The cssR gene of Corynebacterium glutamicum plays a negative regulatory role in stress responses

Yang Liu†, Wenzhi Yang†, Tao Su, Chengchuan Che, Guizhi Li, Can Chen* and Meiru Si*

Abstract
Background: CssR, the product of the Corynebacterium glutamicum nclg1578 gene cotranscribed with nclg1579, is a TetR (tetracycline regulator) family repressor. Although many TetR-type regulators in C. glutamicum have been extensively described, members of the TetR family involved in the stress response remain unidentified.

Results: In this study, we found thatCssR regulated the transcription of its own gene and the nclg1576-ncgl1577 operon. The nclg1576-ncgl1577 operon, which is located upstream of cssR in the orientation opposite that of the cssR operon, encodes an ATP-binding cassette (ABC), some of which are involved in the export of a wide range of antimicrobial compounds. The cssR-deletion (ΔcssR) mutant displayed increased resistance to various stresses. An imperfect palindromic motif (5′-TAA(G)TGN13CA(G)TTA-3′; 25 bp) located at the intergenic region between cssR and nclg1577 was identified as the sole binding site for CssR. Expression of cssR and nclg1577 was induced by antibiotics and heavy metals but not H2O2 or diamide, and the DNA-binding activity of CssR was impaired by antibiotics and heavy metals but not H2O2. Antibiotics and heavy metals caused CssR dissociation from target gene promoters, thus derepressing their transcription. Oxidant treatment neither altered the conformation of CssR nor modified its cysteine residues, indicating that the cysteine residues inCssR have no redox activity. In the ΔcssR mutant strain, genes involved in redox homeostasis also showed increased transcription levels, and the NADPH/NADP+ ratio was higher than that of the parental strain.

Conclusion: The stress response mechanism of CssR in C. glutamicum is realized via ligand-induced conformational changes of the protein, not via cysteine oxidation-based thiol modification. Moreover, the crucial role of CssR in the stress response was demonstrated by negatively controlling the expression of the nclg1576-ncgl1577 operon, its structural gene, and/or redox homeostasis-related genes.

Keywords: Stress response, TetR, Transcription regulation, Ligand binding, Corynebacterium glutamicum

Background
Corynebacterium glutamicum, a well-known L-amino acid producer in industry and a model organism for systems biology, unavoidably generates or encounters a series of unfavorable circumstances during fermentation [1, 2], including pH and temperature fluctuations, osmotic variation, and nutrient shortages. Diverse environments inevitably produce excessive reactive oxygen species (ROS) [3]. Massive amounts of ROS are presumed to be toxic to cells such that they damage diverse cellular
components, including DNA, lipids, proteins, iron sulfur clusters and the amino acids cysteine and methionine [4]. However, one of the most remarkable features of C. glutamicum is its striking survivability under excessive ROS production. As a result, the defense systems of C. glutamicum against stress-causing factors have attracted considerable attention from scientists, and their molecular mechanisms are now being revealed.

Corynebacterium glutamicum shows strong survivability that is attributed to two elaborate defense mechanisms, including enzymatic and nonenzymatic systems. In response to ROS, C. glutamicum mainly activates low-molecular-weight (LMW) substances as nonenzymatic systems, including β-carotene, vitamins (vitamins C and E), NAD(P)H, and LMW thiols, such as MSH (mycothiol; chemically, 1D-myo-inositol-2-[N-acetyl-l-cysteinyl] amido-2-deoxy-α-d-glucopyranoside), cysteine, and coenzyme A [1, 5]. During the course of the defense response against ROS, the cellular concentration of NAD(P)H is critical because NAD(P)H serves as the defense response against ROS, the cellular concentration of MSH constitutes a buffer to maintain intracellular redox homeostasis, allow the proper functioning of a variety of biological molecules and prevent disulfide stress [1]. Along with the use of nonenzymatic systems, C. glutamicum leverages various direct ROS-scavenging terminal enzymes, oxidized protein-repairing oxidoreductases, and regulatory proteins. The enzymatic system for scavenging ROS involves a number of enzyme-catalyzed reactions with different mechanisms, such as superoxide dismutase (SOD), catalase (Kat) and peroxidases [7–11]. A series of peroxidases constitute a large family, including mycothiol peroxidase (MPx), peroxiredoxin (Prx), cysteine-based organic hydroperoxide resistance protein (Ohr), and osmotically inducible protein C (OsmC), which have been found to contribute to organism resistance to inorganic peroxide and organic hydroperoxide by detoxifying peroxides [8–11]. Peroxidases metabolize peroxides via a conserved NH$_2$-terminal cysteine residue, which undergoes oxidation. To complete the catalytic cycle, the Cys residue must be reduced. Various peroxidases rely on different reducing systems, such as thioredoxin (Trx) and thioredoxin reductase (TrxR); mycoredoxin-1 (Mrx1), mycothione reductase (Mtr), mycothiol (MSH); alkyl hydroperoxide reductase subunit D (AhpD); dihydroliopamide dehydrogenase (Lpd); and dihydrolipoamide succinyltransferase (SucB) [12–14]. Notably, when bacteria encounter stress, the expression levels of terminal peroxidases and oxidoreductases are altered, and this process is generally considered a stress response. To realize this response, the genes encoding these enzymes are regulated at the transcriptional level by stress response transcription factors. Each of these regulators senses a specific stress and responds to it by activating or derepressing a specific set of genes under its control. Frequently, the sensing of stress is mediated by oxidation of one or more regulator protein thiolates [15]. Certainly, regulator proteins also sense environmental stress by directly accommodating small ligands, such as salicylic acid (SA), antibiotics, and benzoate [16].

In C. glutamicum, many stress-sensing regulators from different transcription factor families, such as the LysR (DNA-binding transcriptional dual-lysine regulator) family regulator OxyR (the thiol-based redox sensor of peroxides) [17]; zinc-associated extracytoplasmic function (ECF)-type sigma factor H (SigH) [18]; MarR (multiple antibiotics resistance regulator) family of regulators, including RosR (regulator of oxidative stress response), OhsR (organic hydroperoxides stress regulator), CosR (C. glutamicum oxidant-sensing regulator), QorR (quinone oxidoreductase regulator), MalR (malic regulator), and OasR (organic peroxide- and antibiotic-sensing regulator) [19–24]; TetR (tetracycline repressor protein) family regulator OsrR (oxidative stress response regulator) [25]; and XRE (xenobiotic-response element) family regulator OsrR (oxidative stress negative regulator) [26], have been well studied. Among these regulatory protein families, the TetR family is one of the major transcription factor families in C. glutamicum [27]. In many cases, TetR family transcriptional regulators act as sensors to monitor the cellular environment in bacteria and provide a very common switch for the regulation of gene expression [28]. Many of these regulators control the expression of genes required for bacteria to adapt to environmental stresses [28]. However, research on TetR-type regulatory proteins in C. glutamicum is still very limited. Only several TetR-type regulators, including OsrR, the l-methionine biosynthesis regulator MchR, the resorcinol regulator RolR, the aconitase repressor AcrN, the C. glutamicum multidrug-responsive transcriptional repressor CgmR, the phenylacetic acid repressor PaaR, the biotin biosynthesis and transport repressor BioQ, and the ammonium assimilation and transport regulator AmtR, have been reported [25, 29–35]. Structural and functional analyses of these novel TetR family proteins can promote the elucidation of drug resistance mechanisms in bacteria. C. glutamicum ncfg1578 encodes a protein that belongs to the helix-turn-helix DNA-binding motif-containing TetR family. NCgl1578, named CssR (C. glutamicum stress-sensing regulator) on the basis of the results of this study, is located immediately downstream and is oriented in the direction opposite that of the ncfg1576-ncfg1577 operon, encoding the ATP-binding cassette (ABC). Importantly, CcssR contains two cysteine residues. This characteristic allowed us to investigate the function of C. glutamicum CcssR as a transcriptional repressor of putative toxic
compound transporters critical for increasing resistance to environmental stresses. In the present study, CssR serving as a transcriptional repressor was found to directly control the expression of the cssR-negl1579 and negl1576-negl1577 operons and to indirectly negatively control the genes involved in redox homeostasis. In addition, we showed that the responses of the cssR-negl1579 and negl1576-negl1577 operons were affected by antibiotics and heavy metals but not hydrogen peroxide (H$_2$O$_2$) or diamide. To our knowledge, this is the first report demonstrating the ability of CssR to sense intracellular stress.

