

Aromatic Biosynthesis in *Archaeoglobus fulgidus*

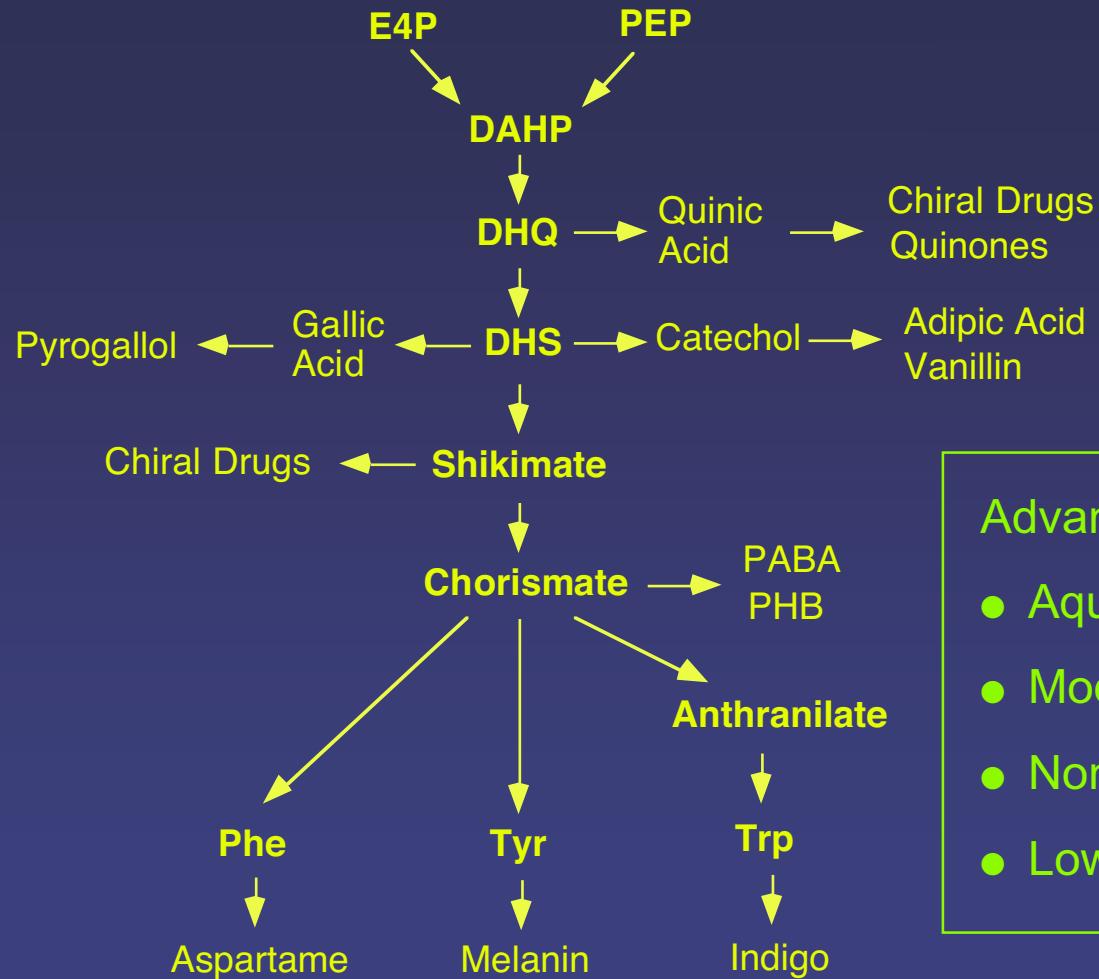
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- Hyperthermophilic Archaea, a source of robust enzymes for biosynthesis
- An extremely stable and highly active shikimate dehydrogenase (SDH)
- A novel trifunctional polypeptide exhibiting chorismate mutase (CM), prephenate dehydrogenase (PDH), and prephenate dehydratase (PDT) activity
- Analysis of gene expression under Trp⁺/Trp⁻ conditions using a whole-genome DNA microarray for *A. fulgidus*



Aromatic Biosynthesis Pathways Lead to Industrial Products



Advantages

- Aqueous solvent
- Moderate T, p
- Non-toxic intermediates
- Lower raw material cost

Not all metabolites (bold) are shown. **E4P**, D-erythrose-4-phosphate; **PEP**, phosphoenolpyruvate; **DAHP**, 3-deoxy-D-arabino-heptulosonate-7-phosphate; **DHQ**, 3-dehydroquinate; **DHS**, 3-dehydroshikimate; **PABA**, *p*-aminobenzoic acid; **PHB**, *p*-hydroxybenzoic acid; **Phe**, phenylalanine; **Tyr**, tyrosine; **Trp**, tryptophan.

