JOURNAL OF BACTERIOLOGY, July 2005, p. 4984–4991 Vol. 187, No. 14
0021-9193/05/$08.00+0 doi:10.1128/JB.187.14.4984–4991.2005

Systematic Characterization of the ADP-Ribose Pyrophosphatase Family in the Cyanobacterium *Synechocystis* sp. Strain PCC 6803

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Received 22 November 2004/Accepted 15 April 2005

We have characterized four putative ADP-ribose pyrophosphatases Sll1054, Slr0920, Slr1134, and Slr1690 in the cyanobacterium *Synechocystis* sp. strain PCC 6803. Each of the recombinant proteins was overexpressed in *Escherichia coli* and purified. Sll1054 and Slr0920 hydrolyzed ADP-ribose specifically, while Slr1134 hydrolyzed not only ADP-ribose but also NADH and flavin adenine dinucleotide. By contrast, Slr1690 showed very low activity for ADP-ribose and had four substitutions of amino acids in the Nudix motif, indicating that Slr1690 is not an active ADP-ribose pyrophosphatase. However, the quadruple mutation of Slr1690, T73G/I88E/K92E/A94G, which replaced the mutated amino acids with those conserved in the Nudix motif, resulted in a significant (6.1×10^2-fold) increase in the k_{cat} value. These results suggest that Slr1690 might have evolved from an active ADP-ribose pyrophosphatase. Functional and clustering analyses suggested that Sll1054 is a bacterial type, while the other three and Slr0787, which was characterized previously (Raffaelli et al., FEBS Lett. 444:222–226, 1999), are phylogenetically diverse types that originated from an archaeal Nudix protein via molecular evolutionary mechanisms, such as domain fusion and amino acid substitution.

Nudix hydrolases are a family of proteins that catalyze the hydrolysis of a nucleoside diphosphate that is linked to some other moiety, such as a phosphate, sugar, or nucleoside (2). These proteins are defined by the Nudix motif, GX_5EX_7 REUXEEXGU, where U is a hydrophobic amino acid, usually Ile, Leu, or Val (2). At present, a Pfam database search for the Nudix motif reveals that there are more than 1,700 open reading frames containing this motif in eukaryotes, prokaryotes, archaea, and viruses. Substrates for Nudix hydrolases include (deoxy)ribonucleoside triphosphates (14, 15, 16), nucleotide sugars (5, 6, 32), coenzymes (4, 28, 30), diadenosine polyphosphates (1, 3), and diphosphoinositol polyphosphate (23). Since the accumulation of these substrates is often toxic to the cell, their intracellular levels need to be regulated precisely by Nudix hydrolases.

ADP-ribose, a substrate for Nudix hydrolase, is an intermediate that is produced during the metabolism of NAD^+, mono- or poly-ADP-ribosylated proteins, and cyclic ADP-ribose (10). Little is known about the role of free ADP-ribose in cell physiology. High levels of ADP-ribose in the cell cause nonenzymatic ADP-ribosylation, which inactivates various proteins and interferes with metabolic regulation via enzymatic ADP-ribosylation (10). To prevent such nonenzymatic ADP-ribosylation, control of the level of ADP-ribose is essential. The ADP-ribose pyrophosphatases, which constitute a subfamily of the Nudix hydrolases, hydrolyze ADP-ribose to AMP and ribose-5-phosphate. Thus, it is likely that ADP-ribose pyrophosphatases function in general to eliminate potentially deleterious ADP-ribose.

In recent studies, several ADP-ribose pyrophosphatases have been characterized biochemically (5) and, in some cases, the three-dimensional structure of the protein has been determined (7, 12). In addition, the determination of the genome sequence of many organisms has suggested the presence of various types of ADP-ribose pyrophosphatases, and their characterization is needed to understand the overall function of the ADP-ribose pyrophosphatase family.

Cyanobacteria are prokaryotes that perform oxygenic photosynthesis, which is similar to that of higher plants. These organisms are able to adapt to various kinds of environmental stresses. Little is known about Nudix hydrolases in cyanobacteria, although two Nudix hydrolases have been characterized in cyanobacteria to date. NuhA is a novel type of ADP-ribose pyrophosphatase in *Synechococcus* sp. strain PCC 7002, which has a C-terminal domain responsible for oligomerization (18). Slr0787 is an ADP-ribose pyrophosphatase in *Synechocystis* sp. strain PCC 6803 that not only hydrolyzes ADP-ribose but also synthesizes NAD^+ from nicotinamide mononucleotide (22). In the genome of *Synechocystis* sp. strain PCC 6803 (11), there are a total of eight genes that encode putative Nudix hydrolases. Five of them, including Slr0787, have a proline as the 14th to 16th amino acid from the C terminus of the Nudix motif, which is conserved in the ADP-ribose pyrophosphatase family (5).

In the present study, we have systematically characterized putative ADP-ribose pyrophosphatases in *Synechocystis* sp. strain PCC 6803. The recombinant proteins, which were overexpressed in *Escherichia coli* and purified, are shown to possess different levels of hydrolytic activity against ADP-ribose. Clustering analysis of these proteins suggested that they might have diversified via molecular evolution.

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MATERIALS AND METHODS

Plasmid construction for overexpression. Genes of interest were amplified from the genomic DNA of *Synechocystis* sp. strain PCC 6803 by PCR with

synthetic oligonucleotide primers. These primers provided an NdeI site at the start of the gene and an XhoI site at its end. The amplified gene was ligated into the pGEM-T Easy vector (Promega, Madison, WI). The insert was prepared by digestion with NdeI and XhoI and ligated into the pET-21b vector (Novagen, Darmstadt, Germany), which provided a 6×His tag at the C terminus. The cloned genes and their accession numbers were as follows: *sll1054* (NP_439968), *slr0920* (NP_442398), *slr1134* (NP_440605), and *slr1690* (NP_441705). The resultant plasmid constructs were designated pET/*sll1054*, pET/*slr0920*, pET/*slr1134*, and pET/*slr1690*, respectively. The resultant plasmids were used to transform *E. coli* BL21(DE3)/pLysS for overexpression.