**Methods**

**Strains and culture conditions**

The bacterial strains and plasmids used in this study were listed in Additional file 1: Table S1. *Escherichia coli* and *C. glutamicum* RES167 were cultured in Luria–Bertani (LB) medium as previously reported [36]. For generating and maintaining *C. glutamicum* RES167 mutants, brain heart infusion containing 0.5 M sorbitol (BHIS) medium was used [36]. ΔcssR and Δnegl1576-negl1577 in-frame deletion mutants were generated by means of the method described [36]. For complementation, the pXMI19-cssR and pXMI19-negl1576-negl1577 derivatives were transformed into ΔcssR and Δnegl1576-negl1577 mutants by electroporation, respectively. The transformants were selected on LB plates supplemented with nalidixic acid (NAL) and chloramphenicol (CHL) and the expression was induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) into medium. All chemicals were of Analytical Reagent Grade purity or higher. Antibiotics were added at the following concentrations: Kanamycin (KAN), 50 µg ml$^{-1}$ for *E. coli* and 25 µg ml$^{-1}$ for *C. glutamicum*; NAL, 40 µg ml$^{-1}$ for *C. glutamicum*; CHL, 20 µg ml$^{-1}$ for *E. coli* and 10 µg ml$^{-1}$ for *C. glutamicum*.

**Plasmid construction**

The cssR and negl1576-negl1577 genes were amplified by PCR from genomic DNA of *C. glutamicum* RES167 strain with corresponding primer pairs listed in Additional file 1: Table S2. These DNA fragments were digested and subcloned into similar digested vectors, obtaining pET28a-cssR, pXMI19-cssR and pXMI19-negl1576-negl1577.

The suicide plasmids pK18mobsacB-ΔcssR and pK18mobsacB-Δnegl1576-negl1577 were prepared by overlap PCR with primer pairs listed in Additional file 1: Table S2 according to the method described by Shen et al. [36]. Site-directed mutagenesis was constructed as described [21].

The lacZY fusion reporter vectors pK18mobsacB-PcssR::lacZY, pK18mobsacB-Pnegl1577::lacZY, pK18mobsacB-PmshC::lacZY, and pK18mobsacB-Psoda::lacZY were made by the fusion of the promoter DNA fragments of cssR (152-bp, from −140 to 12 bp), negl1577 (155-bp, from −143 to 12 bp), mshC (305-bp, from −293 to 12 bp), and sodA (612-bp, from −597 to 15 bp) [all distances were with respect to the start codon of the open reading frame (ORF) of the target gene] to the lacZY reporter gene via overlap PCR [21].

For obtaining pK18mobsacB-P$_{cssRM}$::lacZY, 152-bp cssR promoter DNA containing mutagenesis sequence of the identified CssR binding site (P$_{cssRM}$) was first directly synthesized by Shanghai Biotechnology Co., Ltd.. Mutagenesis sequence was shown in blue below the promoter sequence (Additional file 1: Figure S2a). P$_{cssRM}$ had the same nucleotide sequence as 152-bp P$_{cssR}$ in P$_{css}$::lacZY except for mutation sites. Then, the resulting 152-bp P$_{cssRM}$ was fused to a lacZY reporter gene. Finally, P$_{cssRM}$::lacZY was inserted into similar digested pK18mobsacB. A similar process was used to construct pK18mobsacB-P$_{negl1577M}$::lacZY.

The fidelity of all constructs was confirmed by DNA sequencing (Sangon Biotech, Shanghai, China).

**Overexpression and purification of recombinant protein**

To express and purify soluble His$_6$-tagged recombinant proteins, pET28a derivatives were transformed into BL21(DE3) cells. Recombinant proteins were purified with the His-Bind Ni–NTA resin (Novagen, Madison, WI) according to manufacturer's instructions. Eluted recombinant proteins were dialyzed against PBS at 4 °C and concentrated for further experiments (>95% purity as estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)). Cleavage of the His$_6$ tag was performed by adding 10 units of Enterokinase-Max (Invitrogen, Karlruhe, Germany) and incubation at 4 °C overnight to conduct subsequent isothermal titration calorimetry (ITC) analysis. Ni–NTA agarose was used to remove the cleaved tag and uncleaved protein from the tag-free protein. All enzymes were purchased from Sigma-Aldrich (St. Louis, MO).

**Sensitivity assays**

For measuring the response to antibiotic, heavy metal and oxidant, experiment was performed according to Helbig et al. [37]. To measure the response to various environmental stress conditions, overnight cultures of *C. glutamicum* strains grown in LB broth medium at 30 °C were diluted 100-fold with LB broth medium, and the diluted cells
were exposed to various antibiotics (1 \(\mu \text{g ml}^{-1}\) GEN, 3.5 \(\mu \text{g ml}^{-1}\) ERY, 1.2 \(\mu \text{g ml}^{-1}\) CIP for 60 min), oxidants (100 mM \(\text{H}_2\text{O}_2\), 25 mM diamide, 5.5 mM CHP, and 15 mM \(t\)-BHP for 30 min) and heavy metals (0.15 mM \(\text{CdCl}_2\), 6 mM \(\text{NiSO}_4\), 0.5 mM \(\text{K}_2\text{Cr}_2\text{O}_7\) for 30 min) at 30 °C with shaking (100 rpm). After treatment, the cultures were serially diluted and plated onto LB agar plates, and colonies were counted after 36 h of growth at 30 °C. Percentage survival was calculated as follows: \([\text{CFU (Colony-Forming Unit) ml}^{-1}\) after challenge at different stresses]/\([\text{CFU ml}^{-1}\) before stress challenge]\) \(\times 100\).

**Ligand binding assays**

Ligand binding was measured using isothermal titration calorimetry (ITC) at 25 °C with a NANO-ITC 2G microcalorimeter (TA Instruments, New Castle, DE, USA) [24].

**Construction of chromosomal fusion reporter strains and \(\beta\)-galactosidase assay**

The lacZY fusion reporter plasmids \(pK18mob\)acB-\(P_\text{cssRM}\)lacZY, \(pK18mob\)acB-\(P_\text{ncgl1577}\)lacZY, \(pK18mob\)acB-\(P_\text{ncgl1577M}\)lacZY were transformed into corresponding \(C.\ glutamicum\) RES167 strains by electroporation. The transformants were selected by plating on LB agar plates containing 40 \(\mu\text{g ml}^{-1}\) NAL, 25 \(\mu\text{g ml}^{-1}\) KAN, and 10 \(\mu\text{g ml}^{-1}\) CHL [24]. The resulted strains were grown in LB broth medium to an optical density at 600 nm of 0.6–0.7 and then treated with different reagents of various concentrations at 30 °C for 30 min. \(\beta\)-Galactosidase activities were assayed with \(O\)-nitrophenyl-\(\beta\)-d-galactopyranoside (ONPG) as the substrate [38]. All \(\beta\)-galactosidase experiments were performed with at least three independent biological replicates.

