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Regulation of the Expression of Genes Involved in NAD *De Novo* Biosynthesis in *Corynebacterium glutamicum*▽

Haruhiko Teramoto, Masako Suda, Masayuki Inui, and Hideaki Yukawa*

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

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Three genes, *nadA*, *nadB*, and *nadC*, involved in NAD *de novo* biosynthesis are broadly conserved in the genomes of numerous bacterial species. In the genome of *Corynebacterium glutamicum*, *nadA* and *nadC* but not *nadB* are annotated. The *nadA* and *nadC* genes are located in a gene cluster containing two other genes, designated *ndnR* and *nadS* herein. *ndnR* encodes a member of the Nudix-related transcriptional regulator (NrtR) family. *nadS* encodes a homologue of cysteine desulfurase involved in Fe-S cluster assembly. The gene cluster *ndnR-nadA-nadC-nadS* is genetically characterized herein. Mutant strains deficient in *nadA*, *nadC*, or *nadS* required exogenous nicotinate for growth, and the nicotinate auxotrophy was complemented by introduction of the corresponding gene in *trans*, indicating that each of these genes is essential for growth in the absence of an exogenous source of NAD biosynthesis. The results of reverse transcriptase PCR analyses and *ndnR* promoter-*lacZ* expression analyses revealed that the expression of *ndnR*, *nadA*, *nadC*, and *nadS* genes was markedly and coordinately repressed by nicotinate. The expression of these genes was enhanced by the disruption of *ndnR*, resulting in the loss of the nicotinate-responsive regulation of gene expression. These results suggest that NdnR acts as a transcriptional repressor of NAD *de novo* biosynthesis genes and plays an essential role in the regulation of the response to nicotinate.

NAD plays a crucial role in living organisms because it acts as a cofactor for numerous redox reactions in cellular metabolism. The biochemistry and genetics of NAD metabolism have been well studied in the enteric bacteria *Salmonella enterica* serovar Typhimurium and *Escherichia coli* (1–3, 7, 16, 22, 27, 30). NAD is synthesized from L-aspartate through the intermediate metabolite nicotinate mononucleotide (Fig. 1). The NAD *de novo* biosynthesis pathway converting L-aspartate to nicotinate mononucleotide proceeds via three enzymatic reactions catalyzed by L-aspartate oxidase, quinolinate synthetase, and quinolinate phosphoribosyltransferase. L-Aspartate oxidase encoded by *nadB* catalyzes the conversion of L-aspartate to iminoaspartate. Quinolinate synthetase encoded by *nadA* catalyzes the condensation of iminoaspartate with dihydroxyacetone phosphate. Quinolinate phosphoribosyltransferase encoded by *nadC* catalyzes the reaction between quinolinate and 5-phosphoribosyl-1-pyrophosphate to give nicotinate mononucleotide, pyrophosphate, and CO_2. Nicotinate mononucleotide is also generated from nicotinate. Extracellular nicotinate and its derivatives, which are derived from pyridine nucleotide degradation, are taken up by bacteria and utilized for NAD biosynthesis. Thus, the salvage pathway complements the *de novo* biosynthesis of NAD, and the relevant pathways must be coordinately regulated for maintaining homeostasis of the NAD cofactor pool. While the NAD *de novo* biosynthesis genes are found in the genomes of a number of bacterial species, some microbes lack these genes and depend entirely on salvage of NAD precursors (4).

In *S. enterica* serovar Typhimurium, the expression of *nadB* and *nadA* but not *nadC* is regulated by the transcriptional regulator NadR in response to the intracellular NAD level (6, 23). However, NadR-mediated regulation is limited to a compact phylogenetic group of enterobacteria (5). In *Bacillus subtilis*, a different transcriptional regulator, NiaR (YrxA), regulates the expression of the *nadABC* operon in response to the intracellular nicotinate level (26). NiaR homologues are broadly found in the bacterial genomes of the *Bacillus*/*Clostridium* group and in the deeply branching *Fusobacteria* and *Thermotogales* lineages (25). A recent comparative genomics study based on several hundred bacterial species anticipates that members of a novel family of transcriptional regulators, the Nudix-related transcriptional regulator (NrtR) family, are responsible for the regulation of various aspects of NAD metabolism, including the *de novo* biosynthesis, and that the regulatory networks are highly diverse among bacteria (24). However, the physiological role of the NrtR family regulators *in vivo* remains unclear.

Corynebacterium glutamicum, a Gram-positive soil bacterium, is widely used for the industrial production of amino acids, such as glutamate and lysine (13, 15). We have developed a bioprocess for lactate, succinate, and ethanol production using *C. glutamicum* (9, 10, 19–21). It is important to understand the regulation of NAD metabolism to enable optimal engineering based on the genome sequence of this industrially important microorganism (8, 12, 36). In the genome of *C. glutamicum*, putative *nadA* and *nadC* genes have been annotated, but no *nadB* orthologue has been identified to date. Thus, the functionality of *C. glutamicum nadA* and *nadC* is of particular interest.

In this study, we genetically characterized the *nad* gene cluster *cgR_1153* (*ndnR*)-*cgR_1152* (*nadA*)-*cgR_1151* (*nadC*)-*cgR_1150* (*nadS*) in the genome of *C. glutamicum* (Fig. 2). *ndnR* encodes an NrtR family protein. *nadS* encodes a homo-

* Corresponding author. Mailing address: Research Institute of Innovative Technology for the Earth, 9-2 Kizugawadai, Kizugawa, Kyoto 619-0292, Japan. Phone: 81-774-75-2308. Fax: 81-774-75-2321. E-mail: mmg-lab@rite.or.jp.

