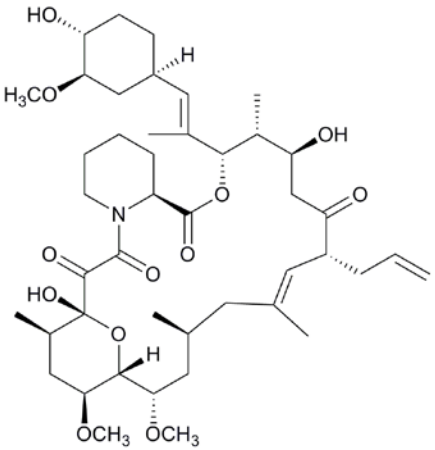
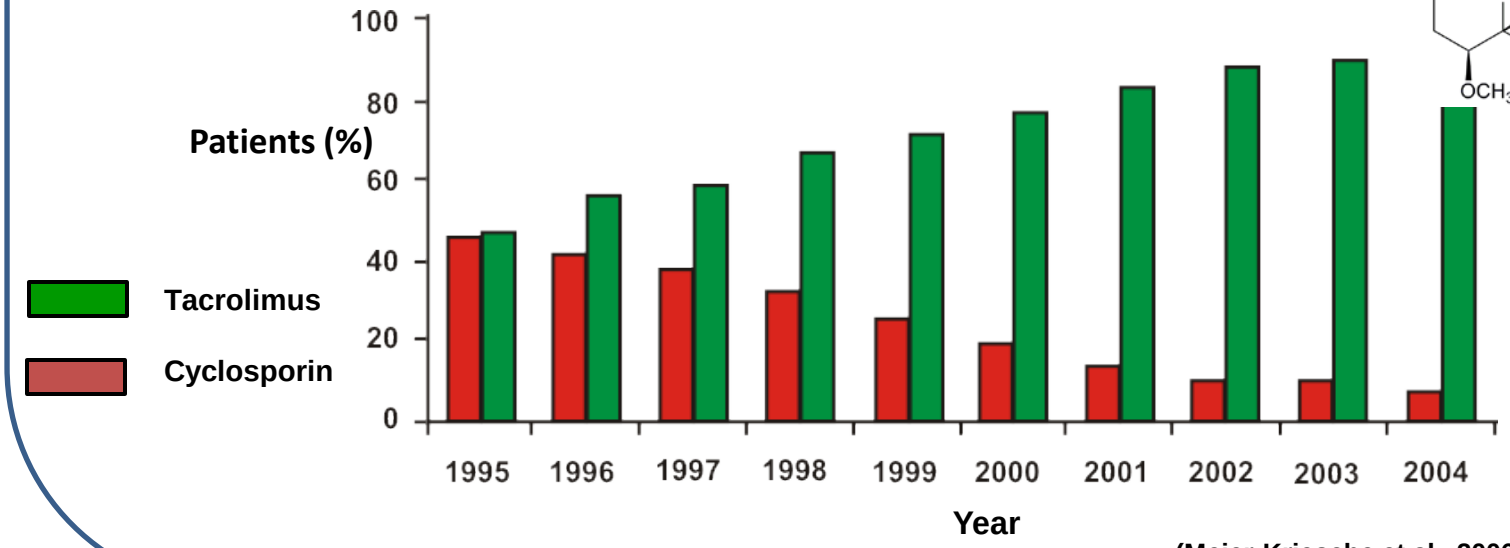
Tacrolimus (FK506) is an immunosupressant produced by *Streptomyces tsukubaensis*