**Quantitative real-time polymerase chain reaction (qRT-PCR) analysis**

Total RNA was isolated from exponentially growing strains exposed to different toxic agents of indicated concentrations for 30 min using the RNeasy Mini Kit (Qiagen, Hilden, Germany) along with the DNase I Kit (Sigma-Aldrich, Taufkirchen, Germany). Purified RNA was reverse-transcribed with random 9-mer primers and MLV reverse transcriptase (TaKaRa, Dalian, China). Quantitative RT-PCR analysis (7500 Fast Real-Time PCR; Applied Biosystems, Foster City, CA) was performed as described previously [20]. The primers used were listed in Additional file 1: Table S2. To obtain standardization of results, the relative abundance of 16S rRNA was used as the internal standard. The experiment was performed with at least three independent biological replicates.

**Electrophoretic mobility shift assay (EMSA)**

EMSA was performed using the method of Si et al. [24]. Briefly, 131-bp cssR DNA promoter sequence \([P_{\text{cssR}}]\) covering the putative promoter sequences of the cssR-\(n\)cgl1579 and \(n\)cgl1576-\(n\)cgl1577 operons and corresponding to nucleotides −131 to −1 relative to the translational start codon (ATG) of the cssR ORF was amplified using primer pair EcssR-F/EcssR-R (Additional file 1: Table S2). A 131-bp mutation promoter DNA sequence \((P_{\text{cssRM}})\) was directly synthesized by Shanghai Biotechnology Co., Ltd. \(P_{\text{cssRM}}\) contained the mutated sequence of the identified CssR-binding site (the mutated sequence was shown in blue below the promoter sequence in Additional file 1: Figure S2a) and had the same nucleotide sequence as 131-bp \(P_{\text{cssR}}\) except for mutation sites. The binding reaction mixture (20 μl) contained 10 mM Tris–HCl (pH 7.4), 5 mM MgCl\(_2\), 50 mM KCl, 5% glycerol, 0.1% Nonidet P-40 (NP40), 1 μg poly(dI:dC), 0.5 mM ethylene diamine tetraacetic acid (EDTA), 20 ng \(P_{\text{cssR}}\) or \(P_{\text{cssRM}}\) and 0–80 nM of CssR. After the binding reaction mixture was incubated at room temperature for 30 min, the mixture was subjected to electrophoresis on 8% nondenaturing polyacrylamide gel, and stained with a 10,000-fold diluted SYBR Gold nucleic acid staining solution (Molecular Probes) for 30 min. The DNA bands were visualized with UV light at 254 nm. Fragments amplified from the cssR coding region with primers Control-F and Control-R instead of the 131-bp cssR promoter or BSA instead of His\(_6\)-CssR in the binding assays were used as negative controls to determine the binding specificity of CssR.

The loss of binding due to xenobiotics inducer was tested as follows. Indicated concentration of xenobiotics were added to CssR solution, immediately aliquots were taken and was cultivated with 20 ng \(P_{\text{cssR}}\) for EMSA. All aliquots were incubated in binding buffer for 30 min at room temperature and separated on 8% nondenaturing polyacrylamide gel and the gel was stained using SYBR Gold nucleic acid staining solution. The experiment was performed in triplicate.

For the determination of apparent \(K_D\) values, increasing concentrations of the CssR (0–100 nM) were incubated for 30 min at room temperature with 20 ng \(P_{\text{cssR}}\). The samples were applied onto an 8% native polyacrylamide gel and separated at 180 V for 1 h on ice. The gels stained with GelRed™ and photographed were quantified using ImageQuant software (GE Healthcare), and the percentage of shifted DNA was calculated. These values were plotted against the CssR concentration in log\(_{10}\) scale, and a sigmoidal fit was performed using GraphPad.
Prism software (GraphPad Software, San Diego California USA), considering the error bars as well as 0 and 100% shifted DNA as asymptotes, the turning point of the curve was defined as the apparent $K_D$ value. All determinations were performed in triplicate.

**DNase I footprinting assay**

DNase I footprinting assays were performed as described [39].

**Size exclusion chromatography**

The size of purified His$_6$-CssR was estimated by gel filtration on Superdex 75 10/300 GL column (GE Healthcare, Piscataway, NJ) using a buffer (50 mM potassium phosphate [pH 7.4], 0.15 M NaCl) with a gel filtration calibration kit (low molecular weight; GE, United Kingdom). The calibration curve was plotted by use of the $K_{av}$ versus the logarithm of the molecular weight.

**Quantification of intracellular NADPH/NADP$^+$ and NADH/NAD$^+$ ratios**

NADPH/NADP$^+$ and NADH/NAD$^+$ ratios were detected according to the cold methanol quenching method described by Jeong et al. using the NADPH/ NADP$^+$ and NADH/NAD$^+$ assay kit (Bioassay systems, USA) [26]. Intracellular nucleotides were extracted according to the manufacturer’s protocol. The assays utilized alcohol dehydrogenase and glucose dehydrogenase cycling reactions for NADP(H) and NAD(H) quantification, respectively. Colorimetric changes were measured at 565 nm using a Shimadzu UV-1650 PC spectrophotometer. The experiment was performed with at least three independent biological replicates.

**Western blot analysis**

Western blot analysis was performed as described previously [20]. The primary antibody at 4 °C overnight: anti-NCgl1577 rabbit polyclonal antibody, 1:1000; anti-NCgl1579 rabbit polyclonal antibody, 1:1000; anti-cyto- solic RNA polymerase α (α-RNAP), 1:5000. The α-RNAP was used as a loading control. The anti-NCgl1577 and anti-NCgl1579 rabbit polyclonal antibodies were generated and affinity-purified according to the method described previously [40]. The density of bands on Western blots was quantified by Image Lab (Bio-Rad, California, USA).

**Quantitative analysis of sulfhydryl groups**

Free thiol content of CssR was measured by using 5,5’-dithio-bis (2-nitrobenzoic acid) (DTNB) [41, 42].

**The redox state of CssR**

The redox state of CssR (20 μM) was analyzed by incubating the proteins with 50 mM DTT, 100 μM H$_2$O$_2$, 80 μM CHP, and 60 μM diamide for 30 min before separating on nonreducing 15% SDS-PAGE. For nonreducing conditions, the loading buffer [250 mM Tris–HCl (pH 6.8), 0.5% bromophenol blue (BPB), and 50% glycerol] was added to treated protein samples. All the samples were boiled for 5 min prior to electrophoresis and then stained with Coomassie Brilliant Blue (CBB). The experiment was performed in triplicate.

**Statistical analysis**

Statistical analyses of survival rate, transcription level and protein level were determined with paired two-tailed Student’s t-test. GraphPad Prism Software was used to carry out statistical analyses (GraphPad Software, San Diego California USA).

**Results**

The TetR-type regulator CssR was conserved in corynebacteria

It has been reported that TetR family regulators function as negative regulators of physiological processes such as efflux pumping and biosynthesis of antibiotics, osmotic stress, and solvent resistance [28]. The ngl1578 gene of C. glutamicum, which was renamed cssR (C. glutamicum stress-sensing regulator) on the basis of the observed phenotypes described herein, encodes a putative TetR-type transcriptional regulator of 198 amino acids (mass, 21,786 Da). A Pfam analysis showed that the deduced CssR protein possessed a TetR-type helix-turn-helix motif located near the N-terminal region (amino acid residues 12 to 50). A sequence comparison showed that CssR putative homologs were present in several species of the genus Corynebacterium, such as C. deserti, C. crudilactis, C. callunae, and C. halotolerans (Additional file 1: Figure S1a). Notably, the genomic organization of the cssR gene in C. glutamicum was almost identical to that of C. deserti, C. crudilactis, C. callunae, and C. halotolerans (Additional file 1: Figure S1b).

