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# The use of microarrays for studying the pathogenesis of *Helicobacter pylori*

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**ABSTRACT.** At present, the genomes of various microorganisms have been completely sequenced, and many others are in progress. The availability of this level of information and the computational analysis of the described sequences have led to the development of new genomic areas such as: analysis *in silico*, comparative genomics, functional genomics, transcriptomics, proteomics, and pharmacogenomics.

Microarray technology is a powerful tool for analyzing the expression profile of thousands of genes in a global way and can be applied to the study of various biological systems. Using the complete sequences for both the *H. pylori* and human genome that are available in the data bases, a number of researchers have revealed important information. Some of these data offer a glimpse into the great genetic diversity of *H. pylori*, the differential genetic expression between the strains that shows the complexity of the response of microorganisms to different conditions of development, and into the association of gene cluster expression with clinical outcome. Other groups have examined the global transcriptional response of gastric epithelial cells to *H. pylori*. The majority of these studies report an alteration in gene expression related to transcription functions, transduction signals, cell cycle regulation and differentiation, development factors, proliferation/apoptosis balance, expression of membrane proteins, and inflammatory response.

**Key words:** *Helicobacter pylori*, microarray, gene expression, cag-pathogenicity island, gastric epithelial cells.

**RESUMEN.** La disponibilidad de las secuencias de los genomas de varios microorganismos, a través de bases de datos y del análisis computacional de las mismas, ha permitido el desarrollo de nuevas áreas tales como: el análisis *in silico*, la genómica comparativa, la genómica funcional, la transcriptómica, la proteómica y la farmacogenómica. Con la tecnología de microarreglos, se ha logrado analizar, de manera global, la expresión de miles de genes y puede ser aplicada a diversos sistemas biológicos. El uso de esta tecnología y el acceso a las secuencias de los genes que conforman los genomas de dos cepas de *H. pylori* y del genoma humano, ha permitido a varios grupos de investigación reportar resultados importantes en los últimos años. Estos reportes muestran, por un lado, la gran diversidad de contenido genético en diferentes cepas de *H. pylori*, la diferente expresión de genes del microorganismo en diferentes condiciones de desarrollo, así como la asociación de la expresión de grupos de genes bacterianos con diferentes patologías gástricas. Por otro lado, analizan la respuesta transcripcional global de la célula epitelial gástrica inducida por la infección con *H. pylori*, reportando alteración en la expresión de genes relacionados con funciones de transcripción, con señales transduccionales, con la regulación y la diferenciación del ciclo celular, con factores de desarrollo, con la alteración en el balance apoptosis/proliferación, con la expresión de proteínas de membrana y con la respuesta inflamatoria del huésped.

**Palabras clave:** *Helicobacter pylori*, microarreglos, expresión de genes, Isla de patogenicidad *cag*, Células epiteliales gástricas.

## INTRODUCTION

*Helicobacter pylori* is a gram-negative spiral-shaped bacillus that colonizes the gastric mucosa of humans. The presence of *H. pylori* leads to chronic inflammation, which increases risk for gastric and duodenal ulcers, gastric adenocarcinoma and gastric lymphoma.[7,9,11,18,20,37] In spite of the increased incidence of *H. pylori* infection worldwide, not all of those infected by this bacteria develop disease; propensity to develop disease may depend on the genotype of the bacterial strain, the genetic factors of the host, or to the specific interactions between host and microbe.

Several virulence mechanisms have been proposed for *H. pylori* associated with several clinical outcomes. Among these factors are the production of urease,[13,17,35,36] lipopolysaccharide (LPS) which possess the carbohydrates Lewis "x" or Lewis "y" or both, "O" antigen,[4,5] and a *cag* pathogenicity island (*cag*-PAI), which encodes a type IV secretion system.[10,34] This island is also essential in the translocation of the bacterial protein CagA[9,11,18,34] into the gastric epithelial cells where it is tyrosine phosphorylated by a kinase of the Src family from the host cell, and where it induces a rearrangement of the cytoskeleton of the host's cells during the formation of an actin pedestal. The production of a vacuolating cytotoxin (VacA) is responsible for the formation of vacuoles in gastric epithelial cells *in vivo*, as seen in different cultured cell lines *in vitro*.[6,12,18,20,22,37] Other components implicated in virulence include: *iceA*1 and *iceA*2, the genes that code for flagellin A (HP0601) and flagellin B (HP0115), adhesins *babA* (HP0896) and *babB* (HP1243) and for a large number of enzymes such as phospholipases, catalase, superoxide dismutase and homologues of mucinase.[32,35]

In *H. pylori* pathogenesis, the acid pH of gastric secretion is essential in order to establish and colonize the stomach mucosa. Following brief exposures of the bacteria to a pH of 3.5, the expression of proteins of thermal shock has been seen to increase, these proteins range between 68 and 90 kDa, and stomach colonization is increased as a response to the given chemical and physical signals through genetic regulation.

