Corynebacterium glutamicum cgR_1435 (cg1552) encodes a protein of the DUF24 protein family, which is a novel family of transcriptional regulators. CgR1435 (QorR) is a negative regulator of cgR_1436 (qor2), which is located upstream of cgR_1435 (qorR) in the opposite orientation, and its structural gene. QorR binds to the intergenic region between qor2 and qorR to repress their expression, which is induced by the thiol-specific oxidant diamide. The DNA-binding activity of QorR is impaired by oxidants such as diamide, H2O2 and cumene hydroperoxide in vitro, and its lone cysteine residue (Cys-17) is essential for redox-responsive regulation of QorR activity both in vivo and in vitro. Moreover, a disruptant of qor2, which is a homologue of the ytfG gene of Escherichia coli encoding quinone oxidoreductase, shows increased sensitivity to diamide. It is concluded that the redox-sensing transcriptional regulator QorR is involved in disulfide-stress response of C. glutamicum by regulating the qor2 expression.

Proteins that belong to the DUF24 protein family of winged-helix DNA-binding proteins are widely distributed among prokaryotes. However, their biochemical characterization as transcriptional regulators has so far been limited to only two proteins, HxIR and YodB, of Bacillus subtilis. HxIR is a positive regulator of hxlAB, an operon that is involved in formaldehyde fixation (1). Although HxIR is necessary for formaldehyde-induced expression of the operon, the finer details as to how formaldehyde modulates hxlAB expression are still not clear. YodB negatively controls expression of spx, azoRl and yodC, genes involved in oxidative stress response (2,3). The DNA-binding activity of YodB is inhibited by thiol-reactive compounds, and its cysteine residues function in redox sensing (2).

Transcriptional regulators involved in oxidative stress response sense cellular redox conditions via oxidation of their cysteine residues, which leads to disulfide bond formation or S-thiolation. For instance, OxyR of Escherichia coli is activated by H2O2 through the formation of an intramolecular disulfide bond between Cys-199 and Cys-208 and then induces the transcription of genes necessary for the bacterial defense against oxidative stress (4,5). Likewise, the redox-regulated transcriptional regulator CprK of Desulfotobacterium dehalogenans controls expression of the cpr gene cluster involved in dehalorespiration (6). When CprK is inactivated by oxidation, it dimerizes through a disulfide bond formation between Cys-11 and Cys-200, at the same time as Cys-105 and Cys-111 form an intramolecular disulfide bond (7). Recently, Cys-11 has been shown to play a dual role as a redox switch and in maintaining the correct tertiary structure that promotes DNA binding (8). In B. subtilis, the lone cysteine residue (Cys-15) of the organic peroxide sensor OhrR is essential for redox sensing (9). Oxidation of Cys-15 by organic peroxides does not lead to intermolecular disulfide bond formation, but leads to a sulfenic acid-containing intermediate that retains DNA-binding activity, and then OhrR is inactivated by S-thiolation by cysteine, coenzyme A or an unknown thiol of molecular weight 398 Da (10).

Corynebacterium glutamicum is a nonpathogenic, GC-rich, gram-positive bacterium that belongs to actinobacteria. It has been widely used for the industrial production of various amino acids and nucleic acids (11,12). Meanwhile, the species is of increasing interest as a model organism for closely related pathogenic species such as Corynebacterium diphtheriae and...
Mycobacterium tuberculosis (13,14). In C. glutamicum, the extracytoplasmic-function (ECF) sigma factor SigM is involved in response to disulfide stress, a subcategory of oxidative stress that causes the accumulation of nonnative disulfide bonds in the cytoplasm (15). Induction of trxB1 and trxC encoding thioredoxins and trxB encoding thioredoxin reductase by the thio-specific oxidant diamide is abolished in a sigM disruptant. Disruption of sigM causes a reduction of cell viability after disulfide, heat and cold stresses. Another ECF sigma factor SigH controls transcription of sigM. Disruption of sigH also makes cells sensitive to diamide and high temperature (16).

C. glutamicum cgR_1435 (cg1552) encodes a protein of the DUF24 protein family. CgR1435 protein contains a lone cysteine residue, which is conserved among CgR1435 homologues, suggesting its involvement in oxidative stress response. In the present study, CgR1435 (QorR) was shown to be a redox-sensing transcriptional regulator of qor2, encoding a quinone oxidoreductase, and its structural gene. This is the first report on a DUF24 family protein other than those of B. subtilis as far as we know.

