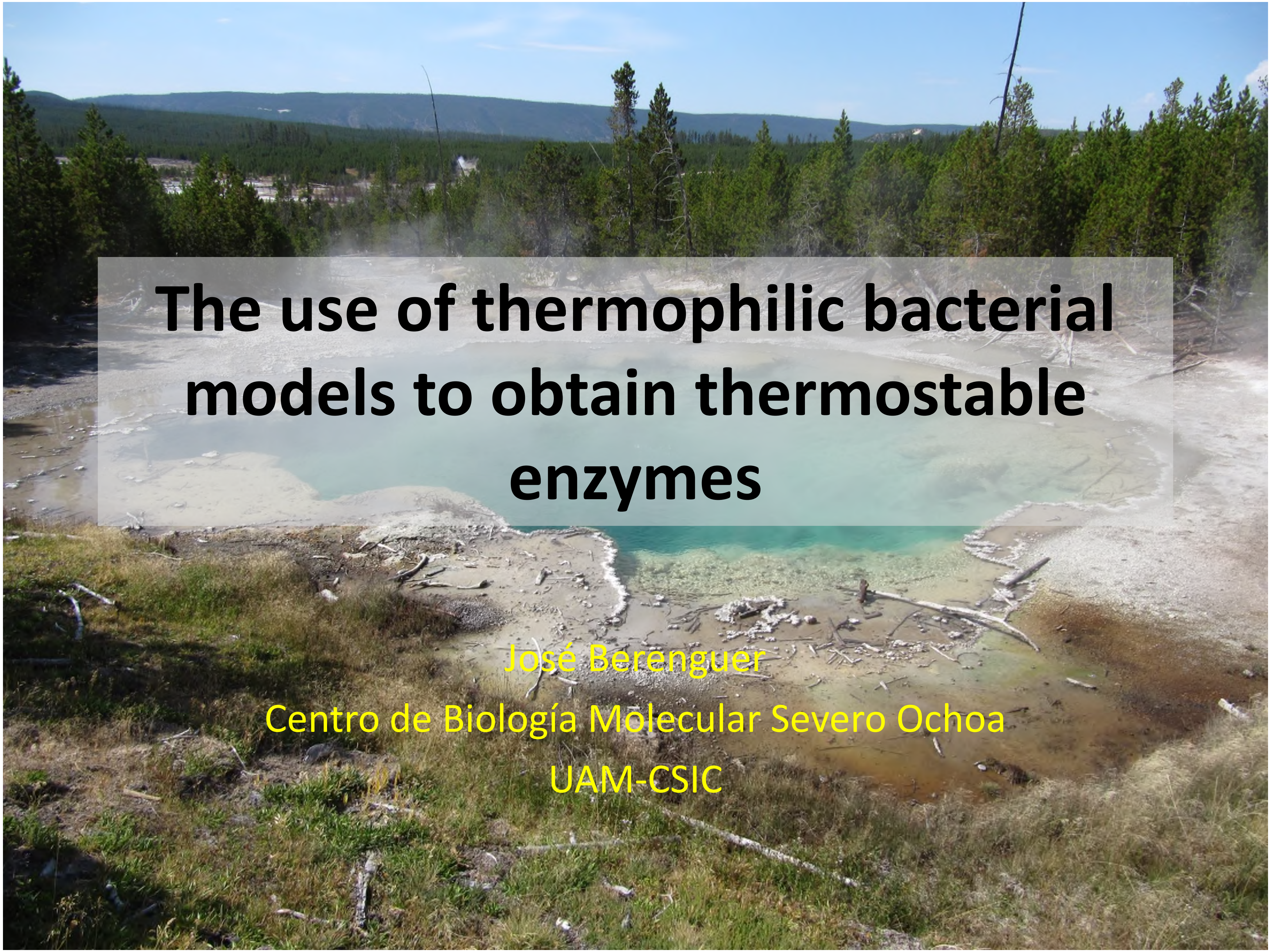


The use of thermophilic bacterial models to obtain thermostable enzymes

José Berenguer
Centro de Biología Molecular Severo Ochoa
UAM-CSIC

Thermostable enzymes

- Easier transport and conservation
- Increased diffusion of substrates and products
- Longer operational half-lives
- Lesser contamination
- Lesser requirement for cooling
- Associated resistances to detergents and organic solvents

Getting thermostable enzymes

- Isolation of thermophiles with new activities
- Isolation of the genes encoding new enzymes from metagenomes of thermal environments
- Selection of thermostable variants from known enzymes of industrial interest (directed evolution)

But..

- Most thermophiles are difficult to grow under lab conditions.... *>> very low success*
- Only a fraction of the genes of a given thermophile can be expressed in an enzyme active form in mesophilic hosts like *E. coli*.... *>> low success*

But..

- Most thermophiles are difficult to grow under lab conditions…. *>> very low success*
- Only a fraction of the genes of a given thermophile can be expressed in an enzyme active form in mesophilic hosts like *E. coli*…. *>> low success*

↓

- *Laboratory-adapted thermophiles can be used instead*
  - *To detect and overexpressed thermozymes*
  - *To select for thermostable variants of non-thermostable enzymes*

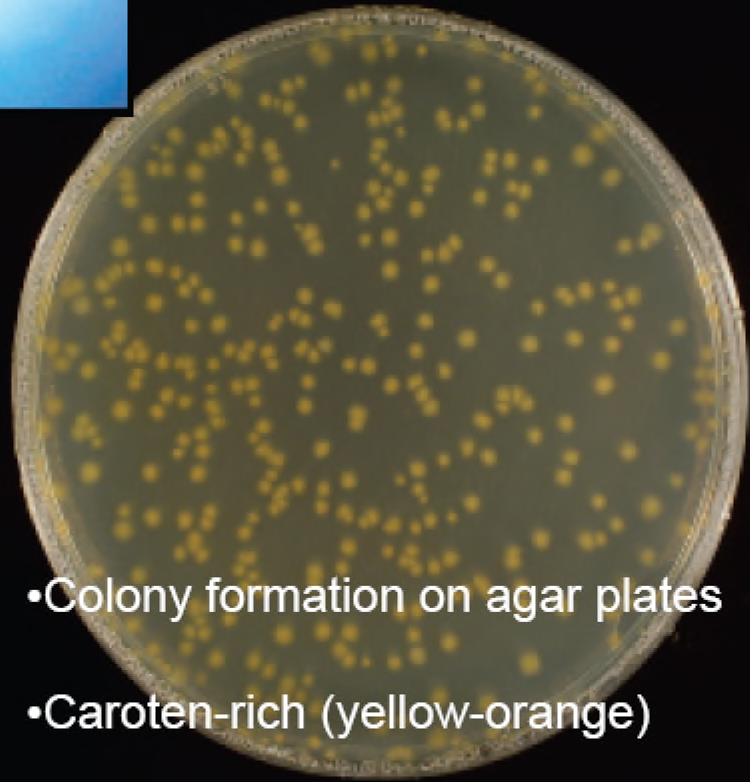
Current extreme thermophilic models as putative cell factories

