CbpA Acts as a Modulator of HspR Repressor DNA Binding Activity in Helicobacter pylori

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The ability of pathogens to cope with disparate environmental stresses is a crucial feature for bacterial survival and for the establishment of a successful infection and colonization of the host; in this respect, chaperones and heat-shock proteins (HSPs) play a fundamental role in host-pathogen interactions. In *Helicobacter pylori*, the expression of the major HSPs is tightly regulated through dedicated transcriptional repressors (named HspR and HrcA), as well as via a GroESL-dependent post-transcriptional feedback control, acting positively on the DNA binding affinity of the HrcA regulator itself. In the present work we show that also the CbpA chaperone participates to the post-transcriptional feedback control of the *H. pylori* heat shock regulatory network. Our experiments suggest that CbpA specifically modulates HspR *in vitro* binding to DNA, without affecting HrcA regulator activity. In particular, CbpA directly interacts with HspR, preventing the repressor to bind to its target operators. This interaction takes place only when HspR in not bound to DNA, being CbpA unable to affect HspR once the repressor is bound to its operator site. Accordingly, *in vivo* overexpression of CbpA compromises the response kinetics of the regulatory circuit, inducing a failure to restore HspR-dependent transcriptional repression after heat-shock. The data presented in this work support a model in which CbpA acts as an important modulator of HspR regulation, by fine-tuning the shut-off response of the regulatory circuit that governs HSP expression in *H. pylori*.
INTRODUCTION

Chaperones, a class of functionally related and conserved proteins found in virtually all living organisms, are involved in many different cellular processes. Their main function is to assist protein folding, assembly, transport and degradation under normal conditions of growth, and even more, during stress. Bacteria rely on chaperones to rapidly adapt to sudden changes of the environment and their survival is directly dependent on the proper regulation of chaperones’ expression. For this purpose, microorganisms have evolved complex regulatory strategies that are highly diversified among different bacterial species (20).

In *H. pylori*, a highly pervasive Gram-negative human gastric pathogen, chaperone and heat shock proteins (HSPs), besides their general role in protection of the bacterium from the extremely hostile environment of the stomach, are involved in more specific pathogenic processes such as regulation of urease enzyme activity and adhesion to epithelial cells ((11), (13), (18), (22)). The main HSPs of *H. pylori* are clustered in three multicistronic operons (Figure 1), transcriptionally controlled by three distinct upstream promoters (namely P*gro*, P*hrc* and P*cbp*). While many Gram-negative bacteria adopt a positive mechanism of HSPs transcriptional regulation through a specialized RNA polymerase σ factor (for example *Escherichia coli* σ^32^), *H. pylori* negatively controls chaperones’ expression by employing two dedicated transcriptional repressors (reviewed in (9)). In particular, it has been demonstrated that two regulators, named HrcA and HspR (homologues of two transcriptional repressors found in *Streptomyces coelicolor* and *Bacillus subtilis*, respectively ((5), (14), (21), (26), (33)), are both necessary to repress transcription from *Phrc* and *Pgro* promoters in normal growth conditions, while HspR alone controls its own promoter (P*cbp*) (27). Upon heat shock, the repression is released and the HSPs transcription is induced. Intriguingly, on co-regulated promoters, although HrcA and HspR are both necessary to repress
transcription under physiological conditions (27), they bind independently to adjacent operators and
do not directly interact each other (23).

In many bacterial species, an emerging feature of heat shock genes’ regulation is the
involvement of chaperones themselves in the feedback modulation of transcriptional regulators’
activity. This additional level of control can be found, for example, in B. subtilis. In this system, the
HrcA repressor is unable to bind its operators if not assisted by the chaperonin GroE. Under stress
conditions, the GroE chaperonin is titrated away by misfolded proteins that accumulate inside the
cell, and HrcA loses relative DNA binding affinity, thereby releasing transcriptional repression
(19). In S. coelicolor the DnaK chaperone protein takes part in the heat shock genes’ regulatory
circuit. During normal conditions of growth, DnaK associates with the HspR repressor, forming a
complex able to bind DNA and to repress transcription. Upon heat shock stress, denatured proteins
sequester DnaK, leaving HspR alone and unable to bind and repress heat shock genes promoter ((4),
(3)). Also in H. pylori chaperone proteins participate in HSPs transcriptional regulation, as HrcA
binding affinity is positively modulated by the GroESL complex (23), possibly through a similar
mechanism as in B. subtilis.

Just upstream hspR, in one of the heat shock operons, cbpA (HP1024) gene encodes a
protein with 30% amino acid identity to CbpA (for curved DNA binding protein A) from E. coli
(30). Firstly identified as a DNA binding protein, E. coli CbpA binds DNA efficiently, with a
preference for curved DNA, and has been localized to the nucleoids of stationary phase cells ((1),
(16), (30)). More recent studies have revealed that E. coli CbpA can function as a co-chaperone for
the DnaK/Hsp70 chaperone system, and its activity can be modulated by CbpM, an 11-kDa protein
that has structural homology to DafA of Thermus thermophilus ((2), (6), (7), (8)).