A. fulgidus: A Well-Characterized Hyperthermophilic Archaeon

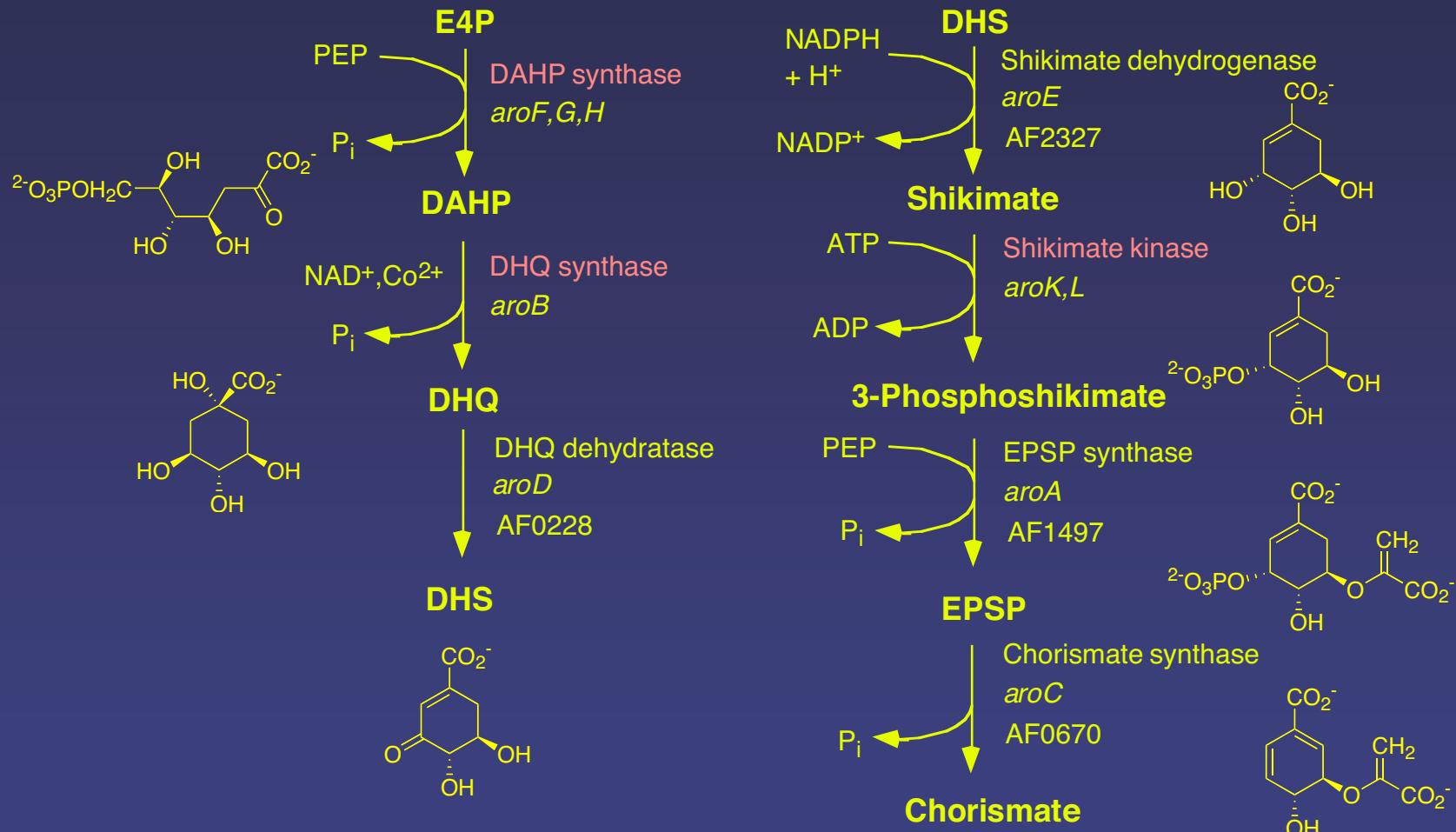
- *Archaeoglobus* = ancient sphere; coccoid
fulgidus = shining
- Marine archaeon
 - Isolated from hydrothermal vents, oil wells
- Optimal anaerobic growth temperature 83 °C
- Energy metabolism:
 $\text{lactate} + 1.5 \text{SO}_4^{2-} + 4 \text{H}^+ \longrightarrow 3 \text{CO}_2 + 1.5 \text{H}_2\text{S} + 3 \text{H}_2\text{O}$
- Genome is sequenced
 - 2,493 ORFs
 - 1,290 with no assigned biological role



Stetter, *System. Appl. Microbiol.*, 1988, 10, 172-173

Klenk *et al.*, *Nature*, 1997, 390, 364-370

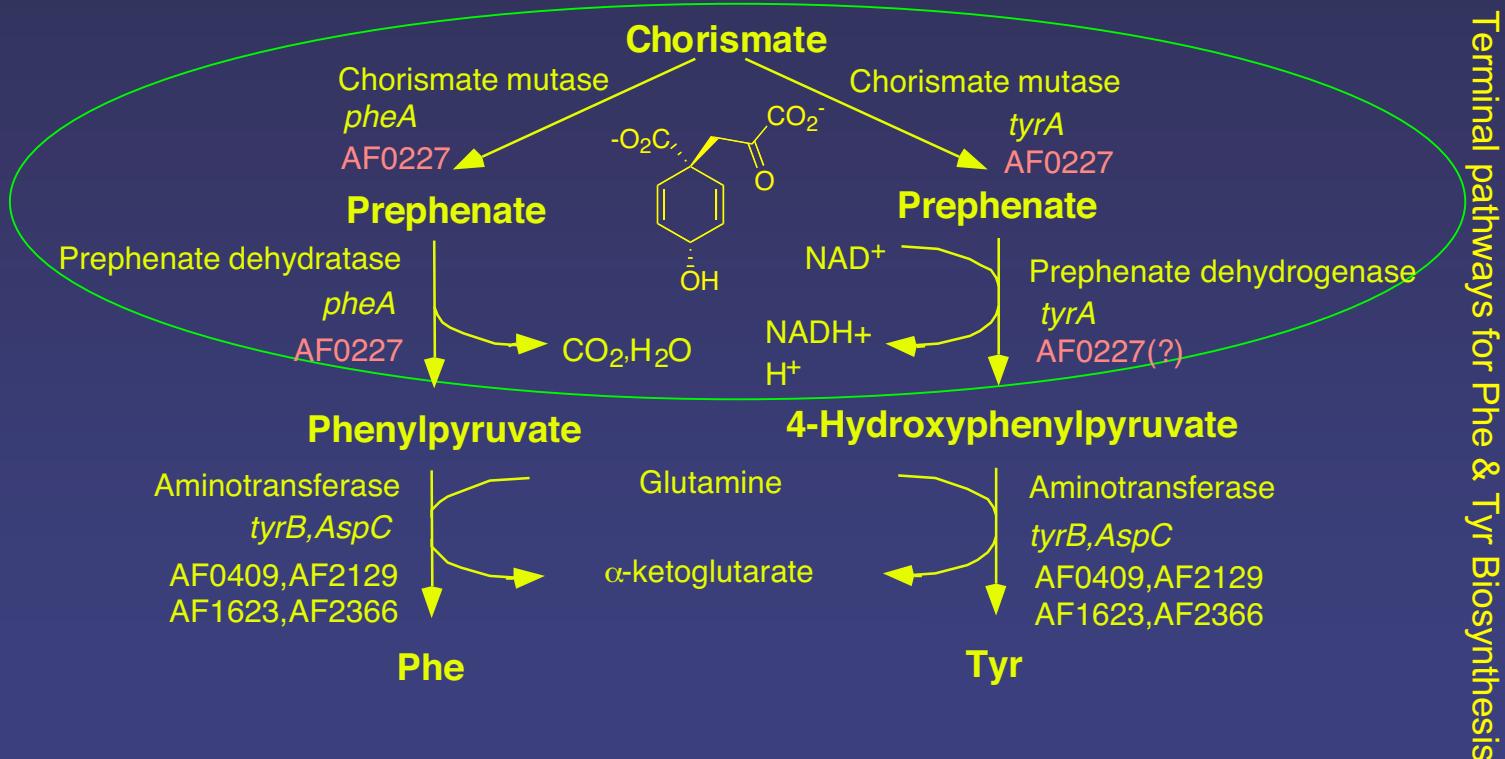
Shikimate Pathway Likely Contains Three Novel Enzymes



A. fulgidus ORFs (AF#) where identified. **E4P**, D-erythrose-4-phosphate; **PEP**, phosphoenolpyruvate; **DAHP**, 3-deoxy-D-arabino-heptulosonate-7-phosphate; **DHQ**, 3-dehydroquinate; **DHS**, 3-dehydroshikimate; **EPSP**, 5-enolpyruvylshikimate-3-phosphate



A Novel Trifunctional Chorismate Mutase/Prephenate Dehydratase/Prephenate Dehydrogenase?