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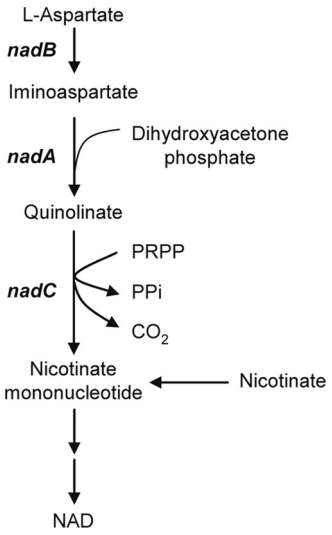

FIG. 1. The NAD *de novo* biosynthesis pathway. L-Aspartate is converted to nicotinate mononucleotide through the three reactions catalyzed by L-aspartate oxidase, encoded by *nadB*, quinolinate synthetase, encoded by *nadA*, and quinolinate phosphoribosyltransferase, encoded by *nadC*. Nicotinate mononucleotide, which is also generated from nicotinate, is subsequently converted to NAD. PRPP, 5-phosphoribosyl-1-pyrophosphate; PPi, inorganic pyrophosphate.

logue of cysteine desulfurase, which is believed to be required in *E. coli* and *B. subtilis* for the maturation of NadA, an Fe-S protein (14, 29, 31). Each of the *nadA*, *nadC*, and *nadS* genes was shown to be essential for growth when *C. glutamicum* cells are incubated in the absence of an exogenous source of NAD. Disruption of *ndnR* enhanced the levels of *nadA*, *nadC*, and *nadS* mRNAs, as well as the expression of the *ndnR* promoter-*lacZ* reporter gene fusion. As a result, the marked repression of these genes by nicotinate which was exhibited in the wild-type strain was completely eliminated, indicating that the regulation of the NAD *de novo* biosynthesis genes is primarily mediated by NdnR.

MATERIALS AND METHODS

Bacterial strains. *C. glutamicum* R (36) was used as a wild-type strain for our experiments. The *cgR_1150* (*nadS*)-deficient strain was obtained from a single-gene-disruptant mutant library constructed by transposon-mediated mutagenesis (32). In this strain, the transposon is inserted 449 bases downstream of the 5′ end of the initiation codon of the *nadS* gene.

Culture conditions. For genetic manipulations, *E. coli* and *C. glutamicum* strains were grown as described previously (33).

For analytical purposes, the *C. glutamicum* cell starter culture was grown aerobically in 10 ml nutrient-rich A medium (10) containing 4% glucose at 33°C in a 100-ml test tube overnight. The cells were harvested by centrifugation at 4,000 g for 10 min at 4°C. The cell pellet was subsequently washed twice with minimal BT medium (10). The washed cells were suspended with 100 ml BT medium containing glucose at 40 mM and supplemented with nicotinate at the concentrations indicated below and then aerobically cultured at 33°C in a 500-ml flask. To assess nicotinate auxotrophy, minimal BTM medium (BT medium supplemented with 1.0 mg liter^{-1} $ZnSO_4 \cdot 7H_2O$, 0.2 mg liter^{-1} $CuSO_4 \cdot 5H_2O$, 10 mg liter^{-1} $CaCl_2 \cdot H_2O$, 0.02 mg liter^{-1} $NiCl_2 \cdot 6H_2O$, and 20 μM protocatechuate) was used.

Cell growth was monitored by measuring the optical density at 610 nm using a spectrophotometer (DU640; Beckman Coulter, CA).

DNA techniques. Chromosomal DNA and plasmid DNA were prepared from *C. glutamicum* and the target DNA regions were amplified by PCR as described previously (33).

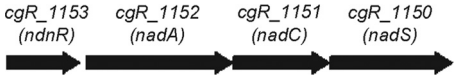

FIG. 2. The *nad* gene cluster of *C. glutamicum* strain R. *cgR_1153* (*ndnR*) encodes an NrtR family transcriptional regulator. *cgR_1152* (*nadA*) encodes quinolinate synthetase. *cgR_1151* (*nadC*) encodes quinolinate phosphoribosyltransferase. *cgR_1150* (*nadS*) encodes a cysteine desulfurase-like protein.

C. glutamicum cells were transformed by electroporation as described previously (34). *E. coli* cells were transformed by the $CaCl_2$ procedure (28).

DNA sequencing was performed with an ABI Prism 3100xl genetic analyzer (Applied Biosystems, Foster City, CA). DNA sequence data were analyzed with the Genetyx program (Software Development, Tokyo, Japan).

Construction of mutants. For gene deletion, the upstream and downstream regions of the gene to be deleted were amplified using the sets of primers summarized in Table 1. The resultant amplicons were fused and cloned into pCRA725 (9), a suicide vector for markerless gene disruption. The resultant plasmids, pCRC303, pCRC304, and pCRC305, were used for the deletion of *nadA*, *nadC*, and *ndnR*, respectively. *C. glutamicum* was subsequently transformed with the respective plasmid DNA, and screening for deletion mutants was performed as previously described (9). Deletion of the target genes was checked by PCR. In all cases, it was observed that each open reading frame (ORF) was deleted except for the 30-bp 5′- and 3′-terminal regions.

Construction of *ndnR* promoter-*lacZ* fusion. A DNA fragment containing the *ndnR* promoter region was amplified by PCR using the *C. glutamicum* chromosomal DNA as a template and a set of primers with appropriate restriction sites (Table 1). The amplified DNA was digested with the appropriate restriction enzyme and inserted into the corresponding site of pCRA741 (11) to construct the *ndnR* promoter-*lacZ* fusion. The *lacZ* fusion was integrated into strain-specific island 7 (SSI7) on the chromosome of *C. glutamicum* R by markerless gene insertion methods as described previously (9).

Plasmids for gene expression. Plasmids for the expression of the *nadA*, *nadC*, *nadS*, and *ndnR* genes were obtained as follows. The region for each of the ORFs was amplified by PCR using the *C. glutamicum* chromosomal DNA as a template and a set of primers with appropriate restriction sites (Table 1). The amplified ORF region was digested with the restriction enzymes and was inserted into the corresponding sites of the *Escherichia coli-Corynebacterium* shuttle vector pCRB1 (18), yielding pCRC306, pCRC307, pCRC308, and pCRC309 for the expression of *nadA*, *nadC*, *nadS*, and *ndnR*, respectively, under the control of the *lac* promoter.

Quantitative RT-PCR. Total RNA was prepared from *C. glutamicum* cells using an RNeasy minikit and RNAprotect bacteria reagent (Qiagen, Hilden, Germany) and quantitative reverse transcriptase PCR (RT-PCR) was performed using an Applied Biosystems 7500 fast real-time PCR system as described previously (33). The primers used are listed in Table 1. Specific amplification of the targeted DNA was confirmed by electrophoresis and sequencing of the PCR product. The relative abundance of the targeted mRNAs was quantified based on the cycle threshold value, which is defined as the cycle number required to obtain a fluorescence signal above the background. To standardize the results, the relative abundance of 16S rRNA was used as the internal standard.