Experimental procedures

Bacterial strains, culture media and growth conditions- C. glutamicum strain R (17) and its derivatives were grown at 33°C in A medium with 4% (wt/vol) glucose as described previously (18). Disruptants of the qorR (cgR_1435) and qor2 (cgR_1436) genes were constructed by the transposon-mediated mutagenesis method as described previously (19). Disruption of the qorR and qor2 genes was confirmed by DNA sequencing of TAIL-PCR products of mutant cells. Transposon was inserted at 160 bases downstream of the 5' end of the qorR gene in Δ1435 and at 30 bases downstream of the 5' end of the qor2 gene in Δ1436.

A DNA fragment containing the promoter region of the bglF2 gene of C. glutamicum strain R was amplified by PCR using the primer pair PbgLF2-F and PbgLF2-R (Table S1) and was cloned between the EcoRI and SacI sites of a shuttle vector pCRC500 (20) to construct pCRC531. DNA fragments containing the qor coding region were excised from pCRD610 and pCRD611 (see below) with NdeI and HindIII, and cloned between the NdeI and HindIII sites of pCRC531 to construct pCRD630 and pCRD631, respectively. The qorR gene is expressed from the bglF2 promoter on these plasmids. A cysteine codon of the qorR gene at position 17 is replaced with a codon for serine on the pCRD631. pCRD630 and pCRD631 were separately used to transform Δ1435 cells, and transformants were selected for resistance to kanamycin and chloramphenicol.

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 (18). Global gene expression analysis was performed with the C. glutamicum R DNA microarray as described previously (18). Microarray analyses were carried out using two sets of RNA samples isolated from independently grown cultures with different combinations of Cy dyes (a dye swap strategy). Since the C. glutamicum R DNA microarray contains two replicates per gene, a total of four replicates per gene were available to determine changes in gene expression. Genes with significantly differential transcript levels (P < 0.05 in a Student’s t test) by at least a factor of two were determined.

Real-time qRT-PCR- A one-step real-time quantitative reverse transcription-PCR (qRT-PCR) was performed with the Power SYBR® Green PCR Master Mix (Applied BioSystems) and a pair of gene-specific primers (Table S1) by using the 7500 Fast Real-Time PCR system (Applied Biosystems) as described previously (18). Relative ratios were normalized with the value for 16S rRNA.

Mapping of transcription initiation sites by rapid amplification of cDNA ends (RACE)-PCR- Transcription initiation sites were determined by using the SMART™ RACE cDNA Amplification Kit (Clontech). 5' RACE-PCRs were carried out as recommended by the supplier with 1 µg of total RNA and gene-specific primers (Table S1). Resulting PCR products were cloned into a pGEM®-T Easy vector (Promega Corporation). At least 10 clones for each 5' RACE-PCR product were sequenced.

Expression and purification of His-QorR and His-QorRC17S- For construction of an
expression plasmid for the histidine-tagged QorR (His-QorR) protein, a DNA fragment containing the qorR gene was amplified by PCR using the primer pair rcgR1435-F and rcgR1435-R (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, pCRD610, contains the qorR gene fused to the His tag sequence. A cysteine residue at position 17 of QorR was replaced with serine by performing site-directed mutagenesis using PrimeSTAR® Mutagenesis Basal Kit (Takara Bio). The pCRD610 plasmid was used as a template for PCR with the primer pair 1435C17S-F and 1435C17S-R (Table S1) to generate a plasmid pCRD611. Escherichia coli BL21 (DE3) cells harboring pCRD610 or pCRD611 were grown at 37°C in 500 ml of Luria-Bertani medium supplemented with kanamycin (50 µg ml⁻¹). The recombinant gene was expressed in exponentially growing cells (optical density at 600 nm of 0.6) by adding 1 mM isopropyl-β-D-thiogalactopyranoside. After 1 h of incubation, the cells were harvested by centrifugation. Recombinant proteins were purified by using Ni-NTA Fast Start Kit (Qiagen). Elution fractions containing purified proteins were loaded onto a PD-10 column (GE Healthcare Bio-Science) equilibrated with buffer A [20 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 10% glycerol], and the protein was eluted with buffer A.

