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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Involvement of the NADH oxidase-encoding *noxA* gene in oxidative stress responses in *Corynebacterium glutamicum*

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Abstract *Corynebacterium glutamicum* ORF NCgl0328, designated *noxA*, encodes an NADH oxidase enzyme. The *noxA* gene, which was preferentially expressed in the log growth phase, was found to be under the control of the *whcA*, *whcB*, and *whcE* genes, which play regulatory roles in cells under oxidative stress. While *noxA* transcription was minimal in *whcE*-deleted mutant cells (Δ*whcE*) during growth, its transcription was maximal even in the stationary phase in Δ*whcA* cells. The transcription levels of *noxA* in Δ*whcB* and *whcB*-overexpressing cells were comparable to the levels only in the log growth phase in Δ*whcA* and *whcA*-overexpressing cells, respectively. Direct binding of purified WhcA to the promoter region of *noxA* was observed in vitro. The DNA-protein interaction was only possible in the presence of the reducing agent dithiothreitol. A *noxA*-deleted mutant strain and a strain overexpressing the *noxA* gene (P_{180}-*noxA*) were established, and these strains were found to exhibit defective cell growth. The Δ*noxA* and P_{180}-*noxA* strains were sensitive to the redox-cycling oxidant menadione, suggesting a role of *noxA* in redox balancing. Accordingly, the purified NoxA enzyme exhibited NADH-oxidizing activity. Taken together, these data show that *noxA* plays a role in oxidative stress responses and also that the gene is under direct control of the WhcA protein, which was shown to be a regulatory DNA-binding protein. Furthermore, the involvement and roles of the *whcA*, *whcB*, and *whcE* genes in regulating the expression of *noxA* were demonstrated.

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Introduction

Corynebacterium glutamicum is a gram-positive organism belonging to the class *Actinobacteria*, which includes the genera *Mycobacterium* and *Streptomyces* (Gao and Gupta 2012). *C. glutamicum* is widely used for the industrial production of amino acids by fermentation processes (Leuchtenberger et al. 2005). During the course of fermentation, microorganisms encounter a variety of cellular stresses including oxidative stress, which is harmful to the fermenting cells. In the past, the oxidative stress response pathways of *C. glutamicum* were analyzed by our group, and it was found that several *whiB*-like genes play important regulatory roles in the oxidative stress responses of *C. glutamicum* (Choi et al. 2009; Kim et al. 2005; Lee et al. 2012, 2013).

The *whiB* gene was originally identified and characterized in *Streptomyces coelicolor* as a developmental regulatory gene required for the sporulation of aerial hyphae (Davis and Chater 1992). *Actinobacteria* species are known to possess *whiB*-like genes that function in diverse cellular processes, including cell division, differentiation, pathogenesis, starvation survival, and stress response (Zheng et al. 2012). Typically, WhiB-like proteins possess a redox-sensitive Fe-S cluster coordinated to four conserved cysteine residues (Alam et al. 2007; Crack et al. 2009; Jakimowicz et al. 2005; Rybniker et al. 2010; Singh et al. 2007; Smith et al. 2010). This cluster plays an important role in controlling protein activity. Upon exposure of the protein to oxygen, for example, the cluster can be lost and the coordinating cysteine thiols are

oxidized, resulting in a loss of protein activity (Crack et al. 2009; Singh et al. 2009). Some WhiB-like proteins possess a helix-turn-helix DNA-binding motif, indicating a role of these proteins as transcription factors (Smith et al. 2010).

C. glutamicum possesses four *whiB*-like genes: *whcE* (*whiB1*), *whcD* (*whiB2*), *whcB* (*whiB3*), and *whcA* (*whiB4*). Of these homologues, three (*whcE*, *whcA*, and *whcB*) have been studied and characterized (Choi et al. 2009; Kim et al. 2005; Lee et al. 2012). The *whcE* gene plays a positive role in the survival of cells exposed to oxidative and heat stresses (Kim et al. 2005), whereas the *whcA* gene plays a negative role in the expression of genes associated with the oxidative stress response (Choi et al. 2009). The WhcA protein interacts with SpiA, an oxidoreductase-like protein (Park et al. 2011, 2012), and the interaction is labile to oxidants. The *whcB* gene plays a regulatory role in the stationary phase of bacterial growth, particularly in electron transfer reactions (Lee et al. 2012). Furthermore, an analysis of the *whc* gene expression network by Lee et al. (2013) revealed that, although the *whc* genes are paralogues, they play distinct regulatory roles during cellular responses to oxidative stress. After all, the oxidative stress response of *C. glutamicum* is regulated in a complex manner involving a hierarchy of several *whc* genes (Lee et al. 2013). Since *whcB* lies at the top of the hierarchy, it is considered the primary regulator of *whc* gene transcription.

Despite recent progress, many questions regarding the mechanisms of oxidative stress responses in *C. glutamicum* remain unanswered. In our previous study, the open reading frame (ORF) NCgl0328 (*noxA*) was detected in analyses involving proteomes of *whc* mutants, but the function and regulation of the *noxA* gene in the oxidative stress response pathway have not been clarified yet (Choi et al. 2009; Lee et al. 2012). In this study, the involvement and roles of the *whcA*, *whcB*, and *whcE* genes in regulating the expression of *noxA* were demonstrated. Direct binding of WhcA to the promoter region of the *noxA* gene was demonstrated for the first time. Furthermore, we report that the purified NoxA protein contains NADH-oxidizing activity. Finally, we propose a role for the *noxA* gene in the oxidative stress response and cell physiology of *C. glutamicum*.

Materials and methods

Bacterial strains and growth conditions

C. glutamicum AS019E12 (Follettie and Sinskey 1986; Yoshihama et al. 1985), a rifampicin-resistant and restriction-deficient derivative of the ATCC13059 strain, was utilized in generating the HL1393 and HL1458 strains. *C. glutamicum* HL1393 carries a ΔNCgl0328 (Δ*noxA*) mutation, whereas *C. glutamicum* HL1458 carries the *noxA*-overexpressing plasmid pSL531 (i.e., P_{180}-*noxA*). *C. glutamicum* HL1171 (Choi et al. 2009), HL1312 (Lee et al. 2012), and HL810 (Kim et al. 2005) carry a Δ*whcA* mutation, a Δ*whcB* mutation, and a Δ*whcE* mutation, respectively, and *C. glutamicum* HL1176, HL1313, and HL1108 carry the pSL432 (Choi et al. 2009), pSL469 (Lee et al. 2012), and pSL395 (Kim et al. 2005) plasmids, respectively. Plasmids pSL432 (i.e., P_{180}-*whcA*), pSL469 (i.e., P_{180}-*whcB*), and pSL395 (i.e., P_{180}-*whcE*) allow for the overexpression of *whcA*, *whcB*, and *whcE* genes, respectively. Cultivation of *Escherichia coli* was performed at 37 °C in Luria-Bertani complex medium (Sambrook et al. 2001). *C. glutamicum* cells were cultured at 30 °C in MB (Follettie et al. 1993) or MCGC (von der Osten et al. 1989). Glucose was added as a carbon source in the minimal medium at a concentration of 1 % (*w*/*v*). Antibiotics were added at the following concentrations (μg ml^{-1}): 50 ampicillin, 20 chloramphenicol, and 25 kanamycin.