β-Galactosidase assay. *C. glutamicum* cells were harvested, washed once with Z buffer (17), resuspended with the same buffer, and treated with toluene. The permeabilized cells were then incubated with *o*-nitrophenyl-β-D-galactopyranoside, and activity was measured in Miller units as previously described (17).

RESULTS

A cluster of NAD *de novo* biosynthesis genes in the genome of *C. glutamicum*. In order to examine the functionality of the *nadA* and *nadC* genes, in-frame deletion mutants (Δ*nadA* and Δ*nadC*) were constructed. A mutant strain deficient in *nadS* (*nadS*::Tn) was obtained from a mutant library previously constructed by transposon-based insertion of a selection marker (32). When Δ*nadA*, Δ*nadC*, and *nadS*::Tn were cultured on plates containing minimal medium, a severe growth defect was observed compared to the growth of the wild-type strain. The growth defect was suppressed by supplementation with nicoti-

TABLE 1. Primers used in this study

Primer and purpose	Target gene	Sequence (5′–3′)[a]	Overhanging restriction site
Plasmid construction for gene deletion			
nadA-U-Fw	*nadA*	AAACTGCAGCCAAAAAGGAAATAAAAGAC	PstI
nadA-U-Rv		GGGGTACCGTTGACAGATGGGGTGATTG	KpnI
nadA-D-Fw		GGGGTACCGTTACTCCTAGCTCCTCGAA	KpnI
nadA-D-Rv		CTCTGAGCTCGCAGAGGCGAGTCTTCCCAC	SacI
nadC-U-Fw	*nadC*	CTCTGTCGACCTCTTTCCAACTTGCCCGCG	SalI
nadC-U-Rv		GGGGTACCGCCAACGATACGGTCAGTAT	KpnI
nadC-D-Fw		GGGGTACCGCACTTGACCTAGGACTCGA	KpnI
nadC-D-Rv		CTCTGAGCTCCTGATCCTCCTCAAACCCCAA	SacI
ndnR-U-Fw	*ndnR*	CTCTGTCGACGGCTGGCCAACATCATGCCC	SalI
ndnR-U-Rv		TCCCCCGGGGGCCATCTGGATTTCAGGTG	SmaI
ndnR-D-Fw		TCCCCCGGGCGCCCACCCAAACTGTTCAG	SmaI
ndnR-D-Rv		CTCTGAGCTCGGGATTCAGGGTGCACGATG	SacI
Plasmid construction for gene expression			
nadA-Fw	*nadA*	CTCTGAGCTCTAGAAGAAAAGACCCCAATC	SacI
nadA-Rv		GGGGTACCTTACGCATCCTTCGAGGAGC	KpnI
nadC-Fw	*nadC*	GGAATTCCCTCGAAGGATGCGTAATTT	EcoRI
nadC-Rv		CTCTGAGCTCTGCATTATCAAGGTAGAGCA	SacI
nadS-Fw	*nadS*	CTCTGAGCTCCTAGGACTCGATATTTTCTA	SacI
nadS-Rv		GGGGTACCGCGTAAACCTCTGACTAGCG	KpnI
ndnR-Fw	*ndnR*	GGAATTCCTTGCCCGCTTCACCTGAAAT	EcoRI
ndnR-Rv		GGGGTACCTTATCTTTGGAATCTGAACA	KpnI
Plasmid construction for chromosomal integration of *lacZ* fusion			
PndnR-Fw	*ndnR* promoter	CTCTTTTAAATCCACGTTGCAACCAGGAGT	DraI
PndnR-Rv		CTCTTTTAAATGAAGCGGGCAAGAAACCAC	DraI
For RT-PCR analyses			
16S-F	16S rRNA	TCGATGCAACGCGAAGAAC	
16S-R		GAACCGACCACAAGGGAAAAC	
nadA-F	*nadA*	TCACCTCAATTTATGGCGATGACAC	
nadA-R		GCGTTCAAACGCCCACTCA	
nadC-F	*nadC*	TGGTGACAGCTTTGAGAC	
nadC-R		CTCTGAATGAAGTTGAGAG	
nadS-F	*nadS*	GTGCGCCTAAAGGGATTGGAGT	
nadS-R		AGGCAGTGGCAAAGGCGATAG	
ndnR-F	*ndnR*	AGCTATCTAGAACAGCTCTACACT	
ndnR-R		CGGACAAGTGCCCAATACAC	
1149-F	*cgR_1149*	CTCATTCCAGCGTCACGA	
1149-R		GCATGTCGTAATCAAATGTTCCTAA	

[a] The restriction site overhangs used in the cloning procedure are underlined.

nate. A plasmid carrying the *nadA*, *nadC*, or *nadS* gene under the control of a constitutive promoter was introduced into the respective mutant strain, Δ*nadA*, Δ*nadC*, or *nadS*::Tn. Each of the complemented strains was cultured in liquid minimal medium, and their growth was compared to that of the mutant strain and the wild-type strain, both of which were transformed with a vector plasmid without the target gene. When the Δ*nadA* cells cultured in nutrient-rich medium were transferred to minimal medium, growth tended to slow down. When an aliquot of the cell culture was subsequently transferred to new minimal medium, the growth was severely defective (Fig. 3A). In contrast, when an aliquot of the cell culture was transferred to medium supplemented with nicotinate, the Δ*nadA* strain grew in a manner comparable to the wild type. The *nadA*-complemented strain in either the presence or absence of nicotinate (Fig. 3B) grew as well as the wild-type strain (data not shown). Similar effects of disruption and complementation on nicotinate auxotrophy were observed in the case of *nadC* (Fig. 3C and D) and *nadS* (Fig. 3E and F). These results indicate that *nadA*, *nadC*, and *nadS* are each essential for growth in the absence of an exogenous source of NAD biosynthesis.