In H. pylori, very little is known about CbpA and, because of its homology with the E. coli
co-chaperone, it has always been considered being a DnaJ-like protein, as part of the DnaK/Hsp70
chaperone system. Here, we present data supporting a novel function for H. pylori CbpA. We show
that *H. pylori* CbpA plays an active role in the chaperones’ transcriptional regulation. We found that CbpA modulates DNA binding activity of HspR through a direct protein-protein interaction with the repressor. The modulation of HspR DNA binding activity seems to be important during the shutoff phase that follows transcriptional derepression in a typical heat shock response.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *H. pylori* strains (Table 1) were recovered from frozen glycerol stocks on Columbia Blood agar plates containing 5% horse blood, 0.2% cyclohexamide and Dent’s or Skirrow’s antibiotic supplement under microaerophilic conditions (Oxoid) for two days. After restreaking on fresh plates, bacteria were cultured in a 9% CO₂–91% air atmosphere at 37°C and 95% humidity in a water-jacketed incubator (Thermo Forma Scientific). Liquid cultures were performed in modified Brucella Broth supplemented with 5% fetal calf serum, 0.2% cyclohexamide and Dent’s or Skirrow’s antibiotic supplement. *E. coli* DH-5α and BL21(DE3) strains, harboring the desired plasmid, were grown on Luria-Bertani Agar plates or LB liquid broth; when required, ampicillin was added to the medium to achieve a final concentration of 100 μg/ml.

**DNA manipulations.** DNA manipulations were performed routinely as described by Sambrook et al. (25). All restriction and modification enzymes were used according to the manufacturers’ instructions (New England Biolabs). Preparations of plasmid DNA were carried out with Qiagen Miniprep Spin kit (Qiagen, Inc.) or NucleoBond Xtra Midi plasmid purification kit...
DNA fragments for cloning purposes were extracted and purified from agarose gel using Qiagen Gel Extraction kit (Qiagen, Inc.). PCR were carried out in a Perkin–Elmer Thermal Cycler using Taq DNA polymerase. In each reaction, 500 ng of H. pylori chromosomal DNA was mixed with 40 pmols of each specific primer in a final volume of 50 μl containing 200 μM of each deoxynucleotide in 1X PCR buffer containing Mg²⁺. A total of 33 cycles were performed by denaturing DNA at 95°C for 45s, annealing at the appropriate temperature for 45s, and extending at 72°C for 1 min.

**Recombinant proteins expression and purification.** Recombinant 6XHis-tagged HrcA and HspR were overexpressed in E. coli BL21(DE3) cells harboring the proper vector (Table 1) and purified through Ni-NTA affinity chromatography as previously described ((24), (29)). For CbpA-His overexpression, *E. coli* BL21(DE3) cells containing the plasmid pET22b-CbpA (Table 1) were inoculated in 250 ml of LB Broth and grown at 37°C with vigorous shaking until mid-exponential phase. The expression of the recombinant protein was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), cells were harvested after 4 h induction at 37°C and stored at -80°C. For CbpA-His purification, cells were resuspended in 25 ml of Lysis Buffer (50 mM NaH₂PO₄; 300 mM NaCl; 10 mM Imidazole, pH 8.0) containing 1 mg/ml lysozyme and 10 μg/ml both DNase and RNase, incubated on ice for 45 min and then disrupted through 2 French Pressure Cell cycles. The soluble protein fraction was mixed with 500 μl of 50% Ni²⁺-NTA slurry (Qiagen, Inc.) and incubated for 90 min at 4°C on a Tilt-Roll. The sample was packed in a polypropylene tube, washed twice with 5 ml of Wash Buffer 20 (50 mM NaH₂PO₄; 300 mM NaCl; 20 mM Imidazole, pH 8.0), twice with 5 ml Wash Buffer 50 (50 mM NaH₂PO₄; 300 mM NaCl; 50 mM Imidazole, pH 8.0) and the bound protein was eluted by adding three times 500 μl of Elution Buffer (50 mM NaH₂PO₄; 300 mM NaCl; 250 mM Imidazole, pH 8.0). Finally, CbpA-His was dialyzed against two changes of 1X FN-Footprinting Buffer (50 mM Tris pH 8.0; 50 mM KCl; 10 mM...
MgCl₂; 1 mM DTT; 50% glycerol) and stored at -20°C. Protein concentration was determined by Bradford colorimetric assay (BioRad) and the purity of the protein preparations was analyzed by SDS-PAGE. To prepare anti-CbpA antiserum, 20 μg of purified protein (CbpA-His) were used to immunize six-week-old CD1 female mice (Charles River Laboratories) and four mice were used. The protein was given intraperitoneally, together with complete Freund’s adjuvant for the first dose and incomplete Freund’s adjuvant for the second (day 21) and third (day 35) booster doses. Bleed out samples were taken on day 49 and used in Western blot analysis by standard methods (25).

