Myxococcus xanthus is a Gram-negative swarming soil bacterium which exhibits a complex life cycle and social behavior (Shimkets, 1999). Upon nutrient limitation, M. xanthus cells form a multicellular fruiting body in which a fraction of the cells develop into myxospores. Diadenosine polyphosphates (ApnA; n = 3–6) found both in prokaryotes and eukaryotes are shown to be involved in stress responses (Kisselev et al., 1998; Lee et al., 1983; Pálfi et al., 1991). In M. xanthus, intracellular Ap4A and Ap5A levels increased several fold after exposure to high temperatures, oxidative and osmotic stresses, and during the early stage of development (Kimura et al., 2017). M. xanthus lysyl-tRNA synthetase generates Ap5A from ATP, and Ap5A from ATP and Ap4 (Oka et al., 2015, 2016). On the other hand, bacteria have two different ApnA hydrolases, ApaH and Nudix (nucleoside diphosphate linked moiety X) (Andreeva and Kutuzov, 2004; McLennan, 2006). The ApaH hydrolase shares sequence similarity with phosphoprotein phosphatases (Andreeva and Kutuzov, 2004; McLennan, 2006). The ApaH hydrolase shares sequence similarity with phosphoprotein phosphatases (Andreeva and Kutuzov, 2004; McLennan, 2006). The ApaH hydrolase shares sequence similarity with phosphoprotein phosphatases (Andreeva and Kutuzov, 2004; McLennan, 2006). The ApaH hydrolase shares sequence similarity with phosphoprotein phosphatases (Andreeva and Kutuzov, 2004; McLennan, 2006).

Nudix hydrolases are found in all types of living organisms; they are encoded by a different number of genes depending upon the organism, and vary in their substrate specificity. Analysis of the complete M. xanthus genomic sequence indicates that it contains 12 Nudix hydrolases. In this study, we cloned M. xanthus Nudix hydrolase genes (Table S1) in the pCold expression vector (Takara Bio.), expressed them in Escherichia coli Novabluve, and determined their hydrolytic activity towards ApnA and their requirements for metal cofactors. In addition, we addressed the role of Nudix hydrolases in M. xanthus by testing their specificity to 11 substrates [ATP, ADP, GTP, GDP, ADP-ribose, GDP-glucose, NADH, Ap4A, Ap5A, ppGpp, and 8-oxo-2′-deoxyguanosine-5′-triphosphate (8-oxo-dGTP)].

Analysis of the complete M. xanthus genomic sequence revealed that this species encoded 12 Nudix hydrolases; among them, eight contained the conserved Nudix box GX5EX7REUXEEXGU and four (MXAN_1246, 3769, 5418, and 6513) had highly similar motifs (Fig. S1). Except for MXAN_4891, 11 His-tagged Nudix hydrolases were expressed in E. coli and designated Nud1 to Nud11 according to the order of gene accession numbers.

To determine the requirements for divalent metal cofactors by M. xanthus Nudix enzymes, we measured their hydrolytic activity towards ApnA in the presence of different metal cations. M. xanthus Nudix hydrolases were stimulated by Co2+, Mn2+, or Mg2+ (Fig. 1), whereas Ca2+, Fe2+, and Fe3+ did not affect the hydrolytic activity towards ApnA (data not shown). Nudix hydrolases require Mg2+ or Mn2+ for activity (McLennan, 2006); however, Co2+ was the most effective in stimulating ApnA hydrolysis by M. xanthus Nud1, Nud6, and Nud11.

*Corresponding author: Yoshio Kimura, Department of Applied Biological Science, Faculty of Agriculture, Kagawa University, Miki-cho, Kagawa, Japan.