Repression of the expression of NAD *de novo* biosynthesis genes in response to nicotinate. Previously, we performed DNA microarray analyses to examine the transcriptional profiles of densely packed *C. glutamicum* cells under oxygen-deprived conditions in minimal medium compared to those under normal aeration conditions in nutrient-rich medium at physiological cell densities (11). Under the former conditions, applicable for a high-productivity bioprocess, 161 genes were upregulated. Among them, the induction levels of all four genes in the NAD *de novo* biosynthesis gene cluster, *ndnR*, *nadA*, *nadC*, and *nadS*, are particularly prominent. This observation suggests that their induction is due to the absence of an exogenous source of NAD biosynthesis. Here, we examined the expression of these genes during aerobic growth of the *C.*

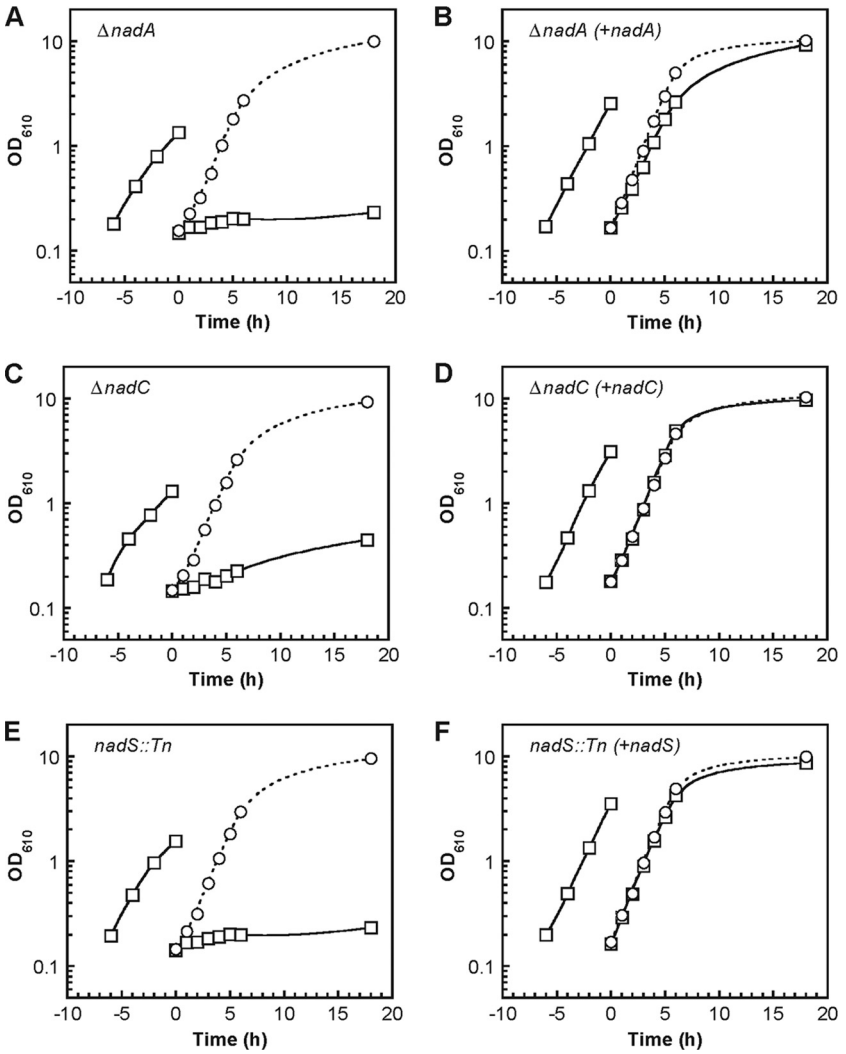

FIG. 3. Effect of disruption of *nadA*, *nadC*, or *nadS* on growth of *C. glutamicum* cells. Strains deficient in *nadA*, *nadC*, or *nadS* containing either a control vector plasmid pCRB1 (A, C, and E, respectively) or a plasmid carrying *nadA*, *nadC*, and *nadS* under the control of a constitutive promoter (B, D, and F, respectively) were grown in minimal BTM medium with (circles) or without (squares) nicotinate supplementation. The optical density at 610 nm (OD_{610}) was monitored. Similar results were obtained from two independent cultivations, and representative results are shown.

glutamicum wild-type strain cultured in minimal medium and in nutrient-rich medium (Fig. 4A). Total RNA was prepared from cells cultured for 2 h, 4 h, and 6 h and was subjected to quantitative RT-PCR analyses. The level of *ndnR* mRNA was markedly upregulated during growth in minimal medium relative to the level in nutrient-rich medium (Fig. 4B). The expression pattern of *nadS* under these conditions was the same as that of *ndnR* (Fig. 4C).

In order to examine the expression of the NAD *de novo* biosynthesis genes in response to nicotinate, *C. glutamicum* wild-type cells were cultured in minimal medium without nicotinate for 4 h and then were supplemented with nicotinate to a final concentration of 41 μM. Quantitative RT-PCR analyses revealed that *ndnR*, *nadA*, *nadC*, and *nadS* mRNAs rapidly decreased to extremely low levels within 12 min of nicotinate supplementation (Fig. 5A). The *ndnR-nadA* and *nadA-nadC* intergenic regions are 48 bp and 3 bp long, respectively. *nadC* and *nadS* overlap by 1 bp. Therefore, it is likely that the *ndnR-nadA-nadC-nadS* genes are transcribed as an operon. The downregulation of the downstream genes in the cluster in response to nicotinate was relatively slow (Fig. 5A). This may be a reflection of a 5′ to 3′ direction of the mRNA degradation process. As a control, the expression of *cgR_1149*, encoding a major facilitator superfamily protein of unknown function, which is located immediately downstream of *nadS* in the opposite direction, was also analyzed in these experiments. In contrast to the *ndnR-nadA-nadC-nadS* genes, the level of *cgR_1149* mRNA was little affected by nicotinate supplementation (Fig. 5A).

The *ndnR* promoter-*lacZ* reporter gene (P*ndnR-lacZ*) fusion was integrated into the genome of the *C. glutamicum* wild-type strain. The resultant strain was cultured in minimal medium supplemented with nicotinate at 0, 0.041, 0.41, 4.1, 41, and 410 μM. Cell growth in the presence of these various concentra-