Site-directed mutagenesis of proteins. Site-directed mutagenesis was performed using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) by PCR with a pair of complementary oligonucleotides of 32 to 44 bases that contained the desired mutation and with pET/*slr1690* as a template. Parental DNA was digested with DpnI to remove the methylated parent strands, and the synthesized plasmid DNA was used to transform *E. coli* XL1-Blue. Multiple mutations of Slr1690 were made by PCR with mutated plasmids of pET/*slr1690* as a template.

Overexpression and purification of the recombinant proteins. Cells were grown at 37°C in 500 ml of LB medium that contained 50 μg ml^{-1} ampicillin, except cells expressing Slr1690 and its mutated proteins, which were grown at 25°C. The expression of proteins was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside to the medium and incubation at the growth temperature for 3 to 8 h. All recombinant proteins were purified as described previously (18). The concentration of purified proteins was determined by the method of Gill and Von Hippel (9). Determinations of molecular mass in solution were made with a gel filtration column of HiLoad 26/60 Superdex 200pg (Amersham Biosciences, Piscataway, NJ) as described previously (18).

Assay of enzymatic activities. The standard reaction mixture of 50 μl contained 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$ or $MnCl_2$, 1 mM dithiothreitol, 0.1 mM nucleotide diphosphate derivatives as the substrate, and various amounts of the recombinant protein. After incubation at 37°C for 30 min, the reaction was stopped by addition of 10 mM EDTA. The enzymatic activity was assayed using high-performance liquid chromatography as described previously (18). The kinetic parameters were determined as described previously (18).

Circular dichroism spectroscopy. Circular dichroism (CD) measurements were carried out with a spectropolarimeter (model J-820; Jasco, Tokyo, Japan). CD spectra in the far-UV region between 200 and 250 nm were measured at 25°C in a mixture that contained 10 μM recombinant protein, 50 mM KH_2PO_4-K_2HPO_4, pH 7.5, and 100 mM KCl in a 1-mm cell.

Phylogenetic analysis. All sequences of the characterized and putative ADP-ribose pyrophosphatases were obtained from NCBI. Multiple sequence alignments and distance analysis using the neighbor-joining method were performed using the ClustalW program. A phylogenetic tree was constructed using TreeView.

Structural modeling of Sll1054. The established three-dimensional structure of ADP-ribose pyrophosphatase (ADPRase; in *E. coli* (PDB ID: 1G0S) was extracted from the Protein Data Bank and was used as the template in the structural modeling of Sll1054 using the comparative modeling program Modeller (24). The calculated three-dimensional model of Sll1054 was stereochemically evaluated using the program Procheck (13). The structural model of Sll1054 was examined using the DeepView/Swiss-PDB Viewer program (http://kr.expasy.org/spdbv).

RESULTS

Selection of putative ADP-ribose pyrophosphatases. A BLAST search revealed that there are a total of eight genes that encode putative Nudix hydrolases in *Synechocystis* sp. strain PCC 6803. To find candidates for ADP-ribose pyrophosphatase, we performed sequence alignment in the regions containing the Nudix motif, since the substrate specificity depends on the regions outside the Nudix motif. A proline at the 14 to 16th amino acid position from the C terminus of the Nudix motif is conserved in the ADP-ribose pyrophosphatase family (5). The sequence alignment revealed that five proteins, Sll1054, Slr0787, Slr0920, Slr1134, and Slr1690, had such a conserved proline, although Slr0787 had the proline at the 20th amino acid position from the C terminus of the Nudix motif (Fig. 1).

Sll1054 consists of 187 amino acids with a predicted molecular mass of 21,030 Da and a pI of 4.71. A search for sequence similarity showed that Sll1054 was 31% identical to ADP-ribose pyrophosphatase in *Mycobacterium tuberculosis* (MT-ADPRase) and 25% identical to an ADPRase in *E. coli* (Fig. 1A). Sll1054 contains two other determinants for substrate specificity. One is tyrosine at the 17th amino acid position from the C terminus of the Nudix motif, which is conserved in the diadenosine polyphosphate hydrolase family (5). The other is the GR2 motif, as part of a Gly-rich domain, which is conserved in the diadenosine polyphosphate hydrolase family (34). However, the whole sequence of Sll1054 did not show any significant identity to the diadenosine polyphosphate hydrolase or diadenosine polyphosphate hydrolase family.

Slr0920 consists of 149 amino acids with a predicted molecular mass of 17,378 Da and a pI of 5.36. Slr1134 consists of 148 amino acids with a predicted molecular mass of 16,525 Da and a pI of 5.58. A search for sequence similarity showed that Slr0920 and Slr1134 were 45% and 42% identical to MJ1149 in *Methanococcus jannaschii*, respectively (Fig. 1B). In addition, the SignalP and PSORT programs suggested that Slr1134 might possess a putative signal peptide of 19 amino acids, which can target it to bacterial inner membranes (Fig. 1B). Furthermore, we found that the ssr2806 hypothetical protein in *Synechocystis* sp. strain PCC 6803 showed 100% identity to amino acids 68 to 148 of Slr1134 (Fig. 1B), suggesting that Ssr2806 is a paralog of Slr1134 derived through gene duplication. The *ssr2806* gene may be a pseudogene, since it lacks half of the amino acid-coding region of Slr1134, including the Nudix motif.