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It is well known that the virulence factors in Enterobacteriaceae are regulated in coordination with a variety of environmental signals. An increased number of acid induced virulence factors in *E. coli* and *S. typhimurium* have been identified. The *tag*E gene, for example, is located in a gene cluster and is regulated by acidic conditions. This gene is required for efficient intestinal colonization by *V. cholerae*. It is known that *H. pylori* presents two orthologous genes to *tag*E (HP1543 and HP 1544).[42] However, the role of the *tag*E gene in pathogenesis has not been demonstrated yet. It is important to determine its expression and participation in the bacteria for surviving the gastric microenvironment, where the pH can be between 2.0 and 3.5.

In spite of much information concerning *Helicobacter pylori*, for technical reasons only a limited subset of genes could be simultaneously analyzed until recently. New molecular tools for the global study of gene expression exist. In fact, the genome of 97 microorganisms has been sequenced completely and a further 142 are in progress. The availability of this level of information and the computational analysis of the described sequences has resulted in the development of new genomic areas such as: analysis *in silico*, functional genomics, transcriptomics, proteomics, pharmacogenomics etc. These allow the application of mutagenesis on a large scale, gene expression profiling and global analysis of proteins as a means to identifying candidate genes for specific diagnostic techniques, vaccine development targets for antimicrobials, and to determine new virulence genes involved in the development of disease.

In fact, a new methodology exists called "Microarrays".[16,21,25,30,39,43] These are glass slides containing an ordered mosaic of an entire genome as a collection of either oligonucleotides (oligonucleotide microarrays), or PCR products representing individual genes (commonly referred to as cDNA microarrays). Later, a hybridization is effected with two populations of cDNA (control and test) labeled with the fluorescent dyes Cy3 and Cy5, in such a way that the control population acts like a template for hybridization with cDNA derived from mRNA of the studied microorganisms. This offers a wide level of information regarding the profile of gene expression and predicts new ways of interaction between them.

The studies carried out using this recent methodology has enabled the detection of specific genes present in each of the complete genome sequence of two strains of *H. pylori*, [1,42] for example: 52 specific genes present in the J99 strain and 110 specific genes in the 26695 strain. These strain-specific genes were compared with genes already identified through computational analysis, allowing the assignation of putative functions for 30 genes, whilst the function of the majority remains unknown.

The work pioneered by Salama et al.[38] using microarray technology revealed the genetic diversity between *H. pylori* strains by comparing the two *H. pylori* sequenced genomes[1,42] with fifteen clinical isolates. In this way, analysis of the genetic pattern of 1643 genes from all of these strains showed that 1281 genes were common for all strains tested, which represent the functional core of the proteome. At least 12% to 18% of the genome of each strain was made up of 160 to 275 strain specific genes, whose function was not determined. The results showed that many of the specific genes of each strain were located in two regions: one being the pathogenicity island and the other being a plasticity zone of the *H. pylori* genome.

The loss and gain of genes in a strain suggests a horizontal transfer of genes in a recombination process that the strains have to carry out in order to adapt themselves and to be able to survive for long periods of time in a specific ecological niche.[27]

With *H. pylori* whole genome microarrays containing 1660 unique *H. pylori* genes, Israel et al.[26] evaluated the genomic content of two *H. pylori* clinical isolates, one from a gastric ulcer patient (B128 strain) and the other from a duodenal ulcer patient (G1.1 strain). Both strains possessed a similar virulence genotypic profile (*cagA*$^+$ *vacA* s1a *iceA1*) to that previously characterized. They found that the B128 strain possesses the *cag* island almost in its entirety (26 of 27 genes) and induces a more intense gastritis in gerbil gastric mucosa, and significantly higher levels of IL-8 *in vitro*, whilst the G1.1 strain presents an important deletion of genes in the *cag* pathogenicity island (*cag-PAI*) such as *picB/cagE*. They also observed an attenuated response of the G1.1 strain in the production of IL-8 in vitro, which that reduces the development of gastritis in animal experiments. These results corroborate previous studies that confirmed the participation of the *picB* and *cagE* genes in the liberation of cytokines in the gastric mucosa. As a consequence, microarrays are a recent and recommendable technology for identifying virulence factors of bacteria in infectious models.

