NdnR is an NAD-responsive transcriptional repressor of the *ndnR* operon involved in NAD *de novo* biosynthesis in *Corynebacterium glutamicum*

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The *Corynebacterium glutamicum ndnR* gene, which is chromosomally located in a gene cluster involved in NAD *de novo* biosynthesis, negatively regulates expression of the cluster genes, i.e. *nadA*, *nadC*, *nadS* and *ndnR* itself. Although *ndnR* encodes a member of the recently identified NrtR family of transcriptional regulators, whether or not the NdnR protein directly regulates these NAD biosynthesis genes remains to be verified. Here, two NdnR binding sites in the promoter region of the *ndnR-nadA-nadC-nadS* operon in *C. glutamicum* were confirmed by *in vitro* DNA binding assay and analysis of *in vivo* expression of the chromosomally integrated *ndnR* promoter–*lacZ* reporter fusion. Electrophoretic mobility shift assay revealed that the NdnR protein binds to the 5′-upstream region of *ndnR*, and that the binding is significantly enhanced by NAD. Mutation in two 21 bp NdnR binding motifs in the *ndnR* promoter region inhibited the binding of NdnR *in vitro*. The mutation also enhanced the promoter activity in cells cultured in the presence of nicotinate, which is utilized in NAD biosynthesis, resulting in the loss of the repression in response to an exogenous NAD precursor; this is consistent with the effect of deletion of *ndnR* reported in our previous study. These results indicate that NAD acts as a co-repressor for the NdnR protein that directly regulates the *ndnR* operon involved in NAD *de novo* biosynthesis; the NAD–NdnR regulatory system likely plays an important role in the control of NAD homeostasis in *C. glutamicum*.

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INTRODUCTION

NAD acts as the primary biological cofactor for numerous oxidation–reduction reactions in cellular metabolism. In many bacteria, NAD is *de novo*-synthesized from L-aspartate. The NAD *de novo* biosynthesis pathway from L-aspartate to the intermediate metabolite, nicotinate mononucleotide, proceeds via the consecutive actions of L-aspartate oxidase, quinolinate synthetase and quinolinate phosphoribosyl-transferase, encoded by *nadB*, *nadA* and *nadC*, respectively (Gazzaniga *et al.*, 2009; Magni *et al.*, 1999). Regulation of the NAD *de novo* biosynthesis genes has been well studied in *Salmonella enterica* serovar Typhimurium. The expression of *nadB* and *nadA* is regulated by the transcriptional regulator NadR (Grose *et al.*, 2005; Penfound & Foster, 1999). NadR represses expression of these genes by binding to their respective promoter regions, and NAD acts as a co-repressor for NadR. This regulatory system is likely to contribute to maintaining the intracellular NAD cofactor level. However, the NadR-dependent regulatory system is limited to a compact phylogenetic group of enterobacteria (Gerasimova

& Gelfand, 2005). On the other hand, in *Bacillus subtilis*, a different type of transcriptional regulator, NiaR (YrxA), represses expression of the *nadBCA* operon by binding to its promoter region (Rossolillo *et al.*, 2005). The binding of NiaR is enhanced by nicotinate, which is derived from pyridine nucleotide degradation, and is utilized for NAD biosynthesis in salvage/recycling pathways through conversion to nicotinate mononucleotide (Rossolillo *et al.*, 2005). NiaR homologues are broadly found in the bacterial genomes of the *Bacillus/Clostridium* group and in the Fusobacteria and Thermotogales lineages (Rodionov *et al.*, 2008b).

Corynebacterium glutamicum is a high-GC Gram-positive bacterium that is widely used for the industrial production of amino acids, and is expected to be a versatile workhorse for biofuels and biochemicals (Hermann, 2003; Inui *et al.*, 2004a; Okino *et al.*, 2008a, b; Smith *et al.*, 2010; Wendisch *et al.*, 2006). In addition, this non-pathogenic soil bacterium is of interest as a model organism for the *Corynebacteriaceae*, an Actinomycetes suborder that includes medically important pathogens such as *Corynebacterium diphtheriae* and *Mycobacterium tuberculosis* (Baumbach *et al.*, 2009; Brune *et al.*, 2005; Liebl, 2005). Elucidation of the regulation of NAD metabolism in *C. glutamicum* is important for

Abbreviation: RACE, rapid amplification of cDNA ends.