Sold as Fujimycin, Prograf, Advagraf, Protopic

Used as Inmunosuppresant /atopic dermatitis/vitiligo

Prograf (astellas Pharma) 261 millions $ USA
Generic in 2009

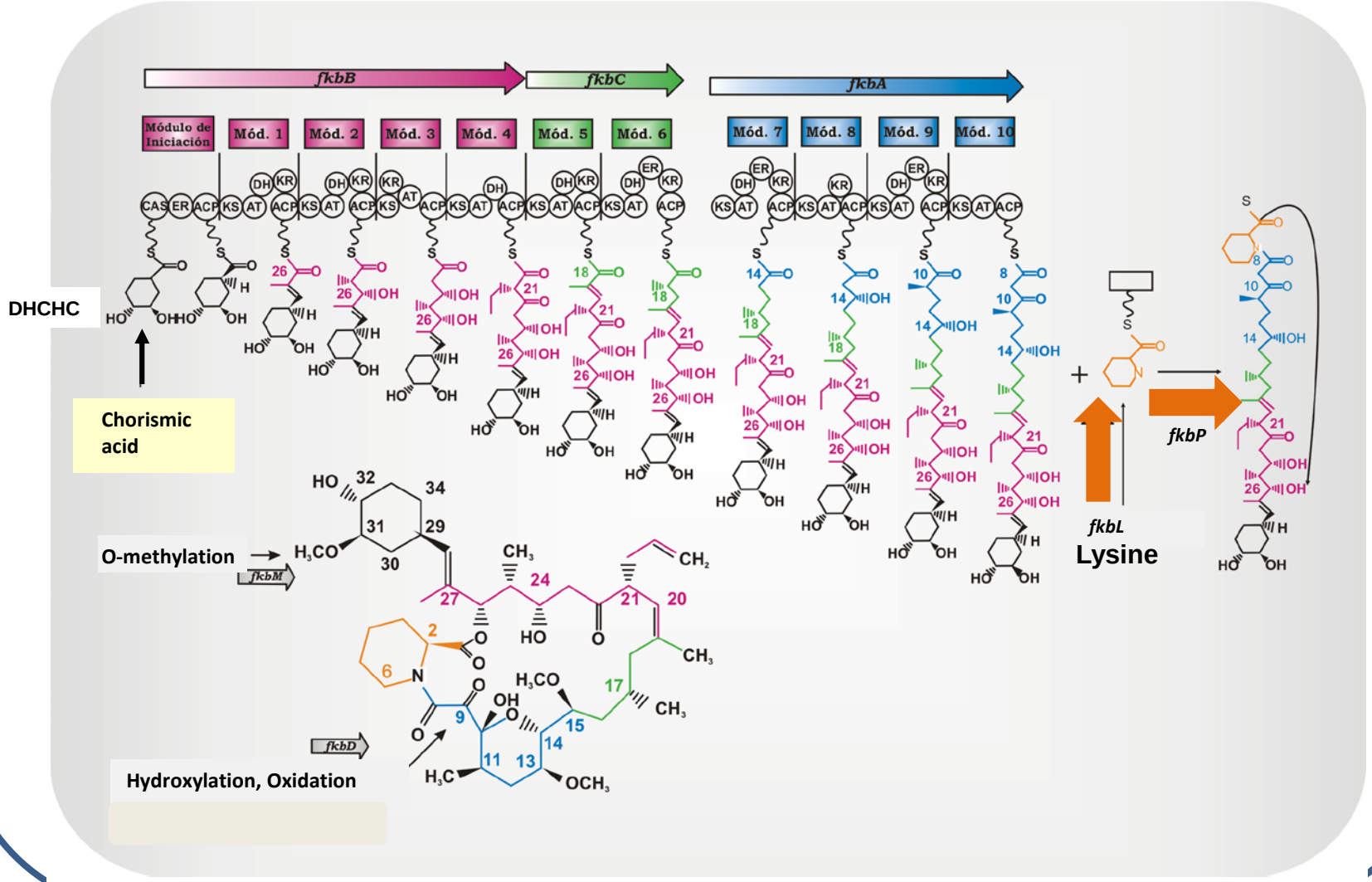




(Meier-Kriesche et al., 2006)

BIOSYNTHESIS OF TACROLIMUS

<u>Tacrolimus</u> is formed by three major PKS´s and a NRPS followed by several modification enzymes



ERA-IB2 PROYECT INMUNOTEC

ROBUST FERMENTATION PROCESS FOR THE PRODUCTION OF TACROLIMUS

PARTNERS

1. Coordinators: **Dr. Paloma Liras and Dr. Juan F. Martín - INBIOTEC Institute of Biotechnology (León,Spain): WP1, WP4**

