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Transcriptional Regulation of Stress Response and Motility Functions in *Helicobacter pylori* Is Mediated by HspR and HrcA▽

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The *hrcA* and *hspR* genes of *Helicobacter pylori* encode two transcriptional repressor proteins that negatively regulate expression of the *groES-groEL* and *hrcA-grpE-dnaK* operons. While HspR was previously shown to bind far upstream of the promoters transcribing these operons, the binding sites of HrcA were not identified. Here, we demonstrate by footprinting analysis that HrcA binds to operator elements similar to the so-called CIRCE sequences overlapping both promoters. Binding of HspR and HrcA to their respective operators occurs in an independent manner, but the DNA binding activity of HrcA is increased in the presence of GroESL, suggesting that the GroE chaperonin system corepresses transcription together with HrcA. Comparative transcriptome analysis of the wild-type strain and *hspR* and *hrcA* singly and doubly deficient strains revealed that a set of 14 genes is negatively regulated by the action of one or both regulators, while a set of 29 genes is positively regulated. While both positive and negative regulation of transcription by HspR and/or HrcA could be confirmed by RNA primer extension analyses for two representative genes, binding of either regulator to the promoters could not be detected, indicating that transcriptional regulation at these promoters involves indirect mechanisms. Strikingly, 14 of the 29 genes which were found to be positively regulated by HspR or HrcA code for proteins involved in flagellar biosynthesis. Accordingly, loss of motility functions was observed for HspR and HrcA single or double mutants. The possible regulatory intersections of the heat shock response and flagellar assembly are discussed.

The heat shock proteins of the gastric pathogen *Helicobacter pylori* have been studied in some detail both because of their general role in protection of the bacteria from the hostile environment of the human stomach and because of their involvement in specific pathogenic processes. The *H. pylori* GroEL homologue (18, 32) has been proposed to play a role in regulating the activity of the nickel-dependent urease enzyme, which generates ammonia ions from the hydrolysis of urea and therefore protects the bacterium from the low pH of the stomach lumen (8, 10). Its cochaperone, GroES, is thought to contribute to this regulation by controlling the availability of nickel ions by means of its intrinsic metal binding activity (17, 32). Although relatively controversial, it has been reported that GroEL, as well as another major heat shock protein, DnaK (Hsp70), can be found in association with the outer membrane, and this surface localization has been suggested to modify the glycolipid binding specificity of *H. pylori* cells at low pH (9, 16, 23).

Because of their functions in the general stress response as well as in specific pathogenic mechanisms, the *H. pylori* heat shock proteins are expected to be tightly regulated in the level of expression. We have previously demonstrated that transcription of the *groESL*, *hrcA-grpE-dnaK*, and *cbpA-hspR-orf* operons encoding the major chaperones of *H. pylori* is negatively regulated by the HspR and/or HrcA repressor protein (28, 31). HspR is a homologue of the repressor of the *dnaK* operon of *Streptomyces coelicolor* that has been shown to bind to inverted repeats in the promoter region designated HAIR (HspR-associated inverted repeat) (4, 12). HrcA is a homologue of the repressor of a set of heat shock genes of *Bacillus subtilis* that binds to an inverted repeat in the promoter region designated CIRCE (controlling inverted repeat of chaperone expression) (20, 27, 37). While HrcA is widely distributed in the prokaryotic kingdom, HspR is found in a restricted number of bacteria (20). Genetic and biochemical studies with different microorganisms have revealed that the chaperone systems directly influence the transcriptional control exerted by HspR and HrcA. For example, in the pathogenic bacterium *Chlamydia trachomatis*, the GroEL protein is able to increase the ability of HrcA to bind to the CIRCE element and to repress transcription (35). In *B. subtilis* the activity of the HrcA repressor is modulated by the GroE chaperonin system (19, 24). Furthermore, detailed in vitro and in vivo studies have provided evidence that DnaK functions as a transcriptional corepressor by binding to HspR at its operator sites in *S. coelicolor* (2, 3).

In *H. pylori*, HspR alone represses transcription of the *cbpA-hspR*-helicase operon, while both HspR and HrcA regulators are required to repress transcription of the *hrcA-grpE-dnaK* and *groES-groEL* heat shock operons (28, 31). Whether HrcA and HspR control transcription of additional genes is unknown. Initial studies indicated that while transcription of the *groESL* and *cbpA-hspR-orf* operons was strongly inducible by treatment with 300 mM NaCl, no induction was observed when cultures were incubated at 45°C (31). Subsequently, Homouth and coworkers (15) showed that transcription of these operons is strongly inducible by a mild heat shock at 42°C, suggesting that HspR can indeed mediate the transcriptional response to a sudden temperature increase, characterized by fast and

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference(s) or source
Strains		
E. coli DH5α	*supE44* Δ*lacU169* (ϕ80*lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*β	14
E. coli BL21(DE3)	*hsdS gal* (λc*Its*857 *ind1 Sam7 nin5 lacUV5-T7 gene 1*)	13
H. pylori G27	Clinical isolate; wild type	36
H. pylori G27(*hrcA*::*km*)	G27 derivative; bp 156 to 375 of *hrcA* replaced by a *km* cassette	28
H. pylori G27(*hspR*::*km*)	G27 derivative; bp 66 to 334 of the *hspR* coding sequence replaced by a *km* cassette	28
H. pylori G27(*hrcA*::*km hspR*::*cm*)	G27(*hrcA*::Km) derivative; bp 66 to 334 of the *hspR* coding sequence replaced by a *cat* cassette	28
H. pylori G27(*hrcA*-HA)	G27(*hrcA*::Km) derivative; *hrcA*-HA complementing strain obtained by double homologous recombination of pVAC-*cat-hrcA*-HA	25, 28
Plasmids		
pGEM-T Easy	Cloning vector, Amp^r	Promega
pGEM-T-Easy-P*hrc*	pGEM-T Easy derivative containing a 308-bp PCR fragment (oligonucleotides hrcA and hrcA1) encompassing the P_{hrc} promoter	25
pGEM-T-Easy-P*gro*	pGEM-T Easy derivative containing a 294-bp PCR fragment (oligonucleotides gro1 and groFP) encompassing the P_{gro} promoter	This study
pGEM-T-Easy-P*mda66*	pGEM-T Easy derivative containing a 250-bp PCR fragment (oligonucleotides mda66PE and mda66rev2) encompassing the P_{mda66} promoter	This study
pGEM-T-Easy-P*flaB*	pGEM-T Easy derivative containing a 323-bp PCR fragment (oligonucleotides fla and fla2) encompassing the P_{flaB} promoter	30
pET22b	Expression vector allowing C-terminal histidine-tagged gene fusion; Amp^r	Novagen
pET22b-GroEL	pET22b derivative containing the *groEL* coding sequence amplified by PCR with oligonucleotides groEL-fwd and groEL-rev on chromosomal DNA of *H. pylori*, digested with restriction enzymes NdeI and XhoI	This study
pET22b-GroES	pET22b derivative containing the *groES* coding sequence amplified by PCR with oligonucleotides groES-fwd and groES-rev on chromosomal DNA of *H. pylori*, digested with restriction enzymes NdeI and XhoI	This study

