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The Extracytoplasmic Function-Type Sigma Factor SigM of *Corynebacterium glutamicum* ATCC 13032 Is Involved in Transcription of Disulfide Stress-Related Genes[▽]

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The gene for the extracytoplasmic function (ECF) sigma factor SigM was deleted from the chromosome of the gram-positive soil bacterium *Corynebacterium glutamicum* to elucidate the role of the SigM protein in the regulation of gene expression. Comparative DNA microarray hybridizations of the *C. glutamicum* wild type and *sigM*-deficient mutant *C. glutamicum* DN1 revealed 23 genes with enhanced expression in the *sigM*-proficient strain, encoding functions in the assembly of iron-sulfur clusters (*suf* operon), thioredoxin reductase (*trxB*), thioredoxins (*trxC*, *trxB1*), chaperones (*groES*, *groEL*, *clpB*), and proteins involved in the heat shock response (*hspR*, *dnaJ*, *grpE*). Deletion of the *sigM* gene rendered the *C. glutamicum* cells more sensitive to heat, cold, and the presence of the thiol oxidant diamide. Transcription of the *sigM* gene increased under different stress conditions, including heat shock, cold shock, and disulfide stress caused by diamide treatment, suggesting a regulatory role for SigM under thiol-oxidative stress conditions. Stress-responsive promoters were determined upstream of the *suf* operon and of the *trxB*, *trxC*, and *trxB1* genes. The deduced SigM consensus promoter is characterized by the −35 hexamer gGGAAT and the −10 hexamer YGTTGR. Transcription of the *sigM* gene is apparently controlled by the ECF sigma factor SigH, since a *sigH* mutant was unable to enhance the expression of *sigM* and the SigM regulon under thiol-oxidative stress conditions. A typical SigH-responsive promoter was mapped upstream of the *sigM* gene. The ECF sigma factor SigM is apparently part of a regulatory cascade, and its transcription is controlled by SigH under conditions of thiol-oxidative stress.

Corynebacterium glutamicum is a gram-positive, nonsporulating soil bacterium that belongs to the order *Actinomycetales*, which also includes the genera *Mycobacterium* and *Streptomyces*. *C. glutamicum* is an industrially relevant producer of several L-amino acids (21, 40). Because of its biotechnological importance, the genetic and biochemical background of amino acid biosynthesis has been studied extensively. In recent years, these studies have been broadened by the completion of the genome sequence of the wild-type strain *C. glutamicum* ATCC 13032 (26, 30). The genome sequence allows the identification of genes by similarity to already known genes in other organisms. However, knowledge about gene expression and its regulation can only be derived from complementary studies. One attempt is based on the detection and classification of transcriptional regulators by bioinformatic methods (6), and the other is based on generating specific regulatory mutants and analyzing changes in gene expression by DNA microarray hybridization and real-time reverse transcription (RT)-PCR assays (25, 54, 36, 7). It is also essential to store this information on transcriptional regulators and their regulatory interactions in the form of specialized databases such as CoryneRegNet, a

data warehouse for corynebacterial transcription factors and gene regulatory networks (4). Moreover, the sigma factors interacting with the RNA polymerase are of high relevance in understanding global changes in gene expression, since they regulate transcription at a higher hierarchical level and can influence the expression of specific target genes and of genes encoding transcription factors or even other sigma factors.

The bacterial RNA polymerase is composed of six subunits, comprising an α dimer; the β, β′, and ω subunits; and a σ factor (9). The sigma factor is responsible for promoter recognition by the RNA polymerase holoenzyme, and it thereby confers specificity on the process of transcription initiation by recognizing promoter elements of genes and operons (28). Under normal growth conditions, the essential sigma factor SigA is used. This sigma factor is apparently responsible for the transcription of housekeeping genes (18). Another category of sigma factors, termed alternative sigma factors, controls the transcription of specialized regulons that are active, for instance, during growth transitions, in the stationary phase, in response to environmental stress conditions, or during morphological development of the bacterial cell.

In 1994, a new alternative sigma factor subgroup designated the extracytoplasmic function (ECF) subfamily was described (42). The ECF sigma factors are small regulatory proteins that are strikingly divergent in amino acid sequence. They control the transcription of genes that are involved in cell envelope functions like protein transport and secretion processes and in response to extracytoplasmic

stress conditions (20). ECF sigma factors can be regulated at the transcriptional, translational, and posttranslational levels (19). Transcriptional control can be complex and can be exerted by a hierarchical regulatory cascade of sigma factors. For instance, transcription of the ECF sigma factor SigJ is under the control of the stress-responsive sigma factor SigH in *Streptomyces coelicolor* (44). The most important regulation of ECF sigma factors, however, is reversible binding of the sigma factor to an anti-sigma factor, holding it in an inactive complex as long as the cognate environmental stimulus is absent. When changes in environmental conditions are sensed by the anti-sigma factor, the complex dissolves, rendering the sigma factor active, resulting in binding of the RNA polymerase core enzyme (24).

Because of the sequencing of more than 300 bacterial genomes in recent years, a large number of ECF sigma factor genes were detected, including 2 in *Escherichia coli*, 7 in *Bacillus subtilis*, 10 in *Mycobacterium tuberculosis*, about 50 in *S. coelicolor* (20), and 5 in *C. glutamicum*, named *sigC*, *sigD*, *sigE*, *sigH*, and *sigM* (30). Concerning the ECF sigma factors, only the *sigH* gene has been studied in more detail and was shown to play a prominent role in the corynebacterial response to heat stress (12) and oxidative stress (34). Furthermore, the nonessential sigma factor SigB of *C. glutamicum* has been characterized. It was shown that this sigma factor controls the expression of genes relevant for the transition phase of growth and the stationary phase (37). In this study, we analyzed the ECF sigma factor SigM of *C. glutamicum* in detail, including its gene regulatory network, the SigM-responsive promoters and the hierarchical regulation by the ECF sigma factor SigH.

MATERIALS AND METHODS

Media and growth conditions. *E. coli* strains were routinely grown at 37°C in Luria-Bertani medium (56). *C. glutamicum* strains were cultivated at 30°C in minimal medium MM1 (MMYE without yeast extract; 32). Different stress conditions were applied to exponentially growing cultures (optical density at 600 nm of 7) of *C. glutamicum* RES167 (63). Where appropriate, diamide (*N,N,N′,N′*-tetramethylazodicarboxamide) was added to a final concentration of 2 mM. To determine the number of viable cells, dilutions of *C. glutamicum* cultures were plated on Luria-Bertani agar and incubated overnight at 30°C.

DNA techniques. *E. coli* DH5αMCR (16) was used for standard cloning experiments. Vector DNA was prepared from *E. coli* cells by alkaline lysis with the QIAprep Spin Miniprep kit (QIAGEN, Hilden, Germany). DNA restriction fragments required for cloning were purified from agarose gels by means of the QIAEX II gel extraction kit (QIAGEN). All recombinant DNA techniques followed standard procedures (56). *E. coli* and *C. glutamicum* cells were transformed by electroporation (62, 63). Chromosomal DNA was extracted from *C. glutamicum* by an alkaline lysis technique with the QIAprep Spin Miniprep kit (QIAGEN) with a 2-h incubation of the cells at 37°C in resuspension buffer P1 containing 50 mg ml^{-1} lysozyme (185,000 U mg^{-1}).

PCR techniques. PCR experiments were carried out with the DNA Engine DYAD thermal cycler (MJ Research, Watertown, MA) and *Pfu* DNA polymerase. Initial denaturation was conducted at 94°C for 2 min, followed by 35 cycles of denaturation for 30 s, annealing for 30 s at a primer-dependent temperature, and extension at 72°C for 45 s, followed by a final extension step at 72°C for 3 min. PCR products were purified by with the QIAquick PCR purification kit (QIAGEN). Cloning of PCR products was performed with *E. coli* TOP10 cells by means of the Zero Blunt TOPO PCR cloning kit (Invitrogen, Karlsruhe, Germany).