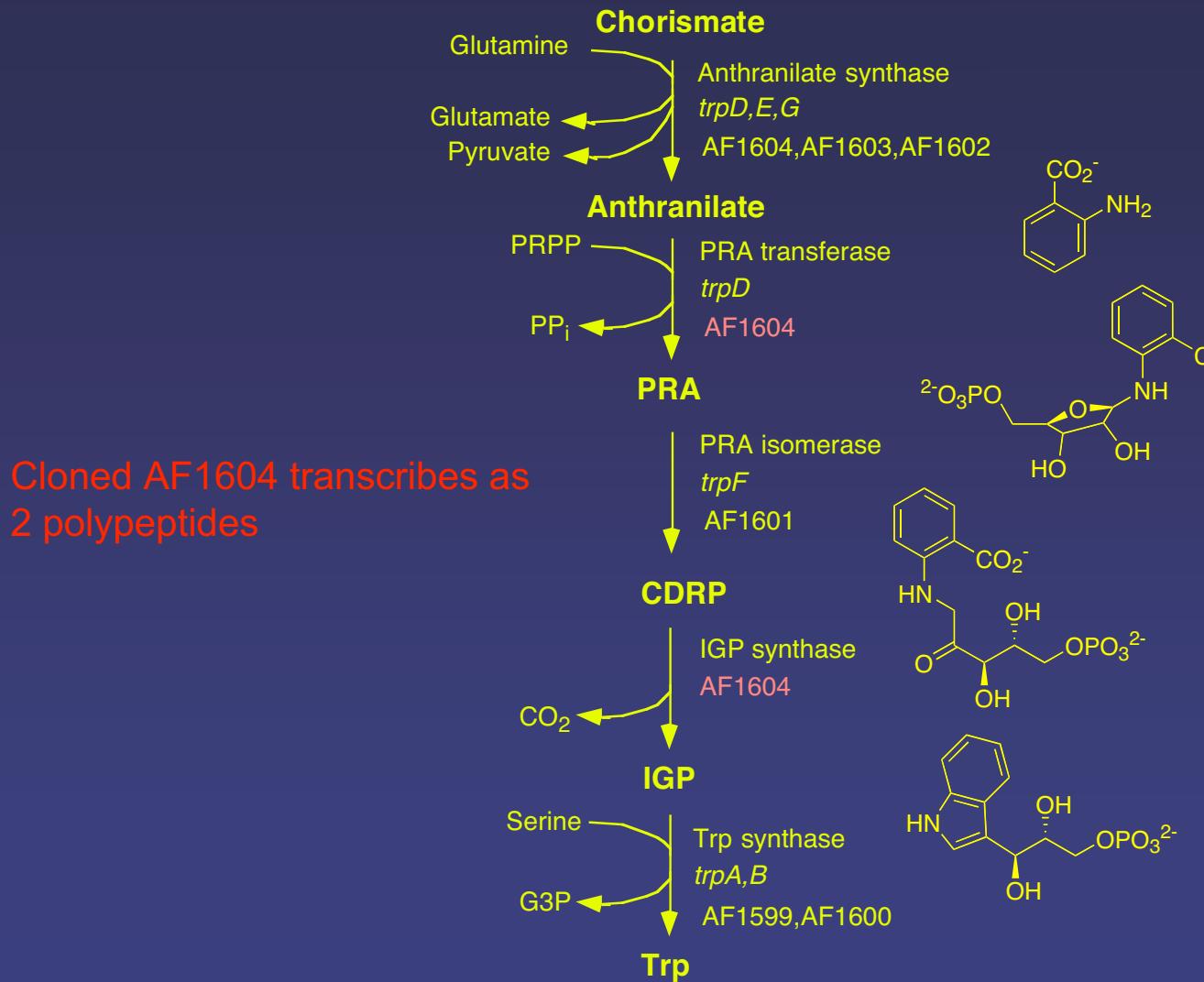


No aromatic aminotransferase genes (*tyrB*) identified on the *A. fulgidus* genome

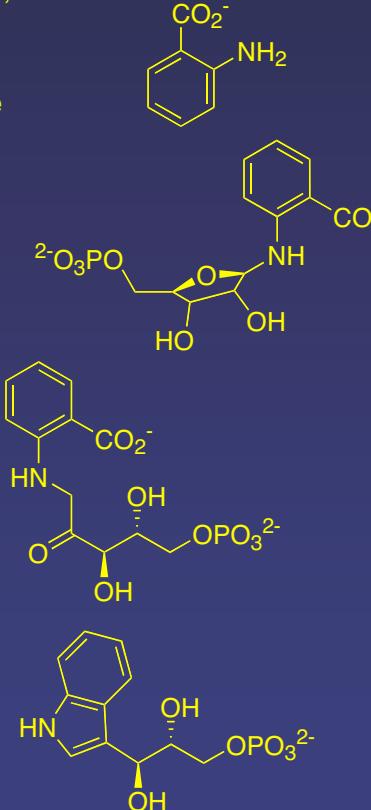


Trp Operon Exists in *A. fulgidus*

Terminal pathway for Trp biosynthesis



Cloned AF1604 transcribes as
2 polypeptides



A. fulgidus ORFs (AF#) where identified. **PRA**, phosphoribosyl anthranilate; **CDRP**, 1-(o-carboxyphenylamino)-1-deoxyribulose-5-phosphate; **IGP**, indoleglycerol phosphate