[a] See Table 2 for oligonucleotide sequences.

strong induction of transcription and a subsequent shutoff phase, whose onset is determined largely by the stability of the respective mRNAs (29). Until recently, the study of HrcA-dependent regulation was hampered by difficulties in purifying a recombinant protein to map the HrcA binding sites on the promoters (25, 31). Thus, so far the interplay between HspR and HrcA at the level of the coregulated promoters, as well as the possible involvement of chaperone proteins, has not been explored. Specifically, HrcA localizes in the inner membrane of *H. pylori* and shows toxic or insoluble properties when it is expressed in *Escherichia coli* (25). However, these effects could be alleviated by expression at 42°C, allowing purification of a recombinant protein suitable for biochemical analyses (25). In the present study we determined the binding sites of HrcA on the coregulated promoters by performing footprinting experiments and showed that under in vitro conditions the binding of HspR and HrcA occurs in an independent manner. The genome-wide regulatory functions of HspR and HrcA were further investigated by transcriptome and phenotypic trait analysis of singly or doubly deficient strains. The results indicate that there is an intimate, although indirect, interconnection between the stress response and motility functions in *H. pylori*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *H. pylori* strains (Table 1) were recovered from frozen stocks by growth on Columbia agar plates containing 5% horse blood, 0.2% cycloheximide, and Dent's or Skirrow's antibiotic supplement (Oxoid) for 2 to 3 days. After passage on fresh plates, bacteria were cultured in a 9% CO_2–91% air atmosphere at 37°C and 95% humidity. Liquid cultures were grown in modified brucella broth supplemented with 5% fetal calf serum, 0.2% cycloheximide, and Dent's or Skirrow's antibiotic supplement. Motility of *H. pylori* strains was assayed by stab inoculating bacteria with a pipette tip into 0.3% agar plates containing brucella broth supplemented with 10% fetal calf serum and Dent's or Skirrow's antibiotic supplement and culturing them as described above.

DNA techniques. DNA manipulations were performed routinely as described by Sambrook and colleagues (26). All restriction and modification enzymes were used according to the manufacturer's instructions (New England Biolabs). Nucleic acid purification was carried with QIAGEN kits (QIAGEN, Inc.). PCRs were carried out using 50 ng of *H. pylori* chromosomal DNA, 40 pmol of each specific primer, and *Taq* DNA polymerase (New England Biolabs) in a 50-μl (final volume) mixture containing 200 μM of each deoxynucleotide in 1× PCR buffer containing Mg^{2+}. A total of 33 cycles consisting of denaturation of the DNA at 95°C for 1 min, annealing at the appropriate temperature for 1 min, and extension at 72°C for 1 min were performed.

RNA preparation. Total *H. pylori* RNA was extracted using a hot-phenol extraction procedure described previously (6). Briefly, 10 ml of exponentially growing cells was added to 1.25 ml of ice-cold ethanol-phenol stop solution (5% water-saturated phenol [pH < 7.0] in ethanol), harvested, and resuspended in 800 μl of a 0.5-mg/ml lysozyme solution in Tris-EDTA (10 mM Tris, 1 mM EDTA; pH 8.0). Then 50 μl of 10% sodium dodecyl sulfate was added, and samples were incubated for 2 min at 64°C. After incubation, 88 μl of 1 M sodium acetate (pH 5.2) and 1 ml of water-saturated phenol (pH < 7.0) were added, and samples were incubated at 64°C for 6 min with occasional shaking on ice for 5 min, spun at 13,000 × *g* at 4°C for 10 min, extracted with 1 volume of chloroform, ethanol precipitated, and stored at −80°C. Prior to use, an aliquot of RNA samples was collected by centrifugation, quantified, and loaded on a 1% agarose gel to assess RNA purity and integrity.

Transcriptome analysis. Prior to reverse transcription, RNA samples were treated with 1 U/μg RQ1 RNase-free DNase (Promega) at 37°C for 30 min, phenol-chloroform extracted, and ethanol precipitated. cDNA synthesis and labeling were carried out with a thermal cycler by combining 25 to 50 μg RNA with 150 pmol random hexamers (Invitrogen) in 28-μl reaction mixtures. After

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′–3′)[a]	Restriction recognition site
hrc1	ATTATTGAATTCTTGGGTTAGGGGGATTTTAAGGG	EcoRI
hrcA	CAAACGCATCTAACAAACTCTC	None
gro1	ATTATTGGATCCAGGGATGATGATGCCTGAACTGG	BamHI
groFP	ATAAGGTTTGTTAATAACGCCCCTTTCTC	None
groEL-fwd	ATTACATACCATATGGCAAAAGAAATCAAATTTTC	NdeI
groEL-rev	ATATATCTCGAGCATCATGCCACCCATGCCTC	XhoI
groES-fwd	ATTACATACCATATGAAGTTTCAGCCATTAGGAG	NdeI
groES-rev	ATATATCTCGAGGTGTTTTTTGTGATCATGAC	XhoI
mda66PE	TGGTCAGTCAAGGTTTCATTG	None
mda66rev2	ATCGTAGAACATGACCACTCCTTA	None
fla	GCATGAGAAGTTAAAGCGGC	None
fla2	ATTATAGAATTCCCTAACATGCCCTTTAGAGGC	EcoRI

[a] Restriction sites added for cloning purposes are underlined.

denaturation for 3 min at 94°C and annealing for 5 min at 37°C, 22 μl of a reverse transcriptase labeling mixture containing 25 U of avian myeloblastosis virus reverse transcriptase (Promega), [α-^{33}P]dATP (2,500 Ci/mmol; Amersham), and 80 U of the RNase inhibitor RNasin (Promega) was added and reverse transcribed at 42°C for 3 h. The reaction was stopped by addition of 2 μl of 0.5 M EDTA, and RNA was degraded by alkaline treatment with 0.15 N NaOH for 15 min at 37°C and then neutralized with 17.5 μl of 1 M Tris-Cl (pH 7.5). cDNA was purified from unincorporated radioactive nucleotides using Chromaspin-TE10 spin columns (Clontech) and was hybridized to *H. pylori* Panorama open reading frame arrays (Sigma-Genosys) according to the manufacturer's instructions. Images were acquired with a Storm phosphorimager (Molecular Dynamics). RNAs were extracted from two independent cultures. Spot intensities on arrays were quantified with ImageQuant 5.2 software (Molecular Dynamics), processed with Microsoft Excel, and normalized by expressing values as percentages of the total gene specific intensity. To avoid background noise, spots with levels of intensity of <0.005% were not considered. For data analysis the statistical significance of expression ratios was determined by running the Web-based Cyber-T application program (http://visitor.ics.uci.edu/genex/cybert), which was specifically developed for array data analysis, based on a Bayesian probabilistic framework. In particular, given our sample size of 1,671 genes, we adjusted the sliding window size to 81 and determined a Bayesian confidence estimate value corresponding to three times the number of experimental replicates. These settings were previously shown to suit transcriptome analysis using macroarrays in *H. pylori* (6). Genes with mutant strain/wild-type strain expression ratios of ≥1.5 or ≤−1.5 and Bayesian *P* values of ≤0.05 were considered to be significantly deregulated.