2. Dr. Wolfgang Wohlleben - Eberhand-Karls-Universität Tübingen (Tübingen, Germany): WP2

3. Dr. Marta Vaz Mendes - IBMC-Instituto de Biología Molecular e Celular (Porto, Portugal): WP3

4. Dr. Lutz Heide - Eberhand-Karls-Universität Tübingen (Tübingen, Germany) : WP5

5. Dr. Tania Velasco - ANTIBIÓTICOS S.A. (León, Spain): WP6

WORK PACKAGEs 1 and 4

PARTNER 1: INSTITUTE OF BIOTECHNOLOGY. LEÓN, SPAIN

Personnel:

Principal Investigator: Prof. Paloma Liras
Prof. Juan F. Martín
Dr. Antonio Rodríguez-García
Dr. Fernando Santos Beneit
María Ordoñez, Ph.D. student

Collaborator :Prof. Hrvoje Petkovic (Slovenia)

WP1: Analysis of the sequences of *S. tsukubaensis* genome. Characterization of the tacrolimus gene cluster and regulatory genes

WP1. THE *S. tsukubaensis* GENOME CHARACTERISTICS

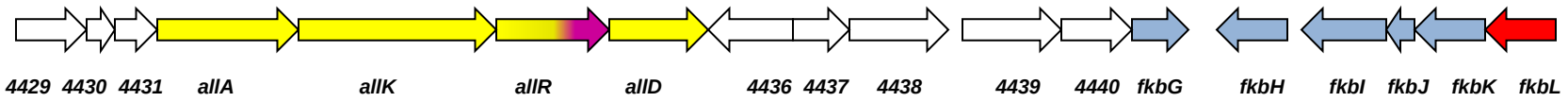
One linear chromosome (7.62 Mbp) and two circular plasmids of 24.7 and 31.1 kbp

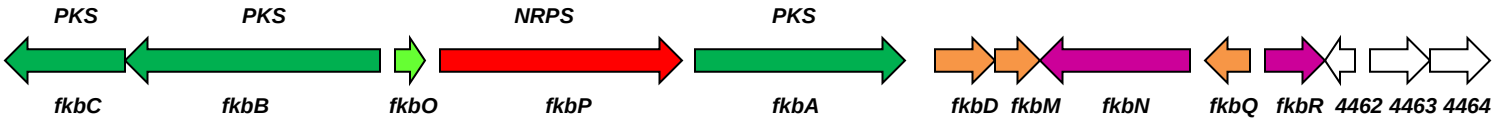
It contains:
6623 protein-encoding large ORFs (>0.8kb)
6 rRNA operons
68 tRNAs
52 Sigma factors
About 20 secondary metabolites clusters

Using the genome sequence WP1 studied and provided genes to the other partners:

- **Genes for tacrolimus biosynthesis subcloned**
- **The *bul* cluster for butyrolactone biosynthesis and regulation**
- **Genes of the ethylmalonyl-CoA pathway**
- **The *pho* regulon. Genes controlled by phosphate. Pho boxes (to WP4)**
- **The nitrogen metabolism structural and regulatory genes (to WP2)**
- **Genes related to oxidative stress (to WP3)**
- **Clusters for other secondary metabolites (to WP5)**