Most Identified Genes Cloned, Many Soluble Overexpression Products

AF#	Enzyme	Gene	Size (bp)	AA's	MW _{calc}	Solubility	Comments
AF0228	DHQ dehydratase	<i>aroD</i>	588	196	22206.99	Soluble	crystalized
AF2327	Shikimate dehydrogenase	<i>aroE</i>	807	269	29136.83	Soluble	homodimer
AF1497	EPSP synthase	<i>aroA</i>	1248	416	45085.29	Insoluble	monomer
AF0670	Chorismate synthase	<i>aroC</i>	1080	360	39262.66	Soluble	homotrimer
AF0227	Chor/preph/preph	<i>pheA</i>	1860	620	70946.38	Soluble	trifunctional
AF0409	Asp aminotransferase	<i>aspB-4</i>	1158	386	43140.16		homodimer
AF2129	Asp aminotransferase	<i>aspB-2</i>	1137	379	42698.89	Soluble	homodimer
AF1623	Asp aminotransferase	<i>aspB-3</i>	1170	390	43615.06	Soluble	homodimer
AF2366	Asp aminotransferase	<i>aspB-1</i>	1119	373	41703.09	Insoluble	homodimer
AF1603	Anthranilate synthase I	<i>trpE</i>	1233	411	46345.54	Insoluble	
AF1602	Anthranilate synthase II	<i>trpG</i>	534	178	19592.77		
AF1604	PRA transf/IGP synth	<i>trpD</i>	1638	546	59327.61	Insoluble	2 polypeptides
AF1601	PRA isomerase	<i>trpF</i>	597	199	22017.2	Soluble	monomer
AF1599	Trp synthase α subunit	<i>trpA</i>	744	248	27299.98	Soluble	
AF1600	Trp synthase β subunit	<i>trpB-2</i>	1191	397	43744.69	Soluble	

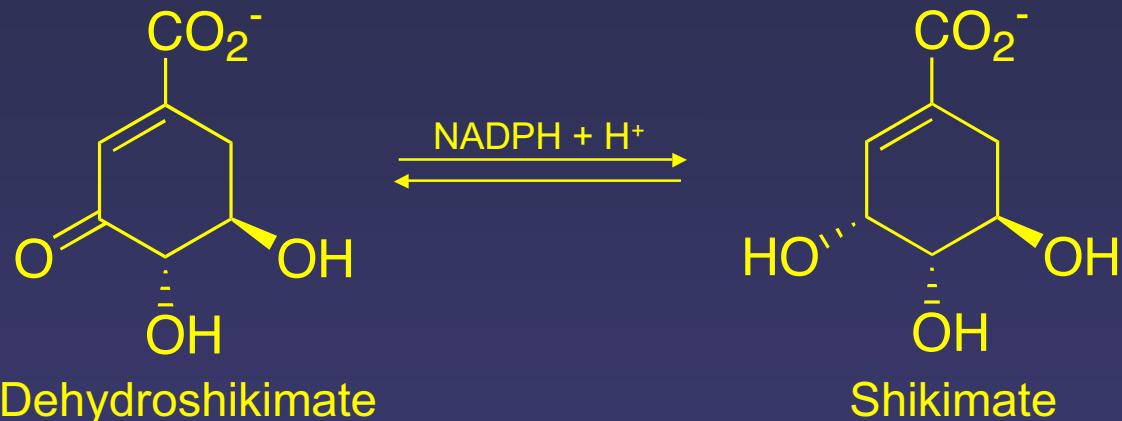
Overexpression strain: BL21(DE3)C⁺ RIL (Stratagene)

Vector: pET-3a (Amp^r Novagen)

Inducer: IPTG (*lac* promoter)



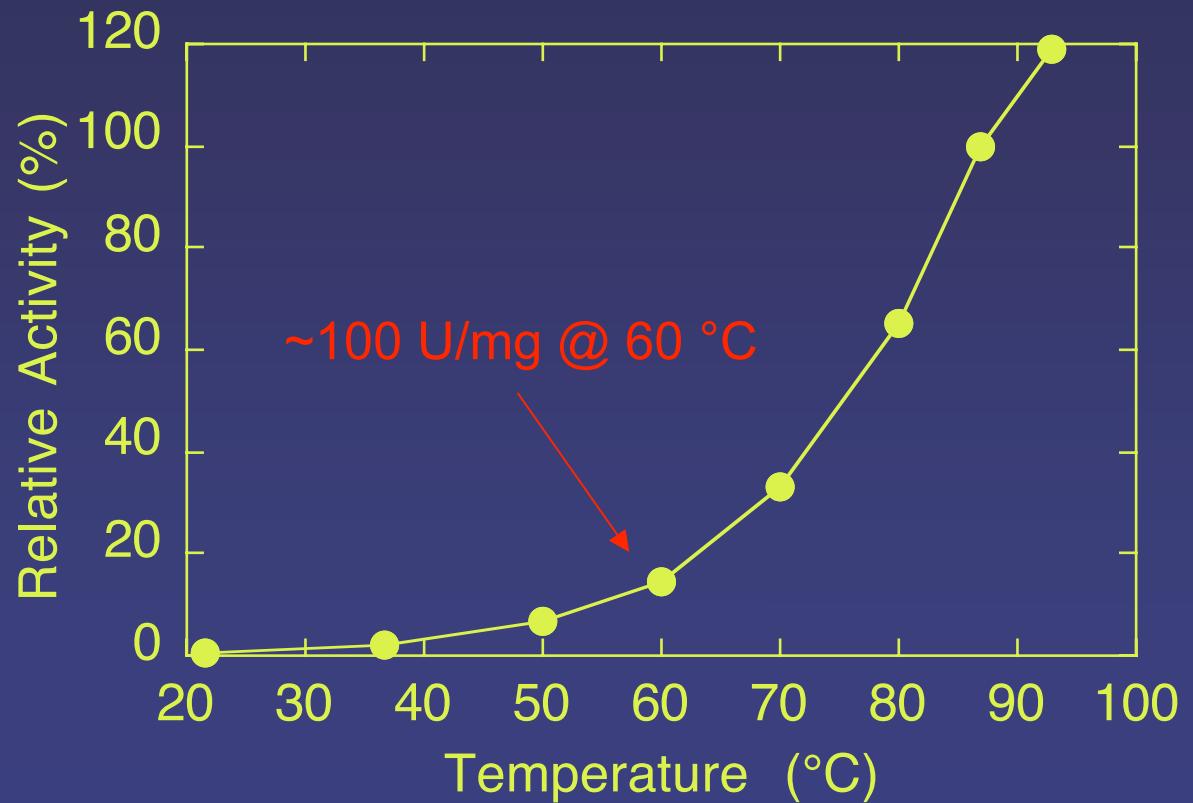
Properties of *A. fulgidus* Shikimate Dehydrogenase