On the other hand, after sequencing the complete genome[1,42] of two strains, 26695 and J99, Jungblut et al.[28] have approached the demanding task of investigating the functional part of the genetic information containing macromolecules, the proteome. The construction of a profile data base of the proteome of three *H. pylori* strains (26695, SS1 and J99) were added to the dynamic 2D-PAGE database (http://www.mpiib-berlin.mpg.de/2D-PAGE/). Silver-stained gels of proteins isolated from cultured bacteria revealed 1863 spots for strain 26695, 1622 spots for strain J99 and 1448 spots for SS1; these profiles reveal a high variability in the products of expression for each strain. In addition, only a small number of proteins were found to be common for the

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*H. pylori* strains studied. One hundred and fifty two proteins were identified, including nine known virulence factors: UreB (HP0072), Cag26 (HP0547), VacA (HP0887), Cag8 (HP0528), Catalase (HP0875), SodB (HP0389), HpaA (HP0410), UreA (HP0073) and GrosES (HP0011) and 28 antigens. The expression of 27 predicted conserved hypothetical open reading frame (ORFs) and six unknown ORFs were confirmed. The growth conditions of the bacteria were shown to have an effect on the presence of certain proteins.

Allan et al. [2] using a high-density array of PCR-amplified random genomic DNA, identified genes differentially expressed when *H. pylori* strain 26695 was exposed to either pH 4.0 or pH 7.0. Among the genes up-regulated at low pH were: *cagA*, which encodes a protein of high molecular weight and is associated with bacterial pathogenicity. HP0681 and HP1289 are considered unique to *H. pylori* and participate in Sec-dependent secretion. HP1052 is an orthologue of *envA* of *E. coli* and catalyses the second step of lipid A biosynthesis and is a point of regulation for LPS synthesis. HP0871 encodes enzymes that participate in the synthesis of phospholipids. HP1459 and HP0919 products participate in the biosynthesis of ribonucleotide and their modification, and *secF* (HP1549) encodes a component of the cytoplasmatic membrane secretion system. Furthermore, the expression of a product of 19.2 kDa has been reported which appears to be specific to *H. pylori*, but no information of a similar product has been found using the databases for other microorganisms. Genes down-regulated at pH 4.0 include those encoding a sugar nucleotide biosynthesis protein, a flagellar protein and an outer-membrane protein. However in another study, Ang et al.[3] constructed membrane-based DNA arrays containing PCR products representing 1543 ORFs from strain 26695.[42] The membranes were used to assess the global transcriptional response of one *H. pylori* strain after growth for 48 hours at pH 5.5 or at pH 7.2. Among 1 534 ORFs, 53 were highly expressed under both neutral and acidic conditions. This implied that these genes have important or essential functions in bacterial physiology. There were 445 ORFs that were stably expressed and whose products are probably needed to maintain the basic physiology in *H. pylori*. However, (contrary to the report by Allan et al) the CagA expression was slightly increased under acidic conditions. There were 952 ORFs that remained undetectable for any signal in this microarray, while the transcript level of 80 genes were increased significantly during acid stress and only 4 genes had decreased expression under this conditions. The induced genes include a number that were already known to be regulated by acid. These include the Omp11 protein-translocating ATPase encoded by HP0472; an arginase (HP1399) that, by analogy to other bacterial systems, is involved in urea cycle regulation; and an iron ABC transporter (HP1562). Other acid-induced genes had no homology to known protein, or had not been previously associated with acid-stress response. Two of the four acid-repressed genes encoded hypothetical proteins. The other two, HP1271 and HP1272, specify the NADII-ubiquinone oxidoreductase component of a multiprotein proton pump. The comparison of the results of these two studies showed that both the numbers of genes found to be differentially regulated in acidic vs. neutral conditions and the identity of these genes varied considerably. The lack of agreement between these studies emphasizes both the complexity of *H. pylori*'s response to acid and the difficulty in comparing single time point experiments for the assessment of global transcription.

