Isolation and Analysis of Mutant Alleles of the Bacillus subtilis HrcA Repressor with Reduced Dependency on GroE Function*

Silke Reischl, Thomas Wiegert‡, and Wolfgang Schumann

From the Institute of Genetics, University of Bayreuth, Universitätsstraße 30, Bayreuth D-95440, Germany

The hrcA gene of Bacillus subtilis codes for a transcriptional repressor protein that negatively regulates expression of the heptacistronic dnaK and the bicistronic groE operon by binding to an operator-element called CIRCE. Recently, we have published data suggesting that the activity of HrcA is modulated by the GroE chaperonin system. Biochemical analyses of the HrcA protein have been hampered so far by its strong tendency to aggregate. Here, a genetic method was used to isolate mutant forms of HrcA with increased activity under conditions of decreased GroE function. One of these mutant forms (HrcA114) containing five amino acid replacements exhibited enhanced solubility when overexpressed. HrcA114 purified under native conditions produced two retarded CIRCE-containing DNA fragments in band shift experiments. The amount of the larger fragment increased after addition of GroEL, GroES, and ATP but decreased when ATP was replaced by the nonhydrolyzable ATP analog ATPγS. DNase I footprinting experiments exhibited full protection of the CIRCE element and neighboring nucleotides in an asymmetric way. An in vitro binding assay using affinity chromatography showed direct and specific interaction between HrcA114 and GroEL. All these experimental data are in full agreement with our previously published model that HrcA needs the GroE chaperonin system for activation.

Bacteria encode genetic systems allowing them to adapt to many stressful situations, including high and low temperature, hyperosmotic and oxidative stress, and severe DNA damage (1). The best-studied stress response is the so-called heat shock response, which is induced after a sudden increase in temperature. This response is characterized by the transiently enhanced synthesis of a group of proteins collectively known as heat shock proteins encoded by heat shock genes. Work carried out over the last 5 years has revealed that in most eubacteria heat shock genes are organized in two and more regulons, where each regulon is either under positive control of an alternative sigma factor or under negative control of a transcriptional repressor (2–5).

In Bacillus subtilis, three different regulons have been identified so far, where Class I heat shock genes are under the negative control of the HrcA transcriptional repressor. This protein binds to an operator designated CIRCE1 (Controlling Inverted Repeat of Chaperone Expression) (6), which precedes the heptacistronic dnaK and the bicistronic groE operon (7–9). Upon a heat shock, HrcA dissociates from its operators leading to a transient induction of the two operons followed by rebinding after about 10 min (7, 8, 10). The pertinent question concerning all heat shock regulators is how the activity of these proteins is modulated after a heat shock. In the present case, we have presented data suggesting that the activity of HrcA is modulated by the GroE chaperonin system (11). HrcA is maintained in an active conformation able to bind to CIRCE through GroE. Under conditions of increased formation of nonnative proteins in the cell, GroE is titrated by these proteins and is no longer available to activate HrcA. This titration model is strengthened by the fact that not only heat shock but also ethanol stress, treatment with puromycin, and artificial overproduction of GroE substrates induce the HrcA regulon (12).

Proof of our model at the molecular level has been hampered so far by the fact that HrcA overproduced in Escherichia coli cells forms inclusion bodies (11). These can be dissolved in the presence of a chaotropic agent such as guanidinium hydrochloride, but upon its removal HrcA molecules aggregate again. Therefore, the objective of the present study was to obtain HrcA in a soluble and active form to investigate the influence of the GroE system on its activity more precisely. Although our attempts to refold and solubilize HrcA by a method published most recently for Bacillus thermoglucosidasius HrcA (13) and by another biochemical method failed, we devised a genetic method to isolate mutant HrcA proteins that show increased repressor binding activity under conditions of reduced GroE function, with the expectation that these proteins also might be less prone to aggregation. Several of these mutants were obtained, and one mutant could indeed be purified under native conditions. This protein was able to specifically retard a DNA fragment containing the CIRCE element in a still GroE-dependent manner, leading to two different complexes. It specifically protected a DNA fragment carrying the CIRCE element in DNase I footprint experiments. In addition, we could show a direct interaction between HrcA114 and GroEL in vitro.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids and Growth Conditions—E. coli strains JM109 (14) and XL1-Blue (Stratagene) were used as recipients in all cloning experiments, and strains MC4100 and its isogenic groE350, groEL100, and groE526 derivatives were obtained by P1 transduction of the mutant alleles from H9253 derivative strains described by Gragerov and coworkers (15). B. subtilis wild-type strains 1012 (16) and AS02 (17) have been published. Strain SR15 contained a deletion of the chromosomal copy of hrcA and the groE operon is under control of the xylose-inducible promoter P xylA (18). Plasmid pQE30 (Qiagen) was used for the production of His-tagged HrcA. Plasmid chaperone expression; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Ni-NTA, nickel-nitrilotriacetic acid; ATPγS, adenosine 5′-O-(thiotriphosphate); wt, wild-type.
The recombinant plasmids were transformed either into E. coli XL1-Blue. HrcA synthesis was driven by the con-stitutive promoter lepA (P_{lepA}), and the activity of the HrcA repressor can be quantified by measuring the β-galactosidase activity encoded by the bgaB gene whose expression is controlled by HrcA via the CIRCE containing dnaK promoter (P_{dnaK}) both in E. coli and B. subtilis.

Transcriptional Analysis and Determination of β-Galactosidase Activities—Preparation of total RNA of B. subtilis and Northern blot analysis were performed as described before (9). To easily monitor HrcA activity, the promoter region of the dnaK operon, including the CIRCE, was transcriptionally fused to the bgaB reporter gene coding for a heat-stable β-galactosidase (21). β-Galactosidase activities were determined as described previously (19).

