl 40u RNase inhibitor, RNase-free H_2O to a final volume of 40 µl. After 2 hours of incubation at 37 °C, 2 µl 1.0u 1u/µl RQ1 RNase-free DNase (Promega) was added and the mixture was incubated for 30 min at 37 °C. 158 µl RNase-free H_2O (+42 µl sample, totally 200 µl) and 200 µl phenol (pH~4.0) were added and the mixture was centrifuged at 13,000 rpm for 5 min. The upper layer was transferred to a new tube, 200 µl chloroform was added and one more centrifugation was performed at 13,000 rpm for 5 min. The upper layer was transferred to a new tube and 1/10 volume of 3 M, pH5.4 NaAc and 2.5 volumes of 99% ethanol were added and the RNA was incubated at –20 °C for 30 min. After precipitation the RNA was collected by centrifugation at 13,000 rpm for 20 min, the supernatant was discarded and the pellet was washed carefully with 200 µl 70% ethanol and centrifuged at 13,000 rpm for 20 min. The supernatant was discarded and the pellet was dried at RT and then resuspended in 20 µl RNase-free H_2O. The RNA concentrations were quantified in a DU 530 Life Science UV/Vis Spectrophotometer (Beckman) and the RNA products were analysed on a 1% agarose gel (0.5 g agarose, 50 ml 0.5x TEB) stained with 3 µl ethidium bromide and visualised with UV-light.

2.2 Preparing glass slides and microarrays for hybridisation

2.2.1 Poly-L-lysine coating of glass slides for microarraying

60 glass slides were coated each time. Throughout the whole process it was important to avoid exposing the glass slides to air any length of time. 30 slides were placed in each slide rack and the slide racks were plunged up and down in two slide chambers containing cleaning solution, which was made the day before. The cleaning solution was prepared as follows: 70 g NaOH was dissolved in 280 ml ddH_2O and 420 ml 95% ethanol was slowly added to the solution under stirring until completely mixed. The slide chambers were shaken on an orbital shaker for 2 hours. The racks were then quickly transferred to fresh chambers filled with ddH_2O and plunged up and down. The rinses were repeated four times with fresh ddH_2O. The racks were transferred to chambers filled with poly-L-lysine solution which was made by: 70 ml poly-L-lysine + 70 ml tissue culture PBS (1 l PBS has 8 g sodium chloride, 0.2 g potassium chloride, 1.44 g sodium phosphate (dibasic, anhydrous) and 0.24 g potassium phosphate (monobasic)) + 560 ml ddH_2O. The chambers were shaken on an orbital shaker for

1 hour. The racks were then transferred to chambers filled with ddH_2O and plunged up and down for 1 min. The slides were spun dry at 500 rpm for 5 min and then placed in a slide box. The slides would be ready for use after a minimum of 4-5 days.

2.2.2 PCR amplification and purification of PCR product of *H. pylori* DNA to print on array

The PCR amplification was performed using 180 ng *H. pylori* DNA of strain 26695 (provided by Lars Engstrand, Karolinska Institute, Stockholm, Sweden) as template and 85 different primer pairs (see table 2, appendix) with the concentration of 1 µM of each primer. The reaction also contained 0.2 mM dNTP (Gibco BRL), 1 µl Taq-polymerase (AmpliTaq Gold, Perkin Elmer), 20 µl 10x PCR buffer containing 15 mM $MgCl_2$ (Perkin Elmer), and ddH_2O was added to a final volume of 200 µl. The amplification was accomplished with following program: (94 °C 6 min) x 1, (94 °C 1 min, 50 °C 1 min, 72 °C 1 min) x 40, (72 °C 10 min) x 1. The genes that were not amplified successfully in the first reaction were reamplified using the same protocol as described above but with one modification: 20 µl 100% DMSO (final concentration 10%) was added to the reaction. The PCR products were analysed on a 2% agarose gel stained with ethidium bromide and visualised with UV-light. The products were purified using QIAquick™ 96 PCR Purification Kit (Merck Eurolab). To estimate the concentrations of the purified PCR products, 1 µl of the products were added to an ethidium bromide plate (25 ml 1% agarose gel, 1 µl ethidium bromide). A dilution series with λ DNA/*Hind* III fragments (Gibco BRL) with concentrations of 200, 100, 50, 25 and 12.5 ng/µl were used as reference concentrations. 1 µl of each reference concentration was also added to the plate. The plate was visualised with UV-light and the PCR products were compared to the dilution series to estimate the concentrations.