Construction of plasmids and strains

Standard molecular cloning, transformation, and electrophoresis procedures were employed. Plasmids were introduced into *C. glutamicum* cells by electroporation (Follettie et al. 1993). All the restriction enzymes and DNA-modifying enzymes were purchased and used according to the manufacturer's instructions (Takara Bio). PCR was carried out as previously described (Kim et al. 2005). The nucleotide sequence of *noxA* (NCgl0328, GenBank GeneID 1021176) was obtained from the published *C. glutamicum* genome sequences (GenBank accession no. NC_003450.3; Ikeda and Nakagawa 2003; Kalinowski et al. 2003). The *C. glutamicum* Δ*noxA* mutant strain was generated according to the method by Schäfer et al. (1994), as follows: the primary PCR products amplified with the F1 5′-CACAGCACCCCA CTTCACATAACC-3′, R1 5′-CCGCCGCGAGGTTTTC CACGCGGGCAACC-3′, and F2 5′-CGTGGAAAACCT CGCGGCGGCTTTTCTA-3′, R2 5′-TTCGATGAGGGT TACTTTGCTGTC-3′ primer pairs were used as templates for secondary PCR. The amplified fragment was cloned into the pGEM-T-easy vector (Promega), after which, the fragment resulting from the digestion with *EcoR*I was isolated and inserted into the *EcoR*I-digested pK19*mobsacB* plasmid (Schäfer et al. 1994). The resulting plasmid, pSL511, was transformed into *E. coli* ET12567 (MacNeil et al. 1992), and the plasmids from this strain were electro-transformed into *C. glutamicum*. Subsequent steps were conducted as described (Hwang et al. 2002). The chromosomal deletion of the *noxA* gene (a 114-bp internal deletion to begin 254 bp downstream of the translation start site and end 214 bp upstream of stop codon) in *C. glutamicum* was validated using PCR, and the mutant bacterial strain was

designated HL1393. Plasmid pSL531 was constructed by amplifying the *noxA* gene using primers 5′-AAAACTGCAGCCAACACATAAAAAGG-3′ and 5′-AAAACTGCAGCCGGTCACAAGCAAAG-3′, followed by ligation of the amplified DNA at the *Pst*I site in the pSL360 plasmid (Park et al. 2004), which allows for the overexpression of the cloned genes. The pSL521 plasmid encoding the maltose-binding protein (MBP)-WhcA-His_6 fusion protein was constructed by amplifying the *whcA* gene using the primers 5′-ATGACGTCTG TGATTCCAGAGC-3′ and 5′-CGCGGATCCTTAGTG GTGATGGTGATGATGAACCCCGGCGATC-3′, followed by the ligation of the amplified DNA at the *Bam*HI site of the pMAL-c2 vector (New England Biolabs). The pSL544 plasmid expressing the His_6-NoxA protein was constructed by introducing the *Bam*HI digested fragment, which was amplified from the *noxA* gene using primers 5′-AATGGGTCGCGGATCCATGTCACT TTCAGTCGT-3′ and 5′-GCTCGAATTCGGATCC-GAGTTAGTAGCTGTTGTC-3′, into the pET28a vector (Novagen).

RNA analysis

5′ Rapid Amplification of cDNA Ends (RACE) was carried out using a 5′/3′ RACE Kit, 2nd Generation (Roche Diagnostics). Total RNA was isolated from the cells at the exponential phase (OD_{600} of 5–15) or stationary phase (OD_{600} of 20) using a NucleoSpin RNA II Kit (Macherey-Nagel). First-strand complementary DNA (cDNA) synthesis was conducted as described previously (Park et al. 2008). cDNA synthesis was carried out using a DyNAmo cDNA Synthesis Kit (Finnzymes), and real-time quantitative PCR (RT-qPCR) was performed as described (Hong et al. 2014), using THUNDERBIRD SYBR qPCR Mix (TOYOBO) and a CFX96™ Real-Time PCR Detection System (Bio-Rad). All the reactions were performed in triplicate. The PCR conditions consisted of an activation step of 15 min at 95 °C, followed by 40 cycles at 95 °C for 20 s, at 60 °C for 20 s, and at 72 °C for 40 s. Data were collected at the 72 °C step of each cycle. Normalized expression and standard error values were calculated using the CFX Manager software ver. 1.5 (Bio-Rad), which employs the $\Delta\Delta C_t$ method. Verification of RT-qPCR products was performed by melting curve and peak analyses. Gene expression levels were normalized to the levels of the 16S rRNA gene, which was used as an endogenous control. The primers used for the detection of *whcA*, *whcB*, *whcE*, *trxB*, and *noxA* were as follows: *whcA*, 5′-ATCGCCCTTGTT ATTGCTACCGGA-3′ and 5′-AGTAGCTGTTGTCGATGC GCCTAT-3′; *whcB*, 5′-ATTGCCTCACCAGCTTCCCG-3′ and 5′-TCGCCGTCCGGGTGATAGAA-3′; *whcE*, 5′-ACGAAGCAATCTGCCGTGAA-3′ and 5′-AGCGGTTG CAGACCATCTTT-3′; *trxB*, 5′-ACCCAACTTGGTGGTC AGATGGAA-3′ and 5′-TTGAGCAGCGGAACCATAGA CCAT-3′; *noxA*, 5′-ATCGCCCTTGTTATTGCTACCGGA-3′ and 5′-AGTAGCTGTTGTCGATGCGCCTAT-3′.

Enzyme assays and protein purification

Cells grown under aerobic conditions to mid-exponential phase were harvested by centrifugation, after which, crude cellular extracts were prepared as previously described (Kim et al. 2004). Protein concentrations were determined by the Bradford method, using bovine serum albumin solutions as standards (Bradford 1976). NADH oxidase activity in the crude cellular extracts was determined by a photometric assay at 340 nm (molar extinction coefficient of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$) using 50 mM potassium phosphate buffer (pH 7.0) containing 10 μM flavin mononucleotide (FMN), 100 μM NADH (Sigma–Aldrich), and a limiting amount of crude extract. β-NADH and FMN were used as the electron donor and the electron acceptor, respectively. The reaction mixture lacking NADH was used as the blank.