A supplementary table, showing the primers used in this study, is available with the online version of this paper.

establishing the fundamental characteristics of the cellular metabolism of this useful micro-organism. We previously reported that, in *C. glutamicum*, deletion of *ndnR*, which is located in a cluster along with the NAD *de novo* biosynthesis genes in the chromosome, enhances expression of these cluster genes when cells are cultured in the presence of nicotinate (Teramoto *et al.*, 2010). This gene cluster is composed of *ndnR*, *nadA*, *nadC* and *nadS* (encoding a putative cysteine desulfurase required for maturation of NadA, an Fe-S protein). Although NdnR belongs to a novel family of transcriptional regulators called the Nudix-related transcriptional regulators (NrtRs), whether or not NdnR directly regulates these NAD biosynthesis genes remains to be verified. A recent comparative genomics study implicated NrtR family members in the regulation of various aspects of NAD metabolism, and suggested that the regulatory networks are highly diverse among bacteria (Rodionov *et al.*, 2008a). In *Shewanella oneidensis* and *Synechocystis* sp. PCC 6803, ADP-ribose, the product of glycohydrolytic cleavage of NAD, was found to suppress the *in vitro* binding of NrtR to its DNA target site (Huang *et al.*, 2009; Rodionov *et al.*, 2008a). However, the physiological role of the ADP-ribose-responsive regulatory system remains to be elucidated.

In this study, we show that *C. glutamicum* NdnR binds *in vitro* to two consensus sites in the promoter region of the *ndnR-nadA-nadC-nadS* operon, and that the binding is enhanced by NAD. In addition, *in vivo* expression of the mutated promoter-*lacZ* reporter gene fusions reveals that both of the two binding sites are involved in the NdnR-dependent regulation.

METHODS

Bacterial strains and culture conditions. *C. glutamicum* R (Yukawa *et al.*, 2007) was used as a wild-type strain.

For genetic manipulations, *Escherichia coli* and *C. glutamicum* cells were grown as described previously (Teramoto *et al.*, 2008).

For analytical purposes, a *C. glutamicum* cell starter culture was grown aerobically in 10 ml nutrient-rich A medium (Inui *et al.*, 2004b) containing 1% glucose at 33 °C in a 100 ml test tube overnight. The cells were harvested, and washed twice with minimal BT medium (Inui *et al.*, 2004b). The washed cells were suspended in 10 ml BTM medium (Teramoto *et al.*, 2010) containing 1% glucose with or without 5 mg nicotinic acid l⁻¹, then cultured at 33 °C in a 100 ml test tube.

Cell growth was monitored by measuring the OD₆₁₀ using a spectrophotometer (DU640, Beckman Coulter).

DNA techniques. Chromosomal and plasmid DNA was prepared from *C. glutamicum* cells, and the target DNA regions were amplified by PCR, as described previously (Teramoto *et al.*, 2008).

C. glutamicum cells were transformed by electroporation as described previously (Vertès *et al.*, 1993). *E. coli* cells were transformed by the CaCl₂ procedure (Sambrook *et al.*, 1989).

DNA sequencing was performed with an ABI Prism 3100xl Genetic Analyzer (Applied Biosystems). DNA sequence data were analysed with the Genetyx program (Software Development, Tokyo, Japan).

RT-PCR and rapid amplification of cDNA ends (RACE). Total RNA was prepared from *C. glutamicum* cells using a Qiagen RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. For RT-PCR analysis, single-stranded cDNA was synthesized from total RNA using PrimeScript Reverse Transcriptase (Takara) with hexadeoxyribonucleotide mixture as a primer. The resulting cDNA was used as a template for PCR with a primer set for the region of interest (Supplementary Table S1). *C. glutamicum* chromosomal DNA was also used as a template for a control PCR with the same primer set. The reaction mixture was electrophoresed on an agarose gel, and the amplified DNA was detected with ethidium bromide.

The 5' end of *ndnR* mRNA was determined by RACE using primers summarized in Supplementary Table S1. Using the 5'-Full RACE Core Set (Takara), single-stranded cDNA synthesized from total RNA using the 5'-phosphorylated primer (ndnR-RA-P) was self-ligated with T4 RNA ligase. The first PCR proceeded using inverted primers (ndnR-RA-F1 and ndnR-RA-R1), and then the second PCR proceeded using nested inverted primers (ndnR-RA-F2 and ndnR-RA-R2). The amplified DNA was digested with *EcoRI* and inserted into the corresponding site of plasmid pHSG398 (Takara). Multiple clones from *E. coli* transformed with the resulting plasmid were sequenced.

