Regulation of *Corynebacterium glutamicum* Heat Shock Response by the Extracytoplasmic-Function Sigma Factor SigH and Transcriptional Regulators HspR and HrcA^{∇§}

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Heat shock response in *Corynebacterium glutamicum* was characterized by whole-genome expression analysis using a DNA microarray. It was indicated that heat shock response of *C. glutamicum* included not only upregulation of heat shock protein (HSP) genes encoding molecular chaperones and ATP-dependent proteases, but it also increased and decreased expression of more than 300 genes related to disparate physiological functions. An extracytoplasmic-function sigma factor, SigH, was upregulated by heat shock. The SigH regulon was defined by gene expression profiling using *sigH*-disrupted and overexpressing strains in conjunction with mapping of transcription initiation sites. A total of 45 genes, including HSP genes and genes involved in oxidative stress response, were identified as the SigH regulon. Expression of some HSP genes was also upregulated by deletion of the transcriptional regulators HspR and HrcA. HspR represses expression of the *clpB* and *dnaK* operons, and HrcA represses expression of *groESL1* and *groEL2*. SigH was shown to play an important role in regulation of heat shock response in concert with HspR and HrcA, but its role is likely restricted to only a part of the regulation of *C. glutamicum* heat shock response. Upregulation of 18 genes encoding transcriptional regulators by heat shock suggests a complex regulatory network of heat shock response in *C. glutamicum*.

Corynebacterium glutamicum is a nonpathogenic, highly G+C gram-positive bacterium that belongs to the order *Actinomycetales*, which includes the genera *Mycobacterium* and *Streptomyces*. *C. glutamicum* has been widely used for the industrial production of various amino acids and nucleic acids (19, 20, 47). We previously demonstrated that growth-arrested *C. glutamicum* cells under conditions of oxygen deprivation exhibit high productivity of ethanol, D-lactic acid and succinic acid (22, 36, 37). Meanwhile, this species is of increasing interest as a model organism for closely related pathogenic species, such as *Corynebacterium diphtheriae*, *Mycobacterium tuberculosis*, and *Mycobacterium leprae* (4, 32, 33).

The heat shock response is the most frequently used model system for studying the impact of stress on biological systems. All organisms respond to a sudden increase in temperature with elevated expression of a set of highly conserved proteins, termed heat shock proteins (HSPs), which include molecular chaperones that assist folding nascent proteins and repairing damaged proteins and ATP-dependent proteases that degrade misfolded proteins (9, 49). Genes with various physiological functions in addition to HSPs have been identified as being part of a heat shock stimulon by whole-genome expression analysis using DNA microarrays (6, 18, 41).

The heat shock response is controlled by a combination of alternative sigma factors that recognize specific heat shock

promoters upstream of heat shock genes and transcriptional regulators that act as the repressors or activators by binding to the promoter regions of certain heat shock genes (48, 52). In *Mycobacterium tuberculosis*, the extracytoplasmic-function (ECF) sigma factor SigH plays a central role in a regulatory network of heat and oxidative stress responses (31, 39). Two transcriptional repressors, HrcA and HspR, are responsible for control of heat shock genes of *M. tuberculosis* in association with DNA elements called CIRCE (for controlling inverted repeat of chaperone expression) and HAIR (for HspR-associated inverted repeat), respectively (45). In *Streptomyces albus* G, another transcriptional repressor, RheA, in addition to HrcA and HspR, is involved in heat shock regulation (15, 16, 43).

In *C. glutamicum*, expression of some HSPs has been shown to be upregulated by heat shock (1, 2, 12). Detailed analysis of transcriptional regulation of the *clpP1-clpP2* operon and the *clpC* gene, each encoding a subunit of ATP-dependent protease, revealed involvement of the ECF sigma factor SigH of *C. glutamicum* in their expression (12). In addition, sequence analyses of promoter regions of *groES*, *groEL2*, and *dnaK* allow the identification of CIRCE and HAIR elements close to the –10 and –35 regions of the promoters, suggesting involvement of HrcA and HspR in regulation of heat shock response in *C. glutamicum* (2). Although our knowledge of *C. glutamicum* heat shock response has increased during the past decade, transcriptional responses to heat shock and its regulatory systems have not been analyzed at the whole-genome level.

In the present study, we defined the heat shock stimulon of *C. glutamicum* using DNA microarray. Expression of about 10% of the genes on the chromosome related to disparate cellular functions was shown to respond to heat shock. We elucidated the regulatory roles of SigH, HrcA, and HspR in

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response to heat shock by combining whole-genome expression analyses with targeted mutagenesis. In identifying the 45 genes that comprise the SigH regulon, we showed that in addition to ClpP1, ClpP2, and ClpC, expression of most HSPs, including DnaK, GrpE, DnaJ, ClpB, DnaJ2, GroES, GroEL1, and GroEL2, was regulated by SigH in concert with HrcA and HspR. Finally, we identified 18 genes encoding transcriptional regulators that were upregulated by heat shock, suggesting complex regulatory systems of *C. glutamicum* heat shock response.