Assay Conditions: 87 °C, pH 7.3

- $M_r \sim 32 \text{ kDa}; M_{\text{calc}} = 29.1 \text{ kDa}$ *E. coli*: $M_{\text{calc}} = 29.4 \text{ kDa}$
- $K_{m, \text{NADP}} = 190 \pm 10 \mu\text{M}$ *E. coli*: pH 8.0, 31 μM
- $K_{m, \text{NAD}} = 11.4 \pm 0.4 \text{ mM}$
- $K_{m, \text{shik}} = 170 \pm 30 \mu\text{M}$ *E. coli*: pH 8.0, 55 μM
- $T_{\text{opt}} \sim 90 \text{ }^\circ\text{C}; \text{pH}_{\text{opt}} \sim 7-7.5$ *E. coli*: pH 9
- Spec. Act. 753 U/mg *E. coli*: 860 U/mg, 25°C

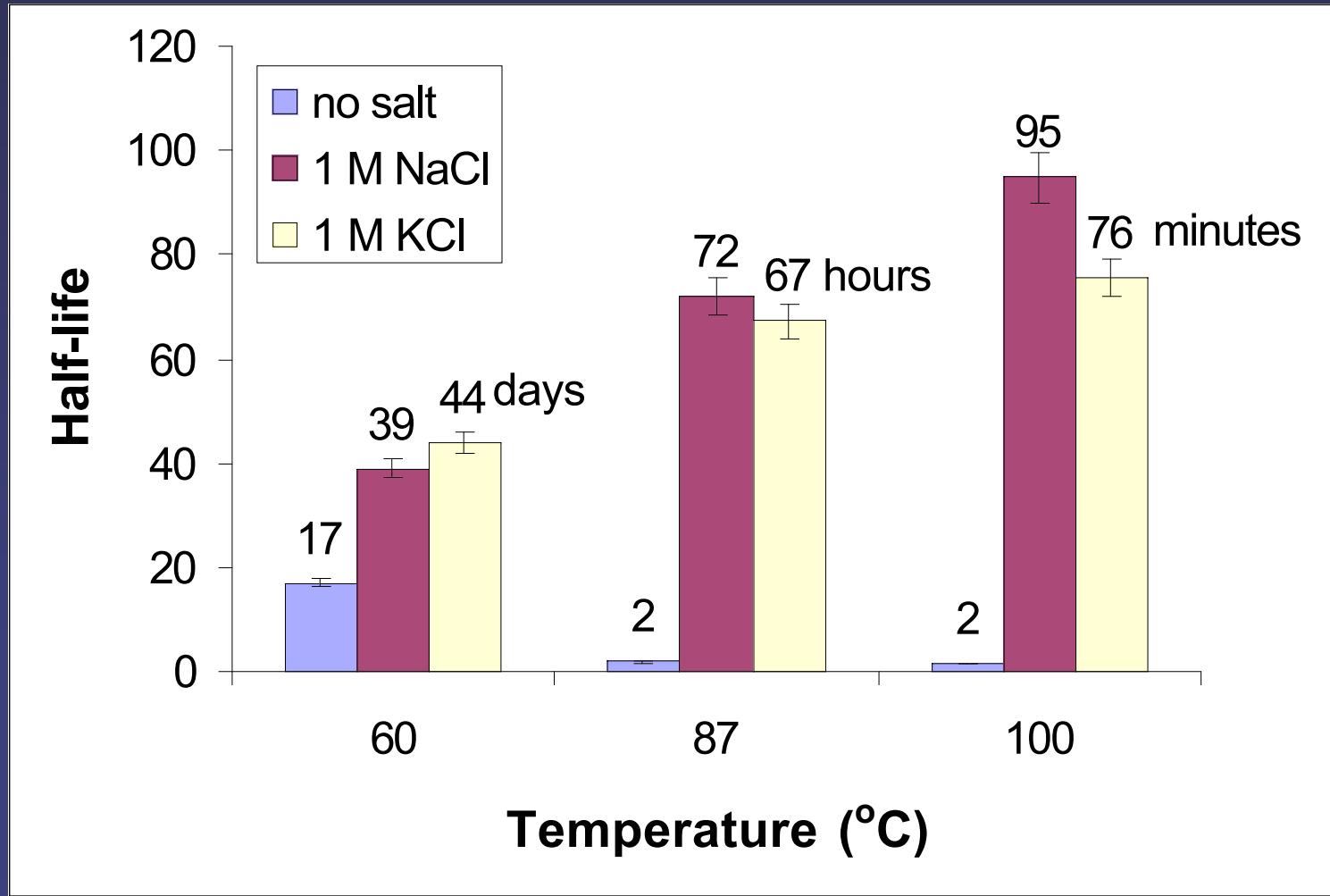
A. fulgidus SDH Very Active at Moderate Temperature



Activity @ 25 °C (2.93 U/mg) is comparable to *P. sativum* (2.25 U/mg)

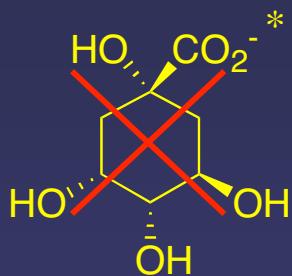


A. fulgidus SDH is Remarkable Stable $t_{1/2}$ @ 60 °C ~ 17 days

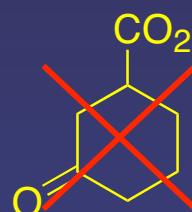
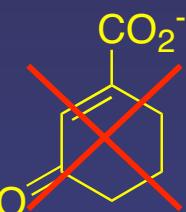
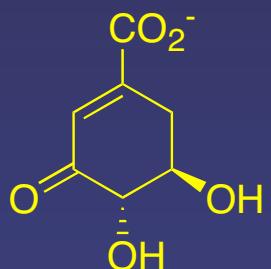


A. fulgidus SDH is Selective

Oxidation:



Reduction:



*PQQ does not serve as electron acceptor for quinate oxidation as observed for the gram negative bacterial SDH.

