A novel redox-sensing transcriptional regulator CyeR controls expression of an Old Yellow Enzyme family protein in *Corynebacterium glutamicum*

Shigeki Ehira,[†] Haruhiko Teramoto, Masayuki Inui and Hideaki Yukawa

Correspondence

Hideaki Yukawa
mmg-lab@rite.or.jp

Research Institute of Innovative Technology for the Earth (RITE), 9-2 Kizugawadai, Kizugawa, Kyoto 619-0292, Japan

Corynebacterium glutamicum cgR_2930 (*cyeR*) encodes a transcriptional regulator of the ArsR family. Its gene product, CyeR, was shown here to repress the expression of *cyeR* and the cgR_2931 (*cye1*)–cgR_2932 operon, which is located upstream of *cyeR* in the opposite orientation. The *cye1* gene encodes an Old Yellow Enzyme family protein, members of which have been implicated in the oxidative stress response. CyeR binds to the intergenic region between *cyeR* and *cye1*. Expression of *cyeR* and *cye1* is induced by oxidative stress, and the DNA-binding activity of CyeR is impaired by oxidants such as diamide and H₂O₂. CyeR contains two cysteine residues, Cys-36 and Cys-43. Whereas mutation of the former (C36A) has no effect on the redox regulation of CyeR activity, mutating the latter (C43A, C43S) abolishes the DNA-binding activity of CyeR. Cys-43 of CyeR and its C36A derivative are modified upon treatment with diamide, suggesting an important role for Cys-43 in the redox regulation of CyeR activity. It is concluded that CyeR is a redox-sensing transcriptional regulator that controls *cye1* expression.

Received 4 December 2009

Revised 26 January 2010

Accepted 27 January 2010

INTRODUCTION

Corynebacterium glutamicum is a non-pathogenic, high-G+C, Gram-positive bacterium that belongs to the actinobacteria, which include the genera *Mycobacterium* and *Streptomyces*. *C. glutamicum* is widely used for the industrial production of various amino acids and nucleic acids (Hermann, 2003; Terasawa & Yukawa, 1993). We have previously demonstrated a high productivity of ethanol and organic acids using *C. glutamicum* (Inui *et al.*, 2004a, b; Okino *et al.*, 2008a, b). Meanwhile, this species is of increasing interest as a model organism for closely related pathogenic species such as *Corynebacterium diphtheriae* and *Mycobacterium tuberculosis* (Brune *et al.*, 2005; Mishra *et al.*, 2007). Since the *C. glutamicum* genomic sequence was determined (Ikeda & Nakagawa, 2003; Kalinowski *et al.*, 2003; Yukawa *et al.*, 2007), knowledge of transcriptional regulation in *C. glutamicum* has drastically increased as a result of combining DNA microarray analysis with targeted mutagenesis (Baumbach *et al.*, 2009). However, of more than 100 genes encoding

transcriptional regulators in its genome (Brune *et al.*, 2005), many remain uncharacterized.

Oxidative stress is an inescapable consequence of aerobic metabolism, which produces reactive oxygen species that can damage cellular components, including nucleic acids, proteins and lipids. Redox-sensing transcriptional regulators play central roles in the cellular response to oxidative stress by regulating expression of resistance genes against oxidative stress (Paget & Buttner, 2003). The reduction-oxidation of the thiol groups of cysteine residues is the basis of various mechanisms that sense changes in cellular redox conditions in response to oxidative stress. For example, a LysR family transcriptional regulator, OxyR, of *Escherichia coli* is activated by oxidation through the formation of an intramolecular disulfide bond between Cys-199 and Cys-208 (Zheng *et al.*, 1998). OxyR regulates the expression of genes involved in peroxide metabolism and protection (*katG*, *ahpCF*, *dps*), and redox balance (*gor*, *grxA*, *trxC*) (Zheng *et al.*, 2001). A MarR family transcriptional regulator, OhrR, of *Bacillus subtilis* is an organic peroxide sensor and represses expression of *ohrA*, which encodes a peroxiredoxin (Fuangthong *et al.*, 2001). The lone cysteine residue (Cys-15) of OhrR is essential for redox sensing (Fuangthong & Helmann, 2002). Oxidation of Cys-15 leads to S-thiolation by cysteine, coenzyme A or an unknown thiol with a molecular mass of 398 Da (Lee *et al.*, 2007).

It has recently been shown that a DUF24 family protein, QorR, of *C. glutamicum* is a redox-sensing transcriptional

[†]Present address: Department of Biological Science, Chuo University, 1-13-27 Kasuga, Bunkyo, Tokyo 112-8551, Japan.