Tel: +81-87-891-3118 Fax: +81-87-891-3021 E-mail: kimura@ag.kagawa-u.ac.jp

None of the authors of this manuscript has any financial or personal relationship with other people or organizations that could inappropriately influence their work.
Ap$_4$A is composed of two adenosine moieties joined by a S'-S'-linked chain of four phosphates. It has been reported that Nudix hydrolases from other organisms catalyze the asymmetric cleavage of Ap$_4$A to ATP and AMP in the presence of Mg$^{2+}$ or Mn$^{2+}$ (Swarbrick et al., 2004). Although \textit{M. xanthus} Nudix enzymes mostly catalyzed a symmetric degradation of Ap$_4$A, producing two ADP molecules in the presence of Co$^{2+}$, they also, with the exception of Nud11, cleaved Ap$_4$A asymmetrically into AMP and ATP in the presence of Mn$^{2+}$ (Figs. 1 and 2A). Among them, Nud4 and Nud5 required only Mn$^{2+}$ for hydrolytic activity towards Ap$_4$A, producing AMP and ADP as Ap$_4$A degradation products, suggesting that ADP and/or ATP were further hydrolyzed to AMP by these enzymes.

To determine the catalytic mode of \textit{M. xanthus} Nudix enzymes, we next examined their hydrolytic activity towards 11 nucleoside diphosphate derivatives in the presence of Mn$^{2+}$ or Co$^{2+}$ at pH 8.0. Nud11 exhibited high hydrolytic activity for Ap$_4$A and Ap$_5$A. Although Nud2 also hydrolyzed Ap$_4$A, its specific activity towards Ap$_4$A and Ap$_5$A was approximately 25–35 times lower than that of Nud11 in the presence of Mn$^{2+}$. Nud11 hydrolyzed Ap$_4$A to ADP and Ap$_5$A to ATP and ADP in the presence of Mn$^{2+}$ or Co$^{2+}$, showing the same digestion patterns as \textit{M. xanthus} ApaH which also required Co$^{2+}$ or Mn$^{2+}$ for Ap$_n$A cleavage (Sasaki et al., 2014). However, the specific activity of Nud11 for Ap$_4$A and Ap$_5$A was approximately 4–10-fold lower than that of \textit{M. xanthus} ApaH. Since an \textit{M. xanthus} apaH mutant still retained about 30–40% of hydrolytic activity towards Ap$_n$A (Kimura et al., 2017), Nud11 may degrade Ap$_n$A in vivo.

On the other hand, the products of Ap$_4$A cleavage by the apaH mutant included AMP and ATP, suggesting that other enzymes may also degrade Ap$_n$A in \textit{M. xanthus} cells. Dunn et al. (1999) have suggested that Nudix hydrolases with Ap$_4$A specificity have a Tyr or Phe residue located 17 amino acids downstream of the Nudix box; however, these residues were not found at the indicated position in \textit{M. xanthus} Nud2 and Nud11 (Fig. S1).
The bacterial alarmone ppGpp synthesized in response to amino acid limitation regulates the transcription of genes involved in important physiological processes, such as cell division, motility, and adaptation. PpGpp is degraded to GDP and pyrophosphate by the SpoT enzyme (Heinemeyer et al., 1978), but can also be a substrate for Nudix hydrolases such as *Thermus thermophilus* Ndx8 (Ooga et al., 2009) and *Arabidopsis* AtNUDX26 (Ito et al., 2012). Although *M. xanthus* Nud3, Nud6, and Nud10 showed hydrolytic activity towards ppGpp, it was significantly lower than that of AtNUDX26 (0.19 ± 0.05 μmol/min/mg) (Ito et al., 2012).

Among Nudix enzymes of *M. xanthus*, Nud2, Nud5, and Nud11 could degrade ATP in the presence of Mn$^{2+}$. In the process, Nud2 and Nud5 produced AMP, and Nud11 ADP. However, Nud2 and Nud5, as well as Nud4 had higher preference for GTP compared to ATP. Although Nud5 did not demonstrate significant hydrolytic activity towards ADP and GDP, it could produce AMP and GMP from ATP and GTP, respectively (Fig. 2B), suggesting that it cleaves these nucleotide triphosphates to nucleotide monophosphates and pyrophosphate.

ADP-ribose is a good substrate for some Nudix hydrolases, but *M. xanthus* Nudix enzymes showed very low hydrolytic activity toward ADP-ribose and UDP-glucose.
The assays were performed in the presence of 1 mM substrate and 5 mM metal ion, and the specific activity (nmol/min/mg) was determined. Data are mean ± SEM values of three independent measurements.

