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The heat-shock response of *Helicobacter pylori*: genomic and molecular characterization of the master repressor HspR

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Abstract

The heat-shock response (HSR) induces the expression of heat-shock proteins, ensuring the bacterial cells to adapt to hostile environmental conditions during stress. In *Helicobacter pylori*, the regulation of the principal genes encoding the heat-shock proteins is under the transcriptional control of two repressor proteins named HspR and HrcA, with the former acting as the master regulator of the circuit. In order to further characterize the HspR regulon and deepen our understanding of HSR in *H. pylori* we used global transcriptome analysis in combination with Chromatin ImmunoPrecipitation of *in vivo* HspR genomic binding sites. These data showed that HspR is involved in the regulation of different cellular crucial functions directly controlling a limited set of target genes. Moreover, to provide further details on HspR-DNA interactions on its genomic targets we performed hydroxyl-radical footprinting experiments. This analysis revealed a peculiar periodic pattern of DNA protection. From a nucleotide sequence alignment of HspR binding sites, DNA sequences with similarities to the HAIR motif were identified. Through site-directed mutagenesis we demonstrated *in vitro* that the HAIR-like motif is essential for the HspR binding to its own promoter region and that non-conserved nucleotides flanking the HAIR-like motif are necessary for the HspR complete binding on its operator sequence.

An important role in resistance against environmental stresses is also played by the ATP-dependent caseinolytic proteases (Clp), a class of serine proteases involved in protein quality control as well as in degradation of regulatory proteins. In order to get more information about the role played by the Clp proteases in *H. pylori* and to directly identify their protein substrates, we implemented a strategy to express *in vivo* a proteolytic inactive form of ClpP, the catalytic subunit of this class of proteases, that will retain but not degrade substrates translocated into its proteolytic chamber.

1. *Introduction*

1.1 Epidemiology and infection of *Helicobacter pylori*

Helicobacter pylori is a gram negative, spiral shaped, microaerophilic, flagellated bacterium, isolated for the first time by Barry Marshall and Robyn Warren in 1983 from a gastric biopsy of a patient suffering from duodenal cancer (Warren et al., 1983). To date, *H. pylori* is one of the most widespread and successfull human pathogen, infecting the gastric mucosa of about 50% of the population in the world and it is recognized as the principal causative agent of chronic active gastritis (Blaser, 1990), gastric and peptic ulcer diseases (Nomura et al., 1994), and lymphoma of the mucosa-associated lymphoid tissue (or MALT-lymphoma). For these reasons, it has been classified as a class 1 carcinogen by the World Health Organization (WHO) (Vogiatzi et al., 2007). Nowadays, *H. pylori* infections can be treated with antibiotics, however, the available therapies are beginning to lose efficacy because of insurgence of antibiotic resistance. For example, due to a constant increase in *H. pylori* resistance to clarithromycin, the triple clarithromycin-based treatment has become progressively less efficacious. Therefore, *H. pylori*, that remains a bacterial pathogen of major medical importance, was recently included by the WHO in a global priority list of 12 antibiotic-resistant bacterial pathogens to help in prioritizing the research, discovery, and development of new antibiotics (World Health Organization, 2017).

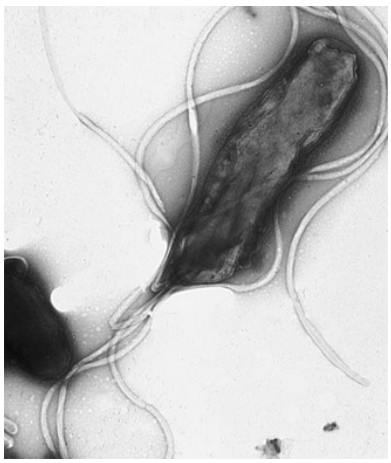


Figure 1. Electron micrograph of *H. pylori* possessing multiple flagella (negative staining); source: Yutaka Tsutsumi, MD, Wikimedia Commons.

In order to establish a persistent infection, *H. pylori* relies on many different virulence factors, that allow the bacterium to contact, enter and persist in the host and to face harsh conditions, typical of the human stomach (Peterson, 1996). Several virulence factors for gastric colonization, tissue damage and survival have been identified in *H. pylori*: among the most important, flagellins (Suerbaum et al., 1993), that allow the bacterium to move into the stomach lumen and through the

viscous mucus layer overlying the gastric epithelium (Suerbaum, 1995), and the urease enzyme, which hydrolyses urea into ammonia and carbon dioxide leading to a pH increase (Labigne et al., 1991; Cussac et al., 1992). Several other bacterial factors are associated to pathogenesis, infection and colonization of the gastric epithelium, like the cytotoxin-associated protein CagA, the vacuolating toxin VacA and various mechanisms of molecular mimicry that allow the bacterium to elude the host immune response (Covacci et al., 1997; de Bernard et al., 1995; Andersen-Nissen et al., 2005). Among these virulence factors, the highly conserved class of stress-induced proteins, known as Heat-Shock Proteins (HSPs), has to be included (Kao et al., 2016). Typically, these proteins play a pivotal role in maintaining cellular homeostasis under physiological and stress growth conditions, by ensuring the correct folding of newly synthetized proteins or by helping to refold damaged proteins (Roncarati et al., 2017). However, at the same time, several evidences show that the heat-shock proteins of *H. pylori* play also non-canonical roles, as for example in the infectious process and interaction with the host (Hoffman et al., 2003; Kao et al., 2016). In particular, the GroEL and GroES homologues of *H. pylori* are considered important modulators of the stability and activity of the urease enzyme (Dunn et al., 1997; Evans et al., 1992; Kansau et al., 1996) and both DnaK and GroEL can be found in association with the outer membrane protein, and this surface localization has been suggested to modify the glycolipid binding specificity of *H. pylori* cells at a low pH (Huesca et al., 1996). In *H. pylori* motility is associated with pathogenesis, and colonization of the gastrointestinal tract depends on the presence of flagellins and also of heat-shock proteins. In this regard, it has been reported that single and double *ΔhspR*/*ΔhrcA* mutant strains of *H. pylori* G27 are non-motile. Probably, the induction of chaperone proteins alters the assembly of the flagellar apparatus which in tourn could led to an inefficient colonization and infection of the gastrointestinal tract (Roncarati et al., 2007a). Furthermore, heat-shock proteins has been identified also as one of the potential immunogens of the bacterium that induces IL-6, IL-8 and tumor necrosis factor alpha (TNF-α). In particular, Hsp60 induces activation of NF-κB via TLR2 and thereby induces human monocytes to secrete IL-8 (Lin et al., 2009; Zhao et al., 2007). Infact, anti-Hsp60 antibodies are consistently detected in *H. pylori*-infected patients (Tanaka et al., 2009). Moreover, it has been reported that IL-12-deficient mice are more permissive for colonization by *H. pylori* SS1 wild-type strain than the wild-type parental mice, and even more by the *H. pylori* SS1 *ΔhspR*/*ΔhrcA* double mutant strain, emphasising the role of Hsp60 and Hsp70 chaperone proteins as potential immunogens of *H. pylori* (Hoffman et al., 2003).

1.2 Heat-shock proteins

The so-called Heat-Shock Proteins (HSPs) or stress proteins constitute a highly conserved family of proteins, expressed in a wide variety of organisms from bacteria to humans (Qiu et al., 2006; Caplan et al., 1993). Usually, this class of proteins accumulates following exposure to different kind of stressful conditions, playing a pivotal role in adaptation and survival of the bacterium (Roncarati and Scarlato 2017). Under physiological growth conditions, these proteins ensure the correct folding, translocation and assembly of cellular proteins acting as molecular chaperones: they mediate protein folding, through ATP-regulated cycles of binding and release, recognizing hydrophobic amino acid residues, exposed by unfolded proteins (Vabulas et al., 2010; Kim et al., 2013). During stress conditions, since spontaneous folding of newly synthetized proteins is inefficient and error-prone, and a large fraction of already folded proteins gets partially or completely denatured, the key role of the HSPs is to prevent protein misfolding and aggregation (Mogk et al., 1999; Vabulas et al., 2010). If refolding is not possible, the degradation of misfolded proteins by cellular proteases is a necessary event to prevent the formation of cytoplasmic amorphous aggregates that could have a severe impact on bacterial survival. Historically, the heat-shock proteins are classified according their molecular weight; among them, the most widely studied and characterized families of HSPs are Hsp60 and Hsp70, well known in *E. coli* and in other bacteria, as DnaK and GroEL and whose ATPase activity is assisted and stimulated by co-chaperone proteins. For example, in both *E. coli* and *H. pylori*, GroES is the co-chaperone of GroEL, while GrpE and DnaJ assist the chaperone activity of DnaK. In *H. pylori* the major encoded heat-shock proteins are listed in Table 1 and can be divided in two main functional groups: chaperone proteins that assist protein folding and assembly, and stress proteases, whose function in the cell includes the removal of misfolded and aggregated polypeptides.

Protein	Genomic locus	Functions
GroEL (Hsp60, Cpn60) GroES (Hsp10, Cpn10)	HPG27_RS00070 HPG27_RS00075	60 kDa molecular chaperonin, protein folding 10 kDa co-chaperone of GroEL chaperonin
DnaK (Hsp70) DnaJ (Hsp40) GrpE CbpA	HPG27_RS00570 HPG27_RS06700 HPG27_RS00575 HPG27_RS02125	70 kDa molecular chaperone, protein folding Co-chaperone of DnaK Co-chaperone and nucleotide exchange factor of DnaK Co-chaperone of DnaK and putative nucleoid associated protein
HtpG (Hsp90)	HPG27_RS01060	Chaperone protein
FtsH HtpX	HPG27_RS01900 HPG27_RS04530	ATP-dependent zinc metallo-protease Membrane-localized metallo-protease
Lon	HPG27_RS06910	ATP-dependent protease
ClpB (Hsp100)	HPG27_RS01325	ATP-dependent protease
HsluV (ClpQ) HsluU (ClpY)	HPG27_RS02475 HPG27_RS02480	ATP-dependent protease, proteolytic subunit ATPase subunit, binds and translocates substrate to HslV
ClpP ClpA ClpS ClpX	HPG27_RS03875 HPG27_RS00180 HPG27_RS00175 HPG27_RS06885	ATP-dependent protease, proteolytic subunit ATP-dependent specificity component of the ClpAP protease ATP-dependent Clp protease adaptor for the ClpAP protease ATP-dependent specificity component of the ClpXP protease

Table 1. *Helicobacter pylori* heat-shock proteins. In the left column, names referred to currently used nomenclature of *H. pylori* HSPs are reported. Alternative names of heat-shock proteins are reported in brackets. In the central column, names of genomic loci, assigned according to the reference genome sequence of *H. pylori* G27 strain. Table adapted from Roncarati and Scarlato 2018a.

1.3 *Helicobacter pylori* chaperone proteins

The *H. pylori* genes encoding almost all the members belonging to the two major chaperone families Hsp60 and Hsp70 are clustered in three multicistronic operons, transcriptionally controlled by three different promoters named P*gro*, P*hrc* and P*cbp* (Figure 2).

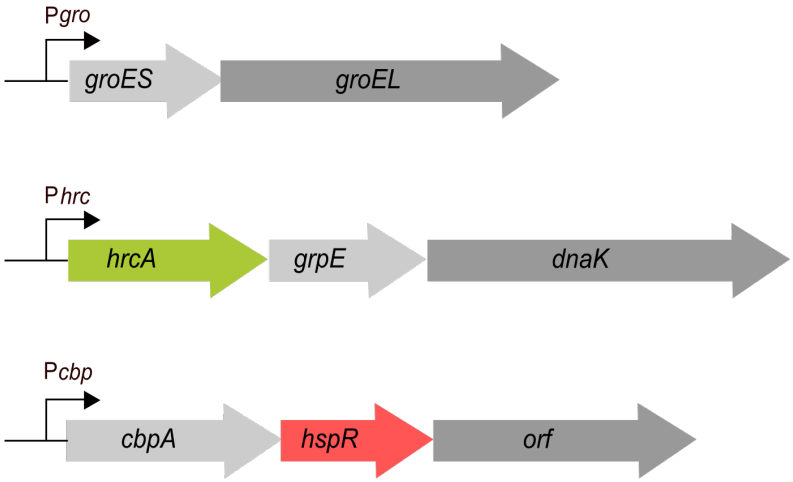


Figure 2. Schematic representation of the three multicistonic operons encoding the major *H. pylori* chaperone proteins. The two regulatory genes are depicted with coloured arrows, while grey arrows represent chaperone genes and the putative helicase encoding gene (named *orf*). The transcriptional start site (TSS) of the three P*gro*, P*hrc* and P*cbp* promoters are indicated by a bent arrow.

In particular, the GroEL chaperone, together with its co-chaperone GroES (Hsp10) (Macchia et al., 1993; Suerbaum et al., 1994), belong to the bi-cistronic operon regulated by the P*gro* promoter; while the coding sequence of the DnaK system members are transcribed by different transcription units. The DnaK co-chaperone GrpE, that acts as nucleotide exchange factor stimulating the rate of ADP release, is in the same operon of the DnaK chaperone, together with the *hrcA* gene (coding for the heat-shock repressor HrcA). Conversely, the gene coding for DnaJ, the principal co-chaperone of DnaK, that enhances its ATPase activity positively regulating the nucleotide-bound (ATP) affinity of the chaperone, maps in a different genomic locus, not represented in Figure 2. Finally, the first gene that belongs to the multicistronic operon regulated by the P*cbp* promoter, encodes for the other DnaK co-chaperone, named CbpA and classified as DnaJ-like protein for its functional homology with the DnaJ co-chaperone (Hinault et al., 2010). The same operon that encodes for the CbpA protein harbours also DNA sequences coding for the heat-shock master repressor HspR and a protein of still poor defined function, with similarities with a putative helicase of *Heamophilus influenzae* (Tomb et al., 1997).

1.4 *Helicobacter pylori* stress proteases

As already mentioned, the other group of heat-shock proteins expressed by *H. pylori* is represented by the class of stress related proteases. This set of proteins with protease activity, plays an important role in resistance against environmental stresses and in the control of bacterium cellular adaptations, by ensuring the proper functioning of protein quality control mechanism as well as by removing mis-folded and denatured polypeptides from stressed cells. Some proteases are composed by a catalytic subunit (e.g. ClpP and HslV) which is associated to different substrate recognition subunits (ClpA or ClpX for ClpP and HslU for HslV). These are ATP-dependent components, harbouring chaperone activity and are able to determine substrate specificity, to remodel target polypeptides upon ATP hydrolysis and deliver them to proteolytic degradation (Missiakas et al. 1996; Wawrzynow, Banecki and Zylicz, 1996). While these stress proteases assemble into complex ring-shaped structures, other members of this group of heat-shock proteins combine on a single polypeptide both chaperone and protease activities (e.g. Lon, FtsH and the membrane bound metalloprotease HtpX). Despite being a member of the Clp ATPase protein family, the oligomeric chaperone ClpB does not associate with a peptidase. Its activity, fundamental for the survival of cells during severe stress, together with DnaK, DnaJ and GrpE, consists in resolubilization of protein aggregates. Specifically, protein binding stimulates ATP hydrolysis, which leads to the unfolding/disaggregation of the denatured protein aggregates (Haslberger et al., 2010). Recently, it has become clear that proteolysis represents a real regulatory mechanism that controls key physiological processes of the cell. In this regard, the class of caseinolytic proteases (ClpPs), in association with the chaperone activity of Clp ATPases, contributes to this type of regulation, controlling stability and activity of central regulatory proteins.

1.4.1 ATP-dependent caseinolytic proteases (ClpPs)

In *H. pylori*, as in the case of the chaperone proteins, the *clp* genes belong to three different multicistronic operons transcribed by different transcription units, represented in Figure 3. The *clpP* gene maps in the same operon that includes the *tig*, *def* and *comM* genes (HPG27_RS03880; HPG27_RS03870; HPG27_RS03865) encoding for a trigger factor, a peptide deformylase and a DNA transformation competence protein, respectively. The ClpA ATPase protein (HPG27_RS00180), together with its adaptor protein ClpS (HPG27_RS00175), belong to the same multicistronic operon, while the coding sequence of the other ClpP ATPase, ClpX (HPG27_RS06885) is transcribed by a different transcription unit.

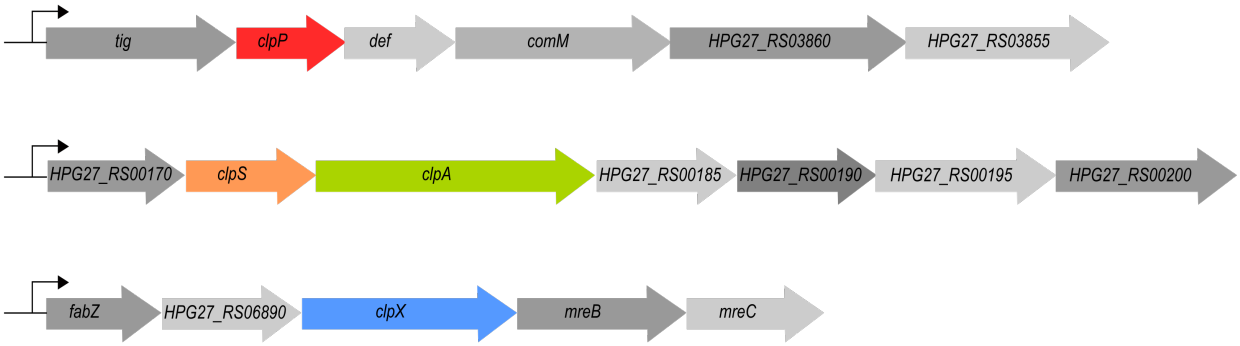


Figure 3. Schematic representation of the three multicistonic operons encoding the *H. pylori clp* genes. The ClpP proteolytic subunits, the ClpS adaptor protein, the ClpA and ClpX ATPases subunits are depicted with coloured arrows, while the other genes that belong to the three operons are represented by grey arrows. Genes names refer to the current nomenclature used in *H. pylori*, according to the *H. pylori* G27 reference genome. The predicted transcriptional start sites (TSS) of the three operons' promoters are indicated by a bent arrow.

As mentioned before, the ATP-dependent ClpP proteases are two-component proteases consisting of separately encoded ATPase and peptidase subunits (Figure 4). The ClpP proteolytic core is represented by 14 serine peptidase subunits, assembled *in vivo* into two heptameric rings forming an internal chamber, composed of 14 proteolytic active sites (represented by the Ser-His-Asp catalytic triad residues) and having a diameter of only 10 Å, a size not sufficient to admit most folded proteins but large enough to accommodate an unstructured polypeptide (Wang et al., 1997). As shown in Figure 4, to gain proteolytic activity, the ClpP multimer associates with one or two hexameric rings of Clp ATPases (ClpA or ClpX), responsible for the recognition, unfolding and translocation of substrates into the ClpP degradation chamber in a process that may be modulated by adaptor proteins, such as ClpS for ClpA.

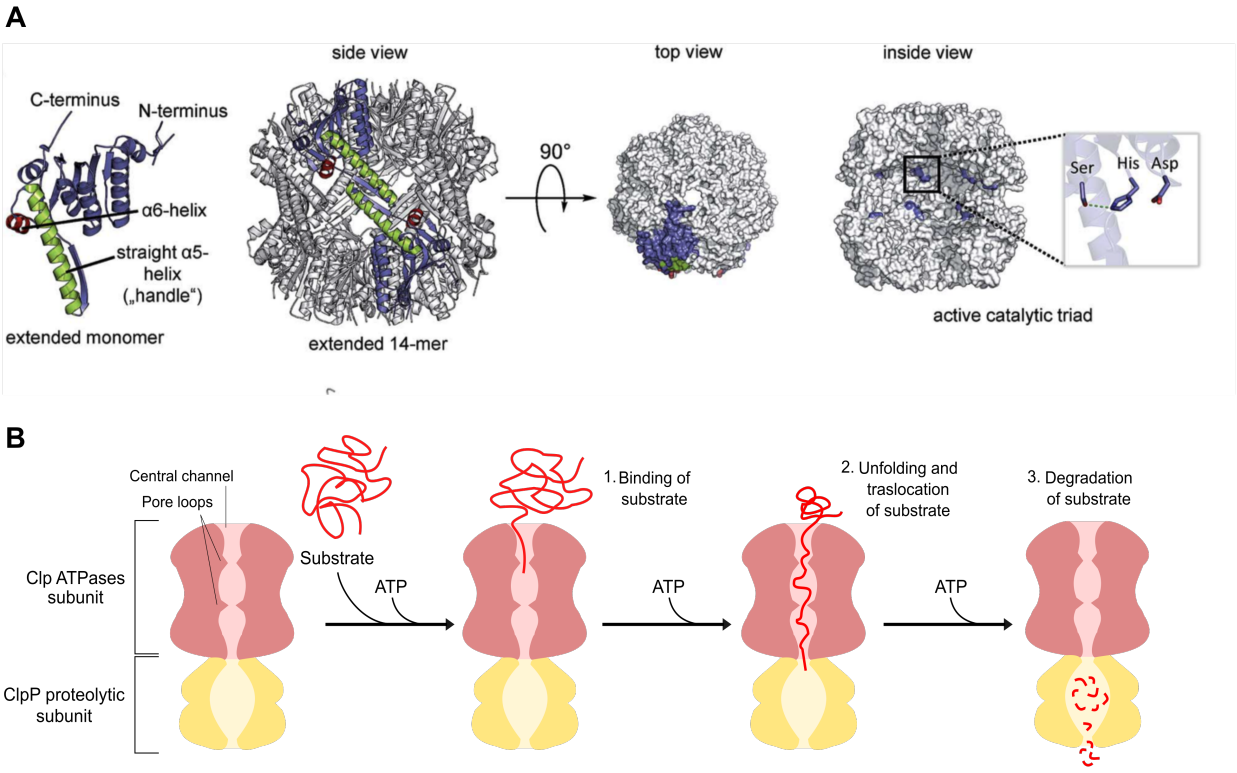


Figure 4. (A) Crystal structure of ClpP proteolytic core from *S. aureus*. The α5 and α6 helices, responsible for the ring–ring connection, are depicted in green and red, respectively. The top and inside views are depicted in a surface fill model representation. The inside view reveals the Ser-His-Asp catalytic triad residues, within the barrel, and the orientations of their side chains (Malik and Br¨otz-Oesterhelt, 2017). **(B)** Schematic representation of the ATP-dependent ClpP proteases, composed by an ATPase and peptidase subunit. Initially, protein substrate interacts with the Clp chaperone component, via the amino-terminal domain and/or the pore loop Tyr residues. Then, the substrate is unfolded as it is translocated through the central channel of the Clp ATPases subunit using the energy from ATP hydrolysis. Finally, unfolded polypeptide is degraded in the central chamber of the ClpP proteolytic subunit. (Figure adapted from Doyle et al., 2013).

Tipically, production of ClpP and most Clp ATPases, is strongly increased in response to heat-shock and other stress conditions. For example, in low GC Gram-positive bacteria, as in the case of *Bacillus subtilis*, the *clp* genes are transcriptionally controlled by the dedicated heat-shock repressor CtsR (Derré et al., 1999). Moreover, the expression of the *clp* genes, in several *Lactobacillus* species, seems to be regulated by the HrcA repressor (Suokko et al. 2005 and 2008), while in *S. coelicolor* and in *Campylobacter jejuni*, is under the transcriptional control of the heat-shock repressor HspR (Bucca et al., 2003; Holmes et al., 2010). On the other hand, in literature there are no data about the transcriptional or post-transcriptional regulation of this class of proteases in *H. pylori*. However, it is known that in the *H. pylori SS1* strain, the Clp proteases seem to have an important role in resistance to antibiotics, oxidative stress, and virulence of the bacterium. Indeed, the *H. pylori SS1 clpA*/*clpP* double mutant strain has fully abolished colonization in murine models

(Loughlin et al., 2009). Accordingly, also among a great number of bacterial species, this conserved class of proteins play pivotal roles in controlling specific stress responses or physiological cellular processes, tightly regulating the protein level of transcriptional regulators, through a molecular mechanism defined regulated proteolysis. For example, in *B. subtilis* and in *E. coli*, Clp proteins are involved in the control of the heat-shock response, degrading the CtsR repressor and the alternative heat inducible sigma factor, named σ^E, respectively (Elsholz et al., 2010; Chaba et al., 2007). In *Lactobacillus lactis* the expression of target genes, upon either heat-shock or DNA damage stimuli, is induced when the dedicated transcriptional repressor HdiR is degraded by the ClpX/P protease complex (Savijoki et al., 2003). Other biologically important examples of regulated proteolysis are presented by the ClpC/P protease complex that, in *B. subtilis*, by degrading the transcriptional activator ComK, controls the expression of genes encoding the DNA uptake apparatus (Msadek et al., 1994; Kong and Dubnau, 1994; Turgay et al., 1997 and 1998), and by the *Staphylococcus aureus* ClpX/P complex, necessary for the timely production of virulence factors involved in the local and systemic spread of this important bacterial pathogen (Frees et al., 2003). In conclusion, considering that the Clp proteases are of great importance to control key physiologycal processes and to establish a persistent infection in several unrelated pathogenic bacteria (Butler et al., 2006), is becoming increasingly frequent the development of experimental approaches aiming at finding new *in vivo* ClpP protein substrates and the identification of small molecules able to inhibit its proteolytic activity (Figure 5). Thus, these chemical compounds, by blocking the ClpP activity, could negatively impact on virulence pathways, representing a promising strategy for the development of novel antimicrobial agents.

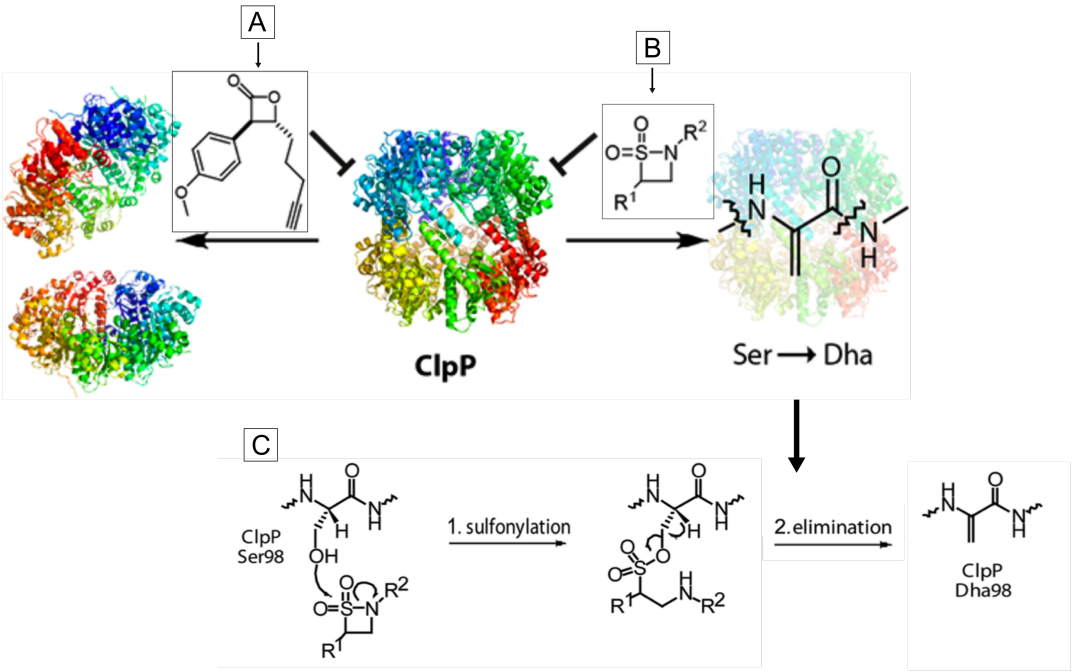


Figure 5. Examples of chemical compounds (β–sultam) that bind ClpP in a substoichiometric fashion, triggering the formation of completely inactive ClpP proteolytic subunit. **(A)** Inhibitor molecule that partially modifies the active sites and changes the ClpP oligomerization to smaller species. **(B)** Inhibitor molecule that induces dehydroalanine formation of the active site serine without changing the ClpP oligomerization state. **(C)** The chemical reaction that induces dehydroalanine formation of the active site serine proceeds through sulfonylation and subsequent elimination, thereby obliterating the catalytic charge relay system. (Ghersh et al., 2013).

1.5 Heat-shock response in bacteria

The well-coordinated expression of various molecular chaperones and stress proteases, essential for survival and adaptation of bacterial species, is tipically regulated upon exposure to different kind of stressful conditions, including heat-shock. In this respect, the heat-shock response is a universal mechanism of cellular protection against sudden hostile environmental conditions widely conserved in both prokaryotes and eukaryotes. It consists in a prompt production of heat-shock proteins that help the cells to survive under conditions that would normally be lethal. Upon a sudden temperature upshift, cellular protein homeostasis is compromised, therefore, the intracellular level of HSPs rapidly increases to assist the folding of newly synthesized polypeptides, to prevent protein aggregation, and to recover proteins that have been partially or completely denatured by the heat stress. Once the organism has adapted to the new temperature, the amount of molecular chaperones and stress proteases decreases to a steady-state level, usually greater than the initial basal level, in order to assist bacterial growth under non-optimal environmental conditions. The molecular mechanisms that govern the regulation of heat-shock genes differ considerably among bacterial species. In particular, many important microorganisms, by combining transcriptional and post-transcriptional mechanisms, employ different regulatory strategies to finely regulate the rapid induction of HSPs synthesis. A very fast heat-shock response, upon signal perception, is provided by mechanism of heat-sensing, in which different classes of biomolecules, including lipids, proteins and nucleic acids, transduce external temperature signals into a transcriptional or post-transcriptional output. For example, after an increase of temperature, the secondary structure of some heat-sensing RNA, known as RNA thermometers, goes through rearrangement or partial melting (Kortmann and Narberhaus, 2012). As a consequence, ribosomes can easily gain access to the 5' mRNA region and translation is enhanced. Alternatively, temperature variations can be directly sensed by a heat sensing transcriptional repressor, that upon heat-shock undergoes a conformational change that lowers relative binding affinity for its operators leading to a derepression of its target genes' transcript levels, as in the case of the *H. pylori* HrcA regulator

(Roncarati et al., 2014). Moreover, sometimes, also the DNA of bacterial cells can act as a thermosensor. Indeed, during stress the metabolic state of the cell, including also the ATP/ADP ratio, is influenced, and consequently, enzymes that require ATP as cofactor will be affected. Considering that, the activity of the DNA-gyrase enzyme is ATP-dependent and strictly linked to the topological state of DNA, changing in the ATP/ADP ratio, caused by external stress stimuli, can have an effect on the global level of DNA supercoiling, thus, influencing genes transcription (Hsieh et al., 1991; Dorman and Corcoran, 2009). However, tipically in bacteria, transcriptional regulation of heat-shock genes could be either positive or negative, according to the kind of regulator involved (Figure 6). Positive transcriptional regulation relies on the use of dedicated alternative sigma factors that, outcompeting the housekeeping σ subunit, normally associated with RNA polymerase, specifically redirect the enzyme to selected heat-shock gene promoters, thereby reprogramming cellular transcription (Figure 6a). This transcriptional control strategy is widely used among bacteria, including the model organism *E. coli* (Schumann, 2016), in which the heat-shock response is governed by the alternative sigma factor σ^{32}, named RpoH (Grossman, Erickson and Gross, 1984). Besides σ^{32}, the master regulator of heat-shock response, in *E. coli* a second heat-shock regulon is governed by another alternative sigma factor named σ^{E} (also known as σ^{24}) (Mecsas et al., 1993). In addition, negative regulation is exerted by dedicated transcriptional repressors, whose DNA-binding activity changes in response to fluctuating environmental temperature. These repressors, under normal growth conditions, bind specific operators of heat-shock promoters, repressing the transcription of heat-shock genes, while upon heat stress, they lose binding affinity for their promoter sequences, ensuring a rapid induction of heat-shock genes expression driven by the RNA polymerase associated to the housekeeping σ subunit (Figure 6b) (Narberhaus, 1999; Roncarati and Scarlato, 2017). Several bacterial species rely on the use of transcriptional repressor to finely regulate the expression of heat-shock genes, including, *B. subtilis*, *S. coelicolor* (Roncarati and Scarlato, 2017), and *H. pylori*.