Determination of the Amount of Soluble HrcA Protein—E. coli MC4100 or its isogenic groES30 or groEL100 derivatives, cells containing the pDN3 or pDN3–114 plasmid were grown at 30 °C. When A_{578} = 0.7 was reached, one culture was transferred to 42 °C, whereas the other one was left at 30 °C. 30 min after the heat shock, 10 ml of each culture was harvested, resuspended in 1 ml of lysis buffer, and lysed by sonication. After the cells were broken, the cells were centrifuged at 4 °C for 1 h at 45,000 rpm. The supernatant was removed, and its protein content was determined. 1 μg of total protein was loaded per lane.

Gel Retardation and DNase I Footprinting Experiments—The DNA fragments used in the gel retardation assays were obtained by enzymatic hydrolysis of pBlueSK-CIRCE with Asp718 and BamHI (CIRCE-AB, 126 bp). Labeling of the DNA fragments with [γ-32P]dATP was performed by fill-in using Klenow enzyme. In gel retardation assays, 10 μg of salmon sperm DNA were added as unspecific competitor and 15 fmol (0.001 μg) of end-labeled DNA fragment was used. When indicated, the reaction mixture was incubated for 15 min at ambient temperatures before addition of the DNA fragment. DNA fragments used for DNase I footprinting were prepared by cutting plasmid pBlueSK-CIRCE either with KpnI and BamHI (resulting in the 122-bp CIRCE-κB non-coding strand) or Asp718 and SacI (CIRCE-AS; 166-bp coding strand). Labeling of the DNA fragments was performed by fill-in using Klenow enzyme. In gel retardation assays, 10 μg of salmon sperm DNA were added as unspecific competitor and 15 fmol (0.001 μg) of end-labeled DNA fragment was used. When indicated, the reaction mixture was incubated for 15 min at ambient temperatures before addition of the DNA fragment. DNA fragments used for DNase I footprinting were prepared by cutting plasmid pBlueSK-CIRCE either with KpnI and BamHI (resulting in the 122-bp CIRCE-κB non-coding strand) or Asp718 and SacI (CIRCE-AS; 166-bp coding strand). Label-
ing of the two fragments was carried out by a fill-in reaction using the Klenow enzyme (BanH1 and Asp718) and [γ-32P]dATP. Reactions were performed in a 25-μl volume and consisted of a 1-μl labeled DNA fragment (130 fmol), 0.8 μl of GroES (13 pmol), 1.3 μl of GroEL (13 pmol; both prepared from E. coli and purchased from Sigma), ATP (0.25 μmol), 1 μl of PUC18 (0.85 μg, unspecific competitor), Sigma, ATP (0.25 μmol), 1 μl of PUC18 (0.85 μg, unspecific competitor), Sigma, and 4 μl of gel shift buffer (50 mM Tris, pH 8.0, 50 mM MgCl2, 50 mM sodium phosphate, pH 8.0, 10 mM dithiothreitol, 5% glycerol, 6.5–26 pmol of HrcA114, and H2O up to 25 μl). The reaction mixture was incubated at room temperature for 10 min, followed by the addition of 0.5 μl of DNAase I (1 unit/μl; Roche Diagnostics). After another incubation for 2 min at room temperature, the reaction was stopped by adding 4 μl of stop buffer (0.025% xylene cyanol, 0.025% bromphenol blue, 10 mM EDTA). 3 μl of the reaction mixture containing about 4 × 104 cpm was loaded onto 6% polyacrylamide/7 M urea sequencing gels. A plus G Maxam and Gilbert reactions (23) were carried out on the appropriate [32P]-labeled DNA fragments and loaded alongside the DNAase I footprint reactions. Gels were analyzed by a PhosphorImager (SI, Amersham Biosciences).

Binding Assays Using Immobilized HrcA114—To demonstrate interactions between HrcA and GroEL, we analyzed binding of GroEL to immobilized HrcA114 following the method described by Enz and coworkers (24). Two Ni-NTA-agarose columns were prepared, one loaded with His-groEL100 and the other left without protein. The binding reactions were performed at 4 °C. 450 μl of crude extract each prepared from B. subtilis strain AS02 (carries a deletion of hrcA) was applied to each column, which was subsequently washed twice with lysis buffer (50 mM NaHPO4, pH 8.0, 300 mM NaCl, 10 mM imidazole) followed by treatment with 200 μl of elution buffer (identical with lysis buffer, but 250 mM imidazole). Each fraction was mixed with 50 μl of sample buffer (incubated for 5 min at 95 °C,) and applied to an SDS-PAGE. Crude extracts of AS02 were prepared by growing the strain to an A600 of 0.7, centrifuging 10 ml of culture, and resuspending the mixture into 1 ml of lysis buffer. Cells were broken by sonification and centrifuged for 15 min at 4 °C at 12,000 rpm. The resulting supernatant was used as a crude extract.

RESULTS

Isolation of hrcA Mutant Alleles Exhibiting Increased Activity in E. coli Strains groES30 and groEL100—It was described that the two E. coli strains Ω392 groES30 and groEL100 expressing hrcA and a reporter fusion of the HrcA-controlled dnaK promoter to the gene of heat-stable β-galactosidase (BgaB) exhibited a high background β-galactosidase activity in the absence of heat stress as compared with wild-type groE cells (11). The temperature-sensitive strains groES30 and groEL100 encode a GroE chaperonin system with reduced activity also under permissive temperature (25). It was reasoned that the decreased repressor activity is due to the failure of GroE to efficiently activate HrcA. This conclusion prompted us to ask whether mutant hrcA alleles exhibiting increased repressor activity can be isolated from groES30 and groEL100 strains. Two classes of mutant alleles could be expected: (i) class I, which results in the synthesis of HrcA protein exhibiting increased activity in the absence of an intact GroE system and (ii) class II mutants in HrcA, which are allele-specific for groES30 or groEL100.

