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The *Helicobacter pylori* HrcA repressor: Study of the Global Transcriptional Response during Heat Shock.

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1. INTRODUCTION

1.1 *Helicobacter pylori* biology

First isolated in 1982, Marshall and Warren (Backert & Blaser, 2016; Warren & Marshall, 1983) described a spiral or curved bacterium from 58 of 100 consecutive histological biopsies of human gastric antral mucosa. This organism demonstrated several similarities to *Campylobacter* and was then referred to and validated in 1985 as *Campylobacter pyloridis*. In 1987 the specific epithet was revised for *Campylobacter pylori* to properly name the latin genus of the pylorus noun.

Only in 1989, given the differences from *Campylobacter* in many molecular and biochemical aspects as the flagella morphology, the content of fatty acids and the sequence of the 16S rRNA, it was named *Helicobacter pylori*.

The etymology of the name *Helicobacter pylori* derives from: helix = spiral; bacterial; pylorus = lower stomach, and currently is described as a Gram-negative bacterium, microaerophilic, spiral or curved shaped and multi-flagellated.

In vitro, *Helicobacter* species grow quite slowly, normally at 37°C and only under microaerobic conditions with 10% CO_2 and rich media supplemented with 5% whole blood or serum.

The natural habitats of this bacterium are the deeper extracts of the gastric lining epithelium, just above the carpet of parietal cells and the successful colonization in this hostile and acid environment is achieved through a combination of several virulence factors.

1.2 Pathogenesis

It is estimated that more than half of the world's population is colonized by *Helicobacter pylori*. The selection of a no-competition habitat with a very acidic pH, which is lethal to most microorganisms, makes *H. pylori* one of the most successful human pathogens, recognized as the main causative agent of chronic gastritis, gastric and peptic ulcers, and persistent colonization is an important factor in the development of gastric lymphoma and adenocarcinoma.

The mechanisms by which the bacterium causes various diseases with different intensities are not fully established. It is believed that the combination of virulence factors expressed by the bacterium with the environment of the host may justify these various clinical evolutions.

For successful colonization, the first and most important step is to survive in the gastric environment. This is achieved through the expression of a large amount of urease enzyme that converts urea into ammonia and bicarbonate. The ammonia protects *H. pylori* against the acidic microenvironment and causes damage to the gastric epithelium (Ansari & Yamaoka, 2017). This enzyme is also responsible for the stimulation of mononuclear phagocyte activation, inflammatory cytokine production and causes toxic effects on gastric epithelial cells (Debowsky, 2017). Therefore, the presence of urea allows *H. pylori* to create a neutral layer around its surface and through adhesion molecules that binds to specific receptors begins the colonization of the gastric mucosa.

Due to its shape and motility caused by the presence of flagella, this microorganism is able to cross the mucus layer of the stomach until it reaches the epithelium, whose degeneration causes the induction of inflammatory infiltration, consisting of leukocytes, neutrophils, lymphocytes and plasma cells. During this process, other enzymes synthesized by the bacterium, such as superoxide dismutase, catalase and arginase confer protection against the lytic activity of macrophages and neutrophils, prevent an efficient response of the host, promoting stimulation of the production and secretion of proinflammatory cytokines by epithelial cells. *Helicobacter pylori* induces humoral and cellular immune responses, promoting an inflammatory reaction that recruits both polymorphonuclear and mononuclear cells with an increased level of pro-inflammatory cytokines such as IL-1β, tumor necrosis factor alpha (TNF-α), IL-8, and IL-6 (Wilson & Crabtree, 2007).

Adherence factors to the gastric epithelium are important for the pathogenicity of *H. pylori* and favor its colonization. In this context, a series of proteins called adhesins interact with receptors present in the gastric epithelial cells and allow the pathogen to anchor itself in a stable way. Among some adhesins are: 1) the neutrophil-activating protein, an HP-NAP gene product that induces neutrophil adhesion to endothelial cells and stimulates the production of reactive oxygen and nitrogen species by neutrophils (Satin *et al*., 2000); 2) *babA*, a blood group antigen adhesin adhesion factor linked to the Lewis blood group that plays a critical role, facilitating the liberation of other bacterial virulence factors and damaging the host tissue (Yamaoka, 2008); 3) the *H. pylori* catalase, a product of the *katA* gene, is related to resistance to oxidative stress, facilitating the

survival of this bacterium, mainly in the presence of hydrogen peroxide. The *katA* knock-out mutant strain is less able to sustain the long-term infection (Harris *et al*., 2003); and 4) recently, the *hopQ* gene has been shown to exploit human carcinoembryonic antigen-related adhesion proteins (CEACAM). CEACAM proteins mediate intercellular adhesion and the external cell signaling events: bacterial proteins that interact with CEACAMs have been related to the inhibition of host immune response activation (Moonens *et al*, 2018).

This bacterium is also associated with the inhibition of the secretory response of mucus cells through the decrease of somatostatin release and consequent increase of gastrin released by the gastric antrum, causing detrimental effect on the primary defense mechanism of the gastric mucosa. In addition, gastric tissue injury is mediated not only by the effects of urease but also by the expression of several genes responsible for the production of cytokines and virulence factors. The ArsS-ArsR two-component system regulates gene expression in response to low-pH and has also been associate to biofilm production (Servetas *et al*., 2016).

The vacuolating cytotoxin, encoded by the *vacA* chromosomal gene, damages the epithelium causing vacuolization in epithelial cells.

After binding to the cell surface, the vacuolating cytotoxin is internalized and forms selective channels for anions in the membranes of endocytic compartments. To compensate the increase in intraluminal chloride, the activity of the vacuolar ATPase is intensified, resulting in an increase in proton pumping and a consequent reduction in pH. Membrane-permeated weak bases, such as ammonia, diffuse into late endocytic compartments and become protonated and trapped in these compartments. The osmotic swelling of these compartments results in cell vacuolization. The overall effects of this toxin on gastric epithelial cells include changes in mitochondrial membrane permeability and apoptosis, stimulation of proinflammatory signaling, as well as the increase of plasma membrane permeability and alterations in the endocytic compartments (Cover and Blake, 2005; Necchi *et al*., 2017).

Another important pathogenic factor of *H. pylori* is the cytotoxin-associated gene A, encoded by the *cagA* gene, mapping in a 38 kb multi-operon locus coding for 28 putative ORFs, called *cag* pathogenicity island (*cag*-PAI), likely acquired by this bacterium from another organism, as the GC content of this island differs

from that observed in the rest of the genome. The cag-PAI also encodes a type IV secretion system (T4SS) that directly injects CagA into host cells. Once inside the host cell, CagA is phosphorylated by host cell kinases and alters multiple host signaling pathways. To induce the production of interleukin (IL)-8 in the epithelial gastric cells, *H. pylori* depends on both mechanisms, the T4SS- and CagA expression. Infection with *cagA*-positive strains increases the risk of gastric cancer by at least one order of magnitude with respect to infection with *cagA*-negative strains (Boonyanugomol *et al*., 2018; Jang *et al*., 2016)

The set of mechanisms that *H. pylori* uses for colonization and infection of the gastric mucosa are illustrated in Figure 1.

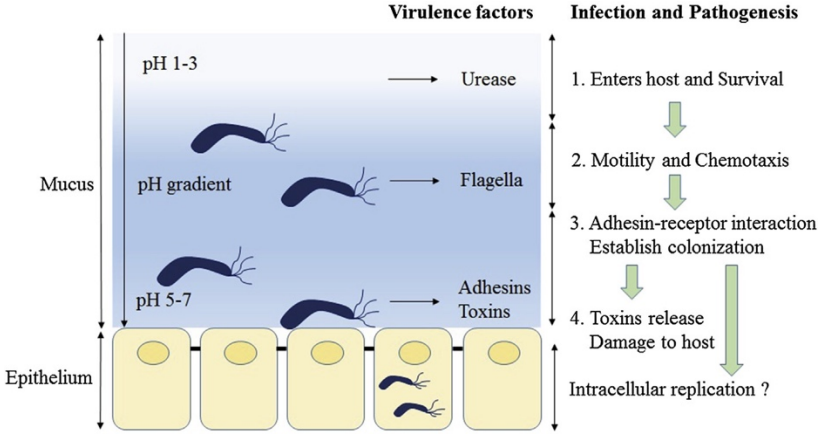

Figure 1. Pathogenesis Model of Helicobacter pylori. The production of urease by *H. pylori* neutralizes the gastric pH, adjusting the medium for colonization and infection by the bacterial cells. The adhesion of the bacteria to the gastric epithelium is mediated by adhesins, allowing the release of the other virulence factors stimulating strong host immune response and inflammation of the gastric mucosa (Kao et al., Biomedical Journal 39 (2016) 14-23. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

H. pylori infections can be successfully cured with antibiotic treatment, associated with a proton pump inhibitor (Megraud and Lamouliatte, 2003). Unfortunately, the available antimicrobial therapies are beginning to lose efficacy, principally because of insurgence of antibiotic resistance. Altered expression of gene products sensitive to antibiotic treatment seems to be especially important for resistance to penicillin, nitroimidazoles and clarithromycin. In 2017 WHO (World Health Organization) classified *Helicobacter pylori* as a high priority pathogen for the production and development of new antibiotics and to explore and identify both bacterial and host markers to diagnose high-risk individuals during severe

infection outcomes, as well as to develop new effective therapeutic strategies. For these reasons, *H. pylori* is a bacterial pathogen of major medical importance.

1.3 The *Helicobacter pylori* Genome

The genomic sequencing of multiple *H. pylori* isolates demonstrated great variation in the sequence and gene content among the analyzed strains, whereas the general genomic organization and the predicted genetic products were quite similar (Alm *et al*., 1999).

The *H. pylori* G27 strain, isolated for the first time at Grosseto Hospital (Tuscany, Italy) from an endoscopic patient has been widely used in research.

The G27 genome is a single circular 1.6 kb chromosome AT rich (61,1%) and contains 1,515 open reading frames (ORFs) and strong similarity to other *H. pylori* genomes of strains 26695, J99 and HPAG with respect to size and composition. G27 contains 58 genes that are not found in 26695, J99 or HPAG and most of these G27-specific genes are predicted to encode hypothetical proteins (Baltrus *et. al*, 2009).

This bacterium has an interesting peculiarity that is the scarcity of transcriptional factors and predicted regulatory proteins, where genomic analyses have identified only 32 gene products of possible regulatory function in which only 17 are predicted to have a role in transcriptional regulation, that is, approximately half of the number of those reported for *H. influenzae*, which has a genome of comparable size to *H. pylori* and less than a quarter of those predicted for *E. coli* (Tomb *et al*., 1997).

However, the small number of transcriptional regulators encoded by *H. pylori* correlates with a small genome size. It has been speculated that the reduction in the number of transcription factors occurred for the absence of selective pressure associated with a restricted and competition-free habitat (Danielli *et al*.,2010; de Reuse and Bereswill, 2007).

The transcriptional machinery of *H. pylori* is similar to the one observed in many other organisms, such as *E. coli*, but with some substantial differences. For instance, the vegetative sigma factor of *H. pylori* σ^{80}, RpoD, of the RNA polymerase enzyme has several differences with respect the σ^{70} subunit of *E. coli* (32% identity, 51% similarity) (Borin BN et al. 2014). Also, *H. pylori* has only

two alternative sigma factors, named σ^{54} (RpoN) and σ^{28} (FliA), and both are involved in controlling the expression of the flagella components (Kao *et al*., 2016). Moreover, mechanisms of post-transcriptional regulation have been described in *H. pylori*, through antisense transcripts and the expression of at least 60 small RNAs, as well as through RNA-binding proteins that modulate mRNA stability and translation efficiency (Sharma *et al*., 2010).

As in other bacteria, coordinated genomic expression is controlled by the transcription regulatory network (TRN), which is commonly organized in hierarchical multilayer structures and composed of regulatory modules (origins) that include the combined activity of transcription factors (TFs) to regulate related physiological functions, so they act as an input signal for the bacteria to respond to changes in the environment (Balazsi and Oltvai, 2005; Danielli *et al*., 2010).

It has been proposed that *H. pylori* adopts a multilayer TRN of low hierarchy, encompassing its four main origons (motility and chemotaxis, metal and ion homeostasis and heat-shock response), in an unsegregated way of its few transcriptional regulators. Thus, different environmental inputs are interpreted by different combinations of small sets of transcription factors or associated proteins. In this microorganism, the TRN is built unequivocally to maintain homeostasis, and colonization and survival in the gastric niche depends on the concerted expression of virulence factors and housekeeping genes.

To initiate the colonization process in the host organism, the *H. pylori* flagella, chemotaxis and motor protein are crucial factors in establishing a successful infection. Their deletion leads to strains with an attenuated or completely defective ability to establish colonization in animal models (Eaton *et al*., 1996; Foynes *et al*., 1999; Josenhans and Suerbaum, 2002).

H. pylori cells usually have a unipolar bundle of two to six flagella with a sheath that allows bacteria to enter and move through the gastric mucosa. Each flagellum exhibits a typical structure as a bulb at its end, being an extension of the outer membrane and has protective function for the flagellar structure at acidic pH of the stomach.

The flagella have three structural elements: a basal body, which is embedded in the cell wall and contains the proteins required for rotation and chemotaxis; an external helically shaped filament that works as a propeller when rotated at its base; and a hook that serves as a joint between the basal body and the flagellar

filament. In *H. pylori,* more than 40 predicted genes are scattered along the genome whose products are involved in the expression, secretion and assembly of this flagellar complex (Spohn & Scarlato, 2001). The flagellar regulatory system adopts a regulatory σ short cascade (σ^{80}> σ^{54}> σ^{28}) and a hierarchical regulation, initiated by the housekeeping σ^{80} factor, where each σ factor activates its dedicated target gene class. In addition, each one is also responsible for the transcription of the next factor in the cascade (Sharma *et al*., 2010).

As in other enterobacteria, flagellar genes in *H. pylori* are positively regulated and organized hierarchically into three classes according to their activating sigma factor: 1) class I, which includes the gene targets transcribed by the RNA polymerase containing the σ^{80} factor and comprises mainly flagellar regulatory genes encoding proteins that form flagellar base structures (Kavermann *et al*., 2003); 2) class II includes specific targets of the σ^{54} factor and encodes components of the basal body and the flagellar hook; finally, class III genes encode late flagellar structures, transcribed by the σ^{28} factor. Thus, the flagellar regulatory cascade of *H. pylori* is appropriate to guarantee the correct sequential expression of early, intermediate and late flagellar components.

Once established in the host, *H. pylori* cells need to respond rapidly and continuously to a severe acidic environment. Therefore, this microorganism adopts a set of acid acclimation genes, and the transcription of these genes is under the control of the housekeeping factor σ80 and regulated mainly by the essential acid response regulator ArsR. This regulator is part of an ArsS-ArsR two-component system and once acidification of the periplasm is detected by ArsS, the signal is transduced by changes in the protonation of various histidine residues in the extracytosolic sensory domain. This stimulus triggers the phosphorylation of ArsR, thus promoting its DNA binding activity towards a specific set of promoters (Danielli *et al*., 2010).

The absorption of metal ions is another crucial aspect for the maintenance of *H. pylori* infection and also for its virulence, since these are important enzymatic cofactors. For example, nickel is a virulence determinant because it is a cofactor of the urease enzyme in the reaction catalyzing the hydrolysis of urea into carbon dioxide and ammonia, which is essential for *in vivo* colonization (Hu *et al*., 2017). However, if present in large intracellular amounts the metal ions are toxic, and their homeostasis must be tightly controlled. *H. pylori* has three systems

dedicated to this fundamental task: the two-component CrdRS system that appears to be involved in copper resistance and apparently is also a sensor for nitrosative stress, the ferric uptake regulator (Fur) involved in iron homeostasis and in detoxification and finally NikR, a homologue of *E. coli* responsive nickel regulator, typically associated with hydrogenase maturation and acidic survival (Pich *et al*., 2012; Hung *et al*., 2015)

1.4 Heat-shock Response

The ability to respond quickly to environmental changes and thus modulate gene expression is a crucial ability exploited by bacteria for their survival. In this context, the widespread human pathogen *H. pylori,* when in stress conditions, induces the synthesis of a class of highly conserved proteins, called Heat Shock Proteins (HSPs).

These proteins, expressed also under physiological growth conditions, accumulate upon a temperature increase and in response to different types of stress and are employed in assisting the correct folding, assembly, transport and degradation of cellular proteins. The ability to perceive and respond quickly to environmental changes is crucial for their survival during infection in a hostile environment such as the human stomach.

When growth conditions are altered, these proteins play a crucial role, acting against the accumulation of incorrectly folded proteins to avoid the formation of amorphous cytoplasmic aggregates, deleterious to the cell (Narberhaus, 1999). Accordingly, sophisticated regulatory circuits guarantee low expression levels of HSPs under physiological growth conditions and a strong and coordinated induction after the perception of various stimuli of stress. This regulatory circuit, therefore, perceives the signals coming from the environment and translate them into a well-defined gene expression pattern.

In bacteria, the transcriptional regulation of heat-shock genes can be regulated by positive or negative regulatory mechanisms. In systems whose transcriptional regulation is negative, as in *H. pylori*, transcription is controlled by dedicated repressors whose DNA binding activity changes in response to stress. Under normal growth conditions, these repressors bind to specific operators and repress the transcription of heat shock genes.

In *H. pylori* the genes coding for the major HSPs are clustered into three multicistronic operons transcribed by the RNA polymerase containing the vegetative sigma factor σ^{80} and their transcription is rapidly induced by heat shock and other stress stimuli (Spohn et al., 2002). Under physiological conditions these operons are repressed by two transcriptional regulators: HrcA and HspR, homologues to well-characterized heat-shock regulators found in many other bacterial species with medical relevance such as *Streptococcus pneumoniae* and *Clostridium difficile* (Jain *et al*., 2017; Chastanet *et al*., 2001). The HspR regulator alone represses the transcription of the *cbpA-hspR-helicase* operon, thereby auto-regulating its own synthesis. On the other hand, both the HspR and HrcA regulators are required to repress transcription of the heat shock *hrcA-grpE-dnaK* and *groES-groEL* operons and this model is represented in Figure 2. Specifically, the HspR and HrcA repressors combine to control the transcription of target genes in a way that the HrcA regulon results embedded within the HspR regulon (Roncarati & Scarlato, 2018).