**Gel mobility shift assay**- His-QorR was incubated with Cy3-labeled probes (2 nM) in 20 µl of the binding buffer [20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol (DTT), 10% glycerol] for 30 min at room temperature. Diamide, H₂O₂ and cumene hydroperoxide were added with concentrations indicated. The mixtures were subjected to electrophoresis on a native 5% polyacrylamide gel, and Cy3-labeled probes were detected by Typhoon Trio+™ Variable Mode Imager (GE Healthcare BioScience).

DNA fragments amplified by PCR using the primer pair RT1436-R and RT1435 and the primer pair RT2930-R and RT2931 were cloned into the HincII site of the pHSG298 (Takara Bio) to construct pCRD620 and pCRD621, respectively. Probes F1 and F2 were prepared by PCR using the primer pair 1436R+51 and 1435R+33 (Table S1). A Cy3-labeled 1436R+51 primer was used for preparation of Cy3-labeled probes.

**Immunoblot analysis**- Cells grown to an optical density at 610 nm of 2.5 was collected by centrifugation, and pellet was wash with PBS (0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄) and mixed with 0.5 g of glass beads and 1 ml of PBS. The cells were disrupted with a sonicator (Bioruptor UCD-250; Cosmo Bio) in a water bath at 4°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 15% polyacrylamide gels by the method of Laemmli (21) using 20 µg of protein. Separated proteins were blotted to polyvinylidene difluoride membranes (Immobilon-P; Millipore) and probed with polyclonal antibodies to His-QorR proteins. The bound antibodies were detected with goat anti-rabbit IgG secondary antibodies conjugated to alkaline phosphatase (Sigma). Chemiluminescence reactions were done using the CDP-Star Detection Reagent (GE Healthcare Bio-Science) and the signal was scanned by a luminescent image analyzer (LAS-3000; FUJIFILM). Protein concentration was determined by using PROTEIN ASSAY (Bio-Rad Laboratories) with bovine serum albumin as the standard.

**Disc diffusion assays**- Approximately 2 × 10⁷ cells of *C. glutamicum* strains were uniformly spread onto plates of A medium with 4% glucose, and a paper disc impregnated with 10 µl of 1 M diamide solution was placed onto the lawn. The susceptibility of *C. glutamicum* strains was determined by measuring of the size of a cleared zone of growth inhibition after 22 h of growth at 33°C using the ImageJ software (National Institute of Health).

**RESULTS**
CgR1435 (QorR) negatively regulates expression of qorR and qor2. To ascertain the physiological role of the DUF24 family protein encoded by cgR_1435 as a transcriptional regulator in C. glutamicum, gene expression profiles during exponential growth were compared between the wild type and a cgR_1435 disruptant (Δ1435) using a DNA microarray, and differential expression was confirmed by qRT-PCR analysis. Expression of only cgR_1436, which is located upstream of cgR_1435 in the opposite orientation (see Fig. 3), was affected by the disruption of cgR_1435. The transcript level of cgR_1436 was more than 500-fold higher in Δ1435 (Table 1). Since promoters of cgR_1435 and cgR_1436 are likely to overlap each other, the transcript level of cgR_1435 was determined by qRT-PCR using the primer pair designed upstream of the transposon insertion site of Δ1435. The transcript level of cgR_1435 in Δ1435 was about five times higher than that of the wild type (Table 1). To confirm that the observed changes in the transcript levels were due to the disruption of cgR_1435, plasmid pCRD630 carrying the coding region of cgR_1435 and the control plasmid pCRC531 were separately used to transform Δ1435 cells. Whereas the transcript level of cgR_1436 in the resultant strain complemented with cgR_1435 was comparable to that of the wild type, the control plasmid did not effect any measurable changes in the transcript level of cgR_1436 (Table 1). These results indicate that CgR1435 negatively controls expression of cgR_1436 and its structural gene.

cgR_1436 is a homologue of the ytfG gene of E. coli with 54% amino acid identity. Given that ytfG codes for a novel type of NADPH-dependent quinone oxidoreductase (QOR2) (22), we designated cgR_1436 as qor2 and cgR_1435 as qorR for quinone oxidoreductase regulator.