Slr1690 consists of 261 amino acids with a predicted molecular mass of 30,044 Da and a pI of 9.24. A search for sequence similarity showed that Slr1690 was 40% identical to NuhA in *Synechococcus* sp. strain PCC 7002 (Fig. 1C). A Pfam database search revealed that Slr1690 contained the Nudix domain in the N terminus and an uncharacterized domain, designated the Pfam-B-3090 domain, in the C terminus. This domain structure resembles that of NuhA. However, there were four substitutions of amino acids in the Nudix motif of Slr1690 (Fig. 1C). The first and the terminal conserved glycine in the Nudix motif were substituted with threonine and alanine, respectively. The second and fourth conserved glutamic acids were substituted with isoleucine and lysine, respectively.

Overexpression and purification of the recombinant proteins. All recombinant proteins were overexpressed with a His tag in *E. coli* and purified from the soluble extract of *E. coli* to homogeneity using an Ni^{2+} chelating column (data not shown). Analysis by gel filtration showed that the molecular masses of Sll1054, Slr0920, and Slr1134 were 40 kDa, 30 kDa, and 30 kDa, respectively (data not shown), suggesting that these proteins are present as dimers in solution. Analysis by gel filtration showed that the molecular mass of Slr1690 was about 155 kDa (data not shown), suggesting that Slr1690 is present as an oligomer, probably a pentamer or a hexamer, in solution.

Enzymatic activities of the recombinant proteins. The recombinant proteins hydrolyzed ADP-ribose to different extents. Table 1 shows kinetic parameters of the recombinant