The MBP-WhcA-His_6 fusion protein was purified as follows. *E. coli* BL21(DE3) (Merck Biosciences) harboring pSL521 was cultivated in LB. Proteins were induced by treating cells with 0.3 mM IPTG at an OD_{600} of 0.5. The cells were cultivated for additional 1 h to fully induce the proteins. The cells were harvested by centrifugation, resuspended in a binding buffer (MBPTrap HP, GE Healthcare), and lysed by sonication on ice (15 times for 3 s with a 6-s rest interval at output level 30 %, VCX-400; Sonics & Materials Inc.). The lysate was centrifuged at 10,000×*g* for 60 min at 4 °C to remove the cell debris. The resulting crude extract was retained for purification. The MBP-WhcA-His_6 fusion protein was purified on an amylose column (MBPTrap HP, GE Healthcare) and subsequently on a Ni^{2+}-NTA column (HiTrap His-FF, GE Healthcare) as described in the Recombinant Protein Purification Handbook (GE Healthcare). The His_6-NoxA fusion protein was purified on a Ni^{2+}-NTA column (HiTrap His-FF, GE Healthcare) after it was overexpressed as described above. Subsequently, the proteins were analyzed by 17 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

DNA-protein affinity purification

Potential DNA-protein interactions were analyzed using a Dynabeads-based magnetic separation protocol (Life Technologies). First, the *noxA* promoter region spanning nucleotides −211 to +70 relative to the transcriptional start site was amplified by PCR using primers 5′-AAGCACCCAACTCG CCAAA-3′ and 5′-GTGGCGCGGCGGTTGGTAAT-3′. The ORF of the *noxA* gene amplified with primers 5′-GGCCTC CCATCAGAAACAA-3′ and 5′-ATCCTCACGCCCGCCG

AGACC-3′ was used as the control DNA. When needed, the primers were appropriately labeled with biotin, according to the manufacturer's instructions (Life Technologies). Next, approximately 30 μg of each biotin-labeled PCR product was mixed with 5 μl (6.7×10^7 beads) streptavidin-coated beads (Dynabeads M-280 Streptavidin) in mobilization buffer (10 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 2 M NaCl, pH 7.5), and the mixture was incubated at room temperature for 3 h to allow for coupling between biotin-labeled DNA and streptavidin-coated beads. Uncoupled DNA was removed by washing the mixture with binding buffer (100 mM HEPES, pH 7.6, 5 mM EDTA, 50 mM $[NH_4]_2SO_4$, 5 mM dithiothreitol (DTT)], 1 % Tween 20 [*w*/*v*], 150 mM KCl). Subsequently, the coupled beads were resuspended in 600 μl binding buffer, and the binding reaction was carried out using 200 μg purified WhcA protein at 15 °C for 3 h with gentle shaking. Salmon sperm DNA was used as a competitor DNA for the reaction. Unbound and nonspecifically bound proteins were removed by magnetic separation using a magnet particle concentrator (Life Technologies), followed by several washes with the binding buffer. Finally, bound proteins were eluted with a binding buffer containing NaCl (0.6 or 1 M). Eluted proteins were separated by 17 % SDS-PAGE and stained with Coomassie Brilliant Blue G-250.

Electrophoretic mobility shift assay

The 220-bp DNA fragment that includes the promoter region of the *noxA* gene (from nucleotides −211 to +9 relative to the transcriptional start site) was amplified using the primers 5′-AAGCACCCAACTCGCCAAA-3′ and 5′-TGTTGGAAAC AGAGTCATGGTAGG-3′. The binding reaction was performed in a total volume of 20 μl (100 mM HEPES, pH 7.6, 5 mM EDTA, 50 mM $[NH_4]_2$ SO_4, 5 mM DTT, 1 % Tween 20 [*w*/*v*], 150 mM KCl) in the presence of 1 μg poly(dI-dC). Binding experiments were also carried out in the absence of DTT. The DNA-protein mixture was incubated at 30 °C for 30 min and was analyzed via electrophoresis using 8 % native polyacrylamide gels. DNA bands were visualized using GelRed nucleic acid stain (Biotium).

Sensitivity to oxidants

The sensitivity of *C. glutamicum* cells to diamide (*N*,*N*,*N*′,*N*′-tetramethylazodicarboxamide), menadione (2-methyl-1,4-naphthoquinone sodium bisulfite), or H_2O_2 was assessed on MB plates as follows. *C. glutamicum* cells (100 μl), which had been cultivated to mid-exponential phase, were mixed with 0.8 % (*v*/*v*) top agar and poured onto MB plates. Paper disks (6.0 mm, Whattman) with 20 μl each of 1 M diamide, 1 M menadione, or H_2O_2 (50 % *w*/*v*) were placed on the plates, after which the plates were incubated at 30 °C for 24 h, until the formation of a clear zone around the disks.

Results

Expression of the *noxA* gene during growth

The NCgl0328 gene (*noxA*) of *C. glutamicum* is annotated to encode NADH oxidase, which belongs to the nitroreductase family of proteins. Theoretically, NADH oxidase (EC 1.6.99.3) catalyzes the oxidation of NADH by simultaneously reducing molecular O_2 to either H_2O_2 in a two-electron reduction or directly to H_2O in a four-electron reduction. These reactions use FMN as a cofactor. Accordingly, a putative FMN-binding site was identified on the N-terminal and central domains of the *noxA*-encoded protein. Since the NoxA protein was identified by 2D-PAGE (Choi et al. 2009; Lee et al. 2012) and suggested to be involved in the oxidative stress response networks, the molecular, regulatory, and physiological properties of the gene were further analyzed.

First of all, we determined the transcription start site of the *noxA* gene using the 5′ RACE PCR technique. As shown in Fig. 1a, the *noxA* transcription start site was identified as a guanine base located 27 bp upstream