Björkholm et al.[8] described one approach for identifying microbial genes that affect the magnitude of host response to infection. Fifteen strains were first phenotyped based on their ability to produce adhesins that recognize two classes of human gastric epithelial receptors. Thirteen binding strains and two non-binding controls were then subjected to whole genome genotyping using *H. pylori* DNA microarrays. Genetically defined and environmentally controlled gnotobiotic transgenic mouse models were used to correlate changes in the expression of selected biomarkers (Hsp70, pIgR, IL-1β) associated with host defenses with the results of whole genome bacterial genotyping. These correlations provide evidence that members of both type I and type II R-M (restriction-modification) systems are linked to a more robust host response. They found that the presence of HP1352, a type II R-M DNA adenine methylase, is associated with induction of a more robust response in the parietal cell-deficient, gnotobiotic transgenic mouse model of chronic atrophic gastritis. A direct correlation between the presence of several *hsdS* genes of type I R-M systems and the intensity of the host (Hsp70) response was also found. These findings raise the possibility that the DNA sequence specificity of HsdS homologues encoded by HP0462, HP1404, HP1383, HP0790 (and/or the colonization associated JHP0726) may be "exploited" by the bacteria to modulate expression of genes that directly or indirectly affect virulence potential.

The application of methods of global analysis of gene expression (microarray studies) resulted in the explosion of information on the molecular pathways activated by *H. pylori* in gastric epithelial cells. New pathways that may play an important role in *H. pylori* carcinogenesis have been discovered in several studies.

Guillemin et al.[19] characterized host cell responses to *H. pylori* infection and identified responses specific to individual virulence determinants. They used human cDNA microarrays to monitor the relative abundance of gastric epithelial cell transcripts over time during coculture with wild type and a panel of isogenic *H. pylori* strains with mutations in *cagN*, *cagA*, *cagE*, or a deletion of the entire *cag PAI*. Infection with the wild type strain induced expression of genes involved in the innate immune response, cell shape regulation, and signal

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transduction. A mutant lacking the *cagA* gene, which encodes an effector molecule secreted by the type IV secretion system (TFSS) and required for the host cell cytoskeletal response, induced the expression of fewer cytoskeletal genes. A mutant lacking *cagE,* which encodes a structural component of the TFSS, failed to up-regulate a superset of host genes, including the *cagA*-dependent genes, and many of the immune response genes and induced transient expression of certain stress and possible adhesion genes, suggesting that the *cagE*$^-$ mutant can achieve a more intimate and proinflammatory interaction with host cells. A mutant lacking the entire *cag PAI* failed to induce both the *cagE*-dependent genes and several transiently expressed *cagE* independent genes. Host cell transcriptional profiling of infection with isogenic strains of *H. pylori* demonstrates that host cells sense and respond to even subtle genetic differences in the bacteria they encounter. Their analysis revealed a subset of the host cell response that depended on the TFSS effector CagA that was enriched for genes encoding regulators of cell shape. Temporal profiling uncovered subtleties in the kinetics of the host response to *H. pylori* virulence determinants, including the late *PAI*-dependent induction of cholesterol biosynthesis genes and the late *cagA*-dependent repression of cell proliferation genes. Statistical analysis revealed consistent low-level differences in the host response to different *H. pylori* mutants, including the *cagA* associate up-regulation of genes encoding cell junction proteins. The analysis also provide specific candidate molecules, such as Lck and Cdc42 effector protein 2, that can be tested for involvement in general classes of CagA-mediated host response, such as CagA signal transduction and small GTPase regulation of the actin cytoskeleton. Host cell transcriptional profiling as a method to dissect the differences between mutant and wild-type bacteria thus provides information about both the nature of bacterial virulence factors and host cell processes in the normal and diseased state.

Sepulveda et al.[40] described the transcriptional profile of the AGS cells induced by infection with a *cag+ H. pylori* strain using an arrangement of 6000 human U95A genes (Affymetrix). They found that approximately 200 genes showed marked changes such as the over regulation of the transcription factors AP-1, c-jun, jun-B, c-fos and cyclin D1, and the induction of the serine theonine-kinase pim-1 and ATF3.

Maeda et al.[31] utilized a high-density cDNA macroarray to characterize the gene expression profile in MKN45 gastric cancer cells and AGS cocultured with a *cag* pathogenicity island (PAI)-positive *H. pylori* strain. The authors found overexpression of 8 of the 2304 genes that were tested, including IL-8, IkBα, A20, ERF-1, keratin K7 and glutathione peroxidase.

Chiou et al.[15] used a microarray of 588 genes on a nylon membrane and identified the overexpression of 21 genes and the suppression of another 17 genes by AGS cells incubated together with *H. pylori*; the majority of these genes were transcription factors such as *c-jun, BTEB2* and *ETR101*. Other genes were involved in transduction signaling such as *MAP* kinases, IL-5 and development factor similar to insulin, with other genes involved in cell cycle regulation and differentiation such as *CDC25B* and *NM23-H2*.[45]

Cox et al.[14] used a high-density cDNA array with 58000 genes to study the changes in gene expression of gastric epithelial cells, Kato 3, induced by a *cag+ H. pylori* strain; they identified an alteration in expression of 208 known genes, 48 novel genes and/or expressed sequence tags of unknown function. Amongst the identified genes were genes that code for development factors, cytokines and chemokines, as well as for their receptors, for proteins involved in apoptosis, in transcription factors, in proteins of the ADAMs family and metalloproteins, and for proteins of the external membrane, to mention just a few. This same team of researchers confirmed the gastric differential expression of amphiregulin and ADAM10 *in vivo* and also a novel gene HPYR1 in patients infected with *H. pylori*.

