High concentrations of intracellular Ap₄A and/or Ap₅A in developing *Myxococcus xanthus* cells inhibit sporulation

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**Abstract**

Diadenosine polyphosphates (Ap₄A) are thought to act as signalling molecules regulating stress responses and biofilm formation in prokaryotes. However, Ap₄A function in *Myxococcus xanthus* remains unknown. Here, we investigated the role of Ap₄A in *M. xanthus*, using the wild-type and Ap₄A hydrolase (apaH) mutant strains exposed to various stress conditions. In both wild-type and apaH mutant cells cultured on starvation medium (CF agar), the levels of intracellular diadenosine tetraphosphate (Ap₄A) and pentaphosphate (Ap₅A) increased several fold during the first 16 h of development and decreased gradually thereafter. The levels of Ap₄A and Ap₅A in the apaH mutant were about 5- and 11-fold higher than those in the wild-type strain at 16 h, respectively. Ap₄A hydrolase activity of the wild-type strain increased 1.5-fold during the first 8 h of development, and it then gradually decreased. The apaH mutant formed spores 1–2 days after the wild-type strain did, and the yield of viable spores was 5.5% of that in the wild-type strain 5 days after inoculation onto CF agar. These results suggest the possibility that high intracellular levels of Ap₄A and/or Ap₅A may inhibit *M. xanthus* sporulation at the early stage of development and that the bacteria reduce intracellular Ap₄A and Ap₅A accumulation through Ap₄A hydrolase activity.

**INTRODUCTION**

Diadenosine 5′,5′″-P₁,P₄-tetraphosphate (Ap₄A) is composed of two adenosine moieties joined in a 5′–5′ linkage by a chain of four phosphates. Ap₄A has been thought to act as a signalling molecule in a variety of biological systems in both prokaryotes and eukaryotes [1]. *Escherichia coli*, *Syn-echococcus* sp. and *Salmonella typhimurium* rapidly accumulate Ap₄A after heat shock and under oxidative or heavy-metal stresses; however, the role of Ap₄A in bacteria under stress conditions is still poorly understood [2, 3]. In prokaryotes, Ap₄A metabolism is also related to the regulation of pathogenesis and biofilm formation [4–6].

Ap₄A is thought to be mainly synthesized by aminocyt-rRNA synthetases, which catalyse aminoclylation of tRNAs, i.e. the attachment of a given amino acid to a particular tRNA molecule. In the absence of tRNAs, the majority of aminocyl-tRNA synthetases produce Ap₄A in a two-step reaction involving a cognate amino acid and ATP [7]. In the first step, the amino acid is activated by condensation with ATP to form aminocly-AMP, and in the second step, ATP reacts with aminocyl-AMP to generate Ap₄A. The presence of tRNA in most cases inhibits Ap₄A synthesis [8]. Since aminocyl-tRNA synthetases are an essential part of each translation system, Ap₄A can be found in all living cells.

In bacteria, diadenosine polyphosphates (Ap₄As) are hydrolysed by two different enzymes, ApaH and Nudix (nucleo-side diphosphate linked to X) hydrolases. ApaH shares sequence similarity with phosphoprotein phosphatases existing only in bacteria [9]. Nudix hydrolases exhibit broad substrate specificity and ambiguity, and many Nudix enzymes hydrolyse Ap₄A into AMP and ATP [10].

*Myxococcus xanthus* is a Gram-negative rod-shaped bacterium that demonstrates complex social behaviour [11, 12]. The bacteria are usually found in topsoil, where they get nutrients preying on other microbes. Under starvation conditions, vegetative cells aggregate and form multicellular fruiting bodies containing differentiated myxospores.

We previously reported that *M. xanthus* lysyl-tRNA synthetase (LysS) demonstrated enzymatic properties different from those of known class II lysyl-tRNA synthetases because LysS does not require Zn²⁺ for the synthesis of Ap₄A [13]. In addition, LysS catalyses the production of diadenosine pentaphosphate (Ap₅A) from Ap₄A [14], which is generated by acyl-CoA synthetase, acetyl-CoA synthetase or adenylate kinase [15–17]. *M. xanthus* contains two Ap₄A hydrolases: ApaH and Nudix hydrolases; ApaH degrades Ap₄A into two ADP molecules in the presence of Co²⁺ or Mn²⁺ [18].
The functional importance of Ap₅As in *M. xanthus* is still unknown. In this study, we investigated the role of Ap₅As in *M. xanthus* by measuring intracellular Ap₅A levels and Ap₅A hydrolase activity in the wild-type and apaH mutant *M. xanthus* grown under various stress conditions.