Primer extension analysis. Transcription from the P_{mda66} and P_{flaB} promoters was assayed by primer extension analysis using oligonucleotides mda66PE and fla, respectively (Table 2). An oligonucleotide (5 pmol) was 5′ end labeled in the presence of [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham) and T4 polynucleotide kinase. The labeled oligonucleotide (0.4 to 1.0 pmol) was coprecipitated with 15 μg of *H. pylori* total RNA and resuspended in 7.5 μl of H_2O, 3.5 μl of 2 mM deoxynucleoside triphosphates, and 3 μl of 5× reverse transcription buffer (Promega). The reaction mixtures were incubated for 3 min at 95°C and for 1 min at 42°C, and then 1 μl of avian myeloblastosis virus reverse transcriptase (10 U/μl; Promega) was added to each sample and reverse transcription was carried out by incubating the samples at 42°C for 45 min. Samples were incubated for 10 min at room temperature with 1 μl of RNase A (1 mg/ml), extracted with phenol-chloroform (1:1), ethanol precipitated, and resuspended in 10 μl of sequencing loading buffer. After denaturation at 95°C for 2 min, samples were subjected to 6% urea–polyacrylamide gel electrophoresis and autoradiographed. To map the P_{mda66} promoter, plasmid pGEM-T-Easy-P*mda66* (Table 1) was sequenced in parallel with oligonucleotide mda66PE, using a T7 sequencing kit (USB).

Overexpression and purification of recombinant proteins. His_6-tagged recombinant HrcA and HspR proteins were overexpressed in freshly transformed *E. coli* BL21(DE3) cells and affinity purified as previously described (25, 31). For overexpression of His_6-tagged recombinant GroES and GroEL proteins, the expression vectors pET22b-GroES and pET22b-GroEL (Table 1) were transformed separately into *E. coli* strain BL21(DE3). Overnight bacterial cultures were diluted 1:50 in 250 ml of LB medium, grown to an optical density at 600 nm of 0.5, induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 37°C, and then centrifuged. Cells were resuspended in 25 ml of ice-cold lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole; pH 8.0), incubated with 1 mg/ml lysozyme at 4°C for 30 min on a Tilt-Roll, disrupted with a French pressure cell (two cycles), incubated on ice for 15 min with 10 μg/ml of DNase I and 10 μg/ml of RNase A, and centrifuged to remove cellular debris (6,000 × *g*, 30 min, 4°C). The soluble fractions were mixed with 750 μl of 50% Ni^{2+}-nitrilotriacetic acid slurry (QIAGEN, Inc.) and incubated for 90 min at 4°C on a Tilt-Roll. Two 10-ml polypropylene columns were then packed with the samples and washed twice with 7.5 ml of wash buffer 20 (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole; pH 8.0) and once with 7.5 ml of wash buffer 50 (50 mM NaH_2PO_4, 300 mM NaCl, 50 mM imidazole; pH 8.0). Recombinant proteins were eluted by applying 1 ml of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole; pH 8.0) three times, dialyzed against storage buffer (50 mM Tris [pH 8.0], 50 mM KCl, 10 mM $MgCl_2$, 0.01% NP-40 Igepal) containing 50% glycerol, and stored at −20°C.

DNase I footprinting. The promoter regions of the *groESL*, *hrcA-grpE-dnaK*, *mda66*, and *flaB* genes were PCR amplified with oligonucleotide pairs gro1/groFP, hrcA/hrcA1, mda66PE/mda66rev2, and fla2/fla (Table 2), respectively, from chromosomal DNA of *H. pylori* G27 and cloned into the pGEM-T Easy vector, resulting in the plasmids listed in Table 1. Promoter DNA fragments obtained by NcoI (for P_{gro}, P_{mda66}, and P_{flaB}) or SalI (for P_{hrc}) digestion were 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase at one extremity and gel purified, and approximately 10,000 cpm of each probe was used for footprinting experiments. Labeled DNA probes were incubated with a purified protein(s) in 50 μl of footprinting buffer (10 mM Tris-HCl [pH 8.0], 50 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 50 mM dithiothreitol, 0.01% NP-40, 10% glycerol) containing 250 ng of sonicated salmon sperm DNA as a nonspecific competitor for 15 min at room temperature. Two microliters of DNase I (0.01 U/μl), freshly diluted in footprinting buffer containing 5 mM $CaCl_2$, was added, and incubation was continued for 75 s at room temperature. DNase I digestion was stopped by addition of 140 μl of stop buffer (192 mM sodium acetate, 32 mM EDTA, 0.14% sodium dodecyl sulfate, 64 μg/ml sonicated salmon sperm DNA). Samples were phenol-chloroform extracted, ethanol precipitated, resuspended in 10 μl of sequencing loading buffer, denatured at 95°C for 2 min, subjected to 6% polyacrylamide–urea gel electrophoresis, and autoradiographed.

RESULTS

HrcA binds CIRCE-like sequences mapping within the P_{gro} and P_{hrc} promoter regions. The structural organization of the three HspR-regulated operons and the respective promoters is schematically depicted in Fig. 1. While transcription of the P_{cbp} promoter is controlled solely by HspR, transcriptional repression of P_{gro} and P_{hrc} requires both the HspR and HrcA repressors (28). In vitro DNase I footprinting experiments with a purified recombinant protein allowed identification of the HspR binding sites within the three promoters (31). In contrast, the binding sites of the HrcA protein have not yet been determined, due to difficulties in obtaining a recombinant purified protein. However, very recently we purified a recombi-

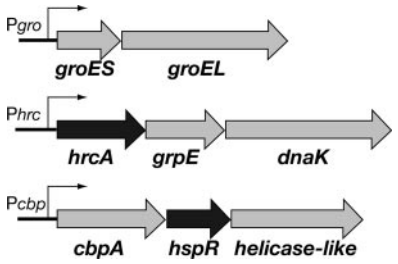

FIG. 1. Structural organization of *H. pylori* chaperone genes. The gray arrows indicate chaperone genes and an open reading frame with a putative helicase-like function, and the black arrows indicate regulatory genes. All genes are labeled based on the genome sequence published by Tomb et al. (33). P_{gro} transcribes one bicistronic mRNA encoding the GroESL chaperonin machinery (32); P_{hrc} transcribes a tricistronic mRNA encoding the membrane-associated repressor HrcA, the chaperone DnaK, and its cochaperone GrpE (15, 25, 31); and P_{cbp} transcribes a tricistronic mRNA encoding the DnaJ homologue CbpA (34), the HspR repressor, and a putative DNA helicase (4, 31).

nant HrcA protein from heat-shocked *E. coli* cells and used it in filter binding assays (25).