AF0520 is 34.4 % identical and 55.6% Similar to *M. jannaschii* Shikimate Kinase

GENOME	Step 1 EC 4.1.2.15 2-Dehydro-3-deoxyphosphoheptonate aldolase <i>aroH, aroG, aroF</i>	Step 2 EC 4.6.1.3 3-Dehydro-quinate synthase <i>aroB</i>	Step 3 EC 4.2.1.10 3-Dehydro-quinate dehydratase <i>aroD</i>	Step 4 EC 1.1.1.25 Shikimate 5-dehydrogenase <i>aroE, ydiB</i>	Step 5 EC 2.7.1.71 Shikimate kinase (Bacterial and Eukaryotic) <i>aroK, aroL</i>	Step 6 EC 2.5.1.19 Phosphoshikimate 1-carboxyvinyl transferase <i>aroA</i>	Step 7 EC 4.6.1.4 Chorismate synthase <i>aroC</i>	(Step 5) (EC 2.7.1.71) Predicted Shikimate kinase (Archaeal)
<i>E. coli</i>	REC01661 REC05569 REC00721	REC05984	REC01650	REC05912 REC01649	REC05985 REC00372	REC00874	REC05421	-
<i>B. subtilis</i>	RBS02969	RBS02266	RBS02304 RBS02442	RBS02559	RBS00316	RBS02256	RBS02267	-
<i>T. maritima</i>	RTM00236	RTM00229	RTM00228	RTM00231	RTM00229	RTM00232	RTM00230	-
<i>S. pneumoniae</i>	RPN00965-66	RPN00386	RPN00384	RPN00385	RPN00391	RPN00390	RPN00387	-
<i>T. pallidum</i>	-	-	-	-	-	-	-	-
<i>S. cerevisiae</i>	RSC01644 RSC08655		RSC06906	(Pentafunctional Enzyme)			RSC05895	-
<i>M. jannaschii</i>	-	-	RMJ05308	RMJ00483	-	RMJ00806	RMJ07769	RMJ07785
<i>A. fulgidus</i>	-	-	RAG18799	RAG27692	-	RAG27692	RAG50410	RAG45918
<i>A. pernix</i>	RAP00399	RAP00398	RAP00397	RAP00396	-	RAP00394	RAP00393	RAP00395
<i>P. furiosus</i>	RPF01413	RPF01411-12	RPF01410	RPF01409	-	RPF01402	RPF01401	RPF01407-08
<i>P. horikoshii</i>	-	-	-	-	-	-	-	-