Abbreviations: AMS, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid; OYE, Old Yellow Enzyme; qRT-PCR, quantitative RT-PCR; RACE-PCR, rapid amplification of cDNA ends-PCR; TIS, transcription initiation site.

A supplementary figure, showing an amino acid sequence alignment of CyeR homologues, and a supplementary table, showing the primers used in this study, are available with the online version of this paper.

regulator, and represses expression of both *qor2*, which encodes a quinone oxidoreductase, and its own structural gene (Ehira *et al.*, 2009a). QorR contains a lone cysteine residue (Cys-17). Under oxidizing conditions, QorR undergoes dimerization and loses DNA-binding activity through the formation of an intermolecular disulfide bond between Cys-17 of each subunit.

In this study, we characterized an ArsR family transcriptional regulator, CgR2930 (CyeR), encoded by the gene which is located immediately upstream of the *cgR_2931* (*cyel*) operon in the opposite orientation. The *cyel* gene encodes an Old Yellow Enzyme (OYE) family protein (Williams & Bruce, 2002). The OYE family members are proposed to be involved in the oxidative stress response in bacteria, e.g. in *B. subtilis* and *Shewanella oneidensis* (Brigé *et al.*, 2006; Fitzpatrick *et al.*, 2003), although the gene regulation mechanism remains unknown. Our present results show that CyeR directly controls expression of the *cyel* operon and its own structural gene as a transcriptional repressor. Expression of *cyeR* and *cyel* is induced by thiol oxidative stress, and the DNA-binding activity of CyeR is impaired by oxidants. Furthermore, site-directed mutagenesis studies show that one of two cysteine residues of CyeR plays a key role in its DNA-binding activity. These findings suggest that CyeR is a redox-sensing transcriptional regulator involved in the oxidative stress response of *C. glutamicum* by regulating *cyel* expression.

METHODS

Bacterial strains, culture media and growth conditions. *C. glutamicum* strain R (Yukawa *et al.*, 2007) and its derivative were grown at 33 °C in A medium (Inui *et al.*, 2007) with 4% (w/v) glucose on a rotary shaker at 180 r.p.m. A disruptant of *cyeR* (Δ 2930) was constructed by the transposon-mediated mutagenesis method, as described previously (Suzuki *et al.*, 2006). Disruption of *cyeR* was confirmed by DNA sequencing of thermal asymmetrical interlaced-PCR products of mutant cells.

RNA isolation and DNA microarray analysis. Total RNA was extracted from *C. glutamicum* cells by using the RNeasy Mini kit (Qiagen) and was treated with DNase I (Takara Bio), as described previously (Ehira *et al.*, 2008). Global gene expression analysis was performed with the *C. glutamicum* R DNA microarray, as described previously (Ehira *et al.*, 2008). Microarray analysis was carried out using two sets of RNA samples isolated from independently grown cultures with different combinations of Cy dyes (a dye swap strategy). Since the *C. glutamicum* R DNA microarray contains two replicates per gene, a total of four replicates per gene were available to determine changes in gene expression. Genes with significantly differential transcript levels ($P < 0.05$ in a Student's *t* test) by at least a factor of two were determined.

Real-time quantitative RT-PCR (qRT-PCR) analysis. A one-step real-time qRT-PCR was performed with the Power SYBR Green PCR Master Mix (Applied Biosystems) and a pair of gene-specific primers (Supplementary Table S1) by using the 7500 Fast Real-Time PCR system (Applied Biosystems), as described previously (Ehira *et al.*, 2008). Relative ratios were normalized with the value for 16S rRNA.

RT-PCR analysis. cDNA was synthesized from total RNA with a reverse primer as follows. An aliquot (1 µg) of total RNA and 1 pmol primer RT2932-R were denatured at 90 °C for 5 min and then gradually cooled to 55 °C. The reverse transcription reaction was performed in 20 µl First-Strand Buffer [50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂] containing total RNA, the primer RT2932-R, 5 mM DTT, 0.5 mM each of dATP, dGTP, dCTP and dTTP, and 200 U SuperScript III RNase H⁻ reverse transcriptase (Invitrogen). The reaction mixture was incubated at 55 °C for 1 h, and then the reaction was stopped by addition of 80 µl TE buffer [10 mM Tris/HCl (pH 8.0), 1 mM EDTA]. An aliquot (1 µl) of the cDNA solution was taken for PCR analysis using the primer pair RT2931-F and RT2932-R.

Mapping of transcription initiation sites (TISs) by rapid amplification of cDNA ends (RACE)-PCR. TISs were determined by using the SMART RACE cDNA Amplification kit (Clontech). 5' RACE-PCRs were carried out as described previously (Ehira *et al.*, 2009b), with 1 µg total RNA and gene-specific primers (Supplementary Table S1). The resulting PCR products were cloned into a pGEM-T Easy vector (Promega). At least 20 clones for each 5' RACE-PCR product were sequenced.