### Table 1. Substrate specificity of 11 Nudix hydrolases from *Myxococcus xanthus*.

| Substrate   | Nud1 (0479) | Nud2 (1246) | Nud3 (2568) | Nud4 (3769) |
|-------------|-------------|-------------|-------------|-------------|
|             | Co          | Mn          | Co          | Mn          | Co          | Mn          | Co          | Mn          |
| Ap4A        | 1.2 ± 0.2   | 0.8 ± 0.2   | 17.5 ± 6.6  | 166.1 ± 8.3 | 6.1 ± 0.7   | 6.1 ± 0.1   | 0           | 3.5 ± 0.2   |
| Ap5A        | 1.4 ± 0.1   | 2.7 ± 0.2   | 21.2 ± 2.0  | 185.7 ± 9.0 | 4.2 ± 0.5   | 5.3 ± 0.6   | 0           | 4.4 ± 0.0   |
| ATP         | 0.1 ± 0.0   | 0.5 ± 0.1   | 69.7 ± 1.8  | 259.3 ± 16.3| 0.8 ± 0.1   | 1.0 ± 0.3   | 0.3 ± 0.0   | 1.1 ± 0.2   |
| ADP         | 1.2 ± 0.2   | 1.0 ± 0.1   | 86.5 ± 19.0 | 129.9 ± 14.6| 0.6 ± 0.0   | 2.2 ± 0.1   | 0           | 13.3 ± 1.8  |
| GTP         | 0           | 0           | 214.0 ± 25.8| 633.1 ± 16.7| 0           | 0           | 27.6 ± 6.3  | 55.2 ± 8.2  |
| GDP         | 0.9 ± 0.0   | 0.6 ± 0.0   | 124.4 ± 7.6 | 378.1 ± 24.9| 0.2 ± 0.0   | 1.7 ± 0.1   | 2.1 ± 0.2   | 10.0 ± 1.2  |
| ADP-ribose  | 4.8 ± 1.4   | 3.2 ± 0.1   | 3.4 ± 0.0   | 0           | 0           | 0           | 7.1 ± 1.4   | 0           |
| UDP-glucose | 0           | 0           | 0           | 0           | 0           | 0           | 0           | 0           |

| Substrate   | Nud5 (3916) | Nud6 (3927) | Nud7 (4215) | Nud8 (4335) |
|-------------|-------------|-------------|-------------|-------------|
|             | Co          | Mn          | Co          | Mn          | Co          | Mn          | Co          | Mn          |
| Ap4A        | 0           | 8.2 ± 0.3   | 31.7 ± 1.0  | 20.5 ± 0.4  | 3.9 ± 0.7   | 5.4 ± 0.6   | 0.3 ± 0.0   | 0.4 ± 0.1   |
| Ap5A        | 0           | 3.3 ± 0.4   | 29.3 ± 2.3  | 16.5 ± 0.9  | 0.4 ± 0.1   | 2.4 ± 0.4   | 0.6 ± 0.0   | 1.0 ± 0.1   |
| ATP         | 3.8 ± 0.6   | 40.0 ± 0.6  | 0.7 ± 0.1   | 14.9 ± 0.5  | 0.2 ± 0.0   | 2.8 ± 0.6   | 0.1 ± 0.0   | 0.2 ± 0.0   |
| ADP         | 0           | 7.3 ± 0.4   | 2.4 ± 0.1   | 6.0 ± 0.7   | 1.1 ± 0.1   | 5.4 ± 0.3   | 0.1 ± 0.0   | 0.4 ± 0.0   |
| GTP         | 0           | 49.2 ± 3.9  | 1.4 ± 0.0   | 7.7 ± 0.4   | 0           | 7.0 ± 0.1   | 0           | 0           |
| GDP         | 0           | 5.3 ± 0.5   | 0.8 ± 0.0   | 3.3 ± 0.3   | 3.9 ± 0.6   | 8.4 ± 1.4   | 0.1 ± 0.0   | 0.2 ± 0.0   |
| ADP-ribose  | 0           | 0.6 ± 0.0   | 3.1 ± 0.9   | 0.2 ± 0.0   | 0           | 0           | 1.0 ± 0.1   | 1.4 ± 0.0   |
| UDP-glucose | 0           | 0           | 0           | 0           | 0           | 0           | 0           | 0           |
| NADH        | 4.2 ± 0.7   | 0.9 ± 0.1   | 0.6 ± 0.1   | 57.6 ± 8.1  | 0           | 4.2 ± 0.2   | 3.1 ± 0.1   | 3.3 ± 0.4   |
| 8-oxo-dGTP  | 0           | 56.8 ± 1.1  | 2.4 ± 0.3   | 21.0 ± 1.6  | 0           | 0.2 ± 0.0   | 0           | 0           |
| ppGpp       | 1.0 ± 0.2   | 0           | 0           | 0.6 ± 0.1   | 2.7 ± 0.1   | 14.4 ± 0.2  | 0           | 0           |