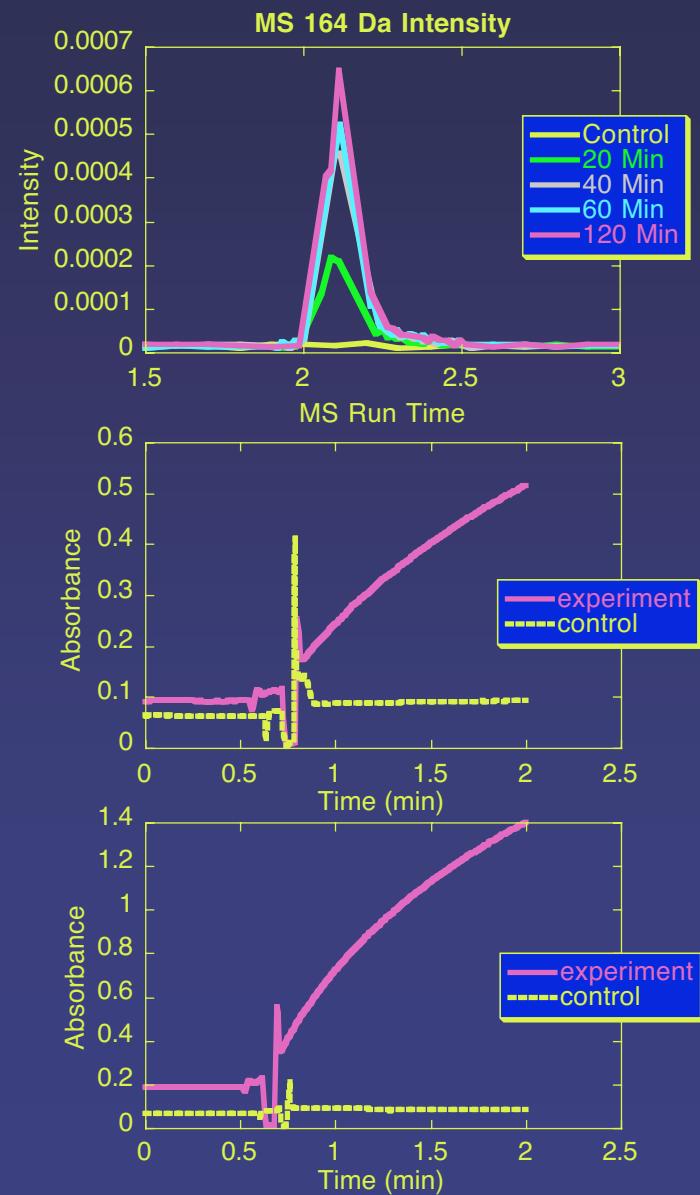
AF0520

Daugherty *et al.*, *J. Bacteriol.*, 2001, 183, 292-300

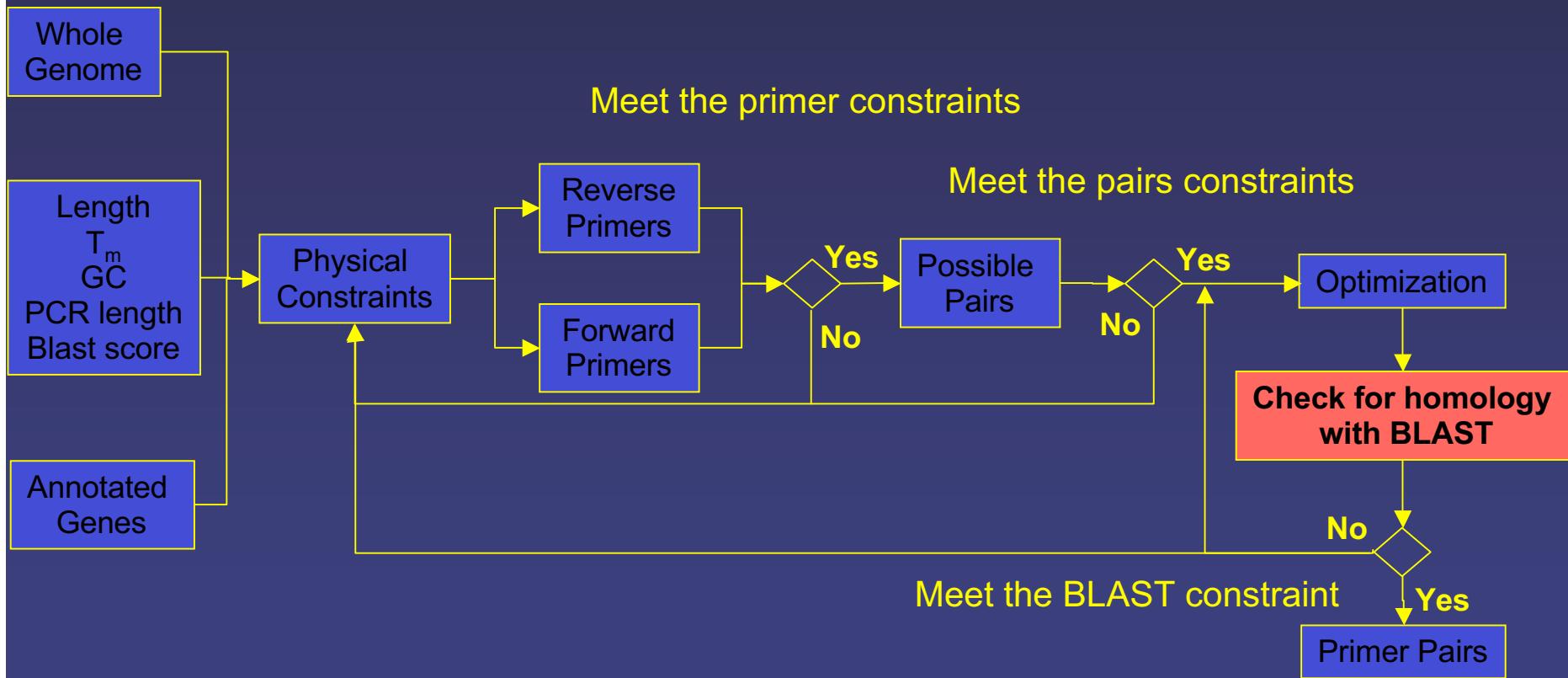


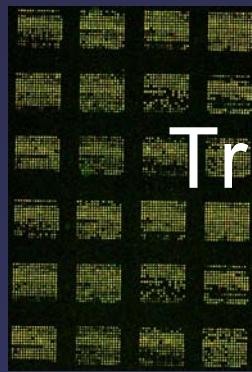
Trifunctional CM / PDT / PDH Polypeptide Confirmed

- PDT activity confirmed
 - LC/MS assay; appearance of phenylpyruvate
- CM activity confirmed using PDH
 - Appearance of NADH @ 340 nm
- PDH confirmed
 - Appearance of NADH @340 nm

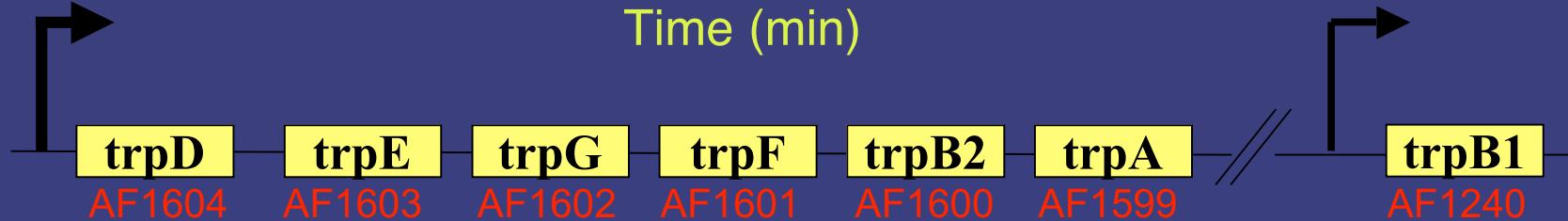
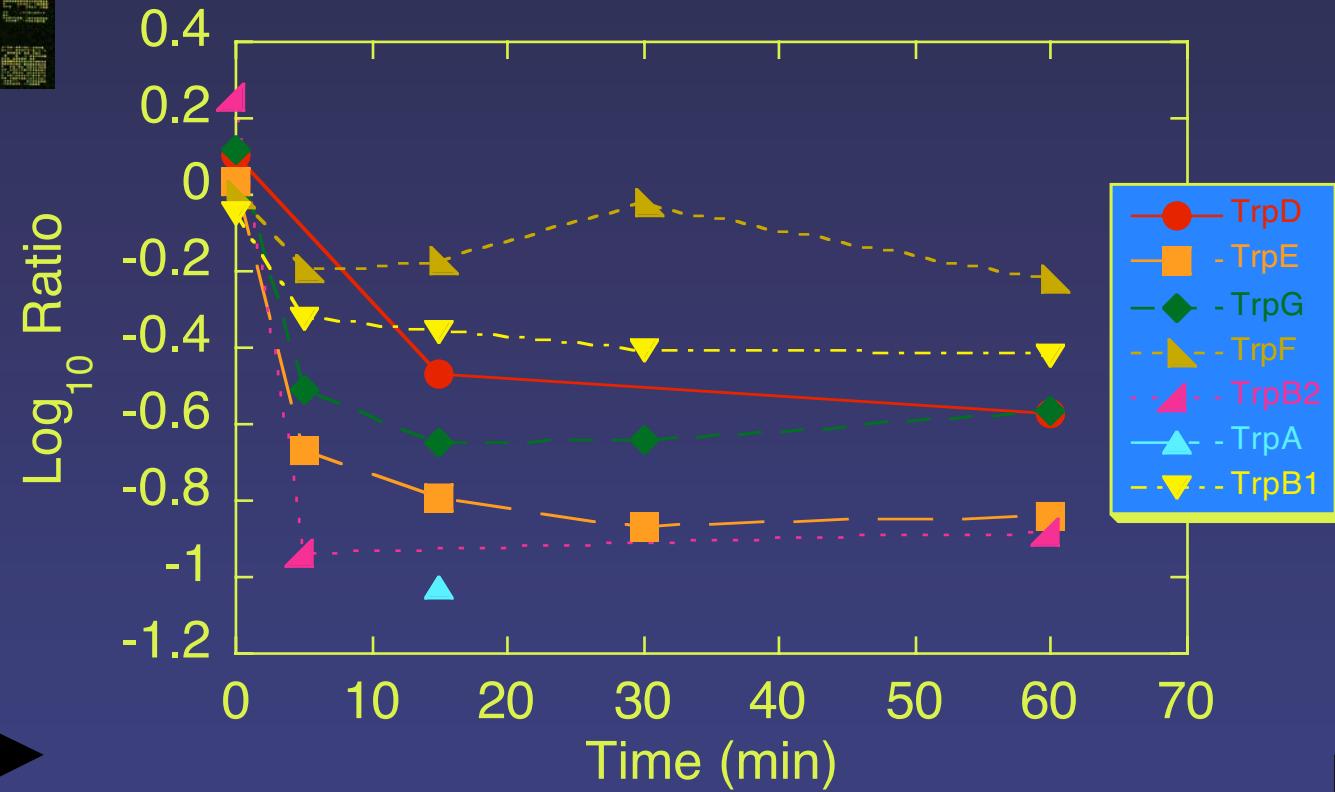