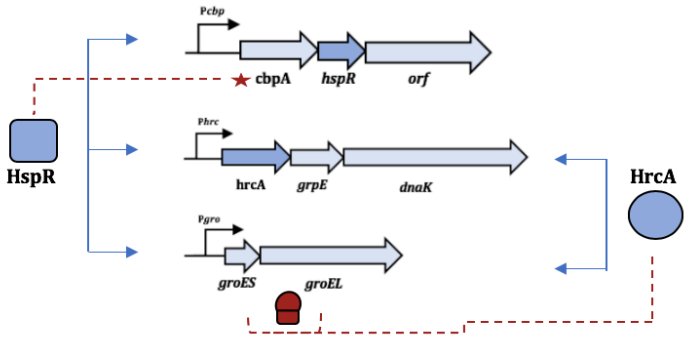

Figure 2. Regulation of H. pylori chaperone expression. Transcriptional repression of the heat shock operons is represented by solid lines that connect HrcA and HspR repressor proteins to their target promoters (P*gro*, P*hrc*, and P*cbp*); dashed lines, linking GroES-GroEL to HrcA and CbpA to HspR, represent the posttranscriptional protein-protein feedback control of the regulators.

While HrcA is widely distributed in the prokaryotic kingdom, HspR is found in a restricted number of bacteria. This latter is a homologue of the repressor that controls the *dnaK* operon of *Streptomyces coelicolor* and has been shown to bind to inverted repeats in the promoter region, designated HAIR (HspR-associated inverted repeat). In *H. pylori*, the HAIR sequence is localized in close proximity to the transcription start site of the *Pcbp* promoter (-59 to +14 on *Pcbp*), while in the *Pgro* and *Phrc* promoters HspR binds far upstream of the core promoter regions,

-120 to -43 on *Pgro*, from -149 to -78 on *Phrc*, respectively, while HrcA binds to operators overlapping the core promoter regions (Roncarati & Scarlato, 2017).

HrcA is a homologue of the repressor of a set of heat shock genes of *Bacillus subtilis* that binds to an inverted repeat in the promoter region designated CIRCE (controlling inverted repeat of chaperone expression) with a consensus motif (TTAGC ACTC-N9-GAGTGCTAA) (Narberhaus and Bahl, 1992). In *H. pylori*, the CIRCE sequence is localized near the transcription start site of the P*gro* and P*hrc* promoters, ranging from -13 to +16 on P*gro* and -59 to -34 on P*hrc* (Roncarati *et al*., 2017).

The presence of both regulators is, therefore, necessary for maintaining P_{gro} and P_{hrc} in the repressed state. Although the binding sites of HrcA and HspR are very close to each other, *in vitro* studies have demonstrated that both bind in an independent manner to their operators, while the HrcA-mediated regulation depends on the presence of HspR. It is not yet known whether this stable complex is due to possible interactions between the repressors with other proteins, so they can form a stable complex capable of repression.

The transcriptional control described, is further assisted by an additional level of post-transcriptional modulation exerted by some HSPs contained in the same operon (GroELS and CbpA), which modulate the binding of HrcA and HspR repressors to DNA (Roncarati *et al*., 2007; Roncarati *et al*., 2011; Roncarati *et al*., 2014).

As in the proposed model for *B. subtilis*, the chaperonine GroE is able to positively influence the DNA binding affinity of HrcA. As illustrated in Figure 3, during physiological growth conditions, the GroE chaperonin establishes a feedback posttranslational regulatory circuit increasing HrcA DNA binding activity. In contrast, in a stress situation the accumulation of misfolded proteins in the cytoplasm competes with GroE causing a loss of DNA binding affinity for HrcA and consequent derepression of heat-shock promoters (Scarlato & Roncarati, 2018).

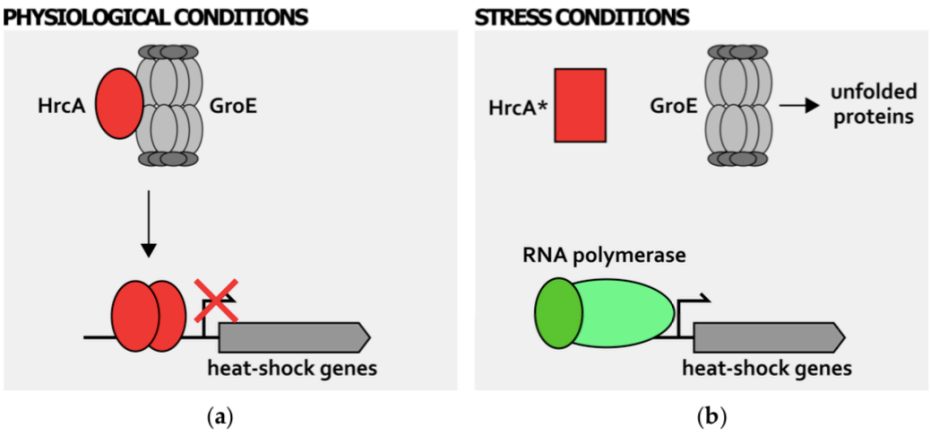

Figure 3. Posttranscriptional GroE-mediated regulation of HrcA activity. (a) During physiological growth conditions the GroE chaperonine (represented in grey) is free to interact and fold the HrcA regulator (red oval). Once properly folded this repressor binds and occupies its target promoters, repressing genes transcription. (b) When cells experience stress growth conditions, GroE is titrated by unfolded proteins that accumulate in the cytoplasm and cannot interact with HrcA; The repressor left alone has a conformation with low DNA-binding affinity (red rectangle) and HrcA-dependent heat-shock genes repression is relieved. The RNA polymerase containing the vegetative σ fator is represented by green ovals and Bent arrow indicates the transcription start site. (Roncarati & Scarlato. *Int. J. Mol. Sci.* **2018**, *19*, 1702. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

It has been demonstrated that HrcA is the direct thermosensor of *H. pylori*. In fact, the DNA binding activity of HrcA is modulated by temperature fluctuations over a very restricted physiological range of temperature (37°C - 42°C).

Under heat stress conditions, due to a major structural alteration, HrcA loses DNA binding activity and the transcription of the chaperone genes is rapidly derepressed. When the ambient temperature drops to physiological levels, GroE chaperonin is able to interact and refold at least a fraction of the heat-inactivated protein and thus, together with the newly synthesized HrcA the repressive state is re-established (Roncarati *et al*., 2014).

Thus, according to the central function of GroE chaperonin in modulating the HrcA binding activity to the DNA, *H. pylori* appears to be able to adjust its transcriptional regulation in response to the perception of various stress signals of different intensities. In particular, depending on the extent of a given stress stimulus, the availability of GroE to the functional interaction with HrcA will be differentiated, influencing restoration levels of heat shock gene repression.

Despite the detailed characterization of the heat shock regulatory circuit described, not enough is known about the global involvement of the heat-shock HrcA regulator in *H. pylori.* A global analysis of the heat-shock HrcA regulator would be highly informative, not only for the characterization of all the effectors

involved in protecting the bacterium against environmental stress, but also for the identification of new virulence factors triggered in response to stress signals perceived by the pathogen.

2. AIM OF THE STUDY

The HrcA protein is a well-known transcriptional regulator of *H. pylori* involved in heat shock response. Despite the detailed characterization of the heat shock regulatory circuit, not enough is known about the role of HrcA at global level in *H. pylori*. In analogy to other bacterial systems most commonly studied, it is expected that exposure of *H. pylori* to heat shock conditions causes an altered expression (increased but also decreased expression) of many proteins. The aim of this project is to carry out an integrated study that will answer a series of questions related to heat shock and stress response in *H. pylori*.

To achieve this goal, we performed a global transcriptome analysis by RNA-sequencing to identify differentially regulated genes by HrcA and, thus, reveal whether this protein is involved in the regulation of other cellular processes and interact with additional genes. Then the second part of this study was aimed to the identification of HrcA binding sites *in vivo* through a genome-wide approach, using Chromatin Immunoprecipitation followed by deep sequencing (ChIP-seq), to assess whether HrcA interacts with other genes to control their expression.

3. HrcA transcriptional response by RNA-seq

3.1 Specific Introduction

In *H. pylori* the heat shock regulon is tightly modulated by the concerted action of two transcriptional repressors, HspR and HrcA. This latter protein negatively regulates transcription from P*hrc* and P*gro* promoters. To study the global heat shock response in *H. pylori* and to identify potential new genes regulated by HrcA, we performed differential transcriptional analysis of heat shock regulation by RNA-seq, comparing the transcriptome of the G27 (hrcA::km) mutant and the parental G27 wild type strain not exposed to heat shock (t0) and at 30 min (t1) after temperature upshift of the culture to 42°C, using total RNA extracts from mid-exponentially growing cultures (OD600= 0.7-0.8), and results have been validated by qRT-PCR on a selection of targets.

3.2 Results

To gain insights into the *H. pylori* heat-shock response and the global role of the HrcA regulator, we performed a transcript profiling experiment using RNA-seq, comparing the transcriptome of the G27 wild type strain and the G27 *(hrcA::km*) mutant isolated from exponential growth phase at 37°C and also from the wild type strain after temperature increase of the culture to 42 °C for 30 min (t1).

This analysis showed that a total of 137 genes were differentially expressed (log2 fold change >1 or <-1) by heat shock and *hrcA* mutation, representing more than 9% of all the *H. pylori* annotated ORFs **(Figure 4 and Supplementary Table 1 and Table 2).** After temperature upshift to 42°C, the heat-shock treatment triggered changes in the transcript levels of 89 of 137 (64,97%) differentially expressed genes (log2FC > 1 *p*adj < 0.01) when compared to the wild type sample not subjected to heat-shock. Of these, 54 genes appeared up-regulated and 35 genes appeared down regulated. As expected, among the up-regulated genes we found the previously characterized heat shock responsive *groES, groEL, grpE, dnaK,* and *cbpA* genes.

In addition, each differentially expressed gene was characterized by a specific category of COG (Cluster of Orthologous Group), a database designed to classify proteins from sequenced genomes based on the orthology concept. From the functional annotation and enrichment analysis, we found that 55% of the up-regulated genes are included in the Unknown Function category. Several up-

regulated genes are involved in “Transcription” and “Post-translational modification, protein turnover, and chaperones and Transcription”. Among the down-regulated genes, also in this case the most of the differentially expressed genes are included in the Unknown Function category but many genes are related with “Cell wall/membrane/envelope biogenesis” and “Replication, recombination and repair” (Figure 4A).

The role of HrcA was established by comparing the transcriptome of the ΔHrcA mutant to that of the *H. pylori* wild type strain, both grown at 37°C (ΔHrcA _vs_WT). Intriguingly, we found several HrcA regulatory targets that are not responsive to heat stress.

This analysis showed a total of 55 deregulated genes (log2FC > |1| *p*adj < 0.01) in the *hrcA* gene deleted condition. Of these, 48 genes were deregulated only in ΔHrcA mutation and not influenced by heat stress response, corresponding to 35% of the differentially expressed genes detected in this study. Among these genes, 33 were down-regulated and 15 were up-regulated (**Figure 4** and **Supplementary Table 1 and Table 2**). Surprisingly, this repressor apparently controls the transcription of several genes, which do not seem to be affected by the heat stress.

The putative HrcA regulon, evidenced by this analysis, is made up of genes coding for proteins involved in several processes. As expected, among the up-regulated genes we found the *groES and groEL* genes (Roncarati *et al*., 2007), which is associated to “Post-translational modification, protein turnover, chaperones” functional enrichment (*p*adj = 0.01). As expected, transcription of the *grpE* and *dnaK* genes (HPG27_RS00570 – HPG27_RS00575) was unchanged in the HrcA mutant, likely due to a polar effect of the first gene of the operon, *hrcA*.

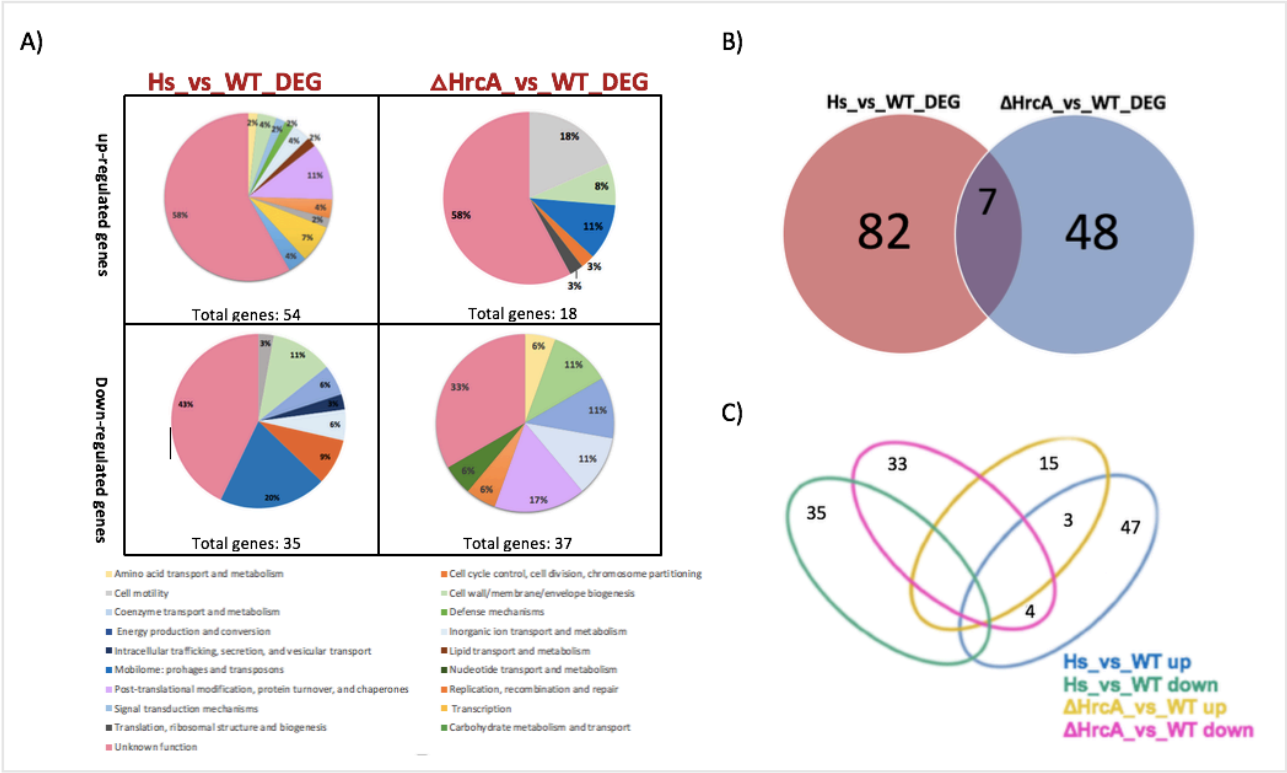

Figure 4. Differential transcriptional study between HrcA and heat-shock response. **(A)** Pie charts showing COGs functional annotation of the differentially expressed genes outlined in the ΔHrcA_vs_WT and HS_vs_WT (Heat-Shock vs. Wilde Type) comparisons, respectively, subdivided into up-regulated (left) and down-regulated (right) groups. The abundance of each category is indicated as a percentage as well as the total number of genes included in each group. **(B)** Venn diagram illustrating the relation between the deregulated expressed genes in the two set of experiments: heat shock (Hs_vs_WT) depicted by red set, ΔhrcA (ΔHrcA_vs_WT) depicted by blue set. **(C)** Venn diagram showing the number of genes in common or peculiar in the four previously described gene groups: Hs_vs_WT_ up, Hs_vs_WT_ down, HrcA_vs_WT_up and HrcA_vs_WT_ down, represented in blue, green, yellow and pink respectively.

In addition to these genes, already known to be the target of HrcA regulation, we noticed some functional groups of particular interest: among the up-regulated genes we found some genes (as the *rfaJ* and HPG27_RS03015 genes) related to "Cell wall/membrane/envelope biogenesis", Coenzyme transport and metabolism or the *dppB* gene, which is part of a multicistronic operon (predicted by DOOR, Database of prOkaryotic OpeRons) related to "Inorganic ion transport and metabolism".

Interestingly, 18% of the down-regulated genes are included in "Cell Motility" category as for example the *flaA*, *flaB*, *flgL*, *fliS*, *flgE*, *fliK* and *fliW* genes. Additionally, many genes not yet included by COG in cell motility and annotated as "Unknown function" are likely to encode proteins for flagellar structure and biosynthesis, such as the *fliD*, *fliT* and *flgK* genes that are detected as down-regulated genes by the HrcA mutant in this analysis.

Several genes involved in the “Cell wall/membrane/envelope biogenesis” and transposase coding genes, classified in the “Mobilome: prophages, transposons” category enrichment were also down-regulated.

Comparison of the transcriptome of the heat-shock response (89 deregulated genes) to that of the Δ*HrcA* mutant (55 deregulated genes) led to the identification of 7 genes that were deregulated in both datasets (**Figures 4-B**). Among these genes, 4 were oppositely regulated (down-regulated in the Δ*HrcA* mutant and up-regulated in heat-shock response) and 3 were similarly up-regulated (**Figure 4C**). The similarly up-regulated genes included the *groES* and *groEL* genes, which are already known to be directly repressed by HrcA and induced by heat- shock (Spohn and Scarlato, 1999). In addition, the HPG27_RS00625 gene coding for a hypothetical protein was up-regulated.

The remaining three genes were induced by heat-shock and repressed in the Δ*HrcA* strain, thus showing opposite behavior. These include HPG27_RS00565, coding for a hypothetical protein, (ykgB) HPG27_RS02740, coding for a membrane protein, and *fliD* (HPG27_RS03660) coding for a flagellar filament capping protein FliD. Since HrcA is a transcriptional repressor, the interaction with these genes probably occurs in an indirect way. Moreover, our transcriptome study shows that 15 genes belonging to the HrcA regulon (made up of 55 genes) are not responsive to heat-shock. In addition, our data suggest that HrcA could play a role in the biosynthesis and regulation of the flagellar apparatus of *H. pylori*.

To validate the results obtained by RNA-seq, the relative abundance of selected RNA transcripts of some selected genes was quantified by Real-Time PCR (qRT-PCR), using specific oligonucleotides for each region of interest listed in Table 3. For data analysis, fold-change ratios of ≥ 2.0 or ≤ 0,5 were considered as significantly up-regulated and down-regulated, respectively (Figure 5).