**METHODS**

*M. xanthus* culture

The wild-type [12] and apaH mutant *M. xanthus* strains were grown at 30 °C in CYE liquid medium (optimal conditions) [19] or on clone fruiting (CF) agar (amino acid starvation) [20]. To investigate *M. xanthus* response to stress conditions, bacteria cultured in CYE medium were exposed to high temperature (37 °C), oxidative stress (30 µM H₂O₂) or osmotic stress (0.25 M NaCl) for different times. Growth of bacterial cell culture was monitored by measuring OD₆₀₀ and cell number was determined using a haemocytometer.

Developmental assays

Wild-type and mutant cells grown in CYE medium were harvested at the mid-logarithmic phase and washed with TM buffer (10 mM Tris/HCl (pH 7.5) and 8 mM MgSO₄), and aliquots (10 µl) of cell suspensions (1 × 10⁶ cells) were spotted on the surface of CF agar plates, which were then incubated at 30 °C for 7 days. Fruiting bodies were harvested in TM buffer and sonicated for 1 min using a Branson sonifier. After incubation for 15 min at 60 °C, the number of viable spores was quantified by plating on casitone/Tris (CT) agar; the total number of spores was determined by microscopy using a haemocytometer. For glycerol induction of spore formation, cultures in the mid-exponential phase were incubated in the presence of 0.5 M glycerol at 30 °C with agitation for 4 h, and the numbers of total and viable spores were determined as described above.

**Assay for Ap₅A accumulation**

*M. xanthus* culture (20 ml, OD₆₀₀=1) was filtered by suction through a 1 µm pore mixed cellulose ester membrane (Advantec), which was then incubated in 50 mM HEPES (pH 8.0) containing 50 % TCA/20 % methanol at 4 °C for 15 min [21]. After centrifugation at 12000 g for 5 min at 4 °C, the supernatant was recovered, washed four times with 0.5 ml of diethyl ether to remove TCA and concentrated to 0.1 ml in a centrifugal concentrator, and a 20 µl aliquot was used for HPLC analysis of Ap₅A levels.

**Ap₅A hydrolase assay**

Wild-type and apaH mutant bacteria were harvested by centrifugation, suspended in 50 mM HEPES (pH 8.0) and sonicated on ice. The suspension was centrifuged, and the supernatant was dialysed three times against 50 mM HEPES (pH 8.0). Ap₅A hydrolase activity was measured in a total volume of 25 µl containing 100 mM Tris/HCl (pH 8.0); 1 mM CoCl₂; 1 mM MnCl₂; 1 mM Ap₅A, Ap₅A or Ap₅A; and 12.5 µl of crude extract solution. The reaction mixtures were incubated at 30 °C for 30 min, and proteins were then precipitated by 25 µl of 20 % TCA/40 % methanol. TCA was then removed as described above, and reaction products were analysed by HPLC.

**HPLC analysis**

Concentrations of intracellular Ap₅As and enzyme reaction products were detected using an ion exchange column Resource Q (1 ml; GE Healthcare) under the following conditions: mobile phase, solvent A (water) and solvent B (0.7 M ammonium bicarbonate); gradient elution: 0 min, 90 % (v/v) solvent A; 0.5 min, 73 % solvent A; 8 min, 61 % solvent A; 10 min, 5 % solvent A; 13 min, 0 % solvent A; flow rate, 1 ml min⁻¹; detection, absorbance at 260 nm.