MATERIALS AND METHODS

Bacterial strains, culture media, and growth conditions. *C. glutamicum* strain R (51) and its derivatives were grown at 33°C in medium A with 4% (wt/vol) glucose as described previously (11). *C. glutamicum* cells were grown at 33°C until they reached an optical density at 610 nm of 2 to 3. The culture temperature was raised to 45°C in a water bath, and then cells were incubated on a rotary shaker at 45°C. The culture was mixed with an equal amount of the RNeasy lysis reagent (Qiagen, Hilden, Germany) 15 min after heat shock and settled at room temperature for 10 min. The cells were harvested by centrifugation and stored at -80°C until they were used to prepare RNA.

Mutant construction. *sigH*, *sigM*, and *hrcA* disruptants were constructed by the transposon-mediated mutagenesis method as described previously (46). Transposon insertion locations were identified based on the sequences of thermal asymmetric interlaced-PCR products of mutant cells. In each disruptant, a transposon was inserted at 426, 162, and 476 bases downstream of the 5' end of the genes *sigH*, *sigM*, and *hrcA*, respectively. Construction of an *hspR* disruptant was carried out as follows. The *hspR* coding region was amplified by PCR using the primer pair 2687-F and 2687-R (see Table S1 in the supplemental material) and cloned between the EcoRI and PstI sites of pHSG398 (Takara Bio, Shiga, Japan). A kanamycin resistance cassette was inserted into the unique EcoO65I site that lies at 144 bases downstream of the 5' end of the *hspR* gene. The resultant plasmid was transferred by electroporation into *C. glutamicum*, and a strain resistant to kanamycin and sensitive to chloramphenicol was selected. Disruption of the *hspR* gene was confirmed by PCR. Construction of *sigH*-overexpressing shuttle vector pCRD601 was carried out as follows. A DNA fragment containing the *sigH* promoter and coding regions was amplified by PCR using the primer pair sigH-F and sigH-R (see Table S1 in the supplemental material) and was cloned between the PstI and EcoRI sites of pCRB1 (23). pCRD601 was transferred by electroporation into *C. glutamicum*.

RNA isolation and DNA microarray analysis. Total RNA was extracted from *C. glutamicum* cells by using the RNeasy mini kit (Qiagen) and treated with DNase I (Takara Bio) as described previously (11). Global gene expression analysis was performed with the *C. glutamicum* R DNA microarray as described previously (11). Microarray analyses were carried out using two sets of RNA samples isolated from independently grown cultures with different combinations of Cy dyes (a dye swap strategy). Since the *C. glutamicum* R DNA microarray contains two replicates per gene, a total of four replicates per gene were available to determine changes in gene expression. Genes with significantly differential transcript levels ($P < 0.05$; Student's *t* test) by at least a factor of two were determined.

Mapping of transcription initiation sites by RACE-PCR. Transcription initiation sites were determined by using the SMART RACE cDNA amplification kit (Clontech, CA) with RNA prepared from cells subjected to heat shock. 5' Rapid amplification of cDNA ends (RACE)-PCR analyses were carried out as recommended by the supplier with 1 µg of total RNA and gene-specific primers (see Table S1 in the supplemental material). Resulting PCR products were cloned into a pGEM-T Easy vector by using the pGEM-T Easy vector system (Promega Corporation, WI). At least 10 clones for each 5' RACE-PCR product were sequenced by the dye terminator method using the BigDye Terminator v3.1 cycle sequencing kit and the 3130xl Genetic Analyzer (Applied Biosystems, CA).

Real-time qRT-PCR. A one-step real-time quantitative reverse transcription-PCR (qRT-PCR) analysis was performed with the Power SYBR green PCR master mix (Applied Biosystems) and a pair of gene-specific forward and reverse primers (see Table S1 in the supplemental material) by using the 7500 Fast real-time PCR system (Applied Biosystems) as described previously (11). Relative ratios were normalized with the value for 16S rRNA and are represented as means of triplicate measurements.

A two-step real-time qRT-PCR was performed to determine the transcript levels of *cgR_2620* and *cgR_2674*. cDNA was synthesized from total RNA with

reverse primers, RT16S-R, RT2620-R, and RT2674-R, as follows. An aliquot (1 µg) of total RNA and 1 pmol each of reverse primers were denatured at 90°C for 5 min and then gradually cooled to 55°C. The RT reaction was performed in 20 µl of first-strand buffer (50 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM MgCl₂) containing total RNA, reverse primers, 5 mM dithiothreitol, 0.5 mM each of dATP, dGTP, dCTP, and dTTP, and 200 U of SuperScript III RNase H⁻ reverse transcriptase (Invitrogen, CA). The reaction mixture was incubated at 55°C for 1 h, and then the reaction was stopped by the addition of 80 µl of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). An aliquot (1 µl) of the cDNA solution was taken for real-time qPCR analysis. For the experiment with 16S rRNA, the cDNA solution was further diluted 10-fold.