Expression and purification of His-CyeR, His-CyeRC36A and His-CyeRC43A. For construction of an expression plasmid for the histidine-tagged CyeR (His-CyeR) protein, a DNA fragment containing the ORF of *cyeR* was amplified by PCR using the primer pair *rcgR2930-F* and *rcgR2930-R* (Supplementary Table S1). The amplified DNA fragment was cloned between the *NdeI* and *EcoRI* sites of the pET-28a expression vector (Merck KGaA). The resulting plasmid, pCRD612, contained *cyeR* fused to the His-tag sequence. A cysteine residue at position 36 or position 43 of CyeR was replaced with alanine by performing site-directed mutagenesis using a PrimeSTAR Mutagenesis Basal kit (Takara Bio). pCRD612 was used as a template for PCR with the primer pair 2930C36A-F and 2930C36A-R or the primer pair 2930C43A-F and 2930C43A-R (Supplementary Table S1) to generate plasmid pCRD613 or plasmid pCRD614, respectively. Expression and purification of recombinant proteins were performed as described previously (Ehira *et al.*, 2009a) using *E. coli* BL21 (DE3) cells harbouring pCRD612, pCRD613 or pCRD614.

Gel mobility shift assay. The gel mobility shift assay was performed with purified recombinant proteins and a Cy3-labelled probe, as described previously (Ehira *et al.*, 2009a). Probes 2930-1 and 1435-6 were prepared by PCR using the primer pair RT2930-R and 2931R+92 and pCRD621 (Ehira *et al.*, 2009a) as a template, and the primer pair 1436R+51 and RT1435-R and pCRD620 (Ehira *et al.*, 2009a) as a template, respectively. A Cy3-labelled 2931R+92 primer was used for preparation of the Cy3-labelled probe.

Thiol redox state analyses. After incubation with 1 mM DTT or diamide for 30 min, His-CyeR and His-CyeRC36A (1 µg) were incubated at room temperature for 2 h in 200 mM Tris/HCl (pH 8.0) containing 1% SDS and 15 mM 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) (Molecular Probes). The mixtures were then separated by non-reducing SDS-PAGE.

RESULTS

CgR2930 (CyeR) negatively regulates expression of the *cyel* operon and *cyeR*

C. glutamicum *cgR_2930* encodes a transcriptional regulator of the ArsR family. To ascertain the physiological role of CgR2930 as a transcriptional regulator in *C. glutamicum*,

gene expression profiles during exponential growth were compared between the wild-type strain and a *cgR_2930* disruptant ($\Delta 2930$) using a DNA microarray. Expression of only *cgR_2931* and *cgR_2932*, which are located upstream of *cgR_2930* in the opposite orientation, was shown to be upregulated in $\Delta 2930$. Differential expression of *cgR_2931* and *cgR_2932* was confirmed by qRT-PCR. In $\Delta 2930$, the transcript levels of *cgR_2931* and *cgR_2932* were more than 20-fold higher (25.9 ± 3.5 and 23.7 ± 5.4 , respectively) than the wild-type levels. As the distance between *cgR_2931* and *cgR_2932* is 12 bp, the two genes are likely to be co-transcribed. The dicistronic transcript of *cgR_2931* and *cgR_2932* was indeed detected by RT-PCR analysis (data not shown). It was not possible to determine the TIS of *cgR_2931* by RACE-PCR and primer extension analysis because of the detection of multiple 5' ends of transcripts. However, putative -10 and -35 promoter sequences were found upstream of *cgR_2931* (Fig. 1). The TIS of *cgR_2930* was determined by RACE-PCR and the -10 and -35 promoter regions were identified (Fig. 1). Since the promoters of *cgR_2930* and *cgR_2931* are supposed to overlap, the transcript level of *cgR_2930* in $\Delta 2930$ was determined by qRT-PCR using a primer pair designed based on a sequence upstream of the transposon insertion site of $\Delta 2930$. The transcript level of *cgR_2930* in $\Delta 2930$ was about 10 times higher (10.9 ± 2.0) than that of the wild-type. These results indicate that *CgR_2930* negatively controls expression of the *cgR_2931*–*cgR_2932* operon and its structural gene.

The *cgR_2931* gene encodes a protein with 42 % amino acid sequence identity to YqjM of *B. subtilis*, which is a member of the OYE family (Fitzpatrick *et al.*, 2003). The entire amino acid sequence encoded by the *cgR_2932* gene does not show high similarity to that of any other protein characterized functionally so far. We designated *cgR_2931* as *cye1* (*Corynebacterium* yellow enzyme 1) and *cgR_2930* as *cyeR* (*Corynebacterium* yellow enzyme regulator).