Construction of *sigM* and *sigH* deletion mutants *C. glutamicum* DN1 and DN2. Defined chromosomal deletions of the *sigM* gene and within the *sigH* coding region of *C. glutamicum* RES167 were constructed with the pK18*mobsacB* vector system, which helps to detect allelic exchange by homologous recombination (57). The respective plasmids (pDN1 and pDN2) were constructed by the GeneSOEing technique (23). Gene replacements in the chromosome of *C. glutamicum* RES167 were verified by PCR experiments.

RNA isolation from *C. glutamicum* cultures and DNA microarray hybridization. Approximately 1×10^9 *C. glutamicum* cells were harvested from exponentially growing cultures by centrifugation at $11,000 \times g$ for 15 s and subsequently transferred into liquid nitrogen. Purification of total RNA was carried out by means of the RNeasy Mini kit (QIAGEN) by following the manufacturer's instructions. The RNase-free DNase set (QIAGEN) was applied for on-column digestion of DNA. A second DNase I digestion of the RNA sample was performed with the DNase I kit (Sigma-Aldrich, Taufkirchen, Germany).

Global transcriptional profiling was performed with the *C. glutamicum* PCR product microarray (25). Synthesis and labeling of cDNA, as well as DNA microarray hybridization, signal detection, and data analysis, followed protocols described previously (25). Two biological replicates were hybridized by the application of a dye swap strategy in which the RNA samples of the two biological replicates were labeled with Cy3/Cy5 in one experiment and Cy5/Cy3 in the other. Since each DNA microarray contains four replicates per gene, a total of eight spots per gene were available to calculate changes in gene expression. Normalization and data evaluation were accomplished by the EMMA microarray data analysis software (11).

Real-time RT-PCR assays. One-step real-time RT-PCR assays were performed with the LightCycler instrument (Roche Diagnostics, Mannheim Germany) and the QuantiTect SYBR green RT-PCR kit (QIAGEN). Analyses were carried out with 1 μg of total RNA as the template and the following cycler program: RT at 50°C for 20 min, initial activation at 95°C for 15 min, and three-step cycling with denaturation at 94°C for 15 s, annealing at 55°C for 40 s, and extension at 72°C for 60 s. Differences in gene expression were determined by comparing the crossing points of two samples measured in eight replicates. The crossing point is defined as the PCR cycle at which the maximum increase in fluorescence within the log-linear phase of the amplification curve occurs. It was calculated by determining the second derivative maximum of the amplification curve by use of the LightCycler software, version 3 (Roche Diagnostics).

Mapping of transcriptional start sites by rapid amplification of cDNA ends (RACE)-PCR assays. Total RNA samples of *C. glutamicum* RES167 grown in MM1 medium and in MM1 medium containing 2 mM diamide were used to determine transcriptional start sites by means of the 5′/3′ RACE kit second generation (Roche Diagnostics). RACE-PCRs were carried out as recommended by the supplier, with 1 μg of total RNA. Resulting PCR products were ligated into the vector pCR2.1 by using the TOPO TA cloning system and chemically competent *E. coli* TOP10 cells (Invitrogen). Sequencing of cloned RACE products was carried out by IIT Biotech (Bielefeld, Germany).

RESULTS

The *sigM* gene is located adjacent to *trx* genes in the genomes of almost all actinobacteria. The genome of *C. glutamicum* ATCC 13032 encodes seven sigma factors, including the essential sigma factor SigA, the nonessential sigma factor SigB, and five alternative sigma factors (SigC, SigD, SigE, SigH, and SigM) that belong to the ECF subgroup (49, 30). The sigma factors of *C. glutamicum* were named according to their closest orthologs in other actinobacteria, in particular, the designations used in the close taxonomic relative *M. tuberculosis*. An unrooted phylogenetic tree of actinobacterial sigma factors was constructed by using different bioinformatic tools. First, a multiple alignment of the amino acid sequences of selected sigma factors was performed with the DIALIGN2 software (45). The unrooted phylogenetic tree was then obtained by using the neighbor-joining algorithm (55) integrated in the CLUSTAL_X software package (64) (Fig. 1A). From the phylogenetic tree, it became apparent that all of the sigma factors of *C. glutamicum* have close orthologs in *M. tuberculosis* and other corynebacteria, with the exception of SigM. The different SigM proteins showed only weak amino acid sequence similarities to each other. This is reflected by the deep branching of the different SigM proteins within the phylogenetic tree. Despite this amino acid sequence divergence, genes homologous to *sigM* are located together with genes encoding thioredoxin (*trxC*) and thioredoxin reductase (*trxB*) in a conserved cluster
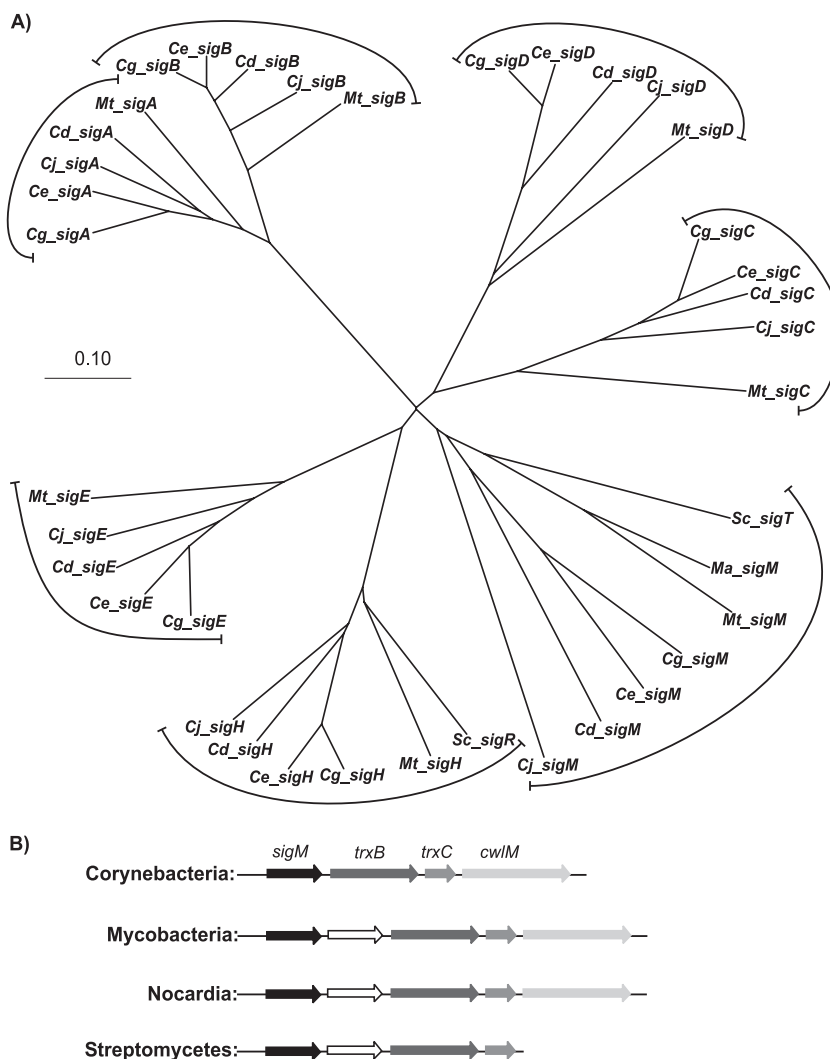
FIG. 1. Comparative analysis of sigma factor proteins and *sigM* gene regions of actinobacterial species. (A) Phylogenetic tree of actinobacterial sigma factors including the seven sigma factors of *C. glutamicum* ATCC 13032. The unrooted phylogenetic tree for different actinobacterial sigma factors is shown. Abbreviations: *Cg*, *C. glutamicum*; *Ce*, *C. efficiens*; *Cd*, *C. diphtheriae*; *Cj*, *C. jeikeium*; *Ma*, *M. avium*; *Mt*, *M. tuberculosis*; *Sc*, *S. coelicolor*. Scale bar, 0.1% amino acid substitution. (B) Comparison of the *sigM* gene regions of different actinobacteria. For corynebacteria, mycobacteria, nocardia, and streptomycetes, the organization of the *sigM* gene region is shown. The locations of the genes *sigM*, *trxB*, *trxC*, and *cwlM* are indicated. In mycobacteria, streptomycetes, and nocardia, a further gene of unknown function is located downstream of *sigM*.