RESULTS

Global changes in gene expression in response to heat shock. There are 13 genes encoding HSPs in the genome of *C. glutamicum*. Present are a single gene each for *groES* (*cgR_0715*), *grpE* (*cgR_2689*), *clpX* (*cgR_2269*), *clpB* (*cgR_2676*), and *clpC* (*cgR_2580*) and two paralogues each for the genes *groEL*, *dnaK*, *clpP*, and *dnaJ*, designated *groEL1* (*cgR_0716*) and *groEL2* (*cgR_2619*), *dnaK* (*cgR_2690*) and *dnaK2* (*cgR_2260*), *clpP1* (*cgR_2308*) and *clpP2* (*cgR_2307*), and *dnaJ* (*cgR_2688*) and *dnaJ2* (*cgR_2162*), respectively. Some of these genes constitute operons, such as *groES-groEL1*, *dnaK-grpE-dnaJ*, and *clpP1-clpP2*. The genes *hspR* and *hrcA* encoding transcriptional regulators are located next to the *dnaK-grpE-dnaJ* operon and the *dnaJ2* gene, respectively.

A gene expression profile of *C. glutamicum* cells subjected to heat shock (45°C) for 15 min was compared to that of cells grown at 33°C. The transcript levels of 157 genes were increased, while those of another 147 genes were decreased at least twofold by heat shock (see Tables S2 and S3 in the supplemental material). In addition to genes such as *clpC*, *clpP1*, *clpP2*, *groES*, *groEL1*, *groEL2*, and the *dnaK* operon that have been shown to be heat inducible in previous studies (2, 12), most of the HSP genes except *dnaK2* and *clpX* were upregulated by heat shock. Genes such as *msrA*, *mtf*, *trxB*, *msrB*, and *trxC*, involved in oxidative stress response, as well as the *cys* genes that are involved in sulfate reduction and the *suf* gene cluster that is involved in iron-sulfur cluster formation were also upregulated. Elevated expression of the genes *sigB* and *sigH* encoding RNA polymerase sigma factors indicates that these sigma factors should take part in regulation of gene expression in response to heat shock.

The downregulated genes included genes of glycolysis (*ptsG*, *gapA*, *glgP1*, *fba*, *eno*, *pyk*, *pfkA*, and *ptsF*), the tricarboxylic acid cycle (*sdhCAB*, *acn*, *lpd*, *fum*, *sucB*, *gltA*, *icd*, and *aceE*), and the pentose phosphate pathway (*gnd*). In addition, the transcript levels of genes involved in transcription and translation (*rpl* and *rps* genes, *infC*, *tig*, *fusA*, *rpoA*, *nusB*, and *efp*) and respiration (*qcrBAC*, *ctaEFC*, *ctaD*, *narkG*, and *ccsA*) and *atp* genes encoding F₁F₀-ATP synthase were decreased. Cell growth was arrested after heat shock (data not shown), resulting in downregulation of these genes.

Regulatory roles of SigH in expression of heat shock-responsive genes. The ECF sigma factor SigH has been shown to control expression of *clpP1-clpP2* and *clpC* in response to heat shock (12). Global regulation of the heat shock response by SigH was elucidated using a DNA microarray. Gene expression profiles of cells subjected to heat shock were compared between the wild type and the *sigH* disruptant. The transcript levels of 19 genes upregulated by heat shock were decreased by

sigH disruption (Table 1). In addition to *clpP1-clpP2* and *clpC*, expression of the *clpB* gene and the *dnaK* operon is downregulated in the *sigH* disruptant. Transcription from the promoters of *clpB*, *dnaK*, *trxB*, and *sufR* has been shown to depend on the other ECF sigma factor, SigM (34). In the *sigM* disruptant, the transcript levels of these genes at 45°C were comparable with or higher than those in the wild type, while they were significantly reduced in the *sigH* disruptant (Fig. 1). Moreover, DNA microarray analyses revealed that *sigM* disruption did not affect the transcript levels of heat-inducible genes (data not shown). These results indicate that SigM is not involved in regulation of gene expression in response to heat shock. Genes regulated by SigH were further identified using the *sigH*-overexpressing strain, in which the *sigH* gene was expressed from a multicopy plasmid, pCRD601. Global changes in gene expression during exponential growth by *sigH* overexpression were examined by a comparison with the strain harboring the control plasmid pCRB1. The transcript levels of 34 genes upregulated by heat shock were increased by the *sigH* overexpression even at 33°C (Table 1). A total of 37 genes that were downregulated in the *sigH* disruptant and/or upregulated in the *sigH*-overexpressing strain were considered to be under the control of SigH.