Expression of *cyeR* and the *cye1* operon is induced by oxidative stress

The OYE family members are implicated in the oxidative stress response in bacteria (Brigé *et al.*, 2006; Fitzpatrick *et al.*, 2003). As expression of *B. subtilis* YqjM and *S. oneidensis* SYE4 is induced in response to oxidative stress, changes in expression of *cyeR* and *cye1* upon oxidative stress were examined. The transcript levels of *cyeR* and

Fig. 2. Changes in the transcript levels of *cyeR* and *cye1* in response to diamide. The relative transcript levels of *cyeR* (squares) and *cye1* (circles) before (0 min) and at 5, 10, 15 and 20 min after addition of 3 mM diamide were determined by qRT-PCR. The transcript level at 0 min was taken as 1. Experiments were repeated twice and representative data are shown.

cye1 in exponentially growing cells were determined by qRT-PCR before and after treatment of cells with the thiol-specific oxidant diamide (Fig. 2). *cyeR* and *cye1* were upregulated within 5 min of the addition of diamide and the transcript levels remained high for another 5 min, before gradually decreasing. The *cye1* transcript level also increased upon addition of H_2O_2 (data not shown).

CyeR binds to the intergenic region between *cyeR* and *cye1*

Gel mobility shift assays were carried out with purified His–CyeR and a DNA probe, 2930-1, for the intergenic region between *cyeR* and *cye1* (Fig. 3). His–CyeR reduced the electrophoretic mobility of probe 2930-1 (Fig. 3, lanes 1–4). The band intensity of the protein–DNA complex was reduced upon addition of a non-labelled 2930-1 fragment (Fig. 3, lanes 5 and 6). However, addition of a probe, 1435-6, that contains the oxidative stress-responsive promoters of *qorR* and *qor2* (Ehira *et al.*, 2009a) did not affect the amount of complex formed (Fig. 3, lanes 7 and 8). It was concluded that His–CyeR binds to the *cyeR* and *cye1* intergenic region in a sequence-specific manner.

```

ACAGCTTGGG CACCAAAGGC TCCATTCAAT CGACTAGTAC GACGTTTACC ATACAACGAG
TGTCGAACCT GTGGTTTCCG AGGTAAGTTA GCTGATCATG CTGCAAAATGG TATGTTGCTC
                                -35 cgR_2931                                -10
TTTGATCGTA CAACTAGCTT GGCTTAAGGG CAAGGTACGA TTAGAGTCCT ACCTAAATTC
AAACTAGCAT GTTGATCGAA CCGAATTCCC GTTCCATGCT AATCTCAGCA TGGATTTAAG
                                -35                                -10
                                cgR_2930
AATACTGTTT TACGAGATCA TTTGGAGGGT GTCATGGCCC GCAAATTGGA ACATCCATCT
TTATGACAAA ATGCTCTAGT AAACCTCCCA CAGTACCGGG CGTTTAACCT TGTAGGTAGA

```

Fig. 1. Nucleotide sequence of the *cgR_2930*–*cgR_2931* intergenic region. The coding regions of *cgR_2930* and *cgR_2931* are shaded in grey. The identified TIS and the putative -10 and -35 promoter regions are indicated by the bent arrow and bold type, respectively.

Fig. 3. Gel mobility shift assays with His-CyeR and the *cyeR-cye1* intergenic region. The binding of His-CyeR to probe 2930-1 was examined. Probe 2930-1 (2 nM) was mixed with His-CyeR in the amounts indicated above each lane, and then the mixtures were subjected to electrophoresis. Non-labelled fragments of 2930-1 (lanes 5 and 6) and 1435-6 (lanes 7 and 8) were added at the indicated amounts. Filled arrow, complex of His-CyeR and probe 2930-1; open arrow, probe 2930-1 alone.

CyeR is a redox-responsive transcriptional regulator

The predicted amino acid sequence of CyeR contains two cysteine residues at position 36 (Cys-36) and 43 (Cys-43). Cys-43 is conserved among uncharacterized CyeR homologues not only in actinobacteria but also in proteobacteria, firmicutes and cyanobacteria (Supplementary Fig. S1). Reduction-oxidation of these cysteine residues is presumed to be involved in the control of CyeR activity. The effect of oxidants on the DNA-binding activity of CyeR was examined by gel mobility shift assays (Fig. 4a). Binding of His-CyeR to probe 2930-1 was prevented by addition of diamide (Fig. 4a, lanes 2–6). The addition of an excess of the reducing agent DTT restored the DNA-binding activity of His-CyeR that was inactivated by diamide (Fig. 4a, lanes 8–12), indicating that the effects of oxidation and reduction on the DNA-binding activity of CyeR are reversible. The DNA-binding activity of CyeR was also impaired by H_2O_2 (Fig. 4b).