| Substrate   | Nud9 (5418) | Nud10 (6513) | Nud11 (7192) |
|-------------|-------------|-------------|-------------|
|             | Co          | Mn          | Co          | Mn          | Co          | Mn          |
| Ap4A        | 4.5 ± 0.8   | 2.8 ± 0.5   | 0.6 ± 0.1   | 3.8 ± 0.4   | 5942 ± 230  | 3957 ± 607  |
| Ap5A        | 0.3 ± 0.0   | 2.1 ± 0.2   | 0.1 ± 0.0   | 7.1 ± 0.4   | 5565 ± 323  | 6290 ± 365  |
| ATP         | 0.1 ± 0.0   | 4.2 ± 0.4   | 0           | 0.1 ± 0.0   | 10.7 ± 0.2  | 52.9 ± 2.6  |
| ADP         | 0.2 ± 0.0   | 1.8 ± 0.4   | 0           | 3.6 ± 0.5   | 3.2 ± 0.1   |
| GTP         | 0           | 21.7 ± 2.7  | 1.7 ± 0.2   | 6.8 ± 1.4   | 0           | 46.2 ± 2.6  |
| GDP         | 2.3 ± 0.1   | 1.1 ± 0.2   | 0           | 1.9 ± 0.3   | 3.4 ± 0.2   |
| ADP-ribose  | 0.5 ± 0.0   | 0.1 ± 0.0   | 7.1 ± 0.5   | 1.7 ± 0.3   | 5.6 ± 0.4   |
| UDP-glucose | 0.4 ± 0.0   | 0.4 ± 0.0   | 0           | 0           | 0           |
| NADH        | 0           | 0           | 0           | 21.2 ± 2.2  | 3.2 ± 0.2   |
| 8-oxo-dGTP  | 0           | 0           | 3.1 ± 0.3   | 2.1 ± 0.2   | 78.9 ± 4.3  | 207.5 ± 1.6 |
| ppGpp       | 2.8 ± 0.1   | 0           | 8.2 ± 0.3   | 0           | 0           |

The assays were performed in the presence of 1 mM substrate and 5 mM metal ion, and the specific activity (nmol/min/mg) was determined. Data are mean ± SEM values of three independent measurements.

cose. NADH was degraded by Nud2, Nud3, Nud6, and Nud11 (Table 1); however, their specific activities were significantly lower than those of *E. coli* Orf186 (21.0 μmol/min/mg; O’Handley et al., 1998) and Npy1 from *Saccharomyces cerevisiae* (2.1 μmol/min/mg; AbdelRaheim et al., 2001). Nudix NADH hydrolases contain the SQWPFPQS motif located 10 residues downstream of the Nudix box (Dunn et al., 1999), which was not found in Nud2, Nud3, Nud6, and Nud11. Although a highly similar motif (SQPWPFGRS) was present in Nud1, this enzyme had very low activity towards NADH. 8-Oxo-dGTP is an oxidized form of dGTP, which causes a replication error because it can pair with both adenine and cytosine (Shibutani et al., 1991). Some Nudix enzymes...
can hydrolyze 8-oxo-dGTP, thus preventing the incorporation of this mutagenic nucleotide into DNA and consequent genetic abnormalities. *E. coli* MutT (Ito et al., 2005) *Mycobacterium tuberculosis* MutT2 (Sang and Varshney, 2013), *M. smegmatis* MutT2 (Sang and Varshney, 2013), and human MTH1 and MTH2 (Takagi et al., 2012) can degrade 8-oxo-dGTP to 8-oxo-dGMP and pyrophosphate, whereas *M. tuberculosis* MutT1 hydrolyzes it to 8-oxo-dGDP and phosphate (Patil et al., 2013). *M. xanthus* Nud2 and Nud11 showed high hydrolytic activity toward 8-oxo-dGTP (Table 1). Nud2 was almost 10 times more efficient compared to Nud11 in degrading 8-oxo-dGTP in the presence of Mn$^{2+}$, converting 8-oxo-dGTP into 8-oxo-dGMP, whereas Nud11 mainly produced 8-oxo-dGDP (Fig. 2C). BlastP search revealed that *E. coli* MutT1 had the highest identity with *M. xanthus* Nud8 (26%); however, Nud8 had no hydrolytic activity towards 8-oxo-dGTP.

