Microarray studies reveal a ‘differential response’ to moderate or severe heat shock of the HrcA- and HspR-dependent systems in Corynebacterium glutamicum

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Genome-wide transcription profile analysis of the heat-shocked wild-type strain under moderate (40°C) and severe heat stress (50°C) revealed that a large number of genes are differentially expressed after heat shock. Of these, 358 genes were upregulated and 420 were downregulated in response to moderate heat shock (40°C) in Corynebacterium glutamicum. Our results confirmed the HrcA/controlling inverted repeat of chaperone expression (CIRCE)-dependent and HspR/HspR-associated inverted repeat (HAIR)-dependent upregulation of chaperones following heat shock. Other genes, including clusters of orthologous groups (COG) related to macromolecule biosynthesis and several transcriptional regulators (COG class K), were upregulated, explaining the large number of genes affected by heat shock. Mutants having deletions in the hrcA or hspR regulators were constructed, which allowed the complete identification of the genes controlled by those systems. The up- or downregulation of several genes observed in the microarray experiments was validated by Northern blot analyses and quantitative (real-time) reverse-transcription PCR. These analyses showed a heat-shock intensity-dependent response (‘differential response’) in the HspR/HAIR system, in contrast to the non-differential response shown by the HrcA/CIRCE-regulated genes.

INTRODUCTION

Interest in the molecular biology of the Gram-positive, non-pathogenic soil bacterium Corynebacterium glutamicum has increased due to its ability to secrete large amounts of amino acids (Martín & Gil, 1999; Hermann, 2003). The availability of its genome sequence (Ikeda & Nakagawa, 2003; Kalinowski et al., 2003) has promoted studies to understand different regulatory pathways using so-called post-genomic techniques such as proteomics (Hermann et al., 2001; Barreiro et al., 2005) and transcriptomics (Hüser et al., 2003; Silberbach et al., 2005).

The heat-shock response is one of the most interesting regulatory networks present in all organisms. It is a universal cellular reaction caused by a sublethal heat treatment, in which cells synthesize several heat-shock proteins that include molecular chaperones and ATP-dependent proteases (Yura & Nakahigashi, 1999). Thermal stress in bacteria has been studied in diverse micro-organisms by using methods such as Northern blotting (Grandvalet et al., 1997), proteomics (Rosen & Ron, 2002) and transcriptomics (Helmann et al., 2001). Thus, different regulatory systems [HrcA/controlling inverted repeat of chaperone expression (CIRCE); HspR/HspR-associated inverted repeat (HAIR); class three stress gene repressor (CtsR); repression of heat-shock gene expression (ROSE)] involved in heat-shock regulation have been described in several micro-organisms, and control hundreds

Abbreviations: CIRCE, controlling inverted repeat of chaperone expression; COG, clusters of orthologous groups; CtsR, class three stress gene repressor; HAIR, HspR-associated inverted repeat; q-RT-PCR, quantitative (real-time) reverse-transcription PCR; ROSE, repression of heat-shock gene expression.

The array data discussed in this paper have been deposited in EBI and are accessible through ArrayExpress series accession number E-MTAB-66.

Tables of microarray data are available as supplementary material with the online version of this paper.
of genes implicated in diverse cellular activities (Chhabra et al. 2006; Koide et al., 2006).

The Gram-positive model bacterium Bacillus subtilis shows a regulatory system involved in thermal stress that employs the secondary sigma factor σB and the regulators HrcA and CtsR (Helmann et al., 2001), and which differs from the actinobacterial mechanisms that have mainly been described in the genus Streptomyces. Streptomyces species present four regulatory systems that govern the induction of heat-shock genes (Bucca et al. 2003): (i) HrcA/CIRCE, regulating the groES-EL1 operon and groEL2 gene; (ii) HspR/HAIR, which controls the dnaK operon and the clpB gene; (iii) RheA, a transcriptional repressor of the hsp18 gene in Streptomyces albus (Servant et al., 2000) and some other Streptomyces species (Ventura et al., 2006); and (iv) PopR, which positively regulates two of the five clpP paralogues in Streptomyces coelicolor.

A first approach to study global gene expression during the heat-shock response in C. glutamicum was developed by using a shotgun DNA macroarray method (Muffler et al., 2002). Later, a transcriptional analysis of the main heat-shock regulatory systems and the transcriptional regulation of some protease genes by the HspR/HAIR system was reported (Barreiro et al., 2004; Engels et al., 2004). A proteomic approach has also been used for such heat-shock analysis (Barreiro et al., 2005). Since the ClgR activator (homologous to PopR) in C. glutamicum is under the control of the HspR system and the rheA–hsp18 system is not present in corynebacteria, it seems that the two other systems described in the Streptomyces genus (HrcA/CIRCE and HspR/HAIR) are the most interesting regulatory mechanisms of the heat-shock response in C. glutamicum. It was therefore of great interest to study the heat-shock regulatory networks that occur in C. glutamicum, using global transcriptomical analysis.