Cys-43 plays a pivotal role in the DNA-binding activity of CyeR

To examine the role of cysteine residues in the redox-responsive regulation of CyeR activity, Cys-36 and Cys-43 were replaced with alanine or serine. His-CyeRC36A reduced the electrophoretic mobility of probe 2930-1 (Fig. 5a, lanes 1–4) and binding of His-CyeRC36A to probe 2930-1 was prevented by addition of diamide (Fig. 5b). However, no interaction between His-CyeRC43A (Fig. 5a, lanes 5–8) or His-CyeRC43S (data not shown) and probe 2930-1 was observed. It was concluded that Cys-36 is dispensable for redox regulation of CyeR activity and that substitutions of Cys-43 inactivate CyeR.

Fig. 4. Redox-sensitive control of DNA-binding activity of CyeR. (a) His-CyeR (300 nM) and probe 2930-1 (2 nM) were incubated in the presence of 1 mM DTT (lanes 2–6) or 1 mM diamide (lanes 8–12) for 30 min, and then diamide (lanes 2–6) or DTT (lanes 8–12) was added in the amounts indicated above each lane. After 30 min, the mixtures were subjected to electrophoresis. Lanes 1 and 7, His-CyeR not added. (b) His-CyeR (300 nM) and probe 2930-1 (2 nM) were incubated in the presence of 1 mM DTT for 30 min, and then H_2O_2 (lanes 2–5) was added in the amounts indicated above each lane. After 30 min, the mixtures were subjected to electrophoresis. Lane 1, His-CyeR not added.

We examined whether Cys-36 and Cys-43 form an inter- or intramolecular disulfide bond under oxidizing conditions. When the His-CyeR protein oxidized with diamide was subjected to non-reducing SDS-PAGE, only one band of a molecular mass of approximately 14 kDa, corresponding to the monomeric form of His-CyeR, was detected (Fig. 6, lane 2), indicating that diamide-inactivated His-CyeR does not undergo dimerization. We next examined whether the thiol groups of cysteine residues of CyeR are modified by treatment with diamide using AMS. AMS covalently modifies free thiol groups, which retards electrophoretic mobility. The electrophoretic mobility of His-CyeR treated with DTT was retarded by AMS modification (Fig. 6, lanes 1 and 3), while AMS did not affect the mobility of diamide-treated His-CyeR (Fig. 6, lanes 2 and 4). When diamide-inactivated His-CyeRC36A was subjected to non-reducing SDS-PAGE, an additional band of molecular mass 28 kDa, which is likely to correspond to the dimeric form of His-CyeRC36A, appeared (Fig. 6, lane 6). AMS modification was not observed for either the monomeric or the dimeric form of diamide-treated His-CyeRC36A (Fig. 6, lane 8). His-CyeRC36A treated with DTT was modified with AMS, although the electrophoretic mobility of the AMS-modified His-CyeRC36A was faster than that of the AMS-modified His-CyeR (Fig. 6, lanes 3 and 7). These results indicate that the diamide treatment prevents AMS modification of two

Fig. 5. DNA-binding activity of His-CyeRC36A and His-CyeRC43A. (a) Gel mobility shift assays were performed with His-CyeRC36A (lanes 1–4) or His-CyeRC43A (lanes 5–8) and probe 2930-1, as described in Fig. 3. (b) His-CyeRC36A (300 nM) and probe 2930-1 (2 nM) were incubated in the presence of 1 mM DTT for 30 min, and then 1 mM (lane 3) or 3 mM diamide (lane 4) was added. After 30 min, the mixtures were subjected to electrophoresis. Lane 1, His-CyeRC36A not added.

cysteine residues, Cys-36 and Cys-43, of His-CyeR, and a lone cysteine residue, Cys-43, of His-CyeRC36A. Therefore, it is likely that modification of Cys-43 under oxidizing conditions inactivates the CyeR activity.