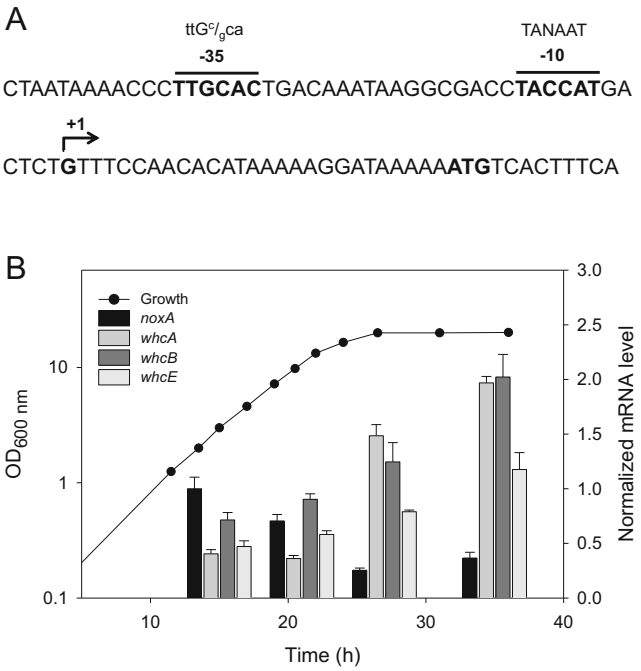

Fig. 1 Promoter region of the *noxA* gene and transcription of *whcA*, *whcB*, *whcE*, and *noxA* during growth. **a** The transcriptional start site (+1) was experimentally determined by 5′ RACE. The translational initiation codon (ATG) for the *noxA* gene is shown in bold. The predicted promoter sequences of −10 and −35, shown in bold, were predicted from known consensus sequences. **b** Cells were grown in MCGC media, and mRNA levels were measured by RT-qPCR. Gene expression levels were normalized to the levels of the 16S rRNA gene, which was used as an endogenous control. Data represent three independent experiments. *Filled circles* indicate the growth of *C. glutamicum*. *Bars* indicate relative mRNA levels of *noxA*, *whcA*, *whcE*, and *whcB*. *OD* optical density

of the translation start codon (ATG). This agreed with the results of Pfeifer-Sancar et al. (2013), which used RNA-Seq for determination of transcription start sites. The putative promoter sequences TTGCAC (-35) and TACCAT (-10) were located in the region upstream of the transcription start site. Purine-rich sequences presumed to be ribosome-binding sites were also located between the ATG start codon and the +1 transcription start site. Next, the transcription profile of *noxA* was assessed and compared with the profiles of the *whcA*, *whcE*, and *whcB* genes, which play regulatory roles in oxidative stress response of *C. glutamicum*. As shown in Fig. 1b, the transcription of *noxA* was found to peak in the log phase and to decrease as the cells entered the stationary phase, at which point, the transcription level was only 30 % of the level observed in log phase. Transcription of the *whcA*, *whcE*, and *whcB* genes, on the other hand, was found to increase by up to two–threefold, as the cells entered stationary phase. As suggested by Choi et al. (2009), these data may indicate that the expression of *noxA* is repressed by the *whcA* gene during the stationary phase of *C. glutamicum*.

The roles of *whc* genes during *noxA* expression

To investigate the potential involvement of the *whc* genes in *noxA* gene expression, the transcription levels of *noxA* were assessed in detail under various genetic conditions. The levels of *noxA* transcription were measured in *whc*-deleted and *whc*-overexpressing cells. Overexpression of *whc* was achieved by employing the P_{180} promoter, which induces overexpression of the cloned gene regardless of growth phase (Park et al. 2004). Typically, a 20-fold overexpression of *whcA*, *whcE*, and *whcB* was achieved as verified by RT-qPCR. First, *noxA* transcription was monitored in *whcA*-deleted (Δ*whcA*) and *whcA*-overexpressing (P_{180}-*whcA*) cells. As shown in Fig. 2a, *noxA* transcription was minimal in P_{180}-*whcA* cells while it was maximal in Δ*whcA* cells regardless of the growth phase. These data clearly indicate that *noxA* is negatively regulated by *whcA*. Next, the transcription levels of *noxA* in Δ*whcE* and P_{180}-*whcE* cells were assessed. While the messenger RNA (mRNA) levels of *noxA* were minimal in Δ*whcE* cells (Fig. 2b), the transcription of *noxA* in P_{180}-*whcE* cells was nearly maximal, with decreased transcription being observed in the log phase. Overall, the levels of *noxA* transcription in P_{180}-*whcE* cells were low compared with the transcription levels in the Δ*whcA* cells, indicating an additional regulation of gene expression by a factor other than *whcE*. Nevertheless, these data clearly show not only that *noxA* is regulated by the *whc* genes but also that the *whcA* and *whcE* genes play negative and positive roles, respectively, in the expression of *noxA*.

Next, the transcription levels of *noxA* were monitored in Δ*whcB* and P_{180}-*whcB* cells to elucidate the role of *whcB*, whose regulatory role in oxidative stress responses is not clear. As shown in Fig. 2c, the mRNA expression levels of *noxA* were repressed in P_{180}-*whcB* cells, especially in the log phase. Repression of *noxA* in P_{180}-*whcB* cells was less evident in the stationary phase. Accordingly, the transcription levels of *noxA* were monitored in the Δ*whcB* cells, and stimulation of *noxA* transcription was only observed during transition from exponential to stationary growth phase. Since *whc* transcription is organized into a hierarchy and *whcB* lies at the top of the hierarchy (Lee et al. 2012), we determined whether the observed repression by *whcB* is exerted via *whcA*. As shown in Fig. 2d, the expression of *whcA* was low in Δ*whcB* cells. Furthermore, the expression of *whcA* in P_{180}-*whcB* cells was comparable to that in the wild-type strain, except in the stationary phase, during which, the transcription of *whcA* was decreased. Taken together, these findings suggest that the effect of *whcB* on *noxA* expression may be an indirect one exerted via *whcA*. It was thus investigated whether WhcA binds to the promoter region of the *noxA* gene in vitro.

Binding of WhcA to the promoter region of *noxA*

The potential binding of WhcA to the promoter region of *noxA* was assessed using DNA-protein affinity purification to visualize bound protein and electrophoretic mobility shift assay (EMSA) to visualize the bound DNA. The WhcA protein was overexpressed as an MBP-WhcA-His_6 fusion protein and then isolated for in vitro analysis. The addition of histidine residues at the C-terminus of the protein was essential for the stability of the purified protein (unpublished data). Next, WhcA was assessed for potential binding to the promoter region of *noxA* (from −211 to +70) by DNA-protein affinity purification. WhcA was allowed to interact with DNA, after which, it was eluted from the DNA for analysis by SDS-PAGE. As shown in Fig. 3a, while the 56.5-kDa WhcA protein was found to bind to the 281-bp DNA fragments harboring the *noxA* promoter region, and could be eluted from the DNA, the protein did not bind to the ORF region of the gene, suggesting the specificity of DNA-binding. The potential binding of WhcB was also assessed; however, specific binding of the WhcB protein to the *noxA* promoter region was not observed (data not shown). These findings indicate that WhcA functions as a transcriptional repressor for the *noxA* gene. Moreover, these findings provide further evidence of *whcB* exerting its effect via *whcA*.