The heat-shock response at different temperatures in Escherichia coli correlates with the translational activation (under moderate heat shock) or transcriptional induction (under severe heat shock, e.g. rapid shift from 28 to 50 °C) of the stress sigma factor RpoH (Rosen & Ron, 2002). This ‘differential response’, which is stress intensity-dependent, affects only a few individual genes described in Gram-positive bacteria (Ventura et al., 2006; Young & Garbe, 1991; Engels et al., 2004). However, it is not known if this also occurs in C. glutamicum.

In this work, null mutants of the main regulators of the heat-shock response (hspR and hrcA) have been constructed by directed gene replacement and used for a microarray analysis of the HspR- and HrcA-dependent networks. This article describes the ‘differential response’ of those regulatory circuits to moderate or severe heat shock in C. glutamicum and the interaction between them.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains, plasmids and primers used in this work are listed in Table 1. C. glutamicum ATCC 13032 was grown in trypticase soy broth (TSB) or TYG (2× TY + 2% glucose) at 30 °C as the standard temperature and shifted to 40 or 50 °C in heat-shock experiments. E. coli transformants were selected in the presence of kanamycin (50 μg ml⁻¹) or apramycin (50 μg ml⁻¹); and C. glutamicum transformants were selected by using kanamycin (30 μg ml⁻¹) or apramycin (25 μg ml⁻¹).

E. coli plasmid DNA was obtained by alkaline lysis. DNA manipulations were performed as described by Sambrook & Russell (2001). E. coli cells were transformed by standard methods and C. glutamicum cells were transformed by conjugation, as described by Schaefer et al. (1994).

Construction of mutant strains. The conjugative vector pK18mob2 (Kirchner & Tauch, 2003) was used to delete the regulatory genes hspR or hrcA by cloning the apramycin-resistance gene [aac(3)IV] controlled by its own promoter and without its terminator region flanked by up- and downstream regions of the selected gene. The aac(3)IV gene was amplified by PCR from the pULVK2A vector (Kumar et al., 1994) (primers: Apr-Sal and Apra-A2). A 983 bp fragment containing the terminator region of the dnaK operon (PCR primers: Ald-Hd and Ald-Sal) and a 919 bp fragment containing the 3’ region of dnaJ (PCR primers: Al-ERI and Al-Bm) were cloned flanking the aac(3)IV gene to delete the hspR regulator. The final vector was named pAhsR.

Similarly, a 975 bp fragment carrying the 5’ region of dnaJ2 (PCR primers: J2-Pst and J2-Sal) and a 846 bp fragment containing the 3’ end of hemN (PCR primers: MN-Bam and MN-ERI) were placed flanking the aac(3)IV gene to delete the hrcA regulator. The resulting vector was named pAhrcA. All the PCR products were sequenced before their location in each vector. The final plasmids were introduced into C. glutamicum by conjugation using the E. coli strain S17-1. Apramycin-resistant mutants were selected and Southern hybridizations were made by using specific probes for the apramycin-resistance (primers: Apr-Sal and Apra-A2), hspR (primers: hspR-5 and hspR-3) and hrcA (primers: hrcA-5 and hrcA-3) genes.

RNA extraction and Northern hybridization. Total RNA from corynebacteria for Northern blots was extracted by the method of Barreiro et al. (2001) at OD₆₀₀ 3–4.

Total RNA from corynebacteria for microarray analyses was obtained as described by Hüser et al. (2005). Cells were disrupted in a Ribolyser instrument (Bio 101). The RNase-free DNase set (Qiagen) was used for on-column digestion of DNA and a second DNase I digestion was performed with the DNase I kit (Sigma–Aldrich). Fluorescently labelled cDNA copies of total RNA were made by an indirect labelling technique. Amino-modified hexamer primers and aminooaryl-modified nucleotides (aa-dUTPs) were incorporated during a first-strand reverse transcription step. The cDNA was labelled with Cy3 and Cy5 monofunctional N-hydroxysuccinimide esters (Amersham Biosciences).