Construction of an *ndnR* promoter-*lacZ* fusion. A DNA fragment containing the *ndnR* promoter region was amplified by PCR using *C. glutamicum* chromosomal DNA as template and appropriate primers (Supplementary Table S1, ndnR-Pr-F1 and ndnR-Pr-R1). The amplified DNA was digested with *DraI*, and inserted into the corresponding site of pCRA741 (Inui *et al.*, 2007) to construct the *ndnR* promoter-*lacZ* fusion. Mutagenesis of the NdnR binding site was performed by overlapping PCR using the plasmid containing the *ndnR* promoter-*lacZ* fusion as template. Primer pairs used are summarized in Supplementary Table S1; ndnR-Pr-F1/ndnR-PrM1-R and ndnR-PrM1-F/*lacZ*-R were used for NdnR site-1 mutation; ndnR-Pr-F1/ndnR-PrM2-R and ndnR-PrM2-F/*lacZ*-R were used for NdnR site-2 mutation. The mutated promoter region was inserted into the *DraI* site of pCRA741 to construct the *lacZ* fusion as described above. The *ndnR* promoter-*lacZ* fusion was integrated into strain-specific island 7 (SSI7) on the chromosome of *C. glutamicum* R by markerless gene insertion methods described previously (Inui *et al.*, 2004a).

Purification of NdnR expressed in *E. coli*. A DNA fragment containing the *ndnR* gene was amplified by PCR using an appropriate primer pair (Supplementary Table S1, ndnR-Ex-F and ndnR-Ex-R). The amplified DNA was digested with *NdeI* and *EcoRI*, and inserted into the corresponding site of the pET-28a expression vector (Merck). The resulting plasmid, pCRC317, contained the *ndnR* gene fused to a His-tag sequence at the N terminus. *E. coli* BL21(DE3) cells transformed with pCRC317 were grown at 26 °C in 100 ml Luria-Bertani medium supplemented with kanamycin (50 µg ml⁻¹). The recombinant gene was expressed in exponentially growing cells (OD₆₀₀ 0.6) by adding 1 mM IPTG. After 4 h of incubation, the cells were harvested by centrifugation. The His-tagged NdnR protein was extracted and purified by affinity column chromatography using a Ni-NTA Fast Start kit (Qiagen). The purified protein was loaded onto a gel filtration column (PD-10 column, GE Healthcare UK) equilibrated with buffer containing 20 mM Tris/HCl (pH 7.5), 20 mM KCl, 10 mM MgCl₂, 0.1 mM EDTA and 1 mM DTT, and the protein was eluted with the same buffer.

Electrophoretic mobility shift assay. The purified His-tagged NdnR protein at the indicated concentrations was incubated with 40 ng of a DNA probe in 20 µl binding buffer containing 10 mM Tris/HCl (pH 7.5), 160 mM KCl, 5 mM MgCl₂, 0.05 mM EDTA, 1.5 mM DTT and 10% (v/v) glycerol for 30 min at 25 °C. Nicotinate,

NAD, NADH, NADP, NADPH, ATP, ADP and ADP-ribose were added at the indicated concentrations. The binding reaction mixture was subjected to electrophoresis on a 6% polyacrylamide gel containing 5% (v/v) glycerol in 0.5× Tris-borate-EDTA (TBE) electrophoresis buffer, and the DNA probe was detected with SYBR Green.

DNA probes were prepared by PCR using the plasmids containing the *ndnR* promoter region as templates. Primers used are summarized in Supplementary Table S1: for the DNA fragment between −278 and +44 bp with respect to the transcription start site of *ndnR*, *ndnR*-Pr-F1 and *ndnR*-Pr-R1 were used; for the fragment between −278 and −105 bp, *ndnR*-Pr-F1 and *ndnR*-Pr-R2 were used; for the fragment between −124 and +44 bp, *ndnR*-Pr-F2 and *ndnR*-Pr-R1 were used.

β-Galactosidase assay. *C. glutamicum* cells were harvested, washed once with Z-buffer (Miller, 1972), resuspended with the same buffer and treated with toluene. The permeabilized cells were then incubated with ONPG, and activity was measured in Miller units as described by Miller (1972).