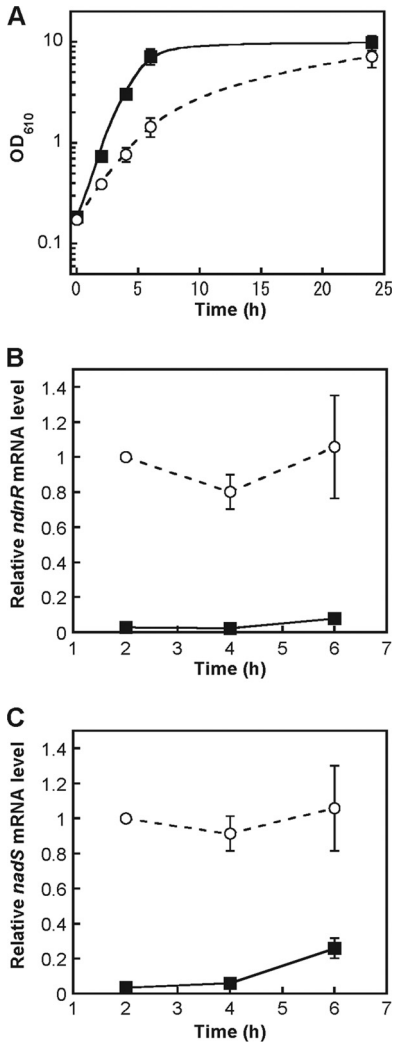

FIG. 4. Expression of the *ndnR* and *nadS* genes during growth in nutrient-rich medium or in minimal medium. The *C. glutamicum* wild-type strain was cultured in nutrient-rich A medium (black rectangles) or in minimal BT medium (white circles). The optical density at 610 nm (OD_{610}) was monitored (A). The levels of *ndnR* (B) and *nadS* (C) mRNAs in the cells were determined by quantitative RT-PCR. The mRNA levels are presented relative to the value from the 2-h culture in minimal medium. The values represent the mean results from three independent cultivations, with standard errors.

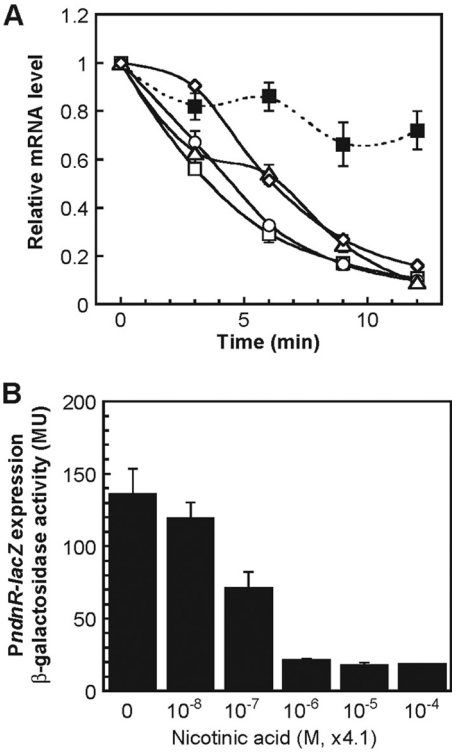

FIG. 5. Expression of the *ndnR*, *nadA*, *nadC*, *nadS*, and *cgR_1149* genes in response to nicotinate. (A) The *C. glutamicum* wild-type strain was cultured in minimal BT medium for 4 h, and then the culture was supplemented with nicotinate to the final concentration of 41 μM. The levels of *ndnR* (white rectangles), *nadA* (white circles), *nadC* (white triangles), *nadS* (white diamonds), and *cgR_1149* (black rectangles) mRNAs in the cells 0, 3, 6, 9, and 12 min after the nicotinate supplementation were determined by quantitative RT-PCR. The mRNA levels are presented relative to the value attained using cells immediately before nicotinate supplementation (0 min). The values represent the means from three independent cultivations with standard errors. (B) *C. glutamicum* wild-type strain carrying the *ndnR* promoter-*lacZ* (P*ndnR-lacZ*) fusion in the chromosome was cultured in minimal BT medium supplemented with nicotinate at 0, 0.041, 0.41, 4.1, 41, and 410 μM. The β-galactosidase activity in the exponentially growing cells was measured in Miller units (MU). The values represent the mean results from three independent cultivations, with standard errors.

tions of nicotinate was mostly similar (data not shown). On the other hand, the β-galactosidase activity of exponentially growing cells was markedly enhanced as the concentration of nicotinate in the medium decreased, with nicotinate levels greater than 4.1 μM resulting in marked repression of the expression of P*ndnR-lacZ* (Fig. 5B).

Involvement of NdnR in control of NAD *de novo* biosynthesis genes. In order to examine the involvement of NdnR, an NrtR family transcriptional regulator, in the expression of *ndnR*, *nadA*, *nadC*, and *nadS*, its in-frame deletion mutant was constructed. The growth of the resultant Δ*ndnR* strain was comparable to that of the wild-type strain in minimal medium in either the presence or absence of nicotinate (data not shown). Analyses of the levels of *nadA* mRNA in exponentially growing cells by quantitative RT-PCR revealed that, in the wild-type strain, *nadA* expression was severely repressed in the presence of nicotinate (Fig. 6). In contrast, in the Δ*ndnR* strain, the level of *nadA* mRNA was markedly high in either the presence or absence of nicotinate. The *nadA* expression in the Δ*ndnR* strain even in the absence of nicotinate was 3 times higher than the induced level in the wild-type strain, and the response to nicotinate was minimal in the Δ*ndnR* strain. The effects of the disruption of *ndnR* on *nadA*, *nadC*, and *nadS* mRNAs were almost the same (Fig. 6).

Moreover, an in-frame deletion mutant of *ndnR* was constructed from a strain chromosomally integrated with P*ndnR-lacZ*, and its β-galactosidase activity was compared to that of the parental strain. These strains grew to similar extents in minimal medium in either the presence or absence of nicotinate (data not shown). In the wild-type background, P*ndnR-lacZ* expression in exponentially growing cells was markedly repressed in the presence of nicotinate relative to that in its

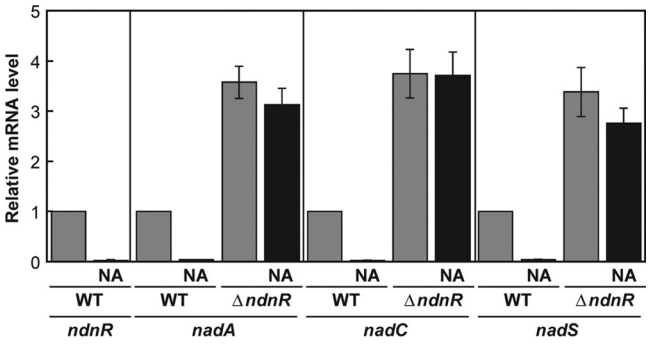

FIG. 6. Effects of disruption of *ndnR* on the expression of the *nadA*, *nadC*, and *nadS* genes. The *C. glutamicum* wild-type strain (WT) and the *ndnR*-deficient mutant strain (Δ*ndnR*) were grown in minimal BT medium with (NA) or without nicotinate supplementation at 41 μM. The levels of *ndnR*, *nadA*, *nadC*, and *nadS* mRNAs in the exponentially growing cells were determined by quantitative RT-PCR. The mRNA levels are presented relative to the value for the wild-type strain grown in the absence of nicotinate. The values represent the mean results from three independent experiments, with standard errors.