Organism	Top	Tran	Plas.	Virus	Antib. Resist.	Aux. Compl.	Rep. Gen.
Sulfolobus solfataricus	80	EP CJ	++	++	Hyg Adh	*pyrEF*	*lacS*
Sulfolobus acidocaldarius	80	CJ $CaCl_2$	+	+	-	*pyrEF*	-
Pyrococcus furiosus	96	$CaCl_2$	+	-	Adh	-	-
Pyrococcus abyssi	103	PEG	+	++	-	*pyrEF*	-
Thermococcus kodakarensis	85	$CaCl_2$ NC	+	-	Sim	*pyrF* *trp*	*lacS*
Thermotoga neapolitana	80	PEG	+/-	-	-	-	-
→ *Thermus thermophilus*	75	NC EP CJ	++	+++	Kan Bleo Hyg Str	*leuB* *trpB* *pyrEF*	*bgaA* *phoA* *sGFP*

Thermus spp

- Brock and Freeze in 1969
  T. aquaticus: Yellowstone
- Ubicuous
- Fast growth in complex media

- Gram negative bacilli



- Colony formation on agar plates
- Caroten-rich (yellow-orange)

Thermus spp

- Brock and Freeze in 1969
  T. aquaticus: Yellowstone
- Ubicuous
- Fast growth in complex media

- Gra

- Natural competence
- Complete genetic toolbox available

- Colony formation on agar plates
- Caroten-rich (yellow-orange)

UNIVERSIDAD AUTONOMA DE MADRID

APPLICATIONS OF *T. thermophilus* IN ENZYME PRODUCTION AND THERMO-SELECTION

- (Meta)genomic libraries
- Protein cell factory

Up to 40% of the genes from a thermophile cannot be expressed in an active form in *E. coli*

- Selection of thermostable protein variants

Functional screening for thermostable enzymes in (meta) genomic libraries

ELSEVIER

Available online at www.sciencedirect.com

ScienceDirect

Systematic and Applied Microbiology 32 (2009) 177–185

SYSTEMATIC AND APPLIED MICROBIOLOGY

www.elsevier.de/syapm

A two-host fosmid system for functional screening of (meta)genomic libraries from extreme thermophiles

Angel Angelov[a,b], Markus Mientus[b], Susanne Liebl[a,b], Wolfgang Liebl[b,*]

[a]*Institut f. Mikrobiologie und Genetik, Georg-August-Universität, Grisebachstr. 8, D-37077 Goettingen, Germany*
[b]*Lehrstuhl f. Mikrobiologie, Technische Universität München, Am Hochanger 4, D-85354 Freising-Weihenstephan, Germany*

frontiers in Microbiology

Identification of novel esterase-active enzymes from hot environments by use of the host bacterium *Thermus thermophilus*

*Benedikt Leis[1], Angel Angelov[1], Markus Mientus[1], Haijuan Li[1], Vu T. T. Pham[1], Benjamin Lauinger[2], Patrick Bongen[2], Jörg Pietruszka[2], Luís G. Gonçalves[3], Helena Santos[3] and Wolfgang Liebl[1]**

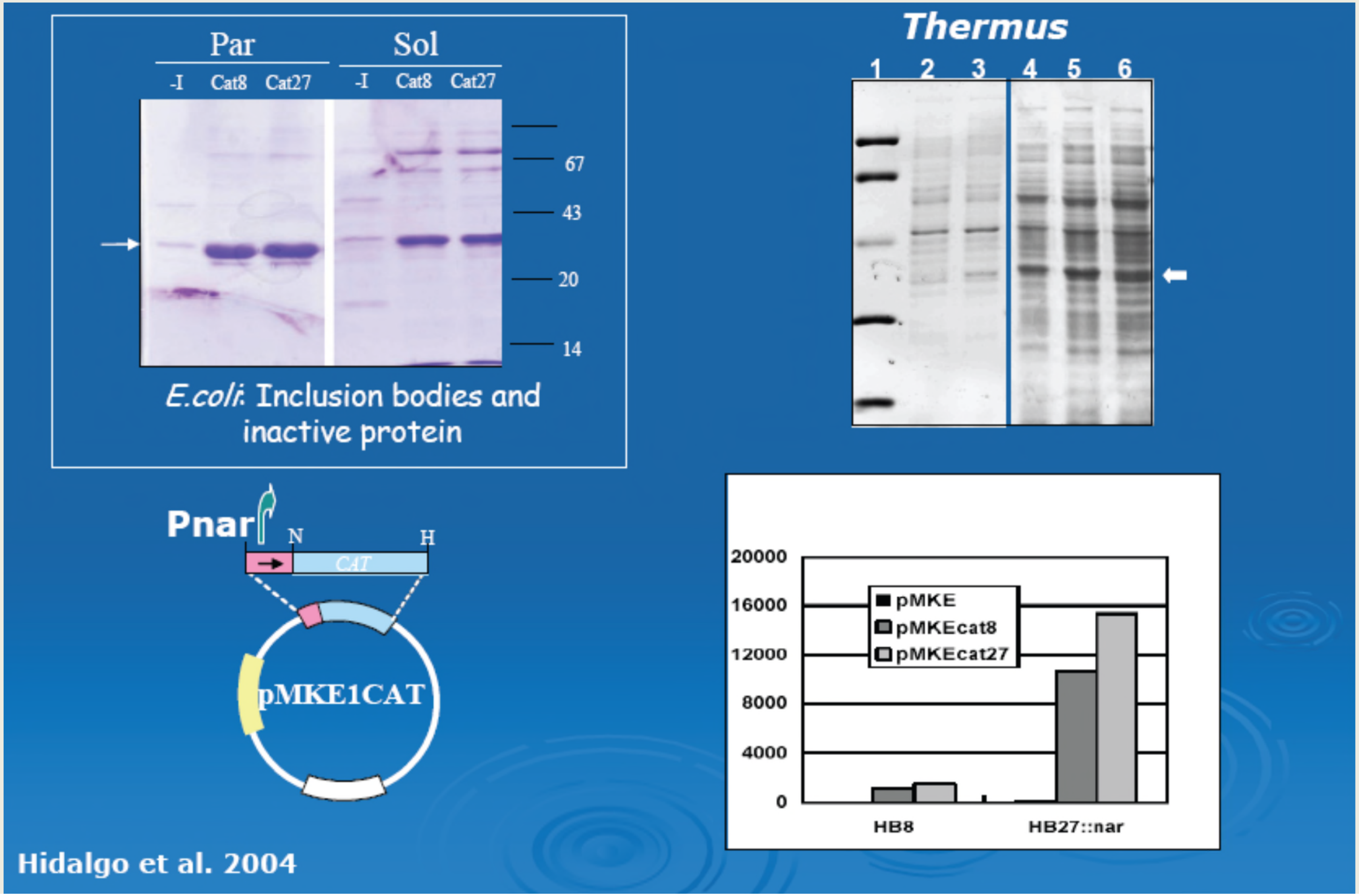
- Fosmid libraries constructed in *E. coli*
- Transferred on 96 well to Tth
- Integration by homologous recombination on *pyrE*
- Many xylanolytic activitiers only detected in *T. thermophilus*