Expression of qor2 and qorR is induced by diamide. As quinone oxidoreductase plays a protective role against oxidative stress (23,24), changes in expression of qor2 and qorR upon oxidative stress were examined. The transcript levels of qor2 and qorR in exponentially growing cells were determined by qRT-PCR before and after treatment of cells with diamide and H$_2$O$_2$ (Fig. 1). The results showed that expression of qorR was induced within 5 min after addition of diamide, with the qorR transcript level increasing about eightfold after 10 min, before gradually decreasing (Fig. 1A). Expression of qor2 was also induced by diamide, with drastic increases in the transcript level of about 200-fold after 5 min of diamide treatment (Fig. 1B). There was no increase observed in qor2 or qorR transcripts in response to the oxidative stress caused by H$_2$O$_2$ (data not shown). Expression of qor2 and qorR was thus induced only by disulfide stress.

QorR binds to the promoter regions of qor2 and qorR. Gel mobility shift assays were carried out with purified His-QorR and a DNA probe F1 of the intergenic region between qor2 and qorR (Figs. 2A and 2C). His-QorR reduced the electrophoretic mobility of probe F1 and the amount of QorR-F1 complex formed increased in proportion to the concentration of His-QorR (Fig. 2A, lanes 1 to 4). Three forms of the QorR-F1 complex with different electrophoretic mobility (C1, C2 and C3) were detected. The band intensity of the QorR-F1 complex was reduced upon addition of a nonlabeled F1 fragment (Fig. 2A, lanes 5 and 6). However, addition of a fragment F2 that contains the oxidative stress-responsive promoters of cgR_2930 and cgR_2931 (Ehira et al., unpublished data) did not affect the amount of QorR-F1 complex formed (Fig. 2A, lanes 7 and 8). These observations invite the conclusion that His-QorR binds to the qor2-qorR intergenic region in a sequence-specific manner.

The genetic organization of qor2 and qorR is conserved among many bacterial species belonging to actinobacteria, proteobacteria and cyanobacteria, based on data from the KEGG Sequence Similarity DataBase (http://www.genome.jp/kegg/ssdb/). For example, the ytfH gene encoding a transcriptional regulator of the DUF24 protein family, which is the sole gene of E. coli belonging to this family, is located upstream of the ytfG gene in the opposite orientation on the genome of E. coli. This suggests that regulatory sequences may also be conserved among these bacteria. Searches for conserved DNA motifs within the intergenic regions between qorR and qor2 homologues were performed using the BioProspector program (25). Sequence QBS1 (QorR-binding site) was
identified as a conserved sequence (Fig. 3A). In addition, similar sequences were found upstream of QBS1 in the opposite orientation (QBS2) and downstream of QBS1 (QBS3) (Fig. 3A). Interactions between His-QorR and the QBS sequences were elucidated by gel mobility shift assays (Figs. 2B and 2C). Deletion of QBS1 abolished the interaction between His-QorR and the qor2-qorR intergenic region (Fig. 2B, lanes 1 to 4). His-QorR bound to QBS2- and QBS3-deficient probes, but complex C3 was not observed with either probe (Fig. 2B, lanes 7 to 12). The inferences that QBS1 is indispensable for QorR binding to the qor2-qorR intergenic region, and that QorR also binds to QBS2 and QBS3 follow. The putative QorR-recognition sequence contains an inverted repeat sequence cTACtN4-5Gttag (Fig. 3B). The QBS1 sequence, ACTTACT5GATAGT, was replaced with AGGCCGT5CGGCTT to generate probe M1. No interaction between His-QorR and probe M1 was observed (Fig. 2B, lanes 5 and 6), confirming that the inverted repeat sequence is recognized by QorR.

The transcription initiation sites of qor2 and qorR were determined by RACE-PCR experiments, and putative -10 and -35 promoter regions, which correspond to those of the SigA-dependent promoter (18), were found upstream of the respective transcription initiation sites (Fig. 3A). Within the qor2 promoter region, QBS1 and QBS2 overlap with the -35 region and QBS3 overlaps with the -10 region. QBS2 also overlaps with the -35 region of the qorR promoter.

