Are the Arginine Kinase functions found in *Myxococcus xanthus* conserved in a sister species, *Myxococcus macrosporus*?

Seula Kim and Dr. Dean Fraga
Biology, The College of Wooster, Ohio

Abstract

Phosphagen kinases (PK) catalyze the reversible phosphorylation of the guanidino substrate to buffer energy in all animal species and some protozoa. Recently, genomic sequencing of bacterial species has revealed that a few of them contain arginine kinases (AK) that phosphorylate arginine and utilize ATP as an energy buffering system. Phylogenetic evidence indicates that closely related bacterial species that carry AK are missing AK homologs, suggesting that these bacterial AK (bAK) are derived through horizontal gene transfer (HGT). Among these bacterial species, the bAK found in *Myxococcus xanthus* (MxAK) was shown to play unique roles in social and developmental behaviors, such as kin discrimination and fruiting body formation. In this study, the role of bAK in *Myxococcus macrosporus*, a bacterial species that is closely related to *M.xanthus*, was studied to determine if the bAK in that species had similar roles in social and developmental behaviors. If the AK of *M.macrosporus* (MmAK) exhibits the same role as MxAK, this might indicate that both species inherited AK from their common ancestor with roles in these behaviors. If MmAK is not responsible for such behaviors, it suggests that MxAk has evolved new functions after getting transmitted to *M.xanthus* through HGT. We first characterized the basic growth and social behaviors of *M.macrosporus* to compare to *M.xanthus*. Several attempts were made to create the MmAK deletion, but the plasmid sequence would not recombine out of the genome, exhibiting very low frequency of plasmid excision compared to MmAK gene. As a consequence, we used the strain that contained the ΔAK construct in addition to the MmAK gene. Both the WT and ΔAK strain exhibited fruiting bodies, but they formed a strict border towards each other, indicating that ΔAK in *M.macrosporus* may participate in the kin recognition.

Arginine Kinase Background

- Arginine kinase (AK) transfers a high energy phosphate from ATP to arginine to buffer energy in eukaryotic and protozoan species (Andrews et al. 2008).
- Recent studies identified several bacterial species that also have AK (bAK) as a result of horizontal gene transfer (HGT) (Fraga et al. 2019).

Myxobacteria Background

Myxobacteria are gram-negative soil bacteria that exhibit complex developmental and social behaviors (Bragg et al. 2012). Several myxobacterial species that carry bAK are syntenic, indicating that they inherited bAK from their common ancestor (Fraga et al. 2019). The developmental behavior that myxobacterial species exhibit is fruiting body formation. When myxobacteria are deprived of nutrients, cells exchange extracellular and physical contact signals to aggregate together and form circular mounds called fruiting bodies to efficiently distribute nutrients (Bragg et al. 2012; Muñoz-Dorado et al. 2016). Then, the cells undergo autolysis and convert to the resting-cell type called myxospores (Bragg et al. 2012). The social behavior that they exhibit is kin recognition. Myxobacterial species are predatory where they hunt in packs to destroy any foreign organisms that they encounter (Gong et al. 2018). During this process, they secrete cell-lyse proteins and hydrolytic enzymes to intoxicate and kill the target cell (Gong et al. 2018). This results in the formation of a demarcation line, or a line of dead cells, that draws strict boundaries between cells (Gong et al. 2018).

Previous characterization of ΔAK

Previous studies knocked out MxAK gene and investigated the role of AK in various behaviors of *M.xanthus.*

- Stress recovery (Bragg et al. 2012):
  - AK deletion strain, ΔAK, exhibited slower generation time under pH and ionic stressors (KCl and NaCl).
- Fruiting body formation (Bragg et al. 2012):
  - Unexpectedly, ΔAK strain was unable to form round shaped fruiting bodies, instead, they showed a web like appearances (Figure 1).
- Kin recognition (Arday 2022; MacLean 2022):
  - Wild Type (WT) and ΔAK strain formed a strict demarcation line, indicating that AK is involved in the signaling pathway

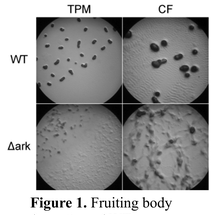

Figure 1. Fruiting body formation of WT and ΔAK strains. The image is adapted from Bragg et al. (2012).