To obtain random point mutations within the hrcA gene, we applied the method of error-prone PCR mutagenesis. PCR products of hrcA were ligated to a plasmid that allows constitutive expression of hrcA and contains a transcriptional fusion of the HrcA/CIRCE-controlled dnaK promoter to bgaB (pDN2) and subsequently transformed to E. coli strains MC4100 groES30 and MC4100 groEL100. Eleven clones with a reproducible white phenotype on X-gal plates out of ~10,000 colonies with groES30 were collected. Plasmids of white colonies were isolated. Respective hrcA genes were cut out of these plasmids, individually recloned into pDN2, and transformed again to MC4100 groES30 and MC4100 groEL100. Ten clones still exhibited a white phenotype on X-gal plates and were chosen for further DNA sequence analysis of respective hrcA genes. All the ten hrcA genes obtained with the groES30 screen contained base pair substitutions; seven carried single and three double point mutations (Table I). Not a single white colony out of ~10,000 could be found using the groEL100 screen. To take advantage of possible additive effects of single point mutations, one mutant hrcA allele of the groES30 screening (hrcA6) was arbitrarily chosen and subjected to a second round of error-prone mutagenesis, and the recombinant plasmids were transformed into groEL100. This procedure resulted in one single mutant hrcA allele designated hrcA11, containing three amino acid replacements altogether (Table I). In summary, ten different alleles were obtained encoding amino acid replacements at twelve different positions, scattered almost over the complete polypeptide with some clustering between amino acid residues 99 and 132 (Fig. 2).

Analysis of the Mutant hrcA Alleles in Different E. coli Strains and in a B. subtilis Wild-type Strain—Repressor activity of the isolated hrcA alleles was analyzed in more detail in E. coli strains MC4100 groES30 and MC4100 groEL100, making use of the bgaB reporter fusion as described above. β-Galactosidase activities were measured 30 min after a shift from 30 to 42 °C and from an unshifted control at the same time point. It turned out that in the groES30 strain the background β-galactosidase activity in the absence of heat stress was slightly reduced with all mutant hrcA alleles (Table II). The mutant alleles hrcA1 and hrcA11 were defective in mediating heat induction of the reporter fusion (Table II). A more distinct picture emerged from the analysis of the E. coli groEL100 strain. With the exception of hrcA8 the background level with all hrcA mutant alleles was clearly reduced compared with the wild-type allele. Again, hrcA11 was active as a repressor almost independently from a heat shock.

Mutant Alleles of the B. subtilis HrcA Repressor

| hrcA allele | Point mutation | Amino acid replacement |
|------------|----------------|------------------------|
| hrcA1      | T20A           | L7Q                    |
| hrcA2      | T386C          | L129P                  |
| hrcA3      | A395G,C529G    | N132S,L177V            |
| hrcA4      | A383G,T386C    | K128R,L129P            |
| hrcA5      | T299C          | H100T                  |
| hrcA6      | G319A          | V107I                  |
| hrcA7      | A728G          | K261R                  |
| hrcA8      | A671G,A710G    | H224R,K237R            |
| hrcA9      | A297C          | K82N                   |
| hrcA10 = hrcA1 | T20A         | L7Q                    |
| hrcA11     | G319A,A710G,A905G | V1071,K237R,D302G |
| hrcA14     | G319A,A383G,T386C,A710G,A905G | V1071,K128R,L129P,K237R,D302G |
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Fig. 2. Location of amino acid replacements within the primary sequence of HrcA. The complete primary sequence of HrcA is given. Amino acids that have been replaced are indicated within the primary sequence by boldface letters, and the changes are marked by arrows pointing to the new amino acids. The dots above the replaced amino acids indicate how often this replacement was found in independent experiments.

TABLE II

| BgaB activity | E. coli MC4100 | E. coli MC4100 |
|---------------|----------------|----------------|
|                | ES30           | EL100          |
| no hrcA        | 1700           | 2450           |
| wt hrcA        | 120            | 1340           |
| hrcA1         | 70             | 70             |
| hrcA2         | 20             | 580            |
| hrcA3         | 30             | 80             |
| hrcA4         | 40             | 350            |
| hrcA5         | 60             | 100            |
| hrcA6         | 40             | 490            |
| hrcA7         | 50             | 100            |
| hrcA8         | 60             | 550            |
| hrcA9         | 90             | 850            |
| hrcA11        | 90             | 110            |

It has to be mentioned that the amount of protein produced in the strains of mutant alleles was the same, as judged by Western blotting with anti-HrcA antibodies (data not shown).

In summary, quantification of the β-galactosidase activities confirmed the plate assays, although there seems to be a difference in β-galactosidase activities on solid plates compared with liquid cultures, because the difference on X-gal plates, with the blue phenotype of the wild-type and the white phenotype of mutant alleles, was more pronounced.