The potential binding of MBP and WhcA to the *noxA* promoter region was also assessed using EMSA to visualize shifted DNA. The target DNA region (bases −211 to +9 relative to the transcriptional start site) was PCR-amplified as described in the "Materials and methods" section. As

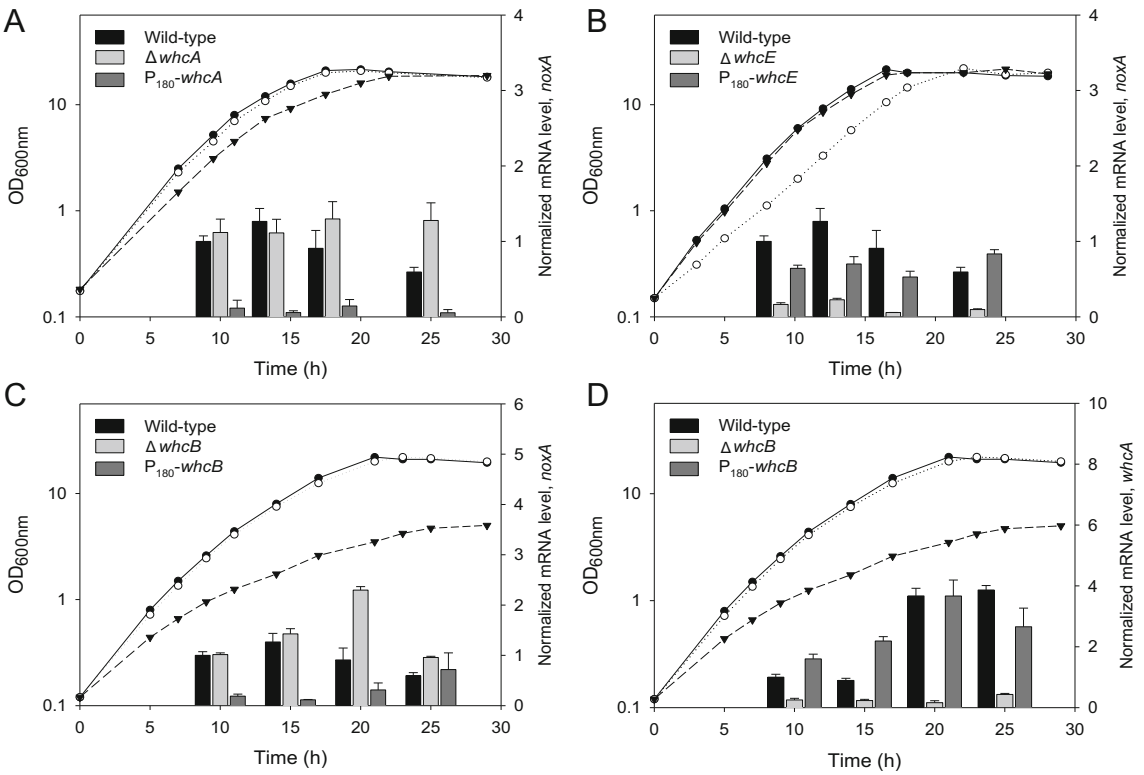

Fig. 2 Transcription of *noxA* and *whcA* in *C. glutamicum* strains. Cells were grown in glucose MCGC media, and mRNA levels were measured by RT-qPCR. Mean values of the mRNA levels and standard deviations are shown based on three independent experiments. Representative growth curves from three independent experiments are shown. *Line graphs* indicate cell growth and *bars* indicate relative mRNA levels. **a** *filled circle* indicates wild-type, *empty circle* Δ*whcA*, and *filled inverted triangle* P_{180}-*whcA*; **b** *filled circle* indicates wild-type, *empty circle* Δ*whcE*, *filled inverted triangle* P_{180}-*whcE*; **c** and **d** *filled circle indicates* wild-type, *empty circle* Δ*whcB*, *filled inverted triangle* P_{180}-*whcB*

shown in Fig. 3b, purified WhcA protein was found to bind to the promoter region of the *noxA* gene, as evidenced by shifted DNA bands. Binding of MBP to the *noxA* promoter region was not observed, suggesting the specificity of the observed protein-DNA binding. Furthermore, shifted bands were not observed in the absence of DTT. Considering the role of WhcA as a repressor of oxidative stress response genes, the binding of the protein only under reducing conditions (i.e., in the presence of DTT) is anticipated. It is also interesting to note that SpiA, which is known to interact with WhcA, was not needed for the DNA binding (see "Discussion").

Growth properties of the *noxA* deletion or overexpression strains

In previous experiments, retarded growth rates were observed in the host cells, when the transcription of *noxA* was decreased to a minimum level (Fig. 2), suggesting that *noxA* transcription is required for proper cell function. Furthermore, the involvement of multiple *whc* genes in the regulation of *noxA* transcription indicates that tight regulation of the *noxA* gene is important for cell physiology. To further analyze these phenomena, a *noxA*-deleted mutant strain (Δ*noxA*) of *C. glutamicum* was generated by gene disruption, and a *C. glutamicum* strain overexpressing *noxA* (P_{180}-*noxA*) was established. Subsequently, the growth rates of both the strains were monitored. Internal deletion of *noxA* in the Δ*noxA* strain was confirmed by PCR (data not shown) and the approximately 20-fold overexpression of *noxA* in the P_{180}-*noxA* strain was confirmed by RT-qPCR (data not shown). First, the growth properties of both strains were assessed using minimal or complex medium. In the minimal medium, the Δ*noxA* mutant strain showed retarded growth with a doubling time of 2.4 h (Fig. 4a), compared with the 2.3 h doubling time measured for the wild-type strain. Furthermore, the P_{180}-*noxA* strain showed much slower growth with a doubling time of 2.5 h, suggesting that excess *noxA* gene expression during the growth phase may interfere with proper cell function. The growth data shown suggests that the *noxA*-encoded protein may function as an enzyme whose cellular concentration must be strictly controlled for proper cell function. Differences in growth rates among the strains were not observed when the cells were grown in a complex medium (data not shown). As suggested by protein sequence homology, the *noxA*-encoded protein may function as an NADH oxidase.