**Generation of CbpA overexpressing strain of H. pylori.** For the generation of a *H. pylori* strain overexpressing the CbpA protein, naturally competent wild type *H. pylori* G27 cells were transformed, as previously described, with the construct pSL1190-cagcbp1/2-CbpA-Cm-cagcbp3/4 (Table 1). Several chloramphenicol resistant colonies were selected and the insertion of the desired fragment in the correct genomic locus was verified by PCR. Constitutive overexpression of CbpA was confirmed by Western blot with an anti-CbpA polyclonal antibody. One of these clones, designated *H. pylori* G27 (*PcagA-cbpA*) (Table 1), was selected for further studies.

**DNase I footprinting.** The region encompassing the *hrcA* promoter was PCR amplified with primers *hrcA* and *hrcA1* from *H. pylori* genomic DNA and cloned into the plasmid pGEM-T Easy (Table 1). Promoter DNA fragments obtained by SalI digestion was 5’end labeled with [γ-32P]-ATP and T4 polynucleotide kinase at one extremity, gel purified, and approximately 10,000 cpm of the 308 bp probe was used for footprinting experiments. Footprinting experiments were essentially performed as described previously (23). Basically, labeled DNA probe was incubated with recombinant purified proteins in 50 μl of 1X FPBH (5mM Tris pH 8.0; 9 mM Hepes pH 8.0; 45 mM NaCl; 14 mM KCl; 5.5 mM MgCl₂; 5 mM DTT; 0.01% NP-40 Igepal; 10% glycerol), containing 200 ng of sonicated salmon sperm DNA as non specific competitor, for 17 min at room
temperature; then, 0.06 Units of DNase I (GE Healthcare), freshly diluted in footprinting buffer containing 5 mM CaCl$_2$, was added to the reaction and the digestion was incubated for 70 seconds at room temperature. The reaction was stopped by adding 140 μl of DNase I Stop Buffer (192 mM NaOAc pH 5.2, 32 mM EDTA, 0.14% sodium dodecyl sulfate, 64 mg/ml sonicated salmon sperm DNA). Samples were phenol:chloroform extracted, EtOH precipitated and resuspended in 10 μl of Formamide Loading Buffer (95% Formamide, 10 mM EDTA, 0.1% Bromophenol Blue). Next, samples were denatured at 95°C for 5 min, snap chilled on ice, subjected to 6% polyacrylamide–urea gel electrophoresis and autoradiographed.