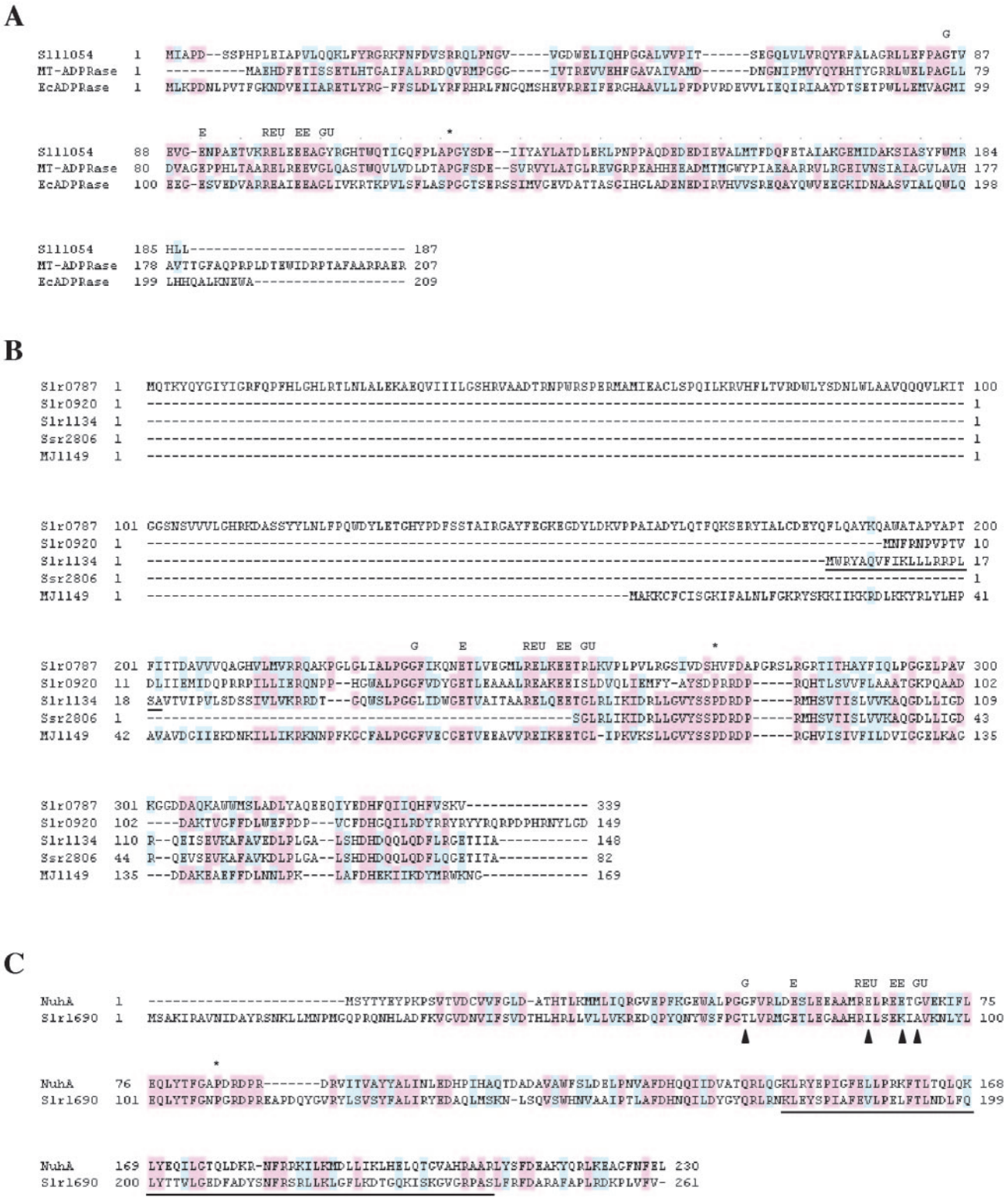

FIG. 1. Sequence alignments of Sll1054 (A), Slr0920, Slr1134, and Slr0787 (B), and Slr1690 (C) with their homologous proteins. Alignments were performed with the ClustalW program. The organisms (GenBank numbers) used here are as follows: *Escherichia coli* (EcADPRase; NP_417506), *Mycobacterium tuberculosis* (MT-ADPRase; NP_216216), *Synechococcus* sp. strain PCC 7002 (NuhA; BAC76070), and *Synechocystis* sp. strain PCC 6803 (Sll1054, NP_439968; Slr0787, NP_442622; Slr0920, NP_442398; Slr1134, NP_440605; Slr1690, NP_441705; and Ssr2806, NP_441686). The Nudix motif is shown above the sequences. The asterisk indicates the position of a proline that is conserved in the ADP-ribose pyrophosphatase family. Numbers to the left and right of the sequences indicate amino acid positions in individual proteins. Amino acids that are fully conserved or substitutive are shaded pink and blue, respectively. The predicted signal peptide in the sequence of Slr1134 is shown by an underline. The arrows below the sequence of Slr1690 indicate the mutated amino acids in the Nudix motif. The Pfam-B-3090 domain of Slr1690 is shown by an underline.

TABLE 1. Kinetic analysis of ADP-ribose pyrophosphatases in *Synechocystis* sp. strain PCC 6803[a]

Enzyme	V_{max} (U mg^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/k_m (M^{-1} s^{-1})
Sll1054	120	20	0.11	1.8×10^5
Slr0787	0.70	0.40	0.27×10^{-3}	1.7×10^6
Slr0920	59	8.6	0.065	1.3×10^5
Slr1134	7.5	2.0	0.29	7.0×10^3
Slr1690	1.7×10^{-3}	1.4×10^{-4}	0.82	1.1×10^{-1}

[a] Kinetic parameters were determined as described in Materials and Methods. A unit of enzyme represents the hydrolysis of 1 μmol of substrate per minute. k_{cat} was calculated from V_{max} assuming one active site per monomer. The kinetic parameters for Slr0787 appear in reference 22.

proteins for ADP-ribose. Both Sll1054 and Slr0920 had a high catalytic activity for ADP-ribose, with catalytic efficiency (k_{cat}/K_m) of 1.8×10^5 s^{-1} and 1.3×10^5, respectively. The k_{cat}/K_m value of Slr1134 was 7.0×10^3 s^{-1}, which was lower than those of Sll1054 and Slr0920. By contrast, Slr1690 showed very low activity, with a k_{cat}/K_m value of 1.1×10^{-1} s^{-1}.

We next examined the substrate specificity of the four proteins (Table 2). Slr0920 hydrolyzed ADP-ribose specifically. Sll1054 was also highly specific for ADP-ribose but it had low activity for adenosine(5′)-tetraphospho-(5′)-adenosine (Ap_4A), adenosine(5′)-pentaphospho-(5′)-adenosine (Ap_5A), and NADH. Slr1134 hydrolyzed not only ADP-ribose but also NADH and flavin adenine dinucleotide (FAD). Slr1690 did not show detectable activity for the other nucleoside diphosphate derivatives examined. None of the four proteins had detectable activity for (deoxy)ribonucleoside triphosphates, which are specific substrates for MutT. For all of the recombinant proteins, a divalent cation was required for the activity, and Mg^{2+} was the most effective cation.

Site-directed mutagenesis of Slr1690. To examine whether the low activity of Slr1690 for ADP-ribose was due to the substitution of the four amino acids in the Nudix motif, we replaced these amino acids with those conserved in the Nudix motif by site-directed mutagenesis. The quadruple mutation T73G/I88E/K92E/A94G resulted in a significant (6.1×10^2-fold) increase in the k_{cat} value (Table 3). Despite the increase in the k_{cat} value due to this mutation, the K_m value for ADP-ribose was not significantly affected (Table 3). We used CD spectroscopy to examine whether these mutations had any effect on the secondary structure elements of the proteins, including α-helix, β-sheet, β-turn, and aperiodic structure elements. The CD spectra of the mutated proteins were not different from that of Slr1690, suggesting that the mutations caused no changes in the overall secondary structure (data not shown).

TABLE 2. Relative substrate specificities of ADP-ribose pyrophosphatases in *Synechocystis* sp. strain PCC 6803[a]

Substrate[b]	Relative activity (% of control)				
	Sll1054	Slr0787	Slr0920	Slr1134	Slr1690
ADP-ribose	100	42	100	100	100
ADP-glucose	<1	<1	<1	<1	<1
GDP-mannose	<1	—	<1	<1	<1
NADH	3	—	<1	43	<1
FAD	<1	—	<1	24	<1
Ap_4A	8	—	<1	<1	<1
Ap_5A	10	—	<1	<1	<1
(d)NTP	<1	—	<1	<1	<1
pADP-ribose	N.D.	100	N.D.	N.D.	N.D.

[a] The hydrolytic activity of the recombinant protein was measured as described in Materials and Methods and is expressed as a % of the activity obtained with ADP-ribose for the hydrolysis of 1 μmol of substrate per minute per mg of protein. The relative activity of Slr0787 appears in reference 22 and is expressed as a percentage of the activity obtained with pADP-ribose. —, not detected. N.D., not determined.

[b] (d)NTP, (deoxy)ribonucleotide triphosphate; pADP-ribose, (2′)monophosphoadenosine 5′-diphosphoribose.

TABLE 3. Kinetic parameters of Slr1690 and its mutated proteins[a]

Mutation	k_{cat}	K_m
None (Slr1690)	1.4×10^{-4}	0.82
I88E/K92E/A94G/T73G	8.5×10^{-2}	0.60

[a] Kinetic parameters were determined as described in Materials and Methods. Results are expressed as the averages of three independent experiments.