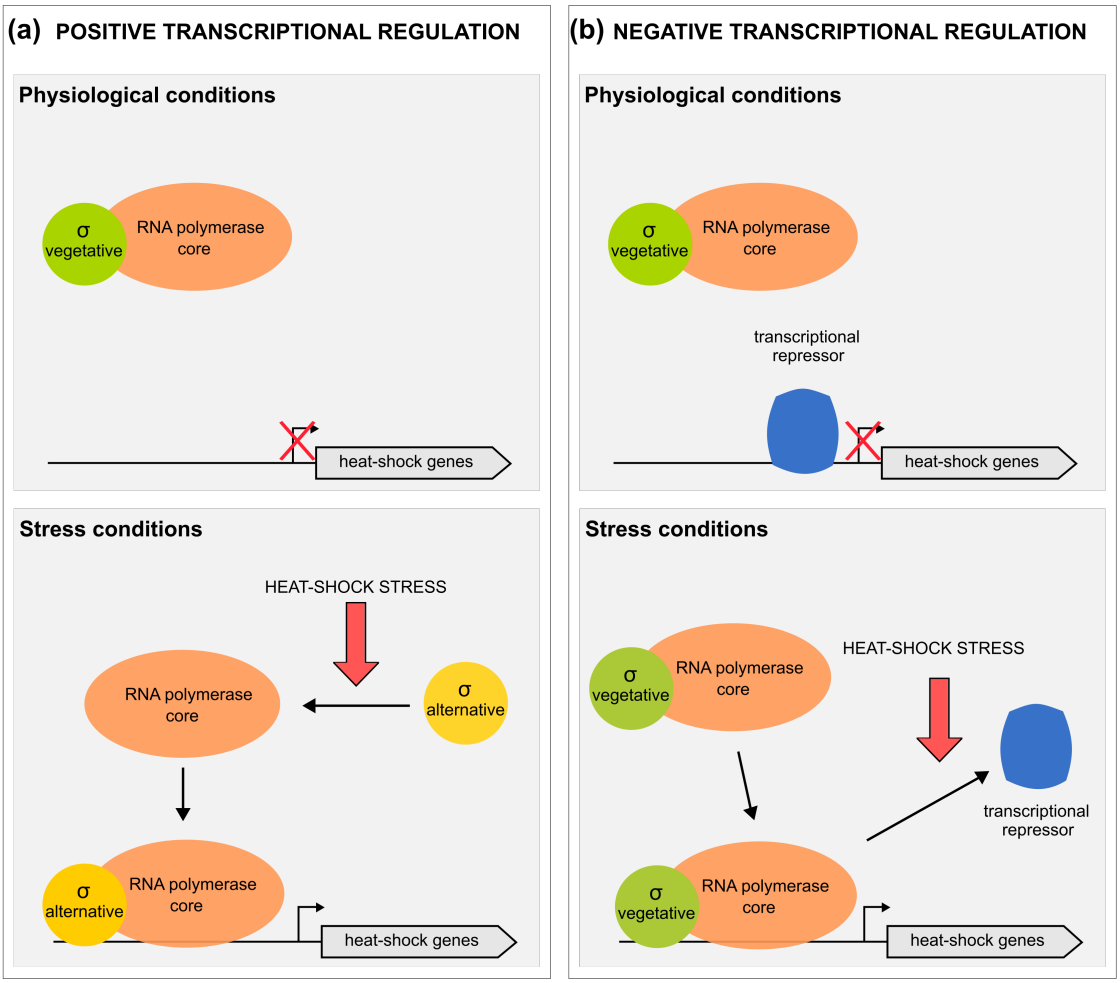


Figure 6. Schematic representation of positive and negative transcriptional regulation mechanisms of heat-shock genes. **(a)** In positive regulation, upon a temperature variation, the heat-shock alternative sigma factor (yellow oval) interacts with the core RNA polymerase (orange oval), driving the transcription enzyme on the promoters of heat-shock genes, thus activating their transcription. **(b)** Negative regulation relies on repressors (blu oval) that keep transcription of heat-shock genes repressed during physiological growth conditions. After the heat-shock stimulus, repressor proteins lose DNA-binding affinity for the promoter sequences of heat-shock genes, whose transcription is driven by the RNA polymerase associated with the vegetative sigma factor (green oval). The transcription start site is indicated by a bent arrow. Adapted from Roncarati and Scarlato 2018b.

1.5.1 Transcriptional regulation of heat-shock genes in *Helicobater pylori*

As mentioned above, *H. pylori* adopts a negative transcriptional regulation of heat-shock genes, thereby controlling their expression, at a transcriptional level, by two dedicated repressors, which are homologues to *Bacillus subtilis* HrcA and to *Streptomyces coelicolor* HspR (Wetzstein et al., 1992; Bucca et al., 1995; Tomb et al., 1997). Both the *hrcA* and *hspR* genes belong to the multicistronic heat-shock operons containing also other important *H. pylori* chaperone proteins,

represented in Figure 2. Their role as negative heat-shock transcriptional regulators was demostrated comparing the transcription of the three chaperone-encoding multicistronic operons in the *H. pylori* wild-type strain and in the *hspR*$^-$ or *hrcA*$^-$ isogenic mutant strains. In particular, the *hspR* gene deletion led to a constitutive strong increase in transcripts associated with the three *cbpA-hspR-helicase*, *hrcA-grpE-dnaK* and *groES-groEL* heat-shock operons. In addition, the *hrcA* deletion specifically provoked the accumulation at a transcriptional level of only the *groES-groEL* and its own operon, *hrcA-grpE-dnaK* (Spohn et al., 1999; Spohn et al., 2004). *In vitro* DNA-binding studies confirmed the *in vivo* studies and demonstrated the direct interaction between the two repressors and the three promoters controlling the heat-shock operons' transcription. Experiments of DNaseI footprinting showed important differences in the position of the HspR and HrcA binding sites, with respect to the core promoter elements (Spohn et al., 1999; Roncarati et al., 2007). In particular, the HrcA repressor binds to a DNA region overlapping the core promoter elements and the transcriptional start site of P*gro* and P*hrc* promoters and spanning from -13 to +16 on P*gro* and from -59 to -34 on P*hrc* (Roncarati et al., 2007a; Roncarati et al., 2007b). On the other hand, the HspR binding site on the promoter region of the *cbpA-hspR-helicase* operon, controlled solely by HspR itself, is located between the – 35 and – 10 core promoter elements, in a typical position for a transcriptional repressor, while on the promoter region of the HspR-HrcA co-regulated operons *groES-groEL* and *hrcA-grpE-dnaK* its binding occurs far upstream of the core promoter elements, in a DNA region centred 72 and 117 bp upstream their specific transcriptional start sites, respectively (Spohn et al., 1999; Roncarati et al., 2007a). Interestingly, whereas upon DNA binding the HrcA regulator covers compact DNA regions of about 30 bp, the HspR repressor covers extended DNA regions, of around 75 bp, despite being a relatively small protein (Roncarati et al., 2007a). Moreover, for both HrcA and HspR, the experimentally-identified operators on the three heat-shock operons' promoters, harbor sequences similar to the CIRCE (Controlling Inverted repeat of Chaperone Expression) and the HAIR motif (HspR Associated Inverted Repeat), proposed as consensus sequence for *Bacillus subtilis* HrcA (Schulz and Schumann, 1996; Zuber and Schumann, 1994) and *Streptomyces coelicolor* HspR, respectively (Bucca et al., 1995; Grandvalet et al., 1997). Peculiar is the role played by the HspR and HrcA repressors on co-regulated operons. While, indeed, the position of the HrcA operator is close to the core promoter region, the HspR binding sites, on *groES-groEL* and *hrcA-grpE-dnaK* operons, are located upstream the -35 and -10 promoter elements. Furthermore, even though HspR and HrcA operators are very close to each other and *in vivo* both repressors are necessary for a full repression, they apperar, at least *in vitro*, to bind indipendently their specific recognition DNA sequences, without direct protein-protein interaction (Roncarati et al., 2007a). However, from these *in vivo* and *in vitro* data, described above,

a model in which HspR alone is able to repress its own transcription was proposed, while both HspR and HrcA are required for dual repression of the other two heat-shock operons *groES-groEL* and *hrcA-grpE-dnaK* (Figure 7).

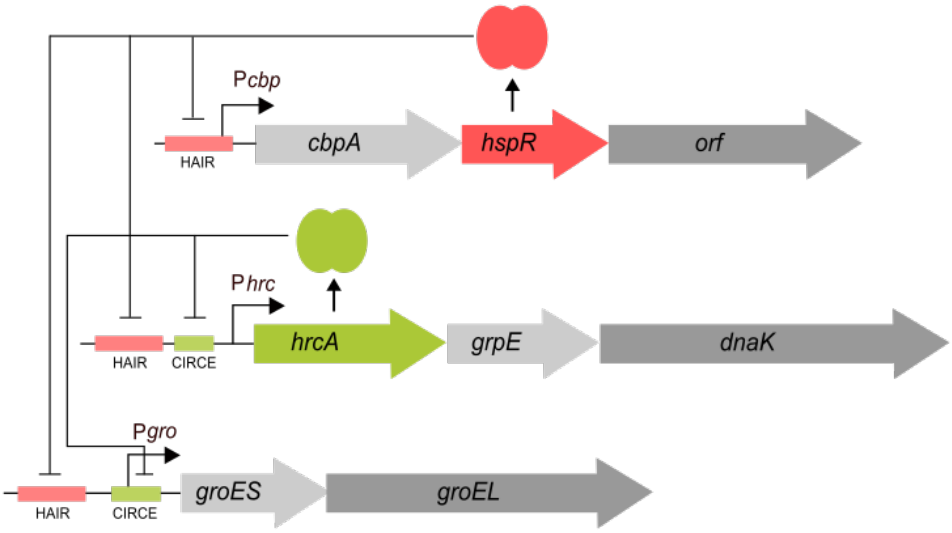


Figure 7. Schematic representation of negative transcriptional regulation of heat-shock genes in *H. pylori*. The HspR repressor negatively regulates, alone, the *cbpA-hspR-orf* operon and in combination with the HrcA regulator repress the transcription of the other two heat-shock operons *hrcA-grpE-dnaK* and *groES-groEL*. The HspR and HrcA consensus sequences, called HAIR and CIRCE respectively, are reported in the promoter region of the three heat-shock operons. Transcriptional repression of the heat-shock genes is represented by solid lines that connect HrcA and HspR repressor proteins to their target promoters.

In this model, under physiological growth condition, transcription of the three heat-shock operons' promoters is driven by RNA polymerase containing the vegetative sigma factor σ^{80} and is maintained repressed by the concerted action of HspR and HrcA. Upon a temperature increase or other stress insults, such as high osmolarity and puromycin treatment (Homuth et al., 2000; Sphon et al., 2002), repression is released, and the heat-shock genes are promptly transcribed with a very rapid kinetic. After temperature upshift, the amount of the transcripts of the three heat-shock operons rapidly increases, starting at 2 minutes and reaching a maximum at 10 to 15 minutes. The ratio of this increase of transcripts is different for the three promoters, ranging from five-fold for P*gro* to seven-fold for P*cbp* and eleven-fold for P*hrc* (Sphon et al., 2002), and is followed by a gradual decrease in the mRNA amounts to a new steady-state level. The rapid derepression of the three heat-shock operons is guaranteed by the logical scheme of the heat-shock response circuit, adopted by *H. pylori*. In this circuit, the master regulator HspR directly controls the expression of the HrcA regulator and the *groESL* operon, which in turn is under the transcriptional control of both HrcA and HspR. Moreover, considering that the three regulatory interactions are repressive, the

heat-shock regulatory module represents an example of an incoherent type-2 feed forward loop (i2-FFL) (Alon, 2007; Danielli et al., 2010). Finally, the rapid kinetic of the heat-shock response circuit is guaranteed also by the involment of two chaperone proteins themselves in the feedback modulation of the transcriptional activity of the two repressors (Figure 8). The HrcA DNA-binding affinity for its CIRCE operators is positively affected by the GroE chaperonine in a feedback regulatoy loop complying with the *B. subtilis* "tritation model" (Mogk et al., 1998). According to this model, GroE interact with HrcA to aid its folding, and upon heat-shock the chaperonin is titrated away by increasing amount of misfolded polypeptides, relieving HrcA transcripional repression of heat-shock target promoters (Roncarati et al., 2007b). In addition, the HspR DNA-binding activity is negatively modulated by the co-chaperone CbpA and is not positively affected by DnaK as has been observed in *S. coelicolor* and *M. tuberculosis* (Bucca et al., 2000; Parijat and Batra, 2015). It has been shown that *H. pylori* CbpA directly interacts with HspR only in solution and with no contact with the DNA, thereby hindering the repressor DNA-binding capabilities to target promoters (Roncarati et al., 2011). Probably, CbpA regulation of HspR binding activity is required to fine-tune the shutoff response of the heat-shock genes in *H. pylori* (Roncarati et al., 2011).

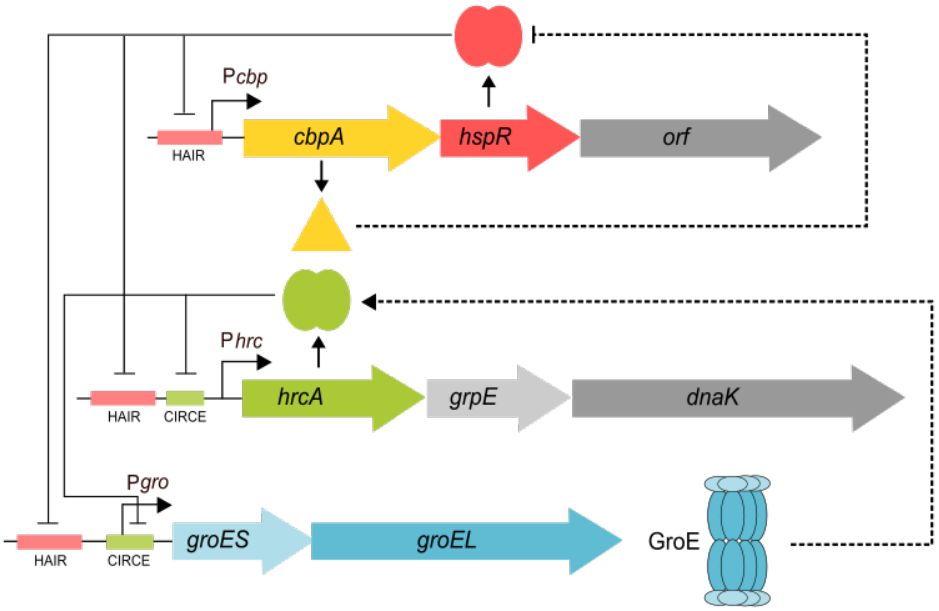


Figure 8. Schematic representation of posttranscriptional regulation of heat-shock genes expression. Dashed lines, linking GroE to HrcA and CbpA to HspR, represent the positive or negative posttranscriptional protein-protein feedback control of the regulators, respectively. Adapted from Roncarati et al., 2011.

Finally, concerning the detection of the stress signals, it has been demonstrated that in *H. pylori* the transcriptional repressor HrcA is the direct heat-sensor of chaperone regolatory circuit (Roncarati et al., 2014). In fact, upon heat-shock, temperature triggers structural changes of the repressor protein,

inducing a complete loss of HrcA DNA-binding affinity for its operators, that causes a rapid induction of heat-shock genes transcription. Then, after temperature challenge, the recovery of the HrcA proper folding and binding activity is mediated by the chaperonin GroE, whose function in this case is to restore HrcA role in heat-shock promoters' repression. In contrast, while HrcA is able to intrinsically sense temperature upshift, the HspR repressor appears to be a stable protein, whose conformation and DNA binding activity are unaffected by temperature variations (Roncarati et al., 2014). Probably, the HspR sensing mechanism could be indirect and mediated by a still unknown interacting partner.

In the last 20 years, by using genome-wide techniques, further studies have been adressed for the identification of additional genes regulated by the HspR and/or HrcA transcriptional repressors both in *H. pylori* (Roncarati et al., 2007a) and in several other bacterial species (Stewart et al., 2002; Hu et al., 2007; Bucca et al., 2009; Holmes et al., 2010). These studies, by combining global gene expression analyses with the search of conserved HAIR and CIRCE elements and with *in vivo* and *in vitro* DNA-binding assays, revealed that both the transcriptional repressors HspR and HrcA control the transcription, in a direct or indirect manner, of a great number of genes involved in the heat-shock stress and also in disparate cellular processes. For example, in the closely-related bacterium *Campylobacter jejuni* or in other bacteria such as *S. coelicolor* and *Mycobacterium tuberculosis* (Stewart et al., 2002; Bucca et al., 2003), *hspR* deletion led to a de-repression of many genes encoding proteins involved also in cellular processes not related to the heat-shock stress. Similarly, in *H. pylori* a previuos DNA microarray-based analysis of gene expression in wild-type strain and in *hrcA* and *hspR* single- and double-mutant strains showed that both regulators affect the transcription of 43 genes encoding proteins involved in stress response, iron metabolism and regulation and assembly of the flagellar apparatus (Roncarati et al., 2007a). Moreover, through an *in vitro* selection of genomic DNA fragments bound by purified *H. pylori* HspR protein, two novel HspR binding sites in the 3' regions of both *speA* and *tlpB* genes, coding for proteins with functions unrelated with those of chaperones, have been identified (Delany et al., 2002b).

2. *Aim of the project*

Bacteria respond to heat-shock and other stress conditions activating a well-coordinated expression of molecular chaperones and proteases, which are able to prevent protein denaturation and avoid the resulting formation of protein aggregates. This response is apparently universal and widely conserved in both prokaryotes and eukaryotes. However, complex strategies have evolved to regulate heat-shock proteins' expression in different bacterial species. In the major human pathogen *Helicobacter pylori*, transcription of chaperone-coding operons is controlled by two auto-regulated repressors, HrcA and HspR, with the latter acting as the master regulator of the heat-shock circuit. During the last years, the molecular mechanism through which these two regulators control the HSPs gene expression at the transcriptional level has been studied in detail. However, the advent of genome-wide techniques could allow the identification of additional genes that are involved in this kind of response and that are directly controlled by the heat-shock transcriptional repressors. In this regard, the first aim of the project was to globally analyse the heat-shock response in *H. pylori* and to further characterize the HspR regulon performing a whole transcriptome analysis (RNA-sequencing) in combination with Chromatin Immunoprecipitation coupled with deep sequencing of HspR *in vivo* genomic binding sites. Moreover, in order to gain more detailed information about the HspR-DNA interactions, *in vitro* DNA-binding assays have been performed by using the high-resolution molecular technique of hydroxyl-radical footprinting.

The other group of heat-shock proteins that plays important roles in resistance against heat-shock stress and in the control of bacterium cellular adaptation, is represented by a class of stress related proteases, called caseinolytic proteases (ClpPs). Little is known about the regulation of this class of proteases in *H. pylori*, as well as about its direct or indirect protein targets. Therefore, the second aim of this project was to get new insights on the role of Clp-mediated proteolysis in *H. pylori* and to identify potential ClpP protein substrates, developing and implementing a "ClpP trapping" strategy.

3. *Part 1 Results*

3.1 *Specific Introduction*

This study is focused on the identification and the detailed investigation of the heat-shock regulon in *H. pylori* and on the direct and indirect contribution of the heat-shock master regulator HspR to its control. In the first part of this work, by combining ChIP-sequencing and RNA-sequencing, we deepen our understanding of the heat-shock response in *H. pylori*, further characterizing the HspR regulon. In the second part, by means of high-resolution hydroxyl-radical footprinting technique, we analyse the HspR-DNA interaction at a molecular level.

3.2 Genome-wide studies

3.2.1 Heat-shock and HspR transcriptome analyses

To gain insight into global changes of transcript levels following a sudden temperature increase (heat-shock stress) in *Helicobacter pylori* and to define the HspR direct and indirect transcriptional contribution to the heat-shock response, we performed a strand specific whole transcriptome analysis. We set up the experiment using *H. pylori* G27 wild-type and *ΔhspR*-mutant strains both liquid-grown up to an exponential growth phase at 37°C and the wild-type strain subjected to 30 min heat-shock stress at 42°C. Globally, 134 genes showed changes in the amount of their transcripts (differentially expressed) in the wild-type strain submitted to heat-shock compared to the wild-type sample grown at 37 °C. Of these differentially expressed genes, 83 were up-regulated and 51 were down-regulated and they mainly belong to the functional categories of "Post-translational modification, protein turnover, chaperones" and "Cell wall/membrane/envelope biogenesis", "Translation, ribosomal structure and biogenesis" respectively (Figure 9A). Predictably, in the wild-type strain subjected to heat stress, a significant up-regulation of the three heat-shock operons *groES-groEL*, *hrcA-grpE-dnaK* and *cbpA-hspR-rarA* was observed. In fact, transcription of these genes, coding for the major chaperonins and heat-shock proteins in *H. pylori*, has been previously shown to respond to the heat-shock stress, under the control of the transcriptional repressor HspR (Spohn and Scarlato, 1999). As one of the roles of the heat-shock response is to monitor and preserve the bacterial membrane during stress, it was not surprising the finding that transcript levels of some genes coding for proteins involved in the biogenesis of cell wall, envelope and bacterial membrane (*csd4*; *murA*; *murZ*; *murL*, see Table S3; Supplementary materials of the annex 1 are avaible here: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01887/full#supplementary-material), were affected by heat stress. In addition, we found changes in the amount of transcripts of

genes implicated in defense mechanism (*napA*), in replication, recombination and repair machinery such as *rarA*, coding for a recombinase and a ribonuclease HII *rnhB*. Intriguingly, various genes, de-regulated during the stress, belong to the class of flagellar apparatus, including *flaA*, *flgR* and *flgM*. Surprisingly, among the protease family of *H. pylori*, only the genes encoding for the ClpB and HtrA proteases were poorly induced after the stress.

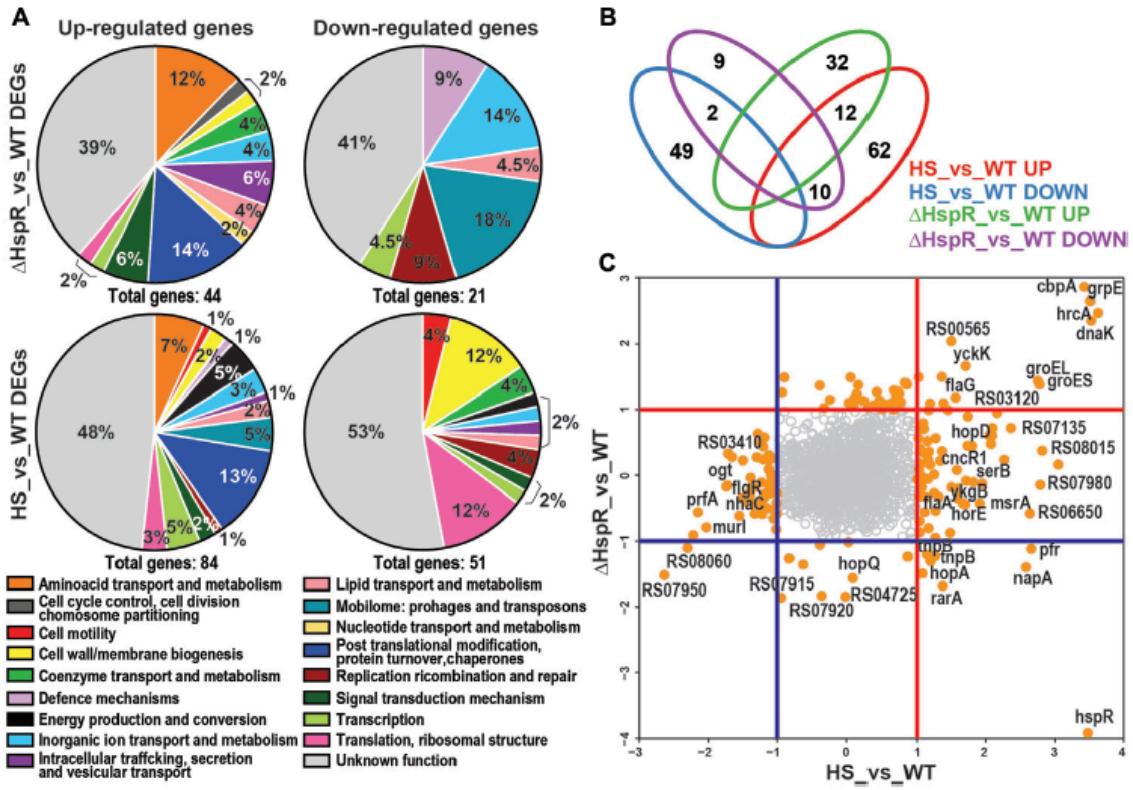


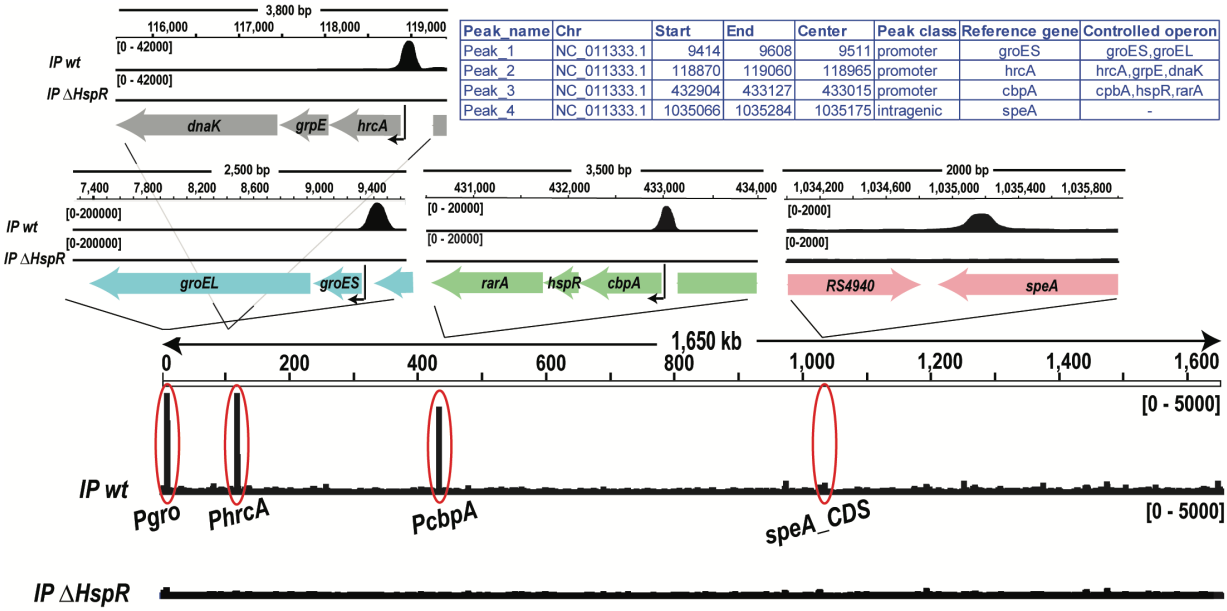
Figure 9. (A) Pie charts showing COGs functional annotation of the differentially expressed genes outlined in the ΔHspR_vs_WT and HS_vs_WT (Heat-Shock vs. Wilde-Type) comparisons, respectively, subdivided into up-regulated (left) and down-regulated (right) groups. The abundance of each category is indicated as a percentage as well as the total number of genes included in each group. **(B)** Venn diagram showing the number of genes in common or peculiar in the four previously described gene groups, red numbers highlight commonly regulated genes. **(C)** log2FC plot showing for each gene HS_vs_WT value on x-axis and HspR_vs_WT value on y-axis. DEGs (log2FC > |1| and padj < 0.01 are represented as orange filled circles; empty gray circles correspond to non-differentially expressed genes). Blue and red lines indicate log2FC < -1 and log2FC > 1 thresholds, respectively, subdividing coherently and incoherently regulated genes. (Pepe et al., 2018)

Subsequently, the transcriptome analysis performed in the *ΔhspR* mutant strain revealed changes in the transcript levels of 65 genes (Figure 9A). As expected, among these the three heat-shock operons (*groES-groEL*, *hrcA-grpE-dnaK* and *cbpA-hspR-rarA*) are negatively regulated by HspR. Moreover, HspR seems to affect the transcript levels, in a positive or negative manner, of additional

59 genes involved in diverse cellular functions not strictly associated to heat-shock. Like, for instance, genes involved in the "Inorganic ion transport and metabolism", some transposons (*tnpB*), the flagellar gene *flaG* and several genes belonging to the superfamily of ABC-type transporters, such as *yckK*, *yckJ*, *dppC* (coding for amino acid and peptide transporters). By comparing the transcriptome of the heat-shock response and of the *hspR* deletion mutant, it was found that only 25 genes were de-regulated in both dataset and, among these, the operons already known to be induced by heat-shock and directly repressed by HspR (Figure 9C). Overall, from these analyses it emerged that in *H. pylori* the heat-shock stress triggers a global cellular response, resulting in a de-regulation of the major heat-shock proteins and of several genes involved in diverse cellular crucial functions. Furthermore, HspR seems to affect, either directly or indirectly, not only the transcription of genes that are responsive to the heat-shock stress (25 out of 134) but also of several genes that are not responsive to heat-shock.

3.2.2 Genome-wide analysis of *in vivo* HspR targets by ChIP-sequencing

To identify the genomic region bound *in vivo* by the HspR repressor protein and to discriminate between a direct effect of the HspR binding on transcription and an indirect effect, on the 59 de-regulated genes in the *hspR* deletion mutant strain, we performed a Chromatin ImmunoPrecipitation assays followed by deep sequencing (ChIP-seq) in *H. pylori* G27 wild-type and *ΔhspR*-mutant strains (Figure 10). Briefly, exponentially liquid-growing *H. pylori* G27 wild-type and *ΔhspR* strains were cross-linked, sonicated, and HspR protein-DNA complexes were immunoprecipitated with a specific HspR polyclonal antiserum. Two sets of biological replicates were employed for the IP analysis, and the ChIP-seq signals, obtained from each wild-type sample (IP *wt*), were compared to those resulting from the two *ΔhspR* mutant samples (IP *ΔhspR*), used as negative control (see materials and methods for details). The significant peaks, identified in both replicates, were reported in the final peak list (Figure 10) and were annotated with respect to the *H. pylori* G27 RefSeq annotation (GCF_000021165.1). In particular, binding sites centered between position -100 and +30 bp with respect to a transcriptional start site were considered associated to a promoter region, while the remaining peaks were classified as internal or intergenic on the basis of the peak central position. Surprisingly, this analysis identified only four HspR *in vivo* genomic binding sites (Figure 10), three of which were associated to the promoter region of the heat-shock operons *groES-groEL*, *hrcA-grpE-dnaK* and *cbpA-hspR-rarA* and the other peak was classified as intragenic because mapping in the coding region of the *speA* gene, coding for an arginine decarboxilase.