All the mutant alleles were also transformed into the wild-type strains E. coli MC4100 and B. subtilis 1012, and the β-galactosidase activities were measured before and after heat shock. It turned out that all ten mutant alleles did not differ from the wild-type allele in both species (data not shown). We conclude from these results that all point mutations belong to the predicted class I, with an HrcA repressor exhibiting increased activity. This assumption is based on the observation that the mutant alleles obtained in the groES30 strain exhibit a comparable phenotype in the groEL100 strain, thereby excluding allele-specific suppressors. Why did we not see an effect in cells producing wild-type GroE chaperonins? We assume that the amount of active HrcA is not limiting in the presence of wild-type GroE proteins. If this conjecture is correct, reducing the amount of wild-type GroE chaperonins should reveal a difference between wild-type and mutant HrcA protein. This was indeed found to be the case (see below).

The Mutant hrcA114 Allele Directs the Synthesis of Soluble HrcA Protein When Overproduced—We observed that overexpression of wild-type HrcA in E. coli cells resulted in the production of inclusion bodies (11). We reasoned that the mutant HrcA proteins might exhibit increased solubility. Therefore, three HrcA alleles (hrcA1, hrcA4, and hrcA11) were fused to a His tag, overproduced, and tested for solubility by a centrifugation step as described under “Experimental Procedures”. Whereas hrcA4 was arbitrarily chosen, hrcA11 was used because it codes for a protein exhibiting increased repressor activity before and after heat shock both in the groES30 and groEL100 strains. The mutant proteins HrcA4 and HrcA11 yielded a higher fraction of soluble protein compared with wild-type HrcA, but the proteins remained inactive in a gel retardation assay (data not shown). As a consequence, it was tested whether a combination of the point mutations found in both alleles would further increase the solubility and, above all, the activity of the protein in gel mobility shifts with the CIRCE element. The respective hrcA allele in the reporter plasmid pMD3 was constructed by standard cloning technology (hrcA114). To test for activity of HrcA114, we transformed pMD3-hrcA114 into the different E. coli strains and into B. subtilis AS02 (ΔhrcA) wild-type as already described for the other hrcA alleles. In the absence of hrcA, the unregulated dnaK promoter directed high level expression of the β-galactosidase fusion in all the four strains analyzed, with some slight increase 30 min after heat challenge (Table III). Addition of the wild-type hrcA allele reduced the background activity in all strains but E. coli groEL100 (Table III). Whereas a clear increase in the β-galactosidase activity after a heat shock was detected in B. subtilis AS02 and E. coli groES30, the increase with E. coli wild-type was low but significant (Table III).
TABLE III

β-Galactosidase activities of B. subtilis 1012, E. coli MC4100, E. coli groES30, and E. coli groEL100 transformed with either the empty vector pDN2 or pDN3 carrying either wild-type hrcA or hrcA114

|                     | B. subtilis | E. coli MC4100 | E. coli ES30 | E. coli EL100 |
|---------------------|-------------|----------------|--------------|---------------|
| αHrcA               | 1600        | 3600           | 3800         | 5600          | 3100         | 4400         | 2700         | 3700         |
| αTF                 | 300         | 2600           | 110          | 200           | 200          | 1900         | 2100         | 3300         |
| hrcA114             | 310         | 3300           | 100          | 120           | 70           | 100          | 60           | 80           |

Other details are as for Table II.

Mutant Alleles of the B. subtilis HrcA Repressor

in an Ultracentrifuge to remove all protein aggregates and submitted to Western blotting with anti-HrcA antibodies and, as an internal control, with anti-trigger factor antibodies. It became apparent that the amount of HrcA114 in unshocked cells remained constant, whereas the amount of wild-type HrcA was reduced in the groES30 and even more in the groEL100 strain (Fig. 3A). Furthermore, after heat shock there was a clear reduction in the amount of soluble wild-type HrcA in the groE wild-type background but not in the amount of the HrcA114 protein.

Next, hrcA114 was fused into the His-tagging vector pQE30 to overproduce and purify His6-HrcA114 in E. coli. Extracts of soluble proteins of the strain overexpressing His6-HrcA114 submitted to SDS-PAGE and Coomassie Blue staining indeed revealed that about 50% of the repressor protein remained soluble (Fig. 3B). Furthermore, HrcA114 proved to be active in gel mobility shift assays (see below). To conclude, the point mutations within hrcA114 result in a repressor protein exhibiting enhanced activity and solubility in both E. coli groES30 and groEL100.

These results further suggest that HrcA114 is less dependent on the GroE chaperonin machine than the two independent mutant proteins HrcA4 and HrcA11. It should be mentioned that hrcA114 carries a total of five amino acid replacements (see Table I) and that we do not know whether all five point mutations contribute to the observed characteristics.

HrcA114 Exhibits Enhanced Stability under GroE Depletion Conditions—As already mentioned, it is difficult to explain why there is such a strong effect in the groES30 and groEL100 strains expressing the hrcA114 allele, but not in the wild-type groE situation in both E. coli and B. subtilis. A possible explanation is, that under groE wild-type conditions the amount of active HrcA in the absence of heat shock is not limiting in contrast to the groES30 and groEL100 background. Therefore, higher amounts of active HrcA do not change the phenotype. This also implies that after a heat shock the amount of the HrcA114 variant becomes limiting and, as a consequence, must have a reduced but still significant dependence on the GroE chaperonin system.

To investigate this possibility, hrcA and hrcA114 were expressed in a B. subtilis strain where the amount of GroE can be controlled by xylose addition to the growth medium. Strain SR15 with a deletion of the chromosomal copy of hrcA and the groE operon expressed from a xylose-regulatable promoter was transformed with the empty vector pDN2 and with pDN3 carrying either the wild-type or the mutant hrcA114 allele. All three strains were grown overnight in the presence of 1% xylose to ensure expression of the groE operon. Each overnight culture was first washed to remove the xylose and then used to inoculate fresh LB medium with and without 1% xylose, respectively. Aliquots were withdrawn after about 2 h of growth at an A670nm of 0.6 for β-galactosidase measurements. Cultures without xylose stopped growing at an A570nm of about 1.