Phylogenetic clustering of ADP-ribose pyrophosphatases. We constructed the phylogenetic tree of ADP-ribose pyrophosphatases on the basis of clustering analysis. Most ADP-ribose pyrophosphatases can be classified into three major branches, designated group I, group II, and group III (Fig. 2). Group I consists of bacterial types of ADP-ribose pyrophosphatase, such as ADPRase in *E. coli* and MT-ADPRase in *M. tuberculosis*. Group II consists of eukaryotic types of ADP-sugar pyrophosphatase, such as NUDT5 in humans and YSA1 in *Saccharomyces cerevisiae*, which hydrolyze ADP-sugars, including ADP-ribose, ADP-mannose, and ADP-glucose (33). Group III consists of archaeal types of ADP-ribose pyrophosphatase, such as MJ1149 in *M. jannaschii* and NuhA in *Synechococcus* sp. strain PCC 7002.

Group III appears to commonly contain an ancestral Nudix domain which is highly homologous to the Nudix domain of MJ1149. Orf186 in *E. coli*, NudE.1 in enterobacterial phage T4, and NUDT9 in humans do not belong to any of the three major branches. Orf186 and NudE.1 might form a different group, since they are orthologous to each other and have a broad substrate specificity (17, 30). NUDT9 might also belong to a distinct group, since its three-dimensional structure and enzymatic properties are very different from those of the ADP-ribose pyrophosphatases belonging to other groups (21, 26). In *Synechocystis* sp. strain PCC 6803, Sll1054 belongs to group I, while the other proteins, Slr0787, Slr0920, Slr1134, and Slr1690, belong to group III (Fig. 2).

DISCUSSION

Based on enzymatic characterization and clustering analysis, we classified most ADP-ribose pyrophosphatases into three major branches (Fig. 2). Group I comprises bacterial ADP-ribose pyrophosphatases, such as ADPRase in *E. coli* and MT-ADPRase in *M. tuberculosis*, which have high catalytic activity for ADP-ribose that is dependent on Mg^{2+} or Mn^{2+} and contain two conserved domains, a Nudix domain that is involved in catalysis and an N-terminal domain that is involved in dimer stabilization (7, 12). Sll1054 belongs to group I (Fig. 2 and 3). Furthermore, homology modeling revealed that the three-dimensional structure of Sll1054 is very similar to that of ADPRase