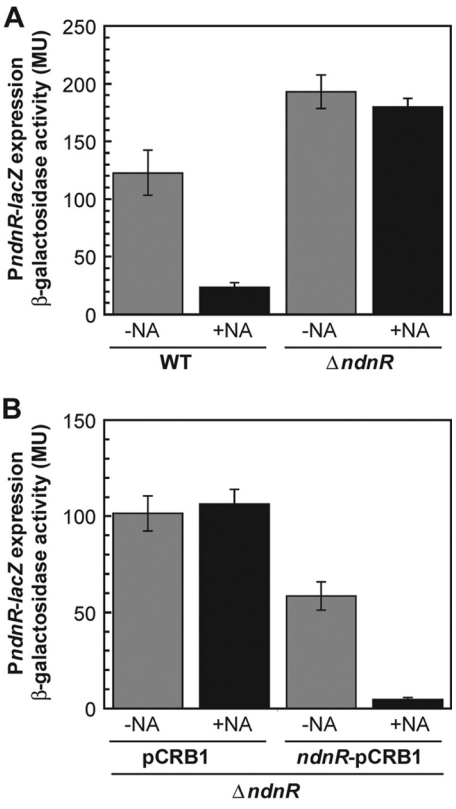

FIG. 7. Effect of disruption of *ndnR* on the expression of the *ndnR* promoter-*lacZ* (P*ndnR-lacZ*) fusion. (A) The *C. glutamicum* wild-type strain (WT) and the *ndnR*-deficient mutant strain (Δ*ndnR*), both of which carry the P*ndnR-lacZ* gene fusion in the chromosome, were cultured in minimal BT medium with (+NA) or without (-NA) nicotinate supplementation at 41 μM. The β-galactosidase activity in the exponentially growing cells was measured in Miller units (MU). The values represent the mean results from three independent cultivations, with standard errors. (B) The *ndnR*-deficient mutant strain (Δ*ndnR*) with the P*ndnR-lacZ* gene fusion containing a control vector plasmid (pCRB1) or a plasmid carrying the constitutive promoter-*ndnR* (*ndnR*-pCRB1) was grown in minimal BT medium with (+NA) or without (-NA) nicotinate supplementation at 41 μM. The β-galactosidase activity in the exponentially growing cells was measured. The values represent the mean results from three independent cultivations, with standard errors.

absence, whereas in the *ndnR*-deficient mutant background, P*ndnR-lacZ* expression was relatively high in either the presence or absence of nicotinate (Fig. 7A). Disruption of *ndnR* eliminated the response of P*ndnR-lacZ* expression to nicotinate. The effects of disruption of *ndnR* on P*ndnR-lacZ* expression were consistent with those on the levels of *nadA*, *nadC*, and *nadS* mRNAs, shown in Fig. 6, suggesting that these genes are probably cotranscribed as an operon under the control of the same promoter. A plasmid carrying the *ndnR* gene under the control of a constitutive promoter was introduced into the *ndnR*-deficient mutant strain containing P*ndnR-lacZ* in the chromosome. The growth of the *ndnR*-complemented strain in the medium tested was comparable to that of the parental strain carrying a vector plasmid without *ndnR* (data not shown). In the *ndnR*-complemented strain, the nicotinate-repressible expression of P*ndnR-lacZ* was restored (Fig. 7B).

DISCUSSION

In this study, we showed that, in *C. glutamicum*, either *nadA* or *nadC* is essential for growth in the absence of an exogenous source of NAD, indicating that these genes are functional for NAD *de novo* biosynthesis *in vivo*. Although *nadA* and *nadC*, along with *nadB*, which are involved in the NAD biosynthesis pathway from L-aspartate, are broadly conserved in numerous bacterial species, *nadB* is not found in *C. glutamicum* (25). In the genome of *C. glutamicum*, *nadC* and *nadA* are located adjacent to each other in the same cluster that also includes two other genes, *ndnR* and *nadS* (Fig. 2). It is noted that, in the genomes of the related species belonging to *Actinobacteria*, such as *Kineococcus radiotolerans* and *Leifsonia xyli*, *nadB* occurs between *nadA* and *nadC* in the same cluster as in *C. glutamicum*. Among *Corynebacterium* species, the gene cluster in *C. efficiens* is the same as that in *C. glutamicum*, while in *C. diphtheriae*, *nadB* occurs between *nadA* and *nadC* but a cysteine desulfurase gene (an *nadS* homologue) does not exist in the cluster. Additionally, in the genome of *C. diphtheriae*, a gene encoding an NrtR family protein (an *ndnR* homologue) is located immediately upstream of *nadA* but in the opposite direction. Interestingly, the same *nad* gene cluster as in *C. diphtheriae* is observed in other species of *Actinobacteria*, such as *Mycobacterium* species and *Nocardia farcinica*. In many bacteria, it is likely that L-aspartate oxidase encoded by *nadB* catalyzes the conversion from L-aspartate to iminoaspartate (Fig. 1). However, the conversion seems to be replaced by a reaction catalyzed by a different type of enzyme, L-aspartate dehydrogenase, the gene of which is located in a gene cluster along with *nadA* and *nadC* in the thermophilic bacterium *Thermotoga maritima* (35). It is possible that the first reaction of NAD *de novo* biosynthesis in *C. glutamicum* is catalyzed by an unidentified enzyme. These findings imply that NAD *de novo* biosynthesis in bacteria is more diverse than previously thought with respect to not only regulation, as described below, but also the structural gene(s).