DISCUSSION

In the present study, we demonstrated that CyeR is a redox-sensing transcriptional regulator that represses expression of the *cye1* operon and its structural gene. Cye1 belongs to the OYE family, members of which are found in yeasts, bacteria, plants and nematodes, and share characteristic biochemical properties: the protein binds flavin and catalyses the reduction of broad substrates, such as α,β -unsaturated aldehydes and ketones, using NADPH as a cofactor (Williams & Bruce, 2002). Expression of bacterial OYE family members such as *B. subtilis* YqjM and *S. oneidensis* SYE4 is induced by oxidative stress. Based on their biochemical and expression properties, the OYE family members are implicated in the control of the cellular redox state in response to oxidative stress, although exact substrates *in vivo* remain elusive (Brigé *et al.*, 2006; Fitzpatrick *et al.*, 2003). We observed that expression of the *cye1* gene is induced by treatment with diamide (Fig. 2) or H_2O_2 . The redox-responsive DNA-binding activity of CyeR suggests that expression of *cye1* is derepressed by inactivation of the transcriptional repressor CyeR, in which

Fig. 6. Oxidation of cysteine residues of CyeR. Purified His-CyeR (lanes 1–4) and His-CyeRC36A (lanes 5–8) were treated as follows. Proteins (1 μ g) treated with 1 mM DTT (lanes 1, 3, 5 and 7) or 1 mM diamide (lanes 2, 4, 6 and 8) were further incubated with (lanes 3, 4, 7 and 8) or without (lanes 1, 2, 5 and 6) AMS. The modified samples were separated by non-reducing SDS-PAGE. Lanes M, molecular mass standard marker.

thiol groups of cysteine residues are modified under oxidizing conditions. The effects of site-directed mutagenesis on the DNA-binding activity imply that Cys-43 plays a pivotal role in the redox regulation of CyeR activity. To our knowledge, this is the first report to characterize a transcriptional regulator that controls expression of OYE family proteins in bacteria. It is noteworthy that a CyeR homologue in *Pseudomonas putida* is located upstream of *xenA*, which encodes a xenobiotic reductase A (Bleher *et al.*, 1999). XenA and Cye1 belong to the YqjM family, a new bacterial subfamily of OYE homologues (Kitzing *et al.*, 2005).

Unlike *C. glutamicum* QorR, which becomes dimeric and inactive under oxidizing conditions (Ehira *et al.*, 2009a), CyeR loses the DNA-binding activity but remains monomeric (Fig. 6, lane 2). Since both Cys-36 and Cys-43 are protected from AMS modification by treatment with diamide (Fig. 6, lane 4), an intramolecular disulfide bond between these two cysteine residues may be formed. However, the DNA-binding activity of CyeRC36A remains redox-responsive (Fig. 5). Most of the diamide-inactivated CyeRC36A protein remains monomeric, although some undergoes dimerization (Fig. 6, lane 6). As Cys-43 of the monomeric form of CyeRC36A is modified upon diamide treatment (Fig. 6, lane 8), CyeR is likely to be inactivated by Cys-43 modification, although the mechanism of CyeRC36A inactivation upon diamide treatment is currently unknown. As reported for *B. subtilis* YodB, a DUF24 family redox-sensing transcriptional regulator, modification of cysteine residues with diamide might occur *in vitro*,

although detection of such molecular species has not yet been realized (Leelakriangsak *et al.*, 2008). Replacement of Cys-43 with alanine or serine inactivates CyeR, suggesting that the thiol group of Cys-43, which is predicted to be positioned in the helix–turn–helix domain, is essential for maintaining the correct structure for DNA binding of CyeR (Fig. 5a). Further study is needed to fully understand the role of Cys-43 in the redox-sensing mechanism of CyeR.

B. subtilis displays a complex adaptive response to oxidative stress that is coordinated by a sigma factor, SigB, along with transcriptional regulators PerR and OhrR (Helmann *et al.*, 2003). In *C. glutamicum*, two extra-cytoplasmic function sigma factors, SigH and SigM, are involved in the response to oxidative stress (Kim *et al.*, 2005; Nakunst *et al.*, 2007). SigH controls transcription of *sigM*, and SigM regulates expression of *trxB1* and *trxC*, encoding thioredoxins, and *trxB*, encoding a thioredoxin reductase. Although there are no *C. glutamicum* homologues of PerR and OhrR, two transcriptional regulators, QorR (Ehira *et al.*, 2009a) and CyeR, contribute to the oxidative stress response. Moreover, *Streptomyces coelicolor* OxyR has been shown to regulate the expression of the *ahpCD* operon, which encodes an alkyl hydroperoxide reductase system, and its structural gene (Hahn *et al.*, 2002). An OxyR homologue is also encoded in the *C. glutamicum* genome. Therefore, the oxidative stress response of *C. glutamicum* as well as other actinobacteria appears to be controlled by a wide variety of regulatory mechanisms, more than is the case for other bacteria that have been examined to date (den Hengst & Buttner, 2008).

ACKNOWLEDGEMENTS

We thank Crispinus A. Omumasaba (RITE) for critical reading of the manuscript. This study was partially supported by a grant from the New Energy and Industrial Technology Development Organization (NEDO), Japan.