For Northern hybridizations, denaturing RNA electrophoresis was performed using 30 μg RNA per sample, as described by Barreiro et al. (2004), using the ‘DIG Northern Starter kit’ (Roche) with exposition times between 30 s and 40 min. The probes used to analyse the transcription of the dnaK and groES-EL1 operons were the ones described by Barreiro et al. (2004).

Quantitative (real-time) reverse-transcription PCR (q-RT-PCR) assays. q-RT-PCR analyses were performed using a QuantiTect SYBR Green RT-PCR kit (Qiagen) and a LightCycler instrument (Roche Diagnostics), as described by Koch et al. (2005). Product verification was performed by melting-curve analysis. The non-normalized relative expression ratios were calculated using the mathematical model described by Pfaffl (2001).
Table 1. Strains, plasmids and oligonucleotides used in this study

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<th>Strain or plasmid</th>
<th>Characteristics</th>
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*In some cases, oligonucleotides were designed to introduce recognition sites for restriction endonucleases (recognition sites underlined; restriction endonucleases indicated).
DNA microarray hybridization, signal detection and data analysis. DNA array studies were performed as described by Hüscher et al. (2003, 2005) using PCR-based genomic DNA microarrays of C. glutamicum ATCC 13032 containing four replicates per gene. For each microarray hybridization, differently labelled cDNA probes, one from the wild-type strain grown under normal conditions and one from the induced or mutant strain, were combined, vacuum-dried, and dissolved in 200 µl DIG EasyHyb hybridization solution (Roche Diagnostics). ‘Dye swap’ was performed. The DNA microarrays were prehybridized with DIG EasyHyb for 45 min at 45 °C, washed in MilliQ H2O for 1 min, immersed in ethanol for 10 s, and finally centrifuged for 3 min at 185 g. The dried DNA microarrays were incubated in preheated (55 °C) hybridization chambers. The combined hybridization probes were denatured by incubation at 65 °C for 5 min, and 70 µl samples placed in the hybridization chambers for 15 h at 45 °C. Following the hybridization procedure, the DNA microarrays were washed and dried as described previously. Two biological replicates (RNAs) were used per assay, which provided eight samples per gene (four gene replicates in each slide).

Microarray signal acquisition was performed with a ScanArray 4000 scanner (Perkin-Elmer). The two fluorophores were excited and detected separately, and the resulting data were stored with a pixel size of 10 µm. Spot finding, signal background segmentation, and intensity quantification were performed with the ImaGene 5.0 software package (BioDiscovery). Normalization was performed with the LOWESS function (Yang et al., 2002), which computes the logarithmic intensity ratio and the logarithmic mean signal intensity for each spot. t-Test statistics were performed with the EMMArray microarray data analysis software (Dondrup et al., 2003). Genes were regarded as being differentially expressed if P \leq 0.05, A \geq 9.0, and M \geq 0.6 or M \leq −0.6.

RESULTS

Transcriptional analyses under different heat-shock conditions

The occurrence of a ‘differential response’ similar to that of E. coli (Yura & Nakahigashi, 1999) was investigated in C. glutamicum, since such differential control has only been reported for the clpC and clpP1P2 genes (Engels et al., 2004). The main systems involved in the heat-shock response of C. glutamicum (HrcA/CIRCE and HspR/HAIR; Barreiro et al., 2004) were studied by Northern blot analysis using specific probes internal to the dnaK (controlled by HspR) and groES-EL1 (regulated by HrcA) operons. Transcriptional analyses were performed under four different combinations of moderately (40 °C) or severely increased (50 °C) temperatures and different treatment times, as follows (Fig. 1): (i) moderate temperature for a long time (40 °C, 60 min); (ii) severe temperature shock for a short time (50 °C, 7 min); (iii) severe induction temperature for a short time (50 °C, 7 min), followed by 30 min of normal growth conditions (recovery time); and (iv) severe temperature of induction for a long period of time (50 °C, 30 min). The growth rates are shown in Fig. 1(c). Results of Northern blot analyses indicated that the dnaK operon (HspR/HAIR system) exhibits a clear overexpression under moderate or severe heat-shock conditions (Fig. 1a). On the other hand, the groES-EL1 operon (HrcA/CIRCE system) showed a stronger response to 40 °C than to 50 °C, and an unexpectedly low expression at 50 °C under prolonged heat-shock conditions (Fig. 1b). These results strongly suggest a ‘differential response’ to heat shock in C. glutamicum, and a negative effect of the severe heat conditions on the HrcA-regulated genes but not on the HspR-dependent system.