UP-REGULATED GENES by HrcA		
Genome ORF	FoldChange_dHRCA	Names
HPG27_RS00070	2,229200929	groEL,hspB,hsp60,mopA
HPG27_RS01500	2,11533277	dppB
HPG27_RS02700	2,158694858	HPG27_RS02700
HPG27_RS03015	5,47573209	HPG27_RS03015
HPG27_RS03495	2,389967774	hopO,omp16
HPG27_RS03940	2,395804082	fecA2,fecA,fecA_2
HPG27_RS05840	2,173383766	gluP
HPG27_RS06930	3,187719853	HPG27_RS06930
HPG27_RS06935	3,858696986	HPG27_RS06935
DOWN-REGULATED GENES by HrcA		
Genome ORF	FoldChange_dHRCA	Names
HPG27_RS00595	0,22454781	HPG27_RS00595
HPG27_RS00600	0,24176078	flaB
HPG27_RS01480	0,24455307	flgL
HPG27_RS01595	0,40777619	babA,omp28
HPG27_RS01870	0,25046842	HPG27_RS01870
HPG27_RS02255	0,25250314	horE,omp11
HPG27_RS02740	0,11950242	ykgB
HPG27_RS02925	0,35321119	flaA
HPG27_RS03665	0,4522008	fliS
HPG27_RS04255	0,24235607	flgE
HPG27_RS04430	0,25000736	fliK
HPG27_RS05560	0,23137699	flgK
HPG27_RS05740	0,48897857	fliw

Figure 5. Summary table of the selected up- and down-regulated genes revealed in our transcriptome analysis including the genome ORF name, the fold-change values and the name of the gene.

To validate the up-regulated genes in the Δ*HrcA* mutant condition, we selected 9 among the 18 genes. Surprisingly, only 2 genes, the HPG27_RS03015 and the known HrcA target *groEL* (HPG27_RS00070), were in agreement between RNA-seq data and qRT-PCR validation (Fig. 6A). With respect to the down-regulated genes, we selected 13 of the 37 of them and we find a good correlation between the results, in which 9 of the 13 selected genes demonstrated a very similar trend between qRT-PCR and RNA-seq, thus validating our data (Fig. 6B).

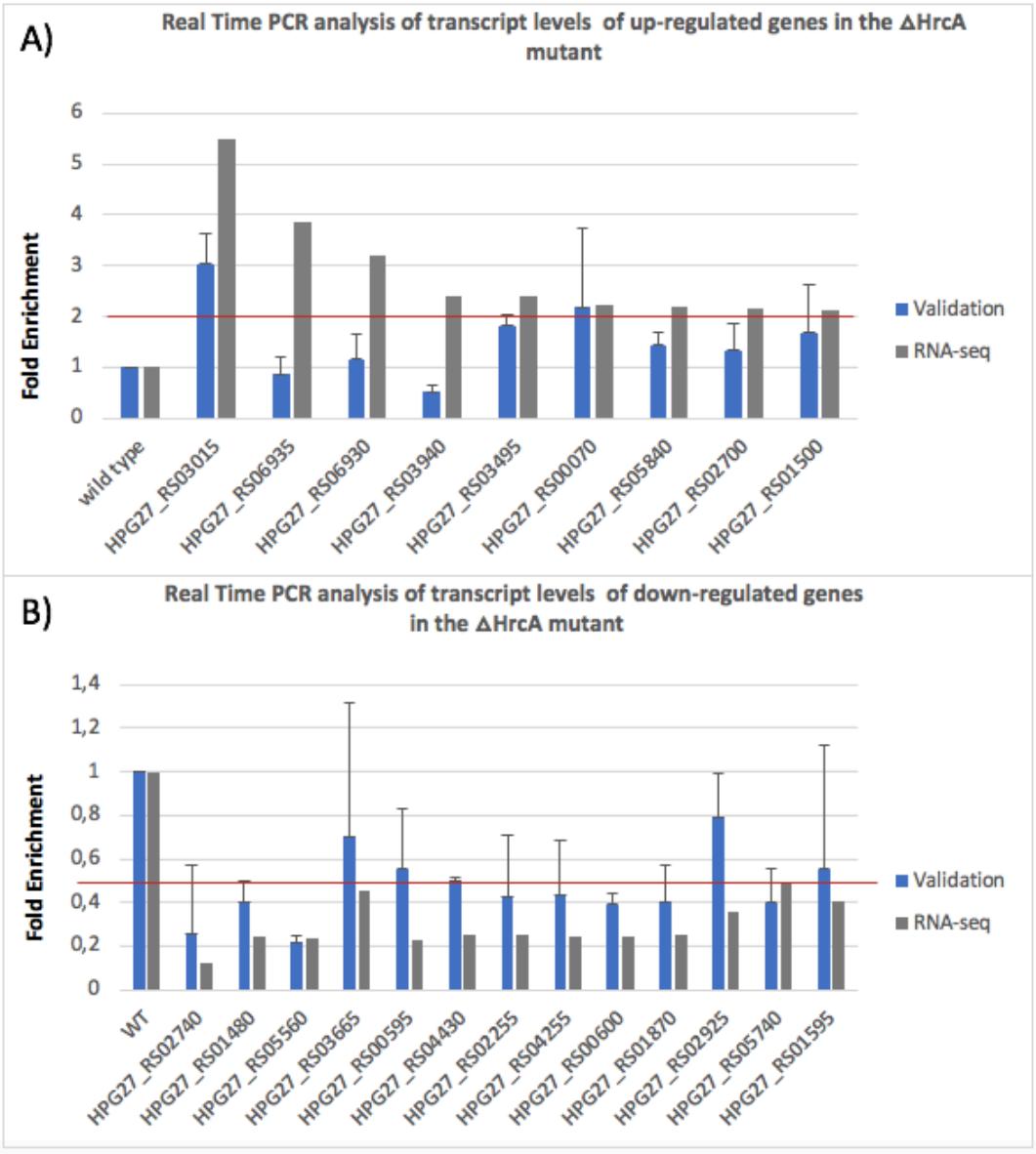

Figura 6. (A) Real Time PCR analysis of transcript levels of 9 selected up-regulated genes in the ΔhrcA mutant compared to the wild type not treated using specific primers for the selected genes (table 3). The blue bars indicate the transcript results of the validation and the light grey bars indicates the fold enrichment indicated in the RNA-seq outcomes. The red line indicates the established threshold value of the enrichment at these regions. **(B)** Real Time PCR analysis of transcript levels of 13 selected down-regulated genes in the ΔhrcA mutant compared to the wild type not treated using specific primers for the selected genes (table 3). The blue bars indicate the transcript results of the validation and the light grey bars indicates the fold enrichment indicated in the RNA-seq outcomes. The red line indicates the established threshold value of the enrichment at these regions.

3.3 Discussion

The Heat-shock response is a universal cellular strategy that allows cells to adapt to their environmental living circumstances and to survive during stress conditions. In *H. pylori*, the HrcA protein is a well-known transcriptional reressor involved in heat shock response, negatively regulating transcription from P*hrc* and P*gro* promoters.

In this study, through differential transcripts analysis of the heat shock response of wild type *H. pylori* and a HrcA isogenic (*hrcA::km*) mutant strain it was possible to verify distinct expression profiles between the heat shock response and the HrcA regulator.

Overall, this analysis highlighted that heat shock treatment of *H. pylori* determined the majority of differentially expressed genes (89 out of 137), while the HrcA mutant showed 48 deregulated genes (**Figure 4**). As expected, within the two sets are the genes encoded by three heat-shock multicistronic operons *groES-groEL*, *hrcA-grpE-dnaK* and *cbpA-hspR-rarA* that were up-regulated. Most of the up-regulated genes belong to the functional class “Post-translational modification, protein turnover, and chaperones”. Similar results were recently obtained by a study of the transcriptional response mediated by HspR (Pepe *et al*., 2018).

One of the most interesting findings that emerged in this study is the interconnection between HrcA and the motility of *H. pylori*, confirming a previous observation that a ΔhrcA mutant strain is non-motile (Roncarati *et al*., 2007). Indeed, several flagellar gene transcripts were downregulated in the G27 (*hrcA::km*) mutant strain and not responsive to heat-shock. Among the down-regulated genes are the *flaA*, *flaB*, *flgL*, *fliS*, *flgE*, *fliK* and *fliW* genes. Transcription of these motility related genesis under the control of the RNA polymerase-containing one of three sigma factors, σ^{80}, σ^{54}, and σ^{28} and code for proteins predicted to be involved predominantly to structural components of the flagella as the filament, hook and basal body. Moreover, interconnections between the heat shock repressors and motility have been observed in the closely related bacterium *Campylobacter jejuni* (Andersen *et al*., 2005). In *H. pylori,* motility is a crucial factor to establish a successful colonization and infection.

Recently, some genes known to regulate the flagellar apparatus, such as *rpoN* (σ^{54}) and *fliA* (σ^{28}), have been related to other important cell functions such as energy metabolism, oxidative stress and lipopolysaccharide synthesis (Sun *et al*., 2013; Baidya *et al*., 2015; De la Cruz *et al*., 2017). Furthermore, the study carried out by De la Cruz *et al*., (2017) reports that in the presence of antibiotics, some of these genes may have altered expression at the transcriptional level. In the same study, the *rpoN*, *fliA*, *flgR* and *crdR* genes were repressed in the presence of kanamycin, chloramphenicol and tetracycline.

Another recent study suggests that the *H. pylori* flagellar apparatus may play an important structural role during biofilm formation (Hathroubi *et al*., 2018). In this study, several genes encoding proteins of the flagellar apparatus as FlgB rod protein, the FlgE flagellar hook protein, the FlgK and FlgL hook-filament junction proteins, the FliK hook length control protein, FlaB and the putative flagellin encoded by FlaG appear up-regulated in presence of biofilm. Interestingly, in the same study, transcript levels showed that HrcA appears up-regulated in biofilm cells, showing a close connection between HrcA, motility and biofilm formation (Hathroubi *et al*., 2018). Similarly, correlation between motility and some flagellar genes were also verified in *E. coli* and *P. aeruginosa* (Domka *et al*., 2007; Sauer *et al*., 2002).

In the present study, comparison between the heat-shock response of the wild tye strain and the Δ*HrcA* mutant identified 7 genes that were deregulated in both transcriptomes (**Figure 4B**). Of these, only 3 were similarly up-regulated (**Figure 4C**), the *groES* and *groEL* genes, which are already known to be directly repressed by HrcA and induced by heat- shock (Spohn and Scarlato, 1999), and the HPG27_RS00625 gene coding for a hypothetical protein.

Among the 4 genes oppositely regulated that were induced by heat-shock and repressed in the Δ*HrcA* strain we found HPG27_RS00565, coding for a hypothetical protein, (*ykgB*) HPG27_RS02740, coding for a membrane protein, and *fliD* (HPG27_RS03660) coding for a flagellar filament capping protein FliD.

The scarcity of deregulated genes in both conditions (heat-shock response and mutant HrcA) strongly suggest that HrcA, independently of the heat-shock circuit, acts in several other cellular processes. Interestingly, a study comparing the transcriptome of the heat-shock response to HspR (Pepe *et al*., 2018) revealed

that 25 genes were deregulated in both datasets, a number approximately 3.5-times greater than our findings for HrcA.

To validate the RNA-seq results the relative abundance of RNA transcripts of selected genes were quantified by Real-Time PCR (qRT-PCR). Surprisingly, only 2 of 9 selected genes, the HPG27_RS03015 and the known HrcA target *groEL* (HPG27_RS00070) genes were in agreement with RNA-seq data(Fig. 6A).

Of note, most of the up-regulated transcripts had a fold-change very close to the established cut-off ratio (Fig. 5), and this may contribute to the observed low rate of validations. In addition, some studies indicate that the validation of results from RNA-seq may depend on the expression levels of the analyzed gene (Wang *et al*., 2014; Yeri *et al*. 2018). A high expression of a gene increases the probability of validation.

By contrast, among the down-regulated genes, 9 out of 13 have been validated by qRT-PCR and RNA-seq (Fig. 6B).

Globally, this study shows that the *H. pylori* HrcA regulon is involved in the regulation of several genes related to crucial cellular processes not responsive to heat-shock and connected to pathogenesis and virulence. In particular, HrcA appears to play a significant role in the biosynthesis and regulation of the flagellar apparatus of *H. pylori*.

4. In seach of new HrcA genomic targets

4.1 Specific Introduction

To better understand the interaction of HrcA with its putative targets and define its role in the regulation of the above identified genes, Chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) was set up. The ChIP-seq assay potentially allows the elucidation of transcriptional networks by measuring the binding of transcription factors throughout the genome at great resolution (Park, 2010). Although it is an excellent tool, the identification of genes directly regulated by a target gene is not simple.

This approach involves cross-linking of proteins to specific DNA elements, DNA fragmentation into small segments, followed by immunoprecipitation with specific antibody direct against the protein–DNA complexes and then, the DNA fragments are purified and used to generate libraries which are high-throughput sequenced (Figure 7).

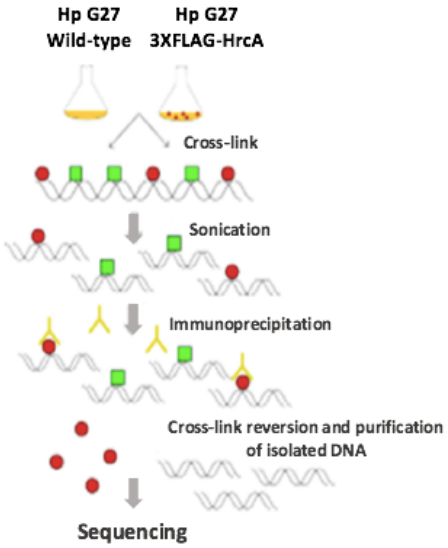

Figure 7. Schematic representation of the ChIP-Seq assay. ChIP-Seq is the most powerful method to investigate *in vivo* DNA-protein interactions in a genome-wide approach. In immunoprecipitation experiments bacterial cultures grow to a mid-exponential phase, protein-DNA complexes are cross-linked with formaldehyde and the genomic DNA is fragmented by sonication. Antibodies direct against the epitope tag are used to select the fusion protein cross-linked to DNA fragments that, upon recovery and purification, are analyzed by Real Time PCR before the sequencing.

Succinctly, the use of a specific antibody is an essential parameter in this technique. Variations in results can be due to differences in affinity and cross-

reactivity of the antibodies, and insufficient enrichment in the expected genomic regions may be masked by protein cross-linking or refolding (Soleimani *et al*., 2013).

Since the *H. pylori* HrcA protein appears to be poorly immunogenic and previous attempts to produce antibodies in mice resulted in a production of antiserum with very low affinity in antigen recognition, it is expected that the use of commercial antibodies will allow us to adopt the best strategy to obtain satisfactory results for the HrcA regulator with this technique.

To these aims, *H. pylori* strains able to express the fusion at the N-terminal of the HrcA protein to an epitope (3XFLAG) were generated and we have set up chromatin immunoprecipitation using α-FLAG antibody (ChIP).

4.2 Results

Transcriptomic analysis performed in this study showed that HrcA seems to be connected to the transcriptional control of other 48 genes involved in diverse cellular processes and not strictly associated to heat-shock. To detect genomic regions, bound *in vivo* by HrcA and possibly genes directly controlled by this protein, we performed a Chromatin Immunoprecipitation assays. To overcome the apparent scarce immunogenicity of HrcA and to allow the use of commercial antibodies, *H. pylori* strains expressing a tagged isoform of HrcA were generated. In circumstances where the use of direct specific antibodies is not available, the protein of interest coupled with a tag that can be recognized by commercial antibodies is an excellent alternative for immunoprecipitation experiments. The most commonly used tags include hemagglutinin (HA), Flag, Myc and V5. However, the efficiency of immunoprecipitation using these tags depends on the nature of the protein to which they were linked and the fusion location, if to amino- or carboxy-terminal regions (Kidder *et al*., 2011).

In the past, different strains of *H. pylori* expressing HrcA isoforms fused to HA, myc, T7 and V5 tags were tested in our laboratory. Results showed that a tag fused to the C-terminal domain of the regulator yielded a non-complementing protein. To obtain a strain expressing a functional fusion protein, in the present study the epitope tags were fused to the N-terminal domain of the protein, which

harbors the DNA-binding domain (Roncarati *et al*., 2007). Specifically, strains of *H. pylori* expressing the HrcA regulator fused to a FLAG, a custom FLAG-FLAG-FLAG, a commercially available 3XFLAG, and a 6XHis epitope at the N-terminal portion of the protein were generated. Functional analyses showed that all N-terminal tag-fused HrcA were complementing the mutant phenotype (data not shown).

The Tag-HrcA complementing strains were obtained by double homologous recombination by transforming the G27 (*hrcA::km*) mutant strain (**Table 1**, Materials and Methods) with the appropriated plasmids **(Table 2)**. The genes coding for the fusion proteins were reintroduced in the genome together with an antibiotic resistance cassette. The schematic representation of the 3XFLAG-HrcA expressing strains, which were selected for further studies, are reported in Figure 8. To find the optimal condition for the immunoprecipitation assays three different strategies aimed to three different expression levels were developed. The first strategy, a strain expressing the 3XFLAG-HrcA fusion protein from its original *locus* and under the transcriptional control of the *hrcA* promoter, P*hrc*, was constructed. Some observations suggested that the HrcA regulator in normal growth conditions is expressed at low levels in the bacterial cells (Martirani *et al*., 2001; Spohn *et al*., 2004).

In the second approach we generated a 3XFLAG-HrcA complementing strains expressed from the *vacA* ectopic and non-essential locus, while maintaining the transcription of *hrcA* driven by its natural P*hrc* promoter. Previous works performed in our laboratory have shown that the *vacA* locus has a positional effect on the inserted gene and usually induces a higher level of proteins compared to the original locus.

Finally, since transcriptional studies highlighted *ureA* as a gene transcribed by a strong promoter (Akada *et al*, 2000; Spohn *et al*, 1997), the *ureA* promoter, P*ureA,* was selected to increase expression of the 3XFLAG-HrcA fusion protein.