in almost all sequenced actinobacteria, including mycobacteria and streptomycetes (Fig. 1B). In order to learn more about the SigM subfamily, the regulatory role in gene expression of the sigma factor SigM was investigated in *C. glutamicum*.

A *sigM*-deficient *C. glutamicum* mutant showed significant differences in global gene expression in comparison to its *sigM*-proficient parental strain. To elucidate the regulatory role of SigM, a defined *sigM* deletion mutant was constructed by gene replacement in *C. glutamicum* RES167 and designated *C. glutamicum* DN1. The chromosomal deletion comprised 618 bp and removed the complete *sigM* coding region (data not shown). To monitor changes in the global gene expression of the *sigM* mutant, DNA microarray hybridizations were performed with a genome-wide *C. glutamicum* microarray (25). *C. glutamicum* RES167 and DN1 cells were grown in minimal medium MM1 at 30°C and harvested during the exponential

growth phase to purify total RNA. The RNA samples were prepared from two independently grown cultures of each strain, and the preparations were used for DNA microarray hybridization by label swapping. Differential gene expression was therefore determined by two DNA microarray hybridizations. Since each DNA microarray contains four replicates per gene, a total of eight replicates per gene were available to calculate changes in gene expression. Labeling of samples, DNA microarray hybridization, and data evaluation were carried out as described previously (25). Normalization by the LOWESS function and *t* test statistics were accomplished by the EMMA microarray data analysis software (11). In all experiments, an *m* value cutoff of ± 1.0 was applied, corresponding to relative expression changes of ≥ 2 or ≤ 0.5. The expression changes were regarded as significant if the *P* values were smaller than 0.05 (25).
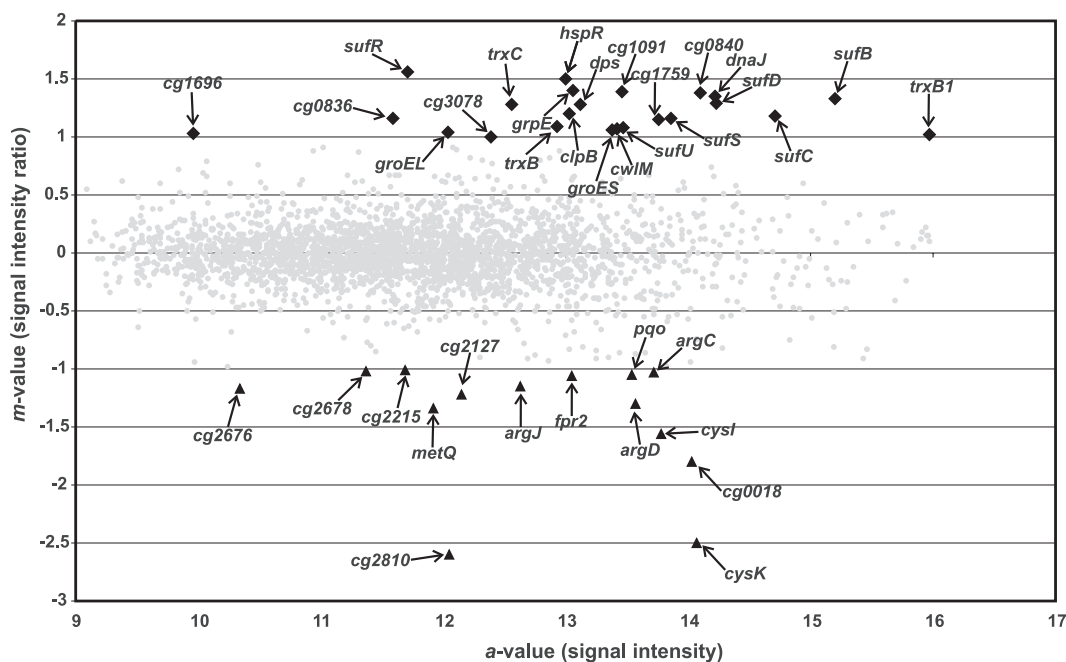
FIG. 2. Ratio-versus-intensity plot of the DNA microarray hybridization comparing the gene expression of *C. glutamicum* parental strain RES167 with that of *sigM* mutant *C. glutamicum* DN1. Genes were regarded as differentially expressed with *m* values of ≥1 or ≤−1. The *m* value is the log₂ normalized expression ratio. Genes with enhanced expression in *C. glutamicum* RES167 are marked by black diamonds; genes with decreased expression are shown as black triangles. Differentially expressed genes of *C. glutamicum* RES167 are labeled by names or identifiers; genes not differentially expressed are shown as gray dots.

The transcriptome of *C. glutamicum* RES167 was compared with that of *C. glutamicum* DN1 to reveal genes that are transcribed with the help of SigM, according to their enhanced expression in the RES167 strain. The results obtained by DNA microarray hybridization are shown in a ratio-versus-intensity plot (Fig. 2). Genes with enhanced expression were of great interest because sigma factors, in principle, function as transcriptional activators of gene expression. The DNA microarray experiment revealed 23 genes with enhanced expression in *C. glutamicum* RES167, compared with the *sigM*-deficient mutant *C. glutamicum* DN1. These genes were therefore considered to represent candidate genes under the transcriptional control of SigM. The differentially expressed genes were classified into the categories (i) disulfide stress-related genes, (ii) heat stress-related genes, and (iii) genes with other functions.

Of interest are disulfide stress-related genes (Table 1, disulfide stress-related genes), e.g., the genes *sufR*, *sufB*, *sufD*, *sufC*, *sufS*, *sufU*, and *cg1759*. The gene products of the *suf* cluster are responsible for the assembly, formation, and repair of iron-sulfur clusters in proteins (29, 38, 48, 68). As known from other organisms, like cyanobacteria (68), the *sufR* gene of *C. glutamicum* encodes the repressor of the *suf* gene cluster, as deduced from the analysis of a *sufR* deletion mutant (data not shown). Another regulated gene cluster is located directly adjacent to the *sigM* gene (Fig. 1B). It contains genes that code for a thioredoxin reductase (*trxB*), a thioredoxin (*trxC*), and a putative cell wall hydrolase (*cwlM*) (10). A further thioredoxin gene, *trxB1*, was also found to be differentially expressed. All of the gene products apparently provide protective functions and are important for the bacterial cell to cope with thiol-oxidizing conditions or misfolded proteins. In particular, the *trx* genes

encode repair functions for oxidized thiol groups in proteins (22) and both thioredoxin and thioredoxin reductase are known to be induced by disulfide stress in different actinobacteria (50, 65). Altogether, 14 genes showed enhanced expression in the mutant strain. These genes encode proteins involved in arginine (*argC*, *argJ*) and cysteine (*fpr2*, *cysI*, *cysK*) biosynthesis, a methionine transporter (*metQ*), a pyruvate:quinone oxidoreductase (*pqo*), and several proteins of hitherto-unknown function.