**Involvement of cssR in antibiotic, heavy metal, and oxidant stress responses**

In the genome of C. glutamicum, cssR was organized in an operon with ngl1579, which encodes a putative protein. An amino acid sequence comparison showed that NCgl1579 has amino acid identities of more than 45% with cystathionine beta-synthase (CBS) domain-containing proteins in C. suranareae, C. glaucum, and C. uterequi (Additional file 1: Figure S1c). CBS is a key enzyme in the metabolic pathway of homocysteine trans- sulfuration [43]. This finding suggested that NCgl1579
was involved in stress resistance by affecting cysteine synthesis. Ninety-three base pairs upstream of cssR constitute the ncgl1576-ncgl1577 locus, which is oriented in the direction opposite that of cssR (Additional file 1: Figure S2a). The ncgl1576-ncgl1577 operon encodes a putative ATP-binding cassette (ABC) transporter permease (NCgl1576) and a putative ABC transporter ATP-binding protein (NCgl1577). Many ABC transport proteins have been found to be involved in the export of a wide range of antimicrobial compounds and have been implicated in the stress response [44]. In several cases, bacterial drug transporter proteins have been described as being controlled by a transcriptional regulatory protein often located in the same operon or in an immediately adjacent region and in the orientation opposite that of the target gene on the chromosome [45]. This genetic organization allowed us to speculate that CssR might also be involved in the stress response.

To elucidate its role in physiology, we constructed C. glutamicum cssR deletion and complement strains through gene disruption and complementation and then analyzed the survival rate of the mutant strains under various stresses (Additional file 1: Figure S2b). Although the C. glutamicum RES167 parental strain (WT) and ΔcssR mutant showed almost identical growth rates (Additional file 1: Figure S3), the sensitivity of the ΔcssR(pXMJ19) mutant (the mutant lacking the cssR gene and expressing the empty plasmid pXMJ19) to various agents was remarkably lower than that of WT(pXMJ19) (the C. glutamicum RES167 parental strain expressing the empty plasmid pXMJ19) (Fig. 1b). These results showed that deletion of the cssR gene led to increased resistance to agents such as antibiotics, heavy metals, and oxidants, further indicating that CssR functioned to combat cellular stress induced by a wide spectrum of environmental cues and played a negative regulatory role in stress response-related genes.

Next, we constructed C. glutamicum ncgl1576 and ncgl1577 deletion and the complement strain and then analyzed their survival rates under various stress conditions (Additional file 1: Figure S2b). Although the Δncgl1576-ncgl1577 mutant did not exhibit changes in growth under normal conditions (Additional file 1: Figure S3), mutants lacking NCgl1576 and NCgl1577 showed obvious sensitivity to gentamicin (GEN), erythromycin (ERY), ciprofloxacin (CIP), cadmium chloride (CdCl2), nickel sulfate (NiSO4), potassium dichromate (K2Cr2O7), hydrogen peroxide (H2O2), diamide, tert-butyl hydroperoxide (t-BHP), but in these cases, we observed no significant differences between the tested strains (Fig. 1b). These results indicated the crucial function of the ncgl1576-ncgl1577 operon for bacteria survival under antibiotic and heavy metal conditions.
CSSR negatively regulated the expression of the divergently oriented operons ncgl1576-ncgl1577 and cssR-ncgl1579

By the online software Virtual Footprint and PROM-Prediction of bacterial promoters, two putative overlapping and divergent promoter sequences in the intergenic region between the start codons of cssR and ncgl1577 were found (Additional file 1: Figure S2a), one of which was located upstream of cssR. Neighboring ncgl1577 had a putative −10 and −35 promoter sequence, which was found to be the ncgl1577 promoter. Moreover, a putative CSSR-binding site in the putative overlapping, divergent promoters of the cssR-ncgl1579 and ncgl1576-ncgl1577 operons was found (Additional file 1: Figure S2a). Thus, we speculated that CSSR negatively regulated the cssR-ncgl1579 operon and repressed the transcription of the adjacent, oppositely oriented ncgl1576-ncgl1577 operon. To verify this speculation, cssR and ncgl1577 transcription levels in the WT (pXMJ19), ΔcssR (pXMJ19), and ΔcssR (pXMJ19-cssR) strains were analyzed by qRT-PCR, and the lacZ activity of the chromosomal promoter fusion reporter was determined. Notably, to study the expression of cssR in the ΔcssR(pXMJ19) mutant by qRT-PCR, a 91-bp cssR transcript (corresponding to nucleotides +1 to +91 relative to the translational start codon (ATG) of the cssR gene) was amplified from the cssR ORF that remained in the ΔcssR(pXMJ19) mutant strain with the primers QcssR-F and QcssR-R (Additional file 1: Figure S4). As expected, the cssR and ΔcssR transcription levels in the ΔcssR(pXMJ19) mutant strain were obviously higher than those in the WT (pXMJ19) and ΔcssR(pXMJ19-cssR) strains (Figs. 2a, b, e, f, 3a, b, e, f). These results indicated that CSSR negatively controlled the expression of the ncgl1576-ncgl1577 operon and its structural gene.

CSSR regulated the expression of the cssR-ncgl1579 and ncgl1576-ncgl1577 operons by directly binding to their promoters

To determine whether CSSR directly regulated the expression of its own gene and ncgl1577, we examined the direct interaction between purified His6-CSSR and

![Fig. 2](image-url)

**Fig. 2** CSSR was negatively autoregulated. a, e β-Galactosidase analyses of the cssR promoter activity by using the transcriptional PcssR-lacZ chromosomal fusion reporter expressed in WT(pXMJ19), ΔcssR(pXMJ19), and ΔcssR(pXMJ19-cssR) strains in the absence of gentamicin (GEN) or CdCl₂ (cadmium chloride) conditions. b, f Quantitative RT-PCR analyses of cssR expression in WT(pXMJ19), ΔcssR(pXMJ19), and ΔcssR(pXMJ19-cssR) strains under GEN or CdCl₂ conditions. The mRNA levels were presented relative to the value obtained from WT(pXMJ19) cells without stress treatment. Relative transcript levels of WT(pXMJ19) strains without stress were set at a value of 1.0. c, g The protein levels of NCgl1579 in WT and ΔcssR in the presence or absence of GEN and CdCl₂. Lysates from stationary phase bacteria exposed to GEN or CdCl₂ for 2 h were resolved by SDS-PAGE, and NCgl1579 was detected by immunoblotting using specific anti-NCgl1579 antibody. For the pellet fraction, RNA polymerase α (α-RNAP) was used as a loading control. Similar results were obtained in three independent experiments, and data shown were from one representative experiment done in triplicate. d, h Relative quantified data for protein levels by Image Lab. Quantified protein expression of Western blots in c and g. Densities of proteins were all justified with α-RNAP. Relative density ratios of C. glutamicum RES167 parental strains (WT) without stress were set at a value of 1.0. Data shown were the averages of three independent experiments, and error bars indicated the SDs from three independent experiments. *P<0.05; **P<0.01; ***P<0.001
131-bp P_{cssR} using EMSAs. The native molecular mass of the purified His\textsubscript{6}-CssR proteins was found to be 52 kDa by size exclusion chromatography (Additional file 1: Figure S5), indicating a homodimeric structure. This size has also been documented for other members of the TetR family, e.g., TetR [46], CamR [47], and EthR [48]. Incubation of 131-bp P_{cssR} with His\textsubscript{6}-CssR caused a clear delay in promoter DNA migration, and the abundance of the delayed migration of P_{cssR} depended on the amount of His\textsubscript{6}-CssR (Fig. 4a). This effect was specific because the combination of His\textsubscript{6}-CssR and 131-bp control DNA fragments amplified from the cssR coding region showed no detectable His\textsubscript{6}-CssR binding (Fig. 4a, lane 8); incubation of BSA with 131-bp P_{cssR} did not lead to retarded mobility (Fig. 4a, lane 9). The apparent K\textsubscript{D} value for P_{cssR} was approximately 17 nMCssR (Fig. 4b), which was within the range found for other transcriptional regulators [19]. These results showed that the CssR-binding site was indeed in the intergenic region between cssR and n Glen77. To further identify the precise CssR-binding site, DNase I footprint analysis was performed (Fig. 4c). A protected DNA region extending from −81 to −56 bp upstream of the initiation codon of the cssR ORF with high affinity for CssR was identified, indicating that the most important part of theCssR-binding site was located within these 26 bp. To confirm the footprint data, a mutated 131-bp promoter DNA sequence (131-bp P_{cssRM}) was used for an EMSA. As shown in Additional file 1: Figure S2c, 131-bp P_{cssRM} abolished the formation of DNA–protein complexes in the EMSA. Consistently, the mutations in the identified CssR-binding site led to the high β-galactosidase activities of the cssR and n Glen77 promoters in the WT(pXMJ19)(P_{cssRM}::lacZY), WT(pXMJ19)(P_{cssRM}::lacZY), ΔcssR(pXMJ19-cssR), and ΔcssR(pXMJ19) strains, similar to those in the ΔcssR(pXMJ19)(P_{cssRM}::lacZY) and ΔcssR(pXMJ19) (P_{cssRM}::lacZY) mutant strains (Additional file 1: Figure S2d). Thus, CssR directly inhibited its own expression and that of the n Glen76-n Glen77 operon by virtue of being located downstream of the −10/35 regions of the proposed promoters, indicating that repression was achieved by inhibition of initiation complex formation. These results further indicated that the corresponding sequence was required for CssR binding.
Identification of the CssR-binding motif