Microarray analysis of the global transcription response under moderate and severe heat-shock conditions

The results of a comparative microarray hybridization analysis of the heat-shock response in C. glutamicum under moderate (40 °C, 60 min) or severe heat-shock conditions (50 °C, 10 min) are shown in Fig. 2. Under moderate heat-shock conditions 358 genes were upregulated, whereas 420 genes were downregulated (Fig. 2a). On the other hand, the severe heat shock induced 555 genes and repressed 519 genes (see supplementary data). These groups included all the previously described genes or proteins involved in heat shock in C. glutamicum (Muffler et al., 2002; Barreiro et al., 2004, 2005; Engels et al., 2004). Thus, the whole set of genes reported by Muffler et al. (2002), including the oxidative stress-related genes, were found to be upregulated under moderate heat-shock conditions (except the protease gene clpP2) (Fig. 2a). Also, the clpC and clpP1 genes, which were initially described as exclusively induced under severe heat-shock conditions (50 °C) (Engels et al., 2004), were also found to be upregulated under moderate conditions. In addition, the ppo gene (formerly poxB), encoding a pyruvate quinone oxidoreductase (pyruvate oxidase) (Schreiner et al., 2006), was upregulated by heat shock, supporting the data previously obtained by proteomics (Barreiro et al., 2005).

The genes of the heat-shock stimulon described in the Gram-positive model bacterium B. subtilis (Helmann et al., 2001; Schumann, 2003), which are present in C. glutamicum, were also upregulated. In addition, the effect of the thermal stress could be observed on hundreds of genes different from those typically involved in the heat-shock response, including several transcriptional regulators. Thus, the regulators Cg0454 and the ArsR-type Cg1211 (Brune et al., 2005) were induced under both conditions (moderate and severe heat shock). On the other hand, some transcriptional regulators showed modified transcriptional patterns only under moderate heat-shock conditions. This group includes the cg2686 gene, encoding a TetR-family transcriptional regulator, and the cg2889 gene, encoding a MerR-family regulator (Brune et al., 2005).

Likewise, other genes were specifically upregulated under severe heat-shock conditions, including the putative anti-sigma factor gene rshA (cg0877), which is connected to the regulation of the sigH (cg0876) and rel (cg1861) genes (Brockmann-Gretza & Kalinowski, 2006). These included the OxyR (cg2109) regulator, which plays an important antioxidant role in E. coli and Pseudomonas putida (Hishinuma et al., 2006), the argR gene (cg1585), which
encodes the main repressor of arginine biosynthesis and uptake genes in *E. coli* (Caldara *et al.*, 2006), and the sulphate metabolism regulator *cysR* (*cg0156*). The *clgR* gene, which is one of the regulators of the ClpCP protease genes (Engels *et al.*, 2004), was also induced under severe stress, but not under moderate heat shock.

In contrast, several genes were found to be downregulated under moderate or severe heat-shock stress. The most interesting group of such genes was the *F₀F¹* ATPase operon (*atpIBEFHAGDC*), which is involved in the formation of ATP using the electrochemical force of the membrane proton gradient. Some transcriptional regulators were specifically downregulated under moderate stress conditions, e.g. the transcriptional regulator WhiB2 (*cg0830*) and the iron-metabolism regulator DtxR (Brune *et al.*, 2006), which is involved in the control of oxidative stress in *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* (Dussurget *et al.*, 1996; Rodriguez *et al.*, 2002) (Fig. 2a). On the other hand, the genes *cg0350* (*glxR*), *cg1648* and *cg2320*, encoding predicted transcriptional regulators (Brune *et al.*, 2005), were downregulated under severe but not under moderate heat-shock conditions. The analysis of the proteome of *C. glutamicum* following heat shock (Barreiro *et al.*, 2005) revealed a downregulation of the *ppiA* gene, which encodes a foldase (peptidyl-prolyl cis-trans isomerase B). The transcriptomic studies (Fig. 2b) confirmed the repression of the *ppiA* gene under severe heat stress.