Peak_name	Chr	Start	End	Center	Peak class	Reference gene	Controlled operon
Peak_1	NC_011333.1	9414	9608	9511	promoter	groES	groES,groEL
Peak_2	NC_011333.1	118870	119060	118965	promoter	hrcA	hrcA,grpE,dnaK
Peak_3	NC_011333.1	432904	433127	433015	promoter	cbpA	cpbA,hspR,rarA
Peak_4	NC_011333.1	1035066	1035284	1035175	intragenic	speA	-

Figure 10. Genome-wide *in vivo* identification of the HspR binding sites. Genome-wide signals of HspR bindings in the wild-type genotype (IP wt, upper track) and in *hspR* deletion mutant used as negative control (IP ΔHspR, lower track). The regions corresponding to the four HspR binding sites revealed by the analysis are magnified in the upper panels showing each peak in its genomic context including annotated genes (thick arrows) and the transcriptional start site (black thin arrow), when present. (Pepe et al., 2018)

These data showed that the HspR regulon is very restricted and limited to three heat-shock multicistronic operons coding for 8 genes. Thus, confirming the HspR direct transcriptional control of these 8 genes coding for the major chaperones and heat-shock proteins in *H. pylori*. Moreover, through ChIP-seq analysis it was confirmed *in vivo* also the existence of at least one intracistronic binding site, apparently not associated to transcriptional regulation.

3.3 Characterization of HspR-DNA binding mechanism

3.3.1 HspR binds with a peculiar binding architecture

Previous *in vitro* DNA-binding studies demonstrated the direct interaction between the HspR repressor protein and the three promoters controlling the heat-shock operons' transcription in *H. pylori*, with important differences in the position of the binding site with respect to the core promoter elements (Spohn and Scarlato, 1999; Roncarati et al., 2007a). In particular, DNaseI footprinting assays revealed that the *H. pylori* HspR repressor binds extended DNA regions of about 70-80 bp, on all the three heat-shock operons' promoters with six bands of enhanced DNaseI hypersensitivity at both adjacent and internal sites. In order to further characterize HspR-DNA interactions, trying to achieve more detailed information about the architecture and the bases involved in HspR DNA-binding, hydroxyl-radical footprinting experiments on the identified genomic targets were performed (Figure 11). Briefly, DNA-probes were radioactively end-labeled with ^{32}P and incubated, *in vitro*, with increasing concentrations of recombinant purified HspR protein and then subjected to hydroxyl-radical ions cleavage to identify DNA regions specifically protected by the HspR binding. The experiments reported in Figure 11 show hydroxyl-radical footprintings performed on the promoter region of the three heat-shock operons P*cbp*, P*gro* and P*hrc* and on the 3' region of the *speA* gene. In agreement with previous observation (Roncarati et al., 2007a; Spohn et al., 1999), HspR binding on the P*cbp* promoter overlaps the – 10 and – 35 boxes (from nucleotide - 63 to + 10), while on the other two heat-shock promoters, P*gro* and P*hrc*, HspR binds upstream of the promoter elements, (from -117 to -44 and from -150 to -82, respectively). Moreover, the HspR binding mapped in the 3' region of *speA* coding sequence, spanning from nucleotide – 298 to – 250 with respect to the translational stop codon. Besides a slight difference between the promotorial and the intragenic binding sites, the HspR binding shows a peculiar periodic pattern of short protected regions from radical digestion. In the promoter region of the three heat-shock operons (P*cbp*, P*gro* and P*hrc*; Fig. 11 panel A), HspR binding generates 7 short protected DNA tracts (indicated with black boxes on the right side of the panel) separated by non-protected regions of 7/8 nucleotides in length, while HspR binding inside the coding region of *speA* target gene differs from the promotorial one as it appears at higher protein concentration and is characterized by 5 short protected regions instead of 7.

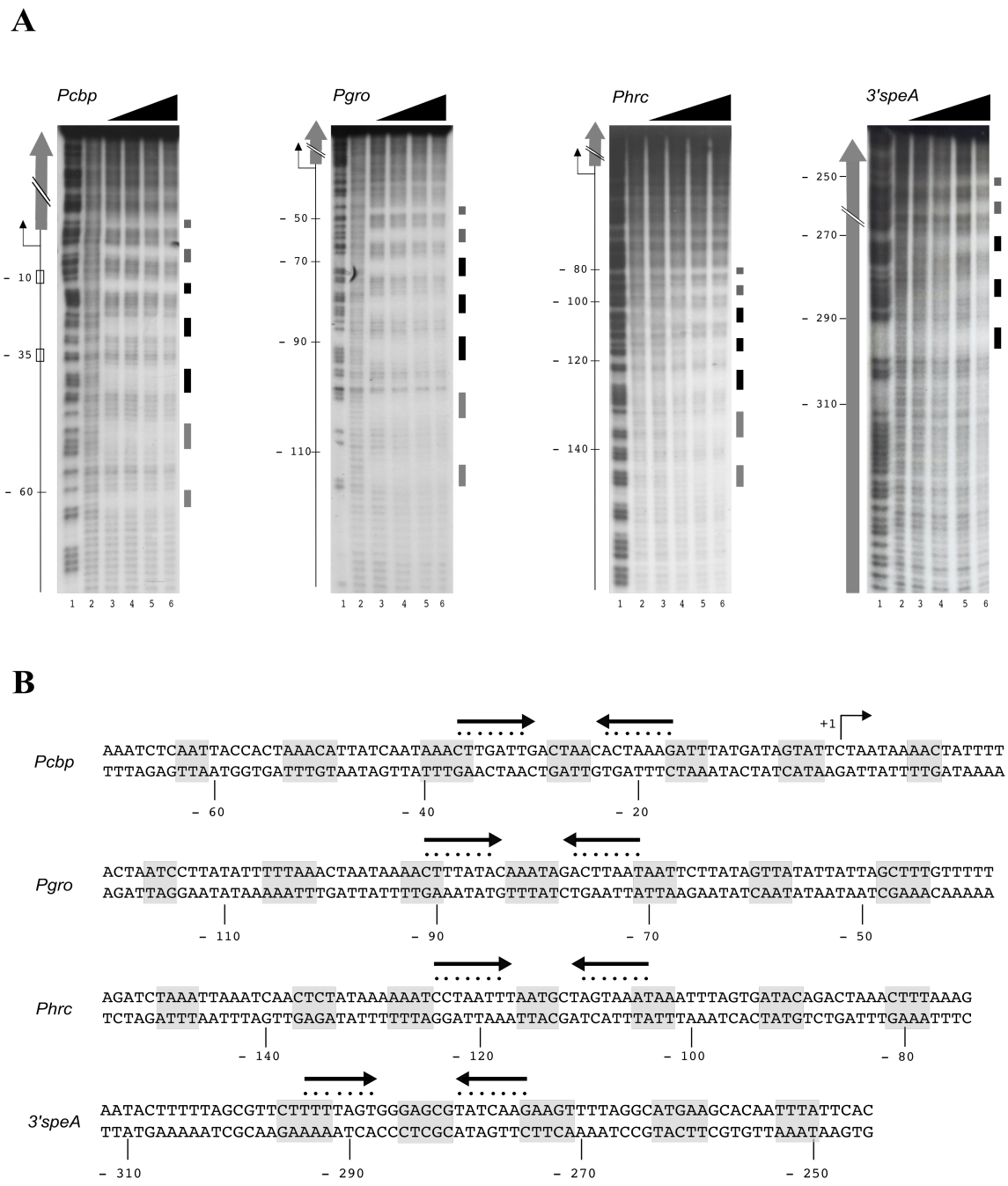


Figure 11. (A) Hydroxyl-radical footprint experiments on HspR positive target genes identified by ChIP-seq analysis. From left to right, specific DNA probes for P*cbp*, P*gro*, P*hrc*, and *speA* fragments were radioactively end-labeled on the coding strands, and incubated with increasing amounts of purified HspR protein prior to hydroxyl-radical digestion. P*cbp*, P*gro*, P*hrc* labeled probes were mixed with 0; 47.5; 95; 190; 380 nM HspR (lanes 2–6); while *speA* probe was mixed with 0; 60.7; 121.5; 243; 486 nM HspR (lanes 2–6). Purified DNA fragments were separated on a polyacrylamide denaturing gel along with a G + A sequence reaction ladder as reference marker (lane 1 in all the panels). On the right of each panel, black and gray boxes represent strong and weak HspR protected regions, respectively. On the left, the –10 and –35 regions and the transcriptional start site (+1, bent arrow) are indicated and the open reading frames are depicted with vertical gray arrows. The relative position of the HspR binding site on the promoter regions of

P*cbp*, P*gro*, P*hrc* is reported with respect to the transcriptional start site, while those mapped on the coding sequence of *speA* gene are reported with respect to the translational stop codon. **(B)** Nucleotide sequences of HspR binding sites on the promoter regions of the three heat-shock operons (P*cbp*, P*gro*, P*hrc*) and on the 3' coding region of the *speA* gene. The HspR protected regions identified in (A) are shaded in gray, while the inverted repeat sequences similar to the HAIR consensus motif are depicted as converging black arrows and each nucleotide of the motif is marked with a dot. Nucleotide positions with respect to the transcription initiation sites are reported on the non-coding strand. (Pepe et al., 2018)

Data obtained from the *in vitro* DNA-binding assay are schematized in Figure 11B, in which the nucleotide sequence of the HspR binding sites (operator) on the promoter region of the three heat-shock operons (P*gro*, P*hrc* and P*cbp*) and on the *speA* coding region are reported. A nucleotide sequence alignment of the protected and spacer DNA region of the HspR operator (data not shown) showed no highly conserved nucleotide sequences among the four HspR binding sites. However, in all the four HspR operators, an inverted repeat with similarities to the HAIR motif, proposed as a consensus sequence for the HspR protein of *Streptomyces coelicolor* (CTTGAGT-N7-ACTCAAG), was clearly identified (Grandvalet et al., 1999). In particular, this identified HAIR-like motif on the promoter region of the three heat-shock operons maps in a central position.

3.3.2 The HAIR-like motif is fundamental for HspR protein-binding

To evaluate the role of the conserved HAIR-like sequences in HspR binding to DNA, bases substitution mutations have been introduced in one or both sequence elements of the HAIR-like motif as well as in non-conserved nucleotides within the binding site of the P*cbp* promoter region. The HspR DNA-binding property on the generated mutants was monitored through hydroxyl-radical footprinting assays. Figure 12 displays a schematic representation of the wild-type and mutant sequences of the HspR operator on its own promoter region P*cbp* (panel A) and the results obtained by hydroxyl-radical footprintings performed on each mutant probe (panel B).

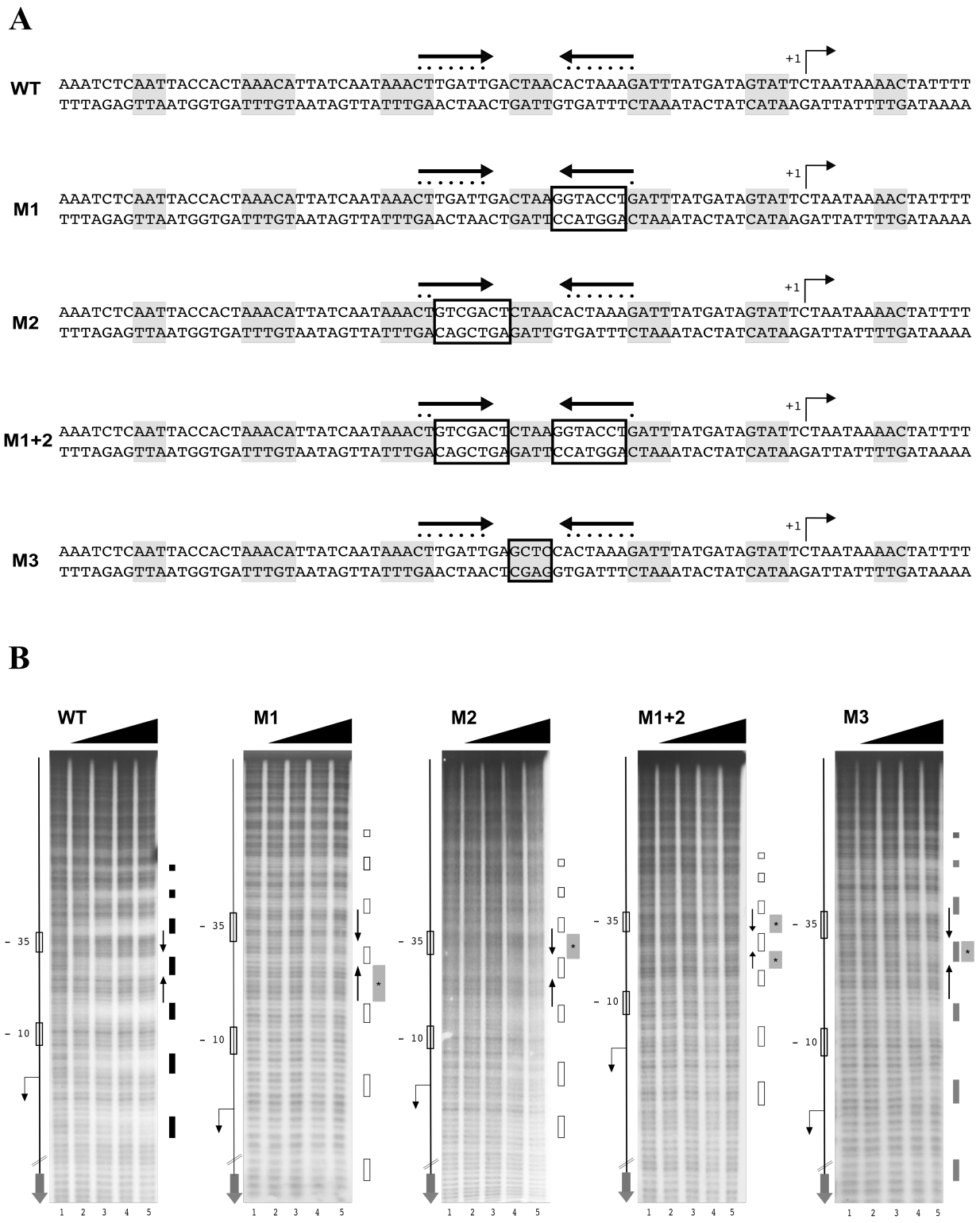


Figure 12. (A) Schematic representation of wild-type and four different P*cbp* mutant probes, in which one (M1 and M2) or both arms (M1 + 2) of the inverted repeat and the protected region between these sequences (M3) have been mutagenized by base substitution. HspR protected regions on P*cbp* are shaded in gray and the inverted repeat sequences (HAIR-like motif) is represented by converging black arrows and their nucleotides marked with dots. In each DNA probe, mutagenized nucleotides are boxed. **(B)** From left to

right, hydroxyl-radical footprint experiments on wild-type and the indicated mutants of the P*cbp* promoter region. Symbols are as described in the legend to Figure 11A. Wild-type (WT) and indicated mutants DNA probes were incubated with increasing amounts of purified HspR protein and submitted to hydroxyl-radical digestion (see legend to Figure 11). Black, gray, and empty boxes to the right of each panel denote strong, weak, and loss of protection by HspR, respectively. Black converging arrows to the right of each panel mark the positions of the HAIR-like inverted repeat sequences, while gray boxes with an internal asterisk indicate the mutagenized regions. (Pepe et al., 2018)

Substitution of one (M1 and M2) or both arms (M1+2) of the HAIR-like sequences with a non-related one, fully abolished, in the protein concentration range tested, the HspR binding to the P*cbp* mutant promoter, compared to the wild-type one (WT), not only in the mutagenized sequence but also in the flanking upstream and downstream regions of the HAIR-like motif. This means that the HAIR-like sequences represent essential nucleotides for the HspR binding and could drive specific DNA-recognition on its own promoter region. Additionally, in the P*cbp* M3 probe, it is possible to appreciate a weak DNA protection, clearly visible only at the highest protein concentration tested (Figure 12A, grey boxes on the right side of the autoradiograph). This is plausible with a substantial loss of HspR protein affinity for this mutant promoter, suggesting that also these non-conserved nucleotides mapping between the inverted repeats are important DNA elements for HspR binding. In light of the *in vitro* results, described above, it can be assumed that the non-conserved nucleotide sequence of the spacer region between the inverted repeats has an influence on HspR DNA-binding affinity. Moreover, the HAIR-like sequences represent crucial DNA elements for the primary DNA-binding of HspR to the P*cbp* promoter region and are indispensable to drive specific protein-DNA recognition.

3.3.3 *In vivo* functional analysis of HAIR-like motif

Since the conserved HAIR-like motif showed to play a crucial role in *in vitro* HspR DNA-binding, we decided to investigate its functional importance also *in vivo*. To this aim, the wild-type and P*cbp* promoter mutants were introduced in the *H. pylori* genome fused to a reporter gene system. Briefly, wild-type and HAIR-like mutants of the P*cbp* promoter region were cloned upstream of a 5' fragment of *luxC1* reporter gene in a suicide vector carrying flanking regions with homology to the *cysS* and *vacA* genes and a *Campylobacter coli cat* cassette conferring selectable chloramphenicol resistance (Figure 13A). After that, the suicide vector was integrated, by double homologous recombination in the *vacA* locus of *H. pylori* G27 wild-type and *ΔhspR* mutant chromosome and the transcript levels were assayed through real-time PCR (qRT-PCR), using specific oligonucleotides mapping within the *luxC1* reporter gene. In the *H. pylori* wild-type background, in comparison to the wild-type construct a significant increase (6.75-fold) of the *luxC1* gene transcript was detected from the HAIR-like mutant P*cbp* promoter (P*cbp*M1+2) (Figure 13B).

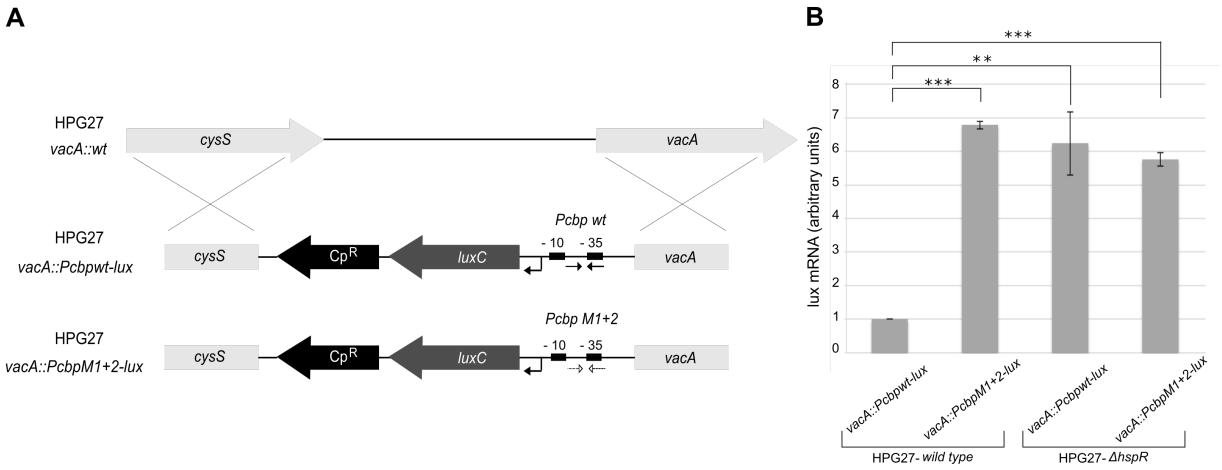


Figure 13. *In vivo H. pylori* transcripts levels from the P*cbp* promoter harboring WT or mutant HAIR-like sequences. **(A)** Schematic representation of P*cbpwt-lux* and *PcbpM1+2-lux* reporter constructs obtained transforming the *H. pylori* G27 wild-type acceptor strain by double homologous recombination in the *vacA* locus, and selected by chloramphenicol resistance (Cp^R). The wild-type (P*cbpwt-lux*) or HAIR-like mutant (P*cbpM1+2-lux*) P*cbp* promoter is inserted upstream of a *luxC* reporter gene. The HAIR inverted repeat sequences are indicated by converging black arrows in the P*cbp* WT promoter (P*cbpwt-lux*) and by converging dotted arrows in the P*cbp* HAIR-like mutant promoter (P*cbpM1+2-lux*). In each reporter construct, the –10 and –35 regions are depicted as black boxes and the transcriptional start site as a bent arrow. **(B)** Transcript levels of P*cbp* wild-type and P*cbp* HAIR-like mutant promoters fused with *lux* reporter gene were assayed by qRT-PCR in the wild-type and *hspR* deletion mutant strains using specific oligonucleotides for the *luxC* gene (LuxRTF/R). Mean values from three independent biological samples are reported in the graph, with error bars indicating standard deviation and asterisks marking statistical significance calculated by a Student's t-test (***p-value < 0.001; **p-value < 0.01). (Pepe et al., 2018)

This experiment showed that *in vivo* the HAIR-like motif plays an essential role, likely driving specific HspR binding to its operator on P*cbp* promoter with concomitant transcriptional repression. Furthermore, we have demonstrated that this *luxC1* transcriptional regulation, from the wild-type or mutant P*cbp* promoter, is HspR dependent. Indeed, in the *hspR*-mutant strain a de-repression of both the wild-type and P*cbp*M1+2 promoters was observed (Figure 13B). Finally, these *in vivo* results are consistent with our *in vitro* observations, highlighting that the central HAIR-like motif provides specific binding for HspR to its operator on P*cbp* with concomitant promoter transcriptional repression.

3.3.4 Non-conserved DNA regions are essential for HspR to fully occupy its binding site

Based on the above reported *in vitro* and *in vivo* results, we decide to further explore the HspR DNA-binding mechanism on the promoter region of its own promoter P*cbp*, to figure out whether non-conserved DNA sequences flanking the HAIR-like motif could also play important roles in HspR binding specificity. To this aim, we generated two additional mutant P*cbp* DNA probes introducing bases substitution mutation in the non-conserved DNA region mapping upstream (M5) and downstream (M4) the inverted repeat. Mutants are schematized in Figure 14 (panel A) and used as probes to verify HspR binding properties using hydroxyl-radical footprinting assays (Figure 14B).

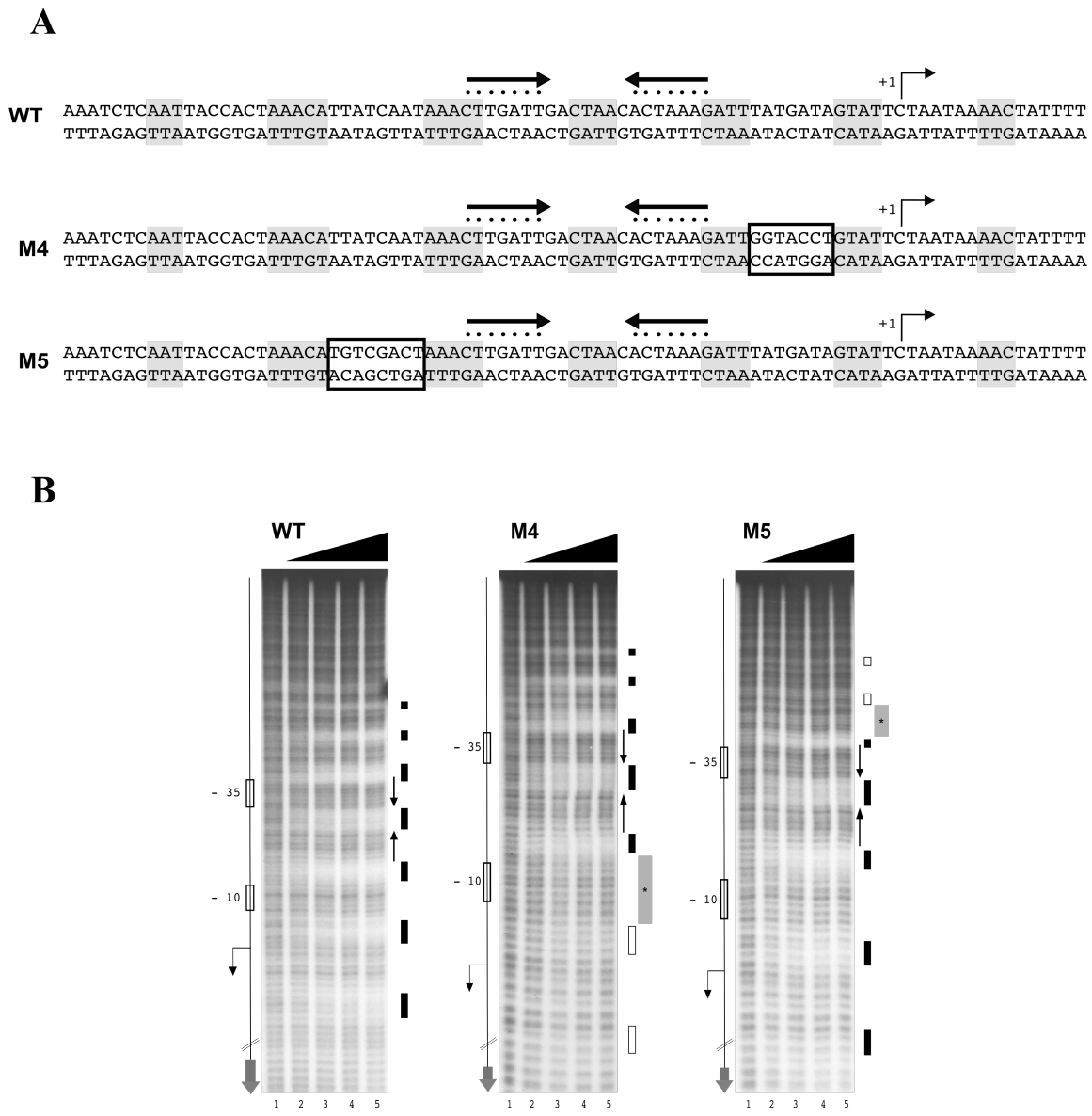


Figure 14. Non-conserved regions surrounding the HAIR-motif are required for HspR binding. **(A)** Schematic representation of WT and two P*cbp* mutant probes, in which the spacer regions highlighted with empty boxes were mutagenized by base substitution. Symbols are as in the legend to Figure 11. **(B)** From left to right, hydroxyl-radical footprint experiments on WT and mutant probes described in (A) (M4 and M5) with increasing amounts of purified HspR protein detailed in the legend to Figure 11. (Pepe et al., 2018)

Both mutations in the spacer regions, between the 4 bp protected tracks on both sides of the HAIR-like motif, abrogated HspR binding on the side of the mutation as shown by the disappearance of the protected nucleotides on the side of the mutated region. By contrast, no changes in the protection were observed on the region harboring inverted repeat and flanking wild-type sequences. In conclusion, taking into account these results and the above described *in vitro* and *in vivo* results,

we propose that HspR binding to its own promoter requires an intact HAIR-like motif surrounded by important DNA elements with no sequence conservation among them.

4. *Part 1 Discussion*

4.1 Genome-wide studies*:* Heat-shock and HspR regulons

The heat-shock response represents a specific gene expression program that enables cells to survive and recover from otherwise lethal conditions, such as a severe heat-shock stress. In *Helicobacter pylori* this specific environmental stress mainly triggers the induction of heat-shock proteins and chaperonins that are tightly regulated by two dedicated transcriptional repressors, HrcA and HspR. Comparative transcriptome analyses of the heat-shock response and of the *ΔhspR* mutant strain in *H. pylori*, has enabled us to grasp information about the specific genes implicated in this stress and the role of the HspR transcriptional regulator in the physiology of the bacterium. The conventional damage generated by the heat-shock stress is accumulation of significant amount of misfolded polypeptides, with subsequent loss of function of one or more essential proteins, so accordingly, from heat-shock transcriptome analysis we detected a significant de-repression of the well-known three heat-shock operons with a subsequent overproduction of heat-shock proteins and chaperones, such as GroEL-GroES, DnaK-DnaJ, able to prevent protein aggregation and to recover protein that have been partially or completely unfolded during the stress. Accordingly, we noticed that the heat-shock stimulus led to the de-regulation of several genes belonging to diverse functional categories that are required to ensure cellular survival also at non-physiological temperatures. It is likely that during stress, the bacterial membrane may be subjected to injury, thus the expression of some proteins involved in the biogenesis of cell wall, envelope and bacterial membrane were also affected. For instance, the *csd4* gene coding for a cell shape determinant protein implicated in maintaining the helical morphology of the bacterium, and genes of the Mur family involved in the biosynthetic pathway of peptidoglycan were also affected by heat-shock. Our analysis indicates also that the heat-shock response maintains and protects the integrity of cellular DNA and RNA, a prerequisite for the survival of the organism at high temperature, controlling the expression of specific ribonucleases and recombinases. Considering that *H. pylori* adopts a negative transcriptional regulation mechanism to control the expression of the heat-shock genes through the HspR and HrcA repressor proteins, it is particularly interesting to note that only the 18% of the overall de-regulated genes were related to the heat-shock response and directly or indirectly controlled by the HspR transcriptional regulator. We speculate that, both in the transcriptome of the *ΔhspR* mutant strain and of the heat-shock stress, the altered expression of chaperones, heat-shock proteins or other crucial regulators could in turn establish different proper transcriptional responses necessary for the survival of the bacterium during adverse environmental growth conditions, as in the case of several genes coding for proteins involved in the assembly and regulation of the flagellar apparatus. In *H. pylori*, the output of the HspR and HrcA module indirectly impairs motility, indeed

both mutant strains are not motile (Roncarati et al., 2007a). Likely, the overproduction of proteins directly regulated by HspR could have a profound impact on the intracellular level of proteins involved in the flagellar biosynthesis. This evidence provides a link between the heat-shock and the flagellar circuits, both involved in the colonization of the gastrointestinal tract of the human stomach that depends on the presence of flagellins and heat-shock proteins acting as virulence factors (Colland et al., 2001; Niehus et al., 2004). Furthermore, since the ChIP-seq analysis clearly revealed only four *in vivo* already known HspR binding sites, three of which (P*cbp*, P*gro* and P*hrc*) associated to transcriptional regulation, the other 59 genes whose transcription was affected in the *ΔhspR* mutant strain were probably indirectly controlled by HspR through a still unknown molecular mechanism. Anyway, this apparent discrepancy between the ChIP-seq and RNA-seq data is supported by other HspR studies conducted in *Streptomyces coelicolor* and *Mycobacterium tuberculosis* (Bucca et al., 2003; Stewart et al., 2002). Also, in these bacteria the HspR direct regulon is limited to a reduced number of genes, usually coding for proteins that play an active role during the thermal stress, and at the same time the HspR deletion led to the de-regulation of a substantial number of genes, both associated and not associated to the heat-shock stress. For instance, we identified several genes coding for ABC transporter proteins, whose role during stress requires further investigation. In addition, we found that few genes, de-regulated in the *ΔhspR* mutant strain, belong to the regulon of Fur protein, a *H. pylori* trancriptional factor involved in the control of transcription of genes implicated in the Fe^{2+} uptake (Delany et al., 2001; van Vliet et al., 2002; Danielli et al., 2009) and detoxification (Bereswill et al., 2000; Ernst et al., 2005). Whether and how HspR can coordinate a proper transcriptional response is still unknown, however, the possibility that up- and/or down-regulated genes in the *ΔhspR* mutant strain might arise from the enhanced synthesis of one or more members of the HspR regulon cannot be ruled out and remains to be elucidated. Furthermore, considering that the *hrcA* gene is one direct target of HspR, it is tempting to speculate that many genes deregulated by the *hspR* deletion and lacking an HspR binding site arise from altered levels of the HrcA regulator in the *ΔhspR* mutant strain. Surprisingly, the transcription of genes coding for the class of stress related proteases seems to be unaffected by heat challenge, except for the *clpB* and *htrA* genes. Unlike *S. coelicolor*, transcription of the *clpB* gene, coding for a protease that interplays with DnaK, DnaJ and GrpE during protein disaggregation, is not directly controlled by the *H. pylori* HspR. Moreover, the *clpB* gene along with the *htrA* gene coding for a protease, weakly respond to the heat stress at a transcriptional level. The latter is a serine-protease associated with pathogenesis: it is secreted by the type IV secretion system with the toxin CagA and contribute to the disruption of the epithelial barrier of the human stomach after the colonization (Pachathundikandi et al., 2013). While in other bacteria, such as *C.*

jejuni and *S. coelicolor* a few proteases, like the Lon and ClpP proteases, are induced by a temperature up-shift and by the deletion of the *hspR* gene (Bucca et al., 2003; Holmes et al., 2010), in *H. pylori* these proteins do not seem to belong to the heat-shock and HspR regulon. Considering that predominant up-regulatory response to thermal upshift in *S. coelicolor* is exerted at the translational rather than transcriptional level (Bucca et al., 2017), an interesting scenario considers that post-transcriptional or post-translational control strategies could provide enhanced levels of these relevant players during adverse environmental growth conditions.