Spirochaeta thermophila DSM 6192
2009

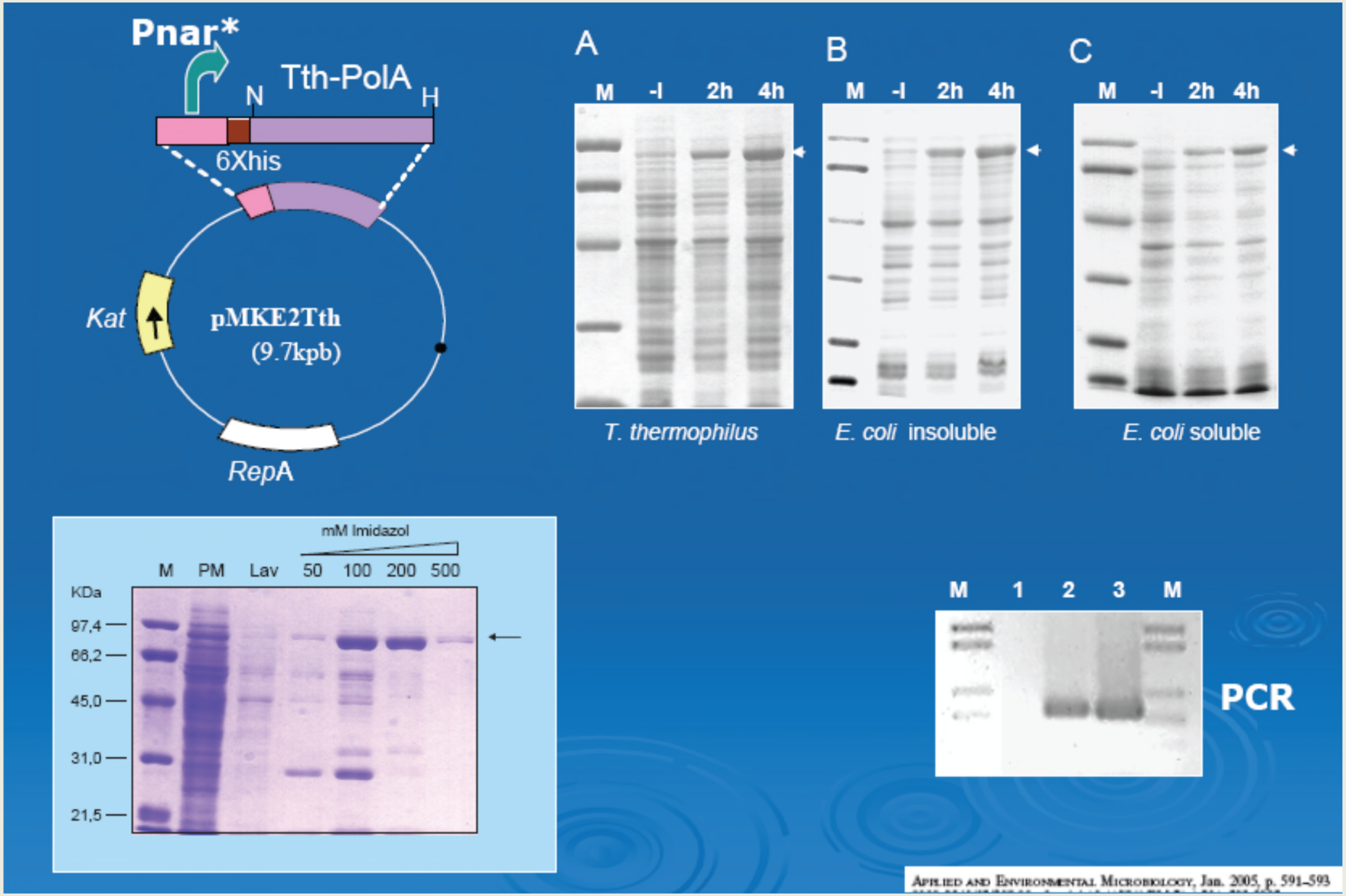
- T. th strain with no esterases as host
- Greater number of esterase-positive clones (6) than with *E. coli* (2)
- Four novel esterases, two of new families

Metagenomic libraries
2015

Host to over-express thermostable enzymes that cannot be expressed in *E. coli*



Cell factories to over-express thermostable enzymes with greater yield than in in *E. coli*



Applied and Environmental Microbiology, Jan. 2005, p. 591–593

Thermostabilization by directed evolution in thermophiles

- **Functional:**
  - Strain growth dependent on the activity

- **Activity independent**:
  - dependent on the functionality of a reporter

Examples of functional selection in *T. thermophilus*

LeuB

Molecular Microbiology (1995) **16**(5), 1031–1036

Screening of stable proteins in an extreme thermophile, *Thermus thermophilus*

Masatada Tamakoshi, Akihiko Yamagishi and Tairo Oshima

nucleotidyltransferase, glucose dehydrogenase, subtilisin and staphylococcal nuclease (Matsumura and Aiba,

Bleo

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 280, No. 12, Issue of March 25, pp. 11422–11431, 2005
Printed in U.S.A.

Engineering a Selectable Marker for Hyperthermophiles*S

Received for publication, December 3, 2004, and in revised form, December 27, 2004
Published, JBC Papers in Press, January 7, 2005, DOI 10.1074/jbc.M413623200

