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

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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 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 regulator that controls cye1 expression.
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 (cye1) operon in the opposite orientation. The cye1 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 (Brígé 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 cye1 operon and its own structural gene as a transcriptional repressor. Expression of cye1 and cye1 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 cye1 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 C. glutamicum was obtained by regulating cyeR gene, which is located immediately upstream of the cgR_2931 (cye1) operon in the opposite orientation. The cye1 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 (Brígé 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 cye1 operon and its own structural gene as a transcriptional repressor. Expression of cye1 and cye1 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 cye1 expression.

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 (Suzuki et al., 2006). Disruption of cyeR was confirmed by DNA sequencing of thermal asymmetrical interlaced-PCR products of mutant cells.

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 of 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 cye1 was amplified by PCR using the primer pair regR2930-F and regR2930-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′-maleimidylstibene-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 cye1 operon and cyeR C. glutamicum cgr2930 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 (Δ2930) 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 Δ2930. Differential expression of cgR_2931
and cgR_2932 was confirmed by qRT-PCR. In Δ2930, 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
Δ2930 was determined by qRT-PCR using a primer pair
designed based on a sequence upstream of the transposon
insertion site of Δ2930. The transcript level of cgR_2930 in
Δ2930 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
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 H₂O₂
(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). How-
ever, 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.
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 H₂O₂ (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.

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). 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
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 \( \alpha,\beta \)-unsaturated aldehydes and ketones, using NADPH as a cofactor (Williams & Bruce, 2002). Expression of bacterial OYE family members such as *B. subtilis* YqiM 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 \( \text{H}_2\text{O}_2 \). The redox-responsive DNA-binding activity of CyeR suggests that expression of cye1 is derepressed by inactivation of the transcriptional repressor CyeR, in which 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 (Blehert et al., 1999). XenA and Cye1 belong to the YqiM 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).

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REFERENCES