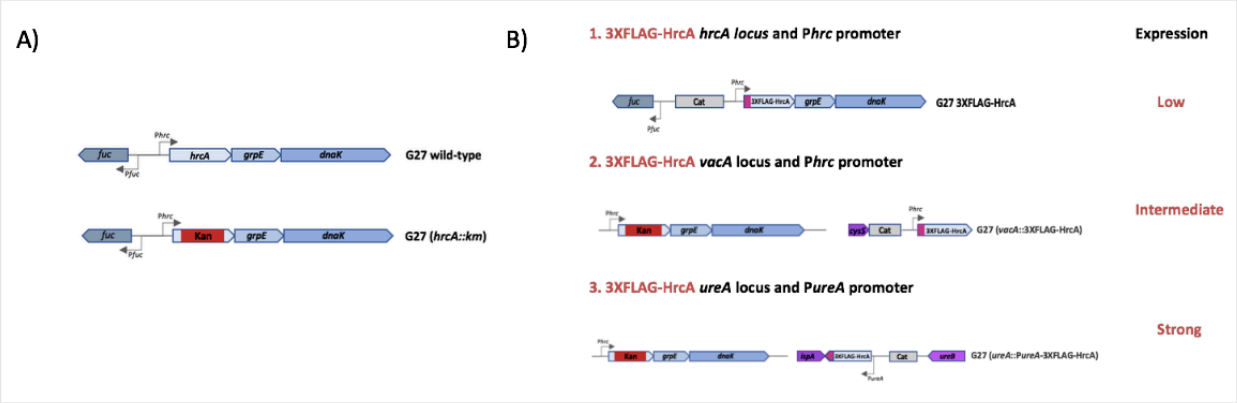

Figure 8. (A) Representation of *hrcA-grpE-dnaK* operon in the G27 wild type strain and the G27 knockout mutant (*hrcA::km*). The arrows indicate the ORFs. The kanamycin resistance cassette (km) is reported in the red box. **(B)** Schematic representation of the three strategies used to construct the complementing 3XFLAG-HrcA strains from the G27 knockout mutant (*hrcA::km*). In these tagged HrcA strains the pink box is used to depict the position of the 3XFLAG epitope tag; the light grey box underlines a chloramphenicol resistance cassette up- and downstream of the *hrcA* gene. The two homologous regions flanking the DNA sequence of interest, necessary for the homologous recombination are: 1) the *fuc* gene (upstream) and the *grpE* gene (downstream) in the G27 3XFLAG-HrcA strain; 2) a portion of *cysS* gene (upstream) and a portion of *vacA* gene in the G27 (vacA::3XFLAG-HrcA) strain; 3) and the *ispA* gene(upstream) and the *ureB* gene (downstream) in the G27 (urea::PureA-3XFLAG-HrcA). The kanamycin resistance cassette (km) is reported in the red box in the overexpressing strains 2 and 3.

To assess the functionality of the 3XFLAG-HrcA fusion proteins expressed from the above described complementing strains we assayed heat shock response of the *groESL* operon, a known target of repression for HrcA. That is, bacteria were liquid grown to mid-exponential phase and total RNA was extracted. Then, transcript amounts of the *groESL* operon was assessed by Real Time PCR and compared to the amount of RNA in the G27 wild type strain). As expected, transcription of the *gro*ESL operon was strongly derepressed in the HrcA mutant strain compared to the wild type strain (data not shown). Interestingly, in the complementing strains, *groESL* repression is restored to almost wild type levels, indicating complementation of the HrcA function by the 3XFLAG-HrcA fusion protein. In contrast, it was observed that in the samples that were submitted to the heat shock, the levels of *groESL* were induced, being similar levels to the HP G27 *hrcA* mutant (*hrcA::km*).

To further confirm expression of the 3XFLAG-HrcA fusion protein in the complementing strains and verify specificity of the antibody, different protein extracts were subjected to Western Blotting analysis with an anti-FLAG tag antibody (Figure 9A).

Subsequently, we immunoprecipitated 3XFLAG-HrcA-bound genomic DNA fragments by chromatin immunoprecipitation assay using α-FLAG antibody. The assays were designed to perform immunoprecipitation of HrcA in the wild-type strain that does not express the tagged protein (negative control) and the strains that expressed the 3XFLAG-HrcA fusion proteins. Following immunoprecipitation, enrichment of a known HrcA target was checked by Real Time PCR, with oligonucleotides specific for the *groESL* binding sites of HrcA (Table 3) with results shown in Figure 9B.

In the first condition tested, that is, in the strain expressing 3XFLAG-HrcA under the Phrc promoter it is possible to appreciate an almost 2,5-fold enrichment of the HrcA binding region (light grey bar) with respect to the wild type strain (dark grey bar). Although promising, this result is not in line with previous observations in our lab that a five-fold enrichment would be the limit required for a significant analysis of the sequencing data.

A similar enrichment level of HrcA binding region (Figure 9B, middle panel) was observed when the immunoprecipitation experiment was carried out on the *vac*::P*hrc*-3XFLAG-hrcA strain in which the levels of the HrcA protein was higher (Figure 9A, middle panel). We hypothesized that overexpression of 3XFLAG-HrcA might alleviate this limitation. Unfortunately, overexpression of 3XFLAG-HrcA showed an even lower amount of immunoprecipitated HrcA binding region (Fig. 9B, right panel). Therefore, as also reported by others works, we conclude that overexpression of a protein does not necessarily lead to more effective immunoprecipitation as it may lead to altered genomic binding profiles due to excess protein in the cell and the abundance of protein tends to produce a high signal-to-noise ratio, which significantly increases the background and hinders a good normalization of the experiment (Kidder *et a*l., 2011).

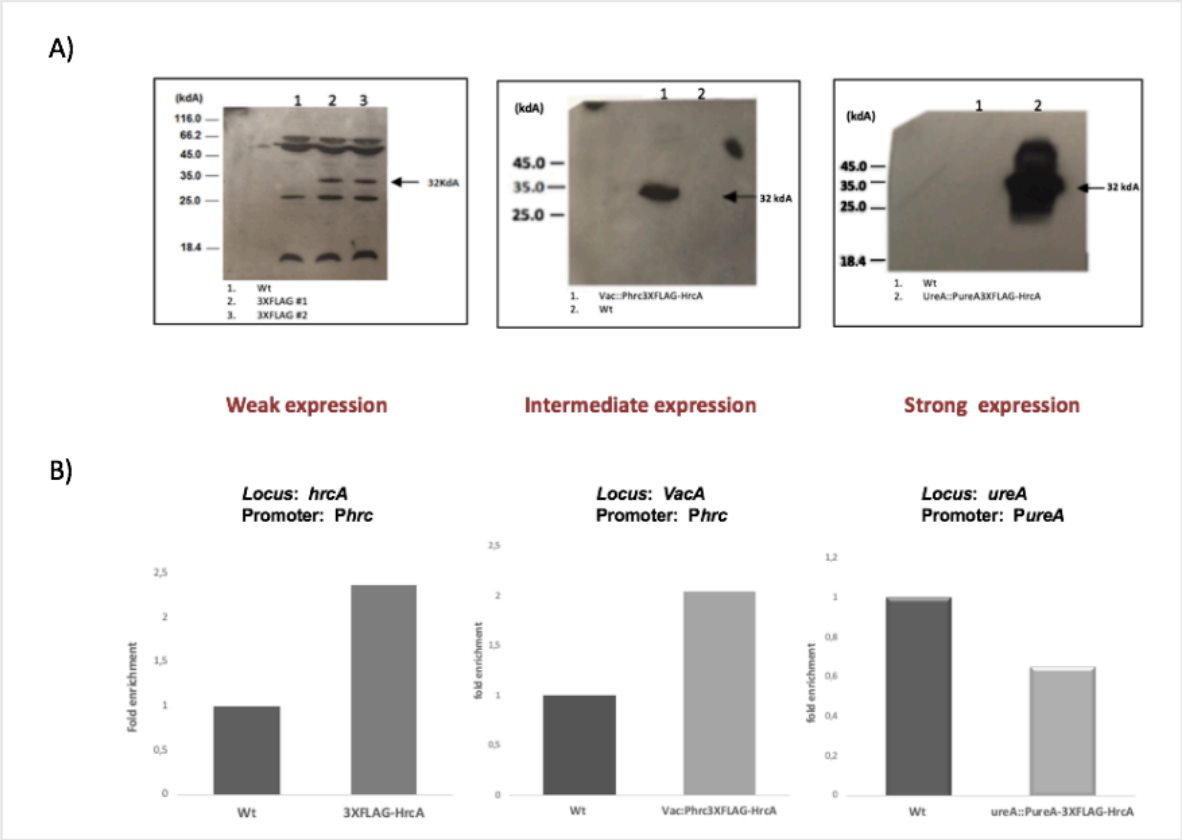

Figure 9. A) About 15 ug of total protein extracts of the *H. pylori* G27 wild type and complementing 3XFLAG-HrcA strains were fractioned in 12% SDS-PAGE. The band is present only in the strains that express the 3XFLAG-HrcA fusion protein and it completely lacks in the wild type strain (negative control). A Pierce Unstained Protein MW Marker (Thermofisher) was used as molecular weight marker and position of the interest band (32 kDa) is reported on the left side of the autoradiogram. **B) Analysis of specific enrichment of known HrcA targets was carried out by Real Time PCR, with oligonucleotides specific for the binding sites of HrcA on *groESL* promoter in immunoprecipitation assay.** Specific primers for the *groESL* promoter (table 3) were used to specifically test the enrichment of the DNA immunoprecipitated. The light grey bar indicates the fold enrichment of the DNA immunoprecipitated in the complementing 3XFLAG-HrcA strains, compared to the negative control (G27 wild type sample, dark grey bar).

Since the ChIP experiments did not allow the identification of new genomic targets, to characterize the HrcA interactions on some putative regulated genes, DNaseI footprinting experiments have been performed on a selection of three validated targets among the list of 18 upregulated genes by HrcA of the transcriptome analysis: the HPG27_RS01495, HPG27_RS03015 and HPG27_RS03495 genes.

The probes were radioactively labeled with ^{32}P and incubated *in vitro* with increasing amounts of recombinant purified His-HrcA and treated with DNaseI to identify HrcA binding sites.

Figure 10 shows DNaseI footprinting experiments performed on the radiolabelled P*hrc* promoter as positive control and the three putative targets (HPG27_RS01495, HPG27_RS03015 and HPG27_RS03495) identified by transcriptome outcomes. Besides the P*hrc* promoter that is known to be a direct target of HrcA, no protection was detected in all the other probes submitted to DNaseI footprinting experiments. We conclude that HrcA probably doesn't bind these regions, at least under the experimental conditions tested.

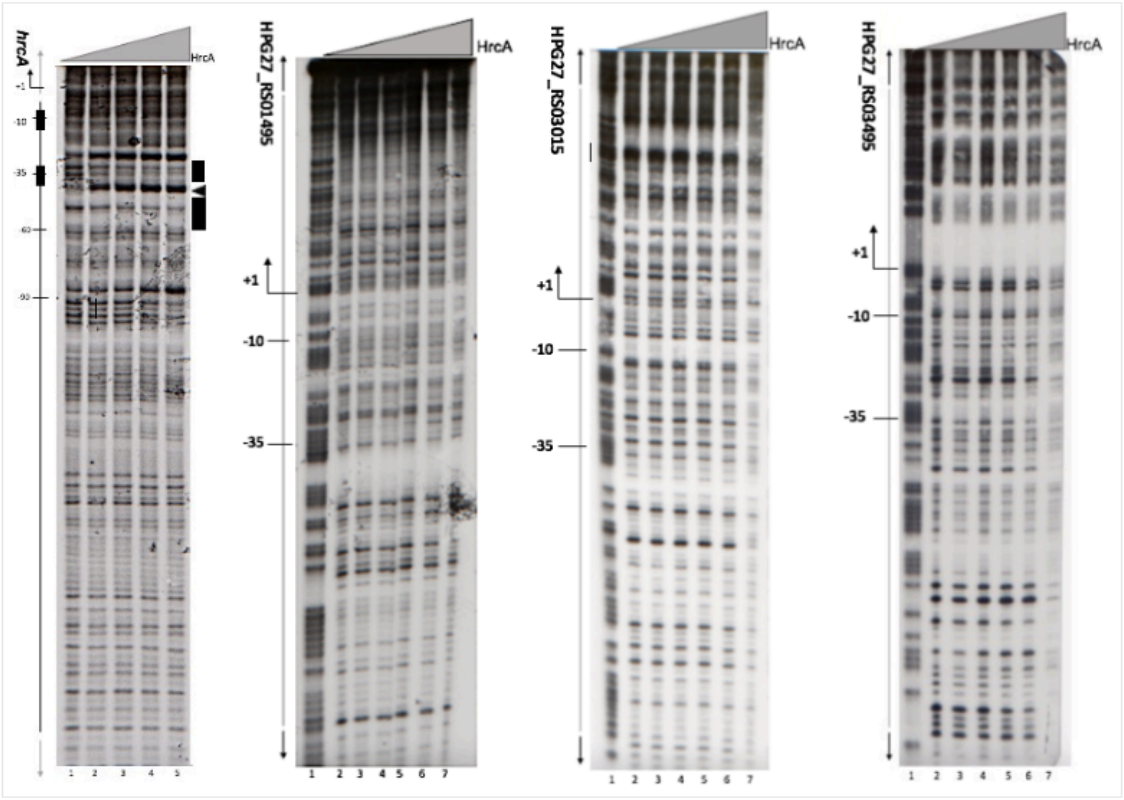

Figure 10. DNaseI footprinting assay with purified recombinant HrcA on P*hrc (A), PhbpA (B), P*HPG27_RS03015 *(C) and PsabB (D)* promoters. **A)** Schematic representation of the P*hrc* promoter region. Transcription start site (bent arrow), −10 and −35 boxes are indicated as well as HrcA open reading frame (open arrow). According to our previously published data (Roncarati *et al*., 2014). HrcA binding site is indicated in black open boxes represent the regions of DNaseI protection, while the black arrowhead indicate bands of hypersensitivity to DNaseI digestion. **B, C and D)** Radiolabelled DNA probes for putative P*hbpa, P*HPG27_RS03015 *and PsabB promoters were* end labeled at their non-coding strands, then were mixed with different amounts of purified HrcA protein and subjected to DNaseI digestion; purified DNA fragments were separated on a polyacrylamide denaturing gel and autoradiographed. Lanes 1 to 7 contain the G+A sequence reaction ladder and 0, 125, 250, 500, 1000 and 2000 ng of His-HrcA, respectively. On the left of each autoradiograph, the numbers refer to the positions with respect to the transcriptional start site (position +1).

4.3 Discussion

In this study, a differential transcriptional analysis allowed us to identify several non-heat shock responsive genes deregulated in the HrcA mutant. Of these, 18 genes appeared up-regulated and 37 genes appeared down regulated by HrcA. These latter are genes are mainly involved in different cellular functions such as cell motility, and transport or cell wall/membrane biogenesis.

To further characterize the HrcA regulon in *H. pylori* and investigate more in detail its direct or indirect contributions in the regulation of these genes, we attempted setting up a ChIP-seq experiments, the most direct way to the genome-wide profiling of protein-DNA interactions. Several attempts to carry out immunoprecipitation assays in diverse conditions showed a poor enrichment of DNA fragments bound by HrcA and the overall results of the experiments performed were not significant for the subsequent step of sequencing, due to a low amount of immunoprecipitated DNA and / or high levels of non-specific signal. It should be pointed out that in bacteria this technique has been used with a limited number of examples and it wasn't possible to obtain the necessary conditions for the *in vivo* study of this regulator using this technique, even if we attempted optimization by changing experimental parameters such as conditions of sonication, antibody concentration, number of cells and levels of expression of the HrcA protein. In all tested conditions the immunoprecipitation results were not sufficient to proceed to genome-wide ChIP and the sequencing.

In literature, it has been reported that the HrcA protein from *B. subtilis*, *B. stearothermophilus, S. aureus*, *C. acetobutylicum* and *B. thermoglucosidasius* shows a common disadvantage characteristic as it is hard to solubilize and has the tendency to form aggregates when expressed in *Escherichia coli* cells (Watanabe., *et al*, 2001). Concerning *Helicobacter pylori*, it has been reported that HrcA is partly toxic and insoluble when expressed in *E. coli*. In addition, there is no structural information about the conformation of the protein, which seems to be associated to the inner membrane fraction of *H. pylori* (Roncarati *et al*., 2007). Possibly this peculiarity contributes to the unsatisfactory results of Chromatin Immunoprecipitation of HrcA-DNA complexes obtained until now.

Since it was not possible to identify new direct targets of HrcA through the ChIP-seq technique, DNaseI footprinting experiments have been performed on a

selection of three targets among the list of 18 upregulated genes by HrcA from the transcriptome analysis with no evidence of DNA-binding (Figure 10). Possibly, regulation of these genes by HrcA is exerted by an indirect mechanism. In *H. pylori*, HrcA represses transcription of the *hrcA-grpE-dnaK* and *groES-groEL* operons by direct binding to the P*gro* and P*hrc* promoters on positions centered at +9 and -42 of the P_{gro} and P_{hrc} promoters, respectively (Roncarati *et al*.,2007). The HrcA binding sites show similarities to a consensus sequence named CIRCE and firstly identified in *B. subtilis* (TTAGC ACTC-N9-GAGTGCTAA) (Narberhaus and Bahl, 1992).

In *H. pylori* and in other organisms, such as *B. subtillis* and *S. aureus*, it has been demonstrated that the GroE chaperonin is required *in vivo* to allow HrcA to fold in its active conformation to bind its target promoters and repress transcription of genes of thermal shock. After heat stress, GroE is titrated by misfolded proteins accumulated in the cell, and HrcA becomes inactive and dissociates from its promoter sequence, inducing the HrcA regulon (Roncarati *et al*., 2014; Chastanet *et al*., 2003; Mogk *et al*., 1997). In this model, the interaction between HrcA and GroESL appears very important. A study in *C. trachomatis* suggested that GroESL is capable not only of increase the binding affinity of HrcA to the CIRCE sequence but also to improve HrcA-mediated transcriptional repression, as HrcA showed efficient bind to the CIRCE in the presence of the GroESL complex (Wilson *et al*., 2005). Accordingly, this direct correlation between the GroESL complex and the HrcA activity suggests that to properly maintain its function *in vivo* HrcA requires the presence of chaperonins (Minder *et al*., 2000). Therefore, the absence of binding on the putative target promoters at the HPG27_RS01495, HPG27_RS03015 and HPG27_RS03495 genes may be explained by the lack of the chaperonin in the invitro technique and the possibility of a direct interaction between HrcA and these genes cannot be *a priori* disregarded.

5. Conclusions

Helicobacter pylori is a widespread human pathogen recognized as the main causative agent of chronic gastritis, gastric and peptic ulcers, and persistent colonization is an important factor in the development of gastric lymphoma and adenocarcinoma.

To respond to stress conditions and survive in a hostile environment such as the human stomach, this bacterium induces the synthesis of a class of highly conserved proteins, known as Heat-shock Proteins, which protect the cell from damage, denaturation, aggregation or folding of proteins.

In *H. pylori*, the genes encoding for the major heat shock proteins are clustered in three multicistronic operons and are repressed by two transcriptional regulators: HrcA and HspR.

In this study, the *H. pylori* HrcA repressor was demonstrated to be involved in the regulation of several non heat-shock responsive genes. With a RNA-seq approach it was possible to identify a total of 48 from 137 deregulated genes, induced or repressed, after the almost complete deletion of HrcA in a mutant strain (*hrcA::km*) and not responsive to heat-shock.