The other *M. xanthus* Nudix hydrolyases (Nud1 and Nud7–10) showed low efficiency in degrading the 11 tested substrates, suggesting that they may have other catalytic preferences, for example for dNTP, dNDP, or CoA.

In summary, in this study we expressed 11 of the 12 identified *M. xanthus* Nudix hydrolyases (except for MXAN_4891) in *E. coli* and analyzed their catalytic activity. The enzymes varied in their requirements for bivalent cations Mn$^{2+}$, Co$^{2+}$, and Mg$^{2+}$, as cofactors for the efficient hydrolysis of Ap$_n$A. Two of the highly conserved amino acids (Gly1 and Glu7) of the Nudix box are replaced in Nud2 by Ala and Gly, respectively; however, Nud2 had high hydrolyse activity towards Ap$_n$A, ATP, GTP, and especially 8-oxo-dGTP. These substrates were also cleaved by Nud11 which had the highest specific activity towards Ap$_n$A. ATP was also hydrolyzed by Nud5, and GTP by Nud4 and Nud5, whereas NADH was mainly degraded by Nud2 and Nud6. Further experiments are in progress to characterize the enzymatic properties of Nud2 and Nud11.

Acknowledgments

This study was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (16K07667).

Supplementary Materials

Supplementary figure and table are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

References

AbdelRahim, S. R., Cartwright, J. L., Gasmi, L., and McLennan, A. G. (2001) The NADH diprophosphatase encoded by the *Saccharomyces cerevisiae* NPY1 *Nudix* hydrolyase gene is located in peroxisomes. *Arch. Biochem. Biophys.*, 388, 18–24.

Andreeva, A. and Kutuzov, M. A. (2004) Widespread presence of “bacterial-like” PPP phosphatases in eukaryotes. *BMC Evol. Biol.*, 4, 47.

Bessman, M. J., Frick, D. N., and O’Handley, S. F. (1996) The MutT proteins or “Nudix” hydrolyases, a family of versatile, widely distributed, “Housecleaning” enzymes. *J. Biol. Chem.*, 271, 25059–25062.

Dunn, C. A., O’Handley, S. F., Frick, D. N., and Bessman, M. J. (1999) Studies on the ADP-ribose pyrophosphatase subfamily of the *Nudix* hydrolyases and tentative identification of *tryg*, a gene associated with tellurite resistance. *J. Biol. Chem.*, 274, 32318–32324.

Heinemeyer, E. A., Geis, M., and Richter, D. (1978) Degradation of guanosine 3’-diphosphate 5’-diphosphate in vitro by the spo7 gene product of *Escherichia coli*. *Eur. J. Biochem.*, 89, 125–131.

Ito, D., Kato, T., Maruta, T., Tami, M., Yoshimura, K. et al. (2012) Enzymatic and molecular characterization of *Arabidopsis* ppGpp pyrophosphohydrolase, AtNUDX26. *Biosci. Biotechnol. Biochem.*, 76, 2236–2241.

Ito, R., Hayakawa, H., Sekiguchi, M., and Ishihashi, T. (2005) Multiple enzyme activities of *Escherichia coli* MutT protein for sanitization of DNA and RNA precursor pools. *Biochemistry*, 44, 6670–6674.