**GST pull-down assay.** For the GST pull-down assay, *H. pylori* HspR (bait) was overexpressed as a fusion protein with Glutathione-S-transferase (GST). As control bait, the GST protein alone was overexpressed and used in the pull-down assay in the same conditions as GST-HspR. In particular, the coding sequence of HspR was excised as an Ndel/XhoI fragment from the plasmid pET22b-HspR (29) and ligated to pGEX$_{NN}$ vector that allow N-terminal glutathione-S-transferase gene fusion (Table 1). The expression of the recombinant GST and GST-HspR proteins in *E. coli* BL21 (DE3) cells was performed as described above for CbpA-His. Then, each bacterial pellet from 250 ml cultures was resuspended in 15 ml of 1X PBS containing 1 mg/ml lysozyme, and 10 μg/ml DNase and RNase, and incubated for 90 min at 4°C. After the addition of 5 mM DTT, cells were sonicated on ice and centrifuged at maximum speed for 30 min. After addition of 1% Triton X-100, each soluble protein fraction was mixed with 400 μl of 50% Glutathione-Sepharose (GSH-Seph) slurry and incubated for 1 h at 4°C on a Tilt-Roll. Samples were packed in a polypropilene tube, washed 5 times with 4 ml of 1X PBS; then, 200 μl of 1X PBS was added to the GSH-Seph slurry with bound GST and GST-HspR. Binding of GST and GST-HspR to GSH-Seph slurry was assessed by SDS-PAGE. Total protein extracts from *H. pylori* G27 (hspR::kan) cells were obtained from 30 ml cultures. In particular, cells were spun down and resuspended in 1.5 ml
of Res-Buffer (10 mM Tris pH 7.0; 100 mM NaCl; 20 mM KCl; 1 mM EDTA; 0.01% NP-40 Igepal), containing 0.5 mg/ml lysozyme and 10 μg/ml both DNase and RNase, and then sonicated on ice, centrifuged at maximum speed for 30 min; the supernatant was collected and stored at 4°C. Pre-clearing of the lysate was performed by mixing 1.5 ml of *H. pylori* G27 (*hspR::kan*) lysate with 50 μl of GST-GSH-Seph slurry and incubated for 90 min at 4°C; then, the suspension was passed through a column and the cleared lysate was collected for the pull-down experiment. For the pull-down assay, the cleared lysate, or a solution containing the purified recombinant CbpA-His protein (the same molar concentration of the bait), were splitted in 2x700 μl aliquots and mixed with GST and GST-HspR-GSH-Seph slurry: the same molar concentration of the two bait proteins (GST or GST-HspR) was used. After 3 h incubation at 4°C, samples were packed in a polypropylene tube and washed 4 times with 150 μl of Res-Buffer; then, 1 bed volume of 1X PBS and the proper amount of 5X SDS Loading Buffer were added to the slurries and samples were boiled for 10 min. For western blot analysis, the same volume of samples coming from GST and GST-HspR-bound column were loaded on a 12% SDS polyacrylamide gel electrophoresis together with 20 ng of purified recombinant CbpA-His protein. After electrophoresis, proteins were blotted onto a PVDF nylon membrane (Amersham) for 17 min at 15V in 0.25X Tris–Glycine buffer (6.25 mM Tris–HCl; 62.5 mM glycine, pH 8.3; 0.025% w/v SDS), containing 20% (v/v) methanol, with a semidy transfer apparatus (Hoefer). After blocking for 1 h at room temperature in 1X PBS containing 5% low-fat milk and 0.05% Tween 20, the membrane was incubated for 16 h at 4°C with a 1:1000 dilution of mouse polyclonal primary antibody (anti-CbpA or anti-GST). After extensive washes in 1X PBS containing 0.05% Tween 20 (PBST), the membrane was incubated for 1 h at room temperature with a 1:5000 diluted peroxidase-conjugated anti-mouse immunoglobulin (Sigma). Once washed several times in PBST, the membrane was developed with an ECL detection system (Amersham).
RNA preparation and primer extension assay. For *H. pylori* heat shock time course, 40 ml of modified Brucella Broth were inoculated with an overnight bacterial culture of the *H. pylori* G27 wild type strain and the *H. pylori* G27 (*PcagA-cbpA*) (Cbpa-overexpressing strain, Table 1) and grown until exponential phase. Then each culture was split into 5 ml aliquots that were heat shocked at 42°C in a water bath for different periods of time (10, 20, 30, 45, 60, 90, 120, 150 min and 0 min – no treatment sample). After treatment, cells were mixed with 0.75 ml of ice-cold EtOH-Phenol Stop Solution (5% Acid Phenol in EtOH) to prevent RNA degradation; cells were then pelleted and stored at -80°C. RNA was extracted as previously described (10). Primer extension experiments with the hrcA oligonucleotide (Table 2) were performed as previously reported (23). Signals of the elongated primer were quantified by means of Phosphorimager and ImageQuant software. For quantitative analysis, four independent experiments were performed.

RNA dot blot analysis. Twenty μg of RNA were ethanol-precipitated, resuspended in 200 μl of RNA Denaturing Buffer (50% formamide; 7% formaldehyde; 15 mM Na$_3$-citrate; 150 mM NaCl), denatured at 65°C for 15 min, chilled on ice, mixed with 400 μl of 20X SSC (0.3 M Na$_3$-citrate; 3 M NaCl; pH 7.0), spotted on a Hybond-N$^+$ nylon membrane by means of a Bio-Dot Microfiltration apparatus (BioRad), and crosslinked to the filter by UV-rays treatment. The filter was prehybridized in 5 ml of Hybridization Buffer (6X SSC; 0.5% SDS; 0.1% Ficoll; 0.1% Polyvinylpyrrolidone; 0.1% Bovine Serum Albumin; 100 μg/ml denatured, fragmented salmon sperm DNA) for 2 h and then hybridized in the same buffer with 1.2 pmol of radioactively labeled cbpe4 oligonucleotide (Table 2) at 45°C for 20 h. The RNA-containing blot was washed twice for 2 min with Wash Buffer (2X SSC; 0.1% SDS) at room temperature, twice for 5 min with Wash Buffer at 45°C, and exposed to Kodak Biomax XAR films.
RESULTS

CbpA negatively modulates *in vitro* HspR binding to the Phrc promoter. In a previous study, addressed to characterize possible regulatory roles of the main chaperones GroESL and DnaKJ-GrpE on the heat shock gene transcriptional control, we have expressed, purified and reconstituted these chaperone machineries *in vitro* and investigated their effects on DNA binding activity of the HrcA and HspR repressors (23). Beside the positive effect of GroESL on HrcA binding, we did not observe significant influence of the DnaK-DnaJ-GrpE system on both HrcA and HspR *in vitro* binding to their operators. Considering the putative DnaJ-like role of CbpA, as inferred by its homology to the well characterized *E. coli* CbpA, we substituted DnaJ with purified His-tagged CbpA, then we performed DNase I footprinting assays with HspR and the DnaK-CbpA-GrpE complex on the labelled Phrc probe. Interestingly, in this case, we observed a negative effect on HspR binding affinity ((23); and data not shown).