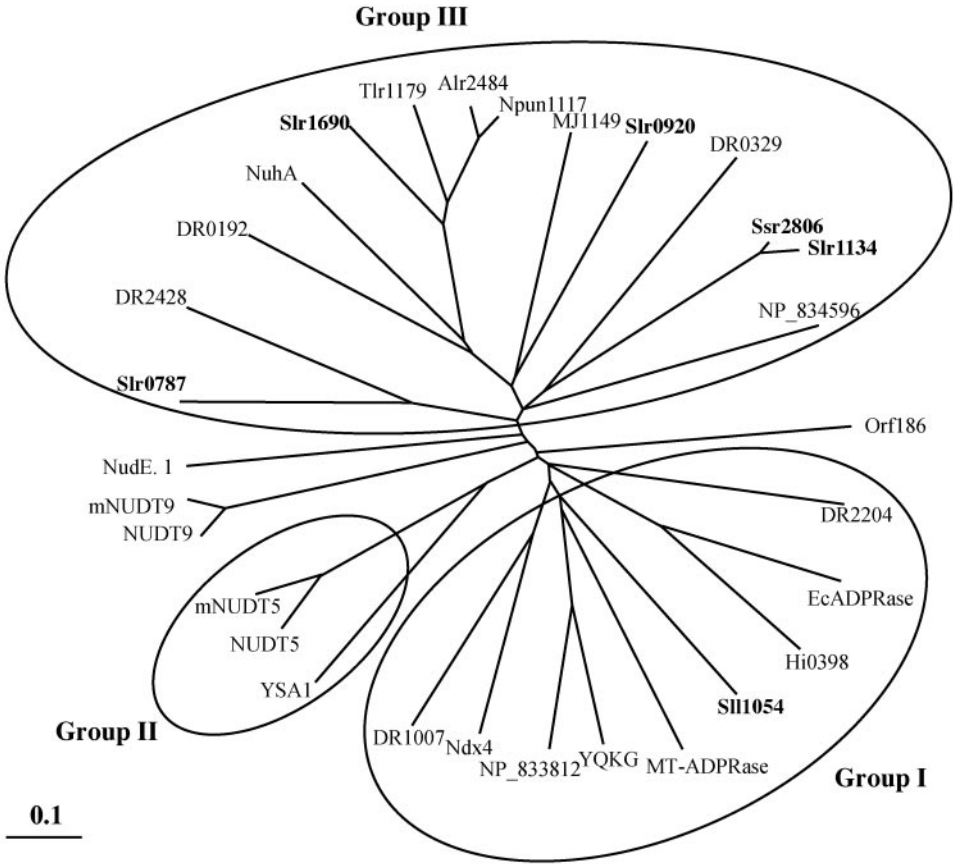

FIG. 2. Unrooted phylogenetic tree diagram of characterized and putative ADP-ribose pyrophosphatases. The phylogenetic tree was constructed as described in Materials and Methods. The bar scale represents the number of substitutions per site. ADP-ribose pyrophosphatases in *Synechocystis* sp. strain PCC 6803 are shown in bold. The organisms (GenBank numbers) used here are as follows: *Bacillus cereus* (NP_834596 and NP_833812) (29), *Bacillus subtilis* (YQKG; NP_390242) (5); *Deinococcus radiodurans* R1 (DR0192, NP_293916: DR0329, NP_294052: DR1007, NP_294731: DR2204, NP_295926: DR2428, NP_296148) (31), *Escherichia coli* (EcADPRase, NP_417506, Orf186, NP_417856) (5); Enterobacteria phage T4 (NudE.1; NP_049737) (30); *Haemophilus influenzae* (Hi0398; P44684) (5); *Homo sapiens* (NUDT5, AAH00025; NUDT9, AAH00542) (21, 33); *Methanococcus jannaschii* (MJ1149; NP_248141) (25); *Mus musculus* (mNUDT5, AAH04571; mNUDT9, AAH33921) (33); *Mycobacterium tuberculosis* (MT-ADPRase; NP_216216) (12); *Nostoc* sp. strain PCC 7120 (Alr2484; NP_486524); *Nostoc punctiforme* (Npun1117; ZP_00106735); *Saccharomyces cerevisiae* (YSA1; S48276) (5); *Synechococcus* sp. strain PCC 7002 (NuhA; BAC76070) (18); *Synechocystis* sp. strain PCC 6803 (Sll1054, NP_439968; Slr0787, NP_442622; Slr0920, NP_442398; Slr1134, NP_440605; Slr1690, NP_441705; Ssr2806, NP_441686) (22); *Thermosynechococcus elongatus* BP-1 (Tlr1179; NP_681969); and *Thermus thermophilus* HB8 (Ndx4; BAC67698) (35).

in *E. coli* (Fig. 4), although their amino acid sequences are only 25% identical. The predicted structure also suggested that Sll1054 contains two putative conserved domains, an N-terminal domain (amino acids 1 to 43) and a Nudix domain (amino acids 44 to 187). The N-terminal domain might be responsible for dimerization of Sll1054, although the sequence identity of the N-terminal domain between Sll1054 and ADPRase in *E. coli* is 21%.

The dimer structures of ADPRase in *E. coli* and MT-ADPRase in *M. tuberculosis* are stabilized by a pair of equivalent interfaces created by swapping the N-terminal domains of monomers (7, 12). The X-ray crystallographic analyses of ADPRase in *E. coli* and MT-ADPRase in *M. tuberculosis* have localized three glutamic acids important for catalysis (8, 12). For example, in *E. coli*, Glu112 and Glu116 in the Nudix motif are involved in the binding of a divalent metal ion, and Glu162 in loop L9 is involved in the deprotonation of a catalytic water molecule. The predicted structure of Sll1054 also has three glutamic acids (Glu100, Glu104, and Glu150) in structurally corresponding positions (Fig. 4). We propose that Sll1054 is an ortholog of bacterial ADP-ribose pyrophosphatase.

Slr0920 shows 45% sequence identity to MJ1149 in *M. jannaschii* among Nudix hydrolases, which have low sequence identity (20% or less) with each other in general. Slr0920 contains only the Nudix domain, and the structure of this domain is similar to that of MJ1149 (Fig. 3). The substrate specificity of Slr0920 is also the same as that of MJ1149 (25). We propose that Slr0920 is an ortholog of MJ1149 in group III.

Slr1134 also shows 42% sequence identity to MJ1149. The domain structure of Slr1134 is similar to that of MJ1149 except for the presence of a putative signal peptide in the N terminus (Fig. 1B and 3). The recombinant Slr1134 hydrolyzed not only ADP-ribose but also NADH and FAD (Table 2). MJ1149 hydrolyzes ADP-ribose and 2′-phospho-ADP-ribose but does not hydrolyze other nucleoside diphosphate derivatives (25). Orf186 in *E. coli*, which consists of only a Nudix domain, has a