The *sigB* gene was also upregulated by heat shock (see Table S2 in the supplemental material). Gene expression profiles of cells subjected to heat shock were compared between the wild type and the *sigB* disruptant. However, disruption of the *sigB* gene did not affect the transcript levels of heat-inducible genes, while genes that have been previously shown to be under the control of SigB (11) were downregulated in the *sigB* disruptant (data not shown). SigB is not involved in regulation of the heat shock response.

Determination of a consensus sequence of SigH-dependent promoters. To determine the SigH-dependent promoter sequence, the operon structures of the genes under the control of SigH were analyzed. Analysis of the genetic organization of the respective genomic regions as well as searches for putative rho-independent transcription terminators was done using the TransTerm program (13), and nine putative operons were discovered among the candidate genes (Table 1). Taking into account these putative operon structures, 45 genes that constitute 29 transcriptional units were regarded as the SigH regulon (Table 1).

The sequence of a SigH-dependent promoter was previously estimated from promoter sequences of three genes, *clpP1*, *clpC*, and *clgR* (12). We tested whether promoter sequences of the newly identified members of the SigH regulon were comparable with the previously defined one by mapping the transcription initiation sites (TISs) using RACE-PCR. TISs of 10 genes that were downregulated by *sigH* disruption and also upregulated by *sigH* overexpression (see genes listed in Table 1) were determined, and promoter sequences were aligned with previously identified promoters (2, 12, 34) (Fig. 2A). Highly conserved -10 and -35 regions were observed within promoter regions of all the genes. Furthermore, these conserved sequences were analyzed for the presence of the upstream regions of genes downregulated by *sigH* disruption or upregulated by *sigH* overexpression (see genes listed in Table 1). Putative promoter sequences with the conserved -10 and -35 regions were found within the upstream regions of most genes, except for *groES-groEL1* and *groEL2* (Fig. 2B). A TIS of

cgR_2451 was determined by RACE-PCR, confirming transcription from the predicted promoter (Fig. 2B). The consensus sequences of the SigH-dependent promoter were defined to be YGTTGAA for the -10 region and GGGAATA for the -35 region.

Identification of the HspR and HrcA regulons. The *hspR* gene constitutes an operon with *dnaK-grpE-dnaJ*, which was induced by heat shock (Table 1). The *hrcA* gene is located immediately upstream of the *dnaJ2* gene, but it was not upregulated by heat shock. The regulons of HspR and HrcA were determined by comparing gene expression profiles at 33°C between the wild type and gene disruptants using a DNA microarray. Expression of the *dnaK* and *clpB* operons was upregulated by *hspR* disruption (Table 2). The HAIR elements are present in the *dnaK* and *clpB* promoter regions (Fig. 3A), indicating that these two operons are directly repressed by HspR. cgR_2674 was identified as belonging to an upregulated gene in the *hspR* disruptant by DNA microarray analysis, but qRT-PCR analysis showed that its transcript level was not affected by *hspR* disruption (data not shown).

In the *hrcA* disruptant, the transcript levels of *groES*, *groEL1*, cgR_1436, and *groEL2* were increased, while the *dps* transcript level was decreased (Table 2). In the promoter regions of *groES-groEL1* and *groEL2*, two CIRCE elements exist (Fig. 3B). A TIS of cgR_1436 was mapped by using RACE-PCR experiments (Fig. 3B). The promoter sequence of cgR_1436 was similar to that of the SigA-dependent promoter, TA(T/C)NNT for the -10 region and TTGACA for the -35 region (11), and a CIRCE element was found within its promoter region. These results indicate that HrcA acts as a transcriptional repressor for *groES-groEL1*, cgR_1436, and *groEL2*. No CIRCE element was found upstream of the *dps*-coding region. The *dps* gene was considered to be subject of an indirect regulation provoked by *hrcA* disruption. By qRT-PCR, cgR_2620 was indicated not to be regulated by HrcA.

Dual control of expression of *clpB* and *dnaK* by SigH and HspR. Expression of the *clpB* and *dnaK* operons was positively regulated by SigH (Table 1) and negatively regulated by HspR (Table 2). Transcriptional regulation of these genes was further investigated. The transcript levels of *clpB* and *dnaK* after heat shock in the wild type and the *sigH* disruptant were determined by qRT-PCR (Fig. 4). Both genes were induced by heat shock, even in the *sigH* disruptant, while the induction levels were reduced compared to that of the wild type. By using RACE-PCR experiments using RNA prepared from cells subjected to heat shock, two TISs were detected for *clpB* (Fig. 3A). The promoter proximal to the 5' end of the gene was the SigH-dependent promoter, and the distal promoter was similar to the SigA-dependent promoter. The *dnaK* operon is transcribed from two promoters, of which one is SigH dependent and the other is SigA dependent (Fig. 3A). The HAIR element is located at the promoter regions of *clpB* and *dnaK* to repress transcription from the SigA-dependent promoter (Fig. 3A). Thus, it is likely that induction of the *clpB* and *dnaK* operons by heat shock is accomplished by derepression of the SigA-dependent promoter and activation of the SigH-dependent promoter.