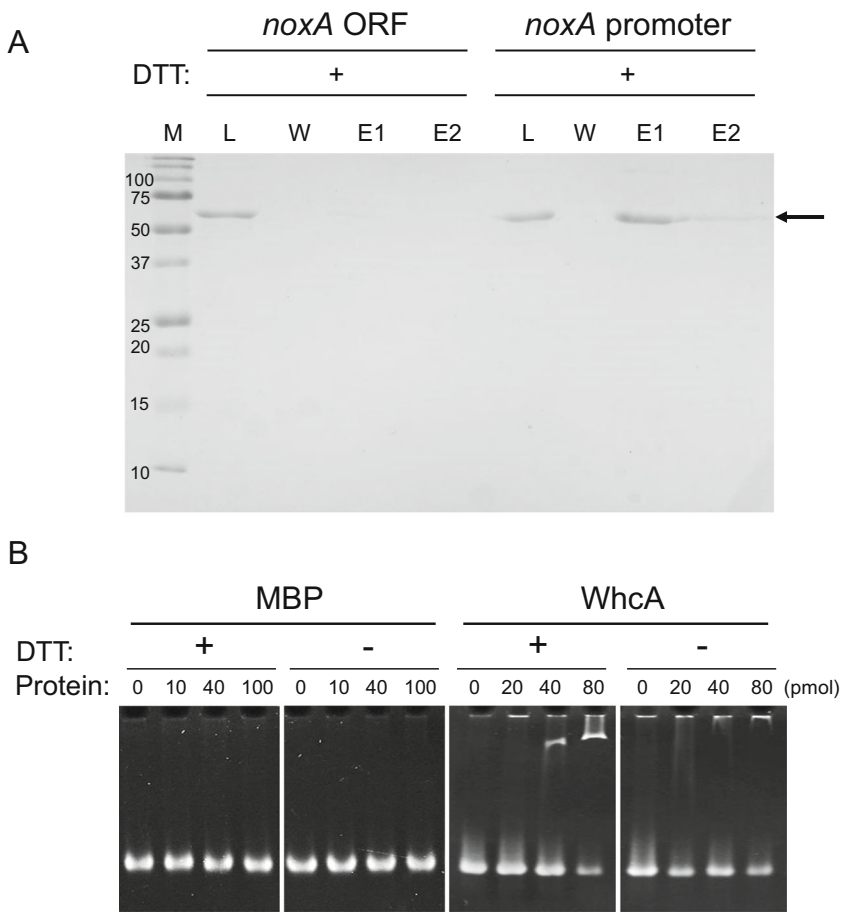

Fig. 3 DNA binding of the purified WhcA protein to the promoter region of the *noxA* gene. DNA affinity purification (**a**) and electrophoretic mobility shift assays (EMSA, **b**) were conducted and analyzed by SDS-PAGE (**a**) and native polyacrylamide gel electrophoresis (**b**), respectively. The *arrow* indicates MBP-WhcA-His_6. *M* molecular weight marker, *L* loaded protein, *W* washed fraction, *E1* protein eluted with 0.6 M NaCl, *E2* protein eluted using 1 M NaCl

Response of *noxA* mutants to oxidative stress

Because the *noxA* gene was found to be under the control of the *whc* genes, it was assumed that *noxA* plays a role in oxidative stress responses. This hypothesis was tested by challenging the Δ*noxA* and P_{180}-*noxA C. glutamicum* strains with various stress-causing oxidants including diamide, menadione, and H_2O_2. The sensitivities of the strains to the reagents were monitored using an agar-diffusion test. As shown in Fig. 4b, the P_{180}-*noxA* strain was found to be highly sensitive to menadione (but not to H_2O_2 or diamide) compared with the wild-type strain, as shown by a larger zone of growth inhibition for P_{180}-*noxA*. Sensitivity of the Δ*noxA* strain to the oxidants was detected, but to a lesser extent than that of the P_{180}-*noxA* strain. Sensitivity of the Δ*noxA* and P_{180}-*noxA* strains to the redox-cycling compound menadione, but not to H_2O_2, may suggest a role for the *noxA* gene in redox balance rather than in the detoxification of reactive oxygen species (see "Discussion"). Assuming that the *noxA*-encoded protein may be membrane-associated, the sensitivity of the Δ*noxA* and P_{180}-*noxA* cells to detergents (Triton X-100 and Tween 20) was assessed. No differences were observed among the strains (data not shown).

The increased sensitivity of the Δ*noxA* and P_{180}-*noxA* strains to oxidants is thought to possibly be due to a faulty oxidation repair system. Thioredoxin reductase reduces oxidized thioredoxin using NADPH as a cofactor, and this reduced thioredoxin functions as a general protein disulfide reductant, which can reactivate previously oxidized proteins (Holmgren 1985). The mRNA levels of ORF NCgl0663, which is assumed to be the *trxB* gene encoding thioredoxin reductase in *C. glutamicum*, were thus measured, and only marginal differences in ORF NCgl0663 expression levels were observed among the *C. glutamicum* wild-type, Δ*noxA*, and P_{180}-*noxA* strains in the log phase (data not shown). This result therefore excludes the possibility that the defective cell growth observed was caused by a defective expression of the *trxB* gene.

NADH oxidase activity

Because *noxA* has been annotated to encode NADH oxidase, and our findings suggest a role for *noxA* in redox balancing, the *noxA*-encoded protein was tested for NADH oxidase activity. Because the presence of multiple NADH oxidases in the annotated genome of *C. glutamicum* could potentially

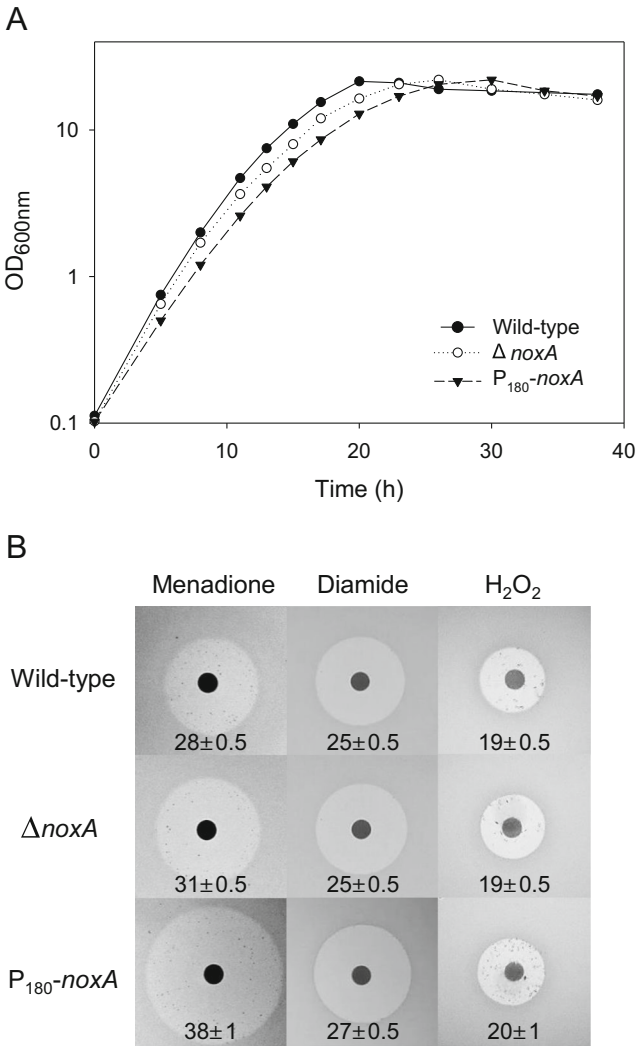

Fig. 4 Growth characteristics of *C. glutamicum* wild-type and *noxA* mutants and sensitivity of the *C. glutamicum* strains to oxidants. **a** Cells were grown on glucose MCGC minimal media. Data represent three independent experiments. *filled circle* indicates wild-type, *empty circle* Δ*noxA*, *filled inverted triangle* P_{180}-*noxA*. **b** A paper disk containing diamide, menadione, or H_2O_2 was placed on each MB plate containing lawns of *C. glutamicum* cells and incubated at 30 °C for 24 h. Numbers denote diameter (mm) of the growth-inhibiting zones. Data represent three independent experiments