Genes that take part in the heat shock response of *C. glutamicum* were also detected as differentially expressed (Table 1, heat stress-related genes). These genes are *groES*, *groEL*, *dnaJ*, *grpE*, and *clpB*, which encode chaperones and *hspR*, which encodes a transcriptional regulator of the heat shock response system (3, 12, 67). Moreover, four genes that encode hypothetical proteins, as well as a putative starvation-induced, DNA-protecting protein, were found to be differentially expressed (Table 1, genes with other functions).

The *sigM*-deficient mutant *C. glutamicum* DN1 was impaired in survival after oxidative and temperature stress. The *sigM*-deficient strain *C. glutamicum* DN1 and the *sigM*-proficient parental strain *C. glutamicum* RES167 were grown in minimal medium MM1. Different stress conditions were applied to exponentially growing cultures for 15 min. These stress conditions were heat shock at 50°C, cold shock at 10°C, disulfide stress caused by the addition of diamide to a concentration of 2 or 10 mM, oxidative stress caused by the addition of hydrogen peroxide to a concentration of 1%, salt stress caused by the addition of NaCl to a concentration of 1 M, and alcohol stress caused by the addition of ethanol to a concentration of 10%. The cells were washed after stress application, and serial dilu-

TABLE 1. Genes with enhanced expression in parental strain *C. glutamicum* RES167 compared with *sigM* mutant *C. glutamicum* DN1

Coding sequence	Gene	Predicted function	*m* value[a]	*a* value[b]
Disulfide stress-related genes				
cg1765	sufR	Transcriptional regulator of *suf* operon	1.56	11.71
cg1764	sufB	Fe-S cluster assembly protein	1.33	15.20
cg1763	sufD	Fe-S cluster assembly membrane protein	1.18	14.71
cg1762	sufC	Fe-S cluster assembly ATPase	1.29	14.23
cg1761	sufS	Fe-S cluster assembly protein	1.16	13.86
cg1760	sufU	Cysteine desulfhydrase	1.08	13.47
cg1759		Fe-S cluster assembly protein	1.15	13.76
cg3299	trxB1	Thioredoxin	1.02	15.97
cg3422	trxB	Thioredoxin reductase	1.09	12.93
cg3423	trxC	Thioredoxin	1.28	12.56
cg3424	cwlM	Cell wall hydrolase (autolysin)	1.07	13.42
Heat stress-related genes				
cg0690	groES	Chaperone	1.06	13.38
cg0691	groEL	Chaperone	1.04	12.04
cg3079	clpB	Chaperone	1.20	13.03
cg3078		Hypothetical protein	1.00	12.39
cg3099	grpE	Chaperone	1.40	13.06
cg3098	dnaJ	Chaperone	1.35	14.22
cg3097	hspR	Transcriptional regulator	1.50	13.00
Genes with other functions				
cg0840		Hypothetical protein	1.38	14.10
cg0836		Hypothetical protein	1.16	11.59
cg1091		Hypothetical protein	1.39	13.46
cg1696		Putative permease	1.03	9.96
cg3327	dps	Starvation-induced DNA-protecting protein	1.28	13.12

[a] *m* value, log$_2$ signal intensity ratio.
[b] *a* value, signal intensity.

tions were plated on Luria-Bertani agar (56). After incubation at 30°C for 24 h, the numbers of CFU were determined for both strains. It turned out that the *sigM* mutant showed decreased cell viability after heat, cold, and disulfide stress (Fig. 3), suggesting that SigM is involved in the respective stress responses of *C. glutamicum*. It was also apparent that the higher diamide concentration (10 mM) had a more severe effect on survival of the *sigM* mutant DN1 than the lower concentration (2 mM), obviously by causing a stronger disulfide stress. Disulfide stress is generally regarded as a subcategory of oxidative stress and is induced by thiol oxidation (39). Moreover, temperature stress and oxidative stress are related, since the induction of both types of stress response may be due to the incorrect folding of proteins which occurs under heat, cold, and oxidative stress conditions (67).

The *C. glutamicum sigM* gene showed enhanced expression in response to stress conditions. To explore the role of *sigM* in more detail, gene expression analyses under different stress conditions were performed by real-time RT-PCR. For this purpose, the *C. glutamicum* RES167 strain was grown in minimal medium MM1 and the already mentioned stress conditions were applied to exponentially growing cultures for 15 min. After stress application, the cells were washed with MM1 medium and total RNA was extracted. The relative amounts of *sigM* transcripts were subsequently determined by real-time RT-PCR and calculated in relation to that of an untreated control culture (Fig. 4). Transcription of the *sigM* gene increased significantly when heat, cold, or disulfide stress was applied. In contrast, transcription of the *sigM* gene was not changed significantly following peroxide, salt, and alcohol stress. These results support the

view that the sigma factor SigM is involved in the transcriptional activation of genes engaged in the cellular response to heat, cold, and disulfide stress.

Most of the SigM-dependent genes are involved in the disulfide stress response of *C. glutamicum*. Since disulfide stress caused the greatest change in the expression of the *sigM* gene, the transcription of SigM-dependent genes, as listed in Table 1, was analyzed as follows. First, the results of the DNA microarray hybridizations, as presented in Fig. 2, were checked by an RT-PCR experiment. For this reason, the mRNA levels of the respective genes (Table 1) of *sigM*-proficient strain RES167 and *sigM*-deficient mutant DN1 were determined and compared by performing RT-PCR assays. The results obtained are incorporated into Fig. 5, presenting most of the SigM-dependent genes of Table 1 in their genomic environment. The results confirmed the findings deduced from the DNA microarray hybridizations shown in Table 1. The main experiment, however, dealt with the question of whether disulfide stress induces the same group of SigM-dependent genes as listed in Table 1. This question was explored by analyzing the gene expression of two *C. glutamicum* RES167 cultures. One culture was treated for 15 min with diamide, whereas the other one was kept untreated and served as a control. The mRNA levels of the genes listed in Table 1 were then compared by RT-PCR experiments. The relative gene expression is incorporated into Fig. 5. Comparing the relative transcript levels from both experiments, it is obvious that nearly all of the genes identified as transcribed SigM dependently also have increased mRNA levels under disulfide stress. It is well established that *dnaK*, *dnaJ*, *grpE*, *hspR*, *groEL*, *groES*, and *clpB* are involved in the bacterial
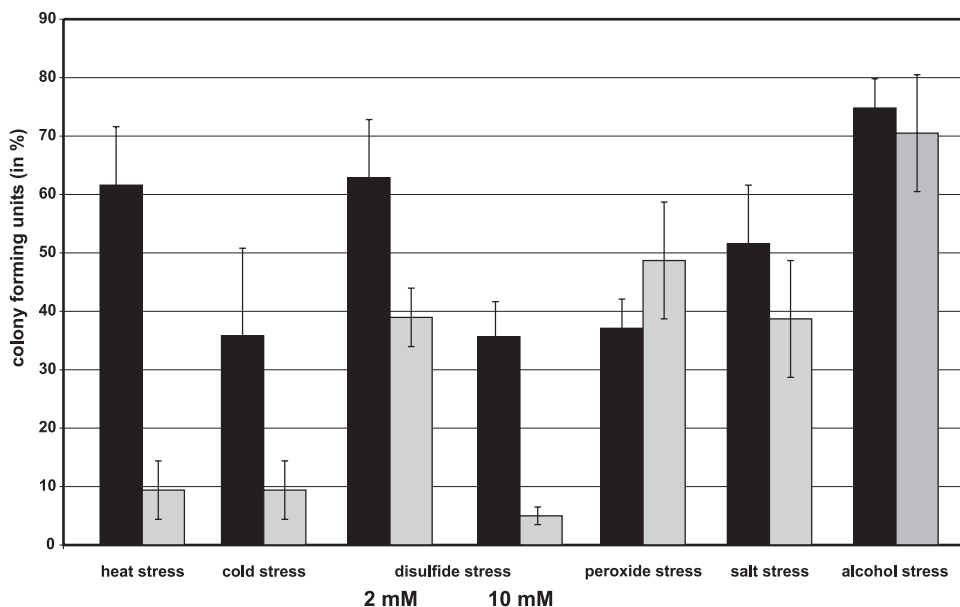
FIG. 3. CFU of *C. glutamicum* parental strain RES167 and *sigM* mutant *C. glutamicum* DN1 after the application of different stress conditions. Cultures of *C. glutamicum* RES167 and DN1 were grown in minimal medium (MM1), and cells were harvested during exponential growth at an optical density of 7. The following stress conditions were applied for 15 min: heat shock at 50°C, cold shock at 10°C, disulfide stress caused by 2 or 10 mM diamide, oxidative stress caused by 1% hydrogen peroxide, salt stress caused by 1 M NaCl, and alcohol stress caused by 10% ethanol. Following stress treatment, the colony-forming ability of the cultures was determined with three biological and two technical replicates. CFU percentages are represented by black (RES167) and gray (DN1) columns.

