Does Arginine Kinase (AK) Bias the Switch? Impact of an AK Deletion on *Myxococcus xanthus* Fruiting Body Formation in Strains within Tan and Yellow Phases

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Background and Significance

- **Phosphagen Kinases (PKs) catalyze the reversible reaction of phosphate transfer between ATP and a guanidino group creating an ATP buffering system [1].**

Figure 1 Reversible reaction catalyzed by Arginine Kinase converting ATP and L-arginine to ADP N$^{\omega}$-phospho-L-arginine in the forward direction.

- **PKs were associated mainly to metabolically demanding eukaryotes but have been found in prokaryotes despite bacteria's preference for a concise genome [1,2].**

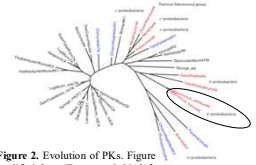

Figure 2. Evolution of PKs. Figure modified from (Fraga et. al, 2019) [2]

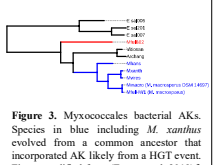

Figure 3. Myxococcales bacterial AKs. Species in blue including *M. xanthus* evolved from a common ancestor that incorporated AK duly from a HGT event. Figure modified from (Fraga et. al, 2019) [2]

- **Arginine Kinase has been found in *Myxococcus xanthus* and was biochemically active *in vitro* and with a role in protective mechanisms including starvation induced fruiting body (FB) formation [3].**

Fluctuation in Phenotype Previously Associated to an AK Loss

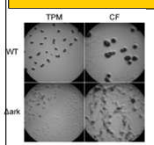

Figure 4. Fruiting to starvation media TPM. Figure modified from (Bragg et. al, 2012) [3]

When subject to rapid starvation, strains lacking AK (dAK) have an arrested developmental phenotype with faint weblike aggregates (Bragg et al., 2019) [3].

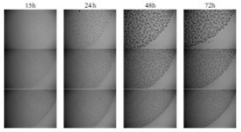

Figure 5. Fruiting body formation of dAK strain is variable even within technical replicates. Images of three dAK-2b Y samples with varying phenotypes selected from the 10 replicates subject to starvation media TPM.

The dAK strain was recently found to fluctuate in terms of colony color flipping to tan phase more readily.

This was simultaneous to a change in developmental phenotype with FB ranging from arrested to rescued phenotype- deviating from previously robust arrested FB formation.

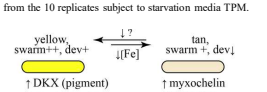

Figure 6. *M. xanthus* phase change key characteristics. Figure modified from (Dziewanowska et. al, 2014) [5]

- Looking further into the color change, *M. xanthus* was found to undergo a spontaneous phase change observed phenotypically by conversion of colony color between yellow and tan [4,5,6].
- The variable phenotype was resemblant to that of tan phased WT that similarly impairs FB development [6].

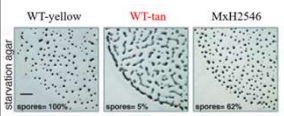

Figure 7. Tan phase impacts developmental phenotype and heat resistance Figure modified from (Furusawa et. al, 2011) [6]

Hypothesis & Research Objectives

Does an Arginine Kinase loss change the color phase preference which may then be attributed to the developmental variability?

- Determine the flipping frequency of dAK strains and if there is a color phase preference.
- Examine developmental phenotype of strains is dependent on initial phase- if the isolated phases prompt rescued or exacerbate arrested FB phenotypes
- Analysis of dAK's genetic expression to identify any changes correlated with arrested development that are linked to phase variation

Hypothesis: *dAK strain's developmental variability is associated to the phase changing mechanism due to resemblance to the tan phase phenotype.*

Loss of AK Leads to a Tan Phase Preference

Table 1. Frequency of Tan Colonies[a]

Sample	Average	Standard Deviation	Variance
Wild type	0	0	0
ΔAK2b-Y	55.32	28.38	805.31
ΔAK2b-Y DF	49.67	22.64	512.78
ΔAK2b-T	89.81	5.84	34.13
ΔAK2b-YT	75.64	16.03	257.01
ΔAK2b-YT2	82.74	9.79	95.90
WT-0228 2cT[b]	100	0	0

[a]Frequency of tan colonies was determined upon averaging the percentages across 4 trials. [b]All the MXAN_0228 samples follow the same trend with only tan colonies formed upon plating on nutrient rich CTTYE media.

dAK strains increase the tan flipping rate. Losing the ATP buffering capacity likely induces the cell to switch towards tan phase to decrease the requirement of energy demanding processes and takes advantage of the limited lysing of cells due to prevention of toxin and antibiotic buildup [5,6].