TACROLIMUS GENE CLUSTER IN *S. tsukubaensis*





Starter Unit: DHCHC

Polyketide Synthase

Methoxymallony-CoA biosynthesis

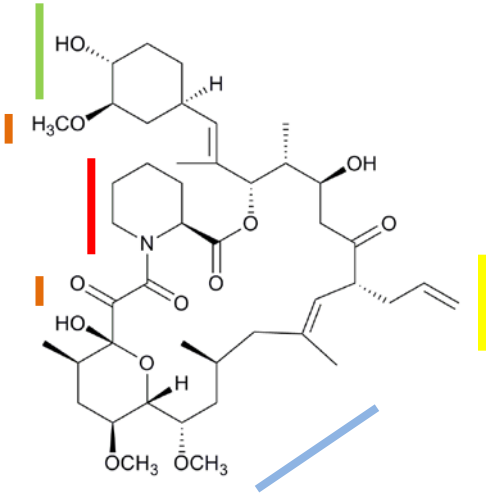
Allylmalonyl-CoA biosynthesis

NRPS-Pipecolic acid

Post-PKS modifications

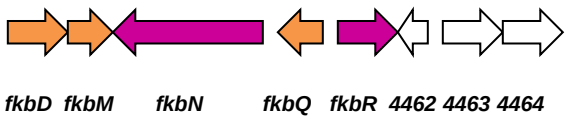
Regulatory genes

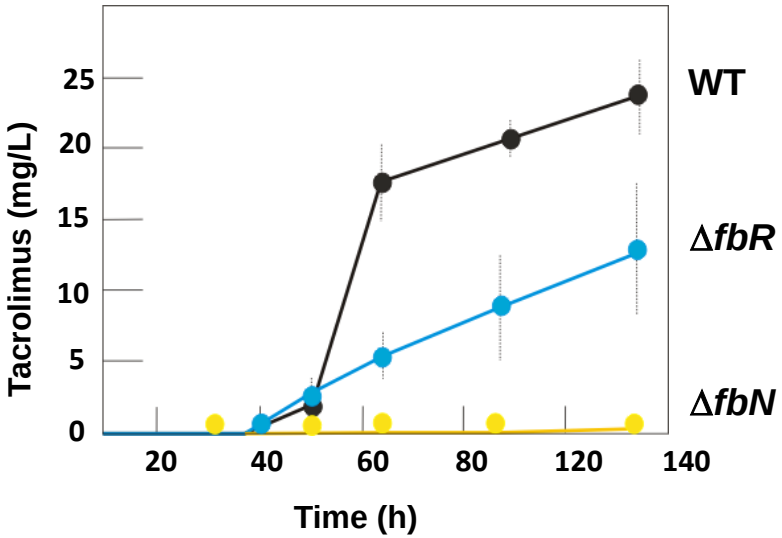
Other genes probably not involved in tacrolimus biosynthesis



Tacrolimus byosinthesis is regulated by two positive regulatory elements in *Streptomyces tsukubaensis*

In colaboration with Prof. H Petkovic





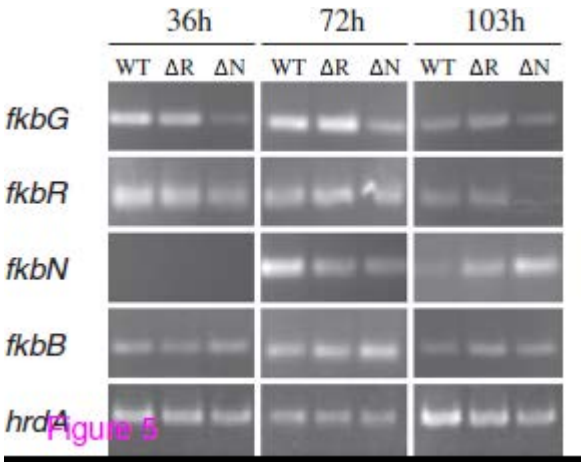
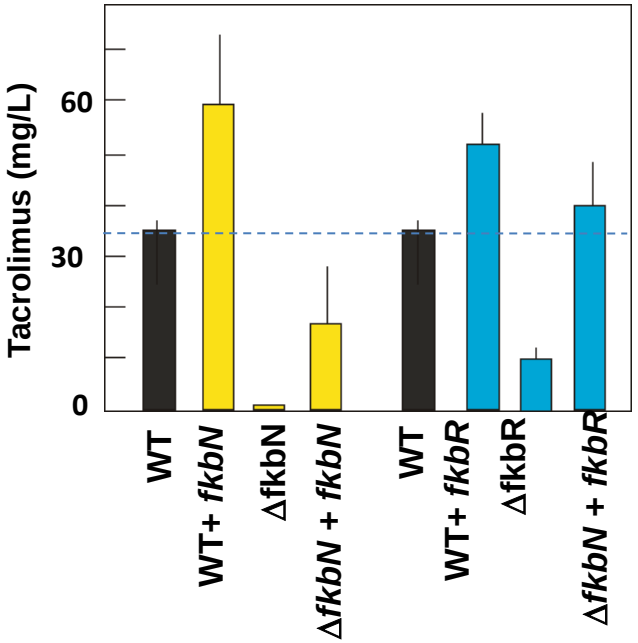