**Construction of the apaH mutant**

The DNA fragment containing the apaH gene from the *M. xanthus* FB genome was amplified by PCR using primers apaH-N (5’-ACTAGTGGATCCCGCTGAGGCGAC- GGAAAA-3’) and apaH-C (5’-GAATTC-TGACGCCCAGGCGTCGTCGTCATGG-3’). The PCR product was inserted into the pBluescript SK vector (Toyobo) using an In-Fusion kit ( Takara Bio), and then the kanamycin resistance-encoding gene (1.25 kb) was inserted into the apaH sequence. The resulting disrupted gene was amplified by PCR using primers apaH-N1 (5’-CGCTGAGGCGACCGGAAA-3’) and apaH-C1 (5’-AGGCTGTCGTCGTCTGATGGCAG-3’) and was introduced into *M. xanthus* FB cells by electroporation [22]. Kanamycin-resistant colonies were selected in CYE medium containing kanamycin (100 µg ml⁻¹), and chromosomal DNA was extracted from the mutants. The insertion of the kanamycin resistance gene into the apaH gene in the *M. xanthus* chromosome was confirmed by PCR and restriction enzyme analysis.

**Reversal period assay**

*M. xanthus* cells (5 µl of 1–2×10⁷ cells ml⁻¹ in CYE medium or TM buffer) were spotted on 1.5 % CYE agar and incubated at 30 °C for 2 h. The frequency of cell reversal was monitored using a Nikon microscope equipped with a CCD camera. At least 15 cells from each strain were followed over a 45 min period.

**RESULTS**

**Intracellular Ap₅A levels in growing cells**

*M. xanthus* wild-type and apaH mutant strains showed similar growth in CYE medium at an optimal temperature of 30 °C (Fig. 1a). In addition, *M. xanthus* wild-type and apaH mutant cells exhibited gliding motility on CYE agar, periodically reversing their movement direction at the frequency of 3.5±0.2 min and 3.8±0.3 min per reversal, respectively. The cell lengths of wild-type and apaH mutant strains were 5.5±0.2 µm and 6.3±0.2 µm, respectively. The intracellular concentrations of Ap₅A and Ap₅A were determined in these *M. xanthus* strains from the exponential growth phase to the late stationary phase. For the wild-type strain, Ap₅A concentration was 1.9-fold (P=0.04, t-test) higher in the early stationary phase compared with that in the exponential phase, after which Ap₅A levels gradually decreased from...
the middle stationary phase to the late stationary phase. In the apaH mutant, ApA levels were about 2.3–3.5-fold (P≤0.03, t-test) higher than those in the wild-type strain, except during the late stationary phase (Fig. 1b). ApA was identified in both, the wild-type and mutant M. xanthus, with the levels about 3–7-fold (P≤0.05, t-test) lower than those of ApA (Fig. 1b). In this assay, ApA could not be detected because the peak corresponding to ApA overlapped with that of ATP in HPLC analysis.

We next determined ApA hydrolase activity in crude extracts prepared from the exponential cultures of the wild-type and mutant strains grown at 30 °C (Fig. 1c). The enzymes expressed by the wild-type and mutant M. xanthus degraded ApA into two molecules of ADP, or AMP and ATP. Since ATP was hydrolysed by crude enzymes, its concentration was lower than that of AMP. Both crude enzymes degraded ApA into ADP and ATP, or ApA and AMP. The total concentration of degradation products generated from ApA as by the apaH mutant was about 40% of that produced by the wild-type strain.

**Intracellular ApA levels generated under stress conditions**

When M. xanthus was incubated at a high temperature (37 °C), the growth of the wild-type strain was faster than that at 30 °C, and the bacteria reached maximum cell density (8×10⁸ cells ml⁻¹) after 18–24 h (Fig. 2a); however, after 28 h, the maximum cell density at 37 °C was about 70% of that at 30 °C. There was no significant difference in the growth at 37 °C between the wild-type and apaH mutant (Fig. 2a). Both wild-type and apaH mutant cells demonstrated an increase in ApA concentration, which peaked at 24 h or 10 h, respectively, and decreased thereafter (Fig. 2c). For both strains, intracellular ApA levels at 37 °C were approximately 2–3-fold (P≤0.036, t-test) higher than those at 30 °C.

ApA hydrolase activity in crude extracts prepared from wild-type M. xanthus and the apaH mutant grown at 37 °C for 10 h increased about 1.3- and 1.4-fold compared with that at 30 °C, respectively (Fig. 2f, g).