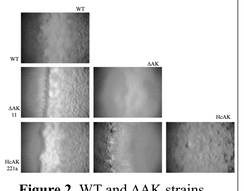

Figure 2. WT and ΔAK strains forming a demarcation line. The image is adapted from MacLean (2022).

How did the MxAK derive its unique function?

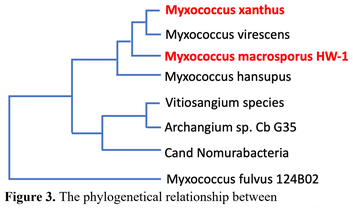

Figure 3. The phylogenetical relationship between *M.xanthus* and *M.macrosporus* HW-1. The image is modified from Fraga et al. (2019).

To determine if the function of MxAK is an inherited trait from the ancestor or independently acquired through HGT, this study examines a phylogenetically closely related bacterial species, *Myxococcus macrosporus* that also possess bAK (Figure 3) (Fraga et al. 2019).

Electroporation and the result

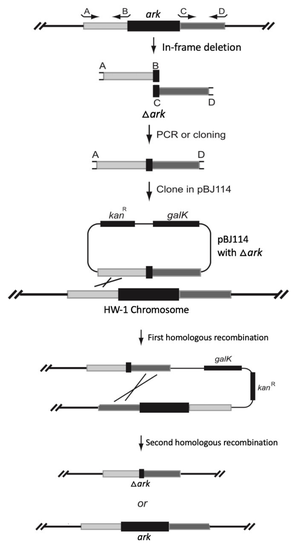

Figure 4. The step-wise representation of the in-frame deletion and subsequent two-step homologous recombination. After the in-frame deletion of the *ark* gene, the plasmid containing the deletion sequence was introduced into the HW-1 chromosome. The two-step homologous recombination then occurs, in which kanamycin is first used to select for kanamycin resistance. Then, galactose is used to counterselect by activating the *galk* gene on the plasmid sequence, leading to the death of the ones still containing the plasmid. This process results in a random ratio of cells either still containing the *ark* gene or successfully deleted (Δark). The figure has been modified from Shi et al. (2008).

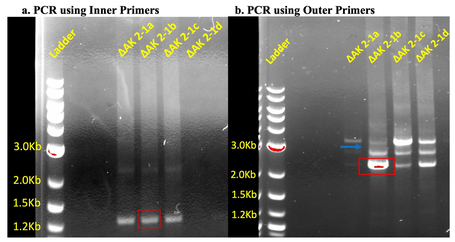

Figure 5. The gel image of the ΔAK 2-1b strain exhibiting band that indicates ΔAK. The blue arrow points to the multiple bands.

After PCR, the gel image showed band size at ΔAK.

- However, there were also multiple bands above (Figure 5).
  - Streaked on Kanamycin plates which indicated that the strain is still containing the plasmid sequence.
  - The later PCR and gel indicated that they also contain AK sequence.
- There were about 180 more strains tested, and only two strains had plasmid out of the genome.
  - They were tested by running PCR and gel, but the result was negative.
- Due to time constraints, I used ΔAK 2-1b throughout the study.

Both WT and ΔAK formed fruiting bodies

- The previous study unexpectedly found that this process is also dependent on MxAK (Bragg et al. 2012).
- Furthermore, the later studies found that it is the natural ability of AK that confers the energy buffering to carry out developmental pathways (MacLean 2022).

- For *M.macrosporus*, both WT and ΔAK 2-1b strain exhibited ridge-shaped fruiting bodies.
  - This could be due to AK present in the ΔAK 2-1b strain.
  - Or MmAK is not necessary for this developmental process.