Quantitative Data Supports Variability Significance

Table 2. Area* of Defined Contours

Sample	15 hours	24 hours	48 hours	72 hours
Wild type	566 ± 727	746 ± 865	829 ± 814	843 ± 845
ΔAK2b Y	149 ± 262	224 ± 362	501 ± 897	533 ± 977
ΔAK2b Y DFs	96 ± 249	154 ± 251	343 ± 643	361 ± 668
ΔAK2b T	64 ± 179	197 ± 278	359 ± 484	372 ± 522
ΔAK2b YT	73 ± 222	271 ± 364	636 ± 724	641 ± 733
ΔAK2b YT2	122 ± 288	200 ± 342	422 ± 802	439 ± 775
WT-0228 2cT	516 ± 714	735 ± 903	813 ± 755	901 ± 866
WT-0228 3bT	451 ± 636	624 ± 750	788 ± 750	789 ± 762
ΔAK2b-0228 3a1	68 ± 222	247 ± 398	557 ± 802	588 ± 865
ΔAK2b-0228 3a5	*113 ± 398*	131 ± 268	388 ± 857	406 ± 951

Table 3. Intensity* of Defined Contours

Sample	15 hours	24 hours	48 hours	72 hours
Wild type	81 ± 19	74 ± 19	61 ± 28	62 ± 29
ΔAK2b Y	95 ± 9	90 ± 11	84 ± 15	83 ± 17
ΔAK2b Y DFs	87 ± 8	87 ± 9	**84 ± 12**	**85 ± 14**
ΔAK2b T	81 ± 8	*76 ± 10*	*71 ± 12*	**73 ± 14**
ΔAK2b YT	91 ± 11	85 ± 13	*75 ± 12*	**74 ± 15**
ΔAK2b YT2	90 ± 13	96 ± 14	**86 ± 11**	**88 ± 12**
WT-0228 2cT	75 ± 12	66 ± 18	51 ± 23	48 ± 22
WT-0228 3bT	82 ± 15	71 ± 17	51 ± 22	51 ± 23
ΔAK2b-0228 3a1	94 ± 14	**91 ± 9**	**78 ± 12**	**76 ± 12**
ΔAK2b-0228 3a5	85 ± 7	88 ± 5	**85 ± 6**	**80 ± 10**

Area of FBs ranged from 5.5 to 9999, averaged across 5 trials with all samples indicating significance when compared to WT and dAK Y baselines except 3a5 identical to dAK Y's initial lag.

Intensity values ranged between 0 to 255 with lower numbers corresponding to darker mounds. All samples show significance except tan phased dAK samples that attain WT FB's darkness 48 hours post starvation confirming tan dAK's rescued phenotype in terms of opacity.

Genetic Expression Changes Govern Developmental Phenotype

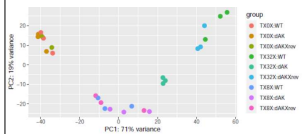

Figure 14. PCA Analysis across WT, dAK (arrested), and dAR (revertant) samples.

Throughout development samples are spread further apart to show FB formation and sporulation require high genetic regulation. Surprisingly, dAR (revertant) clusters closer to WT samples despite AK deletion.

Table 4. Genetic profile throughout development

Samples	Differentially expressed genes	upregulated		downregulated	
		total	LC>1	total	LC>-1
WT 0 vs 8	2268	1055	972	1213	1163
WT 0 vs 32	3808	1858	1538	1950	1737
dAR 0 vs 8	2702	1306	1069	1396	1242
dAR 0 vs 32	3812	1845	1458	1967	1683
dAK 0 vs 8	3815	1873	1490	1942	1605
dAK 0 vs 32	4090	2062	1417	2028	1556

*LC refers to a change in genetic expression with a log fold change of base 2

- DEGs increase throughout development with spore formation and maturation (32 hours) being the most dramatic change.
- dAK (arrested) has ~1.5x WT's DEGs at 8-hour time point, indicating importance to the variability.
- The more consistent revertant phenotype is genetically identical to WT at 0 hours and with minimal changes at 8 and 32 hours.
- dAK upregulates more genes in comparison to WT and dAR, associating the variability and ability to toggle phenotypes with high DEGs

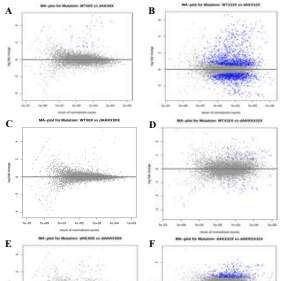

Figure 15. dAK upregulates more genes in comparison to WT and dAR. Significant DEGs are shown with blue dots.