TABLE 1. Heat shock-inducible genes affected by *sigH* disruption or *sigH* overexpression^f

Gene ^c	Product	$\Delta sigH^a$	P^b	SigHox ^a	P^b	Heat shock ^d	P^b
Downregulated in the <i>sigH</i> disruptant and upregulated in the <i>sigH</i> -overexpressing strain							
cgR_0627	Hypothetical protein	-1.34	3.9E-02	2.29	3.2E-04	1.48	1.6E-05
cgR_1297	Thioredoxin domain-containing protein	-1.04	5.4E-03	1.29	2.9E-03	1.02	4.6E-04
cgR_1317 ^{*e}	tRNA-methyltransferase	-1.35	2.1E-02	1.52	4.9E-02	2.64	1.6E-02
cgR_1318 [*]	Hypothetical protein	-0.66	3.4E-03	1.29	1.3E-02	1.21	1.3E-02
cgR_1554	Hypothetical protein	-1.37	2.6E-03	1.21	2.6E-03	1.23	1.2E-03
cgR_1727 (<i>msrB</i>)	Peptide methionine sulfoxide reductase	-2.78	1.1E-03	2.43	9.1E-04	2.08	1.5E-03
cgR_2078	Hypothetical protein	-1.91	5.4E-03	2.30	6.3E-03	1.22	4.7E-02
cgR_2183	Alkanal monooxygenase α chain	-3.05	1.4E-04	3.47	2.2E-05	2.72	4.7E-03
cgR_2308 (<i>clpP1</i>) ^{†c}	ATP-dependent Clp protease, proteolytic subunit	-1.78	4.9E-02	1.85	3.0E-03	1.32	3.3E-03
cgR_2307 (<i>clpP2</i>) [†]	ATP-dependent Clp protease, proteolytic subunit	-1.55	4.3E-02	1.76	8.6E-04	1.22	1.1E-03
cgR_2320	Putative dithiol-disulfide isomerase	-1.41	2.6E-03	2.29	1.7E-04	1.11	1.9E-03
cgR_2580 (<i>clpC</i>)	ATP-dependent Clp protease, ATP-binding subunit	-1.46	2.1E-02	1.13	1.2E-04	2.25	4.9E-03
cgR_2676 (<i>clpB</i>) ^{‡c}	Molecular chaperone	-1.78	3.3E-02	1.82	2.6E-04	4.92	5.8E-04
cgR_2675 [‡]	Hypothetical protein	-1.79	3.4E-02	1.49	1.4E-04	4.16	2.8E-03
cgR_2690 (<i>dnaK</i>) ^{§c}	Molecular chaperone	-1.29	1.2E-01	1.57	7.6E-03	4.20	4.0E-06
cgR_2689 (<i>grpE</i>) [§]	Molecular chaperone	-1.17	6.9E-02	1.23	7.2E-03	4.29	3.2E-06
cgR_2688 (<i>dnaJ</i>) [§]	Molecular chaperone	-1.15	7.7E-03	0.95	2.8E-02	4.20	2.6E-05
cgR_2687 (<i>hspR</i>) [§]	Transcriptional regulator, MerR family	-0.87	2.7E-02	0.35	9.3E-02	4.33	1.6E-05
cgR_2964	Putative oxidoreductase/dehydrogenase	-1.80	6.1E-03	3.33	1.7E-04	1.66	4.3E-02
cgR_2980 (<i>trxB</i>) ^{¶c}	Thioredoxin reductase	-1.76	2.2E-02	2.88	1.0E-03	2.30	2.1E-03
cgR_2981 (<i>trxC</i>) [¶]	Thioredoxin	-1.26	2.2E-03	1.59	1.3E-02	1.79	5.6E-05
cgR_2982 (<i>cwlM</i>) [¶]	<i>N</i> -Acetylmuramoyl-L-alanine amidase	-0.95	1.8E-02	1.33	2.2E-02	1.74	3.0E-04
Downregulated in the <i>sigH</i> disruptant							
cgR_1616 (<i>sufR</i>) ^c	Transcriptional regulator, ArsR family	NA		0.77	3.9E-02	2.34	3.6E-02
cgR_1615 (<i>sufB</i>)	Fe-S cluster assembly protein	-1.62	5.3E-02	0.75	1.5E-01	2.52	2.1E-02
cgR_1614 (<i>sufD</i>)	Fe-S cluster assembly protein	-1.21	3.0E-02	0.65	1.4E-01	2.06	1.7E-02
cgR_1613 (<i>sufC</i>)	Fe-S cluster assembly ATPase	-1.15	2.4E-02	0.78	1.1E-01	1.94	2.3E-02
cgR_1612 (<i>sufS</i>)	Cysteine desulfurase	-0.78	1.9E-02	0.64	1.1E-01	1.47	4.5E-02
cgR_1611 (<i>sufU</i>)	Fe-S cluster assembly protein	-0.77	1.2E-01	0.63	2.7E-01	1.57	4.6E-03
cgR_1610	Hypothetical protein	NA		0.61	2.0E-01	1.75	1.2E-02
Upregulated in the <i>sigH</i> -overexpressing strain							
cgR_0488 (<i>hemA</i>) ^{^c}	Glutamyl-tRNA reductase	-0.17	6.4E-01	1.15	1.0E-03	2.06	9.1E-06
cgR_0489 (<i>hemC</i>) [^]	Porphobilinogen deaminase	-0.20	4.4E-01	0.74	1.3E-04	1.09	3.2E-03
cgR_0715 (<i>groES</i>) ^{◇c}	Molecular chaperone	0.00	9.9E-01	1.14	4.8E-04	1.89	9.7E-03
cgR_0716 (<i>groEL1</i>) [◇]	Molecular chaperone	-0.19	2.6E-01	1.32	7.2E-03	1.92	1.6E-03
cgR_1749 (<i>sigB</i>)	RNA polymerase sigma factor	-0.53	2.6E-01	1.00	4.6E-03	1.34	3.7E-02
cgR_1753	Hypothetical protein	NA		3.66	2.7E-05	3.50	4.4E-03
cgR_1832 (<i>mtr</i>)	Mycothione reductase	NA		3.31	8.4E-03	2.66	1.3E-03
cgR_1848	rRNA large subunit methyltransferase	-1.06	6.0E-02	1.29	2.0E-04	1.19	4.0E-03
cgR_2162 (<i>dnaJ2</i>)	Molecular chaperone	-0.67	1.7E-01	1.69	1.5E-03	1.19	5.3E-04
cgR_2417	Glutamate racemase	NA		1.75	5.7E-03	1.86	2.3E-02
cgR_2451	Na ⁺ /H ⁺ -dicarboxylate symporter	-1.15	1.7E-01	2.65	6.7E-03	4.02	1.8E-03
cgR_2470	Dithiol-disulfide isomerase	NA		2.66	4.3E-04	1.45	5.0E-03
cgR_2619 (<i>groEL2</i>)	Molecular chaperone	NA		1.13	9.3E-04	3.09	1.2E-04
cgR_2830 (<i>msrA</i>) ^{◆c}	Peptide methionine sulfoxide reductase	NA		2.07	1.2E-03	3.39	1.2E-03
cgR_2829 [◆]	Amidohydrolase	NA		NA		1.89	1.1E-03
cgR_2903	NADH dehydrogenase/NAD(P)H nitroreductase	-0.35	8.1E-02	1.45	4.5E-04	1.96	1.3E-02