Our results in this study suggest that *nadS* is also involved in

NAD *de novo* biosynthesis in *C. glutamicum*. NadS exhibits 35% amino acid sequence identity to *E. coli* IscS, which exhibits cysteine desulfurase activity and facilitates Fe-S cluster assembly (14, 29). Disruption of *iscS* leads to a decrease in the activity of a number of Fe-S proteins and results in generally poor growth. One of the growth traits of the *iscS* mutant is auxotrophy for nicotinate, suggesting that IscS is required for the maturation of NadA, an Fe-S protein (14, 22, 29). It is suggested that IscS functions primarily in *de novo* synthesis of Fe-S clusters rather than in the repair of oxidatively damaged clusters (29). In contrast to the *E. coli iscS*-deficient mutant strain, the *C. glutamicum nadS*-deficient mutant strain grew as well as the wild-type strain in nicotinate-supplemented minimal medium, suggesting that NadS is specifically involved in the *de novo* Fe-S cluster synthesis on the nascent NadA apoenzyme. Similar results are observed in the case of a *B. subtilis* mutant strain deficient in a cysteine desulfurase gene homologue, although the gene product has not yet been functionally characterized in detail (31). At present, whether or not the cysteine desulfurases specific for NAD *de novo* biosynthesis are additionally involved in Fe-S cluster repair under oxidative stress conditions remains to be determined.

We also showed that the expression of *nadA* and *nadC*, along with that of *ndnR* and *nadS*, is markedly repressed in cells cultured in the presence of nicotinate. The expression levels decrease rapidly after nicotinate supplementation and remain extremely low during growth in the presence of micromolar levels of nicotinate. These results suggest that *C. glutamicum* efficiently utilizes the exogenous precursor of NAD and strictly regulates NAD *de novo* biosynthesis in response to environmental conditions, resulting in the maintenance of NAD homeostasis. In this context, it is noted that mutant strains deficient in NAD biosynthesis genes grow to some extent in nicotinate-depleted medium, but this is perhaps due to carry over of minimal residual NAD precursors after the washing of cells cultured in nutrient-rich medium (Fig. 3). Disruption of *ndnR*, a gene that encodes a member of the NrtR family of transcriptional regulators, enhances the expression of the *ndnR-nadA-nadC-nadS* genes, suggesting that its product, NdnR, acts as a transcriptional repressor of the NAD *de novo* biosynthesis genes. The autoregulation of *ndnR* may contribute to the fine control of its downstream genes. It should be noted that disruption of *ndnR* completely eliminates the nicotinate response of the expression of the NAD *de novo* biosynthesis genes, indicating that the nicotinate-responsive regulation is primarily mediated by NdnR. NdnR is not homologous to either *S. enterica* serovar Typhimurium NadR or *B. subtilis* NiaR, which are transcriptional regulators of NAD *de novo* biosynthesis genes. Comparative genomics analyses suggest that target genes of the NrtR family members are diverse among bacterial species, since the regulators are predicted to be involved in not only various aspects of NAD metabolism but also sugar pentose utilization and phosphoribosyl pyrophosphate biogenesis (24). The NrtR family regulators are composed of an N-terminal domain homologous to the Nudix (nucleoside diphosphate with large variation of residues) hydrolase family and a C-terminal helix-turn-helix (HTH)-like domain. It is predicted that the Nudix and HTH domains bind to an NAD metabolite as an effector molecule and the promoter regions of the target genes, respectively. Further studies of the NdnR-mediated regulation mechanism in *C. glutamicum* will provide new aspects of the diverse regulation of NAD metabolism in bacteria.

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REFERENCES

1. **Bhatia, R., and K. C. Calvo.** 1996. The sequencing, expression, purification, and steady-state kinetic analysis of quinolinate phosphoribosyl transferase from *Escherichia coli*. Arch. Biochem. Biophys. **325:**270–278.
2. **Flachmann, R., N. Kunz, J. Seifert, M. Gütlich, F. J. Wientjes, A. Läufer, and H. G. Gassen.** 1988. Molecular biology of pyridine nucleotide biosynthesis in *Escherichia coli*. Cloning and characterization of quinolinate synthesis genes *nadA* and *nadB*. Eur. J. Biochem. **175:**221–228.
3. **Foster, J. W., Y. K. Park, T. Penfound, T. Fenger, and M. P. Spector.** 1990. Regulation of NAD metabolism in *Salmonella typhimurium*: molecular sequence analysis of the bifunctional *nadR* regulator and the *nadA-pnuC* operon. J. Bacteriol. **172:**4187–4196.
4. **Gazzaniga, F., R. Stebbins, S. Z. Chang, M. A. McPeek, and C. Brenner.** 2009. Microbial NAD metabolism: lessons from comparative genomics. Microbiol. Mol. Biol. Rev. **73:**529–541.
5. **Gerasimova, A. V., and M. S. Gelfand.** 2005. Evolution of the NadR regulon in Enterobacteriaceae. J. Bioinform. Comput. Biol. **3:**1007–1019.
6. **Grose, J. H., U. Bergthorsson, and J. R. Roth.** 2005. Regulation of NAD synthesis by the trifunctional NadR protein of *Salmonella enterica*. J. Bacteriol. **187:**2774–2782.
7. **Hughes, K. T., A. Dessen, J. P. Gray, and C. Grubmeyer.** 1993. The *Salmonella typhimurium nadC* gene: sequence determination by use of Mud-P22 and purification of quinolinate phosphoribosyltransferase. J. Bacteriol. **175:** 479–486.
8. **Ikeda, M., and S. Nakagawa.** 2003. The *Corynebacterium glutamicum* genome: features and impacts on biotechnological processes. Appl. Microbiol. Biotechnol. **62:**99–109.
9. **Inui, M., H. Kawaguchi, S. Murakami, A. A. Vertès, and H. Yukawa.** 2004. Metabolic engineering of *Corynebacterium glutamicum* for fuel ethanol production under oxygen-deprivation conditions. J. Mol. Microbiol. Biotechnol. **8:**243–254.
10. **Inui, M., S. Murakami, S. Okino, H. Kawaguchi, A. A. Vertès, and H. Yukawa.** 2004. Metabolic analysis of *Corynebacterium glutamicum* during lactate and succinate productions under oxygen deprivation conditions. J. Mol. Microbiol. Biotechnol. **7:**182–196.
11. **Inui, M., M. Suda, S. Okino, H. Nonaka, L. G. Puskás, A. A. Vertès, and H. Yukawa.** 2007. Transcriptional profiling of *Corynebacterium glutamicum* metabolism during organic acid production under oxygen deprivation conditions. Microbiology **153:**2491–2504.
12. **Kalinowski, J., B. Bathe, D. Bartels, N. Bischoff, M. Bott, A. Burkovski, N. Dusch, L. Eggeling, B. J. Eikmanns, L. Gaigalat, A. Goesmann, M. Hartmann, K. Huthmacher, R. Krämer, B. Linke, A. C. McHardy, F. Meyer, B. Möckel, W. Pfefferle, A. Pühler, D. A. Rey, C. Rückert, O. Rupp, H. Sahm, V. F. Wendisch, I. Wiegräbe, and A. Tauch.** 2003. The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. J. Biotechnol. **104:**5–25.
13. **Kinoshita, S., S. Udaka, and M. Shimonō.** 1957. Studies on the amino acid fermentation. Part 1. Production of L-glutamic acid by various microorganisms. J. Gen. Appl. Microbiol. **3:**193–205.
14. **Lauhon, C. T., and R. Kambampati.** 2000. The *iscS* gene in *Escherichia coli* is required for the biosynthesis of 4-thiouridine, thiamin, and NAD. J. Biol. Chem. **275:**20096–20103.
15. **Liebl, W.** 2005. *Corynebacterium* taxonomy, p. 9–34. *In* L. Eggeling and M. Bott (ed.), Handbook of Corynebacterium glutamicum. CRC Press, Boca Raton, FL.
16. **Magni, G., A. Amici, M. Emanuelli, N. Raffaelli, and S. Ruggieri.** 1999. Enzymology of NAD^+ synthesis. Adv. Enzymol. Relat. Areas Mol. Biol. **73:**135–182.
17. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
18. **Nakata, K., M. Inui, P. B. Kos, A. A. Vertès, and H. Yukawa.** 2003. Vectors for the genetics engineering of corynebacteria., p. 175–191. *In* B. C. Saha (ed.), Fermentation biotechnology. American Chemical Society, Washington, DC.
19. **Okino, S., M. Inui, and H. Yukawa.** 2005. Production of organic acids by *Corynebacterium glutamicum* under oxygen deprivation. Appl. Microbiol. Biotechnol. **68:**475–480.
20. **Okino, S., R. Noburyu, M. Suda, T. Jojima, M. Inui, and H. Yukawa.** 2008.