Table 5. Genetic profile differences across strains

Samples	Differentially expressed genes	upregulated		downregulated	
		total	LC>1	total	LC>-1
WT vs dAK 0	39	39	39	0	0
WT vs dAK 8	87	73	70	14	11
WT vs dAK 32	2567	1327	767	1240	469
WT vs dAR 0	0	0	0	0	0
WT vs dAR 8	6	5	5	1	1
WT vs dAR 32	40	24	17	16	11
dAK vs dAR 0	29	1	1	28	28
dAK vs dAR 8	177	18	11	159	123
dAK vs dAR 32	1044	366	119	678	442

*LC refers to a change in genetic expression with a log fold change of base 2

Arginine Kinase Developmental Phenotype Variability Indicates Switch-like Behavior

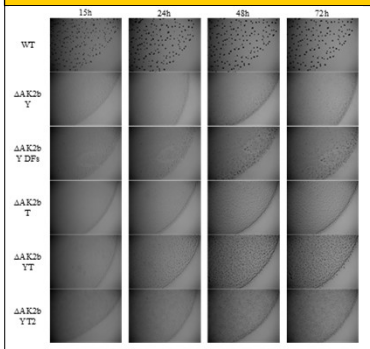

Figure 8. Fruiting body formation of dAK strain varies according to initial phase.

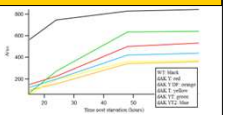

Figure 9. Area of Figure 8's FB mounds.

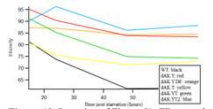

Figure 10. Intensity of Figure 8's FB mounds.

The initial starting phase of dAK strains impacts their FB phenotype such that tan strains progress to late stages of mound formation while the yellow strains reamain arrested in early development with slight variability across replicates.

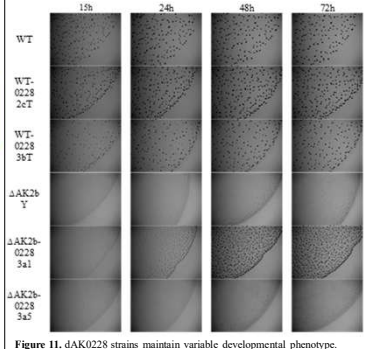

Figure 11. dAK0228 strains maintain variable developmental phenotype.

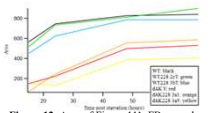

Figure 12. Area of Figure 11's FB mounds.

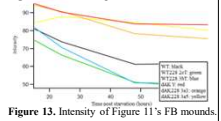

Figure 13. Intensity of Figure 11's FB mounds.

Despite locking within the tan phase, dAK0228 strains maintain variability while WT0228 maintain a consistent FB mound formation with slight deviations from WT's. Thus, the instability is associated to an AK deletion and proposed to be due to yellow phase reversion.

Phase Variation Involved in dAK's Developmental Rescuing Capability

Table 6. DEGs of WT0 vs dAK0

P-value adj.	Log Fold Change*	MXAN #	Annotation
Cell signaling and development response			
1.79E-14	5.18	MXAN_7370	Serine/threonine-protein kinase
1.80E-12	4.98	MXAN_7371	Serine/threonine-protein kinase
3.07E-11	4.26	MXAN_4375*	Serine/threonine-protein kinase
8.52E-08	3.92	MXAN_2840	Serine/threonine-protein kinase
7.16E-07	3.87	MXAN_4475*	Serine/threonine-protein kinase
1.42E-07	2.72	MXAN_4373	Serine/threonine-protein kinase
1.31E-04	2.72	MXAN_3272	Serine/threonine-protein kinase
1.31E-03	2.11	MXAN_4482	Serine/threonine-protein kinase
1.32E-02	1.50	MXAN_7269	Serine/threonine-protein kinase
7.56E-03	1.67	MXAN_7464	Ig-like domain-containing protein
1.58E-06	2.95	MXAN_6104	Response regulator
Ion transport			
3.83E-04	**4.50**	**MXAN_5858**	**Sodium/solute symporter**
1.97E-04	**3.61**	**MXAN_5859**	**Ion transporter**
Metabolic function			
3.07E-11	3.92	MXAN_2399*	Protein kinase
3.92E-04	**3.87**	**MXAN_2352**	**Phosphatase kinase**
9.54E-03	2.03	MXAN_4369	Class I SAM-dependent methyltransferase
2.53E-03	1.96	MXAN_0050	AHH domain-containing protein (toxin)
4.51E-02	**1.76**	**MXAN_1674**	**NAD-dependent epimerase/dehydratase family protein**
DNA regulation proteins and transcription factors			
3.07E-11	4.51	MXAN_6998	HTH domain-containing protein
5.16E-09	3.98	MXAN_4481	HTH domain-containing protein
1.01E-05	3.32	MXAN_4372	HTH domain-containing protein
7.04E-04	2.53	MXAN_4480	HTH transcriptional regulator
1.17E-03	**2.16**	**MXAN_7467**	**HTH domain-containing protein**
1.04E-02	**1.58**	**MXAN_7369**	**HTH domain-containing protein**
2.71E-09	3.70	MXAN_3708	Uma2 family endonuclease
7.56E-03	**1.63**	**MXAN_0519**	**Uma2 family endonuclease**
2.58E-05	3.03	MXAN_7372	Zinc ribbon domain-containing protein
6.25E-06	2.62	MXAN_2371	G/U mismatch-specific DNA glycosylase
4.38E-05	2.33	MXAN_0201	Alpha/beta fold hydrolase
7.56E-03	**1.46**	**MXAN_2897**	**DNA mismatch repair protein MutS**
Genes of unknown function			
8.52E-08	3.88	MXAN_7268	DUF2381 family protein
1.91E-05	2.55	MXAN_4325	DUF2381 family protein
2.16E-03	**2.38**	**MXAN_RS36745**	**Pseudo; DUF2381 family protein**
7.43E-04	2.14	MXAN_4374	DUF2381 family protein
1.32E-02	1.92	MXAN_0048	Hypothetical protein
7.35E-03	**1.86**	**MXAN_0049**	**DUF1629 domain-containing protein**
6.90E-03	1.62	MXAN_4483	DUF2381 family protein
3.99E-02	1.50	MXAN_0047	DUF1629 domain-containing protein
3.40E-02	1.18	MXAN_2297	Hypothetical protein