Inspection of the upstream regions of the cssR gene in *C. deserti*, *C. crudilactis*, *C. callunae*, and *C. halotolerans* revealed that they all contain sequence motifs similar to those protected by CssR in *C. glutamicum*. As shown in the alignment presented in Additional file 1: Figure S6a, a putative CssR consensus sequence was derived, and it contained an imperfect inverted repeat: 5′-TAA(G)TGN_{13}CA(G)TTA-3′ (25 bp). A binding motif of this type and size are typical of TetR-type transcriptional regulators such as TetR [46] or CamR [47].

To confirm the proposedCssR consensus sequence, EMSAs were performed for mutational analysis. In the experimental results shown in Additional file 1: Figure S6b, the shift of six different DNA fragments, which were amplified by PCR, was analyzed, in each case with excess CssR. Fragments M1–M5 represent derivatives of a WT fragment with mutations within or outside the proposed binding motif. Exchange of the three outer
Expression of the cssR-ncgl1579 and ncgl1576-ncgl1577 operons was induced by antibiotics and heavy metals via CssR but not oxidants

To examine whether the expression of the cssR-ncgl1579 and ncgl1576-ncgl1577 operons respond to xenobiotics at the transcriptional level, qRT-PCR profiling was performed, and the lacZY activity of the chromosomal promoter fusion reporter strain was determined. For simplicity, we used GEN, CdCl₂, H₂O₂, and diamide as inducers in the following experiments. As shown in Figs. 2a, e, 3a, e, in the absence of GEN and CdCl₂, the ΔcssR(pXMJ19)(PcssR::lacZY) strains exhibited significantly higher lacZY activity than the WT(pXMJ19)(Pncgl1577::lacZY) or ΔcssR(pXMJ19)(Pncgl1577::lacZY) strains; the ΔcssR(pXMJ19)(Pncgl1577-lacZY) strains exhibited significantly higher lacZY activity than the WT(pXMJ19)(Pncgl1577-lacZY) or ΔcssR(pXMJ19-cssR)(Pncgl1577-lacZY) strains. However, the lacZY activities of the cssR and ncgl1577 promoters in the GEN- and CdCl₂-exposed WT(pXMJ19)(PcssR::lacZY) and WT(pXMJ19)(Pncgl1577::lacZY) strains were obviously higher than that in the untreated WT(pXMJ19)(Pncgl1577::lacZY) or WT(pXMJ19)(Pncgl1577-lacZY) strains. The addition of GEN and CdCl₂ did not change the lacZY activity of the cssR and ncgl1577 promoters in the ΔcssR(pXMJ19)(Pncgl1577::lacZY) and ΔcssR(pXMJ19)(Pncgl1577-lacZY) strains, which was maintained at the same level as that observed in the ΔcssR(pXMJ19)(Pncgl1577::lacZY) and ΔcssR(pXMJ19)(Pncgl1577-lacZY) strains without xenobiotic treatment. Moreover, analysis of the lacZY activity showed dose-dependent expression in the WT (pXMJ19) and ΔcssR (pXMJ19-cssR) strains in response to GEN and CdCl₂ exposure (Figs. 2a, e, 3a, e). Similar CssR regulatory patterns of cssR and ncgl1577 were also observed at the mRNA transcriptional level through qRT-PCR analysis (Figs. 2b, f, 3b, f). Further analysis at the protein level indicated that similar regulation was observed for the production of NCgl1579 and NCgl1577. In the WT strain, GEN and CdCl₂ treatment greatly increased NCgl1577 and NCgl1579 at the cellular level, similar to the ΔcssR strain in the absence of GEN and CdCl₂ (Figs. 2c, g, 3c, g). Interestingly, the transcription of cssR and ncgl1577 was negligibly affected by H₂O₂ and diamide (Additional file 1: Figures S8 and S9). These results clearly demonstrated that cssR and ncgl1577 were upregulated in response to increasing antibiotic and heavy metal concentrations, indicating that antibiotics and heavy metals rendered CssR incapable of binding DNA, instigating the transcription of its own gene, ncgl1579, and that of the ncgl1576-ncgl1577 operon.

The ability of CssR to bind the promoter regions of the cssR-ncgl1579 and ncgl1576-ncgl1577 operons was inhibited by heavy metals and antibiotics but not oxidants

Interestingly, the binding of His₆-CssR to PcssR was prevented by the addition of GEN or CdCl₂ (Fig. 5a). However, 10 mM H₂O₂ did not impair the DNA-binding activity of His₆-CssR (Fig. 5a). Together, these results showed that CssR specifically recognized operators and then directly bound the cssR and ncgl1577 intergenic regions in a sequence-specific manner. In the presence of GEN or CdCl₂, CssR dissociated from the promoter DNA, leading to the upregulation of target genes.

Therefore, to further investigate whether GEN and CdCl₂ are ligands of CssR, isothermal titration calorimetry (ITC) analysis was performed. No binding was observed when buffer was titrated into CssR (Fig. 6a). However, the strength of the interaction between CssR and GEN or CdCl₂ was measured by ITC and possessed negative enthalpic contribution of a typical hyperbolic binding curve (Fig. 6b, c). The stoichiometry N of CssR to GEN or CdCl₂ was between 0.8 and 1.2, which aligned with the standard 1:1 complex formation between a ligand and protein. Moreover, the enthalpic (ΔH) and entropic (ΔS) parameters of CssR binding to GEN or CdCl₂ were −251.9 ± 17.3 kJ mol⁻¹ and −691.9 J mol⁻¹, or −50.4 ± 3.6 kJ mol⁻¹ and −6.1 J mol⁻¹, yielding a dissociation constant, Kₐ, of 0.012 ± 0.005 μM or 0.079 ± 0.004 μM, respectively, indicating that CssR had a high affinity for GEN and CdCl₂. The Kₐ values obtained from C. glutamicum CssR were similar to those of known TetR-type E. coli regulators and the Staphylococcus aureus TetR-type quaternary ammonium compound regulator QacR [46, 49], indicating a high probability that antibiotics and heavy metals bind CssR in vivo. This finding also suggested that CssR can directly bind GEN and CdCl₂, the outcome of which is CssR dissociation from the target DNA sequence and, hence, target regulon activation.

