Abstract: Bacteria respond to different environmental stresses by reprogramming the transcription of specific genes whose proper expression is critical for their survival. In this regard, the heat-shock response, a widespread protective mechanism, triggers a sudden increase in the cellular concentration of different proteins, including molecular chaperones and proteases, to preserve protein folding and maintain cellular homeostasis. In the medically important gastric pathogen Helicobacter pylori the regulation of the principal heat-shock genes is under the transcriptional control of two repressor proteins named HspR and HrcA. To define the HrcA regulon, we carried out whole transcriptome analysis through RNA-sequencing, comparing the transcriptome of the H. pylori G27 wild type strain to that of the isogenic hrcA-knockout strain. Overall, differential gene expression analysis outlined 49 genes to be deregulated upon hrcA gene inactivation. Interestingly, besides controlling the transcription of genes coding for molecular chaperones and stress-related mediators, HrcA is involved in regulating the expression of proteins whose function is linked to several cellular processes crucial for bacterial survival and virulence. These include cell motility, membrane transporters, Lipopolysaccharide modifiers and adhesins. The role of HrcA as a central regulator of H. pylori transcriptome, as well as its interconnections with the HspR regulon are here analyzed and discussed. As the HrcA protein acts as a pleiotropic regulator, influencing the expression of several stress-unrelated genes, it may be considered a promising target for the design of new antimicrobial strategies.

Keywords: heat-shock response; stress conditions; HrcA repressor; Helicobacter pylori; RNA-sequencing; transcriptome
some heat-shock promoters to tightly modulate the expression level of HSPs and, hence, assuring their correct amount inside the bacterial cell. In particular, previous studies by our group showed that HspR alone or in combination with HrcA repress the transcription of the three multicistronic operons (groES-groEL, hrcA grpE dnaK, and cbpA-hspR-rarA), coding for the almost complete chaperone repertoire of H. pylori [5–8]. In fact, HspR alone is able to control its own transcription by binding to the promoter upstream the cbpA gene, while the regulation of groES-groEL and hrcA-grpE dnaK operons’ transcription is dependent on both HrcA and HspR proteins. Considering these regulatory interactions, it appears that the HrcA regulon is totally enclosed inside the regulon of the master repressor HspR, a rare regulatory scheme that resembles the heat-shock network described in Staphylococcus aureus [4].

Although the molecular details of HspR and HrcA regulation on chaperones’ promoters have been extensively studied (recently reviewed in [3]), genome-wide studies addressed at the dissection of the possible involvement of these repressors in controlling other targets, beyond the core heat-shock genes, and other cellular processes have been overlooked until recent years. In a recent study by our group aimed at the further definition of HspR role in H. pylori, ChIP-sequencing and RNA-sequencing approaches have been used to identify the HspR regulon and to discriminate between direct regulation and indirect effects. These data showed that HspR participates in the regulation of tens of genes coding for proteins involved in different cellular crucial functions and is not necessarily connected to stress response. In addition, ChIP-seq data showed that HspR binds to a limited number of genomic sites, supporting the hypothesis that the contribution of HspR to the heat-shock response regulation is largely indirect [9].

In this study, we expanded our analysis to the identification of the HrcA regulon by carrying out a whole transcriptome profiling to globally identify the genes affected by HrcA inactivation. Results were compared to the available heat-shock regulon and to the HspR regulon. This analysis revealed that, besides controlling some major stress-related chaperones, the HrcA protein is engaged in the regulation of different cellular processes including cell motility, transport of small molecules across the membrane and host-pathogen interaction.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

Bacterial cells used in this study (H. pylori G27 wild type strain [10] and the isogenic (hrcA::km) hrcA deletion mutant [6]) were recovered from frozen glycerol stocks on modified Brucella broth agar plates containing 5% fetal calf serum (FCS), in a controlled atmosphere (9% CO2–91% air) at 37 °C in a water-jacketed incubator (Thermo Forma Scientific, Waltham, MA, USA) for two–three days. Liquid cultures were carried out at 37 °C in modified Brucella broth supplemented with 5% FCS, with gentle agitation (130 rpm) for two days, diluted in fresh medium and grown to mid-exponential phase (about 6 h).