RESULTS

The *ndnR*-*nadA*-*nadC*-*nadS* genes form an operon in *C. glutamicum*

Previously, we reported that all of the four genes in the NAD *de novo* biosynthesis gene cluster, *ndnR*, *nadA*, *nadC* and *nadS*, are coordinately downregulated under the control of NdnR in *C. glutamicum* cells cultured in the presence of nicotinate (Teramoto *et al.*, 2010), but whether they are co-transcribed as an operon was not addressed. The *ndnR*-*nadA* and *nadA*-*nadC* intergenic regions are 48 and 3 bp long, respectively. *nadC* and *nadS* overlap by 1 bp. Here, we examined the co-transcribed expression of the flanking genes by RT-PCR. Expression of the *ndnR*-*nadA*, *nadA*-*nadC* and *nadC*-*nadS* regions as well as of their respective coding regions was detected (Fig. 1). The RT-PCR results were not due to genomic DNA contamination, since no amplification of the divergently transcribed *ndnR*-*cgR_1154* region from the same cDNA prepared was detected. These results indicate that *ndnR*, *nadA*, *nadC* and *nadS* are co-transcribed under the control of the *ndnR* promoter.

The transcription start site of the *ndnR* operon was determined by 5'-RACE; it is located 31 bp upstream of the translation start site of *ndnR* (Fig. 1). The consensus sequences of the −10 and −35 regions of the *C. glutamicum* SigA-dependent promoter (Pátek & Nešvera, 2011) are found in the 5'-upstream region of the *ndnR* gene (Fig. 1). The NdnR binding motif was proposed by comparison of the corresponding chromosomal regions found in some closely related species of actinobacteria (Rodionov *et al.*, 2008a). Two putative 21 bp NdnR binding sites exist in the *ndnR* promoter region; one (NdnR site-1) is located between +3 and +23 bp with respect to the transcription start site, and the other (NdnR site-2) between −32 and −12 bp (Fig. 1).

In vitro binding of NdnR to the *ndnR* promoter region

In order to examine the DNA binding ability of NdnR, the His-tagged protein was expressed in *E. coli* and purified by affinity chromatography. Electrophoretic mobility shift assay was carried out with the NdnR protein and the 5'-upstream region of *ndnR* (Fig. 2). When the DNA fragment between −124 and +44 bp with respect to the transcription start site of *ndnR* was used as a DNA probe, the NdnR protein reduced the electrophoretic mobility of this probe; the amount of the NdnR–DNA complex formed increased as the concentration of the NdnR protein increased (Fig. 2, lanes 1–5). In contrast, the mobility of the DNA fragment between −278 and −105 bp was not affected by NdnR (Fig. 2, lanes 6–10). These results confirmed that NdnR binds to the 168 bp *ndnR* promoter region containing the two NdnR binding motifs in a sequence-specific manner.

The effects of candidate effector molecules on the DNA binding of NdnR were examined by electrophoretic mobility shift assay (Fig. 3a). At a concentration of 1 mM, NAD markedly enhanced the binding of NdnR to the *ndnR* promoter region. NADH also acted as a positive effector; the effect was weaker than that of NAD. Other candidate molecules, i.e. nicotinate, NADP, NADPH, ATP, ADP and ADP-ribose, hardly affected the DNA binding activity of NdnR at 1 mM. The binding of NdnR to the *ndnR* promoter region was enhanced as a function of the concentration of NAD, increasing from 10 μM to 1 mM (Fig. 3b).

The 21 bp putative NdnR binding motif consists of a 9 bp inverted repeat separated by 3 bp (5'-TTAnnGTCAnnn-TGACnnTAA-3') (Fig. 4a). The effects of mutations in this motif on the binding of NdnR in the presence of 0.3 mM NAD were examined by electrophoretic mobility shift assay (Fig. 4b). As compared with the *ndnR* promoter region containing both native NdnR site-1 and NdnR site-2 (Fig. 4b, WT) forming a complex with the NdnR protein, the same region containing the mutated NdnR site-1 (Fig. 4b, Mut-1) forming a complex with NdnR showed faster electrophoretic mobility. Mutation in NdnR site-2 gave the same results as mutation in NdnR site-1 (Fig. 4b, Mut-2). The *ndnR* promoter region with mutations in both NdnR site-1 and NdnR site-2 did not bind to the NdnR protein (Fig. 4b, Mut-1/2). These results indicate that both NdnR site-1 and NdnR site-2 are involved in the binding of NdnR to the *ndnR* promoter region.