An efficient succinic acid production process in a metabolically engineered *Corynebacterium glutamicum* strain. Appl. Microbiol. Biotechnol. **81:**459–464.

21. **Okino, S., M. Suda, K. Fujikura, M. Inui, and H. Yukawa.** 2008. Production of D-lactic acid by *Corynebacterium glutamicum* under oxygen deprivation. Appl. Microbiol. Biotechnol. **78:**449–454.
22. **Ollagnier-de Choudens, S., L. Loiseau, Y. Sanakis, F. Barras, and M. Fontecave.** 2005. Quinolinate synthetase, an iron-sulfur enzyme in NAD biosynthesis. FEBS Lett. **579:**3737–3743.
23. **Penfound, T., and J. W. Foster.** 1999. NAD-dependent DNA-binding activity of the bifunctional NadR regulator of *Salmonella typhimurium*. J. Bacteriol. **181:**648–655.
24. **Rodionov, D. A., J. De Ingeniis, C. Mancini, F. Cimadamore, H. Zhang, A. L. Osterman, and N. Raffaelli.** 2008. Transcriptional regulation of NAD metabolism in bacteria: NrtR family of Nudix-related regulators. Nucleic Acids Res. **36:**2047–2059.
25. **Rodionov, D. A., X. Li, I. A. Rodionova, C. Yang, L. Sorci, E. Dervyn, D. Martynowski, H. Zhang, M. S. Gelfand, and A. L. Osterman.** 2008. Transcriptional regulation of NAD metabolism in bacteria: genomic reconstruction of NiaR (YrxA) regulon. Nucleic Acids Res. **36:**2032–2046.
26. **Rossolillo, P., I. Marinoni, E. Galli, A. Colosimo, and A. M. Albertini.** 2005. YrxA is the transcriptional regulator that represses de novo NAD biosynthesis in *Bacillus subtilis*. J. Bacteriol. **187:**7155–7160.
27. **Rousset, C., M. Fontecave, and S. Ollagnier de Choudens.** 2008. The [4Fe-4S] cluster of quinolinate synthase from *Escherichia coli*: investigation of cluster ligands. FEBS Lett. **582:**2937–2944.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
29. **Schwartz, C. J., O. Djaman, J. A. Imlay, and P. J. Kiley.** 2000. The cysteine desulfurase, IscS, has a major role in *in vivo* Fe-S cluster formation in *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **97:**9009–9014.
30. **Seifert, J., N. Kunz, R. Flachmann, A. Läufer, K. D. Jany, and H. G. Gassen.** 1990. Expression of the *E. coli nadB* gene and characterization of the gene product L-aspartate oxidase. Biol. Chem. Hoppe Seyler **371:**239–248.
31. **Sun, D., and P. Setlow.** 1993. Cloning, nucleotide sequence, and regulation of the *Bacillus subtilis nadB* gene and a *nifS*-like gene, both of which are essential for NAD biosynthesis. J. Bacteriol. **175:**1423–1432.
32. **Suzuki, N., N. Okai, H. Nonaka, Y. Tsuge, M. Inui, and H. Yukawa.** 2006. High-throughput transposon mutagenesis of *Corynebacterium glutamicum* and construction of a single-gene disruptant mutant library. Appl. Environ. Microbiol. **72:**3750–3755.
33. **Teramoto, H., T. Shirai, M. Inui, and H. Yukawa.** 2008. Identification of a gene encoding a transporter essential for utilization of C4 dicarboxylates in *Corynebacterium glutamicum*. Appl. Environ. Microbiol. **74:**5290–5296.
34. **Vertès, A. A., M. Inui, M. Kobayashi, Y. Kurusu, and H. Yukawa.** 1993. Presence of *mrr*- and *mcr*-like restriction systems in coryneform bacteria. Res. Microbiol. **144:**181–185.
35. **Yang, Z., A. Savchenko, A. Yakunin, R. Zhang, A. Edwards, C. Arrowsmith, and L. Tong.** 2003. Aspartate dehydrogenase, a novel enzyme identified from structural and functional studies of TM1643. J. Biol. Chem. **278:**8804–8808.
36. **Yukawa, H., C. A. Omumasaba, H. Nonaka, P. Kós, N. Okai, N. Suzuki, M. Suda, Y. Tsuge, J. Watanabe, Y. Ikeda, A. A. Vertès, and M. Inui.** 2007. Comparative analysis of the *Corynebacterium glutamicum* group and complete genome sequence of strain R. Microbiology **153:**1042–1058.