Oxidant neither altered the conformation of CssR nor modified its cysteine residues

The amino acid sequence of CssR contains two cysteine residues at positions 17 and 58. Thus, we thought these
residues might control target gene expression through the action of versatile posttranslational thiol modification mechanisms. Unexpectedly, CssR incubated with H$_2$O$_2$, CHP or diamide migrated as a band of approximately 27 kDa on a 15% nonreducing SDS-PAGE gel, which closely corresponded to the sum of the molecular mass (~22 kDa) of the native CssR protein as deduced from its amino acid sequence and His$_6$ (approximately 5 kDa) (Additional file 1: Figure S10a). This result was confirmed by measuring the thiol content of H$_2$O$_2$. 

![Image of SDS-PAGE gel showing migration of CssR and CssR:C17SC58S with GEN, CdCl$_2$, or H$_2$O$_2$.](image-url)

**Fig. 5** Inhibition of the DNA binding of CssR and CssR:C17SC58S by GEN or CdCl$_2$ but not H$_2$O$_2$. 

**a** GEN, CdCl$_2$, or H$_2$O$_2$ was added to the binding reaction mixture containing CssR and EMSA was performed. 

**b** GEN, CdCl$_2$, or H$_2$O$_2$ was added to the binding reaction mixture containing CssR:C17SC58S and EMSA was performed.
CHP- and diamide-treated CssR with a DTNB assay. The DTNB assay showed that the DTT-treated CssR had $1.795 \pm 0.075$ thiol groups per monomer; H$_2$O$_2$-, CHP-, and diamide-treated CssR monomers contained $1.810 \pm 0.15$, $1.839 \pm 0.126$, or $1.785 \pm 0.107$ thiol groups, respectively. These results indicated that the thiol contents of CssR were unchanged both before and after exposure to oxidant and that there was no thiol modification upon oxidant treatment (Additional file 1: Figure S10b). Our data indicated that oxidative stress did not influence cssR conformation. In addition, analysis of the transcription levels revealed that in the ΔcssR(pXMJ19-cssR:C17SC58S) strain, the expression of the cssR-negl1579 and negl1576-negl1577 operons under GEN and CdCl$_2$ stress conditions remained unchanged (Figs. 2 and 3); an EMSA also revealed that CssR:C17SC58S behaved very similarly to CssR (Fig. 5b). Thus, we speculated that CssR did not regulate genes involved in the stress response via a thiol-based mechanism.

**Cellular redox homeostasis-maintaining reducing systems were negatively regulated by CssR**

Oxidant-resistant strains typically have high levels of ROS-detoxifying enzymes. In *C. glutamicum*, SodA, KatA, MPx, Ohr, Prx, PrxQ, and OsmC have been shown to be the major ROS-detoxifying enzymes and to be important for survival under oxidative stress [7–11]. To test whether CssR controlled the expression of ROS-detoxifying enzymes, the lacZY activity of a chromosomal promoter fusion reporter-expressing strain and qRT-PCR was determined. The findings showed that the transcription levels of the sodA, katA, mpx, ohr, prx, prxQ, and osmC genes in the ΔcssR strain were almost the same as those in the WT strain (Fig. 7a, b). Oxidants have the ability to disturb metabolism, such as carbon metabolism, precursor supply levels, energy metabolism and redox metabolism. Therefore, we postulated that reducing systems might be influenced in the ΔcssR strain, and we measured NADPH levels and the transcription of redox homeostasis-related genes. As shown in Additional file 1: Table S3, the NADPH/NADP$^+$ ratio in the ΔcssR strain (1.29 ± 0.02) was approximately 1.53-fold higher than that in the WT strain (0.84 ± 0.05). In contrast, there was no obvious difference in the NADH/NAD$^+$ ratio in the WT and ΔcssR strains. This result was consistent with the key roles of NADH and NADPH, which are known to act as a pro-oxidant and antioxidant, respectively [6].

The transcription of the genes *trx*, *mtr*, *mrxi*, and *mshC*, which are key members of three alternative physiological reducing systems in *C. glutamicum*, i.e., the MSH system (MSH/Mtr), Mrxi system (Mrxi/Mtr/MSH) and Trx system (Trx/TrxR), as determined by the lacZY activity of the chromosomal promoter fusion reporter-expressing strain and qRT-PCR, was analyzed. Because Trx and TrxR are cotranscribed [12], we measured the transcription of *trx*. As expected, the transcription levels of the *trx*, *mtr*, *mrxi*, and *mshC* genes in the ΔcssR strain were higher than those in the WT strain. These results indicated that the oxidant-resistant ability of the ΔcssR
strain might be attributable to increased reducing power levels (Fig. 7a, b).

To test whether CssR directly regulated the expression of the aforementioned genes involved in supplying reducing power, the direct interaction of CssR with the promoters of the genes *trx*, *mrx1*, *mtr*, and *mshC* was subsequently assessed by EMSAs (Fig. 7c–f). Interestingly, CssR did not bind to the promoter regions of the *trx*, *mrx1*, *mtr*, and *mshC* genes. Moreover, a putative CssR consensus sequence was not found in their promoter regions. These results indicated that they were not direct targets of CssR.

**Discussion**

In this study, we provide insight into the TetR-type regulator CssR in *C. glutamicum*. Recently, members of the TetR regulator family have attracted considerable attention, as some TetR proteins have been shown to contribute to a wide variety of stress-related toxic compound resistance phenotypes [28]. In several cases, TetR-type regulators have been described to control multidrug transporter genes, often located in the same operon or in immediately adjacent upstream regions but with differing orientations. The resulting phenotype of enhanced drug resistance has been considered a result of increased efflux of a toxic compound. In the present study, we demonstrated that CssR was a transcriptional regulator of the TetR family of genes that repressed the expression of the *ncgl1576-ncgl1577* operon and its structural gene. The *ncgl1576-ncgl1577* operon, located immediately upstream and in the opposite direction of *cssR*, encodes an ATP-binding cassette (ABC), some of which are involved in the export of a wide range of antimicrobial compounds and have been implicated in the stress response. Through survival assays, we observed notable resistance of the Δ*cssR* strain and high-level susceptibility of Δ*ncgl1576-ncgl1577* to antibiotics and heavy metals. Further expression property analyses showed that the expression of *cssR* and *ncgl1577* was induced by antibiotics and heavy metals. Moreover, *cssR* and *ncgl1577* expression was derepressed by inactivation of the transcriptional repressor CssR. Remarkably, although many TetR family transcriptional regulators have been reported to control the expression of genes required for bacteria...
to adapt to environmental stresses, it is not clear whether most of these regulators bind ligands and the identity of these ligands [45]. Considering our findings showing that CssR has high affinity for antibiotics and heavy metals, we speculated that CssR binding is affected by one or several antibiotics and/or heavy metals, causing cell stress resistance. These data indicated that antibiotic- or heavy metal-binding to CssR directly interfered with the ability of CssR to recognize DNA, which led to cssR and ncl1577 expression, and then, the product of the ncl1576-ncl1577 operon was increased, and it actively exported toxic compounds.

A sequence alignment assay showed that CssR was conserved in several species in the genus Corynebacterium. Notably, the genomic organization of cssR in C. deserti, C. crudilactis, C. callunae, and C. halotolerans was almost identical to that in C. glutamicum. This indicated that the CssR homologs share a similar regulatory mechanism. Interestingly, a BLAST search also revealed that CssR shared some sequence similarity with the TetR family of bacterial regulator proteins, such as AcrR, EnvR, and KstR. Therefore, we believe that the present study can provide insight into other members of the TetR family of transcriptional regulators that directly bind specific ligands. Our study on the regulatory mechanism of CssR may also lead to a greater understanding of stress response mechanisms involving TetR family transcriptional regulators.