2.2.3 Preparation of *H. pylori* genes and human genes before printing on poly-L-lysine glass slides

51 µl of each purified PCR product (*H. pylori* DNA, section 2.2.2, and human DNA, section 2.1.1) was mixed with 9 µl 20x SSC (a final concentration of 3x SSC) and transferred to a 96-well plate (see table 2 & 3, appendix). The wells which were empty were filled with 51 µl ddH_2O and 9 µl 20x SSC and used as reference wells. The 96-well plate was incubated overnight at 4 °C to allow complete mixing. The *H. pylori* DNA and the human DNA were then printed on poly-L-lysine coated glass slides using a GMS 417 Arrayer robot (Genetic Microsystems Inc.).

2.2.4 Post-processing of printed microarrays

The array boundaries were marked with a diamond scriber. The bottom of a humid chamber (Sigma Diagnostics) was filled with heated ddH_2O with a temperature of 35-40 °C. The arrays were placed face down over the water in the chamber for rehydration. The spots would take up moisture and swell. After approximately 5-15 min the array spots started to glisten, and the slides were transferred quickly to an inverted heating block at 90-100 °C with their array side up. Each array was snap dried for 3-5 s. After denaturation of the arrays, they were placed with their array side up in a GS Gene Linker™ UV chamber (BioRad) and irradiated with 65 mJ. After UV-cross linking the arrays, they were placed in a metal slide rack. Meanwhile 6 g succinic anhydride was dissolved in 335 ml 1-methyl-2-pyrrolidinone with a stir bar on a stir plate. Immediately after the last flake of the succinic anhydride was dissolved, 15 ml sodium borate (1 M, pH8.0) was added into the stirring solution. After the sodium borate solution mixes in, the solution was poured into a slide chamber and the slide rack with the slides was plunged rapidly in the solution and then shaken up and down for a few seconds. The slide

chamber was shaken on an orbital shaker for 10-15 min at maximum speed. The rack of slides was transferred from the blocking solution to 1000 ml ddH_2O heated to 95 °C and plunged in the hot water for 2 min. The rack of slides was then transferred to a slide chamber with 95% ethanol and the slide rack was plunged a few times in the ethanol. The slides were spun dry at 500 rpm for 5 min and were then ready for hybridisations.

2.3 Preparing test (coccoid form) and reference (rod-shaped form) genes (*Helicobacter pylori* RNA)

2.3.1 RNA Preparation (I)

Phenol resistant tubes were filled with 10 ml water-saturated phenol (pH~4.0) and heated to 65 °C. 9 ml sodium acetate buffer (50 mM sodium acetate, 10 mM EDTA pH5.0) and 1 ml 10% SDS were added to the heated phenol. The tubes were immersed in 65 °C bath until heated, which would take approximately 10 min. The frozen *H. pylori* cell pellets, which had been cultured on LB plates and did contain cells with both rod-shaped and coccoid form, (provided by Lars Engstrand, Karolinska Institute, Stockholm, Sweden) were warmed to ~0 °C. 1 ml phenol/buffer mixture was added to each cell pellet, the pellet was dissolved and each mixture was returned to the tube and the tubes were vortexed for 10 s. The tubes were incubated in 65 °C water bath for ~15 min and vortexed every 1-2 min. The next step included tubes with face lock (Eppendorf) that were prepared by centrifuging at 1,500 g for 2 min. The cell/buffer mixtures were transferred to the face lock tubes and centrifuged at 3,000 g for 10 min. The H_2O-phase was transferred to new tubes and 1/10 volume of 3 M, pH5.4 NaAc and 2 volumes of 99% ethanol were added to each tube. Centrifuging at 3,000 g for 30 min precipitated the RNA. The supernatant was discarded and the pellet was washed with 1 ml 70% ethanol and dried at RT. After the pellet had been resuspended in 50 µl RNase-free H_2O, the RNA concentration was quantified in a DU 530 Life Science UV/Vis Spectrophotometer (Beckman).