*LC refers to a log fold change of base 2 [7]upregulation of tan associated genes. *Arginine kinase gene. **Genes upregulated in dAK but not similarly downregulated in dAR**

Table 7. Expression* of DKxanthene Pigment Synthesis Genes

Annotation[7]	MXAN #	WT0 vs WT32	dAK 0 vs dAK32	dAR 0 vs dAK32	dAK32 vs dAR32
Hypothetical protein	MXAN_4288	**2.35**	**0.86**	**1.36**	**0.91**
dkxM; polyketide synthase	MXAN_4292	0.64	**-1.68**	-0.35	**1.64**
dkxK; arsenical-transporting ATPase family	MXAN_4294	0.81	**-1.82**	-0.27	**1.82**
dkxJ; patatin-like phospholipase family	MXAN_4295	**1.26**	**-0.89**	0.33	**1.64**
dkxG; polyketide synthase type 1	MXAN_4298	0.43	**-1.84**	-0.50	**1.76**
dkxG; polyketide synthase type 1	MXAN_4299	0.74	**-1.49**	-0.15	**1.74**
dkxE; polyketide synthase type 1	MXAN_4301	0.15	**-1.95**	**-0.98**	**1.59**

*Differential expression is expressed in log fold changes with base 2. Upregulated genes have a positive log fold change while downregulated genes are negative. Bolded values are correspondent to significantly expressed DEGs. [7]Annotations and pathway *

- The rescued dAK-2b strain downregulates all but 10 DEGs bolded showing increased genetic expression leads to arrested phenotype.
- Upregulation of tan phase genes is found within the arrested phenotype
- Conversely the upregulation of yellow phase genes in the rescued strain show potential yellow phase complementation and synergism of metabolites to allow FB mound maturation

Model of dAK's Instability

- dAK strains push the majority of the population to be within the tan phase due to being the less energy demanding state.

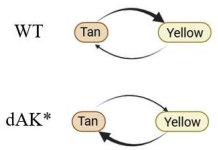

Figure 16. Proposed mechanism of dAK strains' phase preference in relation to WT.

- Variability in developmental phenotype is associated to yellow phase reversion
- Data supports yellow phase synergism forms mature FB leading to a rescued phenotype.
- This supports previous modeling of yellow phase coating of FB mounds

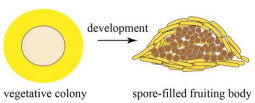

Figure 17. Model of yellow phased colonies' coating of mature FBs to form dark defined spores in WT. Figure modified from (Dziewanowska et. al, 2014) [5]

- dAK's phase variability is masked within MXAN_0228 deletion strains that is likely overwhelmed when the strain is subject to stress indicating a potential threshold.

Figure 18. Proposed mechanism of dAK0228 strains' yellow phase reversion masking being overwhelmed when subject to stress.

Future Research

- Repetition of experiments while refining the code used to collect quantitative data to add more parameters to better quantify results .
- Conduct RNA sequencing during development with WT0228 dAK0228 samples to see if yellow phase masking is overwhelmed during starvation stress by confirmation of increased DKX gene expression that was previously inhibited [6].
- Create double mutants with the 10 dAK upregulated genes (bolded in Table 6) to confirm their role in arrested phenotype
- Look further into pathways the DEGs map onto to create a mechanism for AK action computationally, thus better understanding its role within *M. xanthus* and ultimately evolution within proteobacteria.

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