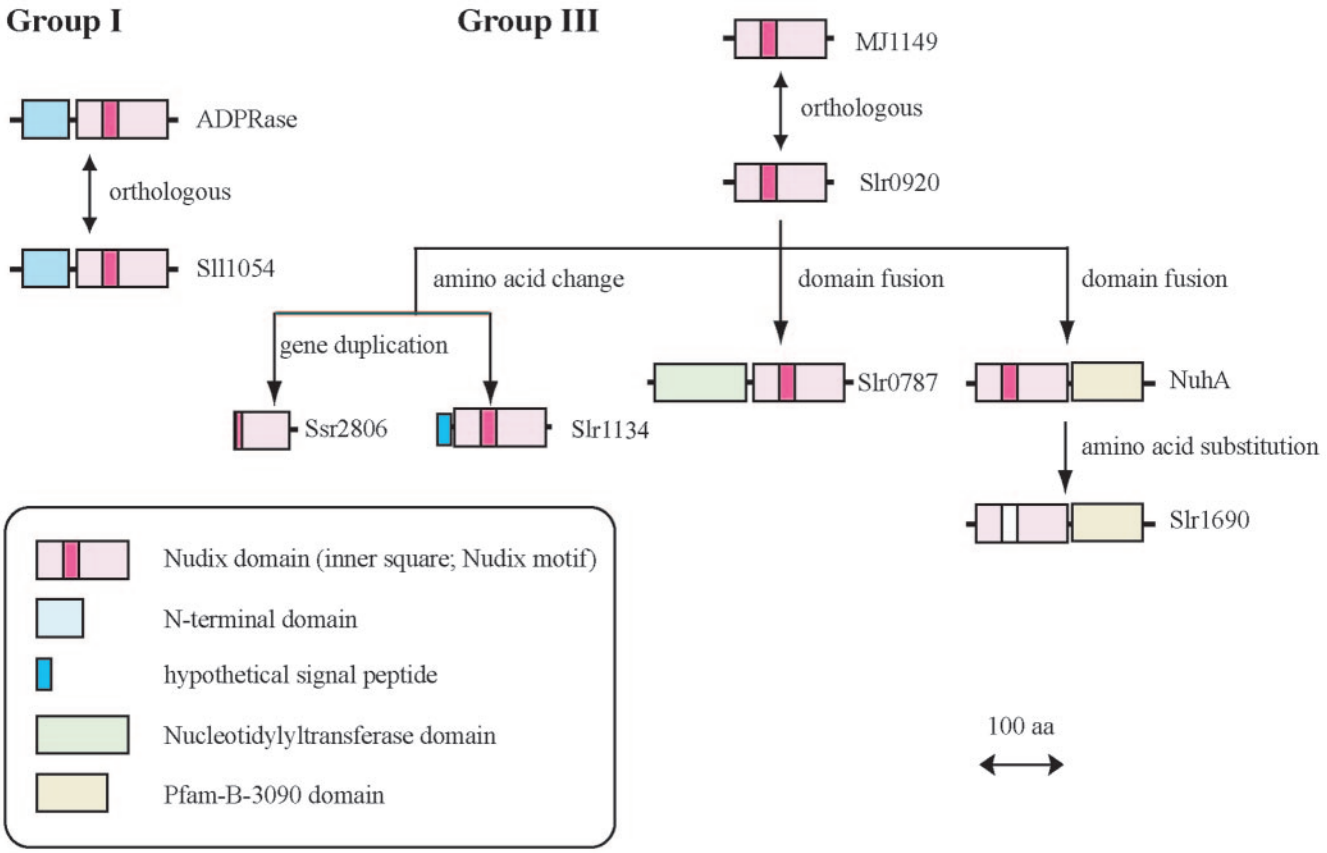

FIG. 3. Predicted domain structures of ADP-ribose pyrophosphatases in *Synechocystis* sp. strain PCC 6803 and a schematic diagram of evolutionary relationships. The domain structure of ADPRase in *E. coli* was inferred from the three-dimensional structure (7). The domain structure of Sll1054 was predicted by homology modeling in this study. The domain structures of group III ADP-ribose pyrophosphatases were predicted by Pfam database searches.

broad substrate specificity for Ap_5A, ADP-ribose, and NADH, which Orf186 hydrolyzes to similar extents (17). Thus, the enzymatic properties of Slr1134 are different from those of MJ1149 and Orf186. We therefore propose that Slr1134 belongs to a distinct subfamily in group III.

Slr1690 shows 40% sequence identity to NuhA, which is an active ADP-ribose pyrophosphatase in *Synechococcus* sp. strain PCC 7002 (18). However, the recombinant Slr1690 showed little catalytic activity for ADP-ribose, with a k_{cat} value of 1.4×10^{-4} s^{-1} and a K_m value of 0.82 mM (Table 1). The kinetic parameters are too low to define Slr1690 as an ADP-ribose pyrophosphatase. This might be in part due to impairment of the Nudix motif. The fully reverse mutation of Slr1690, T73G/I88E/K92E/A94G, showed an increase of the k_{cat} value of 6.1×10^2-fold (Table 3). It appears that Slr1690 might have originally possessed full activity as a NuhA-type ADP-ribose pyrophosphatase. The phylogenetic tree also suggests that Slr1690 is closely related to NuhA (Fig. 2).

The *slr1690* gene and its orthologous genes in cyanobacteria, such as *alr2484* in *Nostoc* sp. strain PCC 7120 and *tlr1179* in *Thermosynechococcus elongatus* BP-1, appear to form an operon with genes that encode a series of enzymes in the NAD^+ salvage pathway. In these operon structures, the *slr1690*-orthologous genes are located between the nicotinate-nucleotide adenylyltransferase gene and the NAD^+ synthetase gene. Thus, Slr1690 might be involved in NAD^+ metabolism. The chemical structures of adenine nucleotide derivatives such as NADH, NAD^+, NADPH, and nicotinic acid adenine dinucleotide that take part in NAD^+ metabolism are very similar to that of ADP-ribose. We found that none of these compounds can be a substrate for Slr1690 (K. Okuda, H. Hayashi, and Y. Nishiyama, unpublished results). Substrates of Slr1690 other than ADP-ribose remain to be identified.

The phylogenetic tree indicates an evolutionary relationship of ADP-ribose pyrophosphatases in *Synechocystis* sp. strain PCC 6803. Sll1054 belongs to group I, which consists of bacterial types of ADP-ribose pyrophosphatase. Group III ADP-ribose pyrophosphatases contain an ancestral Nudix domain which is similar to that of MJ1149. Slr0920 may be closest to an archaeal ADP-ribose pyrophosphatase (Fig. 2 and 3). Given the function and the domain structure, Slr1134, Slr0787, and Slr1690 might have diverged from an archaeal ADP-ribose pyrophosphatase to gain other functions (Fig. 3). Slr1134 might have undergone more amino acid changes in the Nudix domain to gain substrate specificity for NADH and FAD. Slr0787 might have been derived from the fusion of an archaeal Nudix domain and an archaeal nicotinamide mononucleotide adenylyltransferase domain via molecular evolution (22). NuhA might have evolved via the fusion of an archaeal Nudix domain and the Pfam-B-3090 domain, which is related