2.2. RNA Extraction

H. pylori cells were liquid-grown as detailed above until the mid-exponential phase (OD = 0.6–0.7). Then, ten ml of culture was mixed with 1.25 mL of ice-cold EtOH-phenol stop solution (5% acid phenol-95% EtOH) to preserve RNA integrity. Cells were harvested and then lysed to extract total RNA with TRI-reagent (Sigma-Aldrich, St Louis, MO, USA), following the manufacturer’s instructions. Prior to use, RNA samples were analyzed through agarose gel electrophoresis to control RNA purity and integrity.

2.3. RNA-Sequencing: Library Preparation, Sequencing and Analysis

For each condition and replica, rRNAs were depleted by using the RiboZero Gram negative kit (Epicentre, Illumina, Madison, WI, USA) starting from 1 µg of total RNA and strand specific RNA-seq libraries were prepared by using the ScriptSeq™ v2 RNAseq library preparation kit (Epicentre,
Illumina), from 50 ng of previously rRNA-depleted RNA. Then, 85 bp reads were produced on a GAIIx Illumina sequencer obtaining a minimum of 8 Million reads per sample and Bowtie 2 (v2.2.6) [11] was used to align raw reads to H. pylori G27 genome, obtaining between 89% and 94.5% of mapped reads. A modified version H. pylori G27 annotation based on RefSeq GCF_000021165.1, BEDTools (v2.20.1) [12] and SAMtools (v0.1.19) [13] were used to verify the library preparation and sequencing performances and to produce strand specific gene level counts. Ribosomal RNA depletion produced a reduction of ribosomal reads to less than 6% of the total mapping, 99% of the annotated transcripts were covered by at least one strand specific read and a minimum of 100 reads were counted on 90% of them. The R package DESeq2 (v1.4.5) [14] was then used to normalize the counts and to identify differentially expressed genes (DEGs) showing BH (Benjamini-Hochberg) adjusted p-value (padj) lower than 0.01 and log2 fold changes (log2FC) > |1|. To evaluate functional enrichments in the DEGs lists, we retrieved their clusters of orthologous groups (COG) functional classes from the NCBI CDD database [15]. Please refer to Pepe et al. [9] for further details. Raw data are publicly available at the Sequence Reads Archive under accession number BioProject PRJNA421261 and PRJNAXXXXX.

2.4. Quantitative Real Time PCR (qRT-PCR) Analysis

cDNA synthesis and qRT-PCR analysis were carried out as previously reported [16]. Briefly, to eliminate genomic DNA contamination, RNA samples were digested with 1 Unit of DNase I for 45 min at 37 °C. Then, 1 µg of purified RNA was reverse transcribed using 50 ng of random primers (Invitrogen, Carlsbad, CA, USA), dNTPs (1 mM each), AMV-Reverse Transcriptase (Promega, Madison, WI, USA) and incubating the reaction at 37 °C for 60 min. For real time PCR assays, 2 µL of the ten-fold diluted cDNA samples were mixed with 5 µL of 2× qPCRBIO SyGreen Mix LO-ROX (PCR BIOSYSTEMS) and specific primers mapping in the coding sequence of the genes of interest (Table S1) at 400 nM concentration in a final reaction volume of 10 µL. qRT-PCR was carried out as follows: Initial denaturation at 95 °C for 2 min, then 40 cycles consisting of a denaturation step at 95 °C for 5 s followed by 30 s at 60 °C. For each qRT-PCR assay, a melting curve was included at the end of the amplification cycles to check for the specificity of the reaction. Data analysis was done by applying the ∆∆Ct method, in which the 16S rRNA gene was used as internal reference for data normalization. Real Time PCR of 16S rRNA on cDNA samples from H. pylori G27 wild type and ΔhrcA cells gave overlapping amplification plots, indicating that the expression of 16S rRNA was unaffected by hrcA deletion.

2.5. Immunoblot Analysis

Immunoblotting was performed as previously described [17]. Briefly, equal amounts (10 µg) of total protein extracted from H. pylori G27 wild type and hrcA-mutant cells were separated through SDS-PAGE and blotted onto a nylon (PVDF) membrane by means of a wet-transfer apparatus (BioRad). Following 1 h incubation at room temperature in blocking buffer (1× PBS containing 0.05% Tween 20 and 5% low-fat milk), the membrane was stained with a 1:5000 dilution of α-BabA and α-HP1043 antibodies for 16 h at 4 °C in blocking buffer.