Although the cssR-deleted strain (ΔcssR) also exhibited increased resistance to oxidants such as H$_2$O$_2$ and diamide, the apparent failure of cssR and ncl1577 induction was observed under oxidant treatment. Lack of effects from Cys site-directed mutagenesis of CssR on the cssR and ncl1577 expression levels, oxidant exposure on the DNA-binding activity of CssR, or oxidant exposure on the morphology of CssR implied that CssR controlled the target genes expression through a mechanism that does not involve cysteine oxidation-based thiol modification. That is, Cys17 and Cys58 played no role in the regulation of CssR activity. Oxidants, such as diamide and H$_2$O$_2$, have been shown not only to disrupt the cellular redox system but also the defense system against ROS [50]. For example, many antioxidant enzymes remove H$_2$O$_2$, at the expense of reductants such as NAD(P)H and MSH. Diamide specifically oxidized thiol groups such as those in cysteine, resulting in the accumulation of nonnative disulfide bonds [6], which were repaired via the repair reducing system. Interestingly, Mailloux et al. found that metabolism played a key role in Pseudomonas species defenses against oxidative stress [6]. Under H$_2$O$_2$ conditions, the intracellular concentrations of H$_2$O$_2$-scavenging metabolic intermediates and the antioxidant NADPH were increased. Recently, Hong et al. found that TetR-type OsrR in C. glutamicum was not induced by H$_2$O$_2$. However, it was involved in H$_2$O$_2$ resistance, strongly affecting the cellular ratio of NADPH/NADP$^+$, and exhibited a regulatory role for redox homeostasis-related genes, such as trx and mtr [25]. Therefore, we suggested that CssR, despite its moderate sequence similarity to OsrR (approximately 29% identity), may be associated with metabolism, detoxification proteins and repressed system repair. The MSH system (MSH/Mtr), Mrx1 system (Mrx1/Mtr/MSH) and Trx system (Trx/TrxR) have been shown to reduce disulfides in oxidized proteins to maintain intracellular thiol-disulfide homeostasis during bursts of oxidative stress [12, 51, 52]. MSH reportedly acts as a redox buffer and is essential for cellular defenses against ROS and maintaining the reducing state of the cytoplasm [1]. Moreover, MshC was previously found to be necessary for synthesizing MSH, and ΔmsHC mutants lost the ability to produce MSH [1]. Thus, their expression level could reflect the intracellular MSH content to a certain extent. As shown in Fig. 7, genes such as trx, mrx1, mtr, and mshC showed increased transcription in the ΔcssR strain, and the NADPH/NADP$^+$ ratio was higher in the ΔcssR strain. However, detoxification proteins, such as sodA, katA, mpox, prx, prxQ, osmC, and ohr, were not upregulated in the ΔcssR mutant. In C. glutamicum, mpox, prx, prxQ, osmC, and ohr encode peroxidases whose activities are regenerated by Trx, TrxR, Mrx1, Mtr, and MSH [7–11]. Therefore, the protective roles of the genes involved in redox homeostasis in the ΔcssR mutant strain upon oxidant challenge were not realized by supporting peroxidase activity. Additionally, the impact of the enhanced production of redox homeostasis-related genes on oxidative damage restoration should not be very high in the ΔcssR mutant, which may be proven by the oxidant-resistant phenotype of the ΔcssR mutant and indirectly by CssR reliance on redox homeostasis-related genes to a certain extent. Combining these data, we suggested that there might be some oxidant-scavenging metabolic intermediates that act as substrates of CssR, which indirectly caused it to regulate the reduction system. To our surprise, the phenotype of the cssR mutant was almost opposite to that of the osrR mutant. The osrR gene was found to play a positive role in H$_2$O$_2$-detoxifying metabolic systems, except for catalase [25]. Thus, it will be necessary to elucidate how the cssR and osrR genes collaborate with each other to regulate genes involved in oxidative stress responses.

To date, members of the well-known TetR family have been found to form homodimers and then bind to the palindromic sequences of the target gene promoter regions, acting as transcriptional repressors [28]. Our results showed that CssR recognized the 25-bp operator,
which has an imperfect palindromic sequence [TAAG(TGNYGNCNCAATGATT)]. Since, in addition, we found that CsrR occurred as a homodimer in its native form, we proposed that CsrR binds to the target gene promoter as a homodimer.

Overall, CsrR is a TetR family repressor that plays a critical role in the bacterial response to stresses by enhancing the expression of ABC and reductants with ligand-mediated attenuation of DNA binding but not cysteine oxidation-based thiol modifications.

**Abbreviations**

MSH: Mycothiol; TetR: Tetracycline repressor; ABC: ATP-binding cassette; ROS: Reactive oxygen species; DTNB: 5,5′-Dithiobis(2-nitrobenzoic acid); EMSA: Electrophoretic mobility shift assay; CHP: Cumene hydroperoxide; H2O2: Peroxide; NADP+; NADPH: Nicotinamide adenine dinucleotide phosphate; PVDF: Polyvinylidene fluoride; NemR: N-demethylase repressor; OsrR: Oxidative stress response regulator.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s12934-021-01600-8.

**Electrophoretic mobility shift assay; CHP: Cumene hydroperoxide; H2O2: Reactive oxygen species; DTNB: 5,5′-Dithiobis(2-nitrobenzoic acid); MSH: Mycothiol; TetR: Tetracycline repressor; ABC: ATP-binding cassette; ROS:**

**References**

1. Liu YB, Long MX, Yin YJ, Si MR, Zhang L, Lu ZQ, Wang Y, Shen XH. Physiological roles of mycothiol in detoxification and tolerance to multiple poisonous chemicals in Corynebacterium glutamicum. Arch Microbiol. 2013;195:419–29.
2. Shen XH, Zhou NY, Liu SJ. Degradation and assimilation of aromatic compounds by Corynebacterium glutamicum: another potential for applications for this bacterium? Appl Microbiol Biotechnol. 2012;95:77–89.
3. Wheeler GL, Grant CM. Regulation of redox homeostasis in the yeast Saccharomyces cerevisiae. Physiol Plant. 2004;120:12–20.
4. Mannett LJ, Riggins JN, West JD. Endogenous generation of reactive oxidants and electrophiles and their reactions with DNA and protein. J Clin Invest. 2003;111:383–93.
5. Chi BK, Busche T, Laer KV, Baill K, Becker D, Clermont L, Seibold GM, Penicke M, Kalinowski J, Messens J. Protein S-myo-cothiolation functions as redox-switch and thiol protection mechanism in Corynebacterium glutamicum under hypochlorite stress. Antioxid Redox Signal. 2014;20:586–605.
6. Mailloux RJ, Lermire J, Appanna GD. Metabolic networks to combat oxidative stress in Pseudomonas fluorescens. Antonie Van Leeuwenhoek. 2011;99:433–42.
7. Nantapong N, Murata R, Trakulnaleamsai S, Kataoka N, Yakushi T, Matsu-shita K. The effect of reactive oxygen species (ROS) and ROS-scavenging enzymes, superoxide dismutase and catalase, on the thermotolerant ability of Corynebacterium glutamicum. Appl Microbiol Biotechnol. 2019;103:5355–66.
8. Pedre B, Van Molle I, Villadangos AF, Wahni K, Vertommen D, Turell L, Ergodan H, Mateos LM, Messens J. The Corynebacterium glutamicum mycothiol peroxidase is a reactive oxygen species-saving enzyme that shows promiscuity in thiol redox control. Mol Microbiol. 2015;95:1176–91.
9. Su T, Si M, Zhao Y, Liu Y, Yao S, Che C, Chen C. A thioredoxin-dependent peroxiredoxin Q from Corynebacterium glutamicum plays an important role in defense against oxidative stress. PLoS ONE. 2015;10:e0131634. 8. 10. Si M, Su T, Chen C, Wei Z, Gong Z, Li G. Osmc in Corynebacterium glutamicum was a thiol-dependent organic hydroperoxide reductase. Int J Biol Macromol. 2019;136:642–52.
11. Si M, Wang J, Xiao X, Guan J, Zhang Y, Ding W, Chaudhry MT, Wang Y, Shen X. Oct protects Corynebacterium glutamicum against organic hydroperoxide induced oxidative stress. PLoS ONE. 2015;10:e0131634.
12. Che C, Su T, Sun P, Li G, Liu J, Wei Z, Yang G. Thioredoxin and protein-disulfide isomerase selectivity for redox regulation of proteins in Corynebacterium glutamicum. J Gen Appl Microbiol. 2020;66:245–55.
13. Van Laer K, Buts L, Fopolpe N, Vertommen D, Van Belle K, Wahni K, Roos G, Nilsson L, Mateos LM, Kawat M, Van Nuland NAJ, Messens J. Myocredoxin-1 is one of the missing links in the oxidative stress defense mechanism of Mycobacteria. Mol Microbiol. 2012;86:787–804.
14. Su T, Si M, Zhao Y, Yao S, Che C, Liu Y, Chen C. Function of alkyl hydroperoxide ArpH in resistance to oxidative stress in Corynebacterium glutamicum. J Gen Appl Microbiol. 2019;65:72–9.