heat shock response (3, 46, 67) and that the Dps protein protects the DNA from oxidative damage (8). This suggests that the *C. glutamicum* sigma factor SigM plays a major role in the response to disulfide stress.

The transcription of the *dnaK* operon, consisting of the genes *dnaK*, *grpE*, *dnaJ*, and *hspR*, is not induced by disulfide stress (Fig. 5). This means that there is a second class of SigM-dependent genes which do not respond to disulfide
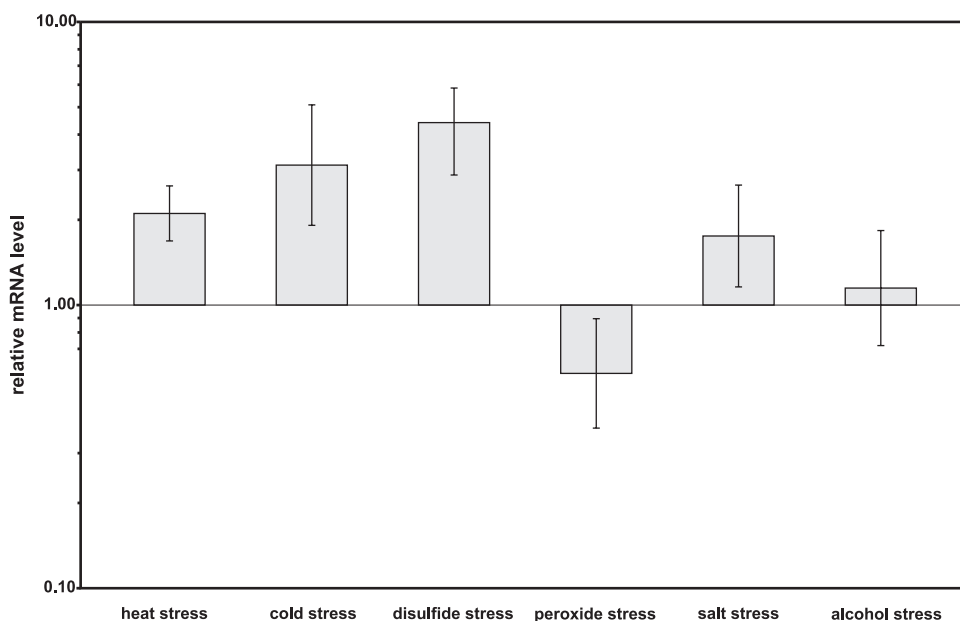

FIG. 4. Relative mRNA levels of the *sigM* gene of a *C. glutamicum* culture following the application of different stress conditions. The stress conditions were heat shock at 50°C, cold shock at 10°C, disulfide stress caused by 2 mM diamide, oxidative stress caused by 1% hydrogen peroxide, salt stress caused by 1 M NaCl, and alcohol stress caused by 10% ethanol. After stress application, the cells were washed with MM1 medium and total RNA was extracted. The relative amounts of *sigM* transcripts were subsequently determined by real-time RT-PCR and calculated in relation to that of an untreated control culture. The measurements were carried out with three biological and two technical replicates.

SigM-dependent and disulfide stress-responsive genes
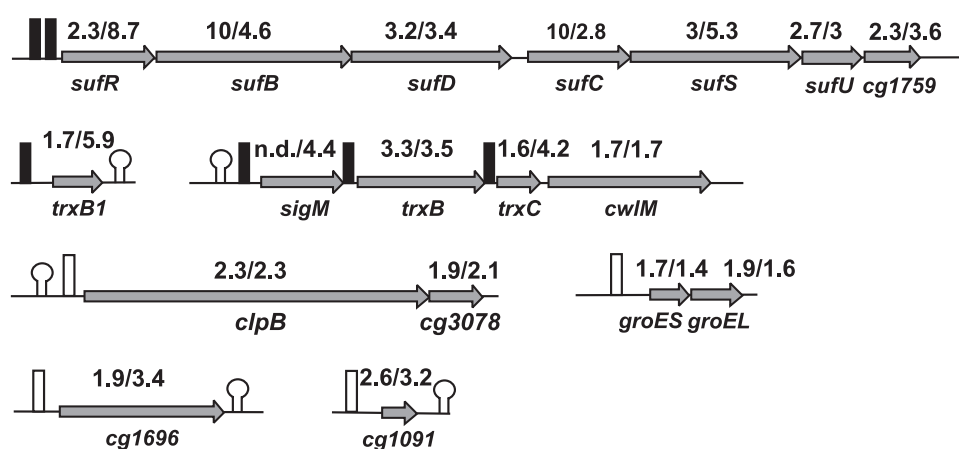
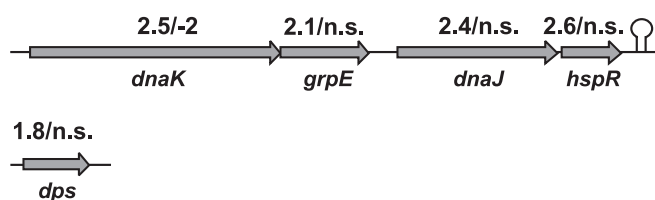## SigM-dependent, disulfide stress non-responsive genes

FIG. 5. Classification of SigM-dependent genes differing in their responses to disulfide stress. The genetic organization of the respective gene regions is shown. Stem-loops denote rho-independent transcriptional terminators (13). Black boxes indicate experimentally mapped promoters, and white boxes indicate promoters that where found by sequence similarity. RT-PCR ratios were deduced from comparisons of gene expression in *C. glutamicum* RES167 and *sigM* mutant *C. glutamicum* DN1 (first number above the genes), as well as from comparison of *C. glutamicum* RES167 treated with 2 mM diamide and a *C. glutamicum* RES167 control (second number above the genes). n.s., no significant change in gene expression.

stress. Also, the *C. glutamicum dps* gene belongs to this second class.