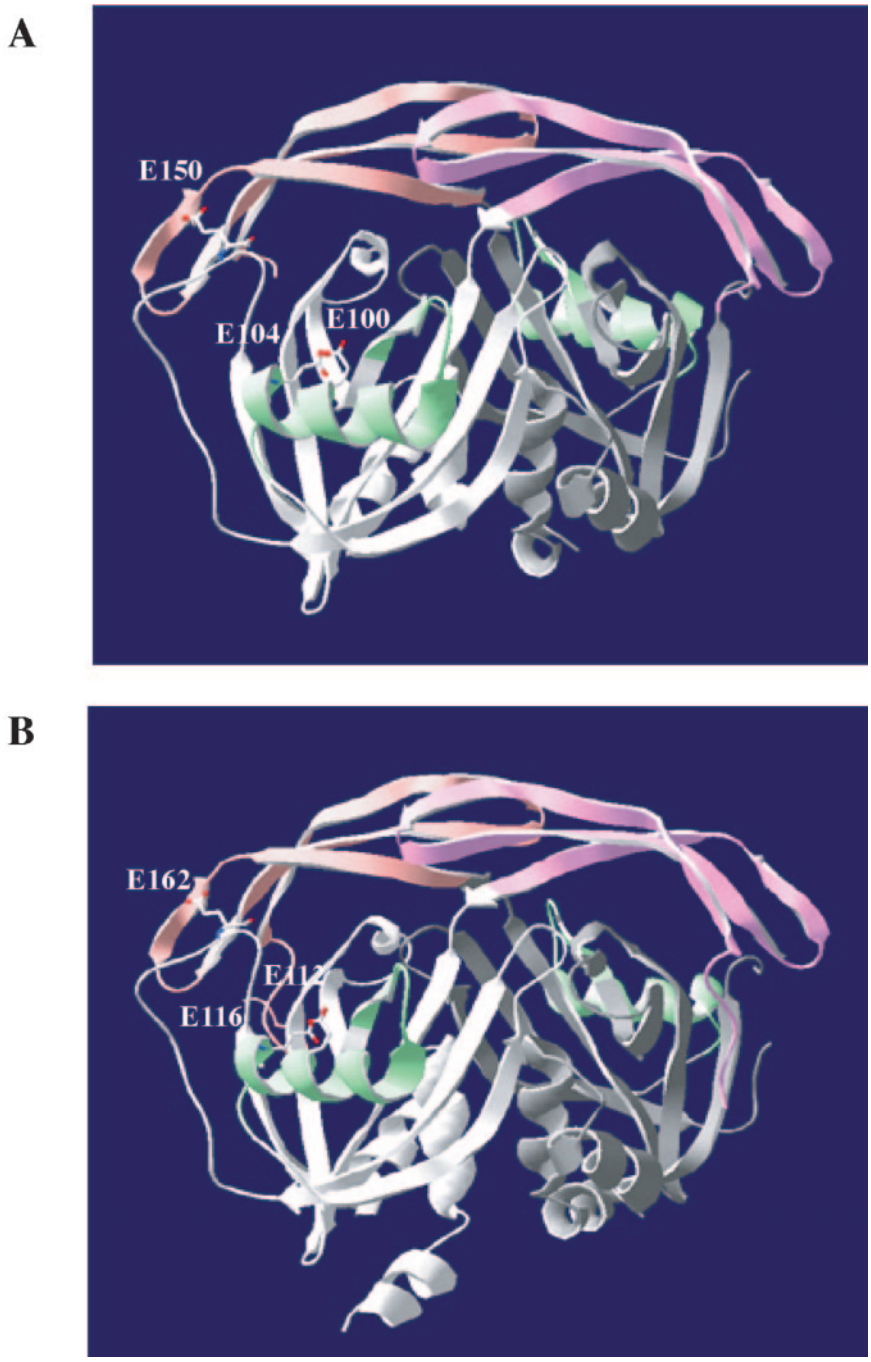

FIG. 4. Prediction of the three-dimensional structure of Sll1054. The prediction was made using a program for comparative protein structure modeling, Modeller, based on the crystal structure analysis of ADPRase in *E. coli* as described in Materials and Methods. (A) The predicted three-dimensional structure of Sll1054. The putative N-terminal domain, Nudix domain, and Nudix motif are shown in pink, white, and green, respectively. Three putative glutamic acids structurally corresponding to glutamic acids involved in the catalysis of ADPRase in *E. coli* are shown in white. (B) The three-dimensional structure of ADPRase in *E. coli* (7). The N-terminal domain, the Nudix domain, and the Nudix motif are shown in pink, white, and green, respectively. Three glutamic acids involved in catalysis are shown in white.

to oligomerization (18). The clustering analysis and site-directed mutagenesis suggest that Slr1690 might have been derived from a NuhA-type ADP-ribose pyrophosphatase (Fig. 2 and 3). Thus, the ADP-ribose pyrophosphatase family in *Synechocystis* sp. strain PCC 6803 appears to comprise a bacterial type and three phylogenetically diverse types (all with an archaeal Nudix domain) that diversified via molecular evolution.

Most organisms appear to have two to four Nudix hydrolases that hydrolyze ADP-ribose. For example, there are two ADP-ribose pyrophosphatases in *E. coli*, one in *M. jannaschii*, and

four in humans. It seems that *Synechocystis* sp. strain PCC 6803 has a relatively large number of ADP-ribose pyrophosphatases. Harmful nonenzymatic ADP-ribosylation can easily occur at micromolar concentrations of ADP-ribose (10). It has been reported that the activity of glutamine synthetase in *Synechocystis* sp. strain PCC 6803 is regulated by endogenous ADP-ribosylation (27). To prevent such nonenzymatic ADP-ribosylation and ensure homeostasis of the cell, control of the level of ADP-ribose is essential. *Synechocystis* sp. strain PCC 6803 may possess various types of ADP-ribose pyrophosphatases, each of which corresponds to a different metabolic pathway of ADP-ribose in the cell. The physiological roles of individual ADP-ribose pyrophosphatases in *Synechocystis* sp. strain PCC 6803 remain to be elucidated.

ACKNOWLEDGMENTS

This work was supported, in part, by a Grant-in-Aid (no. 16013237) to Y.N. from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, by a fund to promote in-house COE of Ehime University to H.H., and by INCS, Ehime University.

We are grateful to Shinobu Okamoto (Kyoto University) for structural modeling and Eiji Ihara (Ehime University) for CD spectroscopy.

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