Figure 5

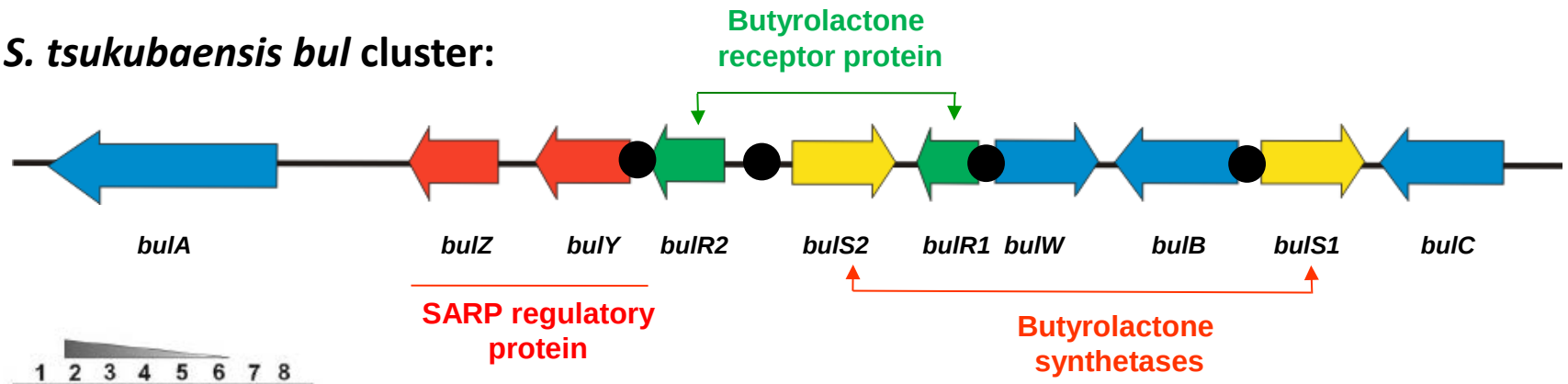


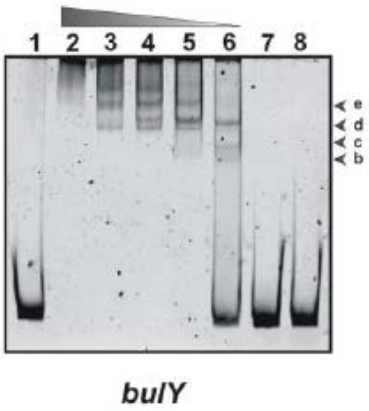
Increasing *fkbN* copy numbers increase 70% production

Goranovic *et al*., 2012

Butyrolactones initiate the production of secondary metabolites by binding butyrolactone receptor proteins