Determination of SigM-dependent promoters. RACE-PCR experiments were conducted to identify SigM-dependent and disulfide stress-responsive promoters of differentially expressed genes detected in *C. glutamicum* RES167. The assays were performed with primers addressing the upstream regions of *sufR*, *trxB*, *trxC*, and *trxB1* (Fig. 6A), with total RNA obtained from a diamide-containing *C. glutamicum* RES167 culture. These assays allowed the mapping of transcriptional start sites in the upstream regions of *sufR*, *trxB*, *trxC*, and *trxB1* (Fig. 6A). By RT-PCR assays, it was shown that the genes of the *suf* cluster form an operon, that the *sigM* and *trxB* transcripts are monocistronic, and that *trxC* and *cwlM* form a bicistronic operon (data not shown). A multiple DNA sequence alignment performed with the CLUSTAL_X tool (64) showed that the −35 and −10 regions of the deduced promoters have similar sequences, enabling the prediction of the promoter consensus sequences GGGAAT for the −35 hexamer and YGTTGR for the −10 hexamer (Fig. 6A). The two hexamers are separated by a spacer sequence of 17 or 18 bp. All of the mapped transcriptional start sites are characterized by a guanine residue located 8 to 10 nucleotides downstream of the −10 box. These promoters were not detected by RACE-PCR experiments in the *sigM* mutant *C. glutamicum* DN1 (data not shown).

By searching with the deduced consensus sequence in the upstream regions of differentially expressed genes, four similar putative promoters were detected in front of *groES-groEL*, *clpB-cg3078*, *cg1091*, and *cg1696* (Fig. 6B). These data supported the assumption that the deduced consensus sequence describes the SigM-dependent disulfide stress-responsive promoter of *C. glutamicum*. The SigM consensus promoter of *C. glutamicum* revealed similarities to ECF sigma factor-dependent promoters of other actinobacteria, especially to that of the ECF-type sigma factor SigR from *S. coelicolor* (Fig. 6A). SigR is known to be responsible for the transcriptional response to disulfide stress in this organism (50, 51).

The transcriptional start site of the *sufR* gene was furthermore mapped with total RNA from *C. glutamicum* RES167 cells harvested during exponential growth in minimal medium MM1 without added diamide. The deduced promoter of the *suf* operon had a sequence different from that of the promoter that was mapped by using RNA from a diamide-treated culture (Fig. 6C). Closer inspection of the promoter elements revealed that the second promoter of the *suf* operon closely resembles housekeeping promoters of *C. glutamicum* (52). Housekeeping promoters of *C. glutamicum* are defined by the consensus sequences TTGACA for the −35 hexamer and TATAAT for the −10 region (52). The two hexamers are generally separated by 17 bp. The housekeeping promoter in front of the *suf* operon matched this consensus sequence well, since five nucleotides

FIG. 6. Promoter sequences of disulfide stress-responsive genes. (A) Alignment of SigM-dependent promoter regions of differentially expressed genes of *C. glutamicum* RES167. The transcriptional start sites (+1) were determined by RACE-PCR, with total RNA from a *C. glutamicum* RES167 culture treated with diamide. *sigR* (Sc), consensus sequence of SigR-dependent promoters from *S. coelicolor* (50). (B) Promoter sequences of other *C. glutamicum* genes potentially transcribed with the help of SigM. The promoter regions (−10 and −35) were detected by similarities to the consensus sequence shown in panel A. (C) Nucleotide sequence of the *suf* operon promoter region of *C. glutamicum*. The transcriptional start sites (+1) and deduced promoter elements (−10 and −35) of the housekeeping promoter (underlined) and the stress-responsive SigM promoter (overlined) were determined by RACE-PCR. (D) Mapped promoter of the *sigM* gene of *C. glutamicum*. *sigH* (Mt), consensus sequence of SigH-dependent promoters from *M. tuberculosis* (33). Abbreviations: R, A or G; Y, C or T.

matched the −35 hexamer and three matched the −10 hexamer. This result confirms the view that promoter mapping with RNA samples from diamide-treated cultures revealed a distinct set of promoters involved in the stress response of *C. glutamicum*.

The sigma factor SigM of *C. glutamicum* is part of a hierarchical regulatory network and is under control of the ECF sigma factor SigH. The next question to be addressed was how *sigM* expression is elicited during the disulfide stress response of the corynebacterial cell. In *M. tuberculosis*, the ECF sigma factor SigH plays a major role in the cellular stress response (43). To analyze the effect of the *C. glutamicum* sigma factor SigH on the expression of the *sigM* gene, a *sigH* deletion mutant of *C. glutamicum* RES167 was constructed by gene replacement, removing an internal fragment of 456 bp from the *sigH* coding region, which is 620 bp in size (data not shown). This mutant was designated *C. glutamicum* DN2. Subsequently, RT-PCR assays were performed to test the hypothesis that *sigM* is transcribed with the help of SigH. Relative expression levels detected in *C. glutamicum* DN1 and *C. glutamicum* DN2 were compared with those measured in *C. glutamicum* RES167 (Fig. 7).

Transcription of *sigH*, *sigM*, *trxB*, *trxC*, *cwlM*, and *trxB1* increased after diamide treatment of a *C. glutamicum* RES167 culture (Fig. 7A). On the other hand, transcription of *sigM*, *trxB*, *trxC* *cwlM*, and *trxB1* was significantly lower in the *sigH* mutant *C. glutamicum* DN2 (Fig. 7B), as was the case when a *C. glutamicum* DN2 culture was treated with diamide (Fig. 7C).

Likewise, transcription of *trxB*, *trxC*, *cwlM*, and *trxB1* decreased in the *sigM* mutant *C. glutamicum* DN1 but that of the *sigH* gene did not (Fig. 7D). Gene expression was also lower in the *sigM* mutant treated with diamide, with the exception of the *sigH* gene, which showed an increased mRNA level (Fig. 7E). These results suggested that the *sigM* gene is under the control of and most probably transcribed with the help of the ECF sigma factor SigH and that SigM then initiates the transcription of its regulon. In order to find further evidence for a regulatory cascade involving SigH and SigM, the promoter of the *sigM* gene was determined by RACE-PCR. The transcriptional start site was mapped at an adenine residue (Fig. 6D), and the deduced −35 and −10 regions were specified by the sequences TGGTGAT and CGTTC, respectively. These −35 and −10 regions are similar to those of the SigH consensus promoter of *M. tuberculosis*, which consists of promoter elements with a CGGRRAY sequence in the −35 region and a CGTTR sequence in the −10 region (33).