Mutations in the NdnR binding sites eliminate nicotinate-dependent repression of the *ndnR* promoter activity in vivo

Effects of mutation in the NdnR binding sites described above on the *ndnR* promoter activity *in vivo* were examined. The native and mutated *ndnR* promoters were fused to the *lacZ* reporter gene, and the respective gene fusions were integrated into the chromosome of the *C. glutamicum* wild-type strain. The resulting strains were

Fig. 1. The *ndnR* operon involved in *de novo* NAD biosynthesis in *C. glutamicum* R. The nucleotide sequence of the *ndnR*-*cgR_1154* intergenic region is shown (upper panel). Translation initiation codons are boxed. The transcription start site (+1) of *ndnR* determined is indicated by an arrow. The -10 and -35 regions are indicated by asterisks above the sequences. NdnR binding motifs (NdnR site-1 and NdnR site-2) are indicated with a grey background. Expression of the *ndnR* operon shown by electrophoresis of the RT-PCR products (lower panel). The genes corresponding to the primers used are indicated above each pair of lanes; the same preparations of *C. glutamicum* genomic DNA (left-hand lane) and cDNA (right-hand lane) were used as templates. Primers (Supplementary Table S1) are as follows: 1154-RT-R/*ndnR*-RT-R1 (1), *ndnR*-RT-F1/R2 (2), *ndnR*-RT-F2/R3 (3), *ndnR*-RT-F2/*nadA*-RT-R1 (4), *nadA*-RT-F1/R2 (5), *nadA*-RT-F2/*nadC*-RT-R (6), *nadC*-RT-F1/R (7), *nadC*-RT-F2/*nadS*-RT-R1 (8), *nadC*-RT-F3/*nadS*-RT-R2 (9), *nadS*-RT-F/R3 (10). M, DNA size marker.

cultured in minimal medium with or without 5 mg nicotinic acid l^{-1} , and their β -galactosidase activity in the exponential and stationary phases was measured (Fig. 5). Cell growth of these strains under these conditions was mostly similar (data not shown). In the exponential growth phase, the activity of the native *ndnR* promoter was markedly repressed in the presence of nicotinate relative to the activity in the absence of nicotinate (Fig. 5, 6 h, WT), as reported previously (Teramoto *et al.*, 2010). The activity of the *ndnR* promoter containing the mutated NdnR site-1 was significantly higher than that of the native promoter in the presence of nicotinate, resulting in a reduced response to exogenous NAD precursor (Fig. 5, 6 h, M1). The effects of mutation in NdnR site-2 on the promoter activity were similar to those of mutation in NdnR site-1 (Fig. 5, 6 h,

M2). The activity of the promoter containing both mutated NdnR binding sites was higher than that of the promoters with mutations in the respective single binding sites when cells were cultured in the presence of nicotinate, and the response to exogenous NAD precursor was eliminated (Fig. 5, 6 h, M1/2). When cells were cultured in the absence of nicotinate, the native promoter activity increased fourfold in the stationary phase relative to that in the exponential phase (Fig. 5, 19 h), while the activity remained strictly repressed in the presence of nicotinate. Similar growth phase-dependent changes were observed with the mutated promoter activity, not only in the absence of nicotinate but also in its presence, suggesting the involvement of another factor independent of the NdnR function in *ndnR* promoter activity. It should be noted that the activity of

Fig. 2. *In vitro* binding of NdnR to the *ndnR* promoter region. Electrophoretic mobility shift assay was carried out with the His-tagged NdnR protein and the 5'-upstream region of *ndnR*. The 20 μ l binding reaction mixture containing 40 ng DNA probe (lanes 1–5, the DNA fragment between –124 and +44 bp with respect to the transcription start site of *ndnR*; lanes 6–10, the DNA fragment between –278 and –105 bp) and the NdnR protein at various concentrations (lanes 1 and 6, no protein; lanes 2 and 7, 120 ng; lanes 3 and 8, 240 ng; lanes 4 and 9, 480 ng; lanes 5 and 10, 960 ng) was subjected to electrophoresis on a 6% polyacrylamide gel. The free DNA and the DNA–protein complex are indicated by white and black arrowheads, respectively.

the promoters with a mutation in either NdnR site-1 or NdnR site-2 was significantly repressed by the presence of nicotinate in the stationary as well as in the exponential growth phase.