4.2 HspR-DNA interaction

Through the ChIP-seq analysis we have confirmed that HspR binds *in vivo* the promoter region of the three heat-shock operons and the coding sequence of *speA* gene, coding for an arginine decarboxylase. Since *in vitro* direct HspR binding on its target genes was already demonstrated by previous DNaseI footprinting assays (Sphon and Scarlato 1999; Roncarati et al., 2007a), we decided to use the high-resolution molecular technique of hydroxyl-radical footprinting to gain additional and novel information on the mechanism of HspR binding on its targets. Despite its small size of only 17 kDa, HspR is able to bind and cover extended DNA regions of about 70/80 bp, with a peculiar DNA binding pattern consisting of short and periodic protected DNA tracts, separated by non-protected regions of 7/8 bp (Section 3.3.1 Figure 11). Interestingly, HspR binds to the three heat-shock operons by protecting seven short DNA tracts, while protection on the coding region of the *speA* was limited to five DNA tracts. This slight difference in the binding pattern could be related to the fact that the HspR intragenic binding site appears not associated to a transcriptional regulation. Indeed, from the RNA-seq analysis it is not possible to appreciate changes in the transcript level of *speA* gene in the *hspR* mutant strain, nor in neighboring genes. This is not surprising, as the advent of the "omics" era highlighted binding of regulatory proteins to a number of sites not associated to regulation, such as the Fur repressor (Danielli et al., 2006; Vannini et al., 2017), the *E. coli* CRP activator and the RNA polymerase enzyme (Grainger et al., 2005). However, among the four HspR binding sites a conserved inverted repeat, similar to the *S. coelicolor* HAIR sequence, was identified. The functional importance of this putative consensus sequence was assessed through *in vitro* and *in vivo* experiments, discovering that it represents an essential and important DNA element for the primary HspR binding on its own promoter with concomitant transcriptional repression. Indeed, mutation of one or both arms of the inverted repeat completely impairs *in vitro* the HspR binding on P*cbp* and prevents *in vivo* HspR-dependent repression of its own promoter, demonstrating for the first time the functional importance, also in *H. pylori*, of the

HAIR-like sequence. In *H. pylori* regulatory proteins, such as HP1043, HrcA and Fur regulators, binds as dimers, protect limited DNA regions and their ability to bind to extended DNA promoter region, as in the case of *H. pylori* Fur regulator, is due to the presence of multiple consensus boxes on the operator of its target genes (Bucca et al., 1995; Delany et al., 2002a; Roncarati et al., 2016). In constrast, HspR-controlled promoters harbor a single, conserved inverted repeat (HAIR-like motif), that drives specific DNA recognition. However, HspR-DNA binding extends over a large portion of the promoter DNA, resulting in the characteristic extended protection in *in vitro* footprinting assays (Roncarati et al., 2007a). We speculate that the DNA-binding mechanism of HspR on its target operators is peculiar and somewhat different from the ones adopted by other HspR homologs that have been characterized at a molecular level. For instance, the ability of *S. coelicolor* HspR to bind to extended DNA regions is due to the presence of multiple consensus boxes (represented by three inverted repeats, IR1, IR2 and IR3), on the operator of its target genes (Bucca et al., 1995). In addition, site directed mutagenesis of the P*cbp* promoter provided useful and novel information about HspR-DNA interactions: the non-conserved spacer regions flanking the central inverted repeat are essential elements for HspR binding to fully occupy its operator on P*cbp* promoter. In fact, mutations in these spacer sequences prevent HspR binding only on the side of the mutated region, with no effects on the protein binding on the opposite side of the mutation and on the DNA region harboring the HAIR-like motif. *In vitro* and *in vivo* experiments provided detailed information of HspR-DNA molecular interactions and, at least for the P*cbp* promoter, enabled us to propose a cooperative mechanism of HspR binding. In our model, repression of transcription by HspR is achieved by a dimer of HspR recognizing the HAIR-like motif for the primary binding and this in turn cooperatively recruits two HspR dimers on both sides of the HAIR motif (Figure 15).

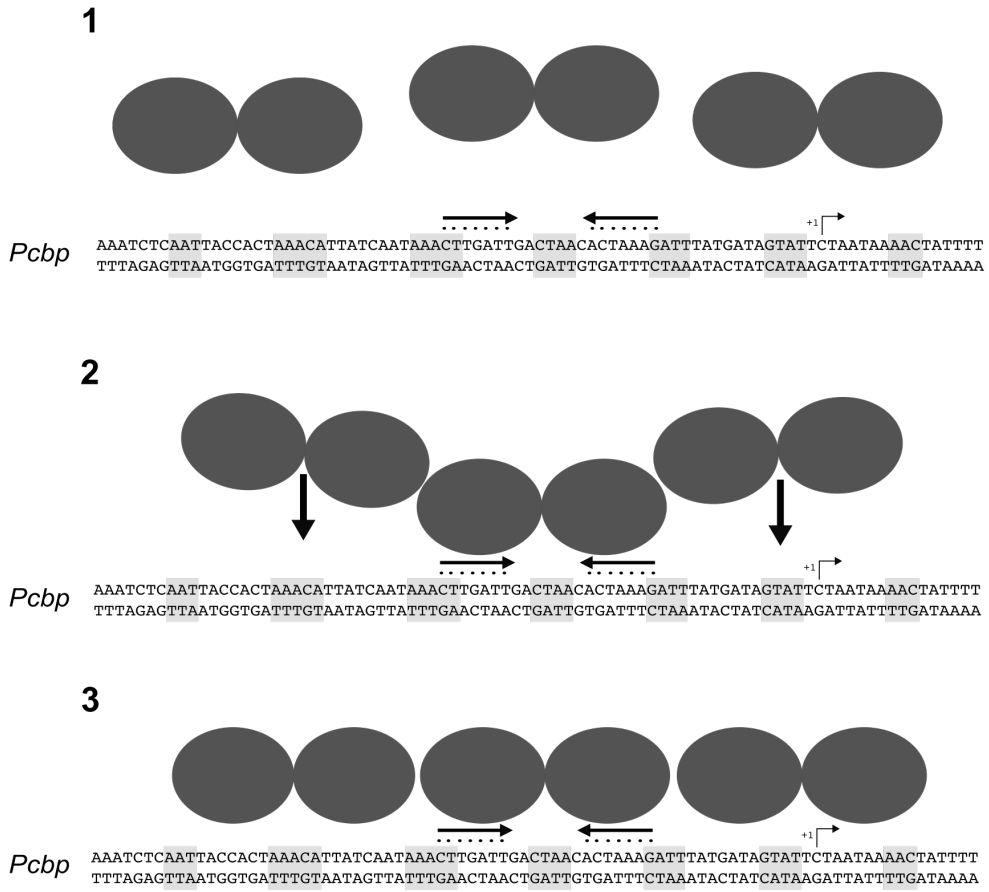


Figure 15. Model of the molecular mechanism for HspR-DNA binding. In solution, HspR dimerizes (1) and binds with high affinity to the DNA region containing the HAIR consensus sequence (2). This binding could act as a nucleation center driving cooperative binding of two HspR dimers on the flanking regions (3), likely recognizing still unknown sequence determinants. (Pepe et al., 2018)

Evidences that HspR could bind its DNA sequences as a dimer came also from previous studies, in which it was found that the *M. tubercolosis* HspR exist in a dynamic state between the monomeric and dimeric form in solution (Parijat and Batra, 2015). From bioinformatic analysis, the HspR repressors of both *H. pylori* and *M. tuberculosis* appear to be members of the MerR superfamily of transcription factors which are known to be dimeric in nature, to have a C-terminal effector binding domain separated by a long coiled-coiled region which acts as the homodimerization domain and to regulate transcription via twisting and bending mechanism. Moreover, Spohn and collaborators (Spohn et al., 2004) provided evidences that *H. pylori* HspR can form high order oligomers. In conclusion, we speculate that an HspR dimer binds to the central DNA region of its operator harboring the HAIR-like motif sequences that acts as a nucleation center for protein-protein interactions, driving cooperative binding of HspR homodimers to the specific and still unknown determinants of the DNA sequences surrounding the inverted repeat.

5. *Part 2 Results*

5.1 *Specific introduction*

The energy-dependent proteases are important components of the heat-shock response in all the organisms, with a major role in degrading proteins that cannot be resurrected by the chaperones induced during heat-shock. A recent study showed the importance of the Clp proteins also in the virulence of *H. pylori*, and their survival within macrophages. The discovery that *clpA*/*clpP* double mutant of *H. pylori* SS1 strain was unable to colonize gastric mucosa in mice is an evidence that removal of damage proteins by the ClpP and ClpA proteases plays an important role in establishing such infection (Loughlin et al., 2009). In several bacteria, the regulation of the heat-shock response seems to affect both the chaperone and protease expression, leading to induction when their steady-state levels are insufficient to deal with damaged proteins. For example, a specific unstable repressor, CtsR, is responsible for heat-shock regulation of the Clp ATPase and protease genes in many gram-positive bacteria (Chastanet et al., 2001; Kruger et al., 2001). Moreover, in other bacteria such as *Streptomyces coelicolor* and *Campylobacter jejuni* few proteases (like Lon and ClpP) are induced by temperature up-shift and transcriptionally regulated by the heat-shock repressor HspR (Bucca et al., 2003; Holmes et al., 2010). In *Helicobacter pylori*, the class of Clp stress induced proteases does not belong to the HspR regulon and, moreover, does not seem to be positively or negatively affected at the transcriptional level during the heat-shock response, as shown by the global transcriptome analysis previously presented and discussed above (paragraph 3.2.1, Figure 9). Little is known about the transcriptional or post-transcriptional regulation of this class of proteases in *H. pylori*, as well as about its direct or indirect protein substrates. In this respect, the second part of this study aims to get new insights on the role of Clp-mediated proteolysis in *H. pylori* and on the identification of potential ClpP protein targets, developing and implementing a ClpP trapping strategy, that potentially could represent also a useful tool to affinity-purify epitope-tagged proteins after homologous expression in *Helicobacter pylori*.

5.2 Construction of a ClpP trap in *Helicobacter pylori*

5.2.1 ClpP protein expression during heat-shock stress in *H. pylori*

To discover if the expression of the *H. pylori* ClpP protease could be positively or negatively affected at a post-translational level during the heat-shock stress, we performed an immunoblot analysis to monitor the ClpP protein level after the heat-shock response. To this aim, we generated a *H. pylori* G27-derivative P*clpP*-ClpP^{WT}-His$_6$-tag strain expressing the *clpP* gene fused with a C-terminal His$_6$-tag in its original genomic locus. Cultures of *H. pylori* G27 wild-type and P*clpP*-

ClpP^{WT}-His$_6$-tag strains were liquid-grown up to the exponential growth phase and the latter strain was exposed to the heat-shock (42°C) for 30 and 60 minutes. Then, total protein extracts of these strains were subjected to an immunoblot analysis with an anti-His$_6$-tag antibody. As negative control, the protein extract of the *H. pylori* wild-type strain was used, because it expresses the native ClpP protein without a His$_6$-tag epitope. In addition, the protein extract of the *E. coli* strain, expressing the ClpP-His$_6$-tag was included as positive control of the anti-His antibody. As shown in Figure 16, we note a band of interest at the expected molecular weight of 22 kDa (black arrow) only in the strain expressing the ClpP-His$_6$-tag fusion protein and not in the wild-type control strain (Fig. 16, lane 2), indicating that the ClpP protein is correctly expressed. Interestingly, no change in the ClpP-His$_6$-tag protein amount is observed in the heat-shocked *H. pylori* P*clpP*-ClpP^{WT}-His$_6$-tag strain (t_1HS lane 4 and t_2HS lane 5) in comparison to the untreated sample (t_0, lane 3), suggesting that in *H. pylori* the *clpP* gene seems not to be heat-shock responsive. However, it cannot be excluded that other environmental inputs could provide enhanced levels of *clpP* gene in *H. pylori*.

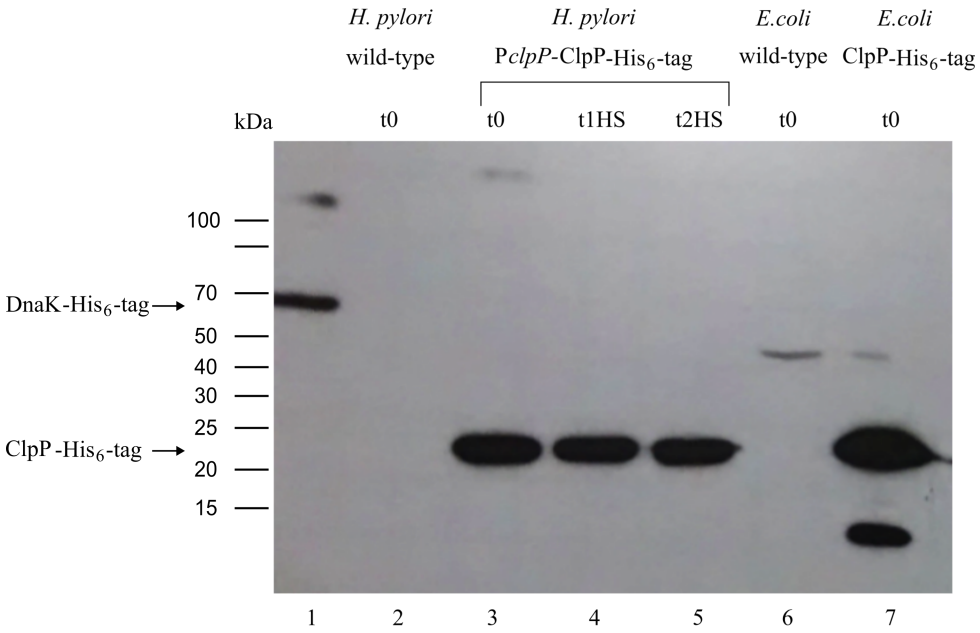


Figure 16. Immunoblot analysis of total protein extracts with an anti-His$_6$ monoclonal antibody. About 12 µg and 8 µg of total protein extract of *H. pylori* and *E. coli* strains, respectively, were separated in a 12% SDS-PAGE. The band of interest, corresponding to the ClpP protein and indicated with a black arrow, appeared in the *H. pylori* P*clpP*-ClpP^{WT}-His$_6$-tag, not treated (t_0, lane 3) and heat-shocked for 30 min (t_1HS, lane 4) and 60 min (t_2HS, lane 5), while, is absent in the *H. pylori* wild-type strain used as negative control (t_0 lane 2). The *E. coli* strain, expressing the ClpP^{WT}-His$_6$-tag (lane 7), was used as positive control, and the DnaK-His$_6$-tag protein (70 kDa) as a marker.

5.2.2 Establishment of the ClpP^{trap} in *H. pylori*

An important and essential role played by ATP-dependent caseinolytic proteases (Clp) is to rapidly adjust the amount of regulatory and metabolic proteins. In this regard, we decided to set up a strategy to directly identify putative ClpP protease substrates. To this aim, we employed a substrate trapping approach that has been previously used for the AAA$^+$ proteases ClpX/P in *E. coli* (Flynn et al, 2003), expressing *in vivo* a proteolytic inactive form of ClpP that can accept and retain but not degrade, substrates translocated into its proteolytic chamber. Briefly, to construct a ClpP^{trap} variant, we inactivated the ClpP catalytic activity by a single point mutation, substituting the active site-containing Serine 98 with an Alanine (ClpP^{S98A}). We also engineered a C-terminal epitope tag (His_6-tag) for affinity purification purposes (see materials and methods for details). There are no reports in literature describing protein purification protocols in *H. pylori*, therefore, before proceeding with the establishment of a ClpP^{trap} variant, we performed a first trial of ClpP purification using the *H. pylori* G27 P*clpP*-ClpP^{WT}-His_6-tag derivative strain. Briefly, bacterial cells were liquid-grown up to late-exponential growth phase (OD 0.7-0.8) and subjected to affinity chromatography using an His-Select-Nickel affinity suspension (50% Ni^{2+}-NTA slurry). Our initial efforts to purify the ClpP^{WT}-His_6-tag protein were unsuccessful, mainly for two reasons. The first one could be related to the low expression level of the ClpP protein governed by its own promoter that does not allow for the production of an adequate amount of His_6-tagged protein, necessary for an optimal interaction with the slurry utilized. The second one is likely due to the presence of a high number of *H. pylori* endogenous proteins with high affinity to nickel ion that saturate the Ni-NTA slurry, thus obtaining not sufficient amount of purified ClpP protein required for subsequent analyses. To overcome these issues, we decided to enhance the *clpP* gene expression and to change the epitope tag.

5.2.3 Generation of *H. pylori* ClpP$^{WT/S98A}$$His_6$-Strep-tag over-expressing strains

In order to obtain a ClpP over-expressing strain, we generated three different constructs in which the ClpP wild-type (ClpP^{WT}) or mutant (ClpP^{S98A}) proteins were expressed under the transcriptional control of three strong *H. pylori* promoters: the *ureA* (P*ureA*), the *cagA* (P*cagA*) and the *cncr1* (P*cncr1*) promoter regions (Fig. 17). Furthermore, to change the epitope-tag we performed an all-around PCR (see materials and methods 8.3.2.5) in which a Strep-tagII epitope was attached at the C-terminal of the ClpP$^{WT/S98A}$ protein, maintaining also the His_6-tag. These vectors were then integrated by double homologous recombination in the *ureA*, *cagA* and *vacA* locus of *H. pylori* G27 wild-type, respectively (Fig. 17A), and the ClpP$^{WT/S98A}$-His_6-Strep-tagII protein levels were assayed

through an immunoblot analysis using an anti His_6-tag antibody. As shown in figure 17B the over-expression of the $ClpP^{WT/S98A}$-His_6-Strep-tagII under the transcriptional control of P*ureA*, P*cagA* and P*cncr1* is 4-6 times higher than the physiological ClpP protein expression driven by its own promoter (Fig. 17B, lane 1).

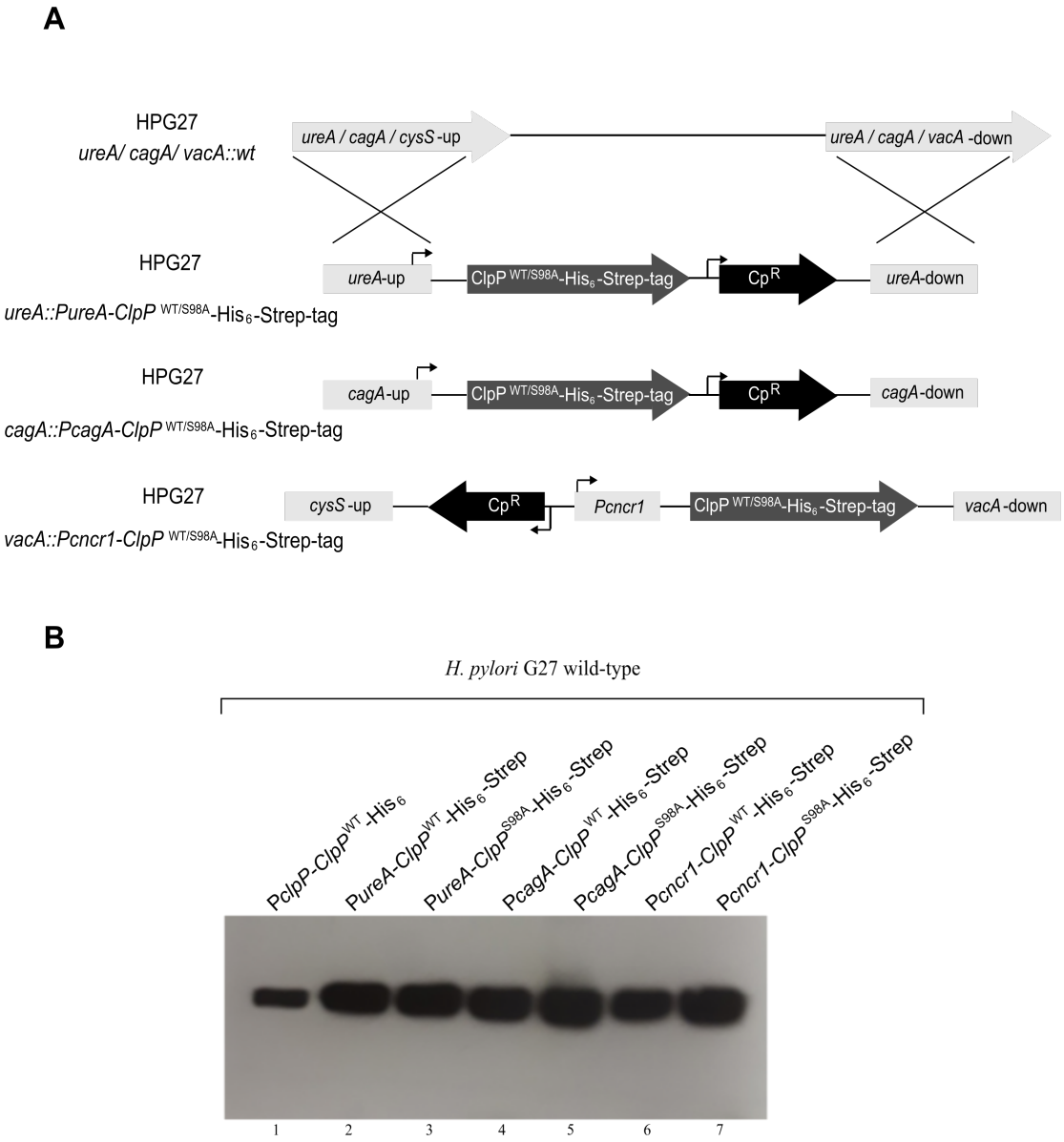


Figure 17. **(A)** Schematic representation of P*ureA*/P*cagA*/P*cncr1*-$ClpP^{WT/S98A}$-His_6-Strep-tagII constructs obtained transforming the *H. pylori* G27 wild-type acceptor strain by double homologous recombination in the *ureA*, *cagA* and *vacA* locus, respectively and selected for chloramphenicol resistance (Cp^R). The transcriptional start site is depicted by a bent arrow. **(B)** Immunoblot analysis of total protein extracts of *H. pylori* $ClpP^{WT/S98A}$-His_6-Strep-tagII over-expressing strains (lane 2 to 7) with an anti-His_6 monoclonal antibody. The *H. pylori* P*clpP*-$ClpP^{WT}$-His_6-tag strain was used as positive control (lane 1). Bands intensity was analyzed using ImageJ.

After generating *H. pylori* G27 derivative strains over-expressing the ClpP$^{WT/S98A}$-His$_6$-Strep-tagII, we performed a ClpP protein purification through affinity chromatography using the Strep-Tactin affinity matrix, which carries an engineered streptavidin. To this aim, we decided to use the ClpP$^{WT/S98A}$ over-expressing strains under the transcriptional control of P*ureA*. Briefly, *H. pylori* P*ureA*-ClpP$^{WT/S98A}$-His$_6$-Strep-tagII strains were liquid-grown up to late-exponential growth phase (OD 0.7-0.8) and subjected to affinity-chromatography purification. Eluted ClpP$^{WT/S98A}$-His$_6$-Strep-tagII proteins were separated on a polyacrylamide denaturing gel and detected through Comassie Blue staining (Fig. 18). Interestingly, in the final eluate (lanes 4 and 5) we note two bands at the expected ClpP molecular weight of 22 kDa. We speculate that the higher molecular weight band could correspond to an unprocessed ClpP precursor protein, while the faster migrating band might represent processed isoforms. Likely, the *H. pylori* ClpP protease could be subjected to a physiological processing, as in the case of *E. coli* ClpP (Michael et al., 1990). In conclusion, by increasing the amount of ClpP protein level and by using the Strep-tagII instead of the His$_6$-tag, we were able to achieve a successful ClpP protein purification in *H. pylori*.

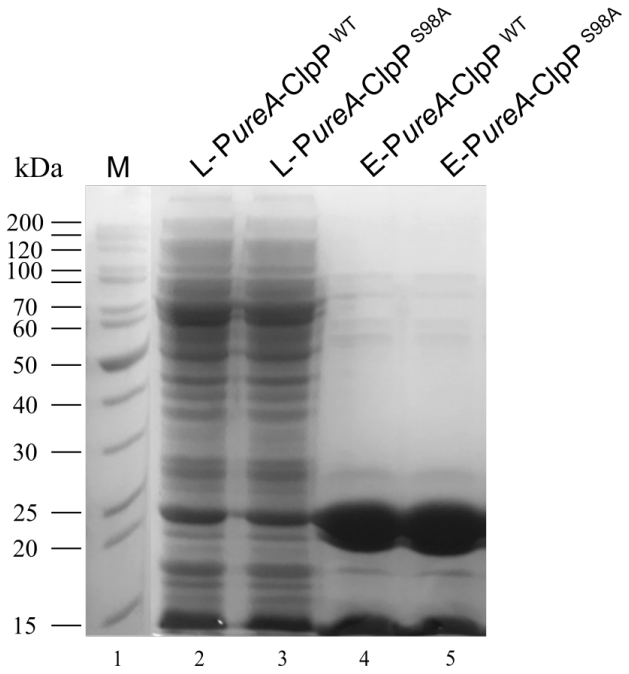


Figure 18. SDS-PAGE of *H. pylori* P*ureA*-ClpP$^{WT/S98A}$-His$_6$-Strep-tagII protein purification through affinity chromatography. After purification, eluted wild-type and mutant ClpP proteins (lane 4 and 5, respectively) were separated on a 12% polyacrylamide denaturing gel and detected through Comassie Blue staining. Protein marker with its corresponding molecular weight standards are indicated in lane 1. In lane 2 and 3 samples of soluble lysate of *H. pylori* wild-type and mutant ClpP over-expressing strains, respectively, recovered before incubation with the Strep-Tactin matrix.

5.2.4 Protein trapping by ClpP *in vivo*

It has been reported that bacterial strains co-expressing $ClpP^{trap}$ and the ClpP wild-type protein determine the incorporation of active $ClpP^{WT}$ subunits in the $ClpP^{S98A}$ oligomer, impairing the efficacy of the ClpP trapping approach (Nowsheen et al., 2013). In fact, depletion of the chromosomally encoded *clpP* gene enriches for inactive $ClpP^{S98A}$ oligomers, improving the recovery of putative ClpP substrates. Unfortunately, we were unable to generate a stable knock-out (KO) mutant of the endogenous *clpP* gene in *H. pylori*. For this reason, we employed a different strategy to inactivate the catalytic activity of the endogenous $ClpP^{WT}$ protease. In other bacterial pathogens, such as for example in *S. aureus*, chemical compounds named *β*-sultams can bind to ClpP in a sub-stochiometric manner and induce irreversible dehydroalanine formation of the active site serine, triggering the formation of completely inactive ClpP heptamers (Gersch et al., 2013). Considering that the catalytic triad of the active site of the ClpP protease family is well conserved among different bacterial species including *H. pylori* that shows 66% of amino acid identity to the *S. aureus* ClpP protein (Fig. 19), we decided to apply this strategy also in *H. pylori*.

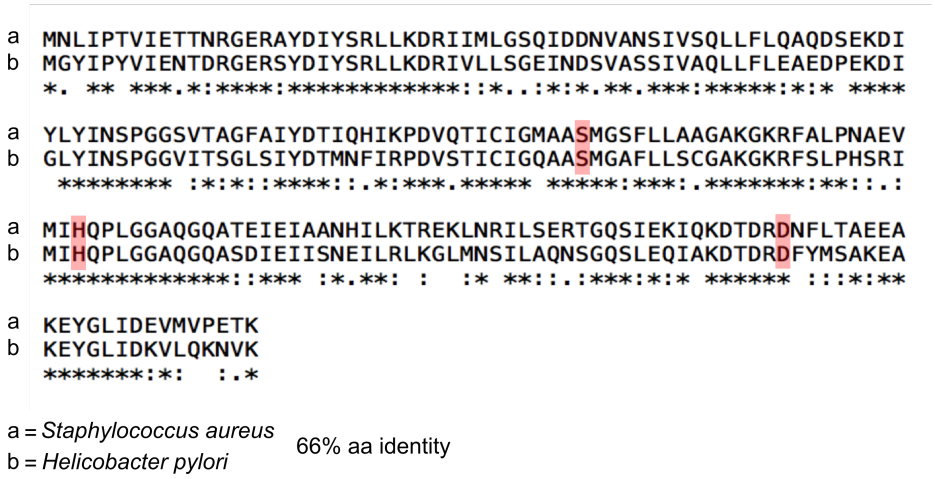


Figure 19. Amino acid sequence alignment of *S. aureus* and *H. pylori* ClpP proteins. The conserved catalytic triad residues, Ser-His-Asp, involved in the ClpP proteolityc activity are highlighted in red. The key below denotes conserved sequence (*), conservative mutation (:), semi-conservative mutations (.), and non-conservative mutation ().

The molecule used to introduce a site-directed mutation into the endogenous ClpP active site is the RSK13, an alkyne–free *β*-sultam, previously tested and applied in *S. aureus* living cells (Gersch et al., 2013). The *H. pylori* G27 derivative strain over-expressing the wild-type and mutant ClpP-His_6-Strep-tagII protein (P*ureA*-$ClpP^{WT/S98A}$-His_6-Strep-tagII), under the transcriptional control of the P*ureA* promoter, was liquid grown up to mid-log phase, split in two subcultures and treated for 4

hours either with 100 µM, 200 µM of RSK13 molecule or with the same volume of dimethyl sulfoxide (DMSO, untreated control sample). Then, the treated bacterial cultures were subjected to affinity chromatography using the Strep-Tactin matrix (see material and methods for details). The eluted $ClpP^{WT/S98A}$-His_6-Strep-tag proteins were analyzed by SDS-PAGE and detected through silver staining with results shown in Figure 20.