### Physiological relevance of the expression changes: clusters of orthologous groups of proteins (COG)

The genes found to be significantly regulated in the microarray experiments were arranged according to the classification of their encoded proteins by the COG system (Tatusov *et al.*, 2000). Fig. 3 shows that the translation-involved genes, including the ribosomal operons (COG class J), were the most intensely repressed at 40 °C. Members of the same ribosomal clusters appear to be repressed in a concerted form, e.g. the *ɛ* cluster (*rpsM*, *rpsK*, *rpsD*, *rpoA* (alpha chain of the RNA polymerase), *rplQ*) (Martin *et al.*, 2003). In addition, the L11 operon (*rplK*, *rplA*) (Barreiro *et al.*, 2001; Wehmeier *et al.*, 2001), which is
Fig. 2. Microarray analysis of the wild-type strain under different heat-shock conditions. Ratio/intensity plot obtained from DNA microarray experiments comparing the transcriptome of the wild-type C. glutamicum ATCC 13032 grown at 30 °C with that of C. glutamicum ATCC 13032 heat-induced at 40 °C (a) or 50 °C (b). Total RNA was isolated from two biological replicates and used for hybridizations. Hybridization data were filtered to identify genes with at least three significant measurements out of the four replicates present on the DNA microarray and an error probability of less than 5% for Student’s t test. Genes showing differential expression are indicated by green diamonds (upregulated) or red triangles (downregulated); those not showing differential expression are indicated by grey spots. WT, wild-type. The relevant genes are indicated by their names.
involved in the stringent response, is also co-ordinately repressed under moderate conditions.

At 50 °C, a larger number of the transcription-involved genes (COG class K) were upregulated. This class includes genes expressed under severe conditions for several transcriptional regulators (cysR, cg0579 and pdxR), the transcription-repair coupling factor Mfd (cg1099) and the DNA/RNA helicase cg1843. Moreover, the gene encoding the sigma factor $\sigma^B$ (cg2102), which plays a role in the response to environmental stress factors (Halgasova et al., 2002; Larisch et al., 2007), was also upregulated under severe conditions.

The upregulation of the amino acid transport and metabolism genes (COG class E) (Fig. 3) at 50 °C is especially interesting, since several amino acid production processes are performed under relatively high-temperature conditions (Delaunay et al., 2002; Uy et al., 2003; Ohnishi et al., 2003). This COG class includes several membrane transporter genes (cg2676, cg2678), aminotransferase genes [cg2680, pat (cg0267)], genes involved in the control of carbon flow through the shikimate pathway [aroF (cg1129), aroG (cg2391)] (Garner & Herrmann, 1985), and those involved in leucine biosynthesis [leuA (cg0303)] (Patek et al., 1994), all of which were only induced under severe thermal conditions (Fig. 2b).

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**Fig. 3.** COG classes of the proteins encoded by the differentially expressed genes. Distribution into COG classes is shown for the proteins encoded by genes that showed enhanced (positive values) or repressed (negative values) expression during growth of *C. glutamicum* at 40 °C (open bars) or 50 °C (filled bars). Grouping into functional categories (described below the histogram) was performed according to the classification scheme of the COG database (Tatusov et al., 2000).
**hrcA and hspR null mutants: Northern analyses of heat-shock genes**

The HrcA regulator binds to the CIRCE motifs of the groES-EL and dnaK operons in B. subtilis (Zuber & Schumann, 1994). In contrast, CIRCE boxes have only been described upstream of the groES-EL1 operon and the groEL2 gene in C. glutamicum (Barreiro et al., 2004). To analyse HrcA/CIRCE regulation, a C. glutamicum ΔhrcA strain was constructed by replacing the chromosomal hrcA gene by an apramycin-resistance marker.

The effect of the deletion of the hrcA gene was tested by analysing the transcriptional pattern of the groES-EL1 operon, which contains two CIRCE sequences within the promoter region. This operon was overexpressed in the C. glutamicum ΔhrcA strain under normal and heat-shock conditions (Fig. 4a), in agreement with the role of HrcA as a repressor.

The dnaK operon of C. glutamicum and other actinobacteria encodes its own autoregulatory repressor designated HspR (Bucca et al., 1995). A C. glutamicum ΔhspR strain was obtained by using the apramycin-resistance gene to delete the chromosomal copy of the hspR gene.

The effect of the deletion of the hspR gene was analysed by means of the transcriptional pattern of the dnaK operon, which contains one HAIR motif in the promoter region. Results showed an overexpression of the dnaK operon in the ΔhspR mutant under normal and thermal stress conditions (Fig. 4b). The deletion of the regulatory genes (hspR or hrcA) did not affect the growth rate of the generated mutant strains, as shown in Fig. 4(c).