REFERENCES

- Baumbach, J., Wittkop, T., Kleindt, C. K. & Tauch, A. (2009). Integrated analysis and reconstruction of microbial transcriptional gene regulatory networks using CoryneRegNet. *Nat Protoc* **4**, 992–1005.
- Bleher, D. S., Fox, B. G. & Chambliss, G. H. (1999). Cloning and sequence analysis of two *Pseudomonas* flavoprotein xenobiotic reductases. *J Bacteriol* **181**, 6254–6263.
- Brigé, A., Van den Hemel, D., Carpentier, W., De Smet, L. & Van Beeumen, J. J. (2006). Comparative characterization and expression analysis of the four Old Yellow Enzyme homologues from *Shewanella oneidensis* indicate differences in physiological function. *Biochem J* **394**, 335–344.
- Brune, I., Brinkrolf, K., Kalinowski, J., Pühler, A. & Tauch, A. (2005). The individual and common repertoire of DNA-binding transcriptional regulators of *Corynebacterium glutamicum*, *Corynebacterium efficiens*, *Corynebacterium diphtheriae* and *Corynebacterium jeikeium* deduced from the complete genome sequences. *BMC Genomics* **6**, 86.
- den Hengst, C. D. & Buttner, M. J. (2008). Redox control in actinobacteria. *Biochim Biophys Acta* **1780**, 1201–1216.
- Ehira, S., Shirai, T., Teramoto, H., Inui, M. & Yukawa, H. (2008). Group 2 sigma factor SigB of *Corynebacterium glutamicum* positively regulates glucose metabolism under conditions of oxygen deprivation. *Appl Environ Microbiol* **74**, 5146–5152.
- Ehira, S., Ogino, H., Teramoto, H., Inui, M. & Yukawa, H. (2009a). Regulation of quinone oxidoreductase by the redox-sensing transcriptional regulator QorR in *Corynebacterium glutamicum*. *J Biol Chem* **284**, 16736–16742.
- Ehira, S., Teramoto, H., Inui, M. & Yukawa, H. (2009b). Regulation of *Corynebacterium glutamicum* heat shock response by the extracytoplasmic-function sigma factor SigH and transcriptional regulators HspR and HrcA. *J Bacteriol* **191**, 2964–2972.
- Fitzpatrick, T. B., Amrhein, N. & Macheroux, P. (2003). Characterization of YqjM, an Old Yellow Enzyme homologue from *Bacillus subtilis* involved in the oxidative stress response. *J Biol Chem* **278**, 19891–19897.
- Fuangthong, M. & Helmann, J. D. (2002). The OhrR repressor senses organic hydroperoxides by reversible formation of a cysteine-sulfenic acid derivative. *Proc Natl Acad Sci U S A* **99**, 6690–6695.
- Fuangthong, M., Atichartpongkul, S., Mongkolsuk, S. & Helmann, J. D. (2001). OhrR is a repressor of *ohrA*, a key organic hydroperoxide resistance determinant in *Bacillus subtilis*. *J Bacteriol* **183**, 4134–4141.
- Hahn, J.-S., Oh, S.-Y. & Roe, J.-H. (2002). Role of OxyR as a peroxide-sensing positive regulator in *Streptomyces coelicolor* A3(2). *J Bacteriol* **184**, 5214–5222.
- Helmann, J. D., Wu, M. F. W., Gaballa, A., Kobel, P. A., Morshedi, M. M., Fawcett, P. & Paddon, C. (2003). The global transcriptional response of *Bacillus subtilis* to peroxide stress is coordinated by three transcription factors. *J Bacteriol* **185**, 243–253.
- Hermann, T. (2003). Industrial production of amino acids by coryneform bacteria. *J Biotechnol* **104**, 155–172.
- Ikeda, M. & Nakagawa, S. (2003). The *Corynebacterium glutamicum* genome: features and impacts on biotechnological processes. *Appl Microbiol Biotechnol* **62**, 99–109.
- Inui, M., Kawaguchi, H., Murakami, S., Vertès, A. A. & Yukawa, H. (2004a). Metabolic engineering of *Corynebacterium glutamicum* for fuel ethanol production under oxygen-deprivation conditions. *J Mol Microbiol Biotechnol* **8**, 243–254.
- Inui, M., Murakami, S., Okino, S., Kawaguchi, H., Vertès, A. A. & Yukawa, H. (2004b). Metabolic analysis of *Corynebacterium glutamicum* during lactate and succinate productions under oxygen deprivation conditions. *J Mol Microbiol Biotechnol* **7**, 182–196.
- Inui, M., Suda, M., Okino, S., Nonaka, H., Puskás, L. G., Vertès, A. A. & Yukawa, H. (2007). Transcriptional profiling of *Corynebacterium glutamicum* metabolism during organic acid production under oxygen deprivation conditions. *Microbiology* **153**, 2491–2504.
- Kalinowski, J., Bathe, B., Bartels, D., Bischoff, N., Bott, M., Burkowski, A., Dusch, N., Eggeling, L., Eikmanns, B. J. & other authors (2003). The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. *J Biotechnol* **104**, 5–25.
- Kim, T. H., Kim, H. J., Park, J. S., Kim, Y., Kim, P. & Lee, H. S. (2005). Functional analysis of *sigH* expression in *Corynebacterium glutamicum*. *Biochem Biophys Res Commun* **331**, 1542–1547.
- Kitzing, K., Fitzpatrick, T. B., Wilken, C., Sawa, J., Bourenkov, G. P., Macheroux, P. & Clausen, T. (2005). The 1.3 Å crystal structure of the flavoprotein YqjM reveals a novel class of Old Yellow Enzymes. *J Biol Chem* **280**, 27904–27913.