2.3.2 RNA Preparation (II)

A/ Harvesting total RNA: The *H. pylori* cells were grown in LB-media (provided by Maria Sjölund, Department of Medical Sciences, Uppsala university, Sweden) for 1, 3, 5 and 7 days. The cells that had been growing for 1 and 3 days were still in the rod-shaped form while the cells that had been growing for 5 and 7 days had transformed to the coccoid form. Harvesting the cells was done by pipetting 30 ml culture into a 50 ml tube containing 3.75 ml of ice-cold ethanol/phenol stop solution (5% water-saturated phenol, pH~4.0 in 99% ethanol) that will stop degradation of mRNA. The cells were spun down at 4,000 rpm for 10 min at 4 °C. To lyse the cells, the pellets were resuspended in a final volume of a fresh solution of 800 µl 0.5 mg/ml lysozyme (Amersham Pharmacia), TE pH7.9. 80 µl 10% SDS was added to the mixture, which was then placed in water bath at 64 °C for 1-2 min until the sample cleared. After incubation, 88 µl 1 M sodium acetate, pH5.4 was added and the sample was mixed.

Hot phenol extraction: An equal volume (1 ml) of water-saturated phenol (pH~4.0) was added to each sample in RNase-free 2 ml microfuge tubes. After inverting 10 times, the tubes were incubated in water bath at 64 °C for 6 min and were inverted 6-10 times every 40 s. The tubes were placed on ice to chill and then centrifuged at 13,000 rpm for 10 min at 4 °C.

Chloroform extraction: The aqueous layer, which contains the RNA, was transferred to a fresh 2 ml microfuge tube containing an equal volume of chloroform. The tubes were inverted 6-10 times and then centrifuged at 13,000 rpm for 5 min at 4 °C.

Ethanol precipitation: For each sample, the aqueous layer was transferred equally into two 1.5 ml microfuge tubes. 1/10 volume of 3 M NaAc pH5.4, 1 mM EDTA, and 2-2.5 volumes of cold 100% ethanol were added and the samples were incubated at –70 °C for 20 min. After precipitation the RNA was collected by centrifugation at 13,000 rpm for 25 min at 4 °C, the supernatant was discarded and the pellet was washed carefully with 1 ml 80% (made with DEPC-treated H_2O) ethanol and centrifuged at 13,000 rpm for 5 min at 4 °C. The supernatant was discarded and the pellet was dried at RT and then resuspended in 50 µl RNase-free H_2O (DEPC-treated). Every four tubes were pooled together.

B/ DNase I treatment of RNA samples: It is essential to remove all contaminating DNA before the reverse transcriptase reaction, therefore, each 200 µl sample was treated with DNase I as follows: 0.6 µl 31 u/µl RNase inhibitor (Promega), 25 µl 10x DNase I buffer (provided with the DNase I enzyme) and 10 µl 1 u/µl RNase-free DNase I (Promega). The reactions were incubated at 37 °C for 30 min. For the purification of the samples the following extractions were performed, following the same procedure as described above:

1 phenol extraction: Water-saturated phenol, pH~4.0 was used for the extraction. The tubes were inverted 6-10 times and then centrifuged at 13,000 rpm for 5 min.

1 phenol/chloroform (50:50) extraction: The tubes were inverted 6-10 times and centrifuged at 13,000 rpm for 5 min.

2 chloroform extractions: The tubes were inverted 6-10 times and centrifuged at 13,000 rpm for 5 min each extraction.