To define the architectural organization of the HrcA and HspR binding sites at coregulated promoters, we purified both recombinant His_6-tagged proteins from *E. coli* as previously described (25, 31) and used them in DNase I footprinting assays for the P_{gro} and P_{hrc} promoters. Figure 2 shows that after addition of HspR (20 nM), large protected regions and bands of enhanced DNase I sensitivity appeared for the P_{gro} (Fig. 2A, lane 4) and P_{hrc} (Fig. 2C, lane 4) promoter probes. In accordance with previous observations (31), the protected regions extended from position −43 to position −120 and from position −78 to position −149 with respect to the transcriptional start sites of the P_{gro} and P_{hrc} promoters, respectively. In contrast, in the presence of HrcA (at a concentration of 18 nM or higher), three bands of DNase I hypersensitivity and proximal regions of weak protection were detected for the P_{gro} promoter probe (Fig. 2B, lanes 4 to 8). While two bands of DNase I hypersensitivity mapped within the area of protection spanning from position −13 to position 16, the other band mapped at position 58 with respect to the transcriptional start site (Fig. 2B). Addition of HrcA to the P_{hrc} promoter probe resulted in a single band of DNase I hypersensitivity in the middle of a protected region spanning from position −34 to position −59 (Fig. 2D, lanes 4 to 8).

Figure 3 shows the sequences of the DNA regions that are protected from DNase I digestion by HspR and HrcA and the positions of the DNase I-hypersensitive sites on the two promoters. The HrcA binding sites map to regions with sequence similarity to the *B. subtilis* CIRCE consensus motif (TTAGC ACTC-N9-GAGTGCTAA) proposed by Narberhaus and Bahl (21). Sequences with similarities to the HAIR consensus motif were found in the middle of the HspR binding regions (2, 7, 11). We thus concluded that while the HspR repressor binds upstream of the P_{gro} and P_{hrc} promoter elements, the HrcA regulator binds to regions overlapping the corresponding −10 and −35 hexamers. Most likely, HrcA represses transcription by interfering directly with the binding of RNA polymerase to these promoter elements. Notably, the distances between the

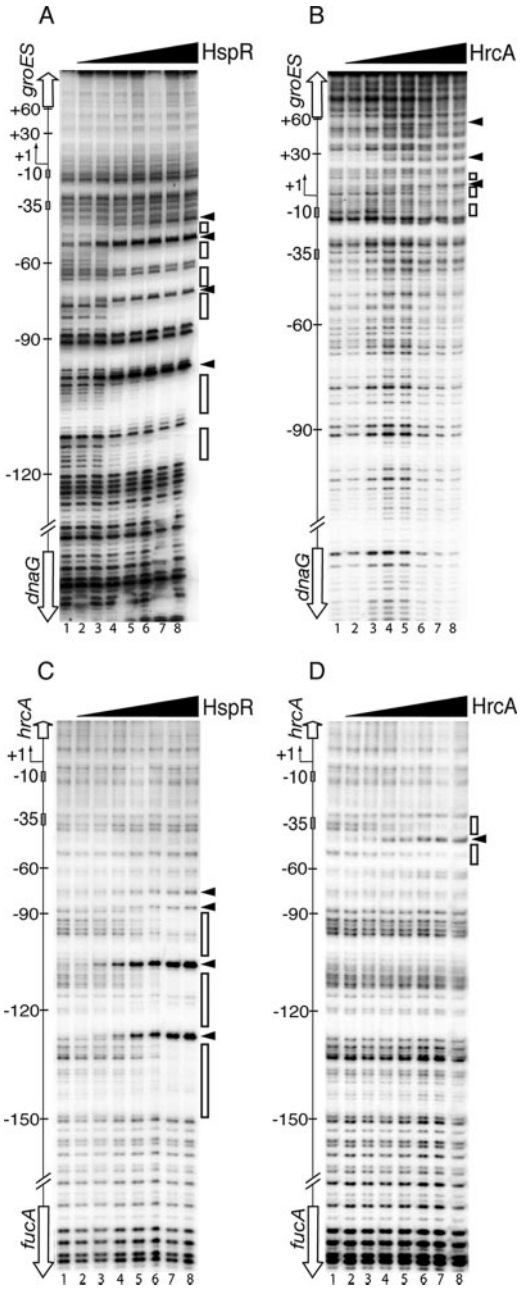

FIG. 2. DNase I footprinting of HspR and HrcA on P_{gro} and P_{hrc} promoters. Specific DNA probes for P_{gro} (A and B) and P_{hrc} (C and D) fragments, end labeled in their noncoding strands, were incubated with increasing amounts of recombinant HspR and HrcA proteins. (A and C) Lanes 1 to 8, 0, 10, 15, 20, 30, 40, 60, and 100 nM HspR added, respectively. (B) Lanes 1 to 8, 0, 5, 10, 20, 30, 60, 90, and 180 nM HrcA added, respectively. (D) Lanes 1 to 8, 0, 1.5, 3, 6, 9, 12, 18, and 24 nM HrcA added, respectively. The open boxes on the right indicate the regions of DNase I protection, while the arrowheads indicate bands of hypersensitivity to DNase I digestion. On the left in each panel, the −10 and −35 regions and the transcriptional start site (bent arrow) are indicated, and the open reading frames are indicated by vertical open arrows. Higher-resolution mapping of HrcA binding at the P_{gro} promoter was carried out by using the same probe end labeled at the opposite extremity (data not shown).