hinder our analysis, NADH oxidase activity was measured and compared in the cell extracts of wild-type, Δ*noxA*, and P_{180}-*noxA* strains. In the Δ*noxA* mutant strain, the activity decreased by approximately 70 % relative to the wild-type strain (Table 1). The activity in the P_{180}-*noxA* strain, however, increased by 20-fold. These data suggest that the *noxA*--encoded protein (NoxA) possesses NADH oxidase activity and NoxA accounts for the majority of the NADH-oxidizing activity in *C. glutamicum*.

To determine if NADH was oxidized specifically by NoxA and not by other enzymes present in the host cell, the NoxA enzyme was purified. First, the *noxA* gene was cloned in to the T7 promoter-based plasmid pET28a to give pSL544 and was

Table 1 Enzyme activities of NADH oxidase in cell extracts of *C. glutamicum* cells

C. glutamicum strains	Total protein (mg)	Total activity[a] (U)[b]	Specific activity (U/mg)	Relative activity[c] (fold)
Wild-type	0.792	0.245	0.31	1
Δ*noxA*	0.799	0.075	0.094	0.3
P_{180}-*noxA*	0.681	4.154	6.1	20

The enzymes were induced by growing *C. glutamicum* AS019E12 cells to late-exponential phase in MCGC minimal medium. Cells were harvested, disrupted, and assayed for activity as described in the "Materials and methods". The activities represent one of the three independent experiments

[a] Activity is based on the amount of whole-NADH oxidase activity

[b] One unit of activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol β-NADH per minute at pH 7.0 at 30 °C

[c] The activity of the wild type strain was arbitrarily set to 1

subsequently expressed in *E. coli* as an NH_2-terminal His_6-tagged protein (His_6-NoxA). Analyses carried out with the extracts of *E. coli* harboring pSL544 revealed high levels of NADH oxidase activity (Table 2). Crude extracts from *E. coli* transformed with pSL544 were analyzed by SDS-PAGE. A protein of 24.8 kDa, a size consistent with the predicted molecular mass for the His_6-NoxA protein, was identified in crude extracts (Fig. 5). The enzyme was purified 3.6-fold to homogeneity with 89 % yield by Ni^{2+}-NTA affinity chromatography (Table 2). Purified His_6-NoxA showed a single band on SDS-PAGE stained with Coomassie Brilliant Blue (Fig. 5). The purified His_6-NoxA dominated the NADH oxidase activity detected in *E. coli* cell-free extracts (Table 2). Purified His_6-NoxA showed high activity in oxidizing NADH in the presence of FMN. The purified protein was colorless and did not display activity in the absence of FMN. These results clearly demonstrate that the NADH oxidase activity observed in crude extracts of the *C. glutamicum* wild-type and P_{180}-*noxA* cells (Table 1) corresponds to that of the NoxA protein.

Discussion

In this study, the role of *whcA* in the regulation of its target gene, *noxA*, was assessed. In accordance with the *noxA* transcription data from the Δ*whcA* and P_{180}-*whcA* strains of *C. glutamicum*, purified WhcA protein was shown to bind to the promoter region of *noxA*. The previously reported presence of the helix-turn-helix DNA-binding motif of WhcA is in agreement with our data. It is interesting to note that SpiA is not required for the DNA-binding of WhcA. Park et al. (2011, 2012) postulated that the WhcA protein forms a complex with the SpiA protein and that the SpiA-WhcA protein complex then binds to its target promoters to repress gene expression in

Table 2 Purification of His_6-NoxA

Steps	Protein (mg)	NADH oxidase activity		Recovery (%)	Purification (fold)
		Total activity (U)[a]	Specific activity[b] (U/mg)		
Crude extract (*E. coli*)	160	319.2	1.995	100	1
Column flow-through[c]	97.3	15.10	0.155	4.73	NA
Ni^{2+}-NTA affinity chromatography	39.7	284.6	7.169	89.2	3.6

NA not applicable

[a] One unit of activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol β-NADH per minute at pH 7.0 at 30 °C

[b] Activity is based on the amount of whole-NADH oxidase activity

[c] Fractions containing proteins that did not bind to the Ni^{2+}-NTA affinity resin

the absence of oxidative stress. However, it was clearly shown in this study that the WhcA protein in fact presents in its DNA-binding configuration and binds to its target gene (*noxA*). Because Park et al. noted that SpiA played both positive and negative roles and that some *whcA*-regulated genes are not under the control of the *spiA* gene, we can speculate that the *noxA* gene is not likely to be part of the *spiA*-regulon. This hypothesis is further supported by the fact that, while the growth of *spiA* mutants is affected by both menadione and diamide (Park et al. 2011), the growth of *noxA* mutants is only affected by menadione. Moreover, unlike in the case of *spiA* mutants, the expression of the *trxB* gene encoding thioredoxin reductase was not affected in the *noxA* mutants—a finding that further supports our hypothesis. Furthermore, the fact that a reducing agent (DTT) is required for the binding of WhcA to DNA is noteworthy, as it suggests that a reducing environment, which is the proposed condition under which WhcA binds DNA as a repressor, is also necessary for the WhcA protein to maintain its DNA-binding activity.