Although about 250 milliunits/mg were determined in the absence of hrcA and xylose, addition of xylose reduced this activity to ~50%, indicating an effect of xylose on the enzyme activity itself, most probably through GroE (Table IV). Addition of HrcA led to a reduction of the β-galactosidase activity to about 50 milliunits/mg (Table IV). When the hrcA114 allele was tested, its activity in the absence and presence of xylose was significantly reduced compared with its wild-type allele (Table IV). These results clearly demonstrate that under GroE-limiting conditions HrcA114 is more active than HrcA and thereby strengthen the hypothesis that the effect of stabilizing amino acid replacements become apparent only under conditions when the GroE chaperonin system becomes limiting. These results further suggest that HrcA114 is not completely independent of the GroE system, because it exhibits enhanced activity in its presence.

To confirm these results by an independent experiment, the amount of chromosomally encoded dnaK-specific transcript...
was analyzed by a slot-blot (Fig. 4A) and Northern blot (Fig. 4B) experiment. As a control in Northern blots, the lepA-specific mRNA was also tested; this gene is not subject to regulation through HrcA (22). In the negative control experiment in the absence of HrcA the amount of dnaK-specific transcript was high and independent of the addition of xylose. With wild-type HrcA, the addition of the inducer xylose reduced the amount of dnaK-specific transcript (Fig. 4). HrcA114 reduced the amount of dnaK transcript in the absence of xylose compared with wild-type HrcA, and addition of the inducer further reduced it significantly (Fig. 4). The amount of the lepA transcript remained unchanged in all six samples (Fig. 4B). These results fully confirm those obtained by measuring β-galactosidase activities.

Purified HrcA114 Specifically Retards a DNA Fragment Containing the CIRCE Element, and This Retardation Is Significantly Increased in the Presence of the GroE Chaperonin System—We already published that purified HrcA is able to specifically retard migration of a CIRCE containing DNA fragment, but this was performed with HrcA of Bacillus steareothermophilus purified under denaturing conditions and refolded (11). Based on all the data obtained with HrcA114 so far, we expected that this mutant protein should exhibit increased activity after purification from E. coli under native conditions. Therefore, HrcA114 was purified as described under “Experimental Procedures” and immediately used in gel retardation assays.

As shown in Fig. 5, addition of GroES/GroEL and ATP alone did not lead to any retardation of the DNA fragment (lane 2). Addition of HrcA114 alone retarded a large portion of the DNA (about 50%) and led to the formation of two different bands (lane 3). The amount of retarded DNA was not altered when GroES and GroEL alone were added (lane 4). In contrast, a significant increase in the upper band was observed upon further addition of ATP (lane 5) but not in the presence of the nonhydrolyzable ATP analog ATPγS (lane 6). Addition of a 100-fold molar excess of competing unlabeled DNA fragment completely prevented binding of HrcA114 to the labeled DNA (lane 7). We also tested whether the incubation temperature influenced the retardation behavior. With HrcA114 and DNA incubation of all components at 25 °C produced the two bands (lane 9), whereas incubation at 37 or 50 °C completely prevented its binding (lanes 10 and 11). When the GroE proteins and ATP were included into the incubation mixture, two bands were still observed at 37 °C (lane 14) and the lower band even at 50 °C (lane 15).

Purified HrcA114 Protects the CIRCE Element and Neighboring Nucleotides—To determine the location and sequence of the HrcA binding site precisely, DNase I footprinting experiments were performed on a DNA fragment carrying the CIRCE element of the B. subtilis groE operon. When the template strand was labeled, HrcA114 protected a region extending from −31 to −15, from −5 to +36, and from +39 to +43 (Fig. 6, A and C) indicating that HrcA114 binding sites are located between positions −31 and +43 of the groE promoter region excluding the −10 box (Fig. 6, A and C). The nontemplate strand was protected from positions +3 to +67 (Fig. 6, B and C). In summary, these results demonstrate that HrcA protects the complete CIRCE element, including its spacer region and binds asymmetrically to the DNA. There are no significant sequence similarities within the flanking regions (Fig. 6C). In conclusion, the DNase I footprinting data identify the CIRCE element as the common binding site present on both DNA strands.

Binding of GroEL to His_{6}-HrcA114 Fixed to a Ni-NTA-Agarose Column—To ascertain physical interaction between HrcA114 and GroEL, Ni-NTA-agarose was used as an affinity matrix for His_{6}-HrcA114 in an in vitro binding assay. This method was chosen, because HrcA114 still tends to aggregate when purified, a fact that also prohibited the estimation of the oligomeric state of the protein (data not shown). Aggregation can be prevented by immobilizing the protein to a gel matrix, which avoids local concentrations that are too high. Two Ni-NTA-agarose columns were prepared, one loaded with His_{6}-HrcA114 and the other left without protein. Crude extracts prepared from B. subtilis strain AS02 (devoid of HrcA and, therefore, overexpressing dnaK and groE operons) were applied onto both Ni-NTA columns. After two washes, both columns were treated with elution buffer, and aliquots of the eluates were analyzed by immunoblotting and probed with different antibodies (Fig. 7). Although the crude extract contained the three chaperones GroEL, DnaK, and HtpG, but no HrcA as expected (lane 1), the eluate obtained from the Ni-NTA column not loaded with HrcA114 only revealed trace amounts of GroEL (lane 2). In contrast, the eluate from the column loaded with HrcA114 contained the represor protein and high amounts of GroEL. DnaK and HtpG were not detectable (lane 3). This experiment nicely proves that GroEL is able to physically interact with HrcA114, but neither are DnaK nor HtpG.
FIG. 5. HrcA114 specifically retards a DNA fragment carrying the CIRCE element in a GroE- and ATP-dependent manner. DNA binding reactions were performed with a DNA fragment carrying the CIRCE element of groE enzymatically hydrolyzed from pBlueSK-CIRCE (from +45 to +82; 13 pmol per reaction) and end-labeled with $^{32}$P. The components of the different reaction mixtures are indicated below the lanes and include 13 pmol of HrcA114, 13 pmol of GroES, 13 pmol of GroEL, 0.25 pmol of ATP, 0.25 pmol of ATP-S, and 1.5 pmol of competing DNA fragment. The reaction mixtures were incubated at the temperatures indicated for 15 min before addition of the CIRCE element. The position of the retarded fragments is indicated by circles, and that of unretarded DNA is indicated by an asterisk.