Hygro

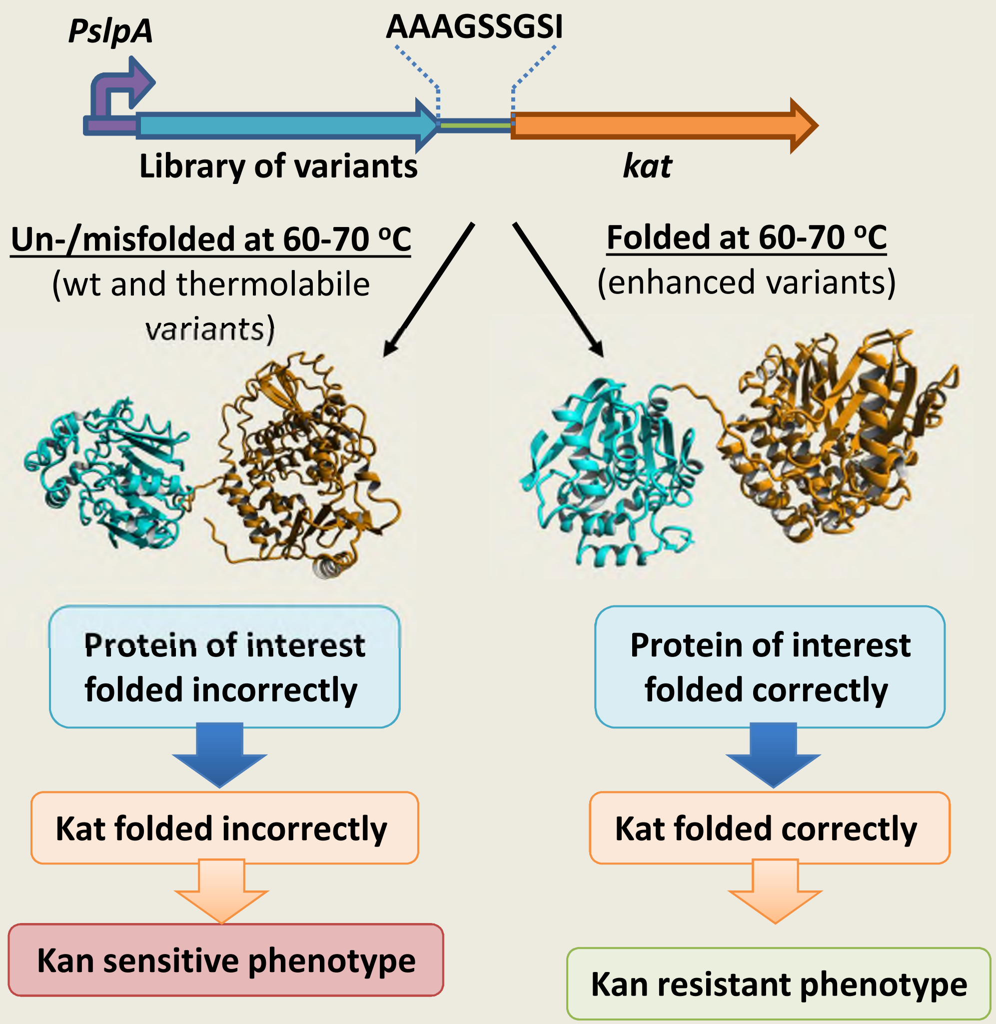
JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 100, No. 2, 158–163. 2005
DOI: 10.1263/jbb.100.158

ACCELERATED PUBLICATION

In vivo Directed Evolution for Thermostabilization of *Escherichia coli* Hygromycin B Phosphotransferase and the Use of the Gene as a Selection Marker in the Host-Vector System of *Thermus thermophilus*

Akira Nakamura,[1*] Yasuaki Takakura,[1] Hideo Kobayashi,[1] and Takayuki Hoshino[1]

In vivo thermoselection (THR)

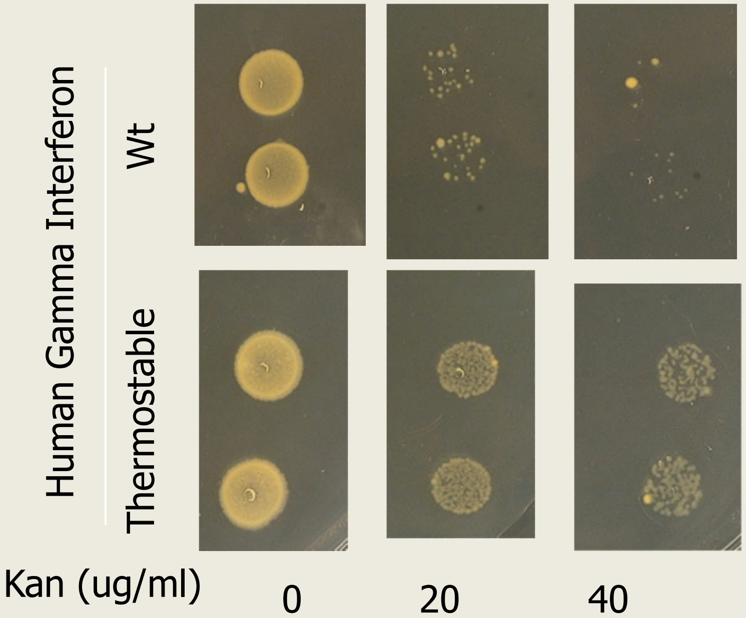


- Constitutive PslpA promoter
- Linker
- Thermostable selection marker: *kat*
- Host: *Thermus thermophilus (50-82 ºC, Topt 72 ºC)*
- Activity INDEPENDENT

Chautard *y cols.* (2007) *Nat. Methods* **4**: 919-921

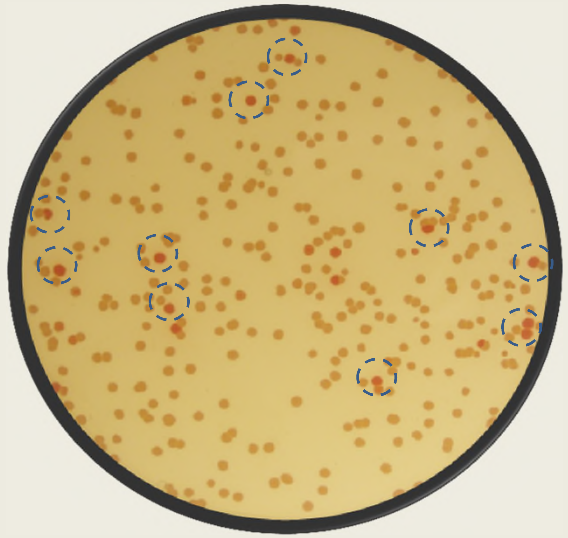
Example of selection at 70ºC

Folding interference primary selection



Cheap high-throughput screening

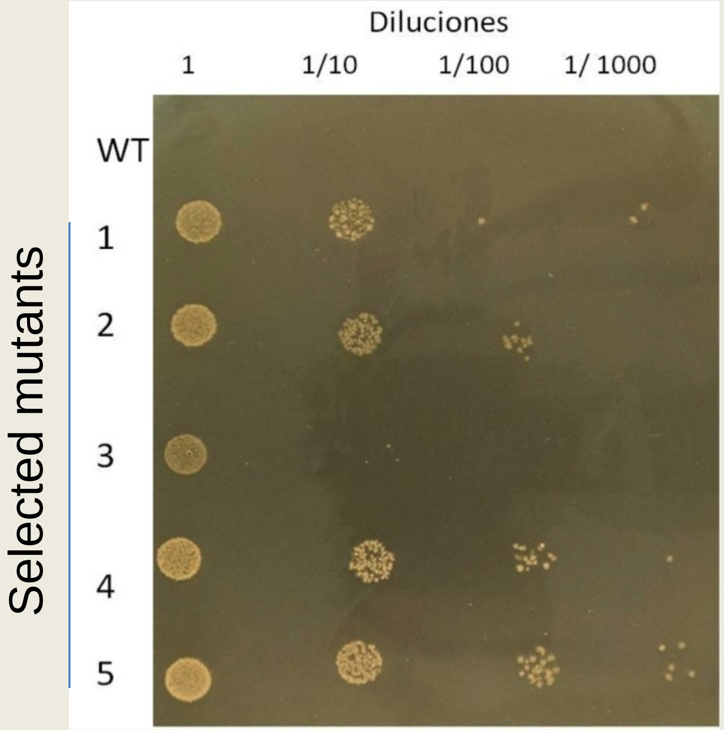
Secondary selection for activity on thermostable clones