Ethanol precipitation: 1/10 volume of 3 M NaAc pH5.4 and 2-2.5 volumes of cold 100% ethanol were added and the samples were incubated at –70 °C for 20 min. After precipitation the RNA was collected by centrifugation at 13,000 rpm for 25 min at 4 °C, the supernatant was discarded and the pellet was washed carefully with 1 ml 80% (made with DEPC-treated H_2O) ethanol and centrifuged at 13,000 rpm for 5 min at 4 °C. The supernatant was discarded and the pellet was dried at RT. After the pellet had been resuspended in 20 µl RNase-free H_2O (DEPC-treated), the RNA concentration was quantified in a DU 530 Life Science UV/Vis Spectrophotometer (Beckman).

2.3.3 RNA Preparation (III)

The RNA preparation was accomplished using RNeasy® Midi and Maxi Kit (Merck Eurolab). The *H. pylori* cells were grown in LB-media (provided by Maria Sjölund, Department of medical sciences, Uppsala university, Sweden) for 1, 3, 5 and 7 days.

2.4 Hybridisation

2.4.1 Labelling of *H. pylori* DNA

Labelling: The labelling of *H. pylori* DNA was based on Bioprime DNA Labelling Kit (Gibco BRL). 2 µg *H. pylori* DNA was added to an eppendorf tube and ddH_2O was added to bring the total volume to 21 µl. 20 µl 2.5x random primer/reaction buffer mix was added and after incubation at 100 °C for 5 min, the sample was placed on ice. 5 µl 10x dNTP (Gibco BRL) mix (1.2 mM each dATP, dGTP and dTTP; 0.6 mM dCTP; 10 mM Tris pH8.0; 1 mM EDTA), 3 µl Cy™5-dCTP (Amersham Pharmacia) and 1 µl 40-50 u/µl Klenow fragment (Gibco BRL) were added to the sample. After incubation at 37 °C for 2 hours, the reaction

was stopped by adding 5 µl 0.5 M EDTA pH8.0. The DNA was purified by adding 450 µl TE pH7.4 to the stopped labelling reaction and transferring it to a microcon YM-30 (Amicon/Millipore). The sample was centrifuged at 12,000 rpm for 9 min. The filter was transferred to a new tube, 500 µl ddH_2O was added and the sample was centrifuged again at 12,000 rpm for 9 min. Then the sample was centrifuged again and the volume was checked every 1 min until appropriate (9.5 µl or less). The filter was transferred to a new tube and inverting and centrifuging the filter for 1 min collected the purified probe.

Microarray hybridisation: To the concentrated sample, 1.8 µl 2.5% SDS, 2.55 µl 20x SSC, 10 µg yeast tRNA (Gibco BRL), 3 µg human Cot-1 DNA (Gibco BRL) were added and the volume was adjusted to 15 µl with ddH_2O. The hybridisation mixture was denatured at 100 °C for 1.5 min and incubated at 37 °C for 30 min. Meanwhile the array was placed in a clean hybridisation chamber and the cover slip was added on top of the array. After centrifugation at maximum speed for 10 s the probe was carefully pipetted to the edge of the cover slip and a drop of 15 µl 3x SSC was pipetted on the slide. The hybridisation chamber was closed and incubated overnight (14-18 hours) at 65 °C. The array was placed into a slide holder containing 1x SSC and 0.2% SDS. The slide was plunged ~20 times, then transferred to 0.4x SSC and plunged ~20 times, then transferred to 0.2x SSC and plunged ~20 times. The array was spun dry at 500 rpm for 5 min and then scanned in a ScanArray®5000 (GSI Lumonics).