**Microarray analysis of hrcA and hspR null mutants**

A microarray approach was used to obtain a complete view of the regulatory systems controlled by the HrcA and HspR regulators. The signal intensity ratio (m)/signal intensity (a) plots deduced from hybridizations using RNA obtained from C. glutamicum wild-type and ΔhspR or ΔhrcA strains growing under normal conditions (30 °C) are shown in Fig. 5. The highest overexpression in the ΔhrcA mutant strain was observed for the groEL2, groES and groEL1 genes (Fig. 5a), which are dependent on the HrcA/CIRCE repression system and contain CIRCE boxes in their promoter regions (Barreiro et al., 2004). The upregulation of the groEL genes in the ΔhrcA strain agrees with the data...
described for the genus *Streptomyces* (Grandvalet et al., 1998; Servant & Mazodier, 2001). Other genes that did not contain heat-shock recognition motifs in their promoter region were weakly overexpressed in the ΔhrcA strain. The upregulation of the *tagA1* (cg3140) gene, which is located 3' to and in the opposite orientation to the *hmp* (cg3140) gene in the *C. glutamicum* genome, could be due to a read-through effect of *hmp*, which is always induced under heat-shock conditions.

The analysis of the *hspR*-deficient strain under normal growth conditions showed a greater number of over-expressed genes than in the ΔhrcA mutant (Fig. 5b). Thus, the genes *grpE* and *dnaJ*, which are located in the dnaK operon, were upregulated. This operon has a HAIR motif upstream of the *dnaK* gene, which is the first gene of the operon (Barreiro et al., 2004). Similarly, the *clpB* operon (*clpB*, cg3078) and *clfR*, which contain two and one HAIR elements, respectively, were upregulated under normal growth conditions in the *hspR*-deficient strain. Similarly, the deletion of the *hspR* gene in *S. coelicolor* (Bucca et al. 2003) upregulates genes homologous to those described here, with the exception of *lon* (not present in *C. glutamicum*) and *clfR*, which has not been described as HspR/HAIR-controlled in *Streptomyces*. The induction of other genes in the *hspR*-deficient strain could be explained by a cascade of regulation due to the induced proteins, or by the presence of a less conserved HAIR motif-like sequence similar to that present in the promoter region of the *dnaJ*-hspR operon (CTTGAAATNNNNNNACTCA AaG, bold type indicates identity with consensus sequence) or in the *groEL2* gene (Barreiro et al., 2004).

**Validation of the differential expression by q-RT-PCR**

The different mechanisms of response to heat shock exhibited by the HAIR- or CIRCE-regulated genes under different stress conditions were further studied by q-RT-PCR (Fig. 6). The genes containing HAIR motifs in their promoter regions exhibited a clear induction under moderate heat-shock conditions. Also, all these genes exhibited the strongest induction levels under severe heat-shock conditions (Fig. 6, WT 50 °C), e.g. the induction factor (ratio 50 °C: 40 °C) of the *clpB* gene is 4.8 times higher under severe conditions. In addition, the *clpC* gene, which is part of the ClpCP protease complex and indirectly regulated by the HspR system through the *clfR* regulator, showed a stronger upregulation, in concordance with the data obtained by microarrays (Fig. 2). Thus, the HAIR-regulated genes show a clear ‘differential response’ that is stimulus strength-dependent.

The whole set of tested HspR-controlled genes were over-expressed at 30 °C in the *hspR*-deficient strain with similar intensities to the expression in the wild-type or ΔhspR strains growing at 40 °C. Also, due to the cross-talk among heat-shock systems, a strong repression was observed in the profile of the *groEL1* gene (Fig. 6, ΔhspR 30 °C). Two genes directly controlled by the HrcA regulator (*groEL1*, *groEL2*) and the repressor of the system (*hrcA*) were analysed by q-RT-PCR. Both *groEL* genes showed upregulation at 40 °C, but did not exhibit stronger expression at 50 °C (Fig. 6), which contrasts with the ‘differential response’ observed for the HspR-controlled genes. Under severe heat-shock conditions, the *groEL2* gene maintains the same high level of induction, whereas the *groEL1* gene seems less overexpressed than at 40 °C (its 40 °C : 50 °C ratio was two times lower), in agreement with the results of Northern blot assays (Fig. 1b).

Under stress conditions, the *groEL2* gene presents an identical level of induction independently of the stress strength or the analysed strain. The presence of a modified HAIR motif in the promoter region in addition to two CIRCE motifs (Barreiro et al., 2004) explains the q-RT-PCR data and suggests the existence of some cross-regulation of the *groEL2* gene.