We also determined the intracellular concentrations of ApA and ApA in M. xanthus cultures exposed to oxidative or osmotic stress conditions (Fig. 2d, e). As shown in Fig. 2(b), the density of the wild-type bacteria cultured under oxidative or osmotic stress conditions for 28 h was about 20–30% of that in the optimal conditions. The apaH mutant grew slightly faster than the wild-type (Fig. 2b). In the wild-type strain, exposure to H₂O₂ or NaCl increased the intracellular accumulation of ApA or ApA [2.3-fold (P=0.05, t-test) or 3.4-fold (P=0.03, t-test)], respectively; however, in the apaH mutant, ApA or ApA levels under these conditions were 6.5-fold (P=0.04, t-test) or 4.1-fold (P=0.01, t-test) higher than those in the wild-type. M. xanthus also demonstrated increased ApA hydrolase activity (1.3–2.2-fold) under stress conditions (Fig. 2f, g).

**Intracellular ApA levels during M. xanthus development**

When the wild-type and apaH mutant bacteria were cultured under starvation conditions (CF agar), wild-type cells moved to aggregation centres and produced spore-filled fruiting bodies within 3 days (Fig. 3a). Within the fruiting bodies of the wild-type M. xanthus, rod-shaped vegetative cells were converted into mature heat- and sonication-resistant myxospores. The apaH mutant cells formed fruiting bodies approximately at the same time as the wild-type cells; however, they were less compact than the dark, mature spore-filled fruiting bodies of the wild-type bacteria (Fig. 3a). As a result, the numbers of mature spores...
Fig. 2. Influence of culture conditions on *M. xanthus* growth, *Ap₄A* accumulation and *Ap₅A* hydrolase activity. (a) Growth curves of the wild-type (open symbols) and *apaH* mutant (closed symbols) strains under optimal conditions and high temperature. Both cell types were inoculated at a concentration of $7 \times 10^7$ cells ml$^{-1}$ in CYE medium and grown at 30°C (circles) and 37°C (squares) with constant shaking. (b) Growth curves of the wild-type (open symbols) and *apaH* mutant (closed symbols) strains under oxidative stress (squares) and osmotic stress (squares). Both cell types were inoculated to $7 \times 10^7\times10^8$ cells ml$^{-1}$ in CYE medium containing 30 µM H$_2$O$_2$ (circles) or 0.25 M NaCl (squares) and grown at 30°C with constant shaking. (c–e) Changes in intracellular *Ap₄A* and *Ap₅A* levels in the wild-type (W) and *apaH* mutant (M) strains at high temperature (c), under oxidative stress (d) and osmotic stress (e). (f, g) *Ap₅A* hydrolase activities in crude extracts of the wild-type (W) and *apaH* mutant (M) strains exposed to stress conditions. Both strains were incubated in CYE medium for 10 h at the optimal conditions (OC), for 10 h at high temperature (37°C), for 6 h under oxidative stress (H$_2$O$_2$) and for 12 h under osmotic stress (NaCl) and were analysed for *Ap₅A* hydrolase activity using 1 mM *Ap₄A* (f) or *Ap₅A* (g) in the presence of 1 mM Co$^{2+}$ and 1 mM Mn$^{2+}$. The data are expressed as the mean±SEM of three independent experiments performed in triplicate.
produced by the mutant at days 4 and 5 were 0.05 and 5.5%, respectively, of those generated by the wild-type strain (Fig. 3b). The final yield of viable spores produced by the apaH mutant at day 7 was 23.3% of that produced by the wild-type strain (data not shown). According to the M. xanthus genome sequence [23], apaH (MXAN_7163) is the last gene in the operon comprising three genes (putative DNA-binding regulatory protein gene, serine/threonine protein kinase gene and apaH gene), suggesting that the insertion of the gene kanamycin resistance-encoding sequence in the apaH gene is unlikely to cause polar effects.