2.4.2 Labelling of *H. pylori* and human RNA, and cDNA synthesis

Labelling: 20 µg of total RNA (coccoid form and rod-shaped form) was aliquoted into two eppendorf tubes and 6 µg random primer (Gibco BRL) was added to each tube. The volume was adjusted to 15 µl with ddH_2O and heated to 65 °C for 1 min, then chilled on ice. To each RNA sample (the coccoid sample and the rod-shaped sample) the following reactants were added: 2 µl reverse transcriptase (Superscript II, Gibco BRL), 6 µl first strand buffer (provided with Superscript II), 3 µl 0.1 M DDT (provided with Superscript II), 0.6 µl labelling mix (25 mM dATP, 25 mM dCTP, 25 mM dGTP, 15 mM dTTP) and 3 µl Cy-dUTP (Cy™3-dUTP to one tube, Cy™5-dUTP to the other)(Amersham Pharmacia). The samples were spun down at maximum speed and incubated at 42 °C for 2 hours. 1.5 µl stop solution (1 M NaOH, 0.1 M EDTA) was added and the samples were heated to 65 °C for 10 min, then 1.5 µl neutralization solution (1 M HCl) was added. Differently labelled samples were mixed and the mix was loaded on a microcon YM-30 (Amicon/Millipore) and 400 µl TE (0.01 M TRIS-HCl, 0.001 M EDTA, pH7.9) was added to the mix. After centrifugation at 12,000 rpm for 9 min, the filter was moved to a new tube and 500 µl ddH_2O was added to the filter. The sample was centrifuged at 12,000 rpm for 9 min. Then the sample was centrifuged again and the volume was checked every 1 min until appropriate (9.5 µl or less). The filter was transferred to a new tube and inverting and centrifuging the filter for 1 min collected the purified probe.

Microarray hybridisation: The concentrated probe volume was adjusted to 9.5 µl with ddH_2O. The following reactants were added to the labelled cDNA: 2.25 µl 20x SSC, 1.25 µl 2.5% SDS, 1 µl 5 mg/ml yeast tRNA (Gibco BRL) and 1 µl 4 mg/ml Cot-1 DNA (Gibco BRL). The probe was denatured by heating to 100 °C for 2 min and then incubated at 37 °C for 10 min. Meanwhile the array was placed in a clean hybridisation chamber and the cover slip was added on top of the array. After centrifugation at maximum speed for 10 s the probe was carefully pipetted to the edge of the cover slip and a drop of 15 µl 3x SSC was pipetted on the slide. The hybridisation chamber was closed and incubated overnight (14-18 hours) at 65 °C. The array was placed into a slide holder containing 1x SSC and 0.2% SDS. The slide

was plunged ~20 times, then transferred to 0.4x SSC and plunged ~20 times, then transferred to 0.2x SSC and plunged ~20 times. The array was spun dry at 500 rpm for 5 min and then scanned in a ScanArray®5000 (GSI Lumonics).

3. Results and discussion

3.1 Preparing negative control genes (human DNA)

3.1.1 PCR amplification and purification of PCR product

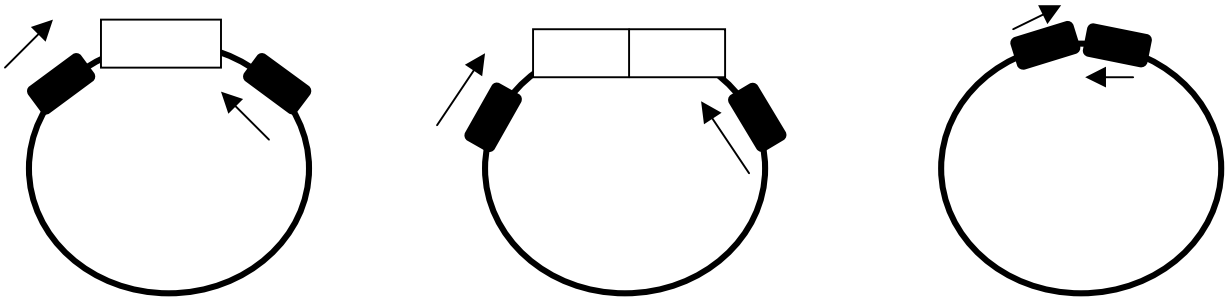
The analysis on a 2% agarose gel verified the presence of each desired human gene in each PCR product. As a negative control, the same reaction mix as described in section 2.1.1 was used, but without the DNA, for each primer pair. The negative control was used together with a 100 base pair (bp) size marker. The analysis also showed thick bands with a size of 40 bp, which indicated the presence of primer dimers. The PCR products were therefore purified, avoiding the primer dimers to ligate to the plasmid (section 2.1.2), instead of the PCR products.