DISCUSSION

The sigma factor SigM is involved in the oxidative stress response of *C. glutamicum*. The viability of *C. glutamicum* RES167 and the *sigM*-deficient strain *C. glutamicum* DN1 after the application of different stresses was measured by determining the numbers of CFU. These experiments revealed that the deletion of the *sigM* gene caused a reduction of the viable cell numbers upon the application of heat, cold, and disulfide
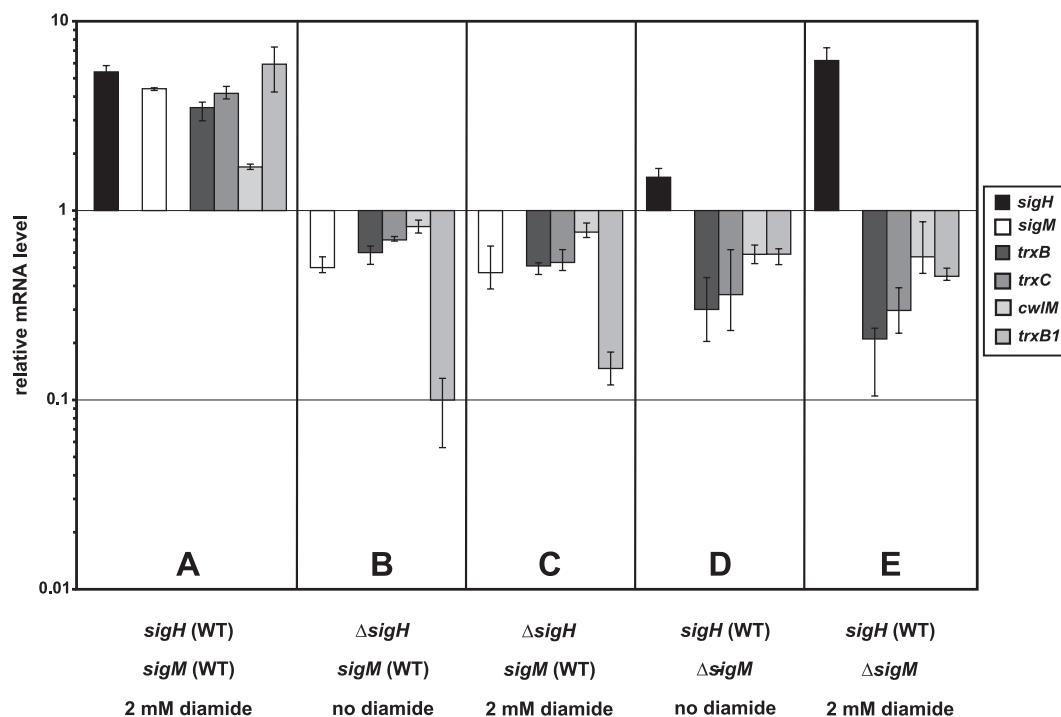
FIG. 7. Relative mRNA levels measured by RT-PCR of differentially expressed genes upon disulfide stress caused by diamide (2 mM) in *C. glutamicum* RES167 (WT [wild type]), the *sigH*-deficient mutant *C. glutamicum* DN2, and the *sigM*-deficient mutant *C. glutamicum* DN1. The mRNA level of an untreated *C. glutamicum* RES167 culture was used as a reference and set to 1. A, diamide-treated *C. glutamicum* parental strain RES167; B, untreated *sigH*-deficient mutant DN2; C, diamide-treated *sigH*-deficient mutant DN2; D, untreated *sigM*-deficient mutant DN1; E, diamide-treated *sigM*-deficient mutant DN1.

stress. Moreover, real-time RT-PCR experiments showed that the transcription of *sigM* increased significantly after the application of heat, cold, and disulfide stress. Both assays suggested that SigM is involved in the oxidative stress response of *C. glutamicum*, especially in the disulfide stress response. Disulfide stress is a subcategory of oxidative stress and is defined as the accumulation of nonnative disulfide bonds in the cytoplasm (2). In this study, disulfide stress was induced by diamide generally reacting with free thiols. The products of this chemical reaction are a disulfide bond and a hydrazine derivative. Heat, cold, and oxidative stresses are mechanistically related because they cause incorrect protein folding (2, 67). However, *sigM* expression did not increase after application of oxidative stress by hydrogen peroxide (H_2O_2). It is likely that the response to hydrogen peroxide stress is controlled not directly by an ECF sigma factor but by a DNA-binding transcriptional regulator. In *E. coli* and *S. coelicolor*, the transcriptional activator OxyR controls the cellular response to hydrogen peroxide stress (17, 60). An OxyR homologue is also encoded in the *C. glutamicum* genome (*cg2109*), but up to now the physiological function of this putative H_2O_2-sensing transcriptional regulator has not been determined.

The SigM-dependent regulon of *C. glutamicum* comprises genes that encode protein maintenance and repair functions. To elucidate the SigM-dependent regulon of *C. glutamicum*, comparative DNA microarray hybridizations of *sigM*-proficient and *sigM*-deficient strains were performed. According to our definition, the SigM regulon comprises genes that appear with enhanced expression in *C. glutamicum* RES167 compared with

that in the *C. glutamicum* DN1 mutant. Accordingly, 23 genes were identified as candidates for SigM-dependent transcription. The screening was validated by the more sensitive real-time RT-PCR assays comparing the *sigM*-deficient strain with the *sigM*-proficient strain and the untreated *sigM*-proficient strain with a diamide-treated *sigM*-proficient strain and also by the determination of SigM-responsive promoters. Components of the SigM-dependent disulfide stress-responsive regulon in *C. glutamicum* are, among others, the genes of the *suf* operon that encode seven proteins, i.e., a transcriptional regulator of the ArsR family, named SufR (6), that functions as a repressor of the *suf* operon (data not shown) and six proteins, SufBDCUS and Cg1759, which are generally required for the assembly and formation of iron-sulfur (Fe-S) clusters and their insertion into various proteins (15). Fe-S clusters are cofactors of different enzymes providing them with the ability to transfer electrons but are otherwise very sensitive to oxidative stress conditions (27). The gene products encoded by the *suf* operon apparently function in the assembly of Fe-S clusters under oxidative stress conditions (47, 61). When Fe-S clusters are damaged by disulfide stress, the expression of the biosynthesis genes increases, thereby enabling the bacterial cell to repair damaged or to assemble new Fe-S clusters.

The genes located directly adjacent to *sigM* are also regulated by the sigma factor SigM in response to disulfide stress. These genes are *trxB*, which encodes a thioredoxin reductase, and *trxC*, which encodes a thioredoxin. Another thioredoxin encoded by *trxB1* that is located elsewhere in the genome of *C. glutamicum* is also part of the SigM regulon. It is known from

B. subtilis (39), *S. coelicolor* (50), and *Staphylococcus aureus* (66) that thioredoxins and thioredoxin reductase take part in the disulfide stress response. Thioredoxins reduce disulfides in proteins and are therefore oxidized themselves. The oxidized form of thioredoxin is then reduced by thioredoxin reductase. The gene located adjacent to *sigM*, *trxB*, and *trxC* is *cwlM*, which encodes a potential cell wall autolysin. Up to now, little was known about cell wall autolysins, although the *M. tuberculosis* CwlM protein is able to lyse whole mycobacterial cells (10).

The heat stress-related genes *groES*, *groEL*, and *clpB*, which encode chaperones, are also part of the SigM regulon. These genes are known to take part in the heat shock response of *C. glutamicum* (3). The integration into the SigM regulon of heat stress-related genes is apparent when considering that the formation of disulfide bonds of cysteine side chains leads to misfolded proteins, as is the case under heat shock conditions (39, 14). The response of *C. glutamicum* to disulfide stress is probably addressing different regulatory networks of the cell, because redox-sensing cysteines play a key role in different signaling pathways, sensing oxidative stress and heat stress. A SigM promoter sequence was also found upstream of the genes *cg1091* and *cg1696*, providing evidence that they are part of the SigM regulon of *C. glutamicum*.

Altogether, 14 genes revealed enhanced transcription in the mutant strain *C. glutamicum* DN1. This set of genes includes, among others, genes involved in cysteine and arginine biosynthesis. Since the mutant is defective in iron-sulfur cluster biosynthesis, more cysteine might be needed to compensate for defective Fe-S clusters. The sulfur for Fe-S clusters is generally mobilized from L-cysteine (69), and cysteine is the most common protein ligand, in accordance with the strong affinity of iron for thiolate residues. Although arginine residues in proteins are possible ligands for Fe-S clusters (5), the connection between oxidative stress and arginine synthesis is not clear.

Genes belonging to the SigM regulon are transcribed from a SigM-dependent promoter. A new type of *C. glutamicum* promoter was recognized upstream of several disulfide-responsive genes, and a consensus sequence for the −35 (gGGAAT) and −10 (YGTTGR) hexamers was deduced from the mapped promoter regions. The consensus sequence of the SigM promoter is very similar to that of SigR from *S. coelicolor*, specified by the −35 region GGAAT and the −10 region GTT (51). The *C. glutamicum* SigM protein revealed 27% amino acid sequence similarity to SigR and 33% similar amino acid sequence similarity to SigT from *S. coelicolor*. Both sigma factors SigR and SigT are involved in the disulfide stress response (20). It is unknown which genes are regulated by SigT upon disulfide stress, whereas the SigR regulon of *S. coelicolor* comprises, among others, genes that encode thioredoxin, thioredoxin reductase, a glutaredoxin-like protein, and some ribosome-associated products, suggesting that the translational machinery of *S. coelicolor* is also modified in response to disulfide stress (51).