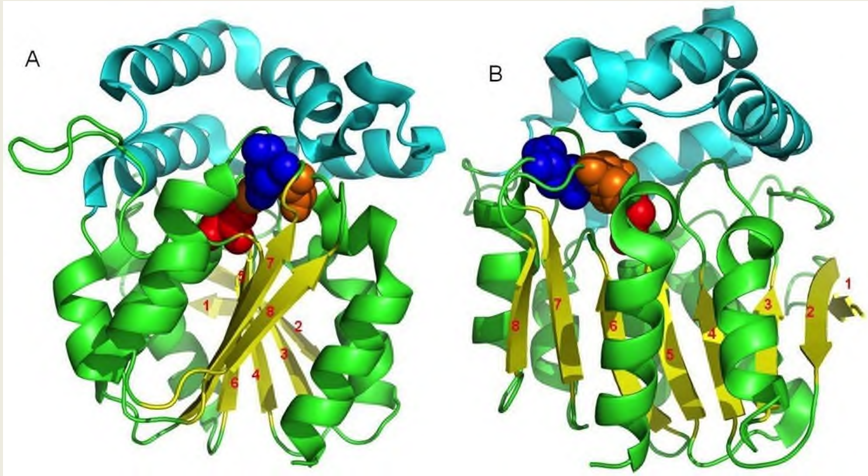
low throughput screening

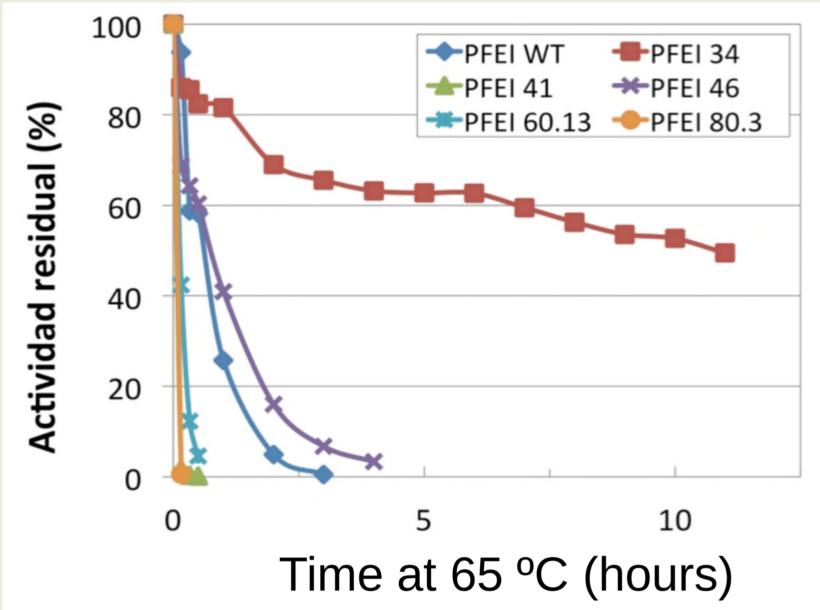
Chautard et al 2007

P. fluorescens Esterase I



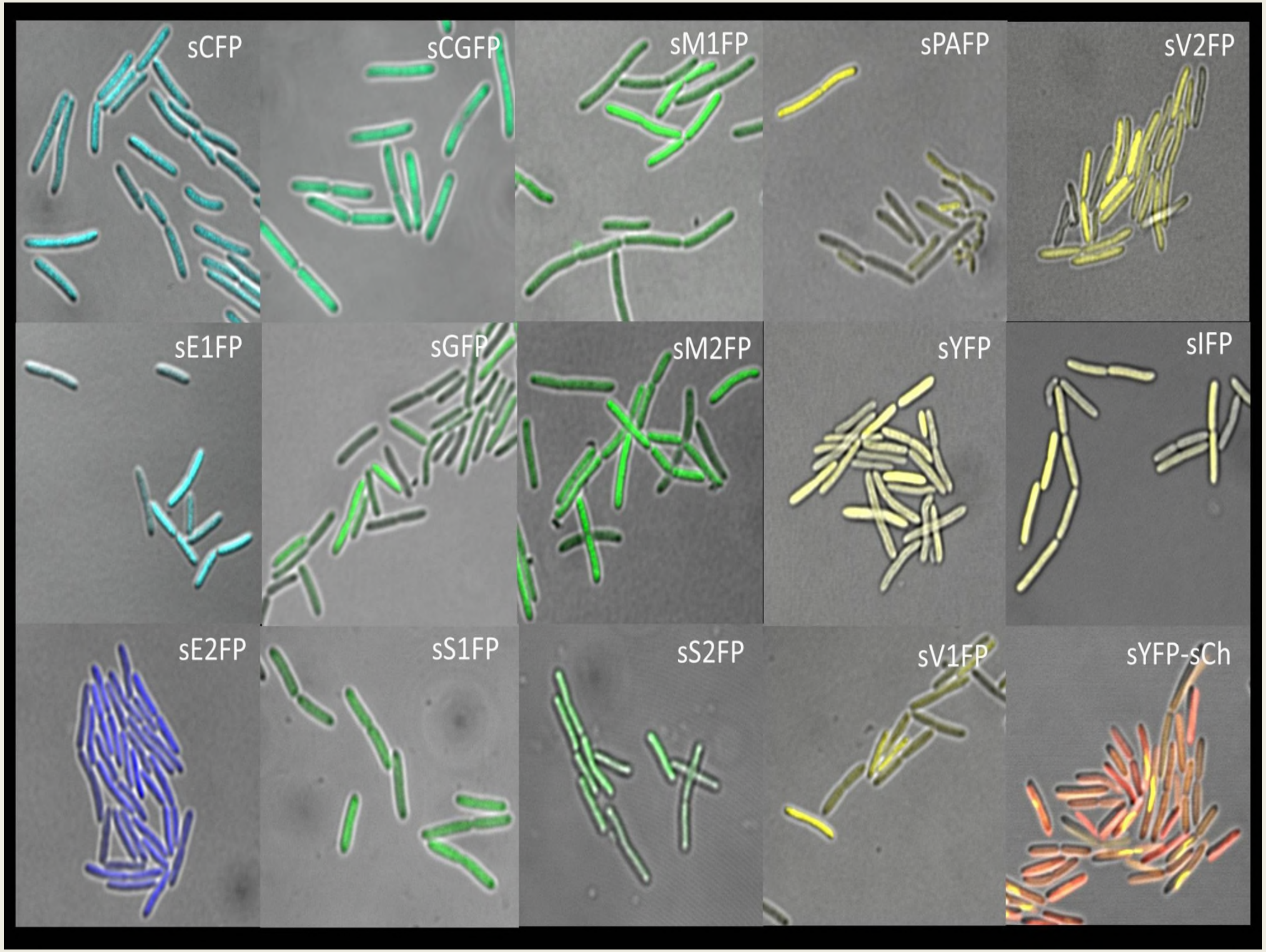
65 ºC- Kn 60 mg/L





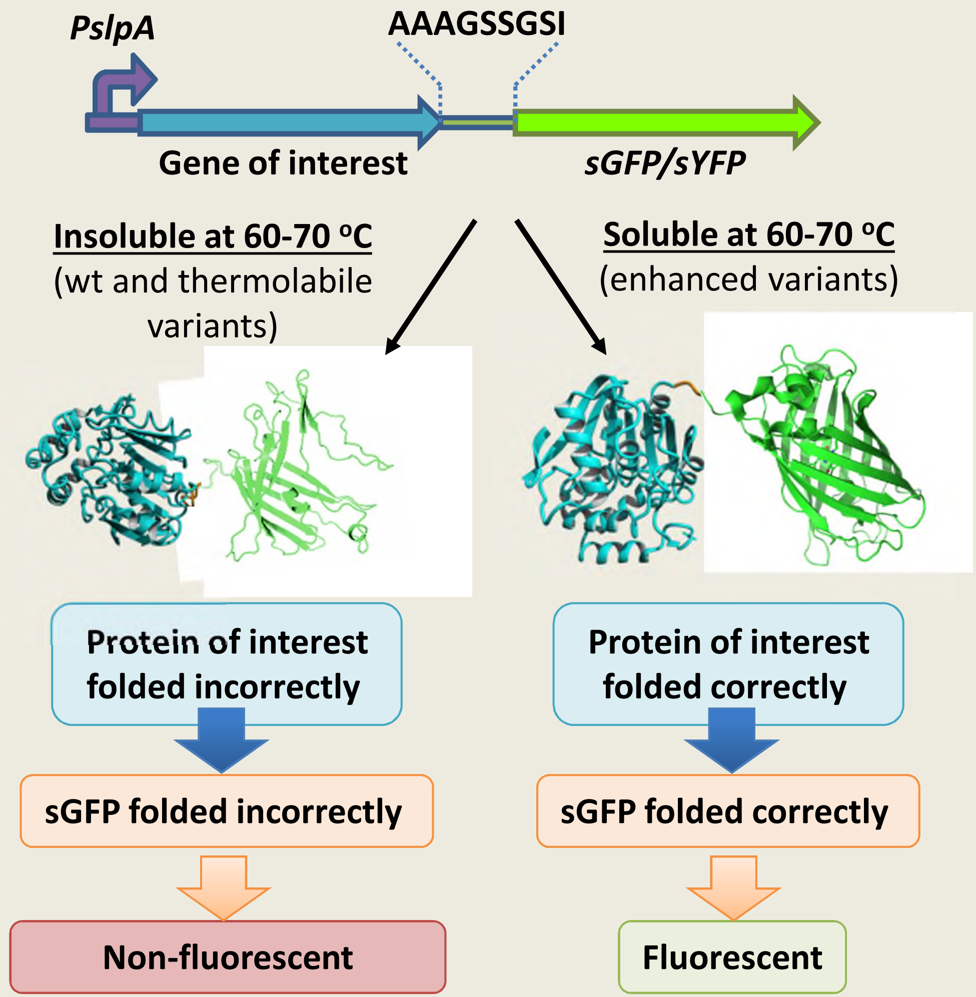
Rivera 2012. Ph.D. Thesis

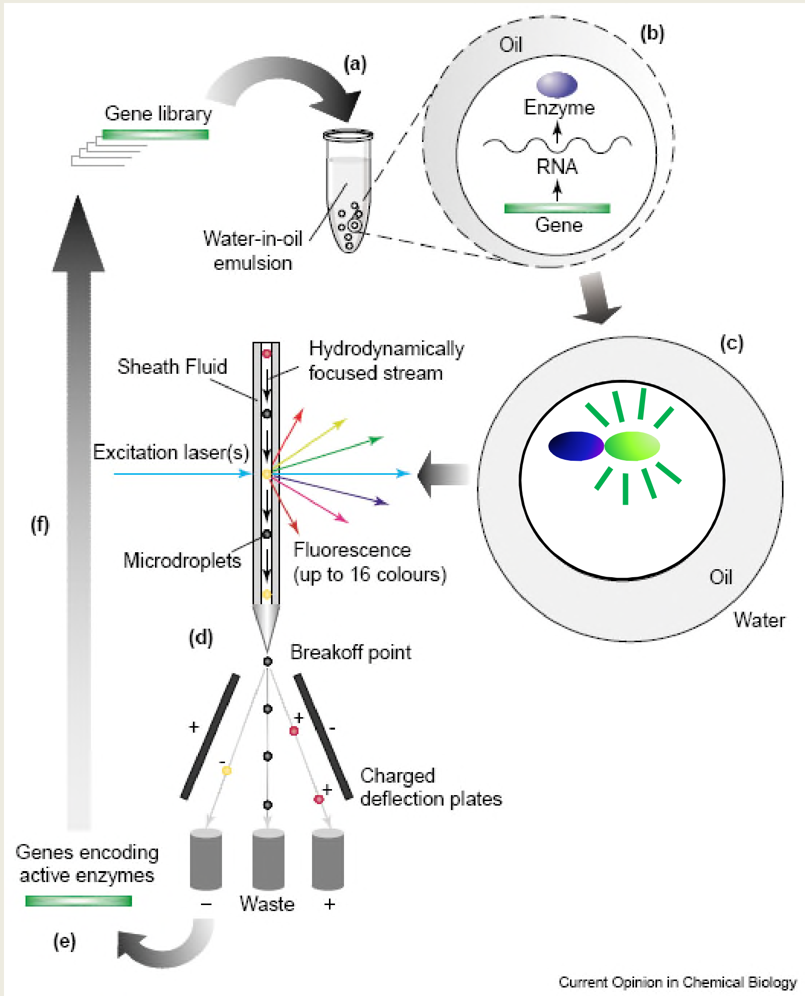
Fluorescent proteins as thermostability reporters in *T. thermophilus*



70ºC

In vitro screening through folding interference with fluorescent reporters






Funded by
European Commission
HOT DROPS
FP7 Marie-Curie EU program
IAPP: Industry Academy Partnership Program
UAM
UNIVERSIDAD AUTONOMA DE MADRID
UNIVERSIDADE DA CORUÑA
Universida de Vigo
UNIVERSITY OF CAMBRIDGE
BIOCHEMIZE
DROP-TECH
Droplets for Diagnostics and Drug Discovery
novozymes
Rethink Tomorrow
GalChimia

Conclussions

- Natural thermostable enzymes can be isolated from thermal environments
- Thermus thermophilus is more efficient for the detection and expression of thermozymes than conventional mesophilic hosts
- The use of folding interference vectors allow the thermostabilization of proteins in an activity independent way, reducing time and costs

Funded by

BERENGUER'S LAB

CBMSO> Microbiology & Virology> Biotechnology & Genetics of extreme thermophilic bacteria