3.1.2 Colony PCR

The analysis on a 2% agarose gel of the products from the colony PCR showed bands with different sizes. The bands which corresponded to the plasmids with inserts equivalent to the five different human genes were ~200 bp larger than each human gene. This is according to the primer pair JS1/JS2 (for sequence, see section 2.1.4), which amplifies a part of the vector along with the insert (figure 6a). This results in bands with a larger size than the actual genes. There were also bands with a very large size that probably originated from two inserts ligated into the same plasmid (figure 6b). Bands smaller than the insert were probably self-ligated plasmid (figure 6c). As a negative control, the same reaction mixes as to the colony PCR was used but without any DNA. A 100 bp size marker was also used.

For quantitating the amount of DNA, readings were taken at 260 nm and 280 nm. The reading at 260 nm allowed the calculation of the concentration of nucleic acid in the sample. The ratio between the readings at 260 nm and 280 nm provides an estimate of the purity of the DNA. Pure preparations of DNA have OD_{260}/OD_{280} values of 1.8. To verify the results from the spectrophotometer a plasmid PCR, with each gene's specific primer pair, was performed. The analysis on an agarose gel showed bands with sizes corresponding to each human gene. Negative controls for each primer pair, but without DNA, was used and also a 100 bp size marker.

Because of too low concentrations of DNA this procedure was repeated a number of times.



a. Plasmid with one insert.

b. Plasmid with two identical inserts.

c. Self-ligated plasmid with no insert.

Figure 6. Three results from cloning into a vector: plasmid with one insert (**a**), plasmid with two identical inserts (**b**), and a self-ligated plasmid with no insert (**c**). The primer will amplify a part of the vector along with the insert. The white box represents the insert; the black box represents the part of the vector that the primer will amplify and the arrows represent from where and at what direction the primer will amplify.

3.1.3 Linearizing of plasmid (I), (II) and *in vitro* transcription

The first experiments of linearizing the recombinant pCR®2.1 vector was performed using the restriction enzyme *Bam* HI. But the analysis on the 0.9% agarose gel, after the linearizing of the plasmid (I) (section 2.1.5), showed bands with sizes corresponding to linearized plasmids but also smaller bands corresponding to small fragments. The restriction enzyme had probably cleaved inside the inserts, leading to small fragments, because of unwanted restriction sites. As a negative control, uncleaved pasmid was used together with a 100 bp size marker. The analysis on the 1% agarose gel, after the *in vitro* transcription, gave the same result. This led to a test where the plasmid was linearized using three different restriction enzymes: *Bam* HI, *Eco* RI and *Hind* III. Analysis on an agarose gel showed that only *Hind* III cleaved the plasmid without cleaving inside the insert. In the following experiments only the restriction enzyme *Hind* III was used.

After the *in vitro* transcription, the RNA concentration was quantified in a DU 530 Life Science UV/Vis Spectrophotometer (Beckman). For quantitating the amount of RNA, readings were taken at 260 nm and 280 nm, as in the case of DNA. But pure preparations of RNA have OD_{260}/OD_{280} values of 2.0. The spectrophotometer showed very low concentrations of RNA and the analysis on agarose gel showed almost no bands at all. A closer look at the gel showed very large bands, much larger than the expected size of the band. The T7-polymerase had probably done a hairpin and been copying itself, leading to very large fragments. This conclusion led to an alteration of the linearization process, described in section 2.1.6. The primer pair JS1/JS2 amplifies a part of the vector along with the insert (described in section 3.1.2) resulting in a PCR product with the wanted gene flanked with a piece of the plasmid. The fragment from the plasmid also contained the site for the T7-polymerase so there was no need for linearizing a whole plasmid. The *in vitro* transcription that followed this experiment gave a higher concentration of RNA. The analysis on agarose gel showed bands with the expected sizes corresponding to the human genes, but also some larger bands. The T7-polymerase had probably done a hairpin and been transcribing the same insert again.