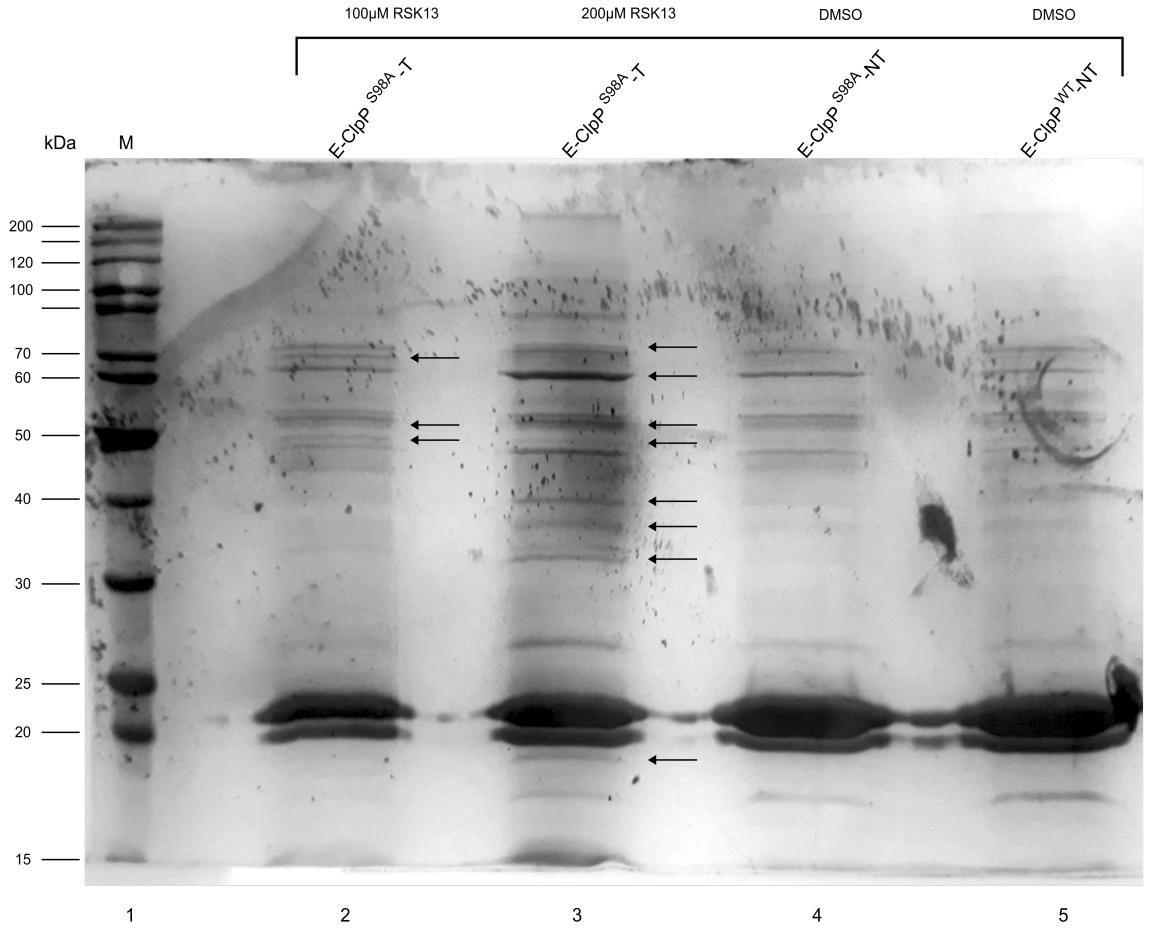


Figure 20. Proteins trapped *in vivo* by the $ClpP^{trap}$ variant. Wild-type and mutant ClpP proteins, treated (lane 2 and 3) and not (lane 4 and 5) with the RSK13 molecule, were eluted from Strep-Tactin affinity column, separated on 12% SDS-PAGE gel and stained with silver staining solution. Proteins co-purified with the mutant $ClpP^{S98A}$ treated with RSK13 (lane 2 and 3) which could represent putative ClpP substrates, are indicated as black arrows. Protein marker with its corresponding molecular weight standards are indicated in lane 1.

It is worth noting that in the control sample ($ClpP^{WT}$ treated with DMSO, lane 5) the co-purified proteins do not represent trapped substrates, but rather proteins that are not specifically associated with the ClpP barrel. No relevant differences in the protein pattern of $ClpP^{WT}$ and $ClpP^{S98A}$ samples treated with DMSO (lane 5 and 4, respectively) have been observed. This is consistent with the fact

that over-expression of the inactive proteolytic form of ClpP ($ClpP^{S98A}$) in a *H. pylori* strain containing also the wild-type endogenous *clpP* gene, impairs the efficacy of the ClpP trapping approach, failing to provide the recovery of putative ClpP substrates. Interestingly, in the mutant $ClpP^{S98A}$ samples treated with 100 μM and 200 μM of RSK13 (lanes 2 and 3, respectively) we observed a dose-dependent appearance of bands, which are not present in the control sample (lane 5). We speculate that proteins co-purified with the mutant $ClpP^{S98A}$ treated with 100 μM and 200 μM of RSK13 (indicated as black arrows in lane 2 and 3, respectively) but not captured by $ClpP^{WT}$ treated with DMSO, could represent putative substrates. It is likely that, the RSK13 molecule inactivates, or at least negatively impairs, the proteolytic activity of the wild-type endogenous *clpP* gene, thus, improving the recovery of putative ClpP substrates. It will be necessary to perform further analyses to confirm and demonstrate that the RSK13 molecule utilized is specific for the *H. pylori* ClpP serine protease. Nevertheless, these results could represent encouraging preliminary data, indicating that the used protocol could be suitable to discover direct ClpP target proteins in *H. pylori*.

6. *Part 2 Discussion*

It is well known that, in a wide range of bacteria, the class of the ClpP protease plays indispensable roles in cellular protein quality control during normal growth conditions, by refolding or degrading damaged proteins, and also during heat-shock or other similar stresses, by removing heat-damaged proteins. While in some bacteria, like *Streptomyces coelicolor* and *Campylobacter jejuni*, the class of ClpP proteases, including the *clpP* gene, are transcriptionally induced by heat-shock, and are directly controlled by dedicated heat-shock regulators (Bucca et al., 2003; Holmes et al., 2010), in *H. pylori* the *clpP* gene does not belong to the heat-shock regulon controlled by the HspR repressor and seems to be unaffected by the heat challenge also at a post-translational level. Clearly, the hypothesis that post-transcriptional or post-translational control mechanisms could provide enhanced levels of the Clp ATPase subunits, like ClpX, ClpA or ClpC, during adverse environmental growth conditions, thus modulating the expression of the ClpP proteolytic subunit cannot be ruled out and needs further investigations.

In recent years, the attention has been focused on the role of proteolysis as a regulatory mechanism. Indeed, regulated proteolysis is essential for biological processes in all the organisms, and ClpP proteases in association with the chaperone activity of Clp ATPases, like ClpA and ClpX, strongly contribute to control stability and activity of central transcriptional regulators. Considering that in some bacterial pathogens, such as in *B. subtilis*, in *S. aureus* and in *C. crescentus* (Gerth et al., 2004; Dorte et al., 2014; Nowsheen et al., 2013), ClpP targets include transcriptional regulators controlling major environmental stress responses infectious and developmental programs, we decided to extend our study on the physiological functions of *H. pylori* ClpP protease. For this purpose, a substrate trapping approach based on a protease-deficient ClpP variant was developed and implemented. During the experiments, we have encountered several problems concerning the tag system and the impossibility of obtaining a stable *clpP* knock-out mutant. However, the use of a strong promoter (P*ureA*) that drives a ClpP over-expression and of a streptavidin as a protein-tag has allowed us to purify enough ClpP protein for trapping purpose (Fig. 18). Moreover, this experimental approach could represent a powerful expression system suitable for the isolation and purification of endogenous proteins from *H. pylori* cells. To this aim, we have generated expression vectors (Fig. 21) suitable to express *in vivo*, under the transcriptional control of a low, medium or strong promoter (P*cagC*, P*cagQ* and P*cncr1* respectively; Fig. 21) an N-terminal or C-terminal Strep-tag protein in *H. pylori*. However, a disadvantage of this system could be represented by the fact that it is not an inducible system, thus resulting in a constitutive protein expression. This may represent a limitation in the case of over-expression of a damaging or toxic protein for the bacterium.

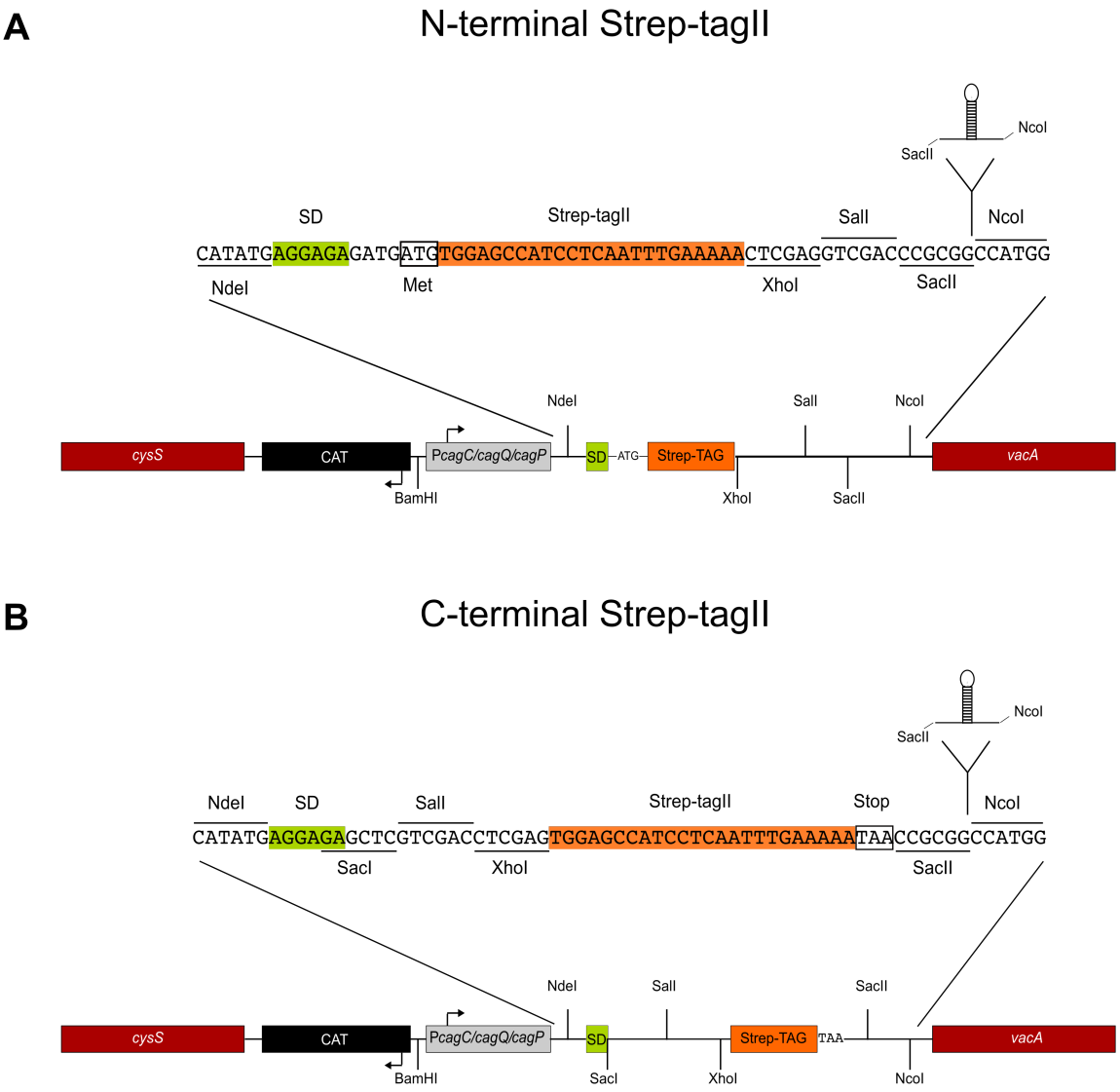


Figure 21. Schematic representation of N-and C-terminal-Strep-tagII expression vectors. These vectors include the promoter region of P*cagC*, P*cagQ* or P*cagP* (low, medium and strong promoter, respectively) and the terminator sequence of HPG27_RS07645 flanking a Multi-Cloning Sites (MCS) region, that allows to clone the protein of interest fused to a Strep-tagII epitope at the N-terminal (**A**) or C-terminal (**B**) protein domain. All the restriction sites included are underlined. The *cysS* and *vacA* regions (red boxes) represent *H. pylori* DNA sequences needed for transforming the *H. pylori* G27 wild-type acceptor strain by double homologous recombination in the *vacA* locus and the derivative strains will be selected by chloramphenicol resistance (Cp^R). The Shine-Dalgarno and the Strep-tagII nucleotide sequences are highlighted in green and orange, respectively, while the start-codon ATG (**A**) and the stop-codon TAA (**B**) are highlighted by empty boxes. The transcriptional start site is depicted as a bent arrow.

A prerequisite for a successful ClpP-trapping is to perform the experiment in a *clpP* knock-out mutant background. While in all the other bacterial species, in which it was performed the $ClpP^{trap}$ experiment, it was possible to delete the endogenous *clpP* gene (Dorte et al., 2014; Nowsheen et el.,

2013), the generation of *clpP* knock-out mutant was unsuccessful in the used *H. pylori* G27 strain. We have tryied to generate a knock-out mutant disrupting the *clpP* gene by completely replacing it with an antibiotic resistance cassette or by insertional mutation to generate a truncated *clpP*. Probably, altering the functionality of the flanking *comM* gene, coding for a DNA transformation competence protein, during the homologous recombination, or the presence of a putative small RNA in this genomic region could impair a successful recombination and the subsequent gene deletion. As all attempts to generate a *clpP* knock-out mutant were unsuccessful, we decided to adopt another strategy, trying to down-regulate or completely inactivate *in vivo* the endogenous ClpP proteolytic activity.

Significant interest in *in vivo* ClpP inactivation started with the discovery of its crucial role in cellular metabolism and in particular in virulence of several bacterial pathogens, including *S. aureus*. To date, several different protease inhibitors are currently applied in the clinic as terapeutic agents, and the most prevalent strategy for protease inhibition is represented by blockage of the active site with covalently or noncovalently active compounds. Recently, in *S. aureus*, it was discovered that a chemical compound (*β*-sultams) termed RSK13, can selectively convert *in vivo* the active site serine into a dehydroalanine, inhibiting the ClpP catalytic activity. This could represent an elegant strategy to introduce a site-directed mutation into a protease active site without affecting other serine residues of the protein and without causing changes in ClpP oligomerization upon ligand binding (Gersch et al., 2013). The amminoacids involved in the proteolytic activity of ClpP are highly conserved among different bacterial species, indeed the ClpP catalytic triad of *H. pylori* is identical to the one of *S. aureus* (Fig. 19). Therefore, we decide to use the RSK13 molecule, previously utilized in *S. aureus*, to inactivate or negatively impair the proteolytic activity of the endogenous *H. pylori* ClpP, thus enhancing the recovery of putative ClpP substrates. Since in the *H. pylori* bacterial strains treated with the RSK13 molecule and over-expressing the $ClpP^{S98A}$ mutant protease the number of co-purified proteins is greater than those found in the control strains (Fig. 20), this approach seems to be promising. Obviously, this represents a preliminary data that requires additional detailed analyses. For example, it will be necessary to monitor if the RSK13 molecule is able to drive the specific inhibition of the *H. pylori* ClpP serine protease and check if the $ClpP^{S98A}$ co-purified proteins are ClpP substrates performing a mass spectrometry analysis and subsequent *in vitro* ClpP degradation assay. However, if it would work properly, this approach could be suitable to investigate the *H. pylori* ClpP contribution to cellular protein homeostasis and metabolism of the bacterium.

7. Conclusions and Perspectives

Comparative transcriptome analysis of the heat-shock response and of the *ΔhspR* mutant strain in *H. pylori* enabled us to obtain additional information about the specific genes implied in this kind of stress response and about the role of the master regulator of this circuit HspR in the physiology of the bacterium. Besides genes encoding for the major heat-shock proteins and chaperones of *H. pylori*, transcripts of several genes belonging to diverse functional categories, required to ensure cellular survival also under a non-physiological growth conditions, seem to be affected by the heat-shock stimulus. By combining the ChIP-seq and RNA-seq analyses we discovered that HspR directly binds, *in vivo*, only four target genes and that controls, probably in an indirect manner, transcripts of a substantial number of genes both associated and not associated to the heat-shock stress. Moreover, the use of the high resolution hydroxyl-radical footprinting technique provided more detailed information of the molecular interactions between HspR and its target DNA sequences, and at least for its own promoter, enabled us to propose a cooperative mechanism of HspR binding.

The identification of potential protein substrates degraded by the ClpP protease during normal growth conditions of the bacterium, could allow us to gain a better understanding on the role of Clp-mediated proteolysis in *H. pylori*. In this regard, the development and implementation of a substrate trapping approach, based on a protease-deficient ClpP variant, provides a good strategy to capture *in vivo* ClpP protein targets also in *H. pylori*. Furthermore, this experimental approach could represent a powerful expression system also suitable for the isolation and purification of endogenous proteins from *H. pylori* cells.

8. *Materials and Methods*

8.1 Bacterial strains and growth conditions

Helicobacter pylori strains (Table 2) were recovered from frozen glycerol stocks on Brucella broth agar plates, containing 5% fetal calf serum (FCS), in a 9% CO_2-91% air atmosphere at 37°C and 95% humidity in a water jacketed incubator (Thermo Scientific). Liquid cultures were grown in Brucella Broth supplemented with 5% fetal calf serum in gentle agitation in glass.

E. coli strains DH5α and BL21 (DE3) (Table 2) were grown on Luria-Bertani (LB) agar plates or LB liquid broth; when required, ampicillin, chloramphenicol and kanamycin were added to the medium to achieve a final concentration of 100 μg/ml, 30 μg/ml and 25 μg/ml, respectively.

8.2 DNA techniques

DNA manipulations as amplification, restriction digestions and ligations were all performed with standard techniques as described by Sambrook et al. (SambrooK et al. 1989). The restriction and modification enzymes were used according to the manufacturers' instructions (New England Biolabs). Preparations of plasmid DNA were carried out with NucleoBond Xtra Midi plasmid purification kit (Macherey-Nagel). DNA fragments for cloning purposes were extracted and purified from agarose gel using a QIagen Gel extraction Kit (QIagen, Inc.).

Experimental methods concerning the Results of part 1 are described in the annex 1.

8.3 Materials and Methods Part 2

8.3.1 Generation of *H. pylori tig-clpP* knock-out mutant strain and P*clpP*-ClpP^{WT}-His$_6$-tag complement strain

H. pylori G27-derivative *tig-clpP* knock-out mutant (*tig-clpP::Km*) was obtained using a pBluescript KSII (+) vector carrying DNA regions flanking the *tig* and *clpP* gene (ORF HPG27_RS03880; HPG27_RS03875) on the *H. pylori* chromosome, and a kanamycine resistance cassette. Specifically, primers ClpPKO5F/R (Table 3) were used to amplify and clone a 622 bp NotI/BamHI DNA fragment encompassing the region upstream the *tig* gene. The oligo pair ClpPKO3F/R (Table 3) was employed for amplification and cloning of a 562 bp BamHI/XhoI DNA

fragment containing the region downstream the *clpP* gene. The kanamycin resistance cassette, deriving as BamHI fragment from pVAC::Km plasmid (Table 2) was cloned in between these two fragments and the final construct was used to transform the *H. pylori* G27 wild-type acceptor strain. The kanamycin-selected mutants were expanded and the correct insertion was confirmed by PCR using oligo pairs ClpPKO5F/ClpPKO3R (Table 3).

H. pylori G27-derivative P*clpP-clpP*^{WT}-His_6-tag strain was obtained by double homologous recombination by transforming the *H. pylori* G27 *tig-clpP* knock-out mutant acceptor strain with the pBS::P*clpP*-ClpP^{WT}-His_6-tag plasmid carrying the genomic regions of *H. pylori* encompassing the *tig* and *clpP* genes amplified with oligonucleotides pair ClpPcF/ClpPcR that adds an His_6-tag epitope to the C-terminal domain of the *clpP* gene.

8.3.2 Generation of *H. pylori* ClpP$^{WT/S98A}$$His_6$-Strep-tag over-expressing strains

8.3.2.1 *Construction of the pBS:: PclpP-ClpP^{S98A}-His_6-tag vector*

Mutagenesis of the *clpP* gene, consisting in the amino acid substitution S98A was generated by all around PCR with oligonucleotides ClpP-S98A-F/ClpP-S98A-R, listed in Table 3, using the pBS::P*clpP*-ClpP^{WT}-His_6-tag plasmid as DNA template. The correct amino acid substitution was assessed by sequencing.

8.3.2.2 *Construction of pVAC::Pcncr1-clpP$^{WT/S98A}$-His_6-tag vectors*

The promoter region of P*cncr1* was PCR amplified from *H. pylori* G27 genomic DNA using specific primers pair PcagPF/PcagPR, listed in Table 3. The generated DNA fragment was digested with appropiate restriction enzymes and cloned into the pVAC-CAT plasmid (Table 2). The wild-type and mutant *clpP*-His_6-tag genes were PCR amplified with oligonucleotides ClpPcagPF/VSCDSCpR and using as DNA template the pBS::*clpP*^{WT}-His_6-tag and pBS::*clpP*^{S98A}-His_6-tag plasmid, respectively. The generated *clpP*$^{WT/S98A}$-His_6-tag DNA fragments were digested with the appropriate restriction enzymes, prior to clone it into the pVAC-CAT plasmid containing the P*cncr1* promoter, obtaining thus the pVAC::P*cncr1-clpP*$^{WT/S98A}$-His_6-tag vectors.

8.3.2.3 *Construction of pCagA::PcagA-clpP$^{WT/S98A}$-His$_6$-tag vectors*

The coding sequence of *clpP*^{WT}-His$_6$-tag and *clpP*-^{S98A}-His$_6$-tag were PCR amplified from the pBS::*clpP*^{WT}-His$_6$-tag and pBS::*clpP*^{S98A}-His$_6$-tag plasmids, respectively, using specific primers pair ClpPcagAF/ClpPcagAR listed in Table 3. Then, the generated DNA fragments were digested with appropriate restriction enzymes and cloned into the pSL1190-cag plasmid (Table 2) carrying the promoter region of P*cagA*.

8.3.2.4 *Construction of pUreA::PureA-clpP$^{WT/S98A}$-His$_6$-tag vectors*

The coding sequence of *clpP*$^{WT/S98A}$-His$_6$-tag were PCR amplified from the pBS::*clpP*^{WT}-His$_6$-tag and pBS::*clpP*^{S98A}-His$_6$-tag plasmids, respectively, digested with the appropriate restriction enzymes and cloned into the PMM682 plasmid (Table 2) carrying the promoter region of P*ureA*.

8.3.2.5 *Generation of H. pylori ClpP$^{WT/S98A}$His$_6$-Strep-tag over-expressing strains*

The pVAC::P*cncr1-clpP*$^{WT/S98A}$-His$_6$-Strep-tag, the pCagA::P*cagA-clpP*$^{WT/S98A}$-His$_6$-Strep-tag and the pUreA::P*ureA-clpP*$^{WT/S98A}$-StrepTag transformation vectors were obtained by all around PCR with oligonucleotides HPStrepTag-F/HPStrepTag-R (Table 3), and using the pVAC::P*cncr1-clpP*$^{WT/S98A}$-His$_6$-tag, the pCagA::P*cagA-clpP*$^{WT/S98A}$-His$_6$-tag and the pUreA::P*ureA-clpP*$^{WT/S98A}$-His$_6$-tag vectors as DNA template, respectively. These final plasmids were used to transform the *H. pylori* G27 wild-type acceptor strain in the *vacA, cagA* and *ureA* locus, respectively. The chloramphenicol-selected mutant strains were expanded and the correct insertion was confirmed by PCR using oligonucleotides pair cys-F/vacA-R for the pVAC::P*cncr1-clpP*$^{WT/S98A}$-His$_6$-Strep-tag strains, BB-F/OrfX/Cag-dx-R for the pCagA::P*cagA-clpP*$^{WT/S98A}$-His$_6$-Strep-tag strains and Ure-up-F/Ure-down-R for the pUreA::P*ureA-clpP*$^{WT/S98A}$-StrepTag strains.

8.3.3 Generation of N-and-C-terminal Strep-tagII expression vectors

The pVAC-Nt-Strep-tag and the pVAC-Ct-Strep-tag plasmids were generated by annealing complementary oligonucleotides pairs C-StrepNtF/C-StrepNtR and C-StrepCtF/C-StrepCtR, respectively, to form a double stranded DNA fragment with compatible overhangs (NdeI-NcoI) required to clone it in the pVAC-CAT plasmid, previously digested with the appropriate restriction enzymes. Then, the promoter regions of P*cncr1*, P*cagQ* and P*cagC* were PCR amplified from *H. pylori* G27 genomic DNA using primer pairs Pcncr1F/R, PcagQF/R and PcagCF/R, respectively,

with either BamHI-NdeI overhangs and cloned into the pVAC-Nt-Strep-tag and the pVAC-Ct-Strep-tag plasmids, previously digested with the appropriate restriction enzymes.

8.3.4 Preparation of protein extracts and immunodetection

Cell pellets were resuspended in 1X PBS buffer (137mM NaCl; 2.7 mM KCl; 10mM NaH_2P0_4; 1.8 mM KH_2P0_4, pH7.4) up to equivalent OD=10 and mixed with protein 5X Loading buffer (60 mM Tris-HCl; 25% glycerol; 2% sodium dodecyl sulfate; 10% βmercaptoethanol; 0.1% methylene blue). After incubation for 10 minutes at 100 °C, samples were loaded on a 12% sodium dodecil sulfate-polyacrylamide gel (SDS-PAGE). After electrophoresis, proteins were blotted onto a PVDF nylon membrane for 45 minutes at 150 V in 0.25X Tris-Glycine buffer (6.25 mM Tris-HCl; 62.5 mM glycine, pH 8.3; 0.0025% w/v SDS) containing 20% (v/v) methanol, with a wet-tranfer apparatus. After blocking for 1h at room temparature in 1X PBS containing 5% low-fat milk and 0.05% Tween, the membrane was incubated for 16h at 4°C with a 1:5000 dilution of anti-His_6-tag monoclonal antibody (PIERCE) in blocking buffer. The membrane was then washed in 1X PBS containing 0.05% Tween and incubated for 1h at room temparature with a 1:10000-diluted peroxidase-conjugated anti-mouse immunoglobulin. The membrane was developed with an ECL detection system.

8.3.5 *In vivo* ClpP trapping

8.3.5.1 *Preparation of cultures*

A total of 60 mL of *H. pylori* ureA::P*ureA-clpP*$^{WT/S98A}$-His_6-StrepTag cultures were liquid-grown up to mid-exponential phase (optical density at 600 nm of 0.4-0.5), then split in two subcultures (25 mL each) and treated with either 100 and 200 μM of RSK13 molecule or equal volume of dimethyl sulfoxide (control sample). Subcultures were grown for 4 hours prior to harvest the bacterial cells.

8.3.5.2 *ClpP purification*

The bacterial pellet was resuspended in wash buffer (100 mM Tris-HCl pH 8.00; 150 mM NaCl; 1 mM EDTA) up to equivalent OD=10. The serine pretease inibitor (PMSF) and lysozime were added at a final concentration of 1 mM and 1 mg/mL respectively, then, the bacterial cells were incubated on a T-roll at 4 °C for 1 hour and disrupted through sonication. After separation of cellular debris, the soluble fraction was incubated with 40 μl of 50% Strep-Tactin superflow and

incubated 120 min at 4°C on a T-roll. Samples were washed 3 times with 2 mL of wash buffer, 2 times with 1.5 mL of wash buffer and 1 times with 0.5 mL of wash buffer, and the bound protein was eluted by adding 20 µl of 5X-SDS-Loading buffer. After incubation for 10 minutes at 100 °C, samples were subjected to SDS gel electrophoresis using standard protocols.

8.3.6 Silver staining

After electrophoresis (SDS-PAGE), the gel was incubated over-night in a fixative solution containing 55% milliQ-water, 5% acetic acid and 40% Ethanol (98%). The gel was washed three times in mQ-water for 15 minutes each and then incubated for 5 minutes in a silver staining solution (41,5 mM silver nitrate, 70 mL milliQ-water, ammonium hydroxide solution 0.42%, 20mM sodium hydroxide). The gel was washed in mQ-water three times for 10 minutes each and developed in a solution containing 100 mL of mQ-water, 50 µl citric acid, 50 µl formaldehyde until the appearance of bands. Finally, the staining of the gel was stopped for 1 minute in a solution composed of 100 mL of milliQ-water and 0.5 mL of acetic acid.