After washing the membrane in PBST (1× PBS; 0.05% Tween-20), it was incubated with 1:5000-diluted horseradish peroxidase-conjugated α-rabbit antibody for 1 h at 25 °C (Thermo Fisher Scientific, Waltham, MA, USA). Following an additional washing step in PBST, the membrane was developed by pouring on it a solution of 1.25 mM luminol containing 0.015% H2O2 and 0.068 mM p-coumaric acid.

3. Results

3.1. RNA-Seq Analysis Reveals the HrcA-Dependent Transcriptome

In order to detect HrcA-dependent changes in gene expression, we carried out whole transcriptome RNA sequencing analysis of the H. pylori G27 wild type and of the isogenic hrcA deletion mutant (hrcA::km) strains. Data analysis revealed that 49 genes were differentially expressed (log2 fold change
>1 or < -1) in the hrcA-mutant (Figure 1, Table 1 and Supplementary Table S2), and among them, 16 were up-regulated and 33 were down-regulated.

Figure 1. Genome-wide HrcA-dependent gene expression. The plot shows log10 scale means of the normalized counts obtained from H. pylori G27 wild type (x axis) and from the isogenic ΔhrcA deletion mutant (y axis) duplicates for each of the expressed genes. Genes found to be up-regulated, down-regulated or unchanged comparing ΔhrcA and wild type in DESeq2 analysis are represented as red, blue and grey dots, respectively.

As HrcA represses the transcription of the groES and groEL genes, their transcripts were accordingly found in the up-regulated list of genes of our analysis (Table 1 and Supplementary Table S2). On the contrary, the transcript amounts of the grpE and dnaK genes (HPG27_RS00570–HPG27_RS00575), belonging to a well-known HrcA target operon, were unaffected by hrcA mutation, likely because of a polar effect of the inactivation of the first gene of the operon (hrcA).

Table 1. List of up- and down-regulated genes.

| Gene Names           | Log2 FC | Common Names | Description                        |
|----------------------|---------|--------------|------------------------------------|
| HPG27_RS00240        | -2.4    | tnpB         | transposase                        |
| HPG27_RS00595        | -2.2    | HPG27_RS00595| motility accessory factor          |
| HPG27_RS00600        | -2.1    | flaB         | flagellin B                        |
| HPG27_RS01480        | -2.1    | fglL         | flagellar hook-associated protein FlgL |
| HPG27_RS01595        | -1.2    | babA, omp28  | membrane protein (adhesin)         |
| HPG27_RS01870        | -1.9    | HPG27_RS01870| hypothetical protein                |
| HPG27_RS00070        | 1.1     | groEL, hspB, hsp60 | molecular chaperone GroEL |
| HPG27_RS00075        | 1.1     | groES, hspA, hsp10 | co-chaperone GroES |
| HPG27_RS00625        | 1.0     | HPG27_RS00625| hypothetical protein                |
Table 1. Cont.