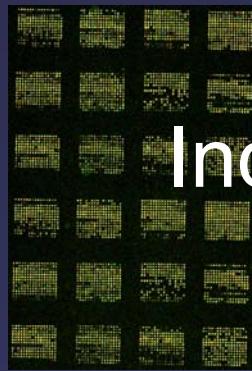
Whole-Genome *A. fulgidus* Microarray: Programmed Primer Pair Optimization



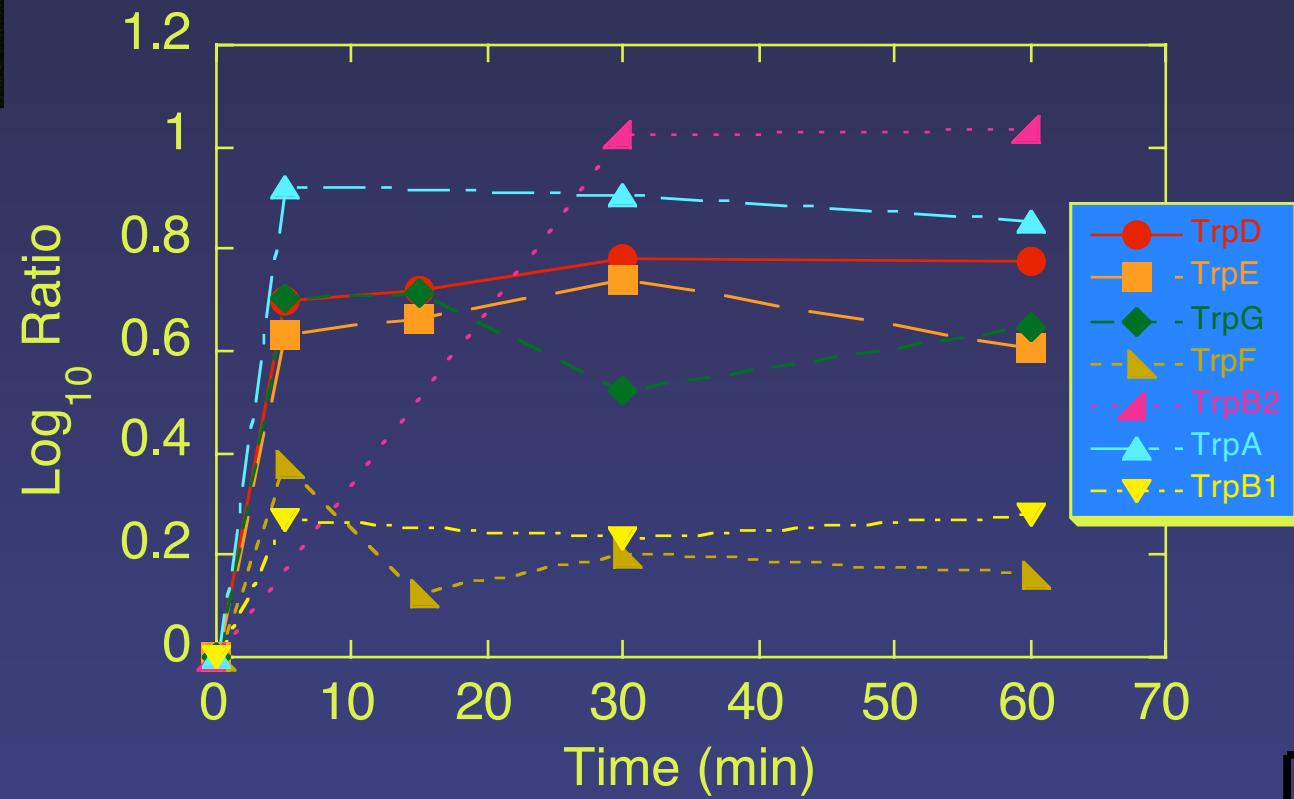


Trp Addition Suppresses the Trp Operon





Indole Acrylate Activates the Trp Operon



Many Similarities in *E. coli* and *A. fulgidus* Microarray Data

	<i>E. coli</i> Trp ⁺	<i>E. coli</i> IAA ⁺	<i>A. fulgidus</i> Trp ⁺	<i>A. fulgidus</i> IAA ⁺
<i>trpE</i>	Down	Up	Down	Up
	Down	Up	Down	Up
	Down	Up	Down	Up
	Down	Up	Down	Up
	Down	Up	Down	Up
<i>trpR</i>	Slightly down	Slightly up	N/A	N/A
	N/A	N/A	Down	Up
	N/A	N/A	Down	Up
<i>aroF</i>	Down	Unchanged	N/A	N/A
	Down	Unchanged	Unchanged	Up AF0227
	Down	Unchanged	N/A	N/A
<i>tyrA</i>	Down	Unchanged	Unchanged	Up AF0227
	Down	Unchanged	N/A	N/A
<i>aroH</i>	Slightly down	Up	N/A	N/A
	Slightly down	Unchanged	N/A	N/A
<i>aroL</i>	Slightly down	Unchanged	N/A	N/A
	Down	Unchanged	N/A	N/A
<i>mtr</i>	Down	Up	N/A	N/A
	Up	Unchanged	N/A	N/A
<i>tnaB</i>	Up	Unchanged	N/A	N/A
<i>tnaA</i>	Up	Unchanged	N/A	N/A



[Known *E. coli* operon regulated by TrpR]

[Known *E. coli* operon regulated by TyrR]

Concluding Remarks

- Hyperthermophilic Archaea provide a source of very active enzymes, highly stable at moderate temperature
- Archaea provide a source of novel enzymes, e.g., trifunctional CM/PDH/PDT polypeptide, shikimate kinase
- LC/MS, a useful tool for enzyme assays, e.g., PDT
- Whole-genome DNA microarray for functional genomics/proteomics, identification of operons, e.g., trp



Acknowledgements



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