Table 2. Strains and plasmid used in this study

Bacterial stains/ Plasmids	Description	Source/ Reference
Strains		
H. pylori G27 wild-type	Clinical isolate, wild-type.	Xiang at al., 1995
H. pylori G27 (*hspR::Km*)	G27 derivative; bp 66 to 334 of the HspR coding sequence replaced by a Kanamycin (Km) cassette; Km^r.	Spohn and Scarlato, 1999
H. pylori G27 (*vacA::*P*cbpwt-lux*)	G27 derivative; containing the P*cbp* wild-type promoter region upstream of the *luxC* gene in the *vacA* locus; Cp^r.	This work
H. pylori G27 (*vacA::*P*cbpwt-lux, hspR::Km*)	G27 derivative; containing the P*cbp* wild-type promoter region upstream of the *luxC* gene in the *vacA* locus and the HspR coding sequence (bp 66 to 334) replaced by a Kanamycin (Km) cassette; Cp^r, Km^r.	This work
H. pylori G27 (*vacA::*P*cbpM1+2-lux, hspR::Km*)	G27 derivative; containing the P*cbp* HAIR-mutant promoter region upstream of the *luxC* gene in the *vacA* locus and the *hspR* coding sequence (bp 66 to 334) replaced by a Kanamycin (Km) cassette; Cp^r, Km^r.	This work
H. pylori G27 (*vacA::*P*cbpM1+2-lux*)	G27 derivative; containing the P*cbp* HAIR-mutant promoter region upstream of the *luxC* gene in the *vacA* locus; Cp^r.	This work
H. pylori G27 (cagA::*PcagA-clpP*$^{WT/S98A}$- His_6-tag-Strep-tag)	G27 derivative; containing the P*cagA* promoter region upstream of the *clpP*$^{WT/S98A}$ gene fused with a C-terminal His_6-Strep-tag in the *cagA* locus; Cp^r.	This work
H. pylori G27 *(tig-clpP::Km*)	G27 derivative; the *H. pylori* DNA region chr:: 822762-819545 was replaced by the *Campylobacter coli* Kanamycin cassette; Km^r.	This work
H. pylori G27 *(*P*clpP::clpP*^{WT}- His_6-tag)	G27 derivative; containing the *clpP* gene fused with a C-terminal His_6-tag in its original locus; Cp^r.	This work
H. pylori G27 (vacA::*Pcncr1-clpP*$^{WT/S98A}$-His_6-tag)	G27 derivative; containing the P*cncr1* promoter region upstream of the *clpP*$^{WT/S98A}$ gene fused with a C-terminal His_6-tag in the *vacA* locus; Cp^r.	This work
H. pylori G27 (cagA::*PcagA-clpP*$^{WT/S98A}$-His_6-tag)	G27 derivative; containing the P*cagA* promoter region upstream of the *clpP*$^{WT/S98A}$ gene fused with a C-terminal His_6-tag in the *cagA* locus; Cp^r.	This work
H. pylori G27 (ureA::*PureA-clpP*$^{WT/S98A}$-His_6-tag)	G27 derivative; containing the P*ureA* promoter region upstream of the *clpP*$^{WT/S98A}$ gene fused with a C-terminal His_6-tag in the *ureA* locus; Cp^r.	This work
H. pylori G27 (vacA::*Pcncr1-clpP*$^{WT/S98A}$-His_6-Strep-tag)	G27 derivative; containing the P*cncr1* promoter region upstream of the *clpP*$^{WT/S98A}$ gene fused with a C-terminal His_6-Strep-tag in the *vacA* locus; Cp^r.	This work
H. pylori G27 (cagA::*PcagA-clpP*$^{WT/S98A}$- His_6-Strep-tag)	G27 derivative; containing the P*cagA* promoter region upstream of the *clpP*$^{WT/S98A}$ gene fused with a C-terminal His_6-Strep-tag in the *cagA* locus; Cp^r.	This work
H. pylori G27 (ureA::*PureA-clpP*$^{WT/S98A}$- His_6-Strep-tag)	G27 derivative; containing the P*ureA* promoter region upstream of the *clpP*$^{WT/S98A}$ gene fused with a C-terminal His_6-Strep-tag in the *ureA* locus; Cp^r.	This work
E. coli DH5α	*supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1.*	Hanahan, 1983
E. coli BL21(DE3)	*hsdS gal (lcIts857 ind1 Sam7 nin5 lacUV5-T7 gene 1).*	Studier et al., 1990

*Plasmid*s		
pGEM-T-Easy	Cloning vector, Amp^r.	Promega
pGEM-T-Easy-RBSgro	pGEM-T-Easy derivative, containing a 147 bp DNA fragment corresponding to the region from 9,452–9,543 of *H. pylori* G27 genome amplified by PCR with oligonucleotides RBS GroF/RBS GroR. This region corresponds to a portion of the promoter region of HPG27_RS00075 (HP0011 according to 26695 annotation).	This work
pGEM-T-Easy-RBShrc	pGEM-T-Easy derivative, containing a 252 bp DNA fragment corresponding to the region from 118,944–119,035 of *H. pylori* G27 genome amplified by PCR with oligonucleotides RBShrcF/PhrcF. This region corresponds to a portion of the promoter region of HPG27_RS00580 (HP0111 according to 26695 annotation).	This work
pGEM-T-Easy-HBScbp	pGEM-T-Easy derivative, containing a 146 bp DNA fragment corresponding to the region from 433,049 to 433,140 of *H. pylori* G27 genome amplified by PCR with oligonucleotides HBSCbpF/HBSCbpF. This region corresponds to a portion of the promoter region and coding sequence of HPG27_RS02130 (HP1024 according to 26695 annotation).	This work
pGEM-T-Easy-HBSspeA	pGEM-T-Easy derivative, containing a 135 bp DNA fragment corresponding to the region from 1,035,104–1,035,195 of *H. pylori* G27 genome amplified by PCR with oligonucleotides HBSspeAF/HBSspeAR. This region corresponds to a portion of the coding sequence of HPG27_RS02130 (HP0422 according to 26695 annotation).	This work
pGEM-T-Easy-HBScbpM1	pGEM-T-Easy derivative, containing a 91 bp DNA fragment corresponding to the region from 432,991–433,071 of *H. pylori* G27 genome generated annealing oligonucleotides Mut1F/Mut1R. This region corresponds to a portion of the promoter region and coding sequence of HPG27_RS02130 (HP1024 according to 26695 annotation).	This work
pGEM-T-Easy-HBScbpM2	pGEM-T-Easy derivative, containing a 146 bp DNA fragment corresponding to the region from 433,049–433,140 of *H. pylori* G27 genome amplified by all around PCR with oligonucleotides Mut2F/Mut2R and using as DNA template the plasmid pGEM-T-Easy-HBScbp. This region corresponds to a portion of the promoter region and coding sequence of HPG27_RS02130 (HP1024 according to 26695 annotation).	This work
pGEM-T-Easy-HBScbpM1+ 2	pGEM-T-Easy derivative, containing a 146 bp DNA fragment corresponding to the region from 433,049–433,140 of *H. pylori* G27 genome amplified by all around PCR with oligonucleotides Mut2F/Mut2DR and using as DNA template the plasmid pGEM-T-Easy-HBScbp. This region corresponds to a portion of the promoter region and coding sequence of HPG27_RS02130 (HP1024 according to 26695 annotation).	This work
pGEM-T-Easy-HBScbpM3	pGEM-T-Easy derivative, containing a 91 bp DNA fragment corresponding to the region from 432,991–433,071 of *H. pylori* G27 genome generated annealing oligonucleotides Mut3F/Mut3R. This region corresponds to a portion of the promoter region and coding sequence of HPG27_RS02130 (HP1024 according to 26695 annotation).	This work

pGEM-T-Easy-HBScbpM4	pGEM-T-Easy derivative, containing a 91 bp DNA fragment corresponding to the region from 432,991–433,071 of *H. pylori* G27 genome generated annealing oligonucleotides Mut4F/Mut4R. This region corresponds to a portion of the promoter region and coding sequence of HPG27_RS02130 (HP1024 according to 26695 annotation).	This work
pGEM-T-Easy-HBScbpM5	pGEM-T-Easy derivative, containing a 91 bp DNA fragment corresponding to the region from 432,991–433,071 of *H. pylori* G27 genome generated annealing oligonucleotides Mut5F/Mut5R. This region corresponds to a portion of the promoter region and coding sequence of HPG27_RS02130 (HP1024 according to 26695 annotation).	This work
pVCC	Vector carrying the *luxCDABE* cassette.	Vannini et al., 2014
pVAC::Km	Cloning vector , Km^r.	Delany et al., 2002c
pVAC::CAT	pVAC::Km derivative, carrying a BglII/BamHI cat cassette from pBS::cat (Vannini et al., 2012).	This work
pVAC-P*cbp*wt-lux	pVAC-CAT derivative, containing a 146 bp DNA fragment amplified by PCR with oligonucleotides HBSCbpF_Eco/ HBSCbpR_BamHI, encompassing the P*cbp* wt promoter region and a 1,000 bp DNA fragment amplified by PCR with oligonucleotides LuxF/LuxR of the *luxC* gene.	This work
pVAC-P*cbp*M1+2-lux	pVAC-CAT derivative, containing a 146 bp DNA fragment amplified by PCR with oligonucleotides HBSCbpF_Eco/ HBSCbpR_BamHI (using as DNA template the plasmid pGEM-T-Easy-HBScbpM1+ 2), encompassing the P*cbp* M1+2 promoter region and a 1,000 bp DNA fragment amplified by PCR with oligonucleotides LuxF/LuxR of the *luxC* gene.	This work
pGEM3-*hspR*::*Km*	pGEM3 vector carrying the *Campylobacter coli* Kanamycin cassette flanked by a 1067 bp fragment comprising the *cbpA* gene (HPG27_RS02130) and a 716 bp fragment comprising the 50 region of the *rarA* gene (HPG27_RS02120).	Spohn and Scarlato, 1999
pET22b	Expression vector, allow C-terminal histidine-tag gene fusion; Amp^r.	Novagen
pET22b-HspR	pET22b derivative, containing the HspR coding sequence amplified by PCR.	Spohn and Scarlato, 1999
pBluescript KS II (+)	Cloning vector, Amp^r	Stratagene
pBS:: *tig-clpP*::*Km*	pBluescript derivative, carrying a 622 bp NotI-BamHI fragment amplified on chromosomal DNA of *H. pylori* G27 (chr:: 822,142-822,762) with oligonucleotides ClpPKO5F_NotI and ClpPKO5R_BamHI, BamHI Km cassette and a 562 bp BamHI-XhoI fragment (chr:: 819,543-820,104) amplified with oligonucleotides ClpPKO3F_BamHI and ClpPKO3R_XhoI; Km^r.	This work
pBS::P*clpP*-$ClpP^{WT}$-His_6-tag	pBluescript *tig-clpP::Km* derivative, carrying a 2063 bp BamHI-BglII fragment corresponding to the region from 820,077-822,139 of *H. pylori* G27 amplified with oligos ClpPcF_BamHI and ClpPcR_BglII and a BamHI-BglII Cloramphenicol cassette; Cp^r.	This work

pBS::P*clpP*-ClpP^{S98A}-His$_6$-tag	pBluescript *tig-clpP::Km* derivative, containing a 2063 bp BamHI-BglII DNA fragment corresponding to the region from 820,077-822,139 of *H. pylori* G27 genome amplified by all around PCR with oligonucleotides ClpP-S98A-F and ClpP-S98A-R and using as DNA template the plasmid pBS::*ClpP*^{WT}-His$_6$-tag; Cp^r.	This work
pSL1190	Cloning vector; Amp^r	Pharmacia
CbpA-pSL1190	pSL1190 derivative, containing the fragment amplified by PCR with oligonucleotides cbphel-1/hspR2 on chromosomal DNA of *H. pylori*, digested with XhoI/BamHI restriction enzymes	Roncarati et al., 2011
pCagA::PcagA-*clpP*^{WT}His$_6$-tag	pSL1190-cagcbp1/2-CbpA derivative, carrying a 623 bp DNA fragment corresponding to the region from 820,077-820,678 of *H. pylori* G27 genome with oligonucleotides ClpPcagAF_XhoI and ClpPcagAR_BamHI; Amp^r, Cp^r. (Roncarati et al., 2011)	This work
pCagA::PcagA-*clpP*^{S98A}His$_6$-tag	pSL1190-cagcbp1/2-CbpA derivative, carrying a 623 bp DNA fragment corresponding to the region from 820,077-820,678 of *H. pylori* G27 genome amplified with oligonucleotides ClpPcagAF_XhoI and ClpPcagAR_BamHI and using as DNA template the plasmid pBS::*ClpP*^{S98A}His$_6$-tag; Ap^r, Cp^r. (Roncarati et al., 2011)	This work
pCagA::PcagA-*clpP*$^{WT/S98A}$His$_6$-Strep-tag	pCagA::PcagA-*clpP*$^{WT/S98A}$His$_6$-tag derivative, carrying a 623 bp DNA fragment corresponding to the region from 820,077-820,678 of *H. pylori* G27 genome amplified by all around PCR with oligonucleotides HPStrepTag-F and HPStrepTag-R and using as DNA template the plasmid pBS::*ClpP*^{WT}His$_6$-tag or pBS::*ClpP*^{S98A}His$_6$-tag; Ap^r, Cp^r. (Roncarati et al., 2011)	This work
pAD1	Vector for introducing recombinant DNA into the *H. pylori ureA* locus; Ap^r, Cp^r.	Ando, Israel et al. 1999; Forsyth and Cover, 2000; Loh, Forsyth et al. 2004; Shaffer, Gaddy et al. 2011
PMM682	*H. pylori* codon-optimized *tetR* cloned into pAD1; Ap^r, Cp^r.	McClain et al., 2013
pUreA::P*ureA*-*clpP*^{WT}His$_6$-tag	PMMB67 derivative, containing a a 623 bp DNA fragment corresponding to the region from 820,077-820,678 of *H. pylori* G27 genome amplified with oligos ClpPureAF_XbaI and ClpPureAR_BamHI; Ap^r, Cp^r.	This work
pUreA::P*ureA*-*clpP*^{S98A}His$_6$-tag	PMMB67 derivative, containing a 623 bp DNA fragment corresponding to the region from 820,077-820,678 of *H. pylori* G27 genome amplified with oligonucleotides ClpPureAF_XbaI and ClpPureAR_BamHI using as DNA template the plasmid pBS::*ClpP*^{S98A}His$_6$-tag; Ap^r, Cp^r.	This work
pUreA::P*ureA*-*clpP*$^{WT/S98A}$His$_6$-Strep-tag	pUreA::PureA-*clpP*$^{WT/S98A}$His$_6$-tag derivative, containing a 623 bp DNA fragment corresponding to the region from 820,077-820,678 of *H. pylori* G27 genome amplified by all around PCR with oligonucleotides HPStrepTag-F and HPStrepTag-R and using as DNA template the plasmid pUreA::PureA-*clpP*^{WT}His$_6$-tag or pUreA::PureA-*clpP*^{S98A}His$_6$-tag; Ap^r, Cp^r.	This work

pVAC::CAT	pVAC::Km derivative, carrying a BglII/BamHI cat cassette from pBS::cat (Vannini et al., 2012).	This work
pVAC::P*cncr1-clpP*^{WT}His$_6$-tag	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 oligonucleotides PcagPF_BamHI and PcagPR_NdeI encompassing the promoter region of *PcagP* and a 631 bp DNA fragment amplified on pBS::*clpP*^{WT}His$_6$-tag with oligonucleotides ClpPcagPF_NdeI and VSCDSCpR_NcoI (chr: 820,077-820,678); Cp^r.	This work
pVAC::P*cncr1-clpP*^{S98A}His$_6$-tag	pVAC::P*cncr1-clpP*^{WT}His$_6$-tag derivative, carrying a 95 bp BamHI-NdeI fragment amplified on chromosomal DNA of *H. pylori* G27 (chr: 536,896-536,990) with oligonucleotides PcagPF_BamHI and PcagPR_NdeI encompassing the promoter region of P*cagP* and a 631 bp DNA fragment corresponding to the region from 820,077-820,678 of *H. pylori* G27 genome amplified with oligonucleotides ClpPcagPF_NdeI and VSCDSCpR_NcoI using as DNA template the plasmid pBS::*ClpP*^{S98A}His$_6$-tag; Cp^r.	This work
pVAC::P*cncr*1-*clpP*$^{WT/S98A}$His$_6$-Strep-tag	pVAC::P*cncr1-clpP*$^{WT/S98A}$His$_6$-tag derivative, carrying a 95 bp BamHI-NdeI fragment amplified on chromosomal DNA of *H. pylori* G27 (chr: 536,896-536,990) with oligonucleotides PcagPF_BamHI and PcagPR_NdeI encompassing the promoter region of *PcagP* and a 631 bp DNA fragment corresponding to the region from 820,077-820,678 of *H. pylori* G27 genome amplified by all around PCR with oligonucleotides ClpP-S98A-F and ClpP-S98A-R and using as DNA template the plasmid pVAC::P*cncr1-clpP*-6xHis; Cp^r.	This work
pVAC-Nt-Strep-tag	pVAC::CAT derivative. Expression vector, allows N-terminal Strep-tag gene fusion. Cp^r.	This work
pVAC-P*cncr1*-Nt-Strep-tag	pVAC-Nt-Strep-tag derivative. Expression vector, allows N-terminal Strep-tag gene fusion, carrying a 95 bp BamHI-NdeI fragment corresponding to the region from 536,896-536,990 of *H. pylori* G27 amplified with oligonucleotides PcagPF_BamHI and PcagPR_NdeI encompassing the promoter region of P*cagP*. Cp^r.	This work
pVAC-P*cagQ*-Nt-Strep-tag	pVAC-Nt-Strep-tag derivative. Expression vector, allows N-terminal Strep-tag gene fusion, carrying a 334 bp BamHI-NdeI fragment corresponding to the region from 536003-536336 of *H. pylori* G27 amplified with oligonucleotides PcagQF_BamHI and PcagQR_NdeI encompassing the promoter region of P*cagQ*. Cp^r.	This work
pVAC-P*cagC*-Nt-Strep-tag	pVAC-Nt-Strep-tag derivative. Expression vector, allows N-terminal Strep-tag gene fusion, carrying a 300 bp BamHI-NdeI fragment corresponding to the region from 547465-547764 of *H. pylori* G27 amplified with oligonucleotides PcagCF_BamHI and PcagCR_NdeI encompassing the promoter region of P*cagC*. Cp^r.	This work
pVAC-Ct-Strep-tag	pVAC::CAT derivative. Expression vector, allows C-terminal Strep-tag gene fusion. Cp^r.	This work
pVAC- P*cncr1*-Ct-Strep-tag	pVAC-Ct-Strep-tag derivative. Expression vector, allows C-terminal Strep-tag gene fusion, 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 P*cagP*. Cp^r.	This work

pVAC-P*cagQ*-Ct-Strep-tag	pVAC-Ct-Strep-tag derivative. Expression vector, allows C-terminal Strep-tag gene fusion, carrying a 334 bp BamHI-NdeI fragment corresponding to the region from 536003-536336 of *H. pylori* G27 amplified with oligonucleotides PcagQF_BamHI and PcagQR_NdeI encompassing the promoter region of P*cagQ*. Cp^r.	This work
pVAC-P*cagC*-Ct-Strep-tag	pVAC-Ct-Strep-tag derivative. Expression vector, allows C-terminal Strep-tag gene fusion, carrying a 300 bp BamHI-NdeI fragment corresponding to the region from 547465-547764 of *H. pylori* G27 amplified with oligonucleotides PcagCF_BamHI and PcagCR_NdeI encompassing the promoter region of P*cagC*. Cp^r.	This work

Table 3. Oligonucleotides used for cloning. Nucleotide added to reconstitute the indicated restriction sites are underlined and written in italics.

Oligonucleotides	Nucleotide sequences (5' to 3')[a]	Restriction recognition site
RBS GroF	TCTTCAAAAAGGTTTGTTAATGACGC	none
RBS GroR	AGCACATTTTTAGGGATAAGTCAAGC	none
RBS hrc F	CGATTTTTCTTTAAAGTTTAGTCTGTATCAC	none
Phrc F	ATATGGATCCTACGTCAAGCAAGCGATAACTTTAC	none
HBS CbpF	AATTCCTTTTAATTGCACTGAAACGGG	none
HBS CbpR	GGTATAAACTCTTGCTCATGAATCACC	none
HBS speAF	CCACGAAGCCCTTGTTTTTGC	none
HBS speAR	CGCTAAATTCCGTAGGGTGC	none
Mut1 F	GATCCAAAATAGTTTTATTAGAATACTATCATAAATCAGGTACCTTAGTCAA TCAAGTTTATTGATAATGTTTAGTGGTAATTGAGATTTG	none
Mut1 R	GTTTTATCAAAATAATCTTATGATAGTATTTAGTCCATGGAATCAGTTAGTT CAAATAACTATTACAAATCACCATTAACTCTAAA*CTTAA*	EcoRI
Mut2 F	*AGTCGAC*AGTTTATTGATAATGTTTAG	SalI
Mut2 R	CTAACACTAAAGATTTATGATAGTATTC	none
MUTD2R	CTAA*GGTACC*TGATTTATGATAGTATTC	KpnI
Mut3 F	GATCCAAAATAGTTTTATTAGAATACTATCATAAATCTTTAGTGGAGCTCAA TCAAGTTTATTGATAATGTTTAGTGGTAATTGAGATTTG	none
Mut3 R	GTTTTATCAAAATAATCTTATGATAGTATTTAGAAATCACCTCGAGTTA GTTCAAATAACTATTACAAATCACCATTAACTCTAAACTTAA	none
Mut4 F	GATCCAAAATAGTTTTATTAGAATACAGGTACCAATCTTTAGTGTTAGTCAA TCAAGTTTATTGATAATGTTTAGTGGTAATTGAGATTTG	none
Mut4 R	GTTTTATCAAAATAATCTTATGTCCATGGTTAGAAATCACAATCAGTTAGTT CAAATAACTATTACAAATCACCATTAACTCTAAACTTAA	none
Mut5 F	GATCCAAAATAGTTTTATTAGAATACTATCATAAATCTTTAGTGTTAGTCAA TCAAGTTTTAGTCGACTGTTTAGTGGTAATTGAGATTTG	none
Mut5 R	GTTTTATCAAAATAATCTTATGATAGTATTTAGAAATCACAATCAGTTAGTT CAAAATCAGCTGACAAATCACCATTAACTCTAAACTTAA	none
LuxF	ATAT*GGATCC*CAGGCTTGGAGGATACGTATGAC	BamHI
LuxR	ATAT*GGATCC*GGCATTCGGTAATATATGCGC	BamHI
LuxRTF	ATCATCCGATAACGCGCTCTT	none
LuxRTR	ACCGCCCAATTAATCGCATC	none
cbpEco	ATAT*GAATTC*AATTCCTTTTAATTGCACTGAAACGGG	EcoRI

cbpBam	ATAT*GGATCC*GGTATAAACTCTTGCTCATGAATCACC	BamHI
hspRC	ATATAT*CTCGAG*TTTTTTAAATAAAATCAGTTCATA	XhoI
cys-F	CGTTTTAGGGACTTTGGGAGG	none
vacA-R	GCTGGTTTTATGCTCTAAACTGG	none
ppk RT-F	CGCGCCTTTCTAAATTTCTGGGCA	none
ppk RT-R	CCCAAGTCAAAGGCTTGAGCGAAA	none
ClpPKO5F	TATGCGGCCGCTTATGGCACAAGCGTGGTGCAAATG	NotI
ClpPKO5R	TATGGATCCAACTAAAAATCATAAGTTGAAGTGAGATAGACAG	BamHI
ClpPKO3F	TATGGATCCTGATAAAGTGTTACAGAAAAATGTGAAGTG	BamHI
ClpPKO3R	TATCTCGAGGGTTACTTGTGTTTTTGTTTTTTTTGCAGC	XhoI
ClpPcF	TATGGATCCTTAAATTTTTGAAAACTAGAATTAAAACCGC	BamHI
ClpPcR	TATAGATCTTTAATGATGATGATGATGATGCTTCACATTTTTCTGTAACACTT TATCAATC	BglII
ClpP-S98A-F	CAAGCGGCTGCTATGGGGGCGTTTTTAC	none
ClpP-S98A-R	GCCGATGCAAATCGTGGAAACATCAGG	none
ClpPcagAF	ATATCTCGAGGCTAAAAAGGAGAGATGATG	XhoI
ClpPcagAR	ATATGGATCCTTAATGATGATGATGATGATGCTTCAC	BamHI
ClpPureAF	ATATTCTAGAATGGGATACATTCCTTATGTAATAGAG	XbaI
ClpPureAR	ATATGGATCCGGATAATCTCTAATAACGCC	BamHI
PcagPF	ATATGGATCCCCAGGTCTTATAGTGTTATG	BamHI
PcagPR	ATATCATATGCACAAATCCATTATATAGAAATAATC	NdeI
ClpPcagPF	ATATCATATGGCTAAAAAGGAGAGATGATG	NdeI
VSCDSCpR	TATCCATGGGGATAATCTCTAATAACGCC	NcoI
HPStrepTag-F	TGGAGCCATCCTCAATTTGAAAAATAAAGATCTTGATAAAGTGTTACAG	none
HPStrepTag-R	ATGATGATGATGATGATGCTTCAC	none
Cys-F	CGTTTTAGGGACTTTGGGAGG	none
VacA-R	GCTGGTTTTATGCTCTAAACTGG	none
BB-F/OrfX	GCAACTCCATAGACCACTAAAG	none
Cag-dx-R	GAGATCGGCTAACGCTTGCTCTA	none
Ure-up-F	GCTCAGTTGGTAGAGCACTACCTTG	none
Ure-down-R	ACGGCTTTTTTGCCTTCGTTGATAG	none
C-StrepNtF	TATGAGGAGAGATGATGTGGAGCCATCCTCAATTTGAAAAA*CTCGAG*GTCG AC*CCCGGGC*	XhoI; SalI; NcoI
C-StrepNtR	CATGG*CCGCGGG*TCGAC*CTCGAGT*TTTTCAAATTGAGGATGGCTCCACATCA TCTCTCCTCA	XhoI; SalI; NcoI
C-StrepCtF	TATGAGGA*GAGCTC*GTCGAC*CTCGAG*TGGAGCCATCCTCAATTTGAAAAATA A*CCGCGG*C	SacI; SalI; XhoI; SacII
C-StrepCtR	CATGGCCGCGGTTATTTTTCAAATTGAGGATGGCTCCA*CTCGAG*GTCGAC*GA GCTC*TCCTCA	SacI; SalI; XhoI; SacII
PcagQF	ATATGGATCCCTTATGATTCGTTCAAAAATTTC	BamHI
PcagQR	ATATCATATGGCCACCAAACAAGCCATATCC	NdeI
PcagCF	ATATGGATCCTGCTTAAAATGGAGCTTTATTC	BamHI
PcagCR	ATATCATATGGCGTTTCCTTTCAAATTGAAATC	NdeI

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Annex 1

frontiers in Microbiology

ORIGINAL RESEARCH
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The *Helicobacter pylori* Heat-Shock Repressor HspR: Definition of Its Direct Regulon and Characterization of the Cooperative DNA-Binding Mechanism on Its Own Promoter

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The ability of pathogens to perceive environmental conditions and modulate gene expression accordingly is a crucial feature for bacterial survival. In this respect, the heat-shock response, a universal cellular response, allows cells to adapt to hostile environmental conditions and to survive during stress. In the major human pathogen *Helicobacter pylori* the expression of chaperone-encoding operons is under control of two auto-regulated transcriptional repressors, HrcA and HspR, with the latter acting as the master regulator of the regulatory circuit. To further characterize the HspR regulon in *H. pylori*, we used global transcriptome analysis (RNA-sequencing) in combination with Chromatin Immunoprecipitation coupled with deep sequencing (ChIP-sequencing) of HspR genomic binding sites. Intriguingly, these analyses showed that HspR is involved in the regulation of different crucial cellular functions through a limited number of genomic binding sites. Moreover, we further characterized HspR-DNA interactions through hydroxyl-radical footprinting assays. This analysis in combination with a nucleotide sequence alignment of HspR binding sites, revealed a peculiar pattern of DNA protection and highlighted sequence conservation with the HAIR motif (an HspR-associated inverted repeat of *Streptomyces* spp.). Site-directed mutagenesis demonstrated that the HAIR motif is fundamental for HspR binding and that additional nucleotide determinants flanking the HAIR motif are required for complete binding of HspR to its operator sequence spanning over 70 bp of DNA. This finding is compatible with a model in which possibly a dimer of HspR recognizes the HAIR motif overlapping its promoter for binding and in turn cooperatively recruits two additional dimers on both sides of the HAIR motif.

Keywords: heat-shock response, HspR repressor, ChIP-seq, RNA-sequencing, transcriptome

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INTRODUCTION

Helicobacter pylori represents one of the most widespread human pathogens, recognized as the principal causative agent of different gastrointestinal severe diseases such as atrophic gastritis, peptic ulcer, MALT-lymphoma and gastric adenocarcinoma (Gisbert and Calvet, 2011; Salama et al., 2013). A peculiar feature of *H. pylori* is its small genome (1.66 Mb), characterized by a relative low abundance of genes encoding regulators of transcription. In this respect, it has been speculated that this paucity of transcriptional regulators could reflect the adaptation of *H. pylori* to its very restricted niche in the mucus layer of the human stomach and could be linked to the lack of competition from other microorganisms (Marshall et al., 1998). To date, only 17 transcriptional regulators have been identified and shown to control key physiological responses of the bacterium, which are necessary to successfully colonize the gastric niche (Scarlato et al., 2001).

Helicobacter pylori transcriptional regulators appear to be arranged in different regulatory modules, transducing separate environmental inputs. Such regulatory modules are constituted by a master regulator, followed by intermediate regulators and regulatory interactions finally resulting into a coordinated output of target gene's expressions (Danielli et al., 2010). Intriguingly, these regulatory modules do not appear as segregated, but highly interconnected and show a significant crosstalk among different signal transduction pathways. *H. pylori* does not possess a homologue of the *Escherichia coli* heat-shock sigma factor σ^{32}. However, the heat stress response is governed by two dedicated transcriptional repressors, HrcA and HspR that negatively regulate the expression of the highly conserved class of stress-induced proteins, known as Heat-Shock Proteins (HSPs). The main function of HSPs is to assist the folding of newly synthetized polypeptides, as well as assembly, transport and degradation of cellular proteins under both normal and adverse growth condition (Roncarati and Scarlato, 2017). Besides their general role in protecting cellular proteins against different kind of stresses and in maintaining cellular homeostasis, some HSPs and chaperones are considered virulence factors and seem to be involved in specific pathogenic processes. Specifically, several lines of evidence show that the heat-shock proteins of *H. pylori* play also non-canonical roles and some of them seem to have undertaken additional functions during the interaction with the host (Evans et al., 1992; Huesca et al., 1996; Phadnis et al., 1996; Dunn et al., 1997; Kao et al., 2016). Because of these important functions in the cell, *H. pylori* has developed regulatory strategies to tightly modulate HSPs expression level in response to environmental signals.

In previous works (Spohn and Scarlato, 1999; Spohn et al., 2004) it has been demonstrated that the transcription of *groES-groEL*, *hrcA-grpE-dnaK,* and *cbpA-hspR-rarA* operons, encoding the major chaperones of *H. pylori*, is negatively regulated by HspR and/or HrcA repressors, with the former acting as the master regulator of this circuit. Indeed, HspR represses transcription of the *cbpA* operon alone, thereby auto-regulating its own synthesis, while it represses the expression of the other two heat-shock operons *groES-groEL* and *hrcA-grpE-dnaK* in combination with HrcA. Genes with sequence similarity to *H. pylori hspR* were annotated in several other bacteria, including species belonging to the *Streptomyces* genus. In particular, *H. pylori* HspR is a homologue of the repressor that controls the expression of the *dnaK* operon in *Streptomyces coelicolor* by binding three copies of the HspR consensus binding sequence called HAIR (for HspR Associated Inverted Repeat) (Bucca et al., 1995; Grandvalet et al., 1999). Also in *Streptomyces albus* it was demonstrated that HspR binds to an inverted repeat identical to *S. coelicolor* HAIR sequence, mapping in the promoter region of the protease gene *clpB* (Grandvalet et al., 1999). In *H. pylori*, DNaseI footprinting experiments with the purified protein showed that HspR binds extended DNA regions located in the promoters of the three heat-shock operons and recognizes sequences similar to the HAIR motif (Delany et al., 2002c; Roncarati et al., 2007). While in the case of *cbpA* promoter HspR binding occurs in close proximity to the transcription start site, on the *groEL* and *hrcA* promoters HspR binds far upstream of the core promoter region, in an atypical position for a repressor (Roncarati et al., 2007). The identification of additional genes controlled by HspR was pursued through array-based whole transcriptome analyses both in *H. pylori* (Roncarati et al., 2007) and in several other bacterial species (Stewart et al., 2002; Andersen et al., 2005; Schmid et al., 2005; Holmes et al., 2010). Moreover, from an *in vitro* selection of genomic DNA fragments bound by purified *H. pylori* HspR protein, two novel HspR binding sites were identified in the 3′ regions of both *speA* and *tlpB* genes coding for proteins with functions unrelated with those of chaperones (Delany et al., 2002c).

In the present study, by combining ChIP-sequencing and RNA-sequencing we investigated more in detail the direct or indirect contribution of *H. pylori* HspR to the heat-shock regulon. While the heat-shock regulon includes many genes with key cellular functions, HspR only binds to a limited number of genomic sites. Furthermore, by means of the high-resolution hydroxyl-radical footprinting technique, we further characterized the HspR-DNA interactions at the molecular level. The data provide a more detailed comprehension of the interaction between HspR and its target DNA sequences and, at least on its own promoter, let us to propose a cooperative DNA-binding mechanism of three HspR dimers per operator sequence.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Helicobacter pylori strains (**Table 1**) were recovered from frozen glycerol stocks on Brucella broth agar plates, containing 5% fetal calf serum (FCS), in a 9% CO_2-91% air atmosphere at 37°C and 95% humidity in a water jacketed incubator (Thermo Scientific). Liquid cultures were grown in Brucella Broth supplemented with 5% FCS with gentle agitation (120 rpm). *E. coli* strains DH5α and BL21 (DE3) (**Table 1**) were grown on Luria-Bertani (LB) agar plates or LB liquid broth with vigorous agitation (250 rpm); when required, ampicillin was added to the medium to achieve a final concentration of 100 μg/ml.

TABLE 1 | Bacterial strains and plasmids used in this study.