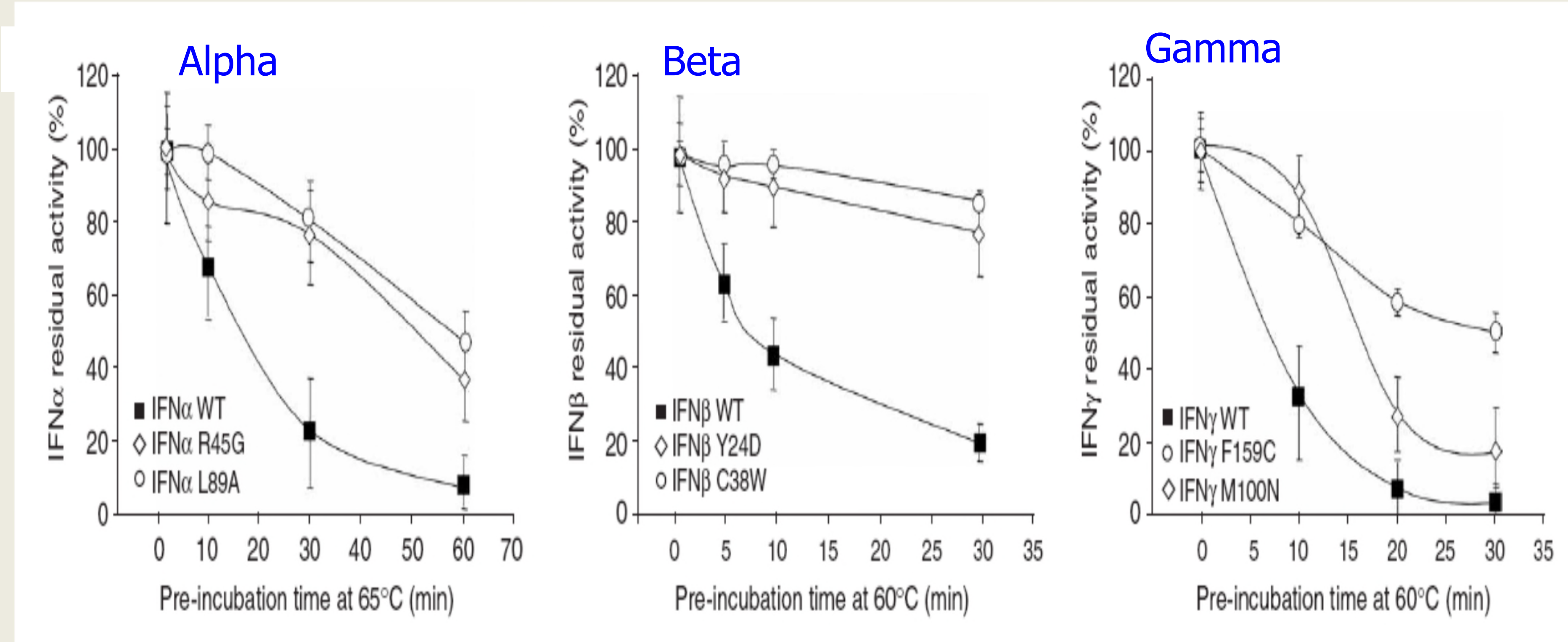
BASIC RESEARCH

- Anaerobic metabolism
- HGT
- Defense systems

APPLIED RESEARCH

- Enzyme thermostabilization
- IVT
- C-C bond enzymatic synthesis
- Metagenomics

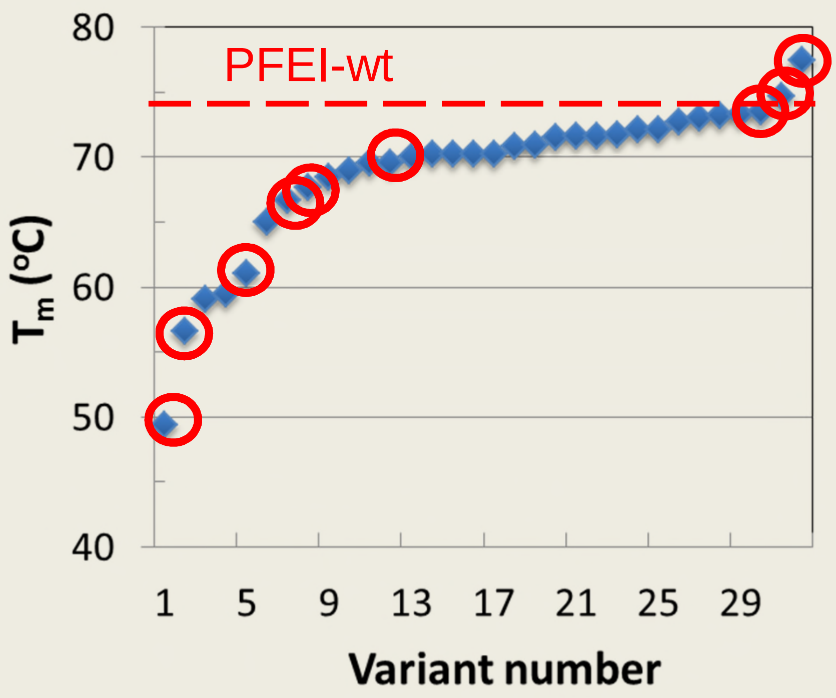
Thermostabilization of proteins without enzymatic activity: Human interferons