| Gene Names Log2 FC | Common Names Log2 FC | Description |
|--------------------|----------------------|-------------|
| HPG27_RS01045 1.0  | HPG27_RS01045        | LPS biosynthesis protein |
| HPG27_RS01500 1.0  | dppB                 | peptide ABC transporter permease |
| HPG27_RS01505 1.2  | dppC                 | peptide ABC transporter |
| HPG27_RS01510 1.1  | dppD                 | ABC transporter ATP-binding protein |
| HPG27_RS01515 1.1  | dppE                 | ABC transporter ATP-binding protein |
| HPG27_RS03015 2.0  | HPG27_RS03015        | LPS biosynthesis protein |
| HPG27_RS03495 1.2  | hopO, omp16, sabB    | Membrane protein (adhesis) |
| HPG27_RS03705 1.0  | HPG27_RS03705        | 5-formyltetrahydrofolate cyclo-ligase |
| HPG27_RS03940 1.2  | fcaA, fcaA, fcaA_2  | ligand-gated channel |
| HPG27_RS05840 1.0  | gluP                 | glucose/galactose MFS transporter |
| HPG27_RS06930 1.6  | HPG27_RS06930        | hypothetical protein |
| HPG27_RS06935 1.9  | HPG27_RS06935        | restriction endonuclease |
| HPG27_RS08000 1.7  | HPG27_RS08000        | hypothetical protein |
| HPG27_RS081870 −1.9| HPG27_RS081870       | hypothetical protein |
| HPG27_RS01990 −1.1 | lpxC, envA           | UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase |
| HPG27_RS02255 −2.0 | horE, omp11          | membrane protein |
| HPG27_RS02575 −2.4 | HPG27_RS02575        | transposase |
| HPG27_RS02740 −3.1 | ykgB                 | membrane protein |
| HPG27_RS02925 −1.5 | flaA                 | flagellin A |
| HPG27_RS03550 −2.4 | inpB                 | transposase |
| HPG27_RS03660 −1.2 | flID                 | flagellar filament capping protein FlID |
| HPG27_RS03665 −1.1 | flIS                 | flagellar chaperone protein FlIS |
| HPG27_RS03670 −1.2 | flIT                 | flagellar chaperone protein FlIT |
| HPG27_RS04250 −1.1 | hopA                 | hydrogenase/urease Ni incorporation protein HypA |
| HPG27_RS04235 −2.1 | flgE                 | flagellar hook protein FlgE |
| HPG27_RS04430 −1.9 | flgK                 | flagellar hook-length control protein FlgK |
| HPG27_RS04645 −2.4 | inpB                 | transposase |
| HPG27_RS04725 −4.5 | HPG27_RS04725        | ATPase |
| HPG27_RS04730 −3.2 | HPG27_RS04730        | hypothetical protein |
| HPG27_RS04735 −2.6 | inpB                 | transposase |
| HPG27_RS04920 −1.2 | HPG27_RS04920        | hypothetical protein |
| HPG27_RS05380 −1.1 | pseC                 | UDP-4-amino-4,6-dideoxy-N-acetyl-β-L-altrosamine transaminase (flagellar modification) |
| HPG27_RS05560 −2.1 | flgK                 | flagellar hook-associated protein FlgK |
| HPG27_RS05565 −2.1 | HPG27_RS05565        | hypothetical protein |
| HPG27_RS05740 −1.1 | flw                  | flagellar assembly protein FlW |
| HPG27_RS06175 −1.8 | HPG27_RS06175        | hypothetical protein |
| HPG27_RS07055 −1.1 | HPG27_RS07055        | hypothetical protein |
| HPG27_RS07080 −1.5 | HPG27_RS07080        | nickel transporter |
| HPG27_RS08050 −2.0 | HPG27_RS08050        | exonuclease VII large subunit |
| HPG27_RS08160 −2.0 | HPG27_RS08160        | hypothetical protein |
| HPG27_RS08320 −1.2 | HPG27_RS08320        | hypothetical protein |

To get a general view of HrcA regulatory function, a functional enrichment analysis was performed, and protein-coding sequences were classified using Clusters of Orthologous Groups of Proteins (COGs) Database [15]. According to this analysis, among the up-regulated genes we noticed some functional groups of particular interest (Figure 2A). For example, we identified genes (HPG27_RS03705 and HPG27_RS08000) belonging to the “coenzyme transport and metabolism” category and a group of genes (dppB, dppC, dppD, dppE) that constitute a multicistronic operon (according to DOOR, the Database of prOkaryotic OpeRons) coding for components of ABC transporters and, hence, related to “inorganic ion transport and metabolism”. Moreover, of particular interest are some genes coding for lipopolysaccharide (LPS) biosynthesis proteins (HPG27_RS01045, HPG27_RS03015), belonging to the “cell wall/membrane/envelope biogenesis” functional group (Figure 2A, Table 1 and Supplementary Table S2).
There were down-regulated in both the hrcA (HPG27_RS00240, HPG27_RS02575, HPG27_RS03550, HPG27_RS04645, HPG27_RS04735) and transposon deletion mutant, hence showing a complex regulatory pattern (Figure 2A).

3.2. Comparison of the HrcA regulon with the HspR and Heat-Shock Regulons

The analysis of transcripts downregulated in the hrcA-mutant shows that the predominant functional category is represented by “cell motility” (Figure 2A), comprising several genes encoding subunits of the flagellar machinery and proteins involved in the proper assembly of the bacterial flagella (flaB, fglL, flaA, fliD, flIS, flgE, fliK, flgK, fliW). This finding is in agreement with previous observations that a H. pylori hrcA deletion mutant shows motility defects when inoculated into semisolid agar plates [7]. In addition, among the down-regulated genes in the hrcA-mutant background, we found four genes coding for transposases and, hence, belonging to the functional category “mobilome: Prophages and transposon” (Figure 2A).