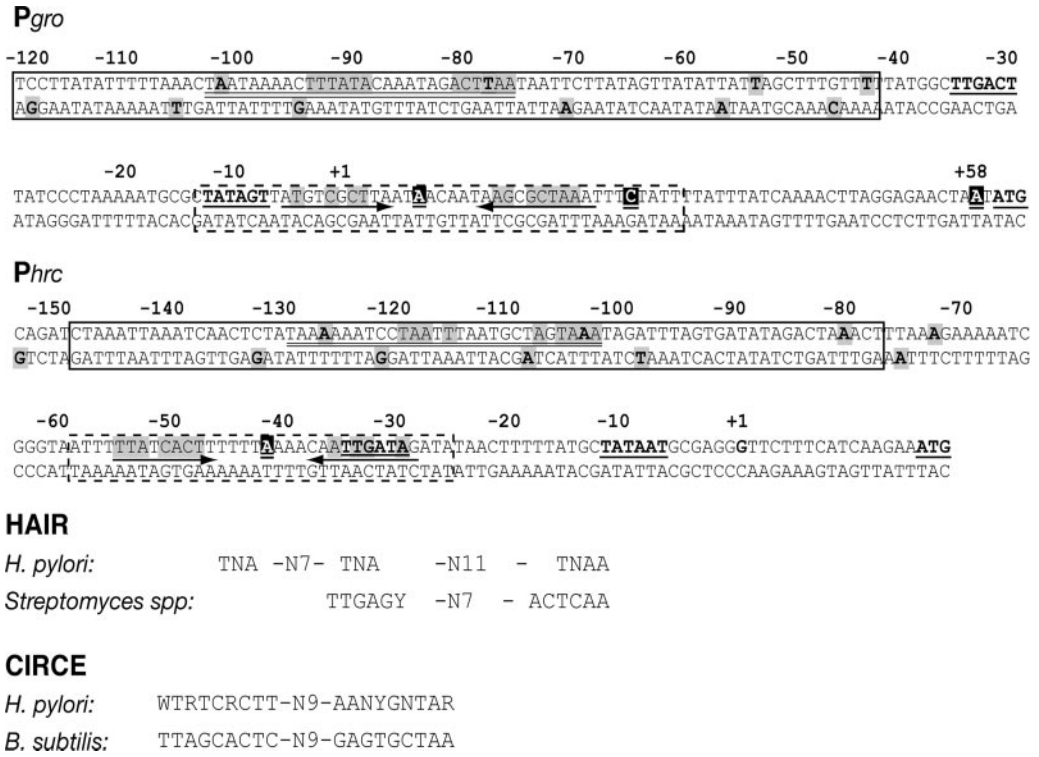

FIG. 3. Features of the P_{gro} and P_{hrc} promoter sequences. For each promoter, the numbers refer to the positions with respect to the transcriptional start site (position 1), and the −10 and −35 promoter elements are in boldface type and underlined. HspR and HrcA binding sites in the P_{gro} and P_{hrc} promoters are enclosed in boxes with solid and dashed lines, respectively. The shaded boldface type indicates sites of hypersensitivity to DNase I digestion after binding of HspR on the coding (Fig. 2) and noncoding DNA strands (31). Sites of DNase I hypersensitivity after binding of HrcA are indicated by a black background (Fig. 2). Known HAIR and CIRCE-like sequences are shown, and nucleotide similarities in the P_{gro} and P_{hrc} promoters are shaded and double underlined and are indicated by converging arrows, respectively. At the bottom, the consensus sequences of HAIR and CIRCE elements are compared. The *H. pylori* CIRCE consensus sequence has been defined by alignment of the two HrcA binding sites on P_{gro} and P_{hrc}.

HspR and HrcA binding sites on the P_{gro} and P_{hrc} promoters are 27 and 18 bp, respectively.

To study possible interactions of HspR and HrcA, we assayed the DNA binding activities of both proteins under competitive conditions. Addition of increasing amounts of HrcA after binding of HspR to the P_{gro} and P_{hrc} promoter probes resulted in footprinting patterns similar to those shown in Fig. 2 (data not shown). Similar results were obtained when HspR was added to HrcA bound to the same promoter probes. Consequently, we concluded that under the in vitro conditions used by us, the two regulatory proteins bind to their operators in an independent manner.

GroESL enhances binding of HrcA and HspR to the P_{hrc} promoter in vitro. Early attempts to purify HrcA from *E. coli* cells were severely hampered by toxicity and/or insolubility of the overexpressed protein. However, these effects were alleviated by induction of HrcA expression at 42°C, suggesting that chaperone proteins were necessary for proper expression and folding of the recombinant HrcA (25). In addition, we observed that the DNA binding activity of the purified recombinant HrcA declined rapidly (within a few days) during storage at −20°C, indicative of loss of folding (not shown). Moreover, as mentioned above, in other bacterial species, the binding activity of the heat shock repressors is stimulated by the chaperone systems that they control. Consequently, we decided to assess the ability of the purified *H. pylori* GroESL chaperone machinery to influence binding of HrcA and HspR to the P_{hrc} promoter.

To do this, we first assessed by footprint analysis the effect of GroES and GroEL on the binding activity of HrcA on the P_{hrc} promoter probe. The results showed that while addition of increasing amounts of each of these proteins in the absence of ATP resulted in no changes in the binding of HrcA on the P_{hrc} promoter, addition of GroEL and ATP resulted in slight enhancement of HrcA binding (data not shown). Finally, the binding of HrcA and HspR was assessed by footprint analysis using both the GroES and GroEL chaperones in the presence of ATP (Fig. 4). With the addition of HrcA, the DNase I-hypersensitive site at position −45 of the P_{hrc} promoter, indicative of HrcA binding, was detected with 30 nM protein and clearly established with 120 nM protein (Fig. 4A, lanes 4 to 6). In the presence of GroESL and ATP, the same hypersensitive site was clearly detected with 7.5 and 15 nM HrcA (Fig. 4A, lanes 8 and 9), indicative of at least a 10-fold increase in the affinity of HrcA for its binding site. Moreover, the intensity of the band increased with increasing amounts of HrcA, also showing the two expected flanking areas of DNase I protection. It is likely that interactions between HrcA and GroESL improve the folding of HrcA and increase its affinity for DNA. Similarly, binding of HspR to the same promoter probe in the

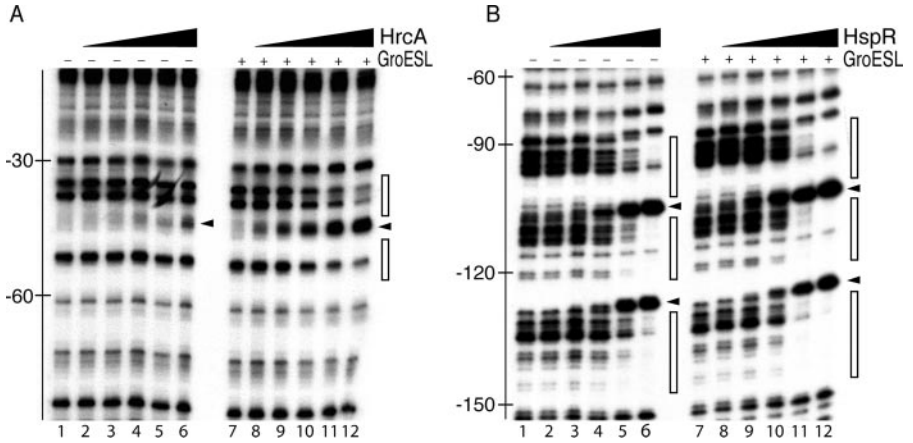

FIG. 4. Effect of GroESL chaperonin on the binding of HrcA and HspR to the P_{hrc} promoter. (A) DNase I footprinting analysis of HrcA on the P_{hrc} promoter in the absence (left panel) and in the presence (right panel) of purified recombinant GroESL complex. A specific end-labeled P_{hrc} fragment was incubated with increasing amounts of purified His-HrcA. Lanes 1 to 6, 0, 7.5, 15, 30, 60, and 120 nM His-HrcA, respectively (in each reaction 240 nM bovine serum albumin was added); lanes 7 to 12, 0, 7.5, 15, 30, 60, and 120 nM His-HrcA, respectively (in each reaction 240 nM GroESL complex and 500 μM ATP were added). (B) DNase I footprinting analysis of HspR on the P_{hrc} promoter in the absence (left panel) and in the presence (right panel) of purified recombinant GroESL complex. A specific end-labeled P_{hrc} fragment was incubated with increasing amounts of purified His-HspR. Lanes 1 to 6, 0, 25, 50, 100, 200, and 400 nM HspR-His, respectively (in each reaction 800 nM bovine serum albumin was added); lanes 7 to 12, 0, 25, 50, 100, 200, and 400 nM HspR-His, respectively (in each reaction 800 nM GroESL complex and 500 μM ATP were added). The open boxes on the right indicate the regions of DNase I protection, while the arrowheads indicate bands of hypersensitivity to DNase I digestion.

presence of GroESL and ATP revealed a slight increase in the patterns of DNase I protection (Fig. 4B, compare lanes 5 and 11), suggesting that binding of HspR could be improved by the action of GroESL. By contrast, incubation of the promoter probes with only GroESL resulted in no changes in the pattern of DNase I digestion (Fig. 4, compare lanes 7 and lanes 1). These data suggest that GroESL directly interacts with HrcA and possibly with HspR to increase their DNA binding affinities for the operators, contributing to the transcriptional repression of the regulated promoters.