15. Lee JW, Soonsanga S, Helmnam JD. A complex thiolate switch regulates the Bacillus subtilis organic peroxide sensor OhrR. Proc Natl Acad Sci USA. 2007;104:8743–8.

16. Wilkinson SP, Grove A. Lipid-responsive transcriptional regulation by members of the MarR family of winged helix proteins. Curr Issues Mol Biol. 2006;8:51–62.

17. Teramoto H, Inui M, Yukawa H. OxyR acts as a transcriptional repressor of hydrogen peroxide-inducible antioxidant genes in Corynebacterium glutamicum. FEBS J. 2013;280:298–312.

18. Bussche T, Silar R, Pcmavanaugh M, Patek M, Kalinowski J. Transcriptional regulation of the operon encoding stress-resistant ECF sigma factor SigH and its anti-sigma factor RshA, and control of its regulatory network in Corynebacterium glutamicum. BMC Genom. 2012;13:445.

19. Busche T, Silar R, Picmanova M, Patek M, Kalinowski J. Transcriptional regulation of the operon encoding stress-responsive ECF sigma factor SigH and its anti-sigma factor RshA, and control of its regulatory network in Corynebacterium glutamicum. BMC Genom. 2012;13:445.

20. Bussche T, Silar R, Picmanova M, Patek M, Kalinowski J. Transcriptional regulation of the operon encoding stress-responsive ECF sigma factor SigH and its anti-sigma factor RshA, and control of its regulatory network in Corynebacterium glutamicum. BMC Genom. 2012;13:445.

21. Krug A, Wendisch VF, Bott M. Identification of AcnR, a TetR-type repressor of the acetate gene acon in Corynebacterium glutamicum. J Biol Chem. 2005;280:585–95.

22. Ito H, Watanabe N, Yao M, Shiraikaya Y, Tanaka I. Crystal structures of the multidrug binding repressor Corynebacterium glutamicum CgmR in complex with inducers and with an operator. J Mol Biol. 2010;403:174–84.

23. Chen X, Kohl TA, Ruckert C, Rodionov DA, Li LH, Ding JY, Kalinowski J, Liu S. Phenylacetic acid catabolism and its transcriptional regulation in Corynebacterium glutamicum. Appl Environ Microbiol. 2012;78:5796–804.

24. Brune I, Gotker S, Schneider J, Rodionov DA, Tauch A. Negative transcriptional control of biotin metabolism genes by the TetR-type regulator BioQ in biotin-auxotrophic Corynebacterium glutamicum ATCC 13032. J Biotechnol. 2012;159:225–34.

25. Jakoby M, Nolden L, Meier-Wagner J, Krammer R, Burkovski A. Amitr, a global repressor in the nitrogen regulation system of Corynebacterium glutamicum. Mol Microbiol. 2000;37:964–77.

26. Shen XH, Jiang CY, Huang Y, Liu ZP, Liu SJ. Functional identification of novel genes involved in the glutathione-independent gentisate pathway in Corynebacterium glutamicum. Appl Environ Microbiol. 2005;71:3442–52.

27. Helbig K, Bleuel C, Krauss GJ, Nies DH. Glutathione and transition-metal homeostasis in Escherichia coli. J Bacteriol. 2008;190:5431–8.

28. Miller JH. A short course in bacterial genetics: a laboratory manual and handbook for Escherichia coli and related bacteria, vol. 1. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 1992.

29. Wang T, Si M, Song Y, Zhu W, Gao F, Wang Y, Zhang L, Zhang W, Ge W, Luo QZ, Shen X. Type VI secretion system transports Zn2+ to combat multiple stresses and host immunity. PLoS Pathog. 2015;11:e1005020.

30. Hubber A, Arasaki K, Nakatsu F, Hardiman C, Lambright D, de Camilli P, Nagai H, Roy CR. The machinery at endoplasmic reticulum-plasma membrane contact sites contributes to spatial regulation of multiple Legionella effector proteins. PLoS Pathog. 2014;10:e1004222.

31. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys. 1959;82:70–7.

32. Gething MJ, Davidson BE. Molar absorption-coefficient of reduced Ellmans reagent -3-carboxylato-4-nitro-thiophenolate. Eur J Biochem. 1972;30:352–3.

33. Saier MH, Paulsen IT. Phylogeny of multidrug transporters. Cell Dev Biol. 2001;12:205–13.

34. Grkovic S, Brown MH, Skurray RA. Regulation of bacterial drug export systems. Microbiol Mol Biol Rev. 2002;66:671–701.

35. Hinrichs W, Kisker C, Duvel M, Muller A, Tovar K, Hillen W, Saenger W. Structure of the Tet repressor-tetracycline complex and regulation of the tetracycline effector proteins. PLoS Pathog. 2014;10:e1004222.

36. Grkovic S, Brown MH, Schumacher MA, Brennan RG, Skurray RA. The staphyloccocal QacR multidrug regulator binds a correctly spaced operator as a pair of dimers. J Bacteriol. 2001;183:7102–9.

37. Helbig K, Bleuel C, Krauss GJ, Nies DH. Glutathione and transition-metal homeostasis in Escherichia coli. J Bacteriol. 2008;190:5431–8.

38. Leichert UJO, Scharf C, Becker M. Global characterization of disulfide stress in Bacillus subtilis. J Bacteriol. 2003;185:1967–75.

39. Si M, Wang T, Pan J, Lin J, Chen C, Wei Y, Lu Z, Wei G, Shen X. Graded response of the multifunctional 2-cysteine peroxiredoxin, CgPrx, to increasing levels of hydrogen peroxide in Corynebacterium glutamicum. Antioxid Redox Signal. 2017;26:1–14.

40. Li X, Liu Y, Zhong J, Che C, Gong Z, Si M, Yang G. Molecular mechanisms of Mycobactin-S in resistance to oxidative stress in Corynebacterium glutamicum. J Gen Appl Microbiol. 2020. https://doi.org/10.1007/s13223-019-00303-8.

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