Figure 2. Analysis of the HrcA regulon and its integration with HspR-mediated regulation and general heat-shock response. (A): Pie charts showing COGs functional annotation of the differentially expressed genes outlined in the ∆hrcA_vs_WT comparison subdivided into up-regulated (left) and down-regulated (right) groups. The abundance of each category is indicated as a percentage, while the total number of up- and down-regulated genes is reported below each chart. (B,C): Venn diagrams showing the number of coherently up-regulated (B) or down-regulated (C) genes specific of the HrcA regulon or in common with the HspR and/or the heat-shock dataset (according to [9]).

Subsequently, the above results were compared to the previously identified deregulated genes upon exposure of the H. pylori G27 wild type strain to heat-shock at 42 °C and to the genes deregulated in the hspR deletion mutant [9]. The Venn diagrams reported in Figure 2 represent the number of genes coherently up- (Figure 2B) or down-regulated (Figure 2C) in the different conditions described above. Only three genes out of 16 were up-regulated by hrcA deletion and by heat-shock treatment and two of them (groES and groEL) were also up-regulated in the hspR-mutant, while one (a hypothetical adhesin coding gene, sabB) was deregulated in the hrcA-mutant and upon heat-shock treatment (Figure 2B). By contrast, four genes (HPG27_RS00625, dppC, fecA2, HPG27_RS08000), not responsive to heat-shock, were up-regulated in both the hrcA and hspR deletion mutants.

Of the 33 down-regulated genes in the hrcA-mutant, none of them were down-regulated also upon heat treatment (Figure 2C), while six genes (HPG27_RS00240, HPG27_RS02575, HPG27_RS03550, HPG27_RS04645, HPG27_RS04725, HPG27_RS04735) of the mobilome/replication functional categories were down-regulated in both hrcA and hspR mutants (Figure 2C).

Surprisingly, we identified 10 genes that were repressed in the hrcA-mutant and induced by heat-shock treatment, thus showing opposite behaviour. Moreover, among these genes, five were also down-regulated in the hspR-deletion mutant, hence showing a complex regulatory pattern (Table S2). These latter include most of the genes mentioned above to be hrcA- and hspR-downregulated (HPG27_RS00240, HPG27_RS02575, HPG27_RS03550, HPG27_RS04645, HPG27_RS04735).
Intriguingly, our transcriptome study shows that only a small fraction of the genes belonging to the HrcA regulon are also heat-responsive (that is, deregulated in the wild type strain subjected to heat-shock), while the major part of them appears to be heat-stress independent.

3.3. RNA-Seq Data Validation

To independently validate the RNA-seq results, RNA transcripts abundance of 14 newly identified HrcA-regulated genes and of groEL (positive control) were measured by quantitative real-time PCR, using specific primers for each sequence of interest. Specifically, as highlighted in grey in Table 1, the transcript levels of five up-regulated genes and of 10 down-regulated genes were compared in the wild type and in the hrcA-mutant strains. For data analysis, ΔhrcA vs wt fold-change ratios of ≥2.0 or ≤0.5 were considered as validated up- and down-regulations, respectively.

For all the genes included in this analysis, we confirmed a significant hrcA-dependent de-regulation in the mutant strain compared to the wild type (Figure 3A,B). To further support our findings, we investigated the expression of the outer membrane protein BabA in the wild type and hrcA-mutant strain through immunoblotting on wild type and ΔhrcA total protein extracts, using a specific α-BabA antibody. According to our RNA-seq results, the BabA encoding gene (HPG27_RS01595, sometimes referred as omp28) appears to be significantly down-regulated in the hrcA-mutant strain (log2 fold change −1.21, Supplementary Table S2). As shown in Figure 3C, immunoblot analysis on three different wild type and ΔhrcA independent biological replicates showed that BabA expression was lower in the hrcA-knockout strain than in the wild type, confirming HrcA involvement in BabA regulation and further supporting our whole transcriptomic analysis.

![Figure 3](image-url)
H. pylori G27 wild type and hrcA-mutant cells and reverse transcribed to cDNA. Transcript levels of genes were quantified by qRT-PCR, using the housekeeping 16S rRNA gene as control. Error bars indicate the standard deviation deriving from three independent biological samples, each analysed in duplicate technical replicates. (C): Immunoblot analysis of total protein extracts of H. pylori G27 wild type and hrcA-mutant cells stained with α-BabA and α-HP1043 antibodies [18]. Immunoblot analysis was carried out on three different H. pylori G27 wild type and ΔhrcA biological replicates.