^a Relative ratios of the transcript levels in the *sigH* disruptant to the wild type 15 min after heat shock ($\Delta sigH$) and those in the *sigH*-overexpressing strain to the wild type at 33°C (SigHox) determined by DNA microarray analyses are shown in the base 2 logarithm.

^b P values for the relative ratio were determined by Student's t test.

^c Genes that constitute a putative operon are indicated with the same character.

^d Relative ratios of transcript levels 15 min after heat shock to 33°C in the wild type.

^e First gene of the operon.

^f Data determined to be significantly changed using the criteria described in Materials and Methods are bold. NA, data not available.

TABLE 2. Genes under the control of HspR and HrcA

Gene ^d	Product	Ratio ^a	P ^b
Differentially expressed between the wild type and the <i>hspR</i> disruptant			
cgR_2674 ^c	Major facilitator superfamily permease	1.30	3.5E-05
cgR_2676 (<i>clpB</i>) ^{*,c}	Molecular chaperone	2.92	4.0E-05
cgR_2675 [*]	Hypothetical protein	2.65	2.5E-05
cgR_2690 (<i>dnaK</i>) ^{†,c}	Molecular chaperone	3.25	2.1E-04
cgR_2689 (<i>grpE</i>) [†]	Molecular chaperone	2.90	1.4E-03
cgR_2688 (<i>dnaJ</i>) [†]	Molecular chaperone	2.24	2.2E-04
Differentially expressed between the wild type and the <i>hrcA</i> disruptant			
cgR_0715 (<i>groES</i>) ^{‡,c}	Molecular chaperone	2.70	1.9E-06
cgR_0716 (<i>groEL1</i>) [‡]	Molecular chaperone	2.90	2.3E-05
cgR_1436	NADPH-dependent quinone oxidoreductase	2.11	3.0E-03
cgR_2619 (<i>groEL2</i>)	Molecular chaperone	3.13	3.3E-06
cgR_2620 ^c	Hypothetical protein	1.73	2.8E-04
cgR_2888 (<i>dps</i>)	Starvation-induced DNA-protecting protein	-1.49	2.5E-03