We next determined intracellular Ap4A and Ap5A levels in the wild-type and apaH mutant cells during starvation-induced development. The time course of Ap4A and Ap5A accumulation in M. xanthus was determined over 120 h culture on CF agar (Fig. 4a, b). Ap4A and Ap5A levels in the wild-type strain increased 2.8-fold ($P=0.002$, t-test) and 2.2-fold ($P=0.07$, t-test) during the first 16 and 24 h of incubation, respectively, and then decreased gradually (Fig. 4a). The apaH mutant strain during the early stage of development demonstrated significantly higher levels of Ap4A and Ap5A (Fig. 4b). Overall, the accumulation of Ap4A and Ap5A in the apaH mutant at 16 h was about 5-fold ($P=0.005$, t-test) and 11-fold ($P=0.003$, t-test) higher, respectively, than that in the wild-type.

Under these conditions, Ap4A hydrolase activity of the wild-type strain was initially 5.2±0.2 pmol min$^{-1}$ per 10$^7$ cells, peaked to 7.8±0.7 pmol min$^{-1}$ per 10$^7$ cells at 8 h and then decreased to the level of the apaH mutant (2–3 pmol min$^{-1}$ per 10$^7$ cells). The apaH mutant also demonstrated an increase in Ap4A hydrolase activity by about 1.5-fold during the first 8 h; however, no significant changes in Ap4A hydrolase activity were observed (Fig. 4c, d).

In M. xanthus, rod-shaped vegetative cells can rapidly convert into spores when exposed to high concentrations of glycerol [24]. In both strains, spherical cells were observed after 1 h of glycerol exposure, and total spore numbers peaked at 3 h of incubation (Fig. 5a). The numbers of total and viable spore produced by the apaH mutant at 3 or 4 h constituted 32 and 7%, or 44 and 41%, respectively, of those generated by the wild-type strain.

Both strains gradually decreased rather than increased the concentration of intracellular Ap4A during glycerol-induced sporulation (Fig. 5b). Intracellular Ap4A levels in the wild-type cells decreased by 50% after 1 h of incubation with glycerol; however, there was no change in Ap4A hydrolase activity during the same incubation period (data not shown), suggesting that Ap4A decrease may be attributed to a reduction in Ap4A synthetase activity. The Ap4A and Ap5A levels in the apaH mutant after 3 h of incubation with glycerol were 10-fold ($P=0.0002$, t-test) and 12-fold ($P=0.0002$, t-test) higher, respectively, than those in the wild-type strain.

**DISCUSSION**

E. coli and Sal. typhimurium dramatically increase intracellular Ap4A concentration (100–200 µM) after exposure to heat shock and oxidizing agents [2]. When subjected to different unfavourable conditions such as elevated temperature and oxidative and osmotic stresses, M. xanthus also accumulated intracellular Ap4A; however, the increase in Ap4A production was not as high as that in E. coli after heat shock (~70-fold) or in Sal. typhimurium after oxidation stress (~10–70-fold) [24, 25]. The M. xanthus cell volume was estimated to be 2.6 µm$^3$ based on a width of 0.75 µm and a length of 6 µm. On the basis of this estimate, the Ap4A concentration (0.16 pmol per 10$^7$ cells) of wild-type cells under the non-stress condition was calculated as 6 µM, and it increased to 17 µM (2.8-fold) and 9 µM (1.5-fold) under conditions of high temperature and oxidative stress, respectively. E. coli expresses two isoforms of class II lysyl-tRNA synthetase, LysS and LysU [26]. While LysS is expressed under regular growth conditions, LysU is the product of a normally silent gene induced by extreme stimuli such as heat shock [27]. M. xanthus also has two lysyl-tRNA synthetases, MXAN_4731 (LysS) and MXAN_5579. While M. xanthus LysS efficiently produces Ap4A from ATP and Ap5A from ATP and Ap4 alone [13, 14], the other lysyl-tRNA synthetase (MXAN_5579) lacks the tRNA-binding domain and has very low Ap4A synthetase activity (unpublished data). We were unable to construct a lysS mutant, suggesting that LysS is essential for the viability of M. xanthus.
The total amount of Ap₄A degradation products generated by vegetative cells of the apaH mutant constituted 30–40% of that in the wild-type, suggesting that, in M. xanthus vegetative cells, about 60–70% and 30–40% of Ap₄A hydrolase activity is provided by ApaH and Nudix hydrolases, respectively. Salmonella enterica also contains ApaH and a Nudix hydrolase, YgdP, which have almost equal Ap₄A hydrolase activities [5]. We have previously reported that M. xanthus recombinant ApaH exhibited high hydrolase activity towards Ap₄A [18]. Thus, the specific activities of ApaH towards Ap₄A and Ap₃A in the presence of 1 mM Co²⁺ were 16.2±0.57 and 54.7±0.60 µmol min⁻¹ mg⁻¹, respectively. M. xanthus has approximately 12 Nudix hydrolases, and we have confirmed that some of the recombinant Nudix proteins exhibit symmetrical and/or asymmetrical Ap₄A hydrolase activity (unpublished data).