HrcA and HspR transcriptome analyses. To identify genes regulated by HrcA, HspR, and both regulators, we employed DNA macroarray analysis of RNA isolated from exponentially growing wild-type and mutant cells. The Δ*hrcA*/wild-type, Δ*hspR*/wild-type, and Δ*hrcA*-Δ*hspR*/wild-type ratios from three hybridization experiments were evaluated to determine statistical significance ($P \leq 0.05$) and compared as described in Materials and Methods. Overall, 43 genes were up- or down-regulated at least 1.5-fold in the double-mutant strain (Δ*hrcA* Δ*hspR*) or in one of the single-mutant strains (Δ*hrcA* or Δ*hspR*), and the results are summarized in Table 3. Fourteen of 43 genes were up-regulated, while 29 genes were down-regulated.

As expected, most of the genes previously shown to be under transcriptional control of HrcA and/or HspR were detected in this analysis. For instance, transcription of the *cbpA-hspR*-helicase operon (HP1024 to HP1026) was derepressed in both the Δ*hspR* and Δ*hspR*-Δ*hrcA* mutant strains but not in the Δ*hrcA* mutant, confirming the transcriptional repression of this operon by HspR (28, 31). Similarly, transcription of the *groES-groEL* operon (HP0010 and HP0011) and of the *grpE* and *dnaK* genes (HP0109 and HP0110) was clearly derepressed in the Δ*hspR* and Δ*hspR*-Δ*hrcA* mutants. Transcription of these genes was apparently not affected in the Δ*hrcA* mutant. The latter observation appears to contrast with previous studies which demonstrated that repression of transcription of these genes is dependent on both HrcA and HspR (28). This discrepancy might have resulted from different experimental designs. While the macroarray technique employed in this study uses open reading frames to measure steady-state levels of cellular transcripts, previous studies focused on primer extension and S1 nuclease mapping analyses which specifically detect RNA 5′ regions. In the case of *groESL* mRNA, the particularly high stability of this RNA (29) might make it more difficult to detect significant differences in the RNA amounts. Nevertheless, there seems to be slight up-regulation of the *groESL* transcript in the Δ*hrcA* mutant. However, transcription of two new genes, the *dcuA* (HP0724; coding for an anaerobic C_4-dicarboxylate transport protein) and *omp16* (HP0722; coding for an outer membrane protein) genes, was clearly derepressed in all three mutant strains, suggesting that repression of transcription is exerted by both regulatory proteins, HspR and HrcA. By contrast, transcription of the *omp6* and *omp27* (coding for putative membrane proteins), *mda66* (coding for a putative NADPH-quinone reductase), and *frpB* (coding for an outer membrane protein) genes was found to be specifically derepressed in the Δ*hrcA* and Δ*hrcA*-Δ*hspR* mutant strains, indicating that there is negative regulation by HrcA alone. Surprisingly, the same analysis highlighted the finding that transcription of 29 genes was decreased in the Δ*hspR*-Δ*hrcA* double mutant and/or in the Δ*hrcA* and Δ*hspR* mutants, suggesting a positive role of HrcA and HspR in transcription of these genes (Table 3). Specifically, transcription of two genes (*omp1* and HP0556) was down-regulated in the absence of HspR, transcription of 17 genes was down-regulated in the absence of HrcA, and transcription of 10 genes was down-regulated in the absence of both HrcA and HspR. Intriguingly, the majority of these positively regulated genes belong to the

TABLE 3. Results of the DNA macroarray hybridization experiments

Genome open reading frame[a]	Fold change in Δ*hrcA*	Fold change in Δ*hspR*	Fold change in Δ*hrcA*-Δ*hspR*	Regulation[b]	Annotation (gene name)
Repressed by HrcA					
HP0229	**1.64**[c]	−1.21	**2.49**		Outer membrane protein (*omp6*)
HP0630	**1.65**	**−1.56**	**1.54**		Modulator of drug activity (*mda66*)
HP0916	**1.57**	1.01	**1.51**		Iron-regulated outer membrane protein (*frpB*)
HP1177	1.29	**−1.56**	**1.61**		Outer membrane protein (*omp27*)
Repressed by HspR					
HP0692	−1.24	**1.53**	**−1.67**		3-Oxoadipate coenzyme A transferase subunit B (*yxjE*)
HP1024	−1.10	**10.09**	**4.12**		Cochaperone-curved DNA binding protein A (*cbpA*)
HP1025	1.15	**−5.50**	**−3.79**		Putative heat shock protein (*hspR*)
HP1026	1.10	**3.58**	**2.35**		Conserved hypothetical helicase-like protein
Repressed by HrcA/HspR					
HP0010	**1.28**	**2.14**	**1.76**		Chaperone and heat shock protein (*groEL*)
HP0011	**1.47**	**2.26**	**1.98**		Cochaperone (*groES*)
HP0109	**−1.39**	**3.87**	**−1.22**		Chaperone and heat shock protein 70 (*dnaK*)
HP0110	**−1.39**	**4.91**	−1.23		Cochaperone and heat shock protein (*grpE*)
HP0722	**3.45**	**1.84**	**2.98**		Outer membrane protein (*omp16*)
HP0724	**4.88**	**2.49**	**3.56**		Anaerobic C_4-dicarboxylate transport protein (*dcuA*)
Induced by HrcA					
HP0295	**−1.96**	−1.23	**−3.40**	σ54	Flagellin B homolog (*fla*)
HP0367	**−1.66**	−1.33	**−2.52**		Predicted coding region
HP0472	**−3.77**	1.03	**−2.62**	σ28	Outer membrane protein (*omp11*)
HP0601	**−3.31**	−1.22	**−1.88**	σ28	Flagellin A (*flaA*)
HP0751	**−2.56**	−1.06	**−2.51**	σ28	Polar flagellin (*flaG*)
HP0752	**−2.96**	−1.10	**−2.86**	σ28	Flagellar hook-associated protein 2 (*fliD*)
HP0753	**−1.75**	−1.02	**−1.75**	σ28	Flagellar protein (*fliS*)
HP0868	**−1.68**	−1.33	**−2.20**	σ54	Predicted coding region
HP0869	**−1.58**	−1.05	**−1.89**	σ54	Hydrogenase expression/formation protein (*hypA*)
HP0907	**−1.75**	−1.14	**−1.87**	σ54	Hook assembly protein, flagella (*flgD*)
HP0908	−1.47	−1.12	**−2.03**	σ54	Flagellar hook (*flgE*)
HP1001	**−1.55**	−1.24	**−1.50**		Predicted coding region
HP1052	**−1.87**	1.05	−1.45	σ28	UDP-3-*O*-acyl-*N*-acetylglcosamine deacetylase (*envA*)
HP1120	**−1.52**	−1.12	**−1.91**	σ54	Predicted coding region
HP1122	**−1.60**	−1.01	−1.46		Anti-sigma factor (*flgM*)
HP1243	**−2.20**	1.08	**−1.54**		Outer membrane protein (*omp28*)
HP1440	**−1.55**	−1.21	−1.46		Predicted coding region
Induced by HspR					
HP0009	−1.24	**−1.77**	−1.46		Outer membrane protein (*omp1*)
HP0556	1.12	**−1.53**	−1.44		Predicted coding region
Induced by HcrA/HspR					
HP0115	**−3.08**	−1.32	**−22.92**	σ54	Flagellin B (*flaB*)
HP0119	**−1.90**	−1.43	−1.40		Predicted coding region
HP0366	**−2.05**	**−1.59**	**−3.86**		Spore coat polysaccharide biosynthesis protein C
HP0870	**−2.78**	−1.45	**−9.71**	σ54	Flagellar hook (*flgE*)
HP0906	**−2.85**	**−1.80**	**−61.08**	σ54	Flagellar hook filament, *fliK*
HP1076	**−2.27**	**−1.84**	**−2.65**	σ54	Predicted coding region
HP1119	**−3.08**	**−1.57**	**−11.65**	σ54	Flagellar hook-associated protein 1 (*flgK*)
HP1188	**−1.69**	**−1.48**	**−1.51**		Predicted coding region
HP1233	**−2.51**	**−2.00**	**−5.96**	σ54	Predicted coding region
HP1559	**−1.53**	**−1.55**	**−1.85**		Flagellar basal body rod protein (proximal rod protein) (*flgB*)