Analysis of the complete genome of *C. glutamicum* revealed seven sigma factor-encoding genes (Kalinowski et al., 2003). The principal sigma factor SigA and the non-essential sigma factor SigB, which is involved in the stress response and modulation of gene expression during the transition growth phase, have been studied previously (Oguiza et al., 1996; Halgasova et al., 2001, 2002; Larisch et al., 2007). In addition, the sigma factor SigH has been described as being involved in the heat- and oxidative-stress responses (Kim et al., 2005). The microarray analysis of the heat-shock response showed upregulation of the *sigB* gene under severe heat-shock conditions (Fig. 2b), whereas the *sigH* gene did not change its expression under any conditions. In contrast, the *sigE* gene was induced under both conditions of stress in the wild-type strain and the *hrcA* mutant (Figs 2 and 5a). The results of q-RT-PCR studies showed that the induction of *sigE* was slightly higher under moderate stress than under extreme conditions (Fig. 6), which agrees with the microarray studies. The deletion of the *hspR* repressor gene influenced the upregulation of *sigE* under heat-shock conditions, since its transcription ratio was smaller. On the other hand, the *hrcA* deletion did not seem to exert a significant effect on the *sigE* expression under heat-shock stress (Fig. 6).

**DISCUSSION**

The present article confirms and expands the group of genes influenced by heat shock in *C. glutamicum*. The set of heat-shock proteins is similar to that observed in other actinobacteria closely related to *C. glutamicum* (Bucca et al. 2003; Stewart et al., 2002; Rezzonico et al., 2007). Novel, interesting genes and regulatory pathways not traditionally thought to be involved in the heat-shock response are described. Thus, the protein Pqo, previously observed to be heat-induced (Barreiro et al., 2005), was confirmed to be upregulated under both studied conditions in the wild-type strain; the pyruvate oxidase-encoding gene of *E. coli* is also
Fig. 5. Microarray analysis of the effect of deletion of the hrcA and hspR regulatory genes. Ratio/intensity plot obtained from DNA microarray experiments comparing the transcriptome of the wild-type C. glutamicum ATCC 13032 grown at 30 °C with that of C. glutamicum carrying an hrcA (a) or hspR gene deletion (b) grown at 30 °C. Total RNA was isolated from two biological replicates. Hybridization data were filtered to obtain genes with at least three significant measurements out of the four replicates present on the DNA microarray and an error probability of less than 5% for Student’s t test. Genes showing differential expression are indicated by green diamonds (upregulated) or red triangles (downregulated); those without differential expression are indicated by grey spots. WT, wild-type. The relevant genes are indicated by their names.
sensitive to environmental osmotic stress (Weber et al., 2006). Two genes of the protease complex ClpCP (clpC and clpP1) were found to be upregulated under moderate heat-shock conditions, in contrast to the results reported by Engels et al. (2004). This induction of the clpC gene was confirmed by q-RT-PCR (Fig. 6, 40 and 50 °C).

The F$_0$F$_1$ ATPase operon is downregulated under heat-shock conditions. In addition, this operon is induced in C. glutamicum under basic pH conditions (Barriuso-Iglesias et al., 2006) and is strongly repressed by phosphate deprivation in S. coelicolor (Rodríguez-García et al., 2007). In E. coli it is sensitive to osmoregulation (Akopyan & Trchounian, 2006). These data show that the F$_0$F$_1$ ATPase operon is an environment-sensitive cluster and HspR- and HrcA-independent, since the regulator-defective strains were not altered in its regulation (Fig. 5).

Several COG classes (except F, N and V) show high upregulation under severe heat-shock conditions. Fifty-six transcriptional regulators (COG class K) that perform 411 regulatory interactions have been recently described in C. glutamicum (Brinkrolf et al., 2007). Changes in the levels of the transcriptional regulators affect the expression of the regulatory cascades present in C. glutamicum and explain the large number of genes affected by heat shock. Thus, numerous genes, not members of known heat-shock operons, have also been identified as heat-induced in B. subtilis (Helmann et al., 2001).

Recent studies of different C. glutamicum strains have shown enhanced amino acid production by growing the cultures at temperatures higher than 30 °C (Delaunay et al., 2002; Uy et al., 2003; Ohnishi et al., 2003; Ohnishi & Ikeda, 2006). These data correlate with the increment of expression of the genes involved in amino acid transport and metabolism under thermal stress (COG class E) (Helmann et al., 2001).