In order to assess a possible effect of CbpA on the DNA binding activity of HspR, we performed DNase I footprinting assays on the labelled Phrc probe with purified recombinant HspR and CbpA alone. As shown in Fig. 2 panel a), the addition of increasing amounts of HspR to the labelled probe led to the appearance of DNase I hypersensitive sites (indicated by black arrows) and of regions of protection from enzymatic digestion (indicated by open boxes), due to HspR binding to the DNA probe (Fig. 2a, lanes 1 to 4). This binding site, spanning from position –149 to –78 with respect to the transcription initiation site (+1, bent arrow on the left side of the autoradiograph) is in agreement with previously published data ((29), (23)). The inclusion of a 4-fold molar excess of CbpA in the binding reaction completely abolished HspR binding to DNA (Fig. 2a, lanes 5 to 8). In this case in fact, upon addition of the same HspR concentrations as in lanes 1 to 4, we could not observe the hypersensitive sites and protected regions typical of HspR binding. It is worth noting
that CbpA alone did not bind Phrc probe (lane 5). The same effect was observed by keeping constant, in the reaction, the amount of HspR, and adding increasing concentrations of CbpA. As clearly evident in Fig. 2a (lanes 9 to 12), upon addition of CbpA to the reaction, the HspR binding pattern (i.e. hypersensitive sites) disappears in a dose-dependent manner. This set of experiments suggests that the heat-shock protein CbpA does not directly bind to the Phrc promoter region, but acts by hindering HspR interaction with its operator.

**CbpA does not affect HrcA binding activity.** To assess whether CbpA can also affect HrcA binding, the HspR repressor partner involved in the regulation of Pgro and Phrc promoters (23), we performed *in vitro* binding experiments on the Phrc probe as described above, using the recombinant purified His-tagged HrcA protein with results shown in Fig. 2 (panel b). As shown above (Fig. 2a, lanes 9-12), increasing amounts of CbpA added to the HspR binding reaction to Phrc promoter fragment determined a decreased binding affinity of HspR to DNA. By contrast, in the presence of constant amounts of both heat-shock repressors, HrcA and HspR, increasing concentrations of the CbpA protein determined no changes in the binding and relative affinity of HrcA to its operator (Fig. 2b, lanes 2 to 5), as highlighted by the DNase I hypersensitive site centred at position -41 (23). Moreover, the negative effect of CbpA on HspR binding remained unchanged also in the presence of HrcA (compare lanes 2 to 5 in Fig. 2b and 9 to 12 in Fig. 2a). This result strongly suggests that CbpA has no influence on HrcA binding to its operator on the Phrc promoter. Moreover, CbpA still modulates HspR binding in the presence of HrcA.

**CbpA acts on unbound HspR, preventing its binding to DNA.** In all the binding experiments described above, CbpA and HspR proteins were added at the same time to the probe in the binding reaction. With this approach, we could investigate only the effect of CbpA on HspR before or during its interaction with DNA. To determine if CbpA could act also on HspR already
bound to its operator, we performed a DNase I footprinting assay in which we added the two proteins sequentially (HspR before CbpA and vice versa). Figure 2 panel c) shows the obtained results. In particular, when HspR was added to the binding reaction containing increasing concentrations of CbpA, we observed a decrease in HspR relative binding affinity for the labelled probe (lanes 2 to 5), in agreement with what we had observed before. On the contrary, when CbpA was added after HspR (lanes 6 to 9), the repressor binding activity remained essentially unaltered. In fact, the DNase I hypersensitive sites, typical of HspR binding to *P* *hrc* (indicated by black arrows), were clearly visible also upon addition of an 8-fold molar excess of CbpA to the reaction (Fig. 2c, compare lanes 5 and 9). We conclude that CbpA exerts no effect on HspR once this repressor is bound to its operator site. This is consistent with the hypothesis that CbpA interacts directly with HspR before DNA binding, thus preventing its binding to the *P* *hrc* operator.