4. Discussion

Whole transcriptome analysis through RNA-sequencing of H. pylori hrcA-mutant strain revealed that this heat-shock repressor is directly or indirectly involved in the regulation of 49 genes (Figure 1) with disparate functions. Considering that H. pylori possesses two heat-shock transcriptional repressors, HrcA and HspR, which are responsive to temperature stress [19,20], we compared the transcriptome of the H. pylori G27 hrcA-mutant strain to the parental strain submitted to heat-shock and to the H. pylori G27 hspR-mutant strain (dataset derived from [9]). Strikingly, the vast majority of genes belonging to the HrcA regulon is not coherently induced by heat-shock, at least in the experimental conditions used, being limited to only three genes up-regulated in both conditions (Figure 2B). Another intriguing observation emerging from this analysis is that the overlap between the HrcA and the HspR regulons is restricted to a limited number of genes, mainly represented by the already known HrcA and HspR co-regulated HSP genes (Figure 2C). The global picture deriving from this study describes the H. pylori HrcA protein as a pleiotropic regulator with a specific (i.e. HspR-independent) regulon, influencing the expression of several stress-unrelated genes (Figure 2A,B,C). In order to define HrcA regulon, genome-wide expression approaches have been applied in other bacterial species, even though such studies are still limited for this repressor. What generally emerges is that many genes coding for proteins involved in diverse cellular processes are dependent on HrcA regulation. For instance, it has been shown that in the food-borne pathogen Listeria monocytogenes, several core cellular functions, including chromosome replication, protein synthesis and stress response, are affected by HrcA [21]. In addition, hrcA gene inactivation provokes a similar pleiotropic gene deregulation also in the Gram-positive lactic acid bacterium Lactobacillus plantarum [22].

One limitation of our study is that several attempts to discriminate between the HrcA direct or indirect contribution in the regulation of the genes belonging to its regulon through genome-wide approaches, such as ChIP-seq assay, were unsuccessful. On the contrary, this kind of analysis has been set up for the other H. pylori heat-shock regulator HspR, leading to the identification of its genomic binding sites, constituted by few promoters driving the transcription of genes coding for chaperones [9]. Further efforts are needed to identify HrcA target promoters, thereby defining the extension of the HrcA direct regulon.

A crucial aspect of HrcA regulation, supported by the data here presented, is the link between this heat-shock repressor and motility of the bacterium. As in many bacterial pathogens, H. pylori relies on the coordinated and hierarchical expression of flagellar genes to successfully colonize the host and to find its proper niche. RNA-sequencing analysis highlights the hrcA-dependent down-regulation of many transcripts coding for components of the H. pylori unipolar flagella (flaA, flaB, fliD, flgK, flgL and flgE) and for proteins involved in the biosynthesis of the motility apparatus (fliK, fliW and fliS) (Figure 4, Table 1 and Supplementary Table S2).
This finding supports a previous observation that the *H. pylori* hrcA-mutant strain shows a non-motile phenotype when assayed on soft-agar plates [7]. In addition, an intimate relationship between motility and heat-shock gene regulation has been proposed in *Campylobacter jejuni*, a close phylogenetic relative of *H. pylori* [23]. Furthermore, a recent work proposes that the *H. pylori* flagella could play a pivotal structural role during biofilm formation [24]. In this latter study, comparative transcriptome profiling between *H. pylori* planktonic and biofilm cells highlighted differential expression of several flagellar genes between the two different modes of growth. Specifically, several flagellar components (such as FlaB and FlaG), other structural components (FlgE flagellar hook protein, FlgB rod protein, FlgK-FlgL hook-filament junction proteins) and flagellar regulatory players (as the hook length control protein FliK) were biofilm up-regulated. A similar correlation between biofilm formation and expression of flagellar genes has also been described in the model organism *Escherichia coli* [25]. Interestingly, in the same study, Hathroubi and colleagues observed that hrcA gene is up-regulated in the biofilm transcriptome, suggesting an intimate link among HrcA, biofilm formation and motility [24].

Of note is the observation that among the up-regulated genes, most of them code for proteins involved in the interaction of *H. pylori* with the external environment and with the host (Figure 4). Indeed, genes coding for subunits of transporters, outer membrane proteins and LPS modifiers belong to the HrcA regulon (Figure 4, Table 1 and Supplementary Table S2). In addition, the babA gene,
encoding a major player in the adhesion process of *H. pylori* to highly glycosylated mucins present in the human stomach [26], is down-regulated upon *hrcA* deletion (Figure 1, Table 1 and Figure 3C). This finding adds further complexity to the role played by the regulator HrcA in *H. pylori* gene regulatory network and encourages further studies to better characterize its involvement in the process of host-pathogen interaction.