DNA-binding activity of QorR is regulated by Cys-17 oxidation. The QorR protein contains a lone cysteine residue at position 17 from the N-terminal (Cys-17). As this cysteine residue is conserved among QorR homologues (Fig. S1A), Cys-17 is likely to be involved in the redox-sensitive control of QorR activity. The effect of oxidants on DNA-binding activity of QorR was thus examined by gel mobility shift assays (Fig. 4). Binding of QorR to probe F1 was prevented by addition of diamide (Fig. 4A, lanes 2 to 6). Addition of an excess of the reducing agent DTT restored DNA-binding activity of QorR that was inactivated by diamide (Fig. 4A, lanes 8 to 12), indicating that the effects of oxidation and reduction on DNA-binding activity of QorR are reversible. When the purified His-QorR protein was subjected to non-reducing SDS-PAGE, two bands of approximate molecular masses of 15 and 30 kDa were observed (Fig. 4B, lane 1). As the theoretical value of the molecular mass of His-QorR is 16.2 kDa, the 15 and 30 kDa bands are likely to correspond to the monomeric and dimeric forms of His-QorR, respectively. Treatment of QorR with DTT resulted in loss of the 30-kDa band, whereas the intensity of the 30-kDa band was increased by diamide addition (Fig. 4B, lanes 2 and 3). The DNA-binding activity of QorR was also impaired by the other oxidants H2O2 or cumene hydroperoxide (Fig. 4C), and they enhanced dimerization of QorR (data not shown). These results support the conclusion that QorR undergoes dimerization and loses the DNA-binding activity under oxidizing conditions.

To ascertain the role of Cys-17 in the redox-sensitive regulation of QorR activity, Cys-17 was replaced with serine to generate His-QorRC17S protein. His-QorRC17S reduced the electrophoretic mobility of probe F1 (Fig. 5A), and required three times as much protein as His-QorR to bind all probe (see Fig. 2A). Unlike His-QorR protein, binding of His-QorRC17S to probe F1 was not inhibited by addition of diamide (Fig. 5B). When His-QorRC17S was treated with diamide, it remained in the monomeric form (Fig. 5C). Thus, Cys-17 of QorR is required for dimerization of proteins and the redox-responsive control of DNA-binding activity.

Cys-17 of QorR is required for regulation of qor2 expression in response to diamide. To investigate the role of Cys-17 of QorR in regulation of qor2 expression, QorR and QorRC17S proteins were expressed from plasmids pCRD630 and pCRD631, respectively, in Δ1435. QorR and QorRC17S were produced at similar levels as shown by immunoblot analysis (Fig. 6A). In Δ1435 expressing QorR, expression of the qor2 gene was induced after treatment with diamide in a similar way to the wild type, while its induction level was 10 times lower than that of the wild type (Figs. 1B and 6B). The qor2 transcript level of the strain expressing QorRC17S was three times higher than that of the QorR-expressing strain in exponentially growing cells (Time 0 in Fig. 6B). However, qor2 was not
induced by diamide in the QorRC17S-expressing strain (Fig. 6B). Expression of the trxB1 gene was induced by diamide in the QorRC17S-expressing strain as previously shown by Nakunst et al. (15), indicating that cells were subjected to disulfide stress (Fig. 6C). These results confirm that the Cys-17 of QorR is required for induction of qor2 in response to diamide.

Qor2 plays a protective role against diamide stress. The role of qor2 in disulfide stress response was investigated by disc diffusion assays using a disruptant of the qor2 gene (Δ1436) (Fig. 7). A paper disc containing diamide was plated onto a lawn of C. glutamicum cells on an agar plate, and the size of a clear zone of growth inhibition surrounding the paper disc was measured. The clear zone of Δ1436 plates was 20% larger than that of the wild type plates, indicating that disruption of the qor2 gene resulted in increased susceptibility to diamide.

DISCUSSION

In the present study, we demonstrated that QorR binds to DNA in a sequence-specific manner and is involved in regulation of gene expression. A DUF24 protein family member was shown to function as a transcriptional regulator in the actinobacterium C. glutamicum, as was previously reported for the firmicute B. subtilis (1,3). More than 1000 proteins of 488 bacterial species are classified into the DUF24 protein family in the Pfam database (26), suggesting that the DUF24 protein family is a regulatory protein family widely spread among bacteria.