To better characterize the HrcA regulon in *H. pylori* and investigate more in detail its direct or indirect contributions in the regulation of these genes, we attempted setting up a ChIP-seq experiments. In the present study the epitope tags were fused to the N-terminal domain of the protein, which harbors the DNA-binding domain (Roncarati *et al*., 2007). However, due to their particular characteristics and the lack of structural information of this protein it wasn't possible to obtain the necessary conditions for the *in vivo* study of this regulator and the overall results of the experiments performed were not significant for the subsequent step of sequencing. For future prospects, it would be interesting to obtain more structural information on this protein in order to reallocate the tag used in a more immunogenic position. Also develop a chromatin tandem affinity purification (ChTAP) strategy could be an effective alternative to ChIP (Soleimani *et al*., 2013; Kolodziej *et al*., 2009).

Furthermore, DNaseI footprinting experiments have been performed on a selection of three targets among the list of 18 upregulated genes by HrcA from the transcriptome analysis with no evidence of DNA-binding. Several studies suggest that in order to properly maintain its functions *in vivo*, HrcA requires the presence of chaperonins. Thus, although HrcA probably interacts with most of

these genes in indirect way, the possible direct interaction between HrcA and these genes can not be *a priori* disregarded, since *in vitro* assays may not be accurate if multiple factors or DNA segments are required for binding (Boyle *et al*., 2017). Perhaps, developing a protocol that allows the use of *in vivo* Footprinting may represent an effective alternative.

Globally, this study reveals the involvement of the *H. pylori* transcriptional regulator HrcA in crucial cellular processes, most of them interconnected between then with functions closely associated to the pathogenesis and virulence of this bacteria.

Most of the up-regulated genes in the ΔHrcA mutant are related to Cell wall/membrane/envelope biogenesis, Coenzyme-, Aminoacid- and Inorganic ion-transport and metabolism. Some studies point out genes related to these functions as potential target for the development of new drugs (Sperandeo *et al*., 2017; Tanaka *et al*., 2018; Thomas and Tampé, 2018).

Interestingly, this study demonstrates a close connection between the HrcA repressor and the motility of *H. pylori*. In fact, it was possible to identify several genes that regulate the biosynthesis and function of the flagellar apparatus. This result is in accordance with previous studies in which the mutant strain HrcA is described with non-motile phenotype. Furthermore, a recent study suggested that the *H. pylori* HrcA regulator and the flagellar apparatus may play an important structural role during biofilm formation (Hathroubi *et al*., 2018).

An interesting aspect to be verified is whether in a deletion condition of *hrcA* there are phenotypic changes in the flagellar structure of *Helicobacter pylori* that agree with its proven loss of motile function.

Although not essential, this study demonstrates that HrcA is interconnected to several cellular functions crucial for the survival, virulence, and maintenance of the *H. pylori* infection state in the human stomach. As a bacterial pathogen of major medical importance and the available antimicrobial therapies are losing efficacy, principally because of insurgence of antibiotic resistance, the globalized performance of HrcA may be the starting point for a search for new targets and therapeutic strategies.

6. Materials and Methods

6.1 Bacterial strains and culture conditions

Helicobacter pylori strains (**listed in Table 1**) were recovered from frozen glycerol stocks on modified Brucella broth agar plates containing 5% fetal calf serum, in a 9% CO_2–91% air atmosphere at 37°C and 95% humidity in a water-jacketed incubator (Thermo Forma Scientific). Liquid cultures were performed in modified Brucella broth supplemented with 5% fetal calf serum, with gentle agitation (120 rpm). *E. coli* DH5-α and BL21 (DE3) strains (**Table 1**), were grown on Luria–Bertani (LB) agar plates or LB liquid broth with vigorous agitation (250 rpm); when required, ampicillin or chloramphenicol was added to the medium to achieve a final concentration of 100 μg ml^{-1}.

6.2 DNA manipulation

DNA manipulations were performed routinely as described by Sambrook *et al*. (1989). All restriction and modification enzymes were used according to the manufacturers' instructions (New England Biolabs). Preparations of plasmid DNA were carried out with Nucleospin plasmid purification kit (Macherey-Nagel). DNA fragments for cloning purposes were extracted and purified from agarose gel using Qiagen Gel Extraction kit (Qiagen, Inc.). PCR were carried out in a Bio-Rad MJ Mini Personal Thermal Cycler using PCRBIO HiFi polymerase. In each reaction, 500 ng of *H. pylori* chromosomal DNA was mixed with 400nM of each specific primer in a final volume of 50 μl containing 1x PCRBIO HiFi Polymerase (2u/μl). A total of 35 cycles were performed by denaturing DNA at 95°C for 75 s, annealing at the appropriate temperature for 15 s, and extending at 72°C for 30 s for each kb.

6.3 Construction of the Tagged-HrcA complementant strains.

The complementing strains were obtained by transforming the mutant G27 (hrcA::km) with the plasmids pBS-KS-(fucFR)-CAT-(PhrcFR-3XFLAG)-(hrcF-grpER), pVAC-Phrc3XFLAG-Hrca and pureA-3xflaghrca(cat^r), respectively **(Table 2)**. Naturally competent *H. pylori* cells, after overnight growth on a plate, were collected in the center of the plate and incubated at 37 °C for 5 hours. At the end of the incubation, 5-10 μg of linearized plasmid DNA were added to the

bacteria and after overnight incubation at 37 °C, bacteria were plated on selective medium and further incubated for 3-4 days at 37 °C. Thereafter, transforming colonies were isolated and analyzed for the correct insertion of the desired fragment through PCR.

6.4 RNA isolation and cDNA synthesis

Helicobacter pylori strains (**Table 1**) were grown with gentle agitation (120 rpm) in 30 ml of Brucella broth at 37°C until the exponential phase (OD = 0.6). For heat-shock treatment, the cultures were split into 15 ml-aliquots and one sample was subjected to heat-shock at 42°C for 30 min (heat-shock sample, HS). A volume of 10 ml cell culture was then added to 1.25 ml of ice-cold EtOH-phenol stop solution (5% acid phenol, in EtOH) to stop growth and prevent RNA degradation. Cells were pelleted, stored at −20°C, and then used to extract total RNA with TRI-reagent (Sigma-Aldrich), according to manufacturer's protocol. Prior to use, an aliquot of each RNA sample was collected by centrifugation, quantified and loaded on a 1% agarose gel to assess RNA purity and integrity. After that, to remove a possible contamination by genomic DNA, 7-8 µg of total RNA were precipitated in ethanol and collected at 14,000 rpm for 30 min at 4°C. The RNA pellet was resuspended in 40 µl of milliQ water. After the quantification, a DNase I digestion reaction was prepared by adding 10 µl of 5X DNase buffer (400 mM Hepes pH 7.5, 50 mM NaCl, 25 mM MgCl2, 50 mM DTT) 1 µl of DNase I (1 u/µl), 5 µg RNA and milliQ water up to the volume of 50 µl. The reaction was incubated 45 min at 37°C and then the RNA was extracted once with an equal volume of phenol-chloroform-isoamyl-alcohol (25:24:1). The tubes were stored at -20°C by adding 0.1 volumes 3M NaOAc pH 5.2 and 2 volumes of 100% ethanol. Then, 2.5 µg of the RNA treated with DNase I was collected by centrifugation at max speed (14,000 rpm) for 30 min at 4°C. The RNA pellet was resuspended in 10 µl of milliQ water. After the quantification, the following reaction was prepared: 2 µl Random primers (25 ng/µl), 1 µg RNA and milliQ water up to a volume of 10 µl. The samples were denatured 5 min at 70°C in order to remove secondary structures and cooled on ice for 5 min. Meanwhile, the reaction of reverse transcription was prepared: 4 µl 5X AMV-RT buffer, 2 µl dNTPs 10 mM, 0,5 µl AMV-RT (10u/µl) and milliQ water up to 10 µl. The reverse transcription reaction

was added to the denaturated RNA and incubated for 60 min at 37°C. The obtained cDNAs were stored at -20°C.

6.5 qRT-PCR Analysis

For qRT-PCR analyses, 2 µL of the diluted (1:10) cDNA samples were mixed with 5 µL of 2X Power Up SYBR Green master Mix (ThermoFisher Scientific) and with 400 nM oligonucleotides specific to transcript genes under study **(Table 3)** in a final volume of 10 µl. Real time PCR was performed using the following cycling protocol: 95°C for 2 min, then 40 cycles consisting of a denaturation for 5s at 95°C followed by 30s at 60°C (annealing and extension steps). Data were analyzed using the ΔΔCt method, using the housekeeping *ppk* gene, known to be constitutively expressed, as internal reference, using oligonucleotides ppk RT-F and ppk RT-R as primers in ChIP experiments (**Table 3**) and in transcriptome studies using the *16S* gene as internal reference, using oligonucleotides 16S-F and 16S-R as primers (**Table 3**) (Muller *et al*., 2011; Agriesti *et al*., 2014).

6.6 Westen Blot assay

Bacteria were liquid grown to the exponential phase and total protein was extracted. The cellular protein extracts were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were blotted onto a PVDF nylon membrane for 45 min at 150V in 0.25X Tris-Glycine buffer (6.25 mM Tris-HCl; 62.5 mM glycine, pH 8.3; 0.025% w/v SDS) containing 20% (v/v) methanol, with a wet-transfer apparatus. After blocking for 2h at room temperature in phosphate-buffered saline (PBS) containing 5% low-fat milk, 1X PBS and 0,05% Tween, the membrane was incubated overnight at 4°C with a 1:3000 dilution of anti-FLAG monoclonal antibody (Sigma-Aldrich) in blocking buffer. The membrane was washed in 1XPBS containing 0.05% Tween and then incubated for 2h a room temperature with a 1:5000 dilution of anti-mouse IgG immunoglobulin (Santa Cruz Biotechnology). Subsequently, the chemiluminescent signal was generated, and the membrane was exposed for 30 minutes to X-ray film for autoradiography.

6.7 Chromatin Immunoprecipitation (ChIP) With α-FLAG Monoclonal Antibody

Bacterial cultures were grown to exponential phase (optical density at 600nm: 0,8) and cross-linked in 1% formaldehyde for 10 min at room temperature. To stop cross-linking, samples were equilibrated with 125 mM glycine and incubated further 10 min at room temperature. Bacteria were pelleted, washed twice in 10 ml of phosphate-buffered saline (PBS) and bacterial pellets were stored at -20°C. The bacterial pellet was resuspended in 1ml of sonication buffer and PMSF (serine protease inhibitor) was added at a final concentration of 1 mM. The suspension was sonicated using the Bioruptor sonicator (60 cycles 30” on and 30” off high power; 60 cycles 30” on and 30” off low power) and centrifuged for 8 min at 6000 rpm in an Eppendorf centrifuge at 4°C to separate the soluble cell extract from the insoluble material. Prior to immunoprecipitation, an aliquot (50 µl) of cell extract was reverted to check the correct size of fragmented DNA as it represents the DNA input for the construction of the sequencing libraries. RNase A (DNase free) was added at a final concentration of 20 µg/ml and the sample was incubated 30 min at 37°C. Proteinase K was then added to a final concentration of 50 µg/ml and the solution was brought to a final concentration of 0.5% SDS; the sample was incubated 6 h at 37 °C and then overnight at 65°C. The sample was extracted with phenol/chloroform and precipitated with 0.1 volume of 3M NaOAc pH 5.2 and 2 volumes of cold 100% ethanol. The sample was washed with 70% ethanol and resuspended in 20 µl of milliQ water. Cell extracts (0.9 ml) were adjusted to RipA Buffer without SDS (140 mM NaCl, 10 mM Tris-Cl pH 8.0, 1 mM EDTA, 0.1% sodium deoxycholate, 1% Triton X- 100) by adding 1.5 volumes of the buffer C-A(166-6 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, 1.66% Triton X-100), precleared with 50 ml of ProtG-Sepharose (50% slurry equilibrated in IP Buffer) for 1h at 4°C, and then incubated for 16 h at 4°C with an anti-FLAG monoclonal antibody (Sigma Aldrich). HrcA-DNA complexes were immunoprecipitated with 50 µl of ProtG-Sepharose slurry (equilibrated in RipA Buffer). Bound protein-DNA complexes were washed 4 times in 1 ml of 1 IP Buffer, and twice in 1 ml of TE. Complexes were finally resuspended in 150 µl of TE and 20 µg/ml of RNase A was added, and the sample was incubated 30 min at 37°C. Then, 50 µg/ml of proteinase K and 0.5% SDS were added, and the mixture was incubated overnight at 37°C. Cross-linking was reverted for 6 h at

65°C. DNA was extracted with phenol/chloroform, and the organic phase was back-extracted with TE; 1 µl of glycogen was added and the samples were stored at -20°C for 2-3 days prior the precipitation. Immunoprecipitated DNA fragments were finally resuspended in 15 µl of sterile water. DNA was analyzed by Real Time PCR before the sequencing.

6.8 RNA-seq: Library Preparation, Sequencing and Analysis

Ribosomal RNAs were depleted starting from 1 µg of total RNA from each of the conditions analyzed by using the RiboZero Gram negative kit (Epicentre, Illumina). Strand specific RNA-seq libraries were prepared by using the ScriptSeq^{TM} v2 RNAseq library preparation kit (Epicentre, Illumina) starting from 50 ng of previously rRNA-depleted RNA from each biological replicate and for all the conditions analyzed. Then, each library was multiplexed in equal amounts and sequenced on a GAIIX Illumina sequencer and 85 bp reads were produced. A minimum of 7 Million reads were obtained for each of the samples and for each replica. Bowtie 2 (v2.2.6) (Langmead and Salzberg, 2012) was used to align raw reads to *H. pylori* G27 genome selecting end-to-end mapping and specifying non-deterministic option. High quality reads were selected requiring: for uniquely mapping reads MAPQ > 30 (mapping quality) and alignment score >−15; for multi-mapping reads alignment score was set ≥−15. *H. pylori* G27 RefSeq annotation GCF_000021165.1) in the version released on sept-2017 was used as the reference for gene annotation to which we manually added validated ncRNAs (Pelliciari *et al*., 2017; Vannini *et al*., 2017) (**Supplementary Table 1 and 2**). We also revised the annotation of protein coding genes that, based on our sequencing data, were improperly annotated as pseudogenes in this version of the reference genome (e.g., *rpoB, rpoA, hspR*), indicating them as “protein-coding*” in **Supplementary Table 1 and 2**. BEDTools (v2.20.1) (Quinlan and Hall, 2010) and SAMtools (v0.1.19) (Li *et al*., 2009) were used to verify the library preparation and sequencing performances. In particular, we measured the level of rRNA depletion, which was very efficient (less than 6% of the mapping reads) and strand specific gene coverage, considering only strand specific reads overlapping for at least 50% of their length to the annotated transcripts. This analysis revealed that 99% of the transcripts were covered by at least one strand

specific read and a minimum of 46 reads were counted on 90% of them. The R package DESeq2 (v1.4.5) (Love *et al*., 2014) was then used to normalize the counts and to identify differentially expressed genes (DEGs) showing BH adjusted *p*-value (*p*adj) lower than 0.01 and log2 fold changes (log2FC) > |1|. To evaluate functional enrichments in the DEGs lists, we retrieved COG functional classes for all the protein coding genes present in our annotation file through the NCBI CDD database (Tatusov *et al*., 1997). We obtained COG records for 1047 genes, 88 of them were annotated as "function unknown" or "general function prediction only" categories, so we considered a final list of 959 COG annotated genes for functional enrichment analysis. The genes classified as: (1) not coding for proteins, (2) coding for proteins but not annotated in COG or (3) annotated in COG to "function unknown" or "general function prediction only" categories were merged together into the "Unknown function" in the annotation file (see **Supplementary Table 1 and 2**).

6.9 Overexpression and Purification of Recombinant HrcA Protein

His6-tagged recombinant HrcA protein was overexpressed in *E. coli* BL21(DE3) cells and affinity purified as previously described (Roncarati *et al*., 2014). The purified His-HrcA protein was dialyzed against two changes of 1X footprinting buffer (10 mM Tris-HCl, pH 8.0; 50 mM NaCl; 10 mM KCl; 5 mM $MgCl_2$; 0.1 mM DTT; 0.01% NP40) avoiding any trace of glycerol, prior to the DNA binding experiment, and stored at −80°C. Protein concentration was determined by Bradford colorimetric assay (BioRad).

6.10 Construction of DNA Probes for *in vitro* DNA-Binding Assays

Genomic regions of *H. pylori* G27 encompassing presumed HrcA binding sites on HPG27_RS01495, HPG27_RS03015 and HPG27_RS03495 promoters were PCR amplified with specific primers (**Table 3**) and cloned into the pGEM-T-Easy plasmid (**Table 2**). The mutant probes were generated by annealing complementary oligonucleotides to form a double stranded DNA fragment with compatible overhangs required to clone it in the pGEM-T-Easy plasmid previously digested with the appropriate restriction enzymes. Then, each probe was 5′-end labelled with [γ-32P]-ATP and T4 polynucleotide kinase at one

extremity. The labeled DNA probe was further digested with another restriction enzyme and purified by native polyacrylamide 4% gel electrophoresis and eluted in 1.5 ml of elution buffer (10mM Tris HCl pH 8.0, 1 mM EDTA, 300 mM sodium acetate pH 5.2, 0,1% SDS) at 37°C overnight.

6.11 DNaseI Footprinting Assay

The Footprinting experiments were essentially performed as described previously (Roncarati *et al*., 2014). The promoter regions of the *hrcA*, *hbpA*, HPG27_RS03015 and *sabB* genes were PCR amplified with oligonucleotide pairs hrca/hrc1, FP-01495-F/ FP-01495-R, FP-03015-F/ FP-03015-R and FP-03495-F/ FP-03495-R (Table 3), respectively, from chromosomal DNA of *H. pylori* G27 and cloned into the pGEM-T Easy vector, resulting in the plasmids listed in Table 2. Promoter DNA fragments obtained by the appropriate restriction enzymes digestion were 5' end labeled with [γ -^{32}P] ATP and T4 polynucleotide kinase at one extremity and gel purified, and approximately 10 fmoles of each probe was used for footprinting experiments. Labeled DNA probes were incubated with a purified HrcA protein in 50 µl of footprinting buffer (10 mM Tris-HCl [pH 8.0], 50 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 50 mM dithiothreitol, 0.01% NP-40, 10% glycerol) containing 250 ng of sonicated salmon sperm DNA as a nonspecific competitor for 15 min at room temperature. Two microliters of DNase I (0.01 U/ µl), freshly diluted in footprinting buffer containing 5 mM $CaCl_2$, was added, and incubation was continued for 75 s at room temperature. DNase I digestion was stopped by addition of 140 µl of stop buffer (192 mM sodium acetate, 32 mM EDTA, 0.14% sodium dodecyl sulfate, 64 µg/ml sonicated salmon sperm DNA). Samples were phenol-chloroform extracted, ethanol precipitated, resuspended in 6µl of FLB (formamide loading buffer), denatured at 95°C for 2 min, subjected to 6% polyacrylamide–urea gel electrophoresis, and autoradiographed.