Although *hrcA* is not essential, this study highlights the importance of this repressor for *H. pylori* virulence, survival and persistence inside the human stomach. With respect to that and considering the major medical threat of increasing *H. pylori* antibiotic resistance, HrcA may be regarded as a promising target for the design of new antimicrobial strategies.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/2076-2607/7/10/436/s1:
Table S1: List of oligonucleotides used in this study, Table S2: Extended table of up- and down-regulated genes.

**Author Contributions:** Conceptualization, D.R., C.P. and V.S.; methodology, D.R., C.P. and V.S.; software, C.P. and E.P.; validation, E.P. and D.R.; formal analysis, D.R. and E.P.; investigation, D.R., E.P., E.F. and S.L.; resources, C.P. and V.S.; data curation, D.R. and E.P.; writing—original draft preparation, D.R. and V.S.; writing—review and editing, D.R., E.P., E.F., S.L., C.P., V.S.; visualization, D.R. and E.P.; supervision, D.R., C.P. and V.S.; project administration, C.P. and V.S.; funding acquisition, V.S. and C.P.

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**References**

1. Gisbert, J.P.; Calvet, X. Review article: Common misconceptions in the management of Helicobacter pylori-associated gastric MALT-lymphoma. *Aliment. Pharmacol. Ther.* 2011, 34, 1047–1062. [CrossRef] [PubMed]
2. Salama, N.R.; Hartung, M.L.; Muller, A. Life in the human stomach: Persistence strategies of the bacterial pathogen Helicobacter pylori. *Nat. Rev. Microbiol.* 2013, 11, 385–399. [CrossRef] [PubMed]
3. Roncarati, D.; Scarlato, V. The interplay between two transcriptional repressors and chaperones orchestrates Helicobacter pylori heat-shock response. *Int. J. Mol. Sci.* 2018, 19, 1702. [CrossRef]
4. Roncarati, D.; Scarlato, V. Regulation of heat-shock genes in bacteria: From signal sensing to gene expression output. *FEMS Microbiol. Rev.* 2017, 41, 549–574. [CrossRef] [PubMed]
5. Spohn, G.; Scarlato, V. The autoregulatory HspR repressor protein governs chaperone gene transcription in Helicobacter pylori. *Mol. Microbiol.* 1999, 34, 663–674. [CrossRef] [PubMed]
6. Spohn, G.; Danielli, A.; Roncarati, D.; Delany, I.; Rappuoli, R.; Scarlato, V. Dual control of Helicobacter pylori heat shock gene transcription by HspR and HrcA. *J. Bacteriol.* 2004, 186, 2956–2965. [CrossRef] [PubMed]
7. Roncarati, D.; Danielli, A.; Spohn, G.; Delany, I.; Scarlato, V. Transcriptional regulation of stress response and motility functions in Helicobacter pylori is mediated by HspR and HrcA. *J. Bacteriol.* 2007, 189, 7234–7243. [CrossRef] [PubMed]
8. Roncarati, D.; Danielli, A.; Scarlato, V. CbpA acts as a modulator of HspR repressor DNA binding activity in Helicobacter pylori. *J. Bacteriol.* 2011, 193, 5629–5636. [CrossRef] [PubMed]
9. Pepe, S.; Pinatel, E.; Fiore, E.; Puccio, S.; Peano, C.; Brignoli, T.; Vannini, A.; Danielli, A.; Scarlato, V.; Roncarati, D. The Helicobacter pylori heat-shock repressor HspR: Definition of its direct regulon and characterization of the cooperative DNA-binding mechanism on its own promoter. *Front. Microbiol.* 2018, 9, 1887. [CrossRef]
10. Xiang, Z.; Censini, S.; Bayeli, P.F.; Telford, J.L.; Figura, N.; Rappuoli, R.; Covacci, A. Analysis of expression of CagA and VacA virulence factors in 43 strains of Helicobacter pylori reveals that clinical isolates can be divided into two major types and that CagA is not necessary for expression of the vacuolating cytotoxin. * Infect. Immun.* 1995, 63, 94–98.
11. Langmead, B.; Salzberg, S.L. Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 2012, 9, 357–359. [CrossRef] [PubMed]
12. Quinlan, A.R.; Hall, I.M. BEDTools: A flexible suite of utilities for comparing genomic features. *Bioinformatics* 2010, 26, 841–842. [CrossRef] [PubMed]
13. Li, H.; Handsaker, B.; Wysoker, A.; Fennell, T.; Ruan, J.; Homer, N.; Marth, G.; Abecasis, G.; Durbin, R. 1000 Genome Project Data Processing Subgroup. The sequence alignment/map format and SAMtools. *Bioinformatics* 2009, 25, 2078–2079. [CrossRef] [PubMed]