3.2 Preparation of glass-slides and microarrays for hybridisation; PCR amplification and purification of PCR product of *H. pylori* DNA to print on array

After the first amplification of the 85 different *H. pylori* genes, there were three genes at the positions C8, D2 and F12 that were not amplified successfully. These genes were reamplified using DMSO (section 2.2.2), but without success. In the following experiments these wells without any DNA were seen as negative controls (table 2, appendix).

To estimate the concentrations of the purified PCR products, they were analysed on an ethidium bromide plate. The analysis showed that most of the DNA samples had a concentration of 100 - 200 ng/µl.

3.3 Preparing test (coccoid form) and reference (rod-shaped form) genes (*Helicobacter pylori* RNA) followed by labelling and hybridisation

The first experiment included labelling and hybridisation of *H. pylori* DNA (section 2.4.1) to control the quality of the glass-slides. The results from the scanner showed clear spots against a black background where only the *H. pylori* DNA had hybridised to the arrays. There were also empty black spots where it should be negative controls from the empty wells and the human DNA. This experiment verified that the glass-slides kept a high quality. The next step was to label and hybridise the *H. pylori* RNA that had been prepared according to RNA preparation (I) (section 2.3.1) and the negative controls (the human RNA). The hybridisation was performed according to the described protocol in section 2.4.2. The results of these experiments showed no hybridisation for either the *H. pylori* RNA or the human RNA. The spots seemed to have disappeared and it was a lot of background. Most alarming was the absence of the signal of the negative controls since they always should generate a signal. This led to one more control of the glass-slides by doing an *H. pylori* DNA hybridisation and also a hybridisation with the negative controls only. The results of these experiments showed good signals from both the *H. pylori* DNA and the human RNA, with no background at all, which indicated good quality of glass-slides, arrays and negative controls. Also other experiments were done where, among other things, the incubation of the hybridisation were shortened with ~4 hours to minimise the background and the reverse transcriptase in the labelling reaction was exchanged for a new batch in case the enzyme was inactive. But these experiments did not improve the results. The conclusion drawn from these experiments indicated that it could only be something in the RNA preparation that inhibited the hybridisations, presumably cell debris that had not been washed away in the hot phenol reaction (section 2.3.1). Further indication of this hypothesis was the grey milky coat on the surface of the glass-slide that did not disappear after the washing step (section 2.4.2). An explanation to the cell debris could be that RNA prepared from cells cultured on LB plates are more difficult to purify than cells cultured in LB-media.

New RNA preparations were made according to a new protocol (section 2.3.2) including cells cultured in LB-media, several phenol/chloroform extractions and RNAse inhibitor. The RNA preparations gave much higher concentrations of RNA from the cells that had been growing for 1 and 3 days than from the cells that had been growing for 5 and 7 days. This verifies the characteristic behaviour of the rod-shaped form and the coccoid form very good. Cells with the rod-shaped form are active and infectious while cells, which has transformed into the coccoid forms are inactive and in a dormant phase [3] and consequently are not reproducing itself.