[a] See reference 32.
[b] Alternative σ factors that control gene transcription (21, 29).
[c] Boldface type indicates change of at least 1.5-fold.

class of alternative σ54 and σ28 transcribed promoters, and 14 of the 29 down-regulated genes code for proteins involved in regulation and assembly of the flagellar apparatus.

Primer extension analysis of novel HrcA- and HspR-regulated genes. Since suppressive as well as enhancing effects of HspR and/or HrcA on transcript abundance were revealed by transcriptome analyses, we selected the *mda66* and *flaB* genes as opposite representative cases to study in detail the transcription regulation exerted by HspR and HrcA. Transcription was assessed by primer extension analysis with RNA extracted from wild-type strain G27 and Δ*hrcA*, Δ*hspR*, and Δ*hspR*-Δ*hrcA* mutant strains grown at 37°C.

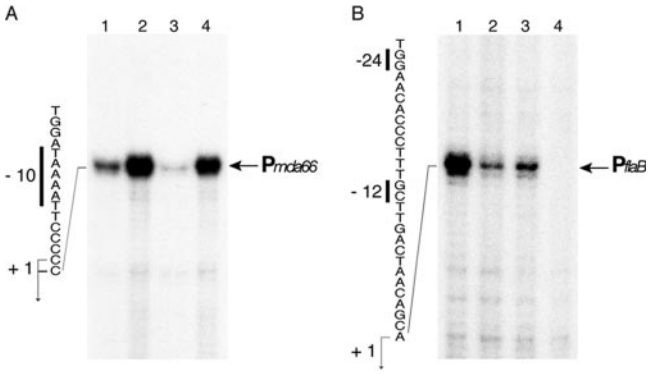

FIG. 5. Primer extension analysis of the promoters of the *mda66* and *flaB* genes. Total RNAs isolated from *H. pylori* strains G27 (lane 1), G27(*hrcA*::*km*) (lane 2), G27(*hspR*::*km*) (lane 3), and G27(*hrcA*::*km hspR*::*cm*) (lane 4) were hybridized to the radiolabeled oligonucleotides mda66PE (A) and fla (B) (Table 2) and elongated with reverse transcriptase. The positions of elongated products are indicated on the right by arrows. The corresponding cloned promoters were sequenced in parallel with the primers used in the primer extension reactions, and the nucleotide sequences upstream of the transcriptional start sites are indicated on the left; the −10 (A) and −12 and −24 (B) motifs are indicated by the vertical bars, and the nucleotides corresponding to position 1 initiation sites are indicated by bent arrows.

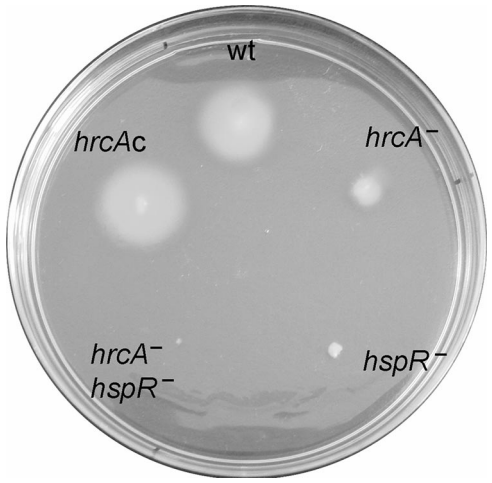

FIG. 6. Bacterial motility assay. Bacteria were stab inoculated with a pipette tip into semisolid agar plates and incubated for 72 h at 37°C under microaerophilic conditions. The strains used in this assay are indicated as follows: wt, G27; *hrcA*⁻, G27(*hrcA*::*km*); *hspR*⁻, G27(*hspR*::*km*); *hrcA*⁻ *hspR*⁻, G27(*hrcA*::*km hspR*::*cm*); and *hrcAc*, G27(*hrcA*-HA).

The *mda66* transcriptional start site was mapped at a position 25 nucleotides upstream of the ATG translation start codon and is preceded by a putative −10 region (TAAAAT), suggesting that *mda66* is transcribed from a promoter (P_{mda66}) recognized by the RNA polymerase containing the vegetative sigma factor σ80 (Fig. 5A). In comparison to the wild-type strain (Fig. 5A, lane 1), the amount of transcript was increased in both the Δ*hrcA* and Δ*hrcA*-Δ*hspR* mutants (Fig. 5A, compare lane 1 to lanes 2 and 4). Interestingly, *mda66* transcription appeared to be down-regulated in the Δ*hspR* mutant (lane 3), possibly due to increased HrcA and GroESL levels arising from transcriptional derepression of P_{hrc} and P_{gro}, which are known to be under negative control of HspR (28).

It was previously reported that transcription of the *flaB* gene starts 25 nucleotides upstream from the ATG start codon at a σ54-dependent promoter (30). Transcription from this promoter resulted in a marked reduction in the amount of transcript in the Δ*hrcA* and Δ*hspR* mutant strains (Fig. 5B, lanes 2 and 3) and was essentially undetectable in the double mutant (lane 4).

We concluded that while transcription from the P_{mda66} promoter appears to be repressed by HrcA, transcription from the P_{flaB} promoter appears to be positively controlled by both regulators (HrcA and HspR), thus confirming the differential regulation patterns observed in the transcriptome analysis. To test whether HspR and HrcA interact with these promoters directly, we carried out DNase I footprinting with labeled DNA fragments encompassing the P_{mda66} and P_{flaB} promoters. Surprisingly, after addition of increasing amounts of HrcA and HspR, no evidence for DNA binding was obtained (data not shown), suggesting that neither of the two proteins binds to these promoters. Therefore, the regulation of these genes is likely to be due to indirect mechanisms.