**CbpA directly interacts with HspR.** To gain further information on the direct interaction between CbpA and HspR, we performed GST-pulldown assays. To this aim, we expressed and purified the recombinant GST-HspR fusion protein which was incubated with an *H. pylori* (**hspR::kan**) (Table 1) total protein extract. As a control, an identical *H. pylori* protein extract was incubated with a recombinant GST protein. After the recovery of the GST and GST-HspR proteins bound to its interaction partner(s), through a GSH-sepharose slurry, we analysed the samples obtained with an immunoblot assay stained with antibody raised against CbpA. Figure 3 panel a) shows the obtained results. By using the GST-HspR fusion protein as bait, a protein band corresponding to the expected molecular weight of CbpA was detected (lane 4). This CbpA-corresponding band was absent in the control sample in which the GST bait was used (lane 3). To further confirm the direct interaction between CbpA and HspR, we performed another GST-pulldown experiment in which the bait proteins (GST-HspR and the control GST) were incubated with the purified CbpA-His protein (see Materials and Methods for details). Figure 3 panel b)
shows the immunoblot stained with both anti-GST and anti-CbpA antibodies. Also with this approach, it was possible to recover a protein band corresponding to the expected molecular weight of CbpA-His in the GST-HspR bait sample (lane 2, marked with *), while it was barely detectable in the control sample (lane 1). These data strongly support the hypothesis of a direct interaction between the *H. pylori* heat shock repressor HspR and the co-chaperone CbpA.

In vivo CbpA overexpression impairs Phrc transcription decline after heat shock. It is well established that all three HSPs promoters (Pgro, Phrc and Pchp), regulated by HspR alone or in combination with HrcA, exhibit a typical heat shock response when cells are exposed to a temperature of 42°C. Their transcription is strongly and rapidly induced after heat shock and this initial induction phase is followed by an adaptation phase in which mRNA amounts decrease to a new steady state level, slightly higher than non-shocked conditions (28). Considering the in vitro results presented above, we hypothesized that CbpA could play a role in the transcriptional response kinetics following heat shock. Accordingly, we decided to compare the levels of Phrc transcripts at fixed time intervals after a sudden increase in temperature, both in wild-type and CbpA overexpressing *H. pylori* G27 strains. To generate the CbpA overexpressing strain, a copy of the CbpA coding sequence was introduced, together with a chloramphenicol-resistance cassette (cat), in the cagA (HP0547) locus by homologous recombination in the G27 *H. pylori* wild-type strain, thereby placing an additional copy of cbpA gene under the control of the strong and heat shock independent PcagA promoter, generating strain G27 (PcagA-cbpA). Total RNA was isolated from *H. pylori* G27 and G27 (PcagA-cbpA) cells grown at 37°C and at different time points after upshift of the culture to 42°C, and subjected to primer extension analysis. Reactions were fractionated on a denaturing polyacrylamide gel and bands were quantified by exposure of the gel to a Phosphorimager. The result of a representative primer extension experiment is reported in Figure 4, panel a). In both wild type and CbpA overexpressing strains, the amount of Phrc transcript
increased rapidly after temperature upshift and reached maximum level after 30-45 minutes. It is worth noting that the transcript levels before heat shock (time 0, physiological growth condition, lane 1) were the same in both strains. Moreover, it is clear in Fig. 4a and b, that the induction kinetics and the maximum transcript levels were very similar in both strains (7-fold increase at 45 min in both strains, lane 5). On the contrary, the adaptation phase that follows the transcript induction peak is different in the two cases. While in the G27 wild type strain the Phrc transcript amount decreased slowly and reached a level of 2-fold higher the unshocked cells after 120 min (lane 8), for the CbpA overexpressing strain the Phrc transcript amount did not decline but remained essentially at the maximum level throughout the analysed time window. These data suggest that CbpA plays a role in the transcriptional shutoff of the Phrc promoter transcript after heat shock induction.

We used the same RNA samples in dot blot experiments to investigated also the heat shock response of the Pcbp promoter controlling cbpA-hspR-helicase operon transcription that is negatively regulated solely by HspR (27). As shown in Figure 4c, we obtained similar results as for Phrc. The CbpA overexpressing strain showed an impaired transcriptional decline phase after heat shock transcription induction. Therefore, we conclude that the H. pylori CbpA chaperone is involved in the transcriptional shut off response after a heat shock. It interacts with HspR, downregulating its DNA binding affinity, in order to modulate the response kinetics that restores the repression of HSP-encoding genes.
DISCUSSION

Microorganisms adopt several strategies to survive and adapt to sudden changes of the environmental conditions they experience during growth. One of the most important of them relies on the coordinated expression of a conserved set of chaperone proteins that protect cells from damages caused by accumulation and aggregation of misfolded polypeptides. Complex mechanisms of regulation of heat shock proteins’ expression, both at the transcriptional and at the post-transcriptional level, are commonly exploited by bacteria to rapidly respond to changing growth conditions. The *H. pylori* heat-shock proteins’ regulatory circuit is governed by the concerted action of two repressors, HrcA and HspR, wired in a what appears a neat example of inocherent feedforward loop (FFL), that controls transcription of the major chaperones in response to stress stimuli (9). We have previously demonstrated that the DNA binding activity of HrcA is modulated by the chaperonine GroE, thereby adding a post-transcriptional level of feedback control to the circuit (23).