Bacterial strains/ Plasmids	Description	Source/ Reference
Strains		
H. pylori G27 wild type	Clinical isolate, wild type	Xiang et al., 1995
H. pylori G27 (*hspR::Km*)	G27 derivative; bp 66 to 334 of the *hspR* coding sequence replaced by a Kanamycin (*Km*) cassette.	Spohn and Scarlato, 1999
H. pylori G27 (*vacA::Pcbpwt-lux*)	G27 derivative; containing the P*cbp* wild type promoter region upstream of the *luxC* gene in the *vacA* locus; Cp^r	This work
H. pylori G27 (*vacA::Pcbpwt-lux*, *hspR::Km*)	G27 derivative; containing the P*cbp* wild type promoter region upstream of the *luxC* gene in the *vacA* locus and the *hspR* coding sequence (bp 66 to 334) replaced by a Kanamycin (*Km*) cassette; Cp^r, Km^r	This work
H. pylori G27 (*vacA::PcbpM1 + 2-lux*, *hspR::Km*)	G27 derivative; containing the P*cbp* HAIR-mutant promoter region upstream of the *luxC* gene in the *vacA* locus and the *hspR* coding sequence (bp 66 to 334) replaced by a Kanamycin (*Km*) cassette; Cp^r, Km^r	This work
H. pylori G27 (*vacA::PcbpM1 + 2-lux*)	G27 derivative; containing the P*cbp* HAIR-mutant promoter region upstream of the *luxC* gene in the *vacA* locus; Cp^r	This work
E. coli DH5α	*supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan, 1983
E. coli BL21(DE3)	*hsdS gal (λcIts857 ind1 Sam7 nin5 lacUV5-T7 gene 1).*	Studier et al., 1990
Plasmids		
pGEM-T-Easy	Cloning vector, Amp^r	Promega
pGEM-T-Easy-RBS gro	pGEM-T-Easy derivative, containing a 147 bp DNA fragment corresponding to the region from 9,452–9,543 of *H. pylori* G27 genome amplified by PCR with oligonucleotides RBS GroF/RBS GroR. This region corresponds to a portion of the promoter region of HPG27_RS00075 (HP0011 according to 26695 annotation).	This work
pGEM-T-Easy-RBS hrc	pGEM-T-Easy derivative, containing a 252 bp DNA fragment corresponding to the region from 118,944–119,035 of *H. pylori* G27 genome amplified by PCR with oligonucleotides RBShrcF/PhrcF. This region corresponds to a portion of the promoter region of HPG27_RS00580 (HP0111 according to 26695 annotation).	This work
pGEM-T-Easy-HBS cbp	pGEM-T-Easy derivative, containing a 146 bp DNA fragment corresponding to the region from 433,049 to 433,140 of *H. pylori* G27 genome amplified by PCR with oligonucleotides HBSCbpF/HBSCbpF. This region corresponds to a portion of the promoter region and coding sequence of HPG27_RS02130 (HP1024 according to 26695 annotation).	This work
pGEM-T-Easy-HBSspeA	pGEM-T-Easy derivative, containing a 135 bp DNA fragment corresponding to the region from 1,035,104–1,035,195 of *H. pylori* G27 genome amplified by PCR with oligonucleotides HBSspeAF /HBSspeAR. This region corresponds to a portion of the coding sequence of HPG27_RS02130 (HP0422 according to 26695 annotation).	This work
pGEM-T-Easy-HBScbpM1	pGEM-T-Easy derivative, containing a 91 bp DNA fragment corresponding to the region from 432,991–433,071 of *H. pylori* G27 genome generated annealing oligonucleotides Mut1F/Mut1R. This region corresponds to a portion of the promoter region and coding sequence of HPG27_RS02130 (HP1024 according to 26695 annotation).	This work
pGEM-T-Easy-HBScbpM2	pGEM-T-Easy derivative, containing a 146 bp DNA fragment corresponding to the region from 433,049–433,140 of *H. pylori* G27 genome amplified by all around PCR with oligonucleotides Mut2F/Mut2R and using as DNA template the plasmid pGEM-T-Easy-HBScbp. This region corresponds to a portion of the promoter region and coding sequence of HPG27_RS02130 (HP1024 according to 26695 annotation).	This work
pGEM-T-Easy-HBScbpM1 + 2	pGEM-T-Easy derivative, containing a 146 bp DNA fragment corresponding to the region from 433,049–433,140 of *H. pylori* G27 genome amplified by all around PCR with oligonucleotides Mut2F/Mut2DR and using as DNA template the plasmid pGEM-T-Easy-HBScbp. This region corresponds to a portion of the promoter region and coding sequence of HPG27_RS02130 (HP1024 according to 26695 annotation).	This work
pGEM-T-Easy-HBScbpM3	pGEM-T-Easy derivative, containing a 91 bp DNA fragment corresponding to the region from 432,991–433,071 of *H. pylori* G27 genome generated annealing oligonucleotides Mut3F/Mut3R. This region corresponds to a portion of the promoter region and coding sequence of HPG27_RS02130 (HP1024 according to 26695 annotation).	This work
pGEM-T-Easy-HBScbpM4	pGEM-T-Easy derivative, containing a 91 bp DNA fragment corresponding to the region from 432,991–433,071 of *H. pylori* G27 genome generated annealing oligonucleotides Mut4F/Mut4R. This region corresponds to a portion of the promoter region and coding sequence of HPG27_RS02130 (HP1024 according to 26695 annotation).	This work

(Continued)

TABLE 1 | Continued

Bacterial strains/ Plasmids	Description	Source/ Reference
pGEM-T-Easy-HBScbpM5	pGEM-T-Easy derivative, containing a 91 bp DNA fragment corresponding to the region from 432,991–433,071 of *H. pylori* G27 genome generated annealing oligonucleotides Mut5F/Mut5R. This region corresponds to a portion of the promoter region and coding sequence of HPG27_RS02130 (HP1024 according to 26695 annotation).	This work
pVCC	Vector carrying the *luxCDABE* cassette	Vannini et al., 2014
pVAC::Km	Cloning Vector, Km^r	Delany et al., 2002b
pVAC::CAT	pVAC::Km derivative, carrying a BglII/BamHI *cat* cassette from pBS::*cat* (Vannini et al., 2012).	This work
pVAC-Pcbpwt-lux	pVAC-CAT derivative, containing a 146 bp DNA fragment amplified by PCR with oligonucleotides HBSCbpFEco/HBSCbpRBamHI, encompassing the P*cbp* wt promoter region and a 1,000 bp DNA fragment amplified by PCR with oligonucleotides LuxF/LuxR of the *luxC* gene.	This work
pVAC-PcbpM1+2-lux	pVAC-CAT derivative, containing a 146 bp DNA fragment amplified by PCR with oligonucleotides HBSCbpFEco/HBSCbpRBamHI (using as DNA template the plasmid pGEM-T-Easy-HBScbpM1 + 2), encompassing the P*cbp* M1 + 2 promoter region and a 1,000 bp DNA fragment amplified by PCR with oligonucleotides LuxF/LuxR of the *luxC* gene.	This work
pGEM3-*hspR::Km*	pGEM3 vector carrying the *Campylobacter coli* Kanamycin cassette flanked by a 1067 bp fragment comprising the *cbpA* gene (HPG27_RS02130) and a 716 bp fragment comprising the 5′ region of the *rarA* gene (HPG27_RS02120).	Spohn and Scarlato, 1999
pET22b	Expression vector, allow C-terminal histidine-tag gene fusion; Amp^r	Novagen
pET22b-HspR	pET22b derivative, containing the HspR coding sequence amplified by PCR	Spohn and Scarlato, 1999

RNA Isolation

Helicobacter pylori strains (**Table 1**) were grown with gentle agitation (120 rpm) in 30 ml of Brucella broth at 37°C until mid-exponential phase (OD = 0.7). For heat-shock treatment, the wild type (WT) culture was 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 −80°C, and then used to extract total RNA with TRI-reagent (Sigma-Aldrich), according to manufacturer's protocol.

RNA-seq: Library Preparation, Sequencing and Analyses

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) (highlighted in yellow in **Supplementary Table S1**). 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 S1**. 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 (see **Supplementary Table S2**). 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| . Raw data are publicly available at Sequence Reads Archive under accession number BioProject PRJNA421261.

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 S1**).

Chromatin Immunoprecipitation (ChIP) With α-HspR Polyclonal Antibody

Available α–HspR polyclonal antibody from immunized mice (Vannini et al., 2016) were purified by 3 sequential precipitations with 35% saturated $(NH4)_2SO_4$ and subsequent resuspension in water. *H. pylori* G27 wild type and *hspR* mutant strains were liquid-grown to an OD_{600} of 0.7, crosslinked, sonicated and immunoprecipitated as previously described (Pelliciari et al., 2017; Vannini et al., 2017). Briefly, protein-DNA complexes were chemically crosslinked with 1% of formaldehyde, and then DNA was sonicated, at high power, with Bioruptor (Diagenode). HspR-DNA complexes were immunoprecipitated by incubating whole cellular extracts with the α-HspR polyclonal antibody at a 1:30 dilution and then captured with Protein-G conjugated sepharose beads. Cross-linking was reverted for 6h at 65°C. DNA was extracted once with phenol-chloroform and further extracted with chloroform. Finally, DNA was ethanol precipitated with the addition of 1% glycogen (Sigma-Aldrich) and resuspended in 50 μl of double-distilled water as previously described (Vannini et al., 2017).

ChIP-seq: Library Preparation, Sequencing and Analyses

Illumina libraries were prepared following the Illumina TruSeq ChIP-seq DNA sample preparation protocol starting from 5 ng of immunoprecipitated-DNA for each of the strains and each of the two biological replicates. Each library was sequenced on a GAIIx or MiSeq Illumina sequencer and 51 bp single stranded reads were produced. At least 2 Million of raw reads were obtained for each IP sample and biological replicate. Bowtie 2 (v2.2.6) (Langmead and Salzberg, 2012) was used to align raw reads deriving from Input (IP Δ*hspR)* and IP (IP *wt*) samples sequencing on the *H. pylori* G27 genome. End-to-end mapping was performed and non-deterministic option was specified. High quality reads were then selected requiring: for uniquely mapping reads MAPQ $>$ 30 (mapping quality) and alignment score $>$ −10 while, for multi-mapping reads, the alignment score was set $\geq$ −10. On average, more than 98% of them mapped on the *H. pylori* G27 reference genome. The ChIP-seq data quality was evaluated by using ENCODE quality metrics[1] and the values obtained are provided in **Supplementary Table S2**. To perform peak calling, the Homer (v4.7.2) (Heinz et al., 2010) algorithm was used with default parameters. Briefly, the Homer algorithm finds non-random clusters of reads by looking at the tested sample alone, then each peak is required to have: (1) 4-fold more normalized reads in the sample experiment than in the background control and a cumulative Poisson *p*-value of 0.0001 to assess the chance that the differences in reads counts between sample and background are statistically significant; (2) read density 4-fold greater than in the surrounding 10 kb region; (3) the ratio between the number of unique positions containing reads in the peak and the expected number of unique positions given the total number of reads in the peak lower than 2. Only the peaks identified in both biological replicates and having overlapping genomic coordinates were considered significantly reliable and included in the final peak list. The peaks having their center within −100/ + 30 bp from a transcription start site (TSS) were defined as promotorial, while the remaining peaks were divided in intragenic, when their center was mapping within a predicted coding region, or intergenic, when it was mapping outside from annotated regions (see **Supplementary Table S1** and RNA-seq analysis paragraph for details). TSS were identified first by blasting 50 bp upstream of each of the transcription initiation sites reported by Sharma et al. (2010) in the HP26695 genome on the G27 genome and then by the positioning of our RNA-seq signals. Raw data are publicly available in Sequence Reads Archive under accession number BioProject PRJNA421261.

[1] https://code.google.com/archive/p/phantompeakqualtools/

DNA Techniques

DNA manipulations were performed 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 NucleoBond Xtra Midi plasmid purification kit (Macherey-Nagel).

Overexpression and Purification of Recombinant HspR Protein

His_6-tagged recombinant HspR protein was overexpressed in *E. coli* BL21 (DE3) cells and affinity purified as previously described (Spohn and Scarlato, 1999; Roncarati et al., 2007). The purified His-HspR 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) and purity assayed by SDS-PAGE.

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

Genomic regions of *H. pylori* G27 encompassing HspR binding sites on *hrcA, groES*, *cbpA* promoters and *speA* coding region were PCR amplified with specific primers (**Table 2**) and cloned into the pGEM-T-Easy plasmid (**Table 1**). The M1, M3, M4 and M5 P*cbp* 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. The M2 and M1+2 P*cbp* mutant probes were generated through site-directed mutagenesis using the plasmid pGEM-T-Easy, harboring the P*cbp* wild type sequence, as DNA template and primers listed in **Table 2**.

TABLE 2 | Oligonucleotides used in this study.

Oligonucleotides	Nucleotide sequences (5' to 3')[a]	Restriction recognition site
RBS GroF	TCTTCAAAAAGGTTTGTTAATGACGC	None
RBS GroR	AGCACATTTTTAGGGATAAGTCAAGC	None
RBS hrc F	CGATTTTTCTTTAAAGTTTAGTCTGTATCAC	None
Phrc F	ATATGGATCCTACGTCAAGCAAGCGATAACTTTAC	None
HBS CbpF	AATTCCTTTTAATTGCACTGAAACGGG	None
HBS CbpR	GGTATAAACTCTTGCTCATGAATCACC	None
HBS speAF	CCACGAAGCCCTTGTTTTTGC	None
HBS speAR	CGCTAAATTCCGTAGGGTGC	None
Mut1 F	GATCCAAAATAGTTTTATTAGAATACTATCATAAATCAGGTACCTTAG TCAATCAAGTTTATTGATAATGTTTAGTGGTAATTGAGATTTG	None
Mut1 R	GTTTTATCAAAATAATCTTATGATAGTATTTAGTCCATGGAATCAGTT AGTTCAAATAACTATTACAAATCACCATTAACTCTAAACTTAA	EcoRI
Mut2 F	AGTCGACAGTTTATTGATAATGTTTAG	SalI
Mut2 R	CTAACACTAAAGATTTATGATAGTATTC	None
MUTD2R	CTAAGGTACCTGATTTATGATAGTATTC	KpnI
Mut3 F	GATCCAAAATAGTTTTATTAGAATACTATCATAAATCTTTAGTGGAGCT CAATCAAGTTTATTGATAATGTTTAGTGGTAATTGAGATTTG	None
Mut3 R	GTTTTATCAAAATAATCTTATGATAGTATTTAGAAATCACCTCGAGTTA GTTCAAATAACTATTACAAATCACCATTAACTCTAAACTTAA	None
Mut4 F	GATCCAAAATAGTTTTATTAGAATACAGGTACCAATCTTTAGTGTTAGT CAATCAAGTTTATTGATAATGTTTAGTGGTAATTGAGATTTG	None
Mut4 R	GTTTTATCAAAATAATCTTATGTCCATGGTTAGAAATCACAATCAGTTA GTTCAAATAACTATTACAAATCACCATTAACTCTAAACTTAA	None
Mut5 F	GATCCAAAATAGTTTTATTAGAATACTATCATAAATCTTTAGTGTTAGT CAATCAAGTTTTAGTCGACTGTTTAGTGGTAATTGAGATTTG	None
Mut5 R	GTTTTATCAAAATAATCTTATGATAGTATTTAGAAATCACAATCAGTTA GTTCAAAATCAGCTGACAAATCACCATTAACTCTAAACTTAA	None
LuxF	ATATGGATCCCAGGCTTGGAGGATACGTATGAC	BamHI
LuxR	ATATGGATCCGGCATTCGGTAATATATGCGC	BamHI
LuxRTF	ATCATCCGATAACGCGCTCTT	None
LuxRTR	ACCGCCCAATTAATCGCATC	None
cbpEco	ATATGAATTCAATTCCTTTTAATTGCACTGAAACGGG	EcoRI
cbpBam	ATATGGATCCGGTATAAACTCTTGCTCATGAATCACC	BamHI
cbpRTRev	TTTAGCTAGGCAATACCACCCGGA	None
hspRC	ATATATCTCGAGTTTTTTAAATAAAATCAGTTCATA	XhoI
cys-F	CGTTTTAGGGACTTTGGGAGG	None
vacA-R	GCTGGTTTTATGCTCTAAACTGG	None
ppk RT-F	CGCGCCTTTCTAAATTTCTGGGCA	None
ppk RT-R	CCCAAGTCAAAGGCTTGAGCGAAA	None

[a] *Nucleotides added to reconstitute the indicated restriction recognition sites are underlined.*

Hydroxyl-Radical Footprinting Assay

Probe DNA fragments obtained by digestion with the appropriate restriction enzymes were 5′ end-labeled with [γ^{32} P]-ATP and T4 polynucleotide kinase and gel purified. Hydroxyl-radical footprinting experiments were performed as previously described (Pelliciari et al., 2017) with some modifications. Approximately 20 fmol of labeled probes were incubated with increasing concentration of HspR protein in hydroxyl-radical 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) for 15 min at room temperature, including 200 ng of sonicated salmon sperm DNA as a non-specific competitor in a final volume of 30 µl. Partial digestions of the labeled probes were achieved using 2 µl each of the following solutions: 125 mM Fe $(NH_4)_2(SO_4)_2$ 250 mM EDTA, 1% H_2O_2 and 100 mM DTT. After 2 min incubation, the reaction was quenched by the addition of 25 µl of OH Stop Buffer (4% glycerol; 600 mM NaOAc, pH 5.2; 100 ng/µl sonicated salmon sperm DNA). Samples were phenol/chloroform extracted, ethanol precipitated and resuspended in 12 µl of Formamide Loading Buffer. Next, samples were denatured at 100°C for 5 min, separated on a 8M urea-8.4% polyacrylamide sequencing gel in TBE buffer and autoradiographed.

Generation of *H. pylori* P*cbp-lux* Reporter Strains

The wild type P*cbp* and mutated P*cbp* M1 + 2 promoter regions were PCR amplified with specific primers listed in **Table 2**, using as template the *H. pylori* G27 genomic DNA

and the plasmid pGEM-T-Easy-HBScbp M1 + 2 (**Table 1**), respectively. Then, the generated DNA fragments were digested with appropriate restriction enzymes and cloned into the pVAC plasmid (**Table 1**). The *luxC* gene was PCR amplified from the pVCC vector (**Table 1**) and cloned downstream the P*cbp* promoters into the pVAC vector. These plasmids were used to transform the *H. pylori* G27 wild type acceptor strain in the *vacA* locus. The chloramphenicol-selected mutant strains were expanded and the correct insertion was confirmed by PCR using oligonucleotides pair cys-F/vacA-R as primers (**Table 2**). The *H. pylori* P*cbp-lux, hspR::Km* reporter strains (**Table 1**) were generated transforming the *H. pylori vacA*::P*cbp* wt-*lux* and the *H. pylori vacA*::P*cbp*M1 + 2-*lux* acceptor strains (**Table 1**) with the plasmid pGEM3-*hspR::Km* (**Table 1**). The kanamycin-selected mutant strains were expanded and the correct insertion was confirmed by PCR using oligonucleotides pair cbpRTRev/hspRC as primers (**Table 2**).

qRT-PCR Analysis

Synthesis of cDNA and qRT-PCR analysis were carried out as previously described (Pelliciari et al., 2015). Briefly, for cDNA synthesis 1 μg of DNA-free RNA was incubated with 50 ng of random hexamers (Invitrogen), dNTPs mix (1 mM each), 5 U of AMV-Reverse Transcriptase (Promega), and incubated for 1 h at 37°C. For qRT-PCR analyses, 2 μl of diluted (1:10) cDNA samples were mixed with 5 μl of 2X Power Up SYBR Green master mix (ThermoFisher Scientific) and specific oligonucleotides for the genes of interest (**Table 2**) at 400 nM concentration in a final volume of 10 μl. qRT-PCR experiments were performed using the following cycling protocol: 95°C for 2 min, then 40 cycles consisting of a denaturation step for 5 s at 95°C followed by 30 s 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 (**Table 2**) (Muller et al., 2011; Agriesti et al., 2014).

RESULTS

RNA-seq Analysis Identifies HspR-Dependent Heat-Shock Gene Transcripts

To define the HspR contribution to the heat-shock response, we performed a strand-specific whole transcriptome analysis of the wild type *H. pylori* G27 strain and of Δ*hspR* mutant both grown to the exponential growth phase at 37°C and of the wild type strain subjected to 30 min heat-shock at 42°C (**Supplementary Table S2**, Materials and Methods).

We defined the role of HspR in standard growth conditions by comparing the transcriptome of the Δ*hspR* mutant to that of the *H. pylori* wild type strain, both grown at 37°C (Δ*hspR*_vs_WT). This analysis showed a total of 65 deregulated genes (log2FC > |1| *p*adj < 0.01) upon *hspR* gene deletion. Of these, 21 were down-regulated and 44 were up-regulated genes (**Figure 1A** and **Supplementary Table S3**, sheet B). As expected, among the up-regulated genes we found *groES, groEL, grpE, dnaK,* and *cbpA* (Spohn and Scarlato, 1999), which strongly contributed to "Post-translational modification, protein turnover, chaperones" functional enrichment among (*p*adj = 0.01) Δ*hspR* de-repressed genes (**Figures 1A,B**), and *hrcA* transcriptional regulator (**Figure 1B**). As expected, because of the *hspR* genomic deletion, this gene appeared as down-regulated. The *rarA* gene, mapping downstream of the *hspR* gene, possibly due a polar effect of the deletion mutant also appeared as a down-regulated gene. Several transposase coding genes, producing the "Mobilome: prophages, transposons" category enrichment (*p*adj = 0.00009), and several genes involved in the "Inorganic ion transport and metabolism" were also down-regulated.

Upon heat-shock at 42°C the transcriptome of the *H. pylori* wild type G27 strain showed a total of 134 differentially expressed genes (log2FC > 1 *p*adj < 0.01) when compared to the wild type sample not subjected to heat-shock (HS_vs_WT). Of these genes, 83 were up-regulated and 51 were down-regulated (**Supplementary Table S3**, sheet A). Functional annotation and enrichment analysis (see Materials and Methods) revealed that up-regulated genes were enriched in "Post-translational modification, protein turnover, chaperones" (*p*adj = 0.0007) and "Mobilome: prophages, transposons" class (*p*adj = 0.008; **Figure 1A**). Several of the 51 down-regulated genes were also annotated to "Cell wall/membrane/envelope biogenesis" and "Translation, ribosomal structure and biogenesis" categories (**Figure 1A**) without statistical significance.

Comparing the transcriptome of the heat-shock response (134 deregulated genes) to that of the *hspR* deletion mutant (65 deregulated genes) we identified 25 genes that were deregulated in both datasets (**Figures 1B,C**, red numbers). Among these genes, 10 were oppositely regulated and 14 were similarly regulated: 2 down-regulated and 12 up-regulated (**Figure 1B**, bottom left and right quadrants of the graph and **Figure 1C**). The similarly up-regulated genes included all the genes of the operons already known to be directly repressed by HspR and induced by heat-shock (Spohn and Scarlato, 1999). In addition, we found genes coding for amino acid transporters *yckK* and *yckJ,* flagellar protein *flaG,* a polyisoprenoid-binding protein and two genes coding for hypothetical proteins, which could be new targets under HspR direct control (**Figures 1B,C**). The remaining eleven genes were induced by heat-shock and repressed in the *hspR* mutant strain, thus showing opposite behavior. Considering that HspR acts as a repressor factor, these genes are probably indirectly controlled by HspR.

Overall, this analysis highlights that HspR could be involved in the control of maximum 18% (25 out of 134) of the genes whose transcription is heat responsive and that it represses the transcription of only 14 genes.

Genome-Wide *in vivo* Identification of HspR Binding Sites Through ChIP-seq

To identify the genomic regions bound *in vivo* by HspR, therefore, the genes directly controlled by HspR binding, we performed a Chromatin Immunoprecipitation assays followed

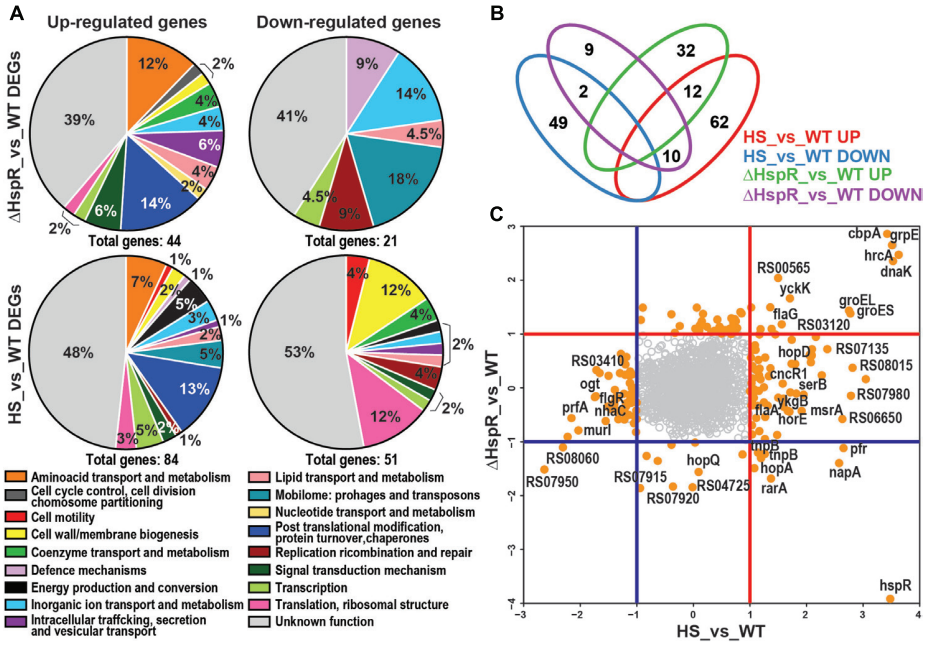
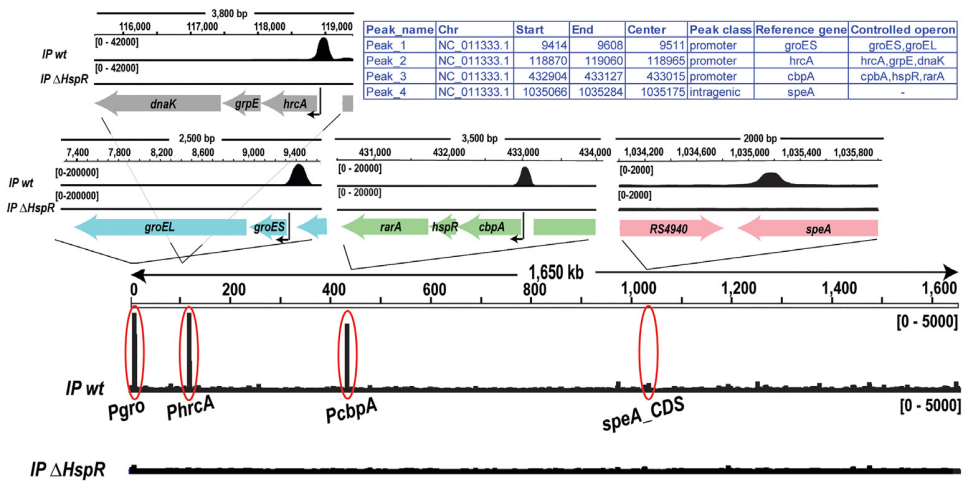


FIGURE 1 | Role of HspR in heat-shock response. **(A)** Pie charts showing COGs functional annotation of the differentially expressed genes outlined in the ΔHspR_vs_WT and HS_vs_WT (Heat-Shock vs. Wilde Type) comparisons, respectively, subdivided into up-regulated (left) and down-regulated (right) groups. 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 showing the number of genes in common or peculiar in the four previously described gene groups, red numbers highlight commonly regulated genes. **(C)** log2FC plot showing for each gene HS_vs_WT value on x-axis and HspR_vs_WT value on y-axis. DEGs (log2FC > |1| and *p*adj < 0.01 are represented as orange filled circles; empty gray circles correspond to non-differentially expressed genes. Blue and red lines indicate log2FC < −1 and log2FC > 1 thresholds, respectively, subdividing coherently and incoherently regulated genes.

by deep sequencing (ChIP-seq) in *H. pylori* G27 wild type and Δ*hspR* strains (**Figure 2**). To identify HspR bound regions (peaks), ChIP-seq signals obtained from each wild type sample (IP wt) were compared to those resulting from the pool of Δ*hspR* mutant samples (IP Δ*hspR*) (see Material and Methods for details). Only the significant peaks in both replicates were considered in the final peak list. Surprisingly, this analysis identified only four reproducible HspR binding regions (**Figure 2**), which were annotated with respect to the latest genome annotation of the *H. pylori* G27 strain (GCF_000021165.1). According to this annotation, three peaks were classified as promotorial and confirmed HspR direct control for 8 genes of the *groES-groEL*, *hrcA-grpE-dnaK* and *cbpA-hspR-rarA* operons. The remaining peak, mapping inside the coding region of *speA* gene, was classified as intragenic. Thus, these data suggest that the HspR direct regulon is very restricted and limited to the three multicistronic heat-shock operons. Moreover, through ChIP-seq analysis we confirmed *in vivo* also the existence of at least one intracistronic binding site, apparently not associated to transcriptional regulation.

HspR Binds Extended DNA Regions and Adopts a Peculiar Binding Architecture

Previous DNase I footprinting assays showed that *H. pylori* HspR binds directly to extended DNA regions of the promoters of the three heat-shock operons (Spohn and Scarlato, 1999; Roncarati et al., 2007). In particular, binding of HspR to its operators resulted in protection of large DNA regions of 70–80 bp with six bands of enhanced DNase I sensitivity at both adjacent and internal sites. In order to further characterize HspR-DNA interactions and to get more detailed information on the HspR DNA-binding architecture, we carried out hydroxyl-radical footprinting experiments on the genomic targets found in ChIP-seq assay. **Figure 3A** shows the results of hydroxyl-radical footprintings performed with increasing concentrations of recombinant purified HspR on the promoter region of the three heat-shock operons (P*cbp*, P*gro*, P*hrc*) and on the 3′ region of the *speA* coding sequence. According to previous observations (Spohn and Scarlato, 1999; Delany et al., 2002c; Roncarati et al., 2007) the protected regions on the P*cbp*, P*gro* and P*hrc* promoters map, respectively, from position −63 to +10, from −117 to −44 and from −150 to −82 with respect to the transcriptional start site. Furthermore, the HspR binding site located in the coding region of *speA* gene spans from nucleotide position −298 to −250 with respect to the translational stop codon. Intriguingly, HspR binding on these targets results in a peculiar periodic pattern of short protected regions from radical digestion, which appears to be slightly different between the promotorial and intragenic binding sites. Indeed, HspR binding on P*cbp*, P*gro* and P*hrc* promoter regions results in 7 short protected DNA tracts separated by non-protected regions of 7/8 nucleotides,



Peak_name	Chr	Start	End	Center	Peak class	Reference gene	Controlled operon
Peak_1	NC_011333.1	9414	9608	9511	promoter	groES	groES,groEL
Peak_2	NC_011333.1	118870	119060	118965	promoter	hrcA	hrcA,grpE,dnaK
Peak_3	NC_011333.1	432904	433127	433015	promoter	cbpA	cpbA,hspR,rarA
Peak_4	NC_011333.1	1035066	1035284	1035175	intragenic	speA	-

FIGURE 2 | Genome-wide *in vivo* identification of the HspR binding sites. Genome-wide signals of HspR bindings in the wild type genotype (IP wt, upper track) and in *hspR* deletion mutant used as negative control (IP ΔHspR, lower track). The regions corresponding to the four HspR binding sites revealed by the analysis are magnified in the upper panels showing each peak in its genomic context including annotated genes (thick arrows) and the transcriptional start site (black thin arrow), when present.

while the binding site located inside the *speA* coding sequence appears at higher protein concentrations and is characterized by 5 short protected regions instead of 7. Data obtained from DNA-binding assays are schematized in **Figure 3B**, which reports the nucleotide sequence of the HspR binding sites (operator) on the P*cbp*, P*gro*, P*hrc* promoters and on the *speA* coding region. Notably, in all the operators we identify one inverted repeat (represented by converging arrows in **Figure 3B**) similar to the HAIR motif proposed as a consensus sequence for the HspR protein of *S. coelicolor* (CTTGAGT-N7-ACTCAAG) (Grandvalet et al., 1999). It is worth noting that in the operators of the three heat-shock operons' promoters the inverted repeat is located in a central position of the HspR binding sites, suggesting that it could play an important role in nucleating HspR binding to DNA. Therefore, the heat-shock transcriptional repressor HspR binds, with a peculiar DNA-binding pattern of short stretches of protected regions, spanning over about 70-80 bp of DNA harboring a conserved inverted repeat similar to the HAIR consensus sequence of *Streptomyces* spp.