HrcA and HspR are required for *H. pylori* motility. It has been reported that a Δ*hspR* mutant strain of *H. pylori* is nonmotile (30) and that the same phenotype was observed in the *hspR*-deficient strain of the closely related microorganism *Campylobacter jejuni* (1). We also tested the motility of the Δ*hrcA* mutant strain of *H. pylori* by assaying the ability of the cells to spread on soft agar plates. To do this, cells were spotted onto low-concentration agar plates and incubated for 72 h at 37°C under microaerophilic conditions. Figure 6 shows that the areas of spreading of the Δ*hrcA*, Δ*hspR* and Δ*hrcA*-Δ*hspR* strains were severely reduced compared with the area covered by the wild-type strain, thus showing a loss of motility functions. Complementation of the HrcA function restored the spreading phenotype to a level similar to that of the wild-type strain. Consequently, we concluded that both heat shock regulators, HspR and HrcA, are required for *H. pylori* motility functions.

DISCUSSION

Bacteria respond to stress conditions by synthesizing chaperones, which protect the cells from damage by preventing protein denaturation, aggregation, or misfolding. *E. coli* and most other gram-negative bacteria use specialized σ factors, which become activated after exposure to stress and direct the RNA polymerase to their target promoters, whereas a subgroup of gram-negative bacteria and all gram-positive bacteria use specialized repressors which become inactivated under stress conditions, leading to derepression of target promoters. We have previously demonstrated that the major chaperone-encoding operons of *H. pylori* are negatively regulated by HspR, the homologue of the repressor of the *dnaK* operon of *Streptomyces* species (31). In addition, two of the HspR-regulated operons, *groESL* and *hrcA-grpE-dnaK*, are also regulated by HrcA (28), the homologue of the repressor of the *groESL* operon of *B. subtilis*. The presence of both regulators is therefore necessary for maintaining P_{gro} and P_{hrc} in the repressed state. HspR binds to large operators located far upstream from these promoters (31) (Fig. 2A and 2C), while the HrcA oper-

ators overlap the core promoter regions (Fig. 2B and 2D). In agreement with a previous hypothesis (28), HrcA binds to sequences that include the CIRCE-related inverted repeats centered at positions 9 and −42 of the P_{gro} and P_{hrc} promoters, respectively (Fig. 3). It is likely that binding of HrcA to these DNA elements represses transcription by steric interference of RNA polymerase binding. Furthermore, the discovery that the affinity of HrcA for its operator is considerably increased in the presence of GroESL (Fig. 4) parallels observations with HrcA proteins from *B. subtilis* and *C. trachomatis*, which showed a positive influence of GroE on the DNA binding activity of the repressor (24, 35). According to a "titration model" proposed for the *B. subtilis* HrcA repressor (19), GroE might interact with *H. pylori* HrcA to aid its folding and enhance its DNA binding activity, thereby efficiently assisting in the repression of transcription of the target promoters. In the presence of stress stimuli, the GroE chaperonin would be titrated away by increasing levels of misfolded proteins, relieving HrcA transcriptional repression of the heat shock promoters. However, HrcA-mediated regulation depends on the presence of HspR, as demonstrated by deletion of the *hspR* gene and deletion of the HspR binding site, both of which lead to promoter deregulation (28). It should therefore be assumed that binding of HrcA to its target sequences is not efficient in vivo for repressing transcription in the absence of a functional HspR repressor. The reason for this dependence might be found in chaperone-mediated protein-protein interactions between the two repressors, which may be a prerequisite for the formation of a stable repression-competent complex. While GroE chaperonin stimulates binding of HrcA to its target, no effects on the binding of HspR have been detected (Fig. 4). The possibility that the DnaK-DnaJ-GrpE chaperone system is involved in the formation of a stable HspR-HrcA repression complex should be considered.

As shown by transcriptome analysis, HspR and HrcA affect transcription of 43 genes in either a positive or negative fashion (Table 3). Of the 29 positively regulated genes, 14 code for proteins involved in regulation and assembly of the flagellar apparatus. Accordingly, loss of motility functions was observed for both mutants (Fig. 6), and transcription of the *flaB* gene was down-regulated both in single mutants and in the HspR-HrcA double mutant (Fig. 5). No binding of HrcA and/or HspR was observed on the promoter, suggesting that positive regulation of this gene is due to indirect mechanisms. Although the possibility was not investigated further, we speculated that induction of chaperone proteins alters the assembly of the flagellar apparatus and/or increases the activity of specialized anti-sigma factors, such as FlgM (5), which in turn establishes negative feedback for the programmed transcription of flagellar and motility genes (22, 30). In *H. pylori*, motility is associated with pathogenesis, and colonization of the gastrointestinal tract depends on the presence of flagellins and heat shock proteins (5, 22). Furthermore, interconnections between the heat shock response and motility have been observed in the closely related bacterium *C. jejuni* (1). In fact, in this bacterium, HspR also controls the expression of genes involved in oxidative stress and motility functions in an indirect fashion (1). Indirect mechanisms might also be responsible for the transcriptional control of other genes of the HspR/HrcA regulon, as highlighted by analysis of the P_{mda66} promoter. Transcription from this promoter appeared to be repressed by HrcA, although the purified protein failed to bind to the promoter region (Fig. 5). Whether similar regulatory feedback mechanisms like those involved in the control of flagellar gene expression or other dedicated systems are active at this promoter has to be established. In this context it is interesting that *mda66*, coding for an NADPH-quinone reductase involved in the oxidative stress response, and the genes coding for proteins that localize to the outer membrane of the bacterium (Omp6, Omp27, and FrpB) appear to be coregulated by the inner membrane-associated protein HrcA, suggesting a putative link between heat shock and oxidative stress responses throughout the bacterial membranes.

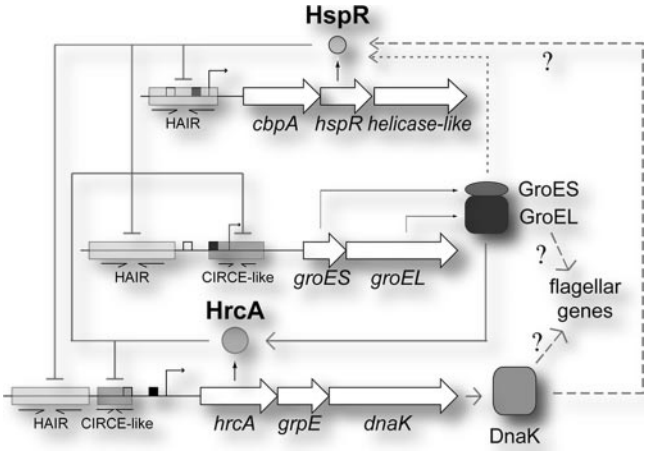

FIG. 7. Regulatory circuit of HspR and HrcA. The dashed lines indicate hypothetical interactions, and the solid lines indicate experimentally supported interactions.

Our results support a model in which, either independently or cooperatively, HspR and HrcA control transcription of chaperone genes by binding to the corresponding promoter regions (Fig. 7). Of crucial importance is the maintenance of chaperone protein homeostasis, as its alteration determines changes in transcription of several genes, including genes involved in motility and flagellar functions. For instance, enhanced synthesis of one or more components of the HspR/HrcA regulon(s), such as the GroESL and/or DnaK chaperones, might alter the programmed assembly of the flagella or other cellular structures, which in turn establishes a proper transcriptional response.

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