Hippo et al.[23] carried out a study using a high-density microarray with oligonucleotides formed from 6800 human genes in which they analyzed gene expression in gastric tissue of patients with and without gastric cancer. In the gastric tissue of cancer patients, they identified overexpression of a group of genes involved in: the cell cycle, development factors, cell motility, cell adhesion and remodelling genes of the matrix. In the gastric tissue of non-cancer patients, they identified the overexpression of genes related to specific gastrointestinal functions and the immune response. Furthermore, various genes associated with metastases to lymphatic nodules were also identified, such as *Oct-2RBP4* and IGF2, as were genes associated with the histological type of gastric cancer, such as *E-cadherine* and *K-sam* in the case of diffused cancer, and β-*katenine* and *c-erbB2* in intestinal cancer.

Other researchers have demonstrated that *H. pylori* infection affects the proliferation/apoptosis balance of gastric epithelial cells with a global increase in cellular turnover, which in turn shows the capacity of *H. pylori* to induce the transcription of cyclin D1, one of the cell cycle regulators of AGS cells.[24]

Meanwhile, other studies show the important participation of genes that make up the *H. pylori cag* island in the transcription of cyclin D1 through an activating cascade that involves induction of MAP kinase activity and that NFκB activation was not required for cyclin D1 activation by *H. pylori* in AGS cells.[24]

Suganuma et al.[41] described the effects of the *H. pylori* membrane protein-1 (MP1) and demonstrated that BAL/3T3 cells transfected with a MP1 expression vector displayed over expression of TNF-α and produced 100% tu-

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mors in nude mice. The suggestion is that this could represent one of the cellular mechanisms involved in the development of gastric cancer.

It is interesting that in cocultures of MKN45 gastric cancer cells with *H. pylori*, Kirikoshi et al.[29] demonstrated that *H. pylori* induces the overregulation of WNT10A, an effect that could be mediated by TNF-α. This could indicate that the induction of WNT10A by TNF-α and *H. pylori* plays a role in the production of human gastric cancer through the activation of the WNT-β-catenin-TCF signaling pathway. Mitsuno et al.,[33] with this same cell line, reported that transactivation of SRE and AP-1 occurs through activation of ERK/MAP and JNK/SAPK pathways.

Another molecular system that could play a role in the pathogenesis of *H. pylori* associated gastric cancer is seen in the changes in the molecular levels of COX-2. The cyclooxygenase (COX)-2 is induced by *H. pylori* in the gastric mucosa. Wambura et al.[44] evaluated the expression of COX-2, cell proliferation and apoptosis indices in gastric biopsies from 160 patients with non-ulcerative dyspepsia or gastric cancer. They found that COX-2 expression in the gastric corpus was significantly higher in *H. pylori* positive patients when compared with *H. pylori* negative patients or those with non-ulcerative dyspepsia. It has been suggested that COX-2 expression is associated with disruption of epithelial cell kinetics in the gastric mucosa.

All of these papers show important results in the study of the global gene expression, as much in *H. pylori* as in gastric epithelial cell lines. However, there are limitations to these results in that the studies looked at the genetic expression of a limited number of *H. pylori* strains with well determined genotypic and phenotypic characteristics, under controlled *in vitro* experimental conditions, without taking into account the great genetic diversity of the microorganism and the fact that bacterial gene expression is modulated by the interaction with the host and the genetic response made by the host to the infection.

The technology of cDNA microarray is a powerful tool for analyzing the expression profile of thousands of genes and could be used in studying various biological systems. The availability and utilization of sequences logged in the data base of the two completely sequenced genomes of *H. pylori*, as well as genes from the human genome project, could allow the design of microarrays that will enable *in vivo* studies of bacterial gene expression during the course of chronic infection of the gastric mucosa. Similarly, such microarrays could be used to identify other virulence genes that participate in the development of different pathologic disorders. Also, it will be feasible to study the alteration in gene expression of host cells *in vivo* when pathologic changes exist that give rise to illnesses such as peptic ulcers and gastric cancer.

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