The Central HAIR-Like Motif Is Essential for HspR Binding to DNA

To ascertain the functional importance of the HAIR-like sequence elements in HspR binding to DNA, we decided to introduce bases substitutions in the inverted repeat and monitor their effects on HspR DNA-binding through hydroxyl-radical footprinting assays. A schematic representation of the wild type and mutant sequences of the HspR binding site on the P*cbp* region is reported in **Figure 4A**. As shown in **Figure 4B**, mutation of one or both arms (M1, M2, or M1 + 2) of the HAIR-like sequence completely abolished HspR binding to the DNA in the concentration range tested. In particular, partial (M1 and M2) or total substitution (M1 + 2) of the HAIR-like sequence prevented HspR binding to both, the mutagenized sequence (central portion of the probe) and the flanking upstream and downstream regions of the HAIR-like motif. It is worth mentioning that regions protected in hydroxyl-radical footprinting experiments reflect limited accessibility of radical ions to the DNA minor groove and, for this reason, these protected regions do not necessarily represent the portions of the probe directly contacted by the HspR protein, but regions surrounding short stretches of contacted nucleotides. Considering that HspR footprint regions surround the conserved HAIR-like element (**Figure 4**), our data suggest that HspR could interact with the HAIR-like element in the DNA major groove narrowing the adjacent minor grooves, which results in the protection observed *in vitro* by hydroxyl-radical footprinting. Furthermore, mutation of the non-conserved sequence in between the two inverted repeats (probe M3 in **Figure 4A**) showed a barely detectable protection of HspR only upon addition of high amount of the protein to the reaction (**Figure 4B**, lane 6 of probe M3). This is likely due to a significant loss of protein affinity to the DNA, suggesting that, besides the HAIR-like motif, also this non-conserved DNA element is important for HspR binding. These data show for the first time that in *H. pylori* the HAIR-like sequence is an essential DNA element for the specific binding of HspR to the P*cbp* promoter region. Likely, HspR binding to the operator is driven by a specific recognition of determinants within the HAIR-like sequence, including the non-conserved spacer between the inverted repeat.

To characterize *in vivo* the functional significance of the central HAIR-like motif, we generated a reporter construct in which the wild type or HAIR-like mutant P*cbp* promoter (P*cbp*M1 + 2) was fused upstream of a 5′ fragment of the *luxCDABE* reporter cassette and, upon integration in *H. pylori* chromosome, transcript level was assayed (through RT–qPCR with *luxC* specific primers) during exponential growth

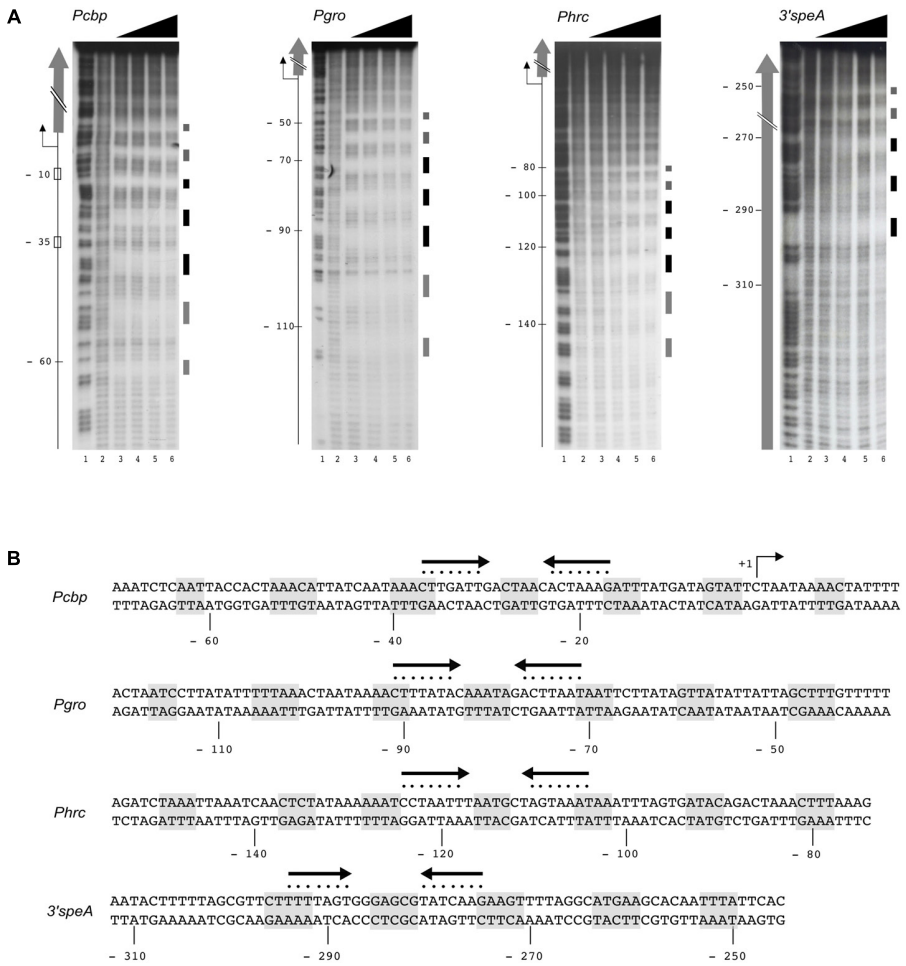


FIGURE 3 | HspR binds extended DNA regions. **(A)** Hydroxyl-radical footprint experiments on HspR positive target genes identified by ChIP-seq analysis. From left to right, specific DNA probes for P*cbp*, P*gro*, P*hrc*, and *speA* fragments were radioactively end-labeled on the coding strands, and incubated with increasing amounts of purified HspR protein prior hydroxyl-radical digestion. P*cbp*, P*gro*, P*hrc* labeled probes were mixed with 0; 47.5; 95; 190; 380 nM HspR (lanes 2–6); while *speA* probe was mixed with 0; 60.7; 121.5; 243; 486 nM HspR (lanes 2–6). Purified DNA fragments were separated on a polyacrylamide denaturing gel along with a G + A sequence reaction ladder as reference marker (lane 1 in all the panels). On the right of each panel, black and gray boxes represent strong and weak HspR protected regions, respectively. On the left, the –10 and –35 regions and the transcriptional start site (+1, bent arrow) are indicated and the open reading frames are depicted with vertical gray arrows. The relative position of the HspR binding site on the promoter regions of P*cbp*, P*gro*, P*hrc* is reported with respect to the transcriptional start site, while those mapped on the coding sequence of *speA* gene are reported with respect to the translational stop codon. **(B)** Nucleotide sequences of HspR binding sites on the promoter regions of the three heat-shock operons (P*cbp*, P*gro*, P*hrc*) and on the 3′ coding region of the *speA* gene. The HspR protected regions identified in **(A)** are shaded in gray, while the inverted repeat sequences similar to the HAIR consensus motif are depicted as converging black arrows and each nucleotide of the motif is marked with a dot. Nucleotide positions with respect to the transcription initiation sites are reported on the non-coding strand.

(**Figure 5**). When the central HAIR-like inverted repeat was mutated in the G27 wild type strain, a consistent increase in the amount of transcript from the P*cbp*M1 + 2 promoter with respect to the wild type P*cbp* promoter was observed (**Figure 5B**). The about 6.5-fold increase of transcript from the P*cbp*M1 + 2 promoter is in line with the previously observed de-repression of the P*cbp* promoter in the *H. pylori hspR*-mutant strain (Spohn et al., 2004). Accordingly, in a *hspR* null background a similar high amount of transcripts from the P*cbpwt* and the P*cbp*M1 + 2 promoters was observed (**Figure 5B**). These *in vivo* data are consistent with the *in vitro* observations, indicating that the central HAIR-like motif drives specific binding of HspR to its operator on P*cbp* with concomitant promoter transcriptional repression.

Non-conserved DNA Regions, Surrounding the HAIR-Like Motif, Are Necessary for HspR to Fully Occupy Its Extended DNA Binding Site

In order to understand if DNA sequences flanking the HAIR-like motif are important for HspR-DNA recognition and binding, we designed two mutant probes of the P*cbp* promoter and assayed for HspR binding by hydroxyl-radical footprintings.

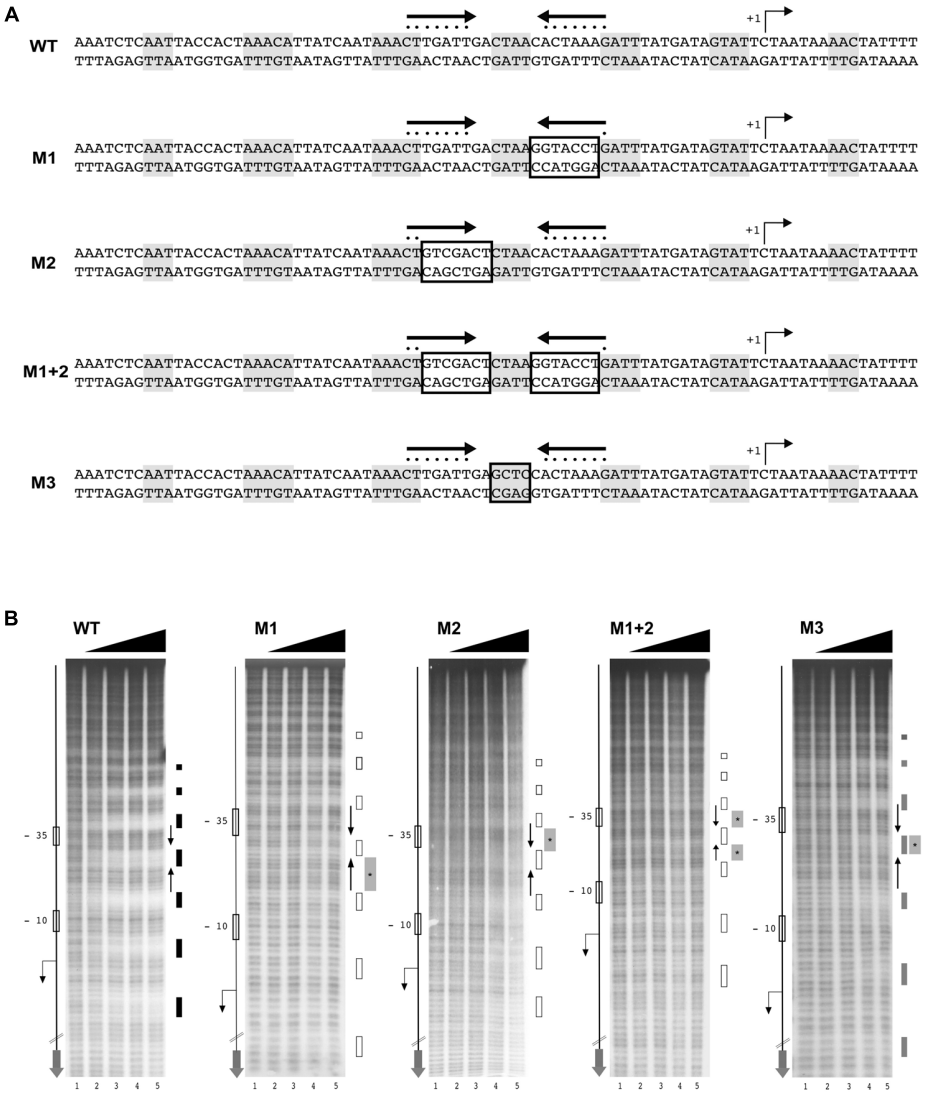


FIGURE 4 | The HAIR-like motif is essential for HspR binding to DNA. **(A)** Schematic representation of wild type and four different P*cbp* mutant probes, in which one (M1 and M2) or both arms (M1 + 2) of the inverted repeat and the protected region between these sequences (M3) have been mutagenized by base substitution. HspR protected regions on P*cbp* are shaded in gray and the inverted repeat sequences (HAIR-like motif) is represented by converging black arrows and their nucleotides marked with dots. In each DNA probe, mutagenized nucleotides are boxed. **(B)** From left to right, hydroxyl-radical footprint experiments on wild type and the indicated mutants of the P*cbp* promoter region. Symbols are as described in the legend to **Figure 3A**. Wild type (WT) and indicated mutants DNA probes were incubated with increasing amounts of purified HspR protein and submitted to hydroxyl-radical digestion (see legend to **Figure 3**). Black, gray, and empty boxes to the right of each panel denote strong, weak, and loss of protection by HspR, respectively. Black converging arrows to the right of each panel mark the positions of the HAIR-like inverted repeat sequences, while gray boxes with an internal asterisk indicate the mutagenized regions.

Mutations were introduced in the non-conserved spacer region between two 4-bp protected tracts on both sides of the inverted repeat (**Figure 6A**, M4 and M5). Surprisingly, as shown in **Figure 6B**, the addition of increasing amounts of HspR to the mutant probes led to the disappearance of regions of protection (marked in light gray) only on the side of the mutated region, while it was unaffected on both the inverted repeat of the HAIR-like sequence and on the opposite side of the mutation (**Figure 6B**, M4 and M5). In conclusion, these data support the pivotal role of the HAIR-like motif for HspR-DNA binding in *H. pylori* and demonstrate that also other non-conserved DNA regions surrounding the HAIR-like motif are important elements that allow HspR to completely occupy its extended binding sites.

DISCUSSION

The heat-shock response is a universal mechanism of cellular protection against sudden adverse environmental growth

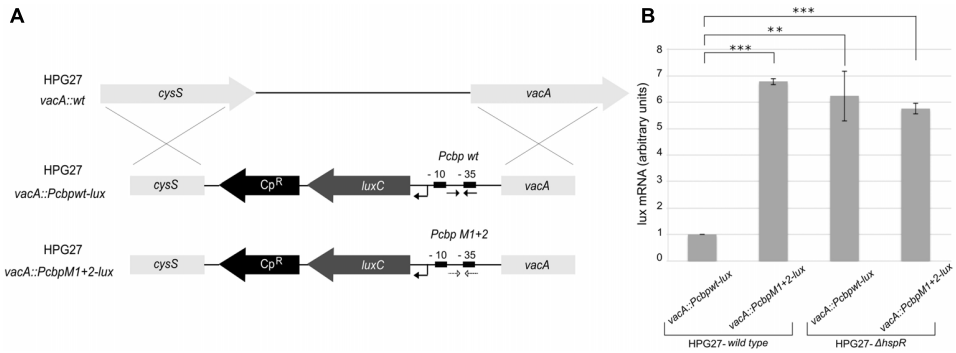


FIGURE 5 | *In vivo H. pylori* transcripts levels from the P*cbp* promoter harboring WT or mutant HAIR-like sequences. **(A)** Schematic representation of P*cbpwt-lux* and P*cbpM1 + 2-lux* reporter constructs obtained transforming the *H. pylori* G27 wild type acceptor strain by double homologous recombination in the *vacA* locus, and selected by chloramphenicol resistance (Cp^R). The wild type (P*cbpwt-lux*) or HAIR-like mutant (P*cbpM1 + 2-lux*) P*cbp* promoter is inserted upstream of a *luxC* reporter gene. The HAIR inverted repeat sequences are indicated by converging black arrows in the P*cbp* WT promoter (P*cbpwt-lux*) and by converging dotted arrows in the P*cbp* HAIR-like mutant promoter (P*cbpM1 + 2-lux*). In each reporter construct, the –10 and –35 regions are depicted as black boxes and the transcriptional start site as a bent arrow. **(B)** Transcript levels of P*cbp* wild type and P*cbp* HAIR-like mutant promoters fused with *lux* reporter gene were assayed by qRT-PCR in the wild type and *hspR* deletion mutant strains using specific oligonucleotides for the *luxC* gene (LuxRTF/R). Mean values from three independent biological samples are reported in the graph, with error bars indicating standard deviation and asterisks marking statistical significance calculated by a Student's *t*-test (***p-value < 0.001; **p-value < 0.01).

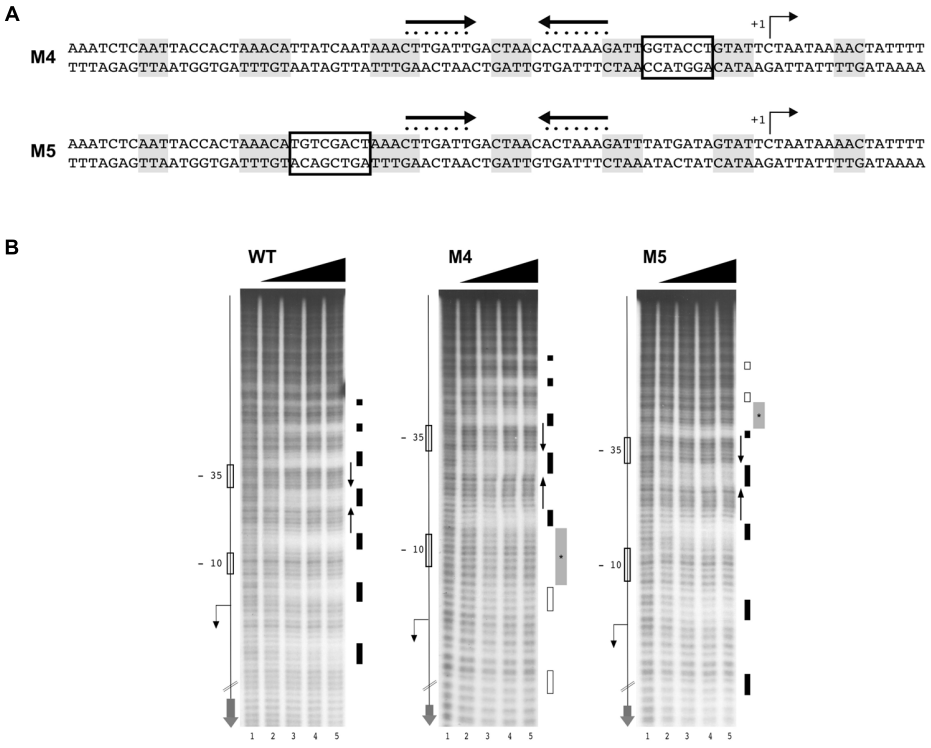


FIGURE 6 | Non-conserved regions surrounding the HAIR-motif are required for HspR binding. **(A)** Schematic representation of WT and two P*cbp* mutant probes, in which the spacer regions highlighted with empty boxes were mutagenized by base substitution. Symbols are as in the legend to **Figure 3**. **(B)** From left to right, hydroxyl-radical footprint experiments on WT and mutant probes described in **(A)** (M4 and M5) with increasing amounts of purified HspR protein detailed in the legend to **Figure 3**.

conditions and has been observed in every bacterial species investigated (Roncarati and Scarlato, 2017). It consists of a set of well-coordinated responses and processes, mostly involving the strictly regulated expression of various heat-shock proteins (HSPs) and chaperones. In *H. pylori* their expression level is tightly modulated by the concerted action of two transcriptional

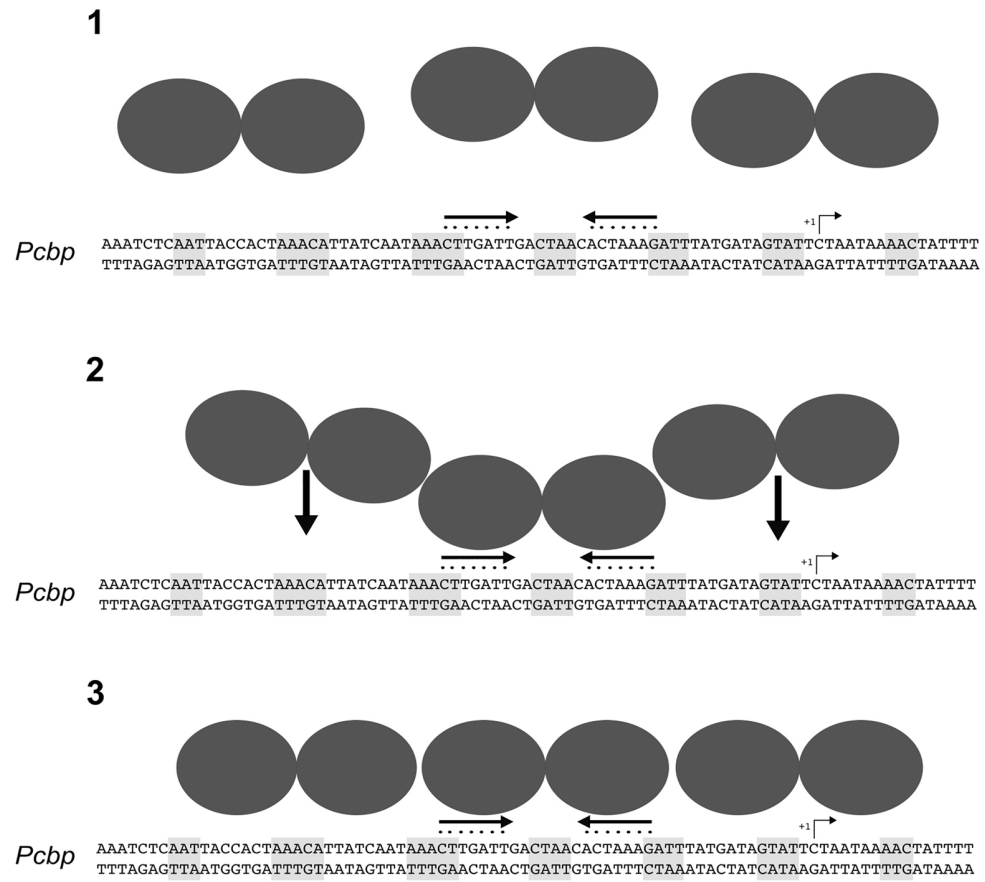


FIGURE 7 | Model of the molecular mechanism for HspR-DNA binding. In solution, HspR dimerizes (1) and binds with high affinity to the DNA region containing the HAIR consensus sequence (2). This binding could act as a nucleation center driving cooperative binding of two HspR dimers on the flanking regions (3), likely recognizing still unknown sequence determinants.

repressors, HrcA and HspR, with this latter acting as the master regulator of this circuit (Danielli and Scarlato, 2010). In the present study, we examined the heat-shock regulon in *H. pylori* and discriminated between direct and indirect transcriptional response mediated by HspR. As shown by transcriptome analyses, heat-shock treatment triggered changes in the transcript levels of 135 genes. Of these, 83 genes appeared up-regulated and 51 genes appeared down regulated (**Figure 1A**). Accordingly, the heat-shock operons *groES-groEL*, *hrcA-grpE-dnaK* and *cbpA-hspR-rarA*, coding for the major chaperones and heat-shock proteins of *H. pylori* were clearly up-regulated. With the exception of the *hspR* and *rarA* genes, these operons, known to be directly repressed by HspR (Spohn and Scarlato, 1999; Roncarati et al., 2007), were coherently up-regulated also in the Δ*hspR* mutant strain. On the other hand, HspR seems to affect in a positive or negative manner the transcription of other 59 genes involved in diverse cellular processes and not strictly associated to heat-shock (**Figure 1A**). In contrast, ChIP-seq analysis showed only four already known *in vivo* HspR genomic binding sites, three of which are associated to the promoter regions of the heat-shock operons and one mapping within the coding sequence of the *speA* gene (**Figure 2** and Spohn and Scarlato, 1999; Delany et al., 2002c; Roncarati et al., 2007). This apparent discrepancy between the RNA-seq and ChIP-seq results is in agreement with other HspR studies conducted in *S. coelicolor* and *Mycobacterium tuberculosis* (Stewart et al., 2002; Bucca et al., 2003). In these bacteria, HspR alone or in combination with other transcriptional regulators controls transcription of a limited number of genes coding for chaperones and heat-shock proteins. In *H. pylori*, *hspR* deletion affects the transcript abundance of a high number of genes not directly controlled by HspR. Likely, regulation by HspR can also be exerted in an indirect manner through a still unknown molecular mechanism. Recalling the presence of the *hrcA* gene among the direct targets of HspR, it is tempting to speculate that many of the genes deregulated by the *hspR* deletion and lacking a HspR binding site arise from altered levels of the HrcA regulator in the Δ*hspR* mutant. Furthermore, the possibility that up- and/or down-regulated genes in the Δ*hspR* mutant strain might arise from the enhanced synthesis of one or more members of the HspR regulon cannot be ruled out and remains to be elucidated.

Although several chaperones and heat-shock proteins of *H. pylori* are induced by a temperature upshift (**Supplementary Table S3**), surprisingly, the transcription of genes coding for stress related proteases seems to be unaffected by the heat challenge. Furthermore, while in some other bacteria, like for example *Campylobacter jejuni* and *S. coelicolor*, several protease-encoding genes have been shown to belong to the HspR regulon (Bucca et al., 2003; Holmes et al., 2010), we demonstrated that in *H. pylori*, these genes are not controlled by the HspR repressor. Considering that the amount of proteases is expected to increase in response to different stress insults encountered by the pathogen, an interesting hypothesis considers the existence of post-transcriptional or post-translational control strategies, which would provide enhanced levels of these crucial players during adverse environmental growth conditions.

Previous DNase I footprinting assays of HspR showed protection of large DNA regions of about 70 bp upstream of the promoters controlling transcription of three heat-shock operons (Spohn and Scarlato, 1999; Roncarati et al., 2007). To deepen our understanding of the molecular mechanisms controlling HspR-DNA interactions, we set up high-resolution hydroxyl-radical footprinting assays on its target gene probes. Results showed that HspR binds extended DNA regions with a peculiar short periodic pattern, which appears to be slightly different between promoter and intragenic regions (**Figure 3A**). Binding of HspR to promoter regions shows 7 short protected DNA tracts spaced by non-protected regions of 7/8 nucleotides, while binding within the *speA* coding sequence appears at higher protein concentrations and shows only 5 short protected regions. This different binding pattern could be related to the fact that the latter binding site on the coding sequence of *speA* gene appears to be not associated to transcriptional regulation. In fact, RNA-seq analysis revealed no changes in the level of *speA* transcript in the *hspR* mutant strain, nor of neighboring genes. This is not surprising, as the advent of the "omics" era highlighted binding of regulatory proteins to a number of binding sites not associated to regulation, such as the *H. pylori* Fur repressor (Danielli et al., 2006; Vannini et al., 2017), the *E. coli* CRP activator, and the RNA polymerase enzyme (Grainger et al., 2005). However, all four *H. pylori* HspR binding sites show an inverted repeat with similarities to the HAIR consensus sequence of *Streptomyces* spp. (Grandvalet et al., 1999) (**Figure 3B**). These HAIR-like motifs map in the central position of the HspR binding sites on the three heat-shock operons' promoters and appear to be an essential DNA element for specificity of protein binding. In fact, mutation of one or both arms of this inverted repeat completely abolished the HspR binding to the operator (**Figure 4**) and prevented *in vivo* HspR-dependent repression of P*cbp* (**Figure 5**), demonstrating for the first time that the HAIR-like sequence is functionally important also in *H. pylori*. The DNA-binding mechanism of HspR on its target operators is unique among *H. pylori* transcriptional regulators characterized so far. Well-studied *H. pylori* regulatory proteins, such as HP1043, HrcA, NikR and Fur appear to recognize conserved sequence motifs as dimers and protect limited DNA regions (Roncarati et al., 2014, 2016; Pelliciari et al., 2017; Vannini et al., 2017). In the case of Fur repressor, however, it has been shown that several Fur-regulated promoters harbor multiple Fur boxes and, upon Fur binding, large regions of the promoter result occupied by this metal-dependent repressor (Delany et al., 2002a; Roncarati et al., 2016). However, data presented in this work suggest a completely different DNA-binding mechanism for HspR. HspR-controlled promoters are characterized by a single, conserved inverted repeat (HAIR-like sequence) that drives DNA specific recognition. In contrast to other *H. pylori* regulators, HspR DNA binding extends over a large portion of the promoter DNA, resulting in the characteristic extended protection in *in vitro* footprinting assays (Roncarati et al., 2007). This peculiar behavior of *H. pylori* HspR appears to be different from the other HspR homologs that have been characterized at the molecular level. For example, in *S. coelicolor* HspR binds extended DNA regions harboring three inverted repeat sequences (IR1, IR2, IR3) in the promoter region of its DNA targets (Bucca et al., 1995), while in *H. pylori* HspR requires only one inverted repeat on the center of the binding site. Also, no additional conserved sequences similar to the HAIR-like motif have been detected. Therefore, we suppose that the molecular mechanism through which *H. pylori* HspR binds to such extended DNA regions is peculiar and differs from the one adopted by the *S. coelicolor* HspR. Moreover, site-directed mutagenesis of the P*cbp* promoter pointed out that additional non-conserved DNA regions located between (**Figure 4B**, M3) and flanking (**Figure 6B**, M4 and M5) the HAIR-like motif are important elements for DNA recognition and binding of HspR to the operator. Intriguingly, mutation of one of these elements impaired binding of HspR on the side of the mutation and showed no effects on the binding on the HAIR-like motif on the opposite side of the mutation (**Figure 6**). This finding is compatible with a cooperative mechanism of HspR binding. Possibly, a dimer of HspR recognizes and binds to the HAIR-like sequence and this in turn drives binding of additional dimers on both sides of the HAIR-like motif (**Figure 7**). Evidences that HspR could bind its DNA sequences as a dimer have been shown in previous studies, in which it was demonstrated that homologs of *H. pylori* HspR repressor protein could exists in a dynamic state between the dimeric and monomeric forms in solution (Parijat and Batra, 2015). Moreover, Spohn et al. (2004) provided evidences that *H. pylori* HspR is able to form high order oligomers. In conclusion, these results provide a more detailed comprehension of the interaction between HspR and its target DNA sequences and, at least for the P*cbp* promoter, let us to propose a cooperative DNA-binding mechanism of three HspR dimers on this operator as schematized in **Figure 7**. In this model, an HspR dimer binds to the central DNA region harboring the HAIR-like motif that acts as a nucleation center for protein–protein interactions driving cooperative binding of HspR homodimers to the specific and still unknown determinants of the DNA sequences surrounding the HAIR-like motif. Giving the low level of sequence conservation of the HAIR-like motifs across the HspR binding sites and the imperfect nature of the inverted repeats (i.e., low conservation of the two arms of the inverted repeats), it can also be speculated that HspR DNA binding could be driven by sequence-dependent local shape variations rather than by a base readout mechanism, or by a combination of these two mechanisms.

In other words, the crucial role of the HAIR-like motifs for HspR binding could be explained by taking into account local DNA shape variations imposed by a peculiar succession of purine/pyrimidine, rather than considering only the unique chemical signatures of the individual DNA bases. This behavior is common among regulators of MerR family, to which the *H. pylori* HspR belongs to, employing indirect (shape) readout as part of their DNA binding mechanisms. These considerations could help also to explain our data on the importance of the central region of the HAIR-like motif and of the non-conserved regions on both sides of the HAIR-like motif (**Figure 4**, M3 and **Figure 6**, M4 and M5).

AUTHOR CONTRIBUTIONS

AD, CP, DR, and VS conceived and designed the experiments. SPE, EP, EF, TB, CP, and AV performed the experiments. SPE, EP, and SPU carried out the data analysis. DR and VS wrote the paper with contributors CP and EP. All the authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01887/full#supplementary-material

TABLE S1 | Annotation of coding genes.

TABLE S2 | Library preparation and sequencing performances.

TABLE S3 | Transcriptome of the wild type and Δ*hspR* strains.

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