DISCUSSION

In this study, we confirmed that *C. glutamicum* NdnR binds to the promoter region of the *ndnR-nadA-nadC-nadS* operon involved in NAD *de novo* biosynthesis, and that NAD significantly enhances the DNA binding activity of the NdnR protein. As shown in our previous study (Teramoto *et al.*, 2010) and discussed below, NdnR negatively regulates the expression of the *ndnR* operon when cells are cultured in the presence of the nicotinate that is utilized for NAD biosynthesis. These findings indicate that NdnR acts as a transcriptional repressor of the *ndnR* operon, and that NAD acts as a co-repressor for NdnR to regulate *de novo* biosynthesis of NAD in order to maintain homeostasis of the NAD cofactor pool. NdnR belongs to the recently identified NrtR family of transcriptional regulators; the

Fig. 3. Effects of pyridine nucleotides on the DNA binding of NdnR. Electrophoretic mobility shift assay was carried out with the His-tagged NdnR protein and the *ndnR* promoter region. The 20 μ l binding reaction mixture containing 40 ng of the DNA fragment between –278 and +44 bp with respect to the transcription start site of *ndnR* and 240 ng NdnR protein was subjected to electrophoresis on a 6% polyacrylamide gel (Cont, control). The binding reaction was supplemented with nicotinate (NA), NAD, NADH, NADP, NADPH, ATP, ADP and ADP-ribose (ADPR) at 1 mM (a). The effects of NAD at 0, 10, 30, 100, 300 and 1000 μ M were also compared (b). Free DNA and the DNA–protein complex are indicated by white and black arrowheads, respectively.

family members are distributed in the genomes of a wide range of bacteria. Comparative genomic analysis suggests that the target genes of NrtR family regulators are diverse among bacterial species; the regulators are predicted to be involved not only in various aspects of NAD metabolism but also in pentose utilization, the pentose phosphate pathway and biosynthesis of phosphoribosyl pyrophosphate, a central precursor for the synthesis of nucleotides and amino acids (Rodionov *et al.*, 2008a). The structure of a typical NrtR family member is composed of an N-terminal effector domain homologous to the Nudix (nucleoside diphosphate with large variation of residues) hydrolase family and a C-terminal DNA binding winged helix–turn–helix domain (Huang *et al.*, 2009; Rodionov *et al.*, 2008a). NAD, which was found to act as a co-repressor for *C. glutamicum* NdnR in this study, presumably binds to the N-terminal Nudix domain to affect NdnR DNA binding activity. Structural characterization of *Shewanella oneidensis* NrtR in complex with target DNA and with ADP-ribose as an effector molecule indicates that ADP-ribose likely plays a critical role in the allosteric regulation of DNA binding and the repressor function of NrtR (Huang *et al.*, 2009). It should be noted that ADP-ribose acts as a negative effector of NrtR in *Shewanella oneidensis* and *Synechocystis* sp. PCC 6803 (Huang *et al.*, 2009; Rodionov *et al.*, 2008a), in contrast to

Fig. 4. NdnR binding sites in the *ndnR* promoter region. The nucleotide sequence of the NdnR binding motif found in the *ndnR* promoter region (NdnR site-1 and NdnR site-2) are aligned (a). An inverted repeat is indicated by arrows at the top of the alignment. Mutated nucleotides in NdnR site-1 (Mut-1) and NdnR site-2 (Mut-2) are shown below the respective native sequences. Effects of mutations in the NdnR binding site were examined by electrophoretic mobility shift assay (b); single mutations in NdnR site-1 (Mut-1) and NdnR site-2 (Mut-2) and a double mutation in these sites (Mut-1/2) were introduced into the *ndnR* promoter region, and the DNA fragment between -278 and +44 bp with respect to the transcription start site of *ndnR* was used as a DNA probe. The 20 μ l binding reaction mixture containing 0.3 mM NAD, 40 ng native (WT) or mutated DNA probe, and the NdnR protein at different concentrations (lanes 1, 4, 7 and 10, no protein; lanes 2, 5, 8 and 11, 120 ng; lanes 3, 6, 9 and 12, 240 ng) was subjected to electrophoresis on a 6% polyacrylamide gel. The free DNA and the DNA-protein complex are indicated by white and black arrowheads, respectively.

the positive effect of NAD on the DNA binding activity of *C. glutamicum* NdnR shown in this study. In addition, the NdnR binding was not affected by ADP-ribose in our

electrophoretic mobility shift assay. These differences are probably due to the different physiological roles of these regulators. It is predicted that *Shewanella oneidensis* NrtR is involved in the regulation of the salvage/recycling pathways from the NAD degradation products, and ADP-ribose, which is derived from enzymic NAD hydrolysis, is assumed to be a signal for NAD turnover in the ADP-ribose-NrtR-dependent regulatory system (Huang *et al.*, 2009; Rodionov *et al.*, 2008a). On the other hand, *C. glutamicum* NdnR plays an essential role in the regulation of the NAD *de novo* biosynthesis operon. Structural characterization with regard to the effect of NAD on the activity of NdnR should provide a new model of this type of transcriptional regulator.