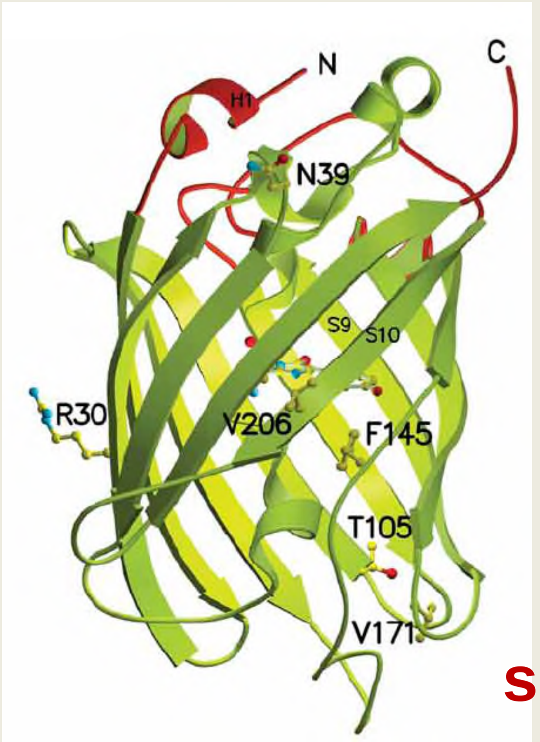
Chautard et al 2007

PFEI: Folding calibration set

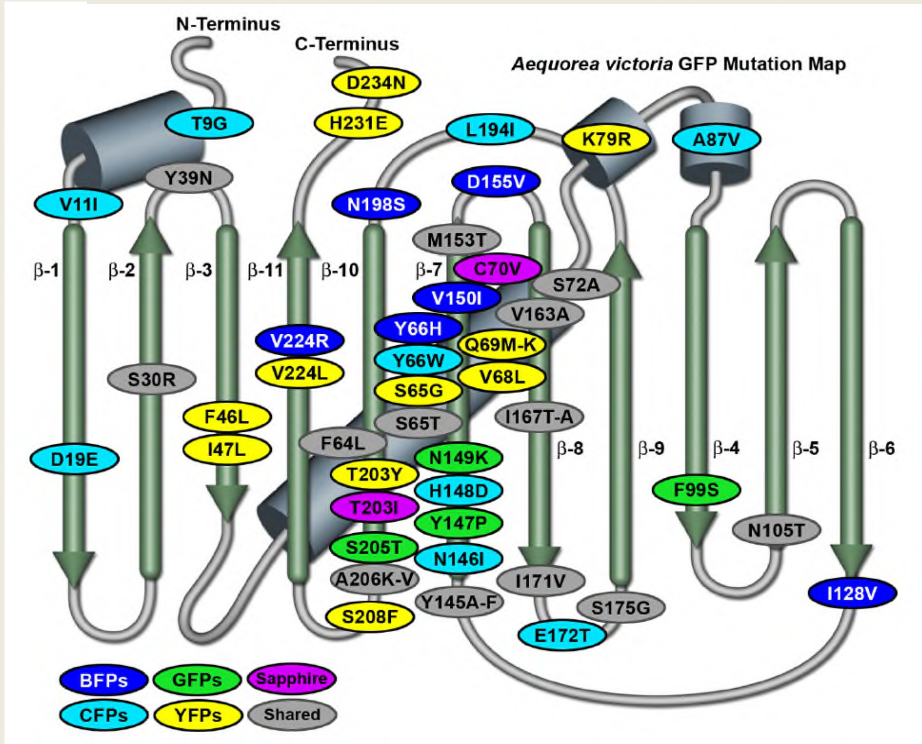
Variant	T_m (ºC)
PFEI-34	78.8
PFEI-16	74.6
PFEI-wt	74.5
PFEI-46	74.4
PFEI-41	69.6
PFEI-60	67.9
PFEI-80	67
PFEI-45	61
PFEI-29	56
PFEI-12	49

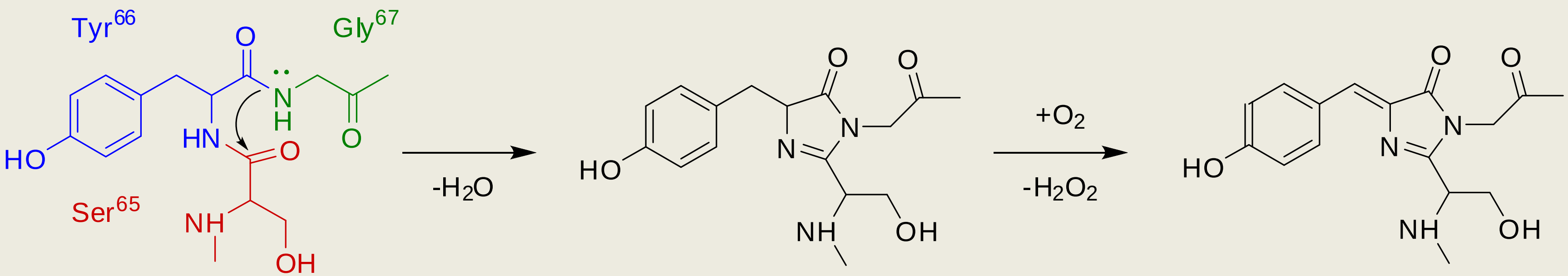


THERMOSTABLE FLUORESCENT REPORTERS

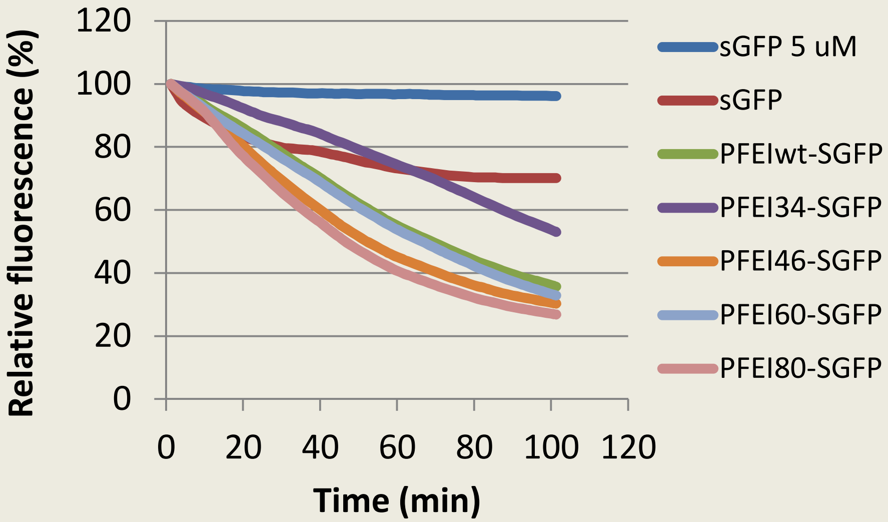


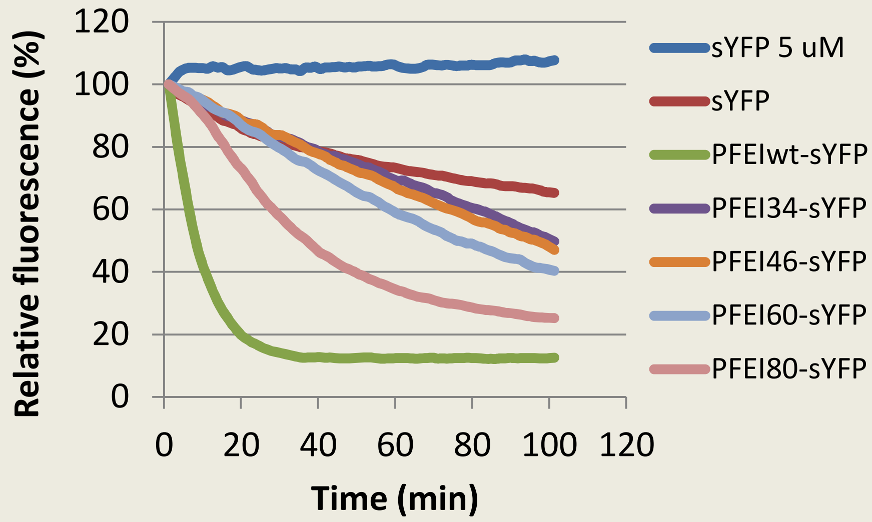
sGFP





Melting 65 °C post-IVTT*Eco* in bulk





Folding interference is also selectable after IVTT with *E. coli* PURE-systems

Aurelio Hidalgo