Table 1. Bacterial strains

Strains	Description	Reference/Source
E. coli DH5α	supE44 lacU169 (80 lacZM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	Hanahan, 1983
E. coli BL21 (DE3)	*hsdS gal (λcIts857 ind1 Sam7 nin5 lacUV5-T7 gene 1)*	Grodberg et al., 1988
H. pylori G27	wild type	Xiang et al., 1995
H. pylori G27 (*hrcA::km*)	G27 derivative; bp 156 to 375 of *hrcA* have been replaced by a kan cassette	Spohn et al., 2004
H. pylori G27 (3XFLAG-hrcA)	G27 (hrcA::km) derivative; 3XFLAG-hrcA complementing strain obtained by double homologous recombination of pBS-KS-(fucFR)-CAT-(PhrcFR-3XFLAG)-(hrcF-grpER) vector	This study
H. pylori G27 (PureA:3XFLAG-HrcA)	G27 (hrcA::km) derivative; 3xFLAG-HrcA-overexpressing strain obtained by double homologous recombination of pureA-3xflaghrca(cat^r) vector; this strain harbors an *3xFLAG-HrcA* under the control of the *ureA* promoter in the *ureA* locus.	This study
H. pylori G27 (VacA::Pcncr1-strep-HrcA	G27 (hrcA::km) derivative; 3XFLAG-overexpressing strain obtained by double homologous recombination of pVAC::Phrc3XFLAG-HrcA vector; this strain harbors 3XFLAG-HrcA under control of its natural *hrc* promoter in the *VacA* locus.	This study

Table 2. Plasmids

Plasmids	Description	Reference/Source
pBluescriptKSII(+) (pBS)	Cloning vector, Amp^r	Stratagene
pBS-KS-(fucFR)-CAT-(PhrcFR-T7)-(hrcF-grpER)	pBS derivative, containing a 397-bp fragments amplified by PCR on chromosomal DNA of H. pylori, digested with BamHI/NdeI restriction enzyme and coding for the T7 epitope tag (aminoacid sequence: MASMTGGQQMG) in the N-terminal position to the *hrcA* gene. Flanked by *fuc* gene and a cat resistant cassette (upstream) and the *grpE* gene (downstream).	Davide Roncarati Personal communication
pBS-KS-(fucFR)-CAT-(PhrcFR-3XFLAG)-(hrcF-grpER)	pBS-KS-(fucFR)-CAT-(PhrcFR-T7)-(hrcF-grpER) derivative, containing a 440-bp fragments amplified by PCR with	

	oligonucleotides Phrc-F/3xflag-R (table 3) on chromosomal DNA of *H. pylori*, digested with BamHI/NdeI restriction enzymes, coding for the 3XFLAG tag (aminoacid sequence: DYKDHDGDYKDHDIDYKDDDDK) and a chloramphenicol (Cat) resistance cassette.	This Study
pVAC::CAT	pVAC::km derivative, carrying a BglII/BamHI cat cassette from pBS::cat.	Vannini et al.,2012
pVAC::P*cncr1-clpP*-6xHis	pVAC::CAT derivative, carrying a 95 bp BamHI-NdeI fragment corresponding to the region from 536,896-536,990 of *H. pylori* G27 amplified with oligos PcagPF_BamHI and PcagPR_NdeI encompassing the promoter region of *PcagP* and a 631 bp DNA fragment amplified on pBS::*clpP*-6xHis with oligos ClpPcagPF_NdeI and VSCDSCpR_NcoI (chr: 820,077-820,678); Cpr.	Simona Pepe Personal Communication
pVAC-P*hrc*3XFLAG-HrcA	pVAC::Pcncr1-*clpP*-6xHis derivative, containing a 1255 bp BamHI-NcoI DNA fragment corresponding to the region from 118,033-119,215 of *H. pylori* G27 genome amplified with oligos Phrc-F and HrcA-R; Cp^r	This Study
pAD1	Vector for introducing recombinant DNA into the H. pylori ureA locus	McClain et al., 2013
pMM682	H. pylori codon-optimized tetR cloned into pAD1	McClain et al., 2013
pureA-3xflaghrca(cat^r)	PMM682 derivative, containing an 896-bp fragments amplified by PCR with oligonucleotides pureA-F(XbaI)/pureA-R(BamHI) on chromosomal DNA of H. pylori, digested with XbaI/BamHI restriction enzymes coding for the 3XFLAG-hrcA. Contains a chloramphenicol (cat) resistance cassette, restriction sites to allow cloning of a gene of interest into a site downstream from the *ureA* promoter and a ribosomal binding site and flanked by upstream and downstream regions for double homologous recombination in the *ureA* locus.	This Study
pGEM-T Easy	Cloning vector, Amp^r	Promega
pGEM-T Easy-P*hrc*	pGEM-T-Easy derivative, containing a 308-bp PCR fragment encompassing the P*hrc* promoter.	Roncarati et al., 2014
pGEM-T Easy-P*hbpA* (HPG27_RS01495)	pGEM-T Easy derivative containing a 234-bp DNA fragment corresponding to the region from 311,051 to 311,264 of *H. pylori* G27 genome amplified by PCR with	This Study

	oligonucleotides FP-277-F and FP-277-R (table 3).	
pGEM-T Easy-HPG27_RS03015	pGEM-T Easy derivative containing a 337-bp DNA fragment corresponding to the region from 632,805 to 633,121 of *H. pylori* G27 genome amplified by PCR with oligonucleotides FP-580-F and FP-580-R (table 3).	This Study
pGEM-T Easy-P*sabB* (HPG27_RS03495)	pGEM-T Easy derivative containing a 314-bp DNA fragment corresponding to the region from 741,362 to 741,655 of *H. pylori* G27 genome amplified by PCR with oligonucleotides FP-677-F and FP-677-R (table 3).	This Study

Table 3. Oligonucleotides

Oligonucleotides	Sequence (5' – 3')
16S RT F	GGAGTACGGTCGCAAGATTAAA
16S RT R	CTAGCGGATTCTCTCAATGTCAA
ppk RT F	CGCGCCTTTCTAAATTTCTGGGCA
ppk RT R	CCCAAGTCAAAGGCTTGAGCGAAA
BShrcA RT F	GTGATACAGACTAAACTTTAAAGAAAAATCGGG
BShrcA RT R	CCATTCTTGATGAAAGAACCCTCGAATTATAGC
Rbs-hrcF	CGATTTTTCTTTAAAGTTTAGTCTGTATCAC
Hrc/fuc-F	GCGGTAATCTATCTTGTCATG
grpE-R	TATCTCGAGTTTAATCGTTTTTAGCAATGCTCA
Abs-groF	GCTTGACTTATCCCTAAAAATGTGC
Abs-groR	AGACCCTTTCTCCTAATGGTTGA
Phrc-F	ATATGGATCCTACGTCAAGCAAGCGATAACTTTAC
3xflag-R	ATATCATATGTTTGTCATCGTCATCTTTATAATCAATATCATGATCTTTAT-AATCACCATCATGATCTTTATAATCCATTATTTGAAAAATCTCGTCAATCACC
pureA-F(XbaI)	ATATTCTAGAATGGATTATAAAGATCATGATGGTG
pureA-R(BamHi)	ATATGGATCCTTATTCCTCCTCAGAAATCGTTTG
Ure-up-F	GCTCAGTTGGTAGAGCACTACCT
Ure-down-R	ACGGCTTTTTTGCCTTCGTTGAT
HrcA-R	ATATCCATGGATTATTCCTCCTCAGAAATCGTTTG

Cys-F	CGTTTTAGGGACTTTGGGAGG
VacA-R	GCTGGTTTTATGCTCTAAACTGG
hrcA	CAAACGCATCTAACAAACTCTC
hrc1	TTCATTATAAAACAAAAAGGATCC
FP-01495-F (XbaI)	ATATTCTAGACATAGATGGCGTTGTGAAG
FP-01495-R (XhoI)	ATATCTCGAGCAAAAACATTATTCATAAGC
FP03015-F (XhoI)	ATATCTCGAGGGGTGCTATTTAAGCCATTATC
FP03015-R (BamHI)	ATATGGATCCCAATAATAGAGTCTTGTTGC
FP-03495-F (XhoI)	ATATCTCGAGGCGTTCTGTTAAGCGTTATC
FP-03495-R (BamHI)	ATATGGATCCGCGTGCAAGAGCGATGAAG
HPG27_RS03015 _F	AATCATAGAGCCGGTGGATATG
HPG27_RS03015 _R	GAAACAGGGCAAGGCAAATAG
HPG27_RS06935 _F	CCGTTTATGACAGCGAGTTTATC
HPG27_RS06935 _R	TTGCTCGCATAGTAGCATTTG
HPG27_RS06930 _F	GCTAAAGATAACAGCACCAAACC
HPG27_RS06930 _R	TCGCCTTCTCCATGCATTT
HPG27_RS03940 _F	CTGGAATGCTAGGCAAACATATAG
HPG27_RS03940 _R	ATAAACCGCATCAAGCAAGTAG
HPG27_RS03495 _F	TGGTCCTGTAACCGACTATG
HPG27_RS03495 _R	TCAGATAGGGTGTGGTTTCTC
HPG27_RS00070_F	TCACGCCATCTTTGGTGATGCTTG
HPG27_RS00070_R	TGAAGGCGTGAGACAACTCCATGA
HPG27_RS05840_F	GTGCTTATCGCTTTGGCTATTC
HPG27_RS05840_R	AAAGATGGTAGGGAACATGATAGAG
HPG27_RS02700_F	GGGAATTTGTAGCAGACATGG
HPG27_RS02700_R	GGTAAAGCGCCAAGCAATAG
HPG27_RS01500_F	ATTAGCGTTGGCGTGTTG
HPG27_RS01500_R	GCATAGAAATCCCGGCTAAAG
HPG27_RS02740_F	ATGACGATCACGACTCTATCC
HPG27_RS02740_R	ACAAGCCTCCAGCAAATAAC
HPG27_RS01480_F	GCGTCTAAACCCAATGAAGTC
HPG27_RS01480_R	CGCTGATTTGTTGGTAGATGG
HPG27_RS05560_F	GAGAGTCCTTGCTTGCTTTG

HPG27_RS05560_R	GGGATGATTTGTTGGATGAGC
HPG27_RS03665_F	CAAACCACCACCCAAGAAAG
HPG27_RS03665_R	GTGTTGCCCTCTTTGGTTAAG
HPG27_RS00595_F	TGAAGACGGATTAAGGGCTAAG
HPG27_RS00595_R	CGCTTCTATCACGCAATCAATC
HPG27_RS04430_F	AATCCCGCTAAAGATCAACAAG
HPG27_RS04430_R	GGTTTCATGCTCATGGTTCTC
HPG27_RS02255_F	ATGGAATTTAGGCGGAAGAATG
HPG27_RS02255_R	CCTACATCTTTGCTGCCTTG
HPG27_RS04255_F	GAGAACTCCCGCTTAAACAAAG
HPG27_RS04255_R	CTGATTGTCCTTAGTGCAAATCTC
HPG27_RS00600_F	AAACTTGACTGAAGTGGGATTG
HPG27_RS00600_R	GCCAGCGCTTGTAGAAATAC
HPG27_RS01870_F	AGCCTTTCAAACCCTAAAGC
HPG27_RS01870_R	CCCTCAAATCATCGCTCAAC
HPG27_RS02925_F	AACGGGCAAGCGTTATTG
HPG27_RS02925_R	CAGAAGTGGTAGAGCCGATAG
HPG27_RS05740_F	GAGGGAATACAGCTTTGTGATAC
HPG27_RS05740_R	CCACGCAATACACCTCAAC
HPG27_RS01595_F	CAACCAAATCCAAACCATCAAC
HPG27_RS01595_R	GCTTGTAACCCACCTGAATAC

7. Bibliography

Agriesti, F., Roncarati, D., Musiani, F., Del Campo, C., Iurlaro, M., Sparla, F., et al. (2014). FeON-FeOFF: the *Helicobacter pylori* Fur regulator commutates iron-responsive transcription by discriminative readout of opposed DNA grooves. *Nucleic Acids Res.* 42, 3138–3151.

Akada, JK., *et al*. (2000) Identification of the urease operon in Helicobacter pylori and its control by mRNA decay in response to pH. Molecular Microbiology **36**(5), 1071-1084.

Alm, RA *et al*. (1999) Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen Helicobacter pylori. Nature | VOL 397 | 14.

Andersen, M. T., Brondsted, L., Pearson, B. M., Mulholland, F., Parker, M., Pin, C., et al. (2005). Diverse roles for HspR in *Campylobacter jejuni* revealed by the proteome, transcriptome and phenotypic characterization of an hspR mutant. *Microbiology* 151, 905–915.

Ansari S, Yamaoka Y. (2017) Survival of Helicobacter pylori in gastric acidic territory. Helicobacter 22:e12386.

Backert, S and Blaser, MJ. (2016) The Role of CagA in the Gastric Biology of *Helicobacter pylori*. Cancer Res; **76**(14).

Baidya, A. K., Bhattacharya, S., and Chowdhury, R. (2015). Role of the flagellar hook-length control protein FliK and sigma28 in cagA expression in gastric cell-adhered *Helicobacter pylori*. *J. Infect. Dis.* 211, 1779–1789. doi: 10.1093/infdis/jiu808

Balázsi, G and Oltvai, ZN. (2005) Sensing Your Surroundings: How Transcription-Regulatory Networks of the Cell Discern Environmental Signals. Sci. STKE 2005, pe20.

Baltrus, DA et al. (2009) The Complete Genome Sequence of Helicobacter pylori Strain G27. Journal of Bacteriology, Jan. 2009, p. 447–448

Borin BN, Tang W, Krezel AM, (2014) Helicobacter pylori RNA polymerase α-subunit C- terminal domain shows features unique to ε-proteobacteria and binds NikR/DNA complexes, *Protein Sci*, 23(4):454-63.

Boyle, AP., Song, L., Lee, BK *et a*l. (2011) High-resolution genome-wide in vivo footprinting of diverse transcription factors in human cells. *Genome Res.* **21**: 456-464

Boonyanugomol, W *et al*. (2018) Synergistic Effects of Helicobacter pylori Outer Inflammatory Protein A (oipA) and cag Pathogenicity Island (cag PAI) on

Interleukin-1β and Interleukin-8 Gene Expression Levels in Gastric Tissues of Thai Gastroduodenal Patients. Journal pure appl microbiol, **12**(2) p. 459-466.

Chastanet, A.; Prudhomme, M.; Claverys, J.P.; Msadek, T. (2001) Regulation of Streptococcus pneumoniae clp genes and their role in competence development and stress survival. *J. Bacteriol.* ***183***, 7295–7307.

Chastanet, A., Fert, J., Msadek, T. (2003) Comparative genomics reveal novel heat shock regulatory mechanisms in Staphylococcus aureus and other Gram-positive bacteria. Molecular Microbiology. **47(4)**, 1061–1073

Chionh, YT., *et al*. (2014) Heat shock protein complex vaccination induces protection against Helicobacter pylori without exogenous adjuvant Vaccine 32, 2350–2358

Cover, TL and Blanke, SR. *Helicobacter pylori* VacA, a paradigm for toxin multifunctionality. Nat. Rev. Microbiol. **3**:320–332.

Danielli, A., Amore, G., and Scarlato, V. (2010) Built shallow to maintain homeostasis and persistent infection: insight into the transcriptional regulatory network of the gastric human pathogen Helicobacter pylori. *PLoS Pathog* **6**: e1000938.

Danielli, A., Roncarati, D., Delany, I., Chiarini, V., Rappuoli, R., and Scarlato, V. (2006) In vivo dissection of the Helicobacter pylori Fur regulatory circuit by genome-wide location analysis. *J Bacteriol* **188**: 4654–4662.

Debowski AW, Walton SM, Chua E-G, Tay AC-Y, Liao T, Lamichhane B, et al. (2017) Helicobacter pylori gene silencing in vivo demonstrates urease is essential for chronic infection. PLoS Pathog **13**(6): e1006464. https:// doi.org/10.1371/journal.ppat.1006464.

Delany I., Ieva, R., Soragni, A., et al. (2005) In Vitro Analysis of Protein-Operator Interactions of the NikR and Fur Metal-Responsive Regulators of Coregulated Genes in *Helicobacter pylori.* JOURNAL OF BACTERIOLOGY, Vol. **187**, No. 22, November p.7703–7715.

De la Cruz, MA., Ares, MA., von Bargen, K., Panunzi, LG., Martínez-Cruz, J., Valdez-Salazar, HA., Jiménez-Galicia, C., and Torres J (2017) Gene Expression Profiling of Transcription Factors of Helicobacter pylori under Different Environmental Conditions. Front. Microbiol. 8:615.

Dian C, *et al*. (2011) The structure of the Helicobacter pylori ferric uptake regulator Fur reveals three functional metal binding sites. Molecular Microbiology **79**(5), 1260–1275.

Domka J., Lee, J., Bansal, T., Wood, TK. (2007). Temporal gene-expression in *Escherichia coli* K-12 biofilms. Environ Microbiol **9:**332-346.

Dunn, B. E., *et al*. (1997) Localization of Helicobacter pylori urease and heat shock protein in human gastric biopsies. *Infect Immun* **65:**1181–1188.

Eaton, K.A., Suerbaum, S., Josenhans, C., and Krakowka, S. (1996) Colonization of gnotobiotic piglets by *Helicobac- ter pylori* deficient in two flagellin genes. *Infect Immun* **64:** 2445–2448.

Evans, D. J., Jr, D. G. Evans, L. Engstrand, and D. Y. Graham. (1992) Ureaseassociated heat shock protein of Helicobacter pylori. *Infect Immun* **60:**2125–2127.

Foynes, S., Dorrell, N., Ward, S.J., Zhang, Z.W., McColm, A.A., Farthing, M.J., and Wren, B.W. (1999) Functional analysis of the roles of FliQ and FlhB in flagellar expression in *Helicobacter pylori*. *FEMS* Microbiol Lett **174:** 33–39.

Hanahan, D. (1983) Studies on transformation of Escherichia coli with plasmids. *J Mol Biol*, **166**, 557-580.

Hanson, BR., Tan, M. Intra-ChiP: studyng gene regulation in an intracellular pathogen (2016) Curr Genet. **62**(3):547-51.

Hanson, BR., Tan, M. Transcriptional regulation of the Chlamydia heat shock stress response in an intracellular infection. (2015) Molecular Microbiology **97**(6), 1158–1167.