The feedback repression of the NAD *de novo* biosynthesis genes mediated by the NAD-responsive transcriptional regulator NdnR in *C. glutamicum* is similar to the NAD-NadR regulatory system in *S. enterica* serovar Typhimurium (Grose *et al.*, 2005; Penfound & Foster, 1999), although these regulators are structurally different. This regulatory strategy is largely different from that of *B. subtilis*, in which availability of the NAD salvage pathways is likely a central signal to regulate expression of the *de novo* biosynthesis genes by the nicotinate-responsive transcriptional regulator NiaR (Rodionov *et al.*, 2008b; Rossolillo *et al.*, 2005). The DNA binding of the multifunctional NadR in *S. enterica* serovar Typhimurium is prevented by ATP, and NAD allows DNA binding in the presence of ATP; it is proposed that NAD represses NadR-dependent transcription by preventing the binding of ATP to the NadR protein (Grose *et al.*, 2005; Penfound & Foster, 1999). In this study, ATP and nicotinate did not affect the binding of *C. glutamicum* NdnR to the *ndnR* promoter region in the electrophoretic mobility shift assay. The newly identified regulatory system of the NAD *de novo* biosynthesis genes in *C. glutamicum* is presumed to be conserved in some species of actinobacteria based on comparative genomic analysis (Rodionov *et al.*, 2008a). However, the predicted binding motifs of the NrtR-family proteins of these species reveal significant variability between two branches of a phylogenetic tree. It should be noted that the binding motif of *C. glutamicum* NdnR has

Fig. 5. Effects of mutation in NdnR binding sites on the *ndnR* promoter activity. Single mutations in NdnR site-1 (M1) and NdnR site-2 (M2) and a double mutation in these sites (M1/2) were introduced into the *ndnR* promoter region. *C. glutamicum* strains carrying the native (WT) or mutated *ndnR* promoter-*lacZ* fusion in the chromosome were cultured in minimal medium with (black bars) or without (white bars) 5 mg nicotinic acid l^{-1} . The β -galactosidase activity in cells cultured for 6 h (exponential phase) and 19 h (stationary phase) was measured. The values represent the mean results from three independent cultivations; error bars, SEM.

low similarity to its counterparts from related pathogenic species, such as *C. diphtheriae* and *M. tuberculosis* (Rodionov *et al.*, 2008a).

The two NdnR binding sites in the promoter region of the *ndnR* operon in *C. glutamicum* were verified by *in vitro* DNA binding assay and analysis of *in vivo* expression of the promoter–*lacZ* reporter fusion. Mutation in both of the binding sites inhibited the binding of NdnR to the *ndnR* promoter region, and markedly enhanced its promoter activity in cells cultured in the presence of nicotinate. The resulting loss of the nicotinate-responsive regulation is consistent with the effects of *ndnR* deletion on the expression levels of the respective genes in this operon, as shown in our previous study (Teramoto *et al.*, 2010). One of the two NdnR binding sites is located between –35 and –10 regions, and the other is located immediately downstream of the transcription start site of *ndnR*. Thus, it is likely that the binding of NdnR prevents RNA polymerase from interacting with the promoter and/or the elongation of transcription. It should be noted that the activity of an *ndnR* promoter with defects in either of the NdnR binding sites was still repressed by nicotinate, although to a lesser extent than the native promoter. These results demonstrate that the two binding sites are required for the strict NAD-responsive regulation of the NAD *de novo* biosynthesis operon. When, in an electrophoretic mobility shift assay, the native *ndnR* promoter containing two binding sites was mixed with the NdnR protein, a DNA–protein complex with faster mobility corresponding to that observed with the mutated promoter with a single binding site was hardly detectable (Fig. 4b). This may be due to a synergistic effect between the two NdnR binding sites in the *ndnR* promoter region; however, further study is needed to elucidate the possibility.

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