QorR was shown to act as a transcriptional repressor of the qor2 and qorR genes (Fig. 1). Three forms of complexes of QorR and the qor2-qorR intergenic region (C1, C2 and C3) were observed (Fig. 2A) and three QorR-binding sites (QBS1 to 3) were identified within the qor2-qorR intergenic region (Fig. 3A). Overlapping of the QBSs with the qor2 and qorR promoter regions is coincident with the function of QorR as a transcriptional repressor. Deletion analyses of QBSs indicated that QorR hierarchically bound to these three sites. Deletion and mutation of QBS1 totally eliminated the binding of QorR to the intergenic region (Fig. 2B). Deletion of QBS2 or QBS3 resulted in loss of complex C3, indicating that QorR can bind to two different sites of the QBS2- or QBS3-deficient probe (Fig. 2B). These results suggest that QorR first binds to QBS1 and then to QBS2 and QBS3. It can be speculated that the QorR-QBS1 complex is responsible for recruiting the second and third QorRs to QBS2 and QBS3. Three QBSs overlap with the qor2 promoter region, while only QBS2 overlaps with the qorR promoter region (Fig. 3A). The drastic induction of the qor2 gene by diamide (about 200-fold) is likely to reflect strict repression of the qor2 expression by QorR.

QorR homologues share the conserved cysteine residue as Cys-17 in C. glutamicum QorR (Fig. S1A). This conserved Cys-17 was shown to be essential for the redox-responsive regulation of QorR activity in vivo and in vitro. DNA-binding activity of QorRC17S proteins was not impaired by diamide, and dimerization of QorRC17S was not observed even in the presence of excess diamide (Fig. 5). In addition, expression of the qor2 gene did not respond to diamide in the QorRC17S-expressing strain (Fig. 6). It is concluded that QorR activity is regulated by oxidation of Cys-17. Under oxidizing conditions, QorR becomes a dimeric and inactive form probably through the formation of an intermolecular disulfide bond between Cys-17 of each subunit, though possibilities that QorR is inactivated by S-thiolation in vivo as was reported for OhrR of B. subtilis (10) cannot be ruled out.

The DNA-binding activity of QorR was impaired by H2O2 in vitro (Fig. 4C), but the transcript levels of qor2 and qorR did not respond to H2O2 (data not shown). It has been shown that the sigM transcript level is also increased by diamide, but not by H2O2 (15). As C. glutamicum is highly resistant to H2O2 (15), H2O2 is supposed to unable to induce C. glutamicum oxidative stress response.

CgR1999 (Cg2320) protein of C. glutamicum is another member of the DUF24 protein family. YodB, HxlR and CgR1999 also contain cysteine residues in their N-terminal region, which correspond to Cys-17 of QorR (Fig. S1B). Cys-6 of YodB has been shown to play a role in DNA binding and response to thiol-reactive compounds (2). Thus, the cysteine residue of the N-terminal region of the DUF24 family proteins is likely to play a regulatory role.
The qor2 disruptant showed increased susceptibility to diamide (Fig. 7). It was previously shown that quinone oxidoreductase homologues of Arabidopsis thaliana confer tolerance toward diamide on yeasts (23). Quinone oxidoreductase catalyzes reduction of quinones, which generate reactive oxygen species as a result of redox cycling between quinones and semiquinone radicals (27). Although exactly how quinone oxidoreductase protects cells from disulfide stress has not been revealed, quinones have been shown to react with protein thiolates leading to thiol-S-adduct formation, which causes disulfide stress (2). In the qor2 disruptant, disulfide stress caused by diamide could be reinforced by quinones.

The genetic organization of qorR and qor2 homologues and the cysteine residue that is essential for the redox-responsive control of QorR activity are conserved among disparate bacteria (Fig. S1A). In addition, putative QorR-recognition sequences are found within the intergenic regions between qorR and qor2 homologues (Fig. S2). These observations strongly suggest the existence of a common regulatory mechanism of Qor2 expression in a wide variety of bacteria.

REFERENCES


**FOOTNOTES**

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The abbreviations used are: ECF, extracytoplasmic function; qRT-PCR, quantitative reverse transcription-PCR; RACE, rapid amplification of cDNA ends; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; QorR, quinone oxidoreductase regulator, QBS, QorR-binding site.