Harris, AG *et al*. (2003) Catalase (KatA) and KatA-associated protein (KapA) are essential to persistent colonization in the Helicobacter pylori SS1 mouse model. Microbiology (2003), 149, 665–672

Hathroubi, S., Zerebinski, J., Ottemann, KM. (2018) The Helicobacter pylori biofilm involves a multi-gene stress-biased response including a structural role for flagella. bioRxiv 412239; doi: https://doi.org/10.1101/412239

Hu, HQ *et al*. (2017) Nickel Ligation of the N-Terminal Amine of HypA Is Required for Urease Maturation in Helicobacter pylori. Biochemistry 2017, 56, 1105−1116

Huesca, M., S. Borgia, P. Hoffman, and C. A. Lingwood. (1996) Acidic pH changes receptor binding specificity of Helicobacter pylori: a binary adhesion

model in which surface heat shock (stress) proteins mediate sulfatide recognition in gastric colonization. *Infect Immun* **64:**2643–2648.

Hung, CL *et a*l (2015) The CrdRS two-component system in Helicobacter pylori responds to nitrosative stress. Molecular Microbiology **97**(6), 1128–1141

Ishijima N, *et al*. (2011) BabA-mediated Adherence Is a Potentiator of the *Helicobacter pylori* Type IV Secretion System Activity. The Journal of Biological Chemistry Vol. **286**, no. 28, pp. 25256–25264.

Jain, S., Graham, C., Graham, R.L., McMullan, G., Ternan, N. (2011) Quantitative proteomic analysis of the heat stress response in Clostridium difficile strain 630. *J Proteome Res* **10**(9): 3880-3890.

Jain, S.; Smyth, D.; O'Hagan, B.M.G.; Heap, J.T.; McMullan, G.; Minton, N.P.; Ternan, N.G. (2017) Inactivation of the dnaK gene in Clostridium difficile 630 Δerm yields a temperature-sensitive phenotype and increases biofilm-forming ability. *Sci. Rep*. *7*, 17522.

Jang, S *et al*. (2016) Dynamic Expansion and Contraction of cagA Copy Number in Helicobacter pylori Impact Development of Gastric Disease. mBio 8:e01779-16.

Josenhans, C *et al.* (2002) Functional characterization of the antagonistic flagellar late regulators FliA and FlgM of Helicobacter pylori and their effects on the H. pylori transcriptome. Molecular Microbiology **43**(2), 307–322.

Kao, CY., Sheu, BS., Wu, JJ. (2016) Helicobacter pylori infection: An overview of bacterial virulence factors and pathogenesis. Biomedical journal 39:14e23

Kavermann, H *et al*. (2003) Identification and Characterization of Helicobacter pylori Genes Essential for Gastric Colonization. J. Exp. Med. Vol197, N 7: 813–822

Kidder, BL., Hu, G., Zhao, K. (2011). ChIP-Seq: technical considerations for obtaining high-quality data. Nature immunology vol12 number 10:918-922.

Kolodziej, K et al. (2009). Optimal use of tandem biotin and V5 tags in ChIP assays. BMC Molecular Biology **10**:6.

Kortmann, J., and Narberhaus, F. (2012) Bacterial RNA thermometers: molecular zippers and switches. *Nat Rev Micro* **10**: 255–265.

Ladeira, SPL., Salvadori, DMF., Rodrigues MAM. (2003) Biopatologia do *Helicobacter pylori.* Jornal Brasileiro de Patologia e Medicina Laboratorial, v. **39**, n. 4, p. 335-342.

Lang, BJ., *et al*. (2016). The Helicobacter pylori cytotoxin CagA is essential for suppressing host heat shock protein expression. Cell Stress and Chaperones 21:523–533.

Langmead, B., and Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9, 357–359.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., et al. (2009). The sequence alignment/map format and SAMtools. *Bioinformatics* 25, 2078–2079.

Li, H., Liao, T., Debowski, AW., Tang, H., Nilsson, HO., Stubbs, KA et al. (2016) Lipopolysaccharide Structure and Biosynthesis in Helicobacter pylori. Helicobacter **21**: 445–461

Lin, C.S., et al. (2010) A potential role for Helicobacter pylori heat shock protein 60 in gastric tumorigenesis. *Biochem Biophys Res Commun* **392**(2): 183-9.

Linden, S., Mahdavi, J., Semino-Mora, C., Olsen, C., Carlst-edt, I., Boren, T., and Dubois, A. (2008) Role of ABO secretor status in mucosal innate immunity and H. pylori infection. PLoS Pathog **4**: e2.

Loh, E., Kugelberg, E., Tracy, A., Zhang, Q., Gollan, B., Ewles, H., Chalmers, R., Pelicic, V., Tang, C. M. (2013) Temperature triggers immune evasion by Neisseria meningitidis. *Nature* **502**(7470):237-40.

Longo, D., Fauci, A., Kasper, D., Hauser, S., Jameson, J., Loscalzo, J. (2011) HARRISON'S Principles of Internal Medicine. Mc Graw Hill. 18th ed (2011).

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15:550. doi: 10.1186/s13059-014-0550-8

Maier, T., Guell, M., Serrano, L. (2009) Correlation of mRNA and protein in complex biological samples. *FEBS Lett* **583**: 3966-3973.

Martirani, L et al (2001) Identification of the DNA-binding protein, HrcA, of Streptococcus thermophilus. *FEMS Microbiology Letters*, Volume 198, Issue 2, 1 May 2001, Pages 177–182.

McClain, MS., *et a*l., (2013). Control of gene expression in *Helicobacter pylori* using the Tet repressor. J Microbiol Methods. December; **95**(3).

Megraud, F and Lamouliatte, H. (2003). Review article: the treatment of refractory *Helicobacter pylori* infection. Aliment Pharmacol Ther 2003; 17: 1333–1343.

Minder, AC., Fischer, HM., Hennecke, H., Narberhaus, F. (2000) Role of HrcA and CIRCE in the Heat Shock Regulatory Network of Bradyrhizobium japonicum. Journal of Bacteriology. Vol. 182, No. 1 p. 14–22.

Mogk, A., Homuth, G., Scholz, C., Kim, L., Schmid, F.X., and Schumann, W. (1997) The GroE chaperonin machine is a major modulator of the CIRCE heat shock regulon of Bacillus subtilis. EMBO J 16: 4579–4590

Moonens, K *et al*. (2018) Helicobacter pylori adhesin HopQ disrupts trans dimerization in human CEACAMs. The EMBO Journal e98665.

Muller, C., Bahlawane, C., Aubert, S., Delay, C. M., Schauer, K., Michaud-Soret, I., et al. (2011). Hierarchical regulation of the NikR-mediated nickel response in *Helicobacter pylori*. *Nucleic Acids Res.* 39, 7564–7575.

Narberhaus, F., and H. Bahl. 1992. Cloning, sequencing, and molecular analysis of the *groESL* operon of *Clostridium acetobutylicum*. J. Bacteriol. **174:**3282–3289.

Narberhaus, F. (1999) Negative regulation of bacterial heat shock genes. *Mol Microbiol* **31**: 1–8.

Necchi, V *et al*. Natural history of Helicobacter pylori VacA toxin in human gastric epithelium in vivo: vacuoles and beyond. Scientific reports | **7**: *14526*

Park, PJ. (2010) ChIP–seq: advantages and challenges of a maturing technology. Nature Reviews Genetic Oct;10(10):669-80

Pelliciari, S., Pinatel, E., Vannini, A., Peano, C., Puccio, S., De Bellis, G., et al. (2017). Insight into the essential role of the *Helicobacter pylori* HP1043 orphan response regulator: genome-wide identification and characterization of the DNA-binding sites. *Sci. Rep.* 7:41063.

Pepe, S., Pinatel, E., Fiore, E., Puccio, S., Peano, C., Brignoli, T., Vannini, A., Danielli, A., Scarlato, V. and Roncarati, D. (2018) The Helicobacter pylori Heat-Shock Repressor HspR: Definition of Its Direct Regulon and Characterization of the Cooperative DNA-Binding Mechanism on Its Own Promoter. Front. Microbiol. 9:1887

Phadnis, S. H., *et al*. (1996) Surface localization of Helicobacter pylori urease and a heat shock protein homolog requires bacterial autolysis. *Infect Immun* **64:**905–912.

Pich, OQ *et al.* (2012) Detailed analysis of Helicobacter pylori Fur-regulated promoters reveals a Fur box core sequence and novel Fur-regulated genes. Molecular Microbiology (2012) 84(5), 921–941

Prelich, G., (2012) Gene Overexpression: Uses, Mechanisms and Interpretation. Genetics, Vol. **190**, 841–854

Quinlan, A. R., and Hall, I. M. (2010). BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* 26, 841–842. doi: 10.1093/ bioinformatics/btq033

De Reuse, H and Bereswill, S. (2007) Ten years after the first *Helicobacter pylori* genome: comparative and functional genomics provide new insights in the variability and adaptability of a persistent pathogen. FEMSImmunolMedMicrobiol50(2007)165–176.

Roncarati, D., Danielli, A., Spohn, Gunther, Delany, I., and Scarlato, V. (2007) Transcriptional regulation of stress response and motility functions in Helicobacter pylori is mediated by HspR and HrcA. *J Bacteriol* **189**: 7234–7243.

Roncarati, D., Spohn, Gunther., Tango, N., Danielli, A., Delany, I., and Scarlato, V. (2007) Expression, purification and characterization of the membrane-associated HrcA repressor protein of *Helicobacter pylori.* Protein Expression and Purification 51 (2007) 267–275.

Roncarati, D., Danielli, A., and Scarlato, V. (2011) CbpA acts as a modulator of HspR repressor DNA binding activity in Helicobacter pylori. *J Bacteriol* **193**: 5629–5636.

Roncarati, D., Danielli, A., and Scarlato, V. (2014) The HrcA repressor is the thermosensor of the heat-shock regulatory circuit in the human pathogen Helicobacter pylori. *Mol Microbiol* doi: 10.1111/mmi.12600. [Epub ahead of print].

Roncarati, D and Scarlato, V (2017). Regulation of heat-shock genes in bacteria: from signal sensing to gene expression output. *FEMS Microbiology Reviews*, Volume 41, Issue 4, 1 July 2017, Pages 549–574,

Roncarati, D. and Scarlato, V (2018) The Interplay between Two Transcriptional Repressors and Chaperones Orchestrates *Helicobacter pylori* Heat-Shock Response. *Int. J. Mol. Sci.* **2018**, *19*, 1702.

Sambrook, J., Fritsch, E., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Satin, B., *et al*. (2000) The Neutrophil-activating Protein (HP-NAP) of *Helicobacter pylori* Is a Protective Antigen and a Major Virulence Factor. Journal of Experimental Medicine, Volume 191, Number 9, May 1, 2000 1467–1476

Sauer, K., Camper, AK., Ehrlich, GD., Costerton, JW., Davies DG. (2002) *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. J Bacteriol **184:**1140-1154.

Servetas, SL *et al*. (2016) Characterization of Key Helicobacter pylori Regulators Identifies a Role for ArsRS in Biofilm Formation. J. Bacteriol. 198, 2536–2548. doi: 10.1128/JB.00324-16

Shapiro, R. S., Cowen, L. E. (2012) Thermal control of microbial development and virulence: molecular mechanisms of microbial temperature sensing. *mBio* **3**(5): e00238-12.

Sharma, CM *et al* (2010) The primary transcriptome of the major human pathogen Helicobacter pylori. Nature|Vol 464|11.

Soleimani, VD., *et. al*. (2013) Chromatin Tandem Affinity Purification Sequencing (ChTAP- Seq). Nat Protoc; 8(8): 1525–1534.

Sperandeo, P., Martorana, AM., Polissi, A. (2017) Lipopolysaccharide biogenesis and transport at the outer membrane of Gram-negative bacteria. Biochimica et Biophysica Acta **1862:**1451–1460.

Spohn, G., and Scarlato, V. (1999) The autoregulatory HspR repressor protein governs chaperone gene transcription in Helicobacter pylori. *Mol Microbiol* **34**: 663– 674.

Spohn, G., and Scarlato, V. (2001) Motility, Chemotaxis, and Flagella. NCBI Bookshelf, Helicobacter pylori: Physiology and Genetics. Washington (DC): ASM Press; chapter 21.

Spohn, G., Danielli, A., Roncarati, D., Delany, I., Rappuoli, R., and Scarlato, V. (2004) Dual control of Helicobacter pylori heat shock gene transcription by HspR and HrcA. *J Bacteriol* **186**: 2956–2965.

Spohn, G., Delany, I., Rappuoli, R., and Scarlato, V. (2002) Characterization of the HspR-mediated stress response in Helicobacter pylori. *J Bacteriol* **184**: 2925–2930.

Stein, SC., Faber, E., Bats, SH., Murillo, T., Speidel, Y., Coombs, N., et al. (2017) Helicobacter pylori modulates host cell responses by CagT4SSdependent

translocation of an intermediate metabolite of LPS inner core heptose biosynthesis. PLoS Pathog **13**(7): e1006514.

Sun, Y., Liu, S., Li, W., Shan, Y., Li, X., Lu, X., et al. (2013). Proteomic analysis of the function of sigma factor sigma54 in *Helicobacter pylori* survival with nutrition deficiency stress in vitro. *PLoS ONE* 8:e72920. doi: 10.1371/journal. pone.0072920

Talarico, S., Whitefield, SE., Fero, J., Haas, R., Salama, NR. (2012) Regulation of Helicobacter pylori adherence by gene conversion. Molecular Microbiology **84** (6), 1050–1061.

Tanaka, A., *et al*. (2009) Helicobacter pylori heat shock protein 60 antibodies are associated with gastric cancer. *Pathol Res Pract* **205**(10): 690-4.

Tanaka, KJ., Song, S., Mason, K., Pinkett, H. (2018) Selective substrate uptake: The role of ATP-binding cassette (ABC) importersin pathogenesis. BBA - Biomembranes **1860**:868–877.

Tatusov, R. L., Koonin, E. V., and Lipman, D. J. (1997). A genomic perspective on protein families. *Science* 278, 631–637. doi: 10.1126/science.278.5338.631

Thomas, C and Tampé, R. (2018) Multifaceted structures and mechanisms of ABCtransport systems in health and disease. Current Opinion in Structural Biology **51**:116–128.

Tomb JF, White O, Kerlavage AR, Clayton RA, Sutton GG, et al. (1997) The complete genome sequence of the gastric pathogen Helicobacter pylori. Nature 388: 539–547.

Vannini, A., Roncarati, D., Spinsanti, M., et al. (2014) In Depht Analysis of the Helicobacter pylori cag Pathogenicity Island Transcriptional Responses. PLoS ONE **9**(6): e98416.

Vannini, A., Agriesti, F., Mosca, F., Roncarati, D., Scarlato, V., Danielli, A. (2012) A convenient and robust in vivo reporter system to monitor gene expression in the human pathogen Helicobacter pylori. *Appl Environ Microbiol* **78**(18): 6524-33

Vannini, A., Pinatel, E., Costantini, P. E., Pelliciari, S., Roncarati, D., Puccio, S., et al. (2017). Comprehensive mapping of the *Helicobacter pylori* NikR regulon provides new insights in bacterial nickel responses. *Sci. Rep.* 7:45458.

Voland, P., *et al*. (2002). Interactions among the seven *Helicobacter pylori* proteins encoded by the urease gene cluster. *Am J Physiol Gastrointest Liver Physiol* 284: G96–G106

Wang, C *et al*. (2014) The concordance between RNA-seq and microarray data depends on chemical treatment and transcript abundance. *Nature Biotechnology* vol. **32**:926–932

Watanabe, K., Yamamoto, T., Suzuki, Y. (2001) Renaturation of *Bacillus thermoglucosidasius* HrcA Repressor by DNA and Thermostability of the HrcA-DNA Complex In Vitro. Journal of Bacteriology, Vol. 183, No. 1, p. 155–161

Weel, J.F., van der Hulst, R.W., Gerrits, et al., (1996) The interrelationship between cytotoxin-associated gene A, vacuolating cytotoxin, and Helicobacter pylori-related diseases. *J Infect Dis*, **173**, 1171-1175.

Wilson, K.T & **Crabtree, J.E.** (2007) Immunology of *Helicobacter pylori*: Insights into the Failure of the Immune Response and Perspectives on Vaccine Studies. Gastroenterology; **133**:288–308

Wilson, AC., Wu, CC., Yates III, JR., Tan, M. (2005) Chlamydial GroEL Autoregulates Its Own Expression through Direct Interactions with the HrcA Repressor Protein. JOURNAL OF BACTERIOLOGY, Vol. **187**, No. 21, p. 7535–7542

Xiang, Z., *et al*., (1995) Analysis of expression of CagA and VacA virulence factors in 43 strains of Helicobacter pylori reveals that clinical isolates can be divided into two major types and that CagA is not necessary for expression of the vacuolating cytotoxin. *Infect Immun*, **63**, 94-98.