- Continued to screen for deletions, but in the absence of true deletion, it was worthwile to test the ΔAK 2-1b strain
  - The caveat would be knowing plasmid is still present in the gene

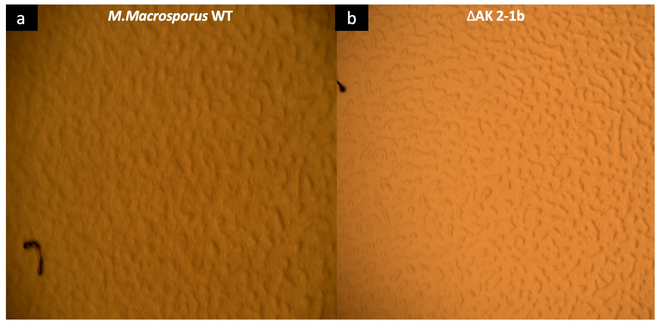

Figure 7. Development of fruiting bodies under starvation condition
Both WT and ΔAK 2-1b strains were grown on CF agar. (a) The image was taken at 63 magnification after one day of growth in the incubator. (b) The image was taken at 32 magnification after two days of growth in the incubator.

Future Research

- To obtain the ΔAK strain of *M.macrosporus*, different counterselectable marker, such as *sacB*, can be used to improve the transformation method (Reyrat et al., 1998).
- Utilize CRISPR/Cas9 induced fragment deletion in *M.macrosporus* that was found to be effective in *M.xanthus.* It can be applied to precisely cut AK gene or plasmid sequence in the ΔAK 2-1b strain (Yang et al., 2017).
- Further tests can be done on *Myxococcus fulvus*, a species that is distantly related to *M.xanthus*, which acquired bAK through HGT. This study can show whether the unique functions of MxAK is also found in MfAK (Fraga et al., 2019).

Border formation between WT and ΔAK

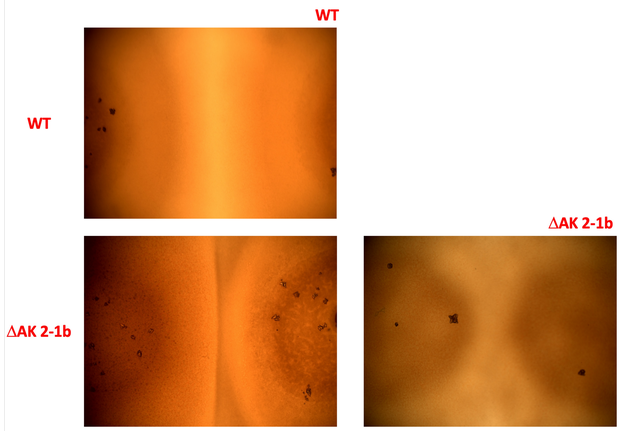

Figure 6. Border formation between *M.macrosporus* WT and ΔAK 2-1b
Each colonies are plated approximately 7mm apart from each other and were grown on CTTYE plates for 6 days. The images were taken at a magnification of 20x.

- Previously, only 9 out of 3,349 genes tested are responsible for kin recognition (Gong et al. 2018).
- In the later studies, WT and ΔAK of *M.xanthus* formed a strong border indicating that the MxAK is vital to this recognition pathway (Arday 2022; MacLean 2022).
- Also found that kin recognition is a phenotype unique to the MxAK that evolved after acquisition of AK(Arday 2022; MacLean 2022).

- In case of *M.macrosporus*, WT and ΔAK 2-1b formed a strict demarcation line, suggesting that they have recognized each other as a different strain.
- Even with the presence of AK, they still formed a border.
  - MmAK is not related to the kin recognition, and the plasmid is mislocated in the ΔAK 2-1b genome that might have disturbed gene expression related to the kin recognition.
  - Or MmAK also participates in the social signaling pathway, but the gene expression of AK is disturbed.

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