**DISCUSSION**

Regulation of heat shock genes by the HrcA repressor protein binding to the CIRCE operator is widespread within the eubacterial kingdom and occurs both in Gram-positive and Gram-negative species (5, 26–28). Common to all regulatory systems involved in the heat shock response is the transient induction of the heat shock genes. This implies that, in the case of positive regulation, the transcriptional regulator has to be transiently activated, and, in the case of negative regulation, to be transiently inactivated. For HrcA of *B. subtilis*, four lines of experimental data strongly suggest that the activity of this repressor protein is modulated by the GroE chaperonin system: (i) depletion of GroES/EL in *B. subtilis* resulted in increased expression of the Class I heat shock genes controlled by HrcA; (ii) overexpression of GroEL in *B. subtilis* reduced both the basal and the induced level of Class I heat shock proteins after a heat shock; (iii) purified GroEL significantly enhanced binding of HrcA to the CIRCE element in gel retardation assays; (iv) the basal level of expression of an HrcA-controlled reporter gene was greatly augmented in both *E. coli* groES30 and groEL100 strains (11). These data led us to suggest a titration model where the amount of active HrcA, which is able to bind to the CIRCE operator, is directly correlated to the amount of available GroE system. Upon a sudden heat shock, denatured proteins titrate the GroE chaperonins, thereby increasing the amount of inactive HrcA repressor, which is unable to interact with the CIRCE element. The more denatured proteins are removed from the cell, the more GroE chaperonins are available to convert inactive HrcA into its active form (12). This in turn leads to the shut off of the Class I heat shock genes under constantly high temperature conditions (7–9). This direct HrcA-GroE interaction model has been challenged by Minder et al. (29) whom did not see a more efficient retardation of target DNA by HrcA of *Bradyrhizobium japonicum* in the presence of added GroEL. Furthermore, researchers performing experiments with HrcA of *Clostridium acetobutylicum* and *Lactococcus lactis* detected an influence of DnaK on the activity of HrcA (30, 31). Therefore, it is of particular importance first to demonstrate a direct interaction between HrcA and the GroE system and second to define the active and inactive forms of HrcA.

The observation that HrcA synthesized in the temperature-sensitive *E. coli* groES30 and groEL100 mutant strains is inactive suggested a screening system to obtain hrcA mutant alleles exhibiting increased repressor activity. The groES30 and groEL100 mutations reduce the overall activity of the GroE system, and HrcA seems to be specifically sensitive to this reduced activity. Whereas the GroES30 protein carries an amino acid replacement within the mobile loop (A314) (32), which is involved in the interaction with GroEL, that in GroEL100 (S201F) (33) is located in the apical domain involved in the binding of nonnative proteins (34, 35). Error-prone PCR mutagenesis produced a total of ten mutant hrcA alleles using *E. coli* groES30 as a recipient. No mutants were directly found in *E. coli* groEL100. This could indicate that single and even double point mutations are not enough or too rare to produce the desired phenotype. This possibility is strengthened by the finding that hrcA6 carrying one point mutation when going through a second round of error-prone mutagenesis resulted in the mutant allele hrcA11, now carrying three point mutations and exhibiting reduced basal activity in the *E. coli* groEL100 strain. That these mutant hrcA alleles display increased independence from the GroE chaperonin machine rather than being allele-specific suppressors is emphasized by the finding that they display comparable activities in both *E. coli* groES30 and groEL100.

Amino acid residues that were replaced in the mutant forms of HrcA were not clustered in a hot spot. They do not reveal a specific conservation among the various HrcAs of different species, taken into account that there is only little sequence conservation between the different HrcA proteins. Stabilization of proteins is often encountered with a change in charge distribution of surface-exposed amino acid residues (36). For example, the point mutation D302G that was found after a second mutagenesis cycle in the groEL100 screen possibly causes such a stabilizing effect, which may be underlined by the fact that the two HrcAs from thermophilic *Bacillus* species (*B. stearobacter* and *B. thermoglucosidasius*) possess a glycine residue at the respective position. Nevertheless, because the three-dimensional structure has yet not been resolved and there is little sequence homology, the nature of the point mutations remains speculative.