^a Relative ratios of the transcript levels in the *hspR* and *hrcA* disruptants to the wild type at 33°C determined by DNA microarray analyses are shown in the base 2 logarithm.
^b P values for the relative ratio were determined by Student's *t* test.
^c qRT-PCR analyses showed that the transcript levels of cgR_2674 and cgR_2620 were not affected by *hspR* and *hrcA* disruption, respectively.
^d Genes that constitute a putative operon are indicated with the same character.
^e First gene of the operon.

of *C. glutamicum* is similar to those recognized by SigH of *M. tuberculosis* (26, 31) and SigR of *Streptomyces coelicolor* (38), which are orthologs of *C. glutamicum* SigH (Fig. 2). The activity of SigH of *M. tuberculosis* and SigR of *S. coelicolor* is regulated by the anti-sigma factors RshA and RsrA, respectively (25, 44). RshA has been demonstrated to bind to SigH to inhibit SigH-dependent transcription, and this interaction is disrupted by elevated temperatures as well as oxidizing conditions (44). An open reading frame encoding a putative anti-sigma factor was identified downstream of the *sigH* gene in the published *C. glutamicum* genome sequences,

FIG. 4. SigH-independent induction of *clpB* and *dnaK* by heat shock. The transcript levels of *clpB* and *dnaK* 15 min after heat shock were compared to those at 33°C in the wild type (white bars) and the *sigH* disruptant (gray bars) by qRT-PCR. Experiments were repeated three times. The transcript levels at 33°C were taken as 1.

although this open reading frame is defined only in the genome of *C. glutamicum* ATCC 13032, reported by Kalinowski et al. (24), but not in *C. glutamicum* ATCC 13032, reported by Ikeda and Nakagawa (21), and *C. glutamicum* strain R, reported by Yukawa et al. (51). The activity of SigH of *C. glutamicum* is probably regulated by an anti-sigma factor in a mechanism similar to those of *M. tuberculosis* and *S. coelicolor* (Fig. 5).

In the *hspR* disruptant, the transcript levels of the *clpB* and *dnaK* operons were upregulated (Table 2). The HAIR elements are present within the promoter regions of *clpB* and *dnaK* (Fig. 3A), suggesting that HspR directly represses expression of these operons. The HspR-HAIR system regulates expression of the *dnaK*, *acr2*, and *clpB* operons in *M. tuberculosis* (45), and the *dnaK* and *clpB* operons and the *lon* gene in

FIG. 3. Regulatory regions of genes under the control of HspR (A) and HrcA (B). Transcription initiation sites are indicated with bent arrows. Solid and dotted lines over the sequences correspond to the SigA- and SigH-dependent promoters, respectively. Double solid and dotted lines under the sequences correspond to the HAIR and CIRCE elements, respectively.

FIG. 5. A model of the regulatory network controlling expression of the HSP genes in response to heat shock. + and – indicate positive regulation and negative regulation, respectively. Indirect regulation of *groES-groEL1* and *groEL2* by SigH is shown with a dotted arrow. Involvement of an anti-sigma factor in regulation of the SigH activity has not been revealed in *C. glutamicum*. See text for details.

S. coelicolor (5). The *acr2* operon and the *lon* gene are found in certain strains of actinobacteria, but *C. glutamicum* does not contain these genes (48). The *dnaK* and *clpB* operons are the ubiquitous regulon of the HspR-HAIR system in actinobacteria. Expression of the *dnaK* and *clpB* operons was positively regulated by SigH and subjected to negative regulation by HspR. These operons were upregulated by heat shock even in the *sigH* disruptant (Fig. 4) and also by the *sigH* overexpression (Table 1). Disruption of *hspR* resulted in upregulation of these operons without affecting expression of other members of the SigH regulon (Table 2). These results indicate that SigH and HspR independently regulate expression of the *dnaK* and *clpB* operons (Fig. 5). In *M. tuberculosis*, the DNA-binding activity of HspR is modulated by chaperones (7). Expression of the *dnaK* and *clpB* operons might be inducible by various stimuli, which are sensed by an anti-sigma factor and chaperones.