**FIGURE LEGENDS**

**Fig. 1.** Changes in the transcript levels of *qor2* and *qorR* in response to diamide. The relative transcript levels of *qorR* (A) and *qor2* (B) before (0 min) and at 5, 10, 15, 20 and 30 min after addition of 3 mM diamide were determined by qRT-PCR. The transcript levels were determined in triplicate measurements using two independently grown cultures. The transcript level at 0 min was taken as 1.

**Fig. 2.** Gel mobility shift assays with His-QorR and the intergenic region of the *qor2* and *qorR* genes. (A) The binding of His-QorR to probe F1, corresponding to the region from +51 with respect to the translation start site of the *qor2* gene to +140 with respect to the translation start site of the *qorR* gene, was examined. Probe F1 (2 nM) was mixed with His-QorR in the amounts indicated above each lane, and then the mixtures were subjected to electrophoresis. Non-labeled fragments of F1 (lanes 5 and 6) and F2 (lanes 7 and 8), which corresponds to the intergenic region between cgR_2930 and cgR_2931, were added at the indicated amounts. (B) Mutation and deletion analyses of QBSs. The binding of His-QorR to DNA probes (2 nM) described below were examined. Lanes 1, 2, 7 and 8; probe F3, corresponding to the region from +51 with respect to the translation start site of the *qor2* gene to +33 with respect to the translation start site of the *qorR* gene, lanes 3 and 4; QBS1-deficient probe D1, lanes 5 and 6; probe M1, in which the QBS1 sequence ACTTACT5GATAGT of probe F3 was replaced with the sequence AGGCCGT 5CGGCTT, lanes 9 and 10; QBS2-deficient probe D2, lanes 11 and 12; QBS3-deficient probe D3. Open and closed arrowheads indicate the positions of free probes and the complexes of QorR and DNA probes, respectively. (C) DNA probes used for gel mobility shift assays. Numbers adjacent to the fragments represent nucleotide positions relative to the start codons of the *qorR* or *qor2* genes.

**Fig. 3.** The nucleotide sequence of the *qor2-qorR* intergenic region. (A) The coding regions of the *qor2* and *qorR* genes were shaded in gray. The identified transcription initiation sites and the -10 and -35 promoter regions were indicated by the bent arrows and boldface letters, respectively.
QorR-binding sites (QBS1 to 3) were enclosed in boxes. (B) Alignment of QBSs. Putative QorR-recognition sequence is given at the bottom.

Fig. 4. Rexox-sensitive control of the DNA-binding activity of QorR. (A) His-QorR (100 nM) and probe F1 (2 nM) were incubated in the presence of 1 mM DTT (lanes 2 to 6) or 1 mM diamide (lanes 8 to 12) for 30 min, and then diamide (lanes 2 to 6) or DTT (lanes 8 to 12) was added in the amounts indicated above each lane. After 30 min, the mixtures were subjected to electrophoresis. Lanes 1 and 7; His-QorR was not added. (B) Purified His-QorR proteins (1 µg) incubated with 1 mM DTT (lane 2) or 1 mM diamide (lane 3) for 30 min were separated by non-reducing SDS-PAGE. Lane M; molecular mass standard marker, lane 1; His-QorR without additives. (C) His-QorR (100 nM) and probe F1 (2 nM) were incubated in the presence of 1 mM DTT for 30 min, and then H$_2$O$_2$ (lanes 3 to 5) or cumene hydroperoxide (CHP) (lanes 6 to 8) was added in the amounts indicated above each lane. After 30 min, the mixtures were subjected to electrophoresis. Lane 1; His-QorR was not added.

Fig. 5. (A) Gel mobility shift assays with His-QorRC17S and the qor2-qorR intergenic region. Probe F1 (2 nM) was mixed with His-QorRC17S in the amounts indicated above each lane, and then the mixtures were subjected to electrophoresis. (B) Effects of diamide on the DNA-binding activity of QorRC17S. His-QorRC17S (300 nM) and probe F1 (2 nM) were incubated in the presence of 1 mM DTT for 30 min, and then diamide (lanes 3 to 5) was added in the amounts indicated above each lane. After 30 min, the mixtures were subjected to electrophoresis. Lane 1; His-QorRC17S was not added. (C) Purified His-QorRC17S proteins (1 µg) incubated with 1 mM DTT (lane 2) or 1 and 3 mM diamide (lanes 3 and 4) for 30 min were separated by non-reducing SDS-PAGE. Lane M; molecular mass standard marker, lane 1; His-QorRC17S was incubated without additives.