Kimura, Y., Tanaka, C., Sasaki, K., and Sasaki, M. (2017) High concentrations of intracellular Ap$_n$A and/or Ap$_n$A in developing *Mycococcus xanthus* cells inhibit sporulation. *Microbiology*, 163, 86–93.

Kisselev, L. L., Justesen, J., Wolfson, A. D., and Frolova, L. Y. (1998) Diadenosine oligophosphates (Ap$_n$A), a novel class of signaling molecules? *FEBS Lett.*, 427, 157–163.

Lee, P. C., Bochner, B. R., and Ames, B. N. (1983) AppppA, heat-shock stress, and cell oxidation. *Proc. Natl. Acad. Sci. USA*, 80, 7496–7500.

McLennan, A. G. (2006) The *Nudix* hydrolase superfamily. *Cell Mol. Life Sci.*, 63, 123–143.

O’Handley, S. F., Frick, D. N., Dunn, C. A., and Bessman, M. J. (1998) Orf186 represents a new member of the *Nudix* hydrolyses, active on adenosine(5’3’)-triphosphenos(5’)-adenosine, ADP-ribose, and NADH. *J. Biol. Chem.*, 273, 3192–3197.

Oka, M., Takegawa, K., and Kimura, Y. (2015) Enzymatic characterization of a class II lysyl-tRNA synthetase, Lys5, from *Mycococcus xanthus*Arch. Biochem. Biophys.*, 579, 33–39.

Oka, M., Takegawa, K., and Kimura, Y. (2016) Lysyl-tRNA synthetase from *Mycococcus xanthus* catalyzes the formation of diadenosine penta- and hexaphosphates from adenine tetraphosphate. *Arch. Biochem. Biophys.*, 604, 152–158.

Ooga, T., Ohashi, Y., Kuramitsu, Y., Koyama, Y., Tomita, M. et al. (2009) Degradation of ppGpp by *nudix* pyrophosphatase modulates the transition of growth phase in the bacterium *Thermus thermophilus*. *J. Biol. Chem.*, 284, 15549–15556.

Palfi, Z., Surányi, G., and Borbély, G. (1991) Alterations in the accumulation of adenylylated nucleotides in heavy-metal-ion-stressed and heat-stressed *Synchococcus sp.* strain PCC 6301, a cyanobacterium, in light and dark. *Biochem. J.*, 276, 487–491.

Patil, A. G., Sang, P. B., Govindan, A., and Varshney, U. (2013) *Mycobacterium tuberculosis* MutT1 (Rv2985) and ADPase (Rv1700) proteins constitute a two-stage mechanism of 8-oxo-dGTP and 8-oxo-GTP detoxification and adenosine to cytidine mutation avoidance. *J. Biol. Chem.*, 288, 11252–11262.

Sang, P. B. and Varshney, U. (2013) Biochemical properties of MutT2 proteins from *Mycobacterium tuberculosis* and *M. smegmatis* and their contrasting antimutator roles in *Escherichia coli*. *J. Bacteriol.*, 195, 1552–1562.

Sasaki, M., Takegawa, K., and Kimura, Y. (2014) Enzymatic characteristics of an ApaH-like phosphatase, PrpA, and a diadenosine tetraphosphate hydrolase, ApaH, from *Mycococcus xanthus*. *FEBS Lett.*, 588, 3395–3402.

Shibutani, S., Takeshita, S., and Grollman, A. P. (1991) Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. *Nature*, 349, 431–434.

Shinkets, L. J. (1999) Intercellular signaling during fruiting-body development of *Mycosycoccus xanthus*. *Ann. Rev. Microbiol.*, 53, 525–549.

Swarbrick, J. D., Buyya, S., Gunawardana, D., Gayler, K. R., McLennan, A. G. et al. (2004) Structure and substrate-binding mechanism of human Ap$_n$A hydrolase. *J. Biol. Chem.*, 280, 8471–8481.

Takagi, Y., Setoyama, D. S., Ito, R., Kamiya, H., Yamagata, Y. et al. (2012) Human MTH3 (NUDT18) protein hydrolizes oxidized forms of guanosine and deoxyguanosine diphosphates: comparison with MTH1 and MTH2. *J. Biol. Chem.*, 287, 21541–21549.