An additional housekeeping promoter apparently recognized by SigA was detected upstream of the *suf* operon of *C. glutamicum*. This indicates that the enzymatic functions encoded by the *suf* operon are necessary under standard growth conditions, under which the operon may be transcribed by the help of SigA. The growing requirement for repair and maintenance of iron-sulfur clusters under environmental stress conditions is apparently satisfied by enhanced expression of the *suf* operon, with the ECF sigma factor SigM. The use of different sigma subunits by RNA polymerase seems to be a general principle by which to ensure efficient expression of stress-related genes, since ECF sigma factor promoters and housekeeping sigma factor promoters appear together also upstream of other stress-responsive genes, for instance, upstream of the *dnaK* operon of *C. glutamicum* (3).

Enhanced transcription of the *sigM* gene is dependent on the ECF sigma factor SigH. When a *C. glutamicum* culture was treated with diamide, transcription of the *sigH* gene was enhanced in both the *sigM*-proficient and the *sigM*-deficient strains, whereas enhanced expression of *sigM* was not detected in a *sigH* mutant. Although a self-activating function of SigM could not be ruled out completely by the experiments performed, the detection of a SigH-type promoter upstream of *sigM* supported the view that SigM is part of a cascade and under the direct transcriptional control of SigH. In *M. tuberculosis*, SigH plays a prominent role in survival following heat shock and oxidative stress conditions (14), since the expression of other sigma factor genes, like *sigB* and *sigE*, is controlled by SigH in this organism (43). Many ECF sigma factors are regulated moreover at the protein level by reversible binding to an anti-sigma factor (24). In particular, the activity of oxidative-stress-responsive sigma factors can be controlled effectively in this way. The anti-sigma factor possesses cysteine residues in its protein sequence that are susceptible to oxidation. In the case of oxidative or disulfide stress, the cysteinyl residues form disulfide bridges, leading to a conformational change in the protein and rendering the anti-sigma factor unable to bind the sigma factor. Release of the sigma factor and its binding to the RNA polymerase core enzyme then cause activation of the transcription of its gene regulatory network. The SigR protein of *S. coelicolor* contains no cysteines that would enable the protein to respond to thiol oxidation and reduction. The activity of the SigR protein is therefore regulated by interaction with the anti-sigma factor RsrA (31). The activity of the *M. tuberculosis* sigma factor SigH is also regulated by its anti-sigma factor, named RshA (58). In this organism, the *sigH* gene forms an operon together with the gene *rshA*, which encodes the anti-sigma factor (58). Likewise, the *C. glutamicum* SigH protein contains no cysteine residues and the *sigH* gene is located together with the *rshA* gene, which encodes a putative anti-sigma factor. The ensemble of SigH and RshA probably senses disulfide stress by oxidation of cysteine residues in the RshA protein, releasing SigH to activate its regulon, including the *sigM* gene. Then, SigM is expressed and able to activate its own regulon. Among others, the SigM regulon encodes repair functions like the thioredoxin system encoded by *trxB1* and *trxBC*, leading to the reduction of the oxidized cysteinyl groups in RshA, and consequently, SigH can be bound again by the anti-sigma factor protein. Such a reactivation has been described for the *S. coelicolor* sigma factor SigR (51) and represents a regulatory mechanism by which to switch off the cellular response to environmental stress conditions.

The regulatory network structure of the *C. glutamicum* disulfide stress response is different from that of *M. tuberculosis* and *S. coelicolor*. Although the regulatory role in gene expression of the *C. glutamicum* SigH protein may be similar to those
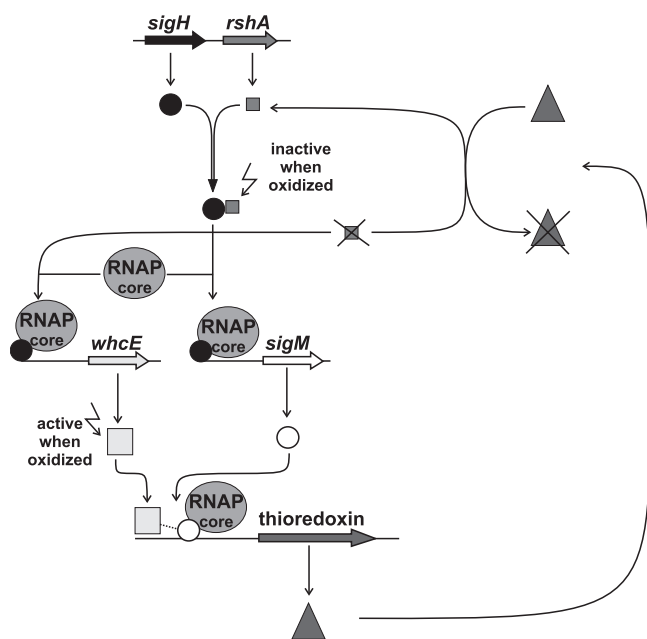
FIG. 8. The regulatory network of the *C. glutamicum* disulfide stress response. The sigma factor SigH is bound to its anti-sigma factor RshA until the anti-sigma factor senses an oxidative stress by being oxidized and releases the sigma factor. SigH binds to the RNA polymerase and initiates the transcription of the *whcE* and *sigM* genes. SigM binds to the RNA polymerase, interacts with WhcE, and initiates the transcription of the SigM-dependent disulfide stress-responsive genes, including thioredoxin-encoding genes. Thioredoxin is able to reduce oxidized groups in RshA, and therefore RshA is again functional and binds SigH.

of SigH and SigR from *M. tuberculosis* and *S. coelicolor*, respectively, the regulatory network structure of the disulfide stress response is different in *C. glutamicum*. In particular, the thioredoxin genes are transcribed with the help of SigH in *M. tuberculosis* (43), and *trx* gene expression is controlled directly by SigR in *S. coelicolor* (50). In contrast to the observations described for *M. tuberculosis* and *S. coelicolor*, *C. glutamicum* regulates the disulfide stress response and *trx* gene expression through a hierarchical network comprising the sigma factors SigH and SigM (Fig. 8). The data presented in this study unraveled a new physiological role for SigM, since the SigM protein of *M. tuberculosis* was shown to regulate genes required for the synthesis of surface or excreted molecules (53, 1). Moreover, expression of the *sigM* gene in *M. tuberculosis* was found to be induced only after heat shock and in the stationary phase and not under cold shock and disulfide stress conditions (53, 1). Furthermore, the promoter elements recognized by the SigM proteins differ in the deduced −10 region when considering the consensus sequence GGGAACC-N$_{17}$-GTCCGA of the *M. tuberculosis* SigM protein (53).

Regulation of *trx* gene expression in *C. glutamicum* is even more complex, since the WhcE protein, a DNA-binding regulator of the WhiB family, is involved in the transcriptional control of *trx* genes and the *whcE* gene is expressed only in the presence of the sigma factor SigH (34). The WhcE protein apparently functions as an activator of *trx* gene expression under heat and disulfide stress conditions (35). The WhiB3

protein of *M. tuberculosis* was demonstrated in a yeast two-hybrid screen to interact directly with the 4.2 region of the principal sigma factor (59) which is involved in recognizing the −35 promoter region (41). It is possible that WhcE interacts with the SigM subunit of the *C. glutamicum* RNA polymerase to trigger the transcription of the SigM regulon under disulfide stress conditions (Fig. 8). This conclusion provides an attractive hypothesis for further examination of the disulfide stress response of *C. glutamicum*.

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