In this work we show that also CbpA takes part in the control of HSPs expression. Our data indicate that CbpA negatively modulates *in vitro* HspR binding to Phrc and Pcbp promoters through a direct interaction with the repressor, without contacting DNA. The repressor partner HrcA, on the contrary, appears to be completely unaffected by CbpA modulation. Due to sequence homology to CbpA of *E. coli*, the *H. pylori* HP1024 gene was annotated as *cbpA* and it has always been considered as a co-chaperone DnaJ-like heat shock protein. In *E. coli*, in fact, CbpA acts as a DnaJ-like co-chaperone: it can assist protein folding and, under some *in vivo* conditions, it can suppress the phenotypes of a *dnaJ* deletion strain ((30), (31)). *E. coli* and *H. pylori* CbpA proteins share an overall 30% aminoacid identity, reaching a value of 43% in the N-terminal portion, which harbours the so-called J domain mediating its interaction with DnaK. Whether CbpA could exert a similar...
function also in *H. pylori* has not been investigated yet. However, in this study, we report a novel function for *H. pylori* CbpA in the regulation of HSP gene expression. Like GroE that, besides its general and well characterized role in protein quality control, is involved in different organisms in the modulation of regulators’ activity (*B. subtilis*, *Chlamydia trachomatis*, *H. pylori*), we show that *H. pylori* CbpA is involved in a feedback negative control of the HspR repressor DNA binding activity. Moreover, the data presented in this study support a model in which CbpA interacts with HspR only when the repressor is not bound to its operators, thereby preventing its immediate re-binding to DNA and repression of transcription. Hence, the effect of CbpA modulation of HspR would reflect on the kinetic of heat shock response, slowing down the shut-off phase that follows a stress mediated transcription induction. Considering that, the presence of both regulators, HspR and HrcA, is necessary for maintaining the co-regulated promoters P*gro* and P*hrc* in the repressed state (27), CbpA modulation of HspR binding activity affects the whole regulation of the major HSPs expression. A recent paper by Elsholz and co-workers (12) describes heat shock gene regulation in low GC Gram-positive bacteria and proposes a model in which the heat shock master regulator CtsR, upon heat stress, loses ability of DNA binding and, only in this un-bound state, it is captured by a kinase protein called McsB that delivers the repressor toward protease-mediated degradation and thereby prevents its immediate re-binding to DNA. Even if we still know very little about the exact mechanism in *H. pylori*, from a functional point of view, the control of CtsR activity and availability after a stress stimulus parallels well with the CbpA control of the HspR repressor. Considering these observations, it would be interesting to investigate and characterize more in detail if also in *H. pylori*, after heat shock, HspR is delivered to controlled protease-mediated degradation and if the CbpA-HspR interaction/complex formation could be a first indispensable pre-requisite for this process.

The findings we present in this work allow to draw a more detailed picture of the complex *H. pylori* heat shock regulatory network. As schematically summarized in Figure 5, while HrcA
activity is positively controlled by the action of GroE (23), HspR binding activity is inhibited in solution soon after heat shock de-repression by its interaction with CbpA, thus preventing its repressive state.

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REFERENCES


FIGURE LEGENDS

FIG. 1. Schematic representation of the three heat shock operones containing the major \textit{H. pylori} HSP and their transcriptional regulation exerted by HrcA and HspR (23).

FIG. 2. DNase I footprintings on the Phrc promoter with CbpA, HspR and HrcA His-tagged recombinant proteins. Radiolabeled Phrc DNA probe was mixed with different amounts and combinations of purified proteins, subjected to DNase I digestion, DNA fragments were separated on a polyacrilamide denaturing gel, and autoradiographed. Panel a), the labeled probe was mixed with 0, 14, 28 and 56 nM HspR in absence (lanes 1 to 4) or in presence of 220 nM CbpA (lanes 4 to 8). In lanes 9 to 12, the Phrc probe was mixed with 0, 28, 110 and 220 nM CbpA together with 28 nM HspR. Panel b), lane 1: no protein added; lanes 2 to 5: same concentrations of CbpA and HspR added as in lanes 9 to 12, panel a), in presence of 200 nM of HrcA protein. Panel c), lane 1: no protein added. Lanes 2 to 5: the Phrc probe was mixed with 0, 28, 110 and 220 nM CbpA together with 28 nM HspR. Lanes 6 to 9: the labeled probe was incubated with 28 nM HspR for 10 min, then increasing amounts of CbpA (as in lanes 2 to 5) were added to the reactions and incubated for additional 7 min prior to DNase I digestion. White open boxes on the right of the autoradiographic film represent the regions of DNase I protection, the black arrowheads indicate bands of hypersensitivity to DNase I digestion. On the left of each panel, the -10 and -35 regions and the transcriptional start site (+1, bent arrow) are indicated, and the open reading frames are indicated by vertical open arrows.