Fig. 6. (A) Immunoblot analysis of QorR proteins. Crude extracts (20 µg of proteins) from Δ1435 cells expressing QorR (left) or QorRC17S (right) were subjected to SDS-PAGE, and QorR derivatives were detected with polyclonal antibodies to His-QorR. Changes in the transcript levels of qor2 (B) and trxB1 (C) by diamide in Δ1435 strains expressing QorR (filled circles) or QorRC17S (open circles). The relative transcript levels before and after addition of 3 mM diamide were determined by qRT-PCR. Mean values and standard deviations of three independent experiments are shown. The transcript level at 0 min in Δ1435 expressing QorR is taken as 1.

Fig. 7. Susceptibility of the qor2 disruptant to diamide. A paper disc impregnated with 10 µl of 1 M diamide solution was placed onto the lawns of the wild type (A) and the qor2 disruptant (Δ1436) (B). The plates were incubated at 33°C for 22 h, and then the size of a clear zone was measured. Experiments were repeated three times with independently grown cultures. Ratio of the size of a clear zone in Δ1436 to the wild type was 1.22 ± 0.02.
<table>
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<tr>
<th>Gene</th>
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<th>Δ1435[1435]/WT[cont]</th>
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<tr>
<td>cgR_1435</td>
<td>4.7 ± 1.8</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>cgR_1436</td>
<td>522.5 ± 224.5</td>
<td>322.2 ± 53.1</td>
<td>1.0 ± 0.1</td>
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*aRelative transcript levels of cgR_1435 and cgR_1436 were determined by qRT-PCR. WT; the wild type strain, Δ1435; the cgR_1435 disruptant. [cont] and [1435] denote strains harboring the control plasmid pCRC531 and a plasmid pCRD630 carrying the coding region of cgR_1435, respectively. Data represent means ± standard deviations of three independent experiments. ND; not determined.*
Fig. 1
Fig. 2

A

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B

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C

\[+140\] \(QBS2\) \(QBS1\) \(QBS3\) \(+51\) F1
\[+33\] \(QBS2\) \(QBS1\) \(QBS3\) \(+51\) F3
\[+33\] \(QBS2\) \(QBS1\) \(QBS3\) \(+51\) D1
\[+33\] \(QBS2\) \(QBS1\) \(QBS3\) \(+51\) M1
\[+33\] \(QBS2\) \(QBS1\) \(QBS3\) \(+51\) D2
\[+33\] \(QBS2\) \(QBS1\) \(QBS3\) \(+51\) D3

Probe
Fig. 3

A
AGAACACATT TGCCGGTTGC TGAATAGGGG AAATATCCAT GGTAGTAATC CTATCCCTAA
S F V N A P Q Q I P S I D M
-10

AGGGATT

S F V N A P Q Q I P S I D M
-10

AAAGTTTCTA ACAAAAGTA T TGCAC TTACT TTTTGATAGT GC
TATCTT CA TTTGTGTACT
-35

TTTCAAAGAT TGTTTT CATA ACGTGAATGA AAAACTATCA CGATAGA AGT AAACACATGA

M R I A V T G A T G S S

B
QBS1 ACTTACTTTTTGATAGT
QBS2 CAATACTTTTT-GTGAAG
QBS3 TCTTCATTTTTGTACCTT
ctTac T T gtagt
Fig. 4
Fig. 5
Fig. 6

A

QorR QorRC17S

B

$qor2$

Relative transcript level vs. Time (min)

C

$trxB1$

Relative transcript level vs. Time (min)
Fig. 7
Regulation of quinone oxidoreductase by a redox-sensing transcriptional regulator QorR in Corynebacterium glutamicum

Shigeki Ehira, Hidetaka Ogino, Haruhiko Teramoto, Masayuki Inui and Hideaki Yukawa

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