Expression of *groES-groEL1*, *cgR_1436*, and *groEL2* was upregulated by *hrcA* disruption (Table 2). CIRCE elements were observed within those promoter regions, suggesting direct regulation by HrcA. The HrcA-CIRCE system regulates expression of the *groES-groEL1* operon and *groEL2* and Rv0991c in *M. tuberculosis* (45). The *groES-groEL1* operon and the *groEL2* gene are also regulated by the HrcA-CIRCE system in *Streptomyces* spp. (10, 15), indicating that these genes constitute the core regulon of the HrcA-CIRCE system in actinobacteria. The transcript levels of *groES-groEL1* and *groEL2* were increased by overexpression of the *sigH* gene (Table 1). As the *groES-groEL1* and *groEL2* genes are transcribed from the SigA-dependent promoters (2), the SigH-associated control over *groES-groEL1* and *groEL2* expression is supposed to be indirect (Fig. 5). In *M. tuberculosis*, the transcript level of *groES* is increased by *hspR* disruption, in which some HSP genes under the control of HspR are derepressed (45). Upregulation of HSP genes by *sigH* overexpression might affect expression of *groES-groEL1* and *groEL2*.

In the gram-positive model bacterium *Bacillus subtilis*, HrcA represses transcription of the *hrcA-grpE-dnaK-dnaJ* and *groEL-groES* operons from SigA-dependent promoters (42, 50). A general stress response sigma factor, SigB, positively regulates expression of the genes *clpP*, *clpC*, and *clpX*, which are also subjected to negative control by CtsR (29). In *C. glutamicum*,

there is no sigma factor homologous to SigB of *B. subtilis* (14), but the ECF sigma factor SigH plays a regulatory role in expression of HSP genes in concert with HspR. On the other hand, the HrcA-CIRCE systems of *C. glutamicum* and *B. subtilis* negatively regulate the genes *groES* and *groEL*, suggesting its ancient origin.

Out of 157 genes upregulated by heat shock, 45 genes were defined to constitute the SigH regulon (Table 1). However, regulation of the remaining 112 genes is not unraveled. We found that some of these genes were transcribed from the SigA-dependent promoter (data not shown), suggesting that hitherto unidentified transcriptional regulators are involved in response to heat shock. Eighteen genes encoding transcriptional regulators were upregulated by heat shock (Table 3). In addition to the *hspR* gene, the transcript levels of the genes *clgR*, *sufR*, *amtR*, *phoR*, and *mcbR* were increased. ClgR activates transcription of *clpP1-clpP2* and *clpC* (12), and SufR is the repressor of the *suf* gene cluster (34). McbR regulates genes involved in sulfur metabolism (40), and AmtR is the master regulator of nitrogen control (3). Some genes under the control of McbR and AmtR were upregulated by heat shock (see Table S2 in the supplemental material). A *phoRS* operon, which is positively regulated by PhoR (28), was included

TABLE 3. Transcriptional regulators upregulated by heat shock

Gene	Product(s)	Ratio ^a	P ^b
cgR_2687 (<i>hspR</i>)	Transcriptional regulator, MerR family	4.33	1.6E–05
cgR_2930	Transcriptional regulator, ArsR family	3.04	1.3E–02
cgR_1792 (<i>clgR</i>)	Transcriptional regulator	3.02	2.4E–02
cgR_1435	Transcriptional regulator, HxlR family	2.70	3.5E–03
cgR_1616 (<i>sufR</i>)	Transcriptional regulator, ArsR family	2.34	3.6E–02
cgR_2613	Transcriptional regulator, MarR family	1.94	7.5E–03
cgR_2858	Transcriptional regulator, MarR family	1.82	6.7E–02
cgR_1024	Transcriptional regulator, TetR family	1.73	1.8E–02
cgR_0978 (<i>amtR</i>)	Transcriptional regulator, TetR family	1.51	2.3E–04
cgR_1106	Transcriptional regulator, MarR family	1.42	2.3E–02
cgR_2511 (<i>phoR</i>)	Two-component response regulator	1.40	1.4E–02
cgR_2866	Transcriptional regulator, PadR family	1.27	1.4E–03
cgR_0024	Transcriptional regulator	1.25	5.3E–04
cgR_2029	Transcriptional regulator, MerR family	1.25	4.5E–02
cgR_0818	Transcriptional regulator, XRE family	1.22	2.9E–03
cgR_0746	Transcriptional regulator, MarR family	1.12	1.5E–03
cgR_0453	Transcriptional regulator, TetR family	1.07	2.2E–04
cgR_2850 (<i>mcbR</i>)	Transcriptional regulator, TetR family	1.02	7.4E–03

^a Relative ratios of the transcript levels 15 min after heat shock to 33°C in the wild type determined by DNA microarray analyses are shown in the base 2 logarithm.

^b P values for the relative ratio were determined by Student's *t* test.

among the upregulated genes (see Table S2 in the supplemental material). Characterization of transcriptional regulators that are upregulated by heat shock would lead to further understanding of the regulatory network of gene expression in response to heat shock.

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