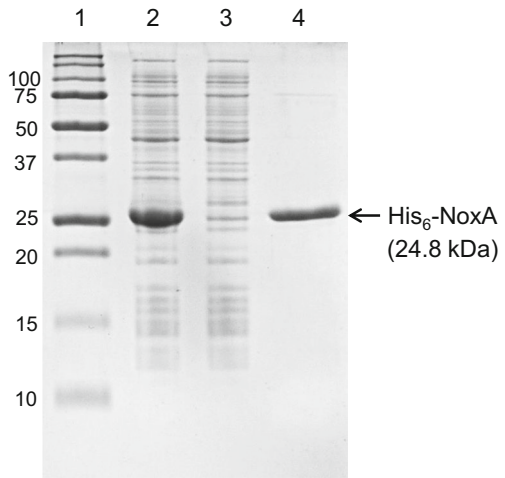

Fig. 5 SDS-PAGE analysis of expression and purification of His_6-NoxA. The purified His_6-NoxA (lane 4, 19 μg) and molecular-weight standards (lane 1) are indicated along with their corresponding molecular masses. *Lane 1* molecular mass standards, *lane 2* crude extract (12 μg), *lane 3* column flow-through fractions (8 μg), *lane 4* His_6-NoxA after Ni^{2+}-NTA chromatography. SDS-PAGE gel (17 %) was stained with Coomassie Brilliant Blue R-250

The regulation of *noxA* gene expression by *whcE* and *whcB* appears to be different from the regulation by *whcA*. Although the transcription of *noxA* was minimal in the Δ*whcE* strain, the stimulatory effect achieved by the overexpression of *whcE* gene was rather limited, suggesting the presence of an additional regulatory component. It is possible that the limited effect is due to the presence of an intact chromosomal copy of *whcA*, which exerts a negative regulatory effect. The overexpression of *whcE* likely stimulates the expression of *whcA*, and the stimulatory effect of *whcE* may be suppressed by the WhcA protein. Overall, our findings, including the evidence for the action of *whcB* being conveyed via *whcA*, are in agreement with the hypothesis proposed by Lee et al. (2013).

The involvement of multiple *whc* genes in the regulation of *noxA* expression suggests that tight regulation of *noxA* gene expression is essential. The severe retardation of the growth rates observed for the P_{180}-*whcA*, P_{180}-*whcB*, and Δ*whcE* strains were associated with low expression levels of *noxA*, suggesting the importance of adequate *noxA* expression. It is also interesting to note that, although the overexpression of *noxA* in the P_{180}-*noxA* cells caused severe growth retardation, overexpression of *noxA* did not cause severe growth defects in Δ*whcA*, P_{180}-*whcE*, and Δ*whcB* cells. This could be due to the level of expression achieved in the P_{180}-*noxA* cells. Approximately 20-fold expression was observed in P_{180}-*noxA* cells, whereas only deregulation was observed in the P_{180}-*whcA*, P_{180}-*whcB*, and Δ*whcE* cells.

The sensitivity of the *noxA* mutant strains to oxidants indicates that NADH oxidase plays a role in oxidative stress responses. It is interesting to note that *noxA* mutants were only sensitive to menadione, and not to H_2O_2 or diamide. The redox-cycling compound menadione exerts its toxic effects by stimulating the intracellular production of superoxide radicals (Afanas'ev et al. 1990). The thiol-specific oxidant diamide, on the other hand, specifically oxidizes sulfhydryl groups, such as those in cysteine residues. The comparable levels of *trx* transcription measured in the Δ*noxA* and P_{180}-

noxA strains correlates with the diamide insensitivity of the strains. The increased sensitivity of P_{180}-*noxA* cells to menadione is suggestive of an overproduction of superoxide radicals in *C. glutamicum* cells.

The *noxA* gene has been annotated to encode NADH oxidase, a member of the nitroreductase family of proteins. In this study, the *noxA*-encoded protein was indeed shown to exhibit NADH oxidase activity. The fact that the basal level of NADH oxidase activity measured was relatively high in the Δ*noxA* mutant strain is indicative of the presence of multiple NADH-oxidizing enzymes in *C. glutamicum*. Possible candidates for such additional nitroreductase enzymes include NCgl2735 and NCgl2913, which are found in the genome of *C. glutamicum*. The similarity of these two gene products with the *noxA*-encoded protein, however, is low (20–24 %). The observed NADH-oxidizing activity may alternatively result from the electron transfer reaction from NADH to oxygen through the components of the electron transport chain. The measured NADH oxidation activity of NoxA would strongly compete with NADH oxidation by the NADH dehydrogenase (*ndh*) of the respiratory chain and thus with oxidative phosphorylation. In addition, although this has not been shown in *C. glutamicum*, many enzymes exhibiting NADH oxidase activity have been reported in other organisms (Kawasaki et al. 2009; Kundu et al. 2012; Singh et al. 2004; Zarepour et al. 2010). NADH oxidase catalyzes the oxidation of NADH to NAD^+ using molecular oxygen as the electron acceptor. One type catalyzes the oxidation of NADH by reducing molecular oxygen to H_2O_2, while the other catalyzes a four-electron reduction of oxygen to H_2O with NADH oxidation. Some enzymes also reduce H_2O_2 to H_2O at the expense of NADH. Although NADH oxidases play an important role in scavenging oxygen in anaerobic bacteria, thus providing defense against oxidative stress, common NADH oxidases of mesophilic organisms are assumed to protect cells from oxidative stress by reducing oxygen to water, without the formation of harmful reactive oxygen species (Chenier et al. 2008; Cortial et al. 2010; Derr et al. 2012; Jia et al. 2010; Kang et al. 2013; Kawasaki et al. 2005; Pesakhov et al. 2007; Yang and Ma 2007). Moreover, NADH oxidases from several bacteria also play important roles in recycling pyridine nucleotides such as NAD^+/NADH during catabolism (Kang et al. 2013; Sauvageot et al. 2012; Yamamoto et al. 2006; Zhang et al. 2012). The fact that Δ*noxA* and P_{180}-*noxA* cells did not show significant difference in their sensitivities to H_2O_2 in this study indicates that NADH oxidase may not be involved in H_2O_2 generation and/or elimination in *C. glutamicum*, thus excluding the possibility of a role for the enzyme in the detoxification of reactive oxygen species. Furthermore, the growth differences that were observed only in minimal medium suggest that NADH oxidase may contribute to sugar metabolism, probably through the oxidization of NADH, as observed in *Enterococcus faecalis* (Sauvageot et al. 2012), suggesting a role in NAD^+/NADH balance. The finding that both deletion and overexpression of the *noxA* gene were harmful to the cell growth also provides evidence for a role of *noxA* in maintaining NAD^+/NADH balance. Although unlikely, NADH oxidase from *C. glutamicum* may play additional roles by transferring electrons to acceptors other than molecular oxygen.

Taken together, our results show that the *noxA* gene of *C. glutamicum* plays important roles in oxidative stress responses as well as in NAD^+/NADH redox balance. Considering the role of *C. glutamicum* cells in metabolite overproduction and the importance of cofactor regeneration such as NAD^+/NADH cycling in metabolic engineering (Hou et al. 2014; Ji et al. 2013; Rocha-Martin et al. 2011; Zhang et al. 2014), the *noxA* gene may be of importance in the field of biotechnology. Toward fully elucidating the function of *noxA* in *C. glutamicum* physiology, additional investigations are underway.

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