Since even the concentrations of the RNA prepared from cells growing for 1 and 3 days were too low, a number of changes of the protocol (section 2.3.2) were made to increase the quantity of RNA. A stepwise decrease of the various phenol- and chloroform extractions was made to increase the concentration. Unfortunately this gave worse results in the hybridisations with no signals and more background, since several phenol- and chloroform extractions result in more purified RNA. To decrease the background, the washing steps after the hybridisation (section 2.4.2) were increased to embrace an extra step where 0.1x SSC was heated to 65 °C. The changed washing procedure gave less background but did not improve the signals. One last attempt to increase the concentration of RNA was to prepare RNA according to the RNeasy® Midi and Maxi Kit (Merck Eurolab) (section 2.3.3). Unfortunately it did not increase the concentration of the RNA. The following experiments gave the same results as before with almost no signal at all and a lot of background. These discoveries resulted in two possible assumptions: 1. There is no RNA in the solutions. It could be degraded RNA, or solely rRNA or sRNA, which was measured in the spectrophotometer and also labelled. 2. During the preparation of RNA, the purification of the RNA may not have been able to wash away contaminating material from the cell debris or the media, which inhibited the hybridisation. An analysis on a 1% agarose gel showed that RNA that was

prepared and purified with a maximum of phenol- and chloroform extractions was the most purified RNA, while RNA that had been purified with a limited number of phenol- and chloroform extractions presented a smear. The analysis also showed a slight degradation of the RNA[2].

3.4 Conclusion and future research prospective

The conclusion to be drawn from these results is that the purity of the RNA plays an important role in the hybridisation process. Presumably there is something from the media or the cell debris that are not washed away in the purification step during the preparation of the RNA, inhibiting the array hybridisation. This assumption is confirmed by Selinger *et al* [15] who found that contaminating salts and sugars from the media were inhibiting the reverse-transcription reaction used to make labelled cDNA. They dramatically improved the yield by removing salts and sugars after the first precipitation by three passes through Centricon PL-20 concentrator columns [15]. Unfortunately this article was published at the end of the project, leaving no time for improvements.

The future research prospective is to improve the preparation and purification of the RNA to avoid the inhibiting of the hybridisations, making possible to detect the levels of the expressed mRNA of the coccoid form. This information will hopefully prove useful in the ongoing work aiming to find a connection between the varying levels of expression of different genes and the emergence of different diseases in the stomach.

[2] The analysis on the agarose gel was an estimation of rRNA. rRNA exists in a much larger amount than the other types of RNA, which will just be seen as a slight smear on the gel. The analysis just gives an indication if the RNA has been degraded or not.

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Appendix

Table 2. The 85 different *H. pylori* genes and its positions in the 96-well plate.

Position in 96-well plate	*H. pylori* genes (each gene amplified with its specific primer pair with the same name)
A1	53334
A2	379
A3	520
A4	521
A5	522
A6	523
A7	524
A8	525
A9	526
A10	527
A11	528
A12	529
B1	530
B2	531
B3	532
B4	535
B5	536
B6	537
B7	538
B8	539
B9	540
B10	541
B11	542
B12	543
C1	544
C2	545
C3	546
C4	547
C5	651
C6	alpAF
C7	alpBF
C8	babaiF (neg)
C9	babA2
C10	CAGA2
C11	icea
C12	inyA
D1	vacB
D2	vacAm1 (neg)
D3	vacAm2
D4	vacAs1/s2

D5	16SRNA
D6	26
D7	27
D8	121
D9	154
D10	176
D11	194
D12	574
E1	588
E2	589
E3	590
E4	591
E5	1088
E6	1099
E7	1100
E8	1101
E9	1166
E10	1227
E11	1261
E12	1264
F1	1265
F2	1266
F3	1267
F4	1268
F5	1272
F6	1273
F7	1345
F8	1346
F9	1385
F10	1386
F11	ICEA1
F12	ICEA2 (neg)
G1	PLP1
G2	RIBA
G3	16SRNA
G4	147
G5	193
G6	280
G7	432
G8	927
G9	930
G10	1025
G11	1444
G12	970
H1	1273

Table 3. The five different human genes, functioning as negative control genes, and its positions in the 96-well plate.

Position in 96-well plate	Human genes (each gene amplified with its specific primer pair with the same name)
H2	AGTR1
H3	ACE14521
H4	REN204
H5	AGT
H6	AMEL
H7-H12	empty wells (neg)