FIG. 3. GST pull-down assays performed with \textit{H. pylori} total protein extracts (panel a) and with purified recombinant proteins (panel b). Panel a), western blot analysis of samples of the GST pull-down assay performed with \textit{H. pylori} total protein extract, stained with an anti-CbpA antibody.
Samples collected from the column containing GST- (lane 3) and GST-HspR-GSH-Sepharose slurry (lane 4), were separated by SDS-PAGE and blotted on a nylon membrane, together with 20 ng of purified His-tagged CbpA, used as positive control (lane 2), and a molecular weigh ladder containing a 50 kDa stained band (lane 1). The bands corresponding to the recombinant His-tagged CbpA (*) and to the endogenous protein (**) are indicated on the right. Panel b), western blot analysis of samples of the GST pull-down assay performed with recombinant purified CbpA-His, stained with anti-CbpA and anti-GST antibodies. Samples collected from the column containing GST- (lane 1) and GST-HspR-GSH-Sepharose slurry (lane 2), were separated by SDS-PAGE and blotted on a nylon membrane. The bands corresponding to recombinant His-tagged CbpA (*), to the GST (**), and to the GST-HspR protein (+) are indicated on the right. For both panels, the positions of the molecular weight standards are shown to the left.

**FIG. 4** RNA analyses of P_{hrc} and P_{cbp}. Wild type and CbpA overexpressing H. pylori cultures were heat shocked at 42°C for different periods of time and total RNA was extracted and used in primer extension analysis and dot blot hybridization. Panel a), the labeled cDNA generated by primer extension using oligonucleotide hrcA (Table 2) were separated by electrophoresis and autoradiographed. The positions of elongated products are indicated on the left by arrows, while the H. pylori strains are shown on the right; the heat shock time points are reported on the top of each lane. This experiment is representative of the four different assays. Panel b), graph representing phorphirimager quantification of the elongated products of four independent experiments. Mean and standard deviation are reported for each time-point of the assay. Panel c), RNA dot blot hybridization analysis of P_{cbp} transcripts. Total RNA was immobilized on a nylon membrane and hybridized with the labeled cbpe4 oligonucleotide (Table 2); then the filter was washed and autoradiographed. The H. pylori strains are shown on the right; the heat shock time points are reported on the top of each sample.
FIG. 5. A model depicting the regulation of *H. pylori* chaperones’ expression. Transcriptional repression of the heat-shock operons is represented by solid lines that connect HrcA and HspR repressor proteins to their target promoters (Pgro, Phrc and Pcbp); dashed lines, linking GroES-GroEL to HrcA and CbpA to HspR, represent the post-transcriptional protein-protein feedback control of the regulators. Arrowheads indicate positive regulation, while hammerheads symbolize negative regulation.
<table>
<thead>
<tr>
<th>Strains or plasmids</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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</thead>
<tbody>
<tr>
<td><strong>Bacterial strains</strong></td>
<td></td>
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<td>E. coli DH5α</td>
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<td>H. pylori G27</td>
<td>Clinical isolate; wild type</td>
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<td>H. pylori G27 ([hspR::kan])</td>
<td>G27 derivative; bp 66 to 334 of the hspR coding sequence have been replaced by a kan cassette.</td>
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<td>H. pylori G27 ([PcagA-cbpA])</td>
<td>G27 derivative; CbpA overexpressing strain obtained by double homologous recombination of pSL1190-cagcbp1/2-CbpA-Cm-cagcbp3/4. This strain harbor an additional copy of cbpA under the control of the cagA promoter in the cagA locus.</td>
<td>This work</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pGEM-T-Easy</td>
<td>Cloning vector for PCR products; Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Promega</td>
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<td>pGEM-T-Easy-P&lt;sub&gt;hrc&lt;/sub&gt;</td>
<td>pGEM-T-Easy derivative, containing a 308-bp PCR fragment (oligonucleotides hrcA and hrcA1) encompassing the P&lt;sub&gt;hrc&lt;/sub&gt; promoter.</td>
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<td>pET22-CbpA</td>
<td>pET22b derivative, containing the CbpA coding sequence amplified by PCR with oligonucleotides cbpAN/cbpAC on chromosomal DNA of H. pylori, digested with NdeI/Xhol restriction enzymes.</td>
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<td>pSL1190</td>
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</tbody>
</table>
Heat Shock Time Course

mRNA accumulation (A.U.)

minutes at 42°C

wild type
P_{cbrA-cbrA}

b)