- Lee, J. W., Soonsanga, S. & Helmann, J. D. (2007). A complex thiolate switch regulates the *Bacillus subtilis* organic peroxide sensor OhrR. *Proc Natl Acad Sci U S A* **104**, 8743–8748.
- Leelakriangsak, M., Huyen, N. T., Töwe, S., van Duy, N., Becher, D., Hecker, M., Antelmann, H. & Zuber, P. (2008). Regulation of quinone detoxification by the thiol stress sensing DUF24/MarR-like repressor, YodB in *Bacillus subtilis*. *Mol Microbiol* **67**, 1108–1124.
- Mishra, A. K., Alderwick, L. J., Rittmann, D., Tatituri, R. V., Nigou, J., Gilleron, M., Eggeling, L. & Besra, G. S. (2007). Identification of an $\alpha(1\rightarrow6)$ mannopyranosyltransferase (MptA), involved in *Corynebacterium glutamicum* lipomanann biosynthesis, and identification of its orthologue in *Mycobacterium tuberculosis*. *Mol Microbiol* **65**, 1503–1517.
- Nakunst, D., Larisch, C., Hüser, A. T., Tauch, A., Pühler, A. & Kalinowski, J. (2007). The extracytoplasmic function-type sigma factor SigM of *Corynebacterium glutamicum* ATCC 13032 is involved in transcription of disulfide stress-related genes. *J Bacteriol* **189**, 4696–4707.
- Okino, S., Noburyu, R., Suda, M., Jojima, T., Inui, M. & Yukawa, H. (2008a). An efficient succinic acid production process in a metabolically engineered *Corynebacterium glutamicum* strain. *Appl Microbiol Biotechnol* **81**, 459–464.
- Okino, S., Suda, M., Fujikura, K., Inui, M. & Yukawa, H. (2008b). Production of D-lactic acid by *Corynebacterium glutamicum* under oxygen deprivation. *Appl Microbiol Biotechnol* **78**, 449–454.
- Paget, M. S. B. & Buttner, M. J. (2003). Thiol-based regulatory switches. *Annu Rev Genet* **37**, 91–121.
- Suzuki, N., Okai, N., Nonaka, H., Tsuge, Y., Inui, M. & Yukawa, H. (2006). High-throughput transposon mutagenesis of *Corynebacterium glutamicum* and construction of a single-gene disruptant mutant library. *Appl Environ Microbiol* **72**, 3750–3755.
- Terasawa, M. & Yukawa, H. (1993). Industrial production of biochemicals by native immobilization. In *Industrial Application of Immobilized Biocatalysts*, pp. 37–52. Edited by A. Tanaka, O. Tosaka & T. Kobayashi. New York: Marcel Dekker, Inc.
- Williams, R. E. & Bruce, N. C. (2002). ‘New uses for an Old Enzyme’ – the Old Yellow Enzyme family of flavoenzymes. *Microbiology* **148**, 1607–1614.
- Yukawa, H., Omumasaba, C. A., Nonaka, H., Kós, P., Okai, N., Suzuki, N., Suda, M., Tsuge, Y., Watanabe, J. & other authors (2007). Comparative analysis of the *Corynebacterium glutamicum* group and complete genome sequence of strain R. *Microbiology* **153**, 1042–1058.
- Zheng, M., Åslund, F. & Storz, G. (1998). Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* **279**, 1718–1721.
- Zheng, M., Wang, X., Templeton, L. J., Smulski, D. R., LaRossa, R. A. & Storz, G. (2001). DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide. *J Bacteriol* **183**, 4562–4570.

Edited by: J.-H. Roe