In previous studies, the concentration of Ap₄A in the E. coli apaH mutant increased 20-fold from 300 µM to 6 mM after 1 h of incubation at 46°C. The E. coli apaH mutant showed similar growth to the wild-type strain at 30–42°C [25]; it also showed increased sensitivity to high temperature (55°C) and lacked motility [28]. Additionally, the Sal. enterica apaH mutant exhibited filamentous growth under the non-stress condition [5]. Here, M. xanthus increased Ap₄A hydrolase activity (1.3–2.2-fold) under stress conditions. The apaH-containing operon also includes the gene encoding a putative DNA-binding regulatory protein belonging to the family of extracytoplasmic function sigma factors [29, 30], suggesting that apaH expression may be controlled by a DNA-binding regulatory protein. Under stress conditions, Ap₄A levels (38–67 µM) in the M. xanthus apaH mutant were approximately four times higher than those in the wild-type. However, the increase of Ap₄A levels in the apaH mutant was also significantly lower than that in the E. coli apaH mutant. The M. xanthus apaH mutant did not show significant growth differences with the wild-type strain under normal and stress conditions, and the mutant cells demonstrated gliding motility on a solid surface. These
ble spores (solid lines) were counted. (b) Changes in intracellular Ap
molecules in 0.5 M glycerol for 4 h, and total spores (dashed lines) and via-
mutant (squares) strains. Cells were incubated in CYE medium con-
temperature and oxidative and osmotic stresses.

Intracellular concentration
Spores

Fig. 5. Accumulation of Ap₄A and Ap₃A in M. xanthus during glycerol-
induced sporulation. (a) Sporulation of the wild-type (circles) and apaH mutant (squares) strains. Cells were incubated in CYE medium containing 0.5 M glycerol for 4 h, and total spores (dashed lines) and viable spores (solid lines) were counted. (b) Changes in intracellular Ap₄A and Ap₃A levels in the wild-type (W) and apaH mutant (M) during spor-
ulation. The data are expressed as the means±SEM of three independent experiments.

results suggest that Ap₄As may not function as signalling molecules in M. xanthus under the conditions of high temperature and oxidative and osmotic stresses.

On the other hand, the apaH mutant under starvation condition had reduced sporulation. Intracellular Ap₄A and Ap₃A concentrations in both wild-type and apaH mutant strains increased at the early stage of amino acid starvation-induced development and decreased gradually. Wild-type M. xanthus increased Ap₄A hydrolase activity during the first 8 h of development and then decreased the Ap₄A hydrolase activity to the level of the apaH mutant. Ap₄A and Ap₃A concentrations were 5 and 11 times higher in the apaH mutant than in the wild-type strain, respectively, at 16 h of development, and the apaH mutation affected spore formation. In addition, the apaH mutant also showed low levels of glycerol-induced sporulation, whereas the differen-
ces in mature spore production between the wild-type and apaH mutant were smaller than those of starvation-induced spores. It is unclear whether Ap₄A and/or Ap₃A act as signalling molecules during M. xanthus sporulation because we were unable to construct mutants deficient in Ap₄A or Ap₃A synthetase activity; however, these results suggest that high levels of intracellular Ap₄A and/or Ap₃A in developing cells mainly hinder the progress of multicellular development, but not formation of mature spores. Therefore, M. xanthus might attempt to decrease Ap₄A levels through activation of Ap₃A hydrolase, especially ApaH, at the early stage of development. Further experiments are in progress to detect proteins regulated by Ap₄A during development.

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

Conflicts of interest
The authors declare that there are no conflicts of interest.

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Edited by: R. Lan and W. Achouak

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