Northern analyses (Fig. 4) and q-RT-PCR (Fig. 6) confirmed the strong derepression of the predicted HrcA/CIRCE-controlled genes (groES-EL1 operon and groEL2 gene) observed in the C. glutamicum hrcA null mutant. This control by the HrcA protein has also been described in other actinobacteria (Grandvalet et al., 1998; Servant & Mazodier, 2001). The HrcA-regulated genes in C. glutamicum and Streptomyces species (Servant & Mazodier, 2001) are fewer than those controlled in the cyanobacterium Synechocystis, in which 38 genes show a modified expression pattern in an hrcA null strain (Singh et al., 2006). Nevertheless, the constant induction level of the groEL2 gene under thermal-stress conditions (strain- and stimulus strength-independent) is highly interesting. The presence of two CIRCE motifs and one modified HAIR box in its promoter region (Barreiro et al., 2004) may explain this constant induction level in contrast with the variations observed in the groEL1 gene (Fig. 6). This modified HAIR element is also present in the promoter region.
of the groEL2 gene of Corynebacterium diphtheriae and Corynebacterium efficiens.

In B. subtilis, GroESL modulates the HrcA regulon by controlling the availability of the active form of HrcA (Mokg et al., 1997). In C. glutamicum, the weak heat induction detected for the hrcA gene (q-RT-PCR, microarrays), the lack of CIRCE sequences in its promoter region and the weakness of its promoter activity (data not shown) suggest that the main influence on the regulation of the HrcA/CIRCE system is due to changes in the co-repressor (GroEL) rather than to drastic changes in the HrcA protein itself.

'Differential response' refers to the intensity-dependent response of some regulatory systems or individual genes to a received stimulus. Thus, the response to heat shock has been described as temperature-dependent in Gram-negative bacteria (Yura & Nakahigashi, 1999). Among Gram-positive bacteria, M. tuberculosis shows a conditional response to heat shock (Young & Garbe, 1991), whereby the groEL gene shows higher induction under moderate heat shock than under severe conditions. In C. glutamicum, the Northern blot (Fig. 1) and q-RT-PCR analyses (Fig. 6) of the groES-EL1 operon showed the highest induction level at 40 °C, which perfectly correlates with that described in M. tuberculosis. On the other hand, the dnaK gene in M. tuberculosis exhibits higher induction under severe heat-shock conditions, which agrees with the result observed by q-RT-PCR in C. glutamicum (Fig. 6). Recently, two new examples of 'differential response' to heat shock in Gram-positive bacteria have been described. Ventura et al. (2006) have reported the 'differential response' to heat stress in the bifidobacterium Bifidobacterium breve, and Musatova et al. (2006) have described a differential expression of the cipB and lon genes correlated with the stress intensity in the human-pathogenic bacterium Mycoplasma genitalium.

The different response to heat shock in C. glutamicum suggests the presence of different thermosensor systems, each measuring a particular level of stress. An interesting question is why the HspR/HAIR-controlled genes exhibit a transcription pattern that is responsive to heat-stress intensity. In C. glutamicum, the HspR-controlled genes have HAIR motifs in their promoter region and $\sigma^H$-specific −10 boxes as common regulatory elements (Engels et al., 2004; Barreiro et al., 2004), and this is also the case in other actinobacteria such as Mycobacterium (Song et al., 2003). However, the observed 'differential response' of the HAIR-containing genes is HspR-independent, since under moderate heat shock, these genes show identical behaviour in the wild-type and the hspR null mutant (Fig. 6). Therefore, the 'differential response' to heat shock might be due to SigH changes rather than to HspR concentrations. The sigH operon of the genus Mycobacterium includes the sigH gene and the anti-sigma H factor (rshA) (Song et al., 2003). An identical arrangement has been described in the genome of C. glutamicum (sigH-rshA; Kalinowski et al., 2003) and Corynebacterium jeikeium (Tauch et al., 2005).

In vitro, analysis of the 'differential response' to heat stress in M. tuberculosis has shown that only severe heat stress (53–55 °C) disrupts the SigH–RshA interaction (Song et al., 2003). The disruption of the SigH–anti-sigma factor complex in C. glutamicum under severe heat-shock conditions might release SigH to modulate the 'differential response' observed in the HspR/HAIR-controlled genes. In addition, transcription of the sigB and sigE genes is induced by SigH in mycobacteria (Song et al., 2003). In C. glutamicum, the transcription of sigE and sigB is enhanced under heat-shock conditions (Fig. 2b); this could be a hint of a regulatory cascade between the sigma factors SigH, SigB and SigE, as found in M. tuberculosis (Song et al., 2003).

The heat-shock response in C. glutamicum demonstrates the cross-talk of several regulons due to the effect of thermal stress on sigma factors, transcriptional regulators and genes involved in different cell activities. The over-expression effect of the HspR/HAIR-regulated genes on the HrcA/CIRCE-controlled genes and vice versa is an example of such cross-talk (see groEL1 transcription pattern; Fig. 6, ΔhspK 30 °C).

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