Yamaoka Y. (2008) Roles of Helicobacter pylori BabA in gastroduodenal pathogenesis. *World J Gastroenterol* **14**(27): 4265-4272

Yeri, A., Courtright, A., Danielson, K et al. (2018) Evaluation of commercially available small RNASeq library preparation kits using low input RNA. BMC Genomics **19**:331

Appendix

Supplementaty table 1. Table of the up-regulated genes revealed in our transcriptome analysis including the genome ORF name, the 2logfold-change values, the name of the gene, description and COG class.

	log2FC_hs30	padj_hs30	log2FC_dHRCA	padj_dHRCA	Names	description(g27New)	COG_class
cncr1	1,17	1,86077E-24	0,42	0,00	cncR1	HsrA regulated ncRNA	NA
HPG27_RS00070	1,98	2,26077E-86	1,14	0,00	groEL,hspB,hsp60, mopA	molecular chaperone GroEL	Posttranslational modification, protein turnover, chaperones
HPG27_RS00075	2,12	2,4529E-93	1,11	0,00	groES,hspA,hsp10, mopB	co-chaperone GroES	Posttranslational modification, protein turnover, chaperones
HPG27_RS00305	2,43	5,59353E-76	0,24	0,14	HPG27_RS00305	hypothetical protein	-
HPG27_RS00565	1,77	2,8753E-28	-1,02	0,00	HPG27_RS00565	hypothetical protein	-
HPG27_RS00570	2,75	5,069E-131	-0,83	0,00	dnaK	molecular chaperone DnaK	Posttranslational modification, protein turnover, chaperones
HPG27_RS00575	2,96	7,6556E-171	-0,83	0,00	grpE	nucleotide exchange factor GrpE	Posttranslational modification, protein turnover, chaperones
HPG27_RS00580	3,22	1,6103E-236	-0,04	0,80	hrcA	HrcA family transcriptional regulator	-
HPG27_RS00625	1,01	2,96086E-11	1,04	0,00	HPG27_RS00625	hypothetical protein	-
HPG27_RS01045	0,30	0,201097804	1,08	0,00	HPG27_RS01045	LPS biosynthesis protein	Cell wall/membrane/envelope biogenesis
HPG27_RS01220	1,63	2,1288E-59	-0,60	0,00	napA	DNA starvation/stationary phase protection protein	Inorganic ion transport and metabolism;Defense mechanisms
HPG27_RS01305	1,01	0,000261794	0,03	0,95	hpyORF260M,hpy ORFJ244M,mod	adenine-specific DNA methyltransferase	Replication, recombination and repair
HPG27_RS01315	1,01	4,17535E-08	0,42	0,05	HPG27_RS01315	endonuclease	-
HPG27_RS01500	0,16	0,333564367	1,01	0,00	dppB	peptide ABC transporter permease	Inorganic ion transport and metabolism;Amino acid transport and metabolism
HPG27_RS01505	0,45	0,007013328	1,18	0,00	dppC	peptide ABC transporter	Amino acid transport and metabolism;Inorganic ion transport and metabolism
HPG27_RS01510	0,29	0,104780086	1,15	0,00	dppD	ABC transporter ATP-binding protein	Inorganic ion transport and metabolism;Amino acid transport and metabolism
HPG27_RS01515	0,38	0,007486747	1,07	0,00	dppF	ABC transporter ATP-binding protein	Posttranslational modification, protein turnover, chaperones
HPG27_RS02120	2,23	1,01973E-37	0,19	0,43	rarA	recombinase RarA	Replication, recombination and repair
HPG27_RS02125	3,30	2,1339E-132	0,08	0,71	hspR	MerR family transcriptional regulator	Transcription
HPG27_RS02130	3,02	1,3264E-151	-0,19	0,21	dnaJ1,CbpA	DnaJ family protein	Posttranslational modification, protein turnover, chaperones
HPG27_RS02345	1,10	0,003711227	0,46	0,31	HPG27_RS02345	hypothetical protein	-
HPG27_RS02500	1,03	1,06589E-13	-0,20	0,24	cag1	cag pathogenicity island protein Cag1	-
HPG27_RS02590	1,20	7,28832E-10	-0,33	0,18	cagP,cag15	hypothetical protein	-
HPG27_RS02655	1,12	4,23754E-26	-0,07	0,64	cagA,cag26	exotoxin CagA	-
HPG27_RS02700	0,62	0,09516705	1,10	0,00	HPG27_RS02700	hypothetical protein	-
HPG27_RS02740	1,15	1,55327E-22	-3,18	0,00	ykgB	membrane protein	Unknown
HPG27_RS03015	-0,38	0,100116522	2,10	0,00	HPG27_RS03015	LPS biosynthesis protein	Cell wall/membrane/envelope biogenesis
HPG27_RS03120	1,47	2,56791E-41	-0,39	0,00	HPG27_RS03120	hypothetical protein	-
HPG27_RS03125	1,49	9,31134E-43	-0,23	0,07	HPG27_RS03125	3-hydroxy acid dehydrogenase	Lipid transport and metabolism
HPG27_RS03175	1,05	3,71005E-16	0,05	0,81	futB,fucTa	fucosyltransferase	-
HPG27_RS03180	2,09	1,1101E-61	-0,24	0,14	serB	phosphoserine phosphatase SerB	Amino acid transport and metabolism
HPG27_RS03185	2,05	2,23475E-59	-0,59	0,00	pfr	non-heme ferritin	Inorganic ion transport and metabolism
HPG27_RS03460	1,11	2,31026E-12	-0,11	0,62	rpoN	RNA polymerase sigma-54 factor	Transcription
HPG27_RS03465	1,25	5,218E-21	-0,73	0,00	HPG27_RS03465	ABC transporter ATP-binding protein	Cell wall/membrane/envelope biogenesis
HPG27_RS03495	0,94	1,50962E-19	1,17	0,00	hopO,omp16	hypothetical protein	-
HPG27_RS03655	1,70	1,31574E-42	-0,49	0,00	flaG	flagellar protein FlaG	Not_defined
HPG27_RS03660	1,01	2,42257E-16	-1,22	0,00	fliD	flagellar filament capping protein FliD	NA
HPG27_RS03705	0,57	1,18624E-05	1,02	0,00	HPG27_RS03705	5-formyltetrahydrofolate cyclo-ligase	Coenzyme transport and metabolism
HPG27_RS03750	1,27	6,06171E-07	0,23	0,49	mobA	molybdenum cofactor guanylyltransferase	Coenzyme transport and metabolism
HPG27_RS03930	1,28	9,3234E-21	0,52	0,00	lex2B	lipooligosaccharide 5G8 epitope biosynthesis-associated protein (lex2B)	Cell wall/membrane/envelope biogenesis
HPG27_RS03940	-0,63	0,000361166	1,17	0,00	fecA2,fecA,fecA_ 2	ligand-gated channel	NA
HPG27_RS04395	1,59	1,18567E-07	0,71	0,04	HPG27_RS04395	hypothetical protein	-
HPG27_RS04590	1,12	2,87489E-11	0,65	0,00	yckK	amino acid ABC transporter substrate-binding protein	Signal transduction mechanisms;Amino acid transport and metabolism
HPG27_RS04815	1,49	6,76297E-13	0,72	0,00	HPG27_RS04815	hypothetical protein	-
HPG27_RS04990	1,30	0,004731167	0,51	0,35	HPG27_RS04990	hypothetical protein	NA
HPG27_RS05335	1,12	6,94509E-10	0,12	0,65	HPG27_RS05335	hypothetical protein	-
HPG27_RS05345	1,33	4,20285E-34	-0,35	0,01	homC	hypothetical protein	-
HPG27_RS05575	1,18	1,97733E-22	-0,66	0,00	flgM	hypothetical protein	-
HPG27_RS05840	-0,05	0,820398658	1,04	0,00	gluP	glucose/galactose MFS transporter	Carbohydrate transport and metabolism
HPG27_RS06650	2,32	1,89229E-52	-0,45	0,02	HPG27_RS06650	hypothetical protein	-
HPG27_RS06720	1,04	9,3952E-13	0,83	0,00	HPG27_RS06720	molecular chaperone DnaJ	-
HPG27_RS06750	1,30	1,60496E-07	0,30	0,34	HPG27_RS06750	hypothetical protein	-
HPG27_RS06930	-0,30	0,213635174	1,61	0,00	HPG27_RS06930	hypothetical protein	Replication, recombination and repair
HPG27_RS06935	-0,20	0,331805443	1,95	0,00	HPG27_RS06935	restriction endonuclease	Unknown
HPG27_RS07135	1,96	2,27499E-36	-0,68	0,00	HPG27_RS07135	hypothetical protein	-
HPG27_RS07245	1,32	4,85103E-27	-0,29	0,04	HPG27_RS07245	cytochrome c biogenesis protein CcsA	Posttranslational modification, protein turnover, chaperones
HPG27_RS07620	1,01	7,39219E-17	-0,07	0,69	hsdR3,hsdR	restriction endonuclease subunit R	Defense mechanisms

HPG27_RS07790	1,31	1,01787E-33	0,56	0,00	rps2,rpsB	30S ribosomal protein S2	Translation, ribosomal structure and biogenesis
HPG27_RS07795	1,17	8,06279E-27	0,17	0,22	tfs,tsf	elongation factor Ts	Translation, ribosomal structure and biogenesis
HPG27_RS07980	2,60	4,6717E-119	0,29	0,03	HPG27_RS07980	replication initiation protein	-
HPG27_RS07985	2,24	7,22088E-84	0,08	0,63	HPG27_RS07985	hypothetical protein	Transcription
HPG27_RS07990	2,15	8,05052E-26	0,38	0,14	HPG27_RS07990	cAMP-induced filamentation protein	Transcription
HPG27_RS07995	0,14	0,526333043	1,01	0,00	HPG27_RS07995	MFS transporter	-
HPG27_RS08000	-0,40	0,008816486	1,66	0,00	HPG27_RS08000	hypothetical protein	Coenzyme transport and metabolism
HPG27_RS08015	3,31	3,48494E-85	0,23	0,34	HPG27_RS08015	plasmid mobilization relaxosome protein MobC	-
HPG27_RS08020	1,50	2,28517E-23	-0,24	0,22	HPG27_RS08020	hypothetical protein	-
HPG27_RS08025	1,51	1,3945E-29	0,17	0,34	HPG27_RS08025	YafQ family addiction module toxin	-
HPG27_RS08160	1,05	3,83502E-07	-2,29	0,00	HPG27_RS08160	hypothetical protein	-
HPG27_RS08345	2,21	2,86537E-82	0,19	0,19	HPG27_RS08345	molybdopterin-guanine dinucleotide biosynthesis protein MobA	-

Supplementary table 2. Table of the down-regulated genes revealed in our transcriptome analysis including the genome ORF name, the 2logfold-change values, the name of the gene, description and COG class.

	log2FC_hs30	padj_hs30	log2FC_dHRCA	padj_dHRCA	Names	description(g27New)	COG class
HPG27_RS00160	-1,1	0,007	-0,6	0,194	bioD	dethiobiotin synthase	Coenzyme transport and metabolism
HPG27_RS00240	0,5	0,007	-2,3	0,000	tnpB	transposase	Mobilome: prophages, transposons
HPG27_RS00410	-1,9	0,000	0,1	0,361	prfA	peptide chain release factor 1	Translation, ribosomal structure and biogenesis
HPG27_RS00565	1,8	0,000	-1,0	0,000	HPG27_RS00565	hypothetical protein	-
HPG27_RS00595	-0,3	0,062	-2,2	0,000	HPG27_RS00595	motility accessory factor	Unknown
HPG27_RS00600	-0,1	0,655	-2,1	0,000	flaB	flagellin B	Cell motility
HPG27_RS00760	-1,0	0,000	-0,5	0,003	HPG27_RS00760	hypothetical protein	-
HPG27_RS00935	-1,7	0,000	-0,7	0,066	HPG27_RS00935	hypothetical protein	-
HPG27_RS01005	-1,5	0,000	-0,7	0,000	HPG27_RS01005	hypothetical protein	-
HPG27_RS01480	0,5	0,000	-2,1	0,000	flgL	flagellar hook-associated protein FlgL	Cell motility
HPG27_RS01545	-1,1	0,000	-0,2	0,615	HPG27_RS01545	hypothetical protein	-
HPG27_RS01595	0,1	0,505	-1,2	0,000	babA, omp28	membrane protein	-
HPG27_RS01870	-0,3	0,213	-2,0	0,000	HPG27_RS01870	hypothetical protein	-
HPG27_RS01990	0,6	0,000	-1,1	0,000	lpxC,envA	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase	Cell wall/membrane/envelope biogenesis
HPG27_RS01995	-0,2	0,431	-1,1	0,000	HPG27_RS01995	hypothetical protein	Translation, ribosomal structure and biogenesis
HPG27_RS02255	0,8	0,000	-2,0	0,000	horE, omp11	membrane protein	-
HPG27_RS02575	0,5	0,001	-2,4	0,000	HPG27_RS02575	transposase	NA
HPG27_RS02665	-1,6	0,000	-0,6	0,006	murI,glr	glutamate racemase	Cell wall/membrane/envelope biogenesis
HPG27_RS02680	-1,0	0,000	-0,3	0,009	rsmI	16S rRNA (cytidine(1402)-2'-O)-methyltransferase	Translation, ribosomal structure and biogenesis
HPG27_RS02740	1,1	0,000	-3,2	0,000	ykgB	membrane protein	Unknown
HPG27_RS02925	0,9	0,000	-1,5	0,000	flaA	flagellin A	Cell motility
HPG27_RS03155	-1,1	0,000	-0,1	0,807	HPG27_RS03155	hypothetical protein	-
HPG27_RS03160	-1,1	0,000	-0,2	0,385	murA,murZ	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	Cell wall/membrane/envelope biogenesis
HPG27_RS03170	-1,7	0,000	0,1	0,547	HPG27_RS03170	hypothetical protein	Replication, recombination and repair
HPG27_RS03285	-1,3	0,000	-0,2	0,628	ogt, dat1	methylated-DNA--protein-cysteine methyltransferase	Replication, recombination and repair
HPG27_RS03325	-1,0	0,000	0,3	0,016	fecA1,fecA,fecA_1	ligand-gated channel	Inorganic ion transport and metabolism
HPG27_RS03400	-1,1	0,000	-0,2	0,392	HPG27_RS03400	hypothetical protein	-
HPG27_RS03410	-1,4	0,000	0,3	0,388	HPG27_RS03410	hypothetical protein	-
HPG27_RS03415	-1,1	0,000	0,5	0,017	HPG27_RS03415	hypothetical protein	NA
HPG27_RS03550	0,5	0,001	-2,4	0,000	tnpB	transposase	Mobilome: prophages, transposons
HPG27_RS03660	1,0	0,000	-1,2	0,000	fliD	flagellar filament capping protein FliD	NA
HPG27_RS03665	0,8	0,000	-1,2	0,000	fliS	flagellar protein FliS	Cell motility;Intracellular trafficking, secretion, and vesicular transport
HPG27_RS03670	0,6	0,008	-1,3	0,000	FliT	hypothetical protein	-
HPG27_RS04060	-1,2	0,000	0,2	0,373	HPG27_RS04060	RNA-binding protein	Translation, ribosomal structure and biogenesis
HPG27_RS04250	0,0	0,918	-1,1	0,000	hypA	hydrogenase/urease nickel incorporation protein HypA	Not_defined
HPG27_RS04255	0,4	0,015	-2,1	0,000	flgE	flagellar hook protein FlgE	Cell motility
HPG27_RS04285	-1,1	0,000	0,3	0,011	frpB1,frpB	membrane protein	Inorganic ion transport and metabolism
HPG27_RS04430	0,5	0,001	-2,0	0,000	fliK	flagellar hook-length control protein FliK	-
HPG27_RS04635	-1,5	0,000	0,0	0,835	nhaC	sodium:proton antiporter	Energy production and conversion
HPG27_RS04645	0,5	0,005	-2,4	0,000	tnpB	transposase	Mobilome: prophages, transposons
HPG27_RS04725	0,0	0,954	-4,5	0,000	HPG27_RS04725	ATPase	Replication, recombination and repair
HPG27_RS04730	0,1	0,865	-4,5	0,000	HPG27_RS04730	hypothetical protein	-
HPG27_RS04735	0,5	0,002	-2,6	0,000	tnpB	transposase	Mobilome: prophages, transposons
HPG27_RS04920	0,9	0,001	-1,4	0,000	HPG27_RS04920	hypothetical protein	-
HPG27_RS05095	-1,1	0,000	0,2	0,401	parA	chromosome partitioning protein ParA	Cell motility
HPG27_RS05380	0,0	0,996	-1,2	0,000	pseC	UDP-4-amino-4,6-dideoxy-N-acetyl-beta-L-altrosamine transaminase	Cell wall/membrane/envelope biogenesis
HPG27_RS05560	0,4	0,001	-2,1	0,000	flgK	flagellar hook-associated protein FlgK	Cell motility
HPG27_RS05565	0,2	0,217	-2,2	0,000	HPG27_RS05565	hypothetical protein	-
HPG27_RS05580	-1,0	0,000	-0,5	0,000	HPG27_RS05580	hypothetical protein	-
HPG27_RS05710	-1,3	0,000	0,6	0,003	trmD	tRNA (guanosine(37)-N1)-methyltransferase TrmD	Translation, ribosomal structure and biogenesis

HPG27_RS05715	-1,3	0,000	0,1	0,417	rimM	16S rRNA-processing protein RimM	Translation, ribosomal structure and biogenesis
HPG27_RS05740	0,0	0,996	-1,1	0,000	fliw	flagellar assembly protein FliW	Cell motility
HPG27_RS05745	-0,2	0,191	-1,0	0,000	murG	undecaprenyldiphospho-muramoylpentapeptide beta-N-acetylglucosaminyltransferase	Cell wall/membrane/envelope biogenesis
HPG27_RS05770	-1,4	0,000	0,1	0,597	ybeY	endoribonuclease YbeY	Translation, ribosomal structure and biogenesis
HPG27_RS06160	-1,0	0,000	-0,4	0,035	HPG27_RS06160	hypothetical protein	-
HPG27_RS06175	0,1	0,483	-1,9	0,000	HPG27_RS06175	hypothetical protein	-
HPG27_RS06655	-1,1	0,000	0,1	0,453	rnhB	ribonuclease HII	Replication, recombination and repair
HPG27_RS07015	-1,2	0,000	0,4	0,175	HPG27_RS07015	hypothetical protein	Cell wall/membrane/envelope biogenesis
HPG27_RS07055	0,4	0,011	-1,1	0,000	HPG27_RS07055	hypothetical protein	-
HPG27_RS07080	0,6	0,000	-1,5	0,000	HPG27_RS07080	nickel transporter	-
HPG27_RS07180	-1,4	0,000	-0,3	0,333	HPG27_RS07180	membrane protein insertion efficiency factor YidD	Cell wall/membrane/envelope biogenesis
HPG27_RS07930	-1,6	0,000	0,5	0,000	pdxJ	pyridoxine 5'-phosphate synthase	-
HPG27_RS07935	-1,6	0,000	0,4	0,007	pdxA	4-hydroxythreonine-4-phosphate dehydrogenase	Coenzyme transport and metabolism
HPG27_RS07940	-1,3	0,000	0,7	0,000	gcp,tsaD	tRNA (adenosine(37)-N6)-threonylcarbamoyltransferase complex transferase subunit TsaD	Translation, ribosomal structure and biogenesis
HPG27_RS07950	-2,8	0,000	-1,0	0,000	HPG27_RS07950	ubiquinol-cytochrome C chaperone family protein	Unknown
HPG27_RS07970	-1,2	0,000	0,6	0,000	HPG27_RS07970	hypothetical protein	NA
HPG27_RS08050	0,5	0,001	-2,0	0,000	HPG27_RS08050	exonuclease VII large subunit	-
HPG27_RS08060	-2,8	0,000	-0,8	0,000	HPG27_RS08060	hypothetical protein	-
HPG27_RS08160	1,0	0,000	-2,3	0,000	HPG27_RS08160	hypothetical protein	-
HPG27_RS08320	0,6	0,014	-1,4	0,000	HPG27_RS08320	hypothetical protein	-
HPG27_RS08340	-1,4	0,004	-0,6	0,252	HPG27_RS08340	flagellar biosynthesis protein FlgG	-
isoB	0,3	0,634	-1,6	0,002	isoB	NA	NA