It is of special interest that the reduced dependence on GroE of at least three mutant forms of HrcA tested (HrcA4, HrcA11, and HrcA114) correlated with enhanced solubility of the overexpressed proteins (data not shown and Fig. 3). Nevertheless, HrcA4 and HrcA11 purified under native conditions were inactive in gel retardation assays with a CIRCE-containing DNA fragment. HrcA114 was active, but the high molar ratio of DNA:protein (1:2000) for a complete retardation of the DNA fragment reveals that the soluble protein is also mainly present...
in an inactive form, what has already been shown for the
*B. japonicum* HrcA purified under native conditions (29). This
underlines that solubility of HrcA is not tantamount to full
activity of the repressor. Recently, a method has been pub-
lished allowing effective renaturation and solubilization of
*B. thermoglucosidasius* HrcA with added DNA, leading to a
fully active HrcA (13). This technique failed to work with wild-
type HrcA of *B. subtilis.* Furthermore, we could detect only a
slight increase of DNA-binding activity of renatured HrcA pro-
duction, when CIRCE-containing DNA was added (data not
shown).

Mutant HrcA114 with five amino acid replacements is of
crucial importance, because its enhanced solubility allowed
further biochemical characterization of *B. subtilis* HrcA in gen-
eral. When expressed in *B. subtilis,* it behaved like wild-type
HrcA with respect to basal activity and induction of a Class I
promoter (Table III). Differences could only be seen under
conditions of GroE limitation, with a reduced but still measur-
able dependence on GroE in a depletion assay (Table IV and
Fig. 4). Addition of GroE together with ATP increased the DNA
binding activity of HrcA114 (Fig. 5, lanes 3–5). Furthermore, what
could be shown for the first time for an HrcA repressor,
HrcA114 displayed a high binding affinity for GroEL compared
FIG. 6. DNase I footprinting analy-
sis of HrcA114 binding to the CIRCE
element. Lanes contain 4 × 10^4 cpm of
labeled template strand (A) and non-tem-
plate strand (B). Fragments were incub-
bated with increasing amounts of purified
HrcA114: lane 1, A plus G Maxam and
Gilbert reactions; lane 2, no HrcA114;
added HrcA114: lane 3, 6.5 pmol; lane 4,
13 pmol; and lane 5, 26 pmol. Regions
protected by HrcA114 are indicated by
brackets. DNase I-protected regions are
drawn schematically (C). The sequence of
the hrcA promoter region is shown, with
the DNase I-protected areas shaded in
gray. Positions are relative to the σ^70-
dependent promoter transcription start
point +1 as indicated by a vertical arrow.
Arrowheads: location of the CIRCE ele-
ment; boldface letters and asterisks above
the nucleotides: potential Shine-Dalgarno
sequence. The −35 and −10 boxes are in
boldface letters and the start codon TTG is
underlined.

with DnaK and HtpG as a control (Fig. 7). On the basis of the
titration model, this means that HrcA114 is indeed more inde-
pendent on GroE function but still binds to the chaperonin and

FIG. 7. GroEL binds specifically to immobilized HrcA. Two Ni-
NTA-agarose columns were prepared, and one was loaded with natively
purified His6-HrcA114, whereas the second was left without protein.
Next, both columns were treated with crude extract prepared from
*B. subtilis* strain AS02 (ΔhrcA) and washed, and the bound proteins
were eluted with imidazole. The elution fraction was analyzed by im-
munoblotting using antibodies against HrcA, GroEL, DnaK, or HtpG,
respectively. Lane 1, crude extract of strain AS02; lane 2, eluate from
the control-column without protein; lane 3, column loaded with
HrcA114.
needs at least some of its activity. In vivo substrates of the E. coli GroE system have been identified (37). They are characterized by a molecular mass of 20–60 kDa and αβ domains that have α-helices and β-sheets with extensive hydrophobic surfaces. HrcA has a molecular mass of 39 kDa and, according to secondary structure predictions, possesses several α-helices and β-sheets (data not shown). The HrcA114 mutant protein should be characterized by a more stable folding and thereby be less dependent on the GroE chaperonin system.

There have been several publications demonstrating specific interaction between purified HrcA and a CIRCE element containing DNA fragment in gel retardation assays, including HrcA from Streptococcus pneumoniae, Streptococcus thermophilus, Bacillus japonicum, B. thiomargaritaceae, and B. stearothermophilus (13, 29, 38–40), but all groups reported only one retarded complex. We cannot exclude that the two forms we see in retardation assays represent two different oligomeric states of HrcA114, but the still high tendency of HrcA like most repressors acts at least as a dimer, as it was reported for HrcA from S. thermophilus and B. thiomargaritaceae (13, 39).

It has been described that HrcA of B. thermoglucosidasius may directly act as a thermosensor. Based on the fact that GroE had only a minor effect on preventing HrcA aggregation at different temperatures compared with DNA, it was suggested that GroE is mainly needed for the activation of newly synthesized repressor (13). Our band shift experiments also demonstrate that the activity of HrcA is influenced by the incubation temperature, but this effect could be largely suppressed by adding the complete GroE system (Fig. 5, lanes 8–15). Although HrcA seems to exhibit an intrinsic thermolability, the fact that the heat shock response is shut off after about 10 min under retention of the heat shock conditions (17) supports the titration model. Therefore, we propose a combined model where HrcA exhibits intrinsic thermolability, but the main modulating system is the titration of the GroE chaperonin machine.

In summary, we have succeeded for the first time to obtain purified soluble HrcA protein of B. subtilis specifically retarding a DNA fragment containing the CIRCE element. Two different complexes were produced never reported so far in band shift experiments with HrcA, and further work has to elucidate the stoichiometry within these complexes and their biological function. We also succeeded for the first time to obtain a footprint with HrcA showing that it completely protected the CIRCE element and neighboring sequences in an asymmetric way. Further work has to reveal whether this footprint correlates with the faster or slower migrating complex seen in the band shift assay. Finally, we succeeded in demonstrating that immobilized HrcA specifically interacts with GroEL from crude extracts. All these results are fully in compliance with our model that HrcA of B. subtilis needs the complete GroE machine for activation. Definition of the active and the inactive state of HrcA awaits further extensive experimentation, which is underway in our laboratory.