14. Love, M.I.; Huber, W.; Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 2014, 15, 50. [CrossRef] [PubMed]

15. Tatusov, R.L.; Koonin, E.V.; Lipman, D.J. A genomic perspective on protein families. *Science* 1997, 278, 631–637. [CrossRef] [PubMed]

16. Roncarati, D.; Pelliciari, S.; Doniselli, N.; Maggi, S.; Vannini, A.; Valzania, L.; Mazzei, L.; Zambelli, B.; Rivetti, C.; Danielli, A. Metal-responsive promoter DNA compaction by the ferric uptake regulator. *Nat. Commun.* 2016, 7, 12593. [CrossRef] [PubMed]

17. Roncarati, D.; Spohn, G.; Tango, N.; Danielli, A.; Delany, I.; Scarlato, V. Expression, purification and characterization of the membrane-associated HrcA repressor protein of *Helicobacter pylori*. *Protein Expr. Purif.* 2007, 51, 267–275. [CrossRef]

18. Pelliciari, S.; Pinatel, E.; Vannini, A.; Peano, C.; Puccio, S.; De Bellis, G.; Danielli, A.; Scarlato, V.; Roncarati, D. Insight into the essential role of the *Helicobacter pylori* HP1043 orphan response regulator: Genome-wide identification and characterization of the DNA-binding sites. *Sci. Rep.* 2017, 7, 41063. [CrossRef]

19. Spohn, G.; Delany, I.; Rappuoli, R.; Scarlato, V. Characterization of the HspR-mediated stress response in *Helicobacter pylori*. *J. Bacteriol.* 2002, 184, 2925–2930. [CrossRef]

20. Roncarati, D.; Danielli, A.; Scarlato, V. The HrcA repressor is the thermosensor of the heat-shock regulatory circuit in the human pathogen *Helicobacter pylori*. *Mol. Microbiol.* 2014, 92, 910–920. [CrossRef]

21. Hu, Y.; Oliver, H.F.; Raengpradub, S.; Palmer, M.E.; Orsi, R.H.; Wiedmann, M.; Boor, K.J. Transcriptomic and phenotypic analyses suggest a network between the transcriptional regulators HrcA and sigmaB in *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 2007, 73, 7981–7991. [CrossRef]

22. Van Bokhorst-van de Veen, H.; Bongers, R.S.; Wels, M.; Bron, P.A.; Kleerebezem, M. Transcriptionome signatures of class I and III stress response deregulation in *Lactobacillus plantarum* reveal pleiotropic adaptation. *Microb. Cell. Fact.* 2013, 12, 112. [CrossRef]

23. Andersen, M.T.; Brondsted, L.; Pearson, B.M.; Mulholland, F.; Parker, M.; Pin, C.; Wells, J.M.; Ingmer, H. Diverse roles for HspR in *Campylobacter jejuni* revealed by the proteome, transcriptome and phenotypic characterization of an *hspR* mutant. *Microbiology* 2005, 151, 905–915. [CrossRef]

24. Hathroubi, S.; Zerebinski, J.; Ottemann, K.M. *Helicobacter pylori* biofilm involves a multigene stress-biased response, including a structural role for flagella. *mBio* 2018, 9, e01973-18. [CrossRef]

25. Domka, J.; Lee, J.; Bansal, T.; Wood, T.K. Temporal gene-expression in *Escherichia coli* K-12 biofilms. *Environ. Microbiol.* 2007, 9, 332–346. [CrossRef]

26. Skoog, E.C.; Padra, M.; Aberg, A.; Gideonsson, P.; Obi, I.; Quintana-Hayashi, M.P.; Arnvist, A.; Linden, S.K. BabA dependent binding of *Helicobacter pylori* to human gastric mucins cause aggregation that inhibits proliferation and is regulated via ArsS. *Sci. Rep.* 2017, 7, 40566. [CrossRef]

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