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REFERENCES

1. Storz, G., and Hengge-Aronis, R. (2000) Bacterial Stress Responses, American Society for Microbiology, Washington, D. C.
2. Narberhaus, F. (1999) Mol. Microbiol. 31, 1–8
3. Yura, T., Kenaneri, M., and Morita, M. (2000) in Bacterial Stress Response (Storz, G., and Hengge-Aronis, R., eds) pp. 3–18, American Society for Microbiology, Washington, D. C.
4. Servant, P., and Mazodier, P. (2001) Arch. Microbiol. 176, 237–242
5. Schumann, W., Hecker, M., and Maedek, T. (2001) in Bacillus Subtilis and Its Closest Relatives: From Genes to Cells (Sonnenhain, A. L., Hech, J. A., and Loesch, R., eds) American Society for Microbiology, Washington, D. C.
6. Zuber, U., and Schumann, W. (1994) J. Bacteriol. 176, 1359–1363
7. Watatobe, K., Yamamoto, T., and Suzuki, Y. (2001) J. Bacteriol. 183, 155–161
8. Yanish-Perron, C., Vieira, J., and Messing, J. (1985) Gene (Amst.) 33, 103–119
9. Gragerov, A., Nucler, E., Komissarova, N., Gatanias, G. A., Gottesman, M. E., and Nikiforov, V. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10341–10344
10. Saito, H., Shibata, T., and Ando, T. (1979) Mol. Gen. Genet. 170, 117–122
11. Schulz, A., Tzschaschel, B., and Schumann, W. (1995) Mol. Microbiol. 13, 451–459
12. Kim, L., Mogk, A., and Schumann, W. (1996) Gene (Amst.) 181, 71–76
13. Mogk, A., Hayward, R., and Schumann, W. (1996) Gene (Amst.) 182, 33–36
14. Fromant, M., Blanquet, S., and Plateau, P. (1995) Anal. Biochem. 224, 377–383
15. Hirata, H., Fukazawa, T., Negoro, S., and Okada, H. (1986) J. Bacteriol. 166, 722–727
16. Homuth, G., Masuda, S., Mogk, A., Kobayashi, Y., and Schumann, W. (1997) J. Bacteriol. 179, 1153–1164
17. Schulz, A., Taschachshel, B., and Schumann, W. (1995) Mol. Microbiol. 13, 451–459
18. Kim, L., Mogk, A., and Schumann, W. (1996) Gene (Amst.) 181, 71–76
19. Mogk, A., Hayward, R., and Schumann, W. (1996) Gene (Amst.) 182, 33–36
20. Fromant, M., Blanquet, S., and Plateau, P. (1995) Anal. Biochem. 224, 377–383
21. Hirata, H., Fukazawa, T., Negoro, S., and Okada, H. (1986) J. Bacteriol. 166, 722–727
22. Homuth, G., Heinemann, M., Zuber, U., and Schumann, W. (1996) Microbiology 142, 1641–1649
23. Maxam, A. M., and Gilbert, W. (1980) Methods Enzymol. 65, 499–560
24. Enz, S., Mahren, S., Strelocher, U. H., and Braun, V. (2000) J. Bacteriol. 182, 635–648
25. Carrillo, N., Caccarelli, E. A., Krapp, A. R., Boggio, R. G., and Ferreyra, R. G. (1999) J. Bacteriol. 181, 417–428
26. Segal, G., and Ron, E. Z. (1996) FEMS Microbiol. Lett. 138, 1–10
27. Ahmad, S., Selvapandian, A., and Bhatnagar, R. K. (2001) Int. J. Syst. Bacteriol. 49, 1387–1394
28. Minder, A. C., Fischer, H. M., Hennecke, H., and Narberhaus, F. (2000) J. Bacteriol. 182, 14–22
29. Rügeling, E., Lauf, T., and Bahl, H. (1999) FEMS Microbiol. Lett. 170, 119–123
30. Koch, B., Kistl, K., Volkmer, G.-F., and Hammer, K. (1998) J. Bacteriol. 180, 3873–3881
31. Landry, S. J., Zeihs-Ryls, J., Foyet, O., Georgopoulos, C., and Giersch, L. M. (1993) Nature 364, 255–258
32. Zeihs-Ryls, J., Foyet, O., Baird, L., and Georgopoulos, C. (1993) J. Bacteriol. 175, 1134–1143
33. Braig, K., Otwinski, Z., Hegde, R., Boisvert, D. C., Joachimiak, A., Horwich, A. L., and Sigler, P. B. (1994) Nature 371, 578–586
34. Fenton, W. A., Kashi, Y., Portak, K., and Horwich, A. L. (1994) Nature 371, 614–619
35. Martin, A., Sieber, V., and Schmid, F. X. (2001) J. Mol. Biol. 309, 717–726
36. Henry, A. M., Frishman, H., Ecker, K., and Hartl, F. U. (1999) Nature 402, 147–154
37. Kim, S.-N., Kim, S.-W., Pyo, S.-N., and Lee, D.-K. (2001) Mol. Cells 11, 360–368
38. Martirani, L., Raniego, N., Racquio, G., Ricca, E., and De Felice, M. (2001) FEMS Microbiol. Lett. 186, 177–182
39. Bannett, F., Hoyt, M. A., McFarlane, L., Kochs, E., and Hershkowith, I. (1986) J. Mol. Biol. 187, 213–224