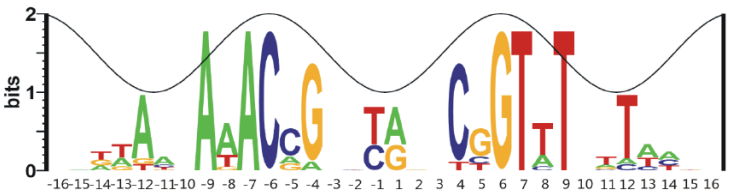
***S. tsukubaensis bul* cluster:**





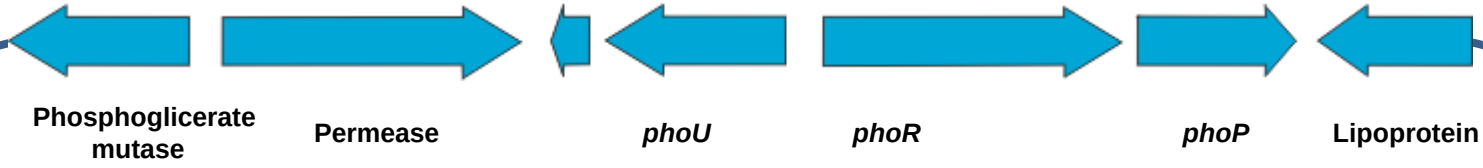
bulY

Purified BulR1 and BulR2
EMSA
Footprinting



The six genes were separatedly deleted. The effect on tacrolimus production was:
***bulR1* is essential. Deletion results in 80% decrease in production. *bulR2* is not required**
***bulS1* or *bulS2* : 35-70% decrease**
***bulZ or bulY:* 50% increase in tacrolimus production, probably by cross-regulation**

Salehi-Najafabadi *et al*. 2014



WP4

Tacrolimus production strongly disminish in the presence of phosphate 10 mM

Phosphate starvation

PstS

PstA PstC

PstB

ATP

PhoU

Cytoplasmic membrane

PhoR PhoR

P

ATP ADP

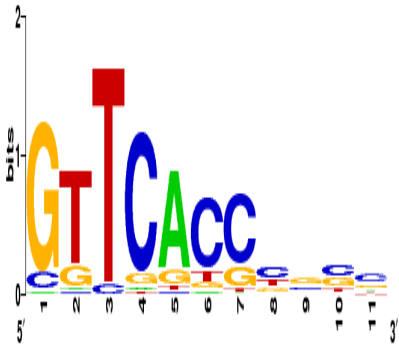
P

PhoP

PhoP binding sites

PhoP

PHO boxes
For PhoP binding



THE TWO COMPONENTS PhoR-PhoP CONTROLLED REGULON

All these genes have been located in *S. tsukubaensis* genome and most of them have been subcloned in cosmids

WP4: Phosphate control of primary metabolism and tacrolimus biosynthesis. Phosphate-deregulated mutants

Attempts to disrupt the *phoP* gene: only single cross-over recombinants

1. **The Redirect approach:** *phoP* replacement by the *aac(3)IV* cassette in a cosmid

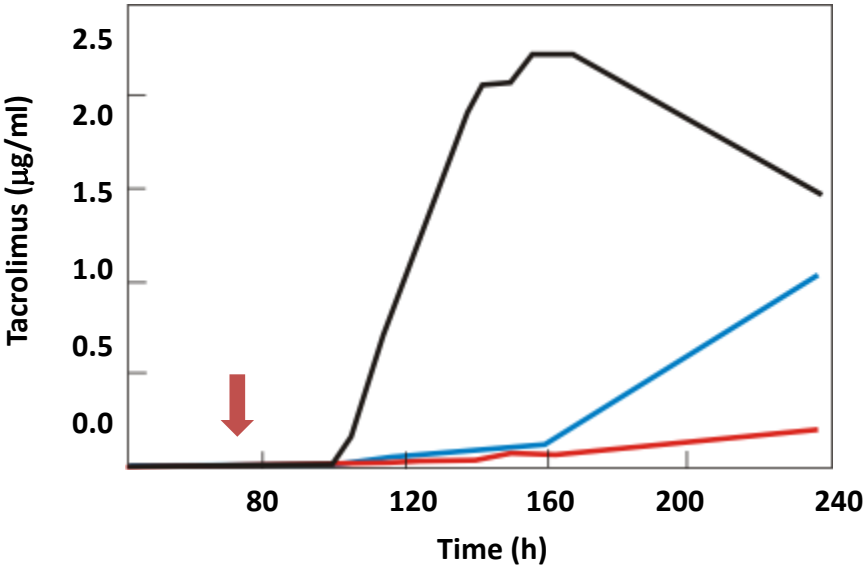
2. **The cytosine deaminase (*codA)* system/5-fluorocytosine:** *phoP* replacement construction

3. **The cytosine deaminase system** *plus* a **thermosensitive replicon (pSG5) (in progress)**

No *phoP*-deleted mutants could be obtained by either approach

Transcriptomic studies to analyze the

Interaction of phosphate and carbon sources exerting carbon catabolite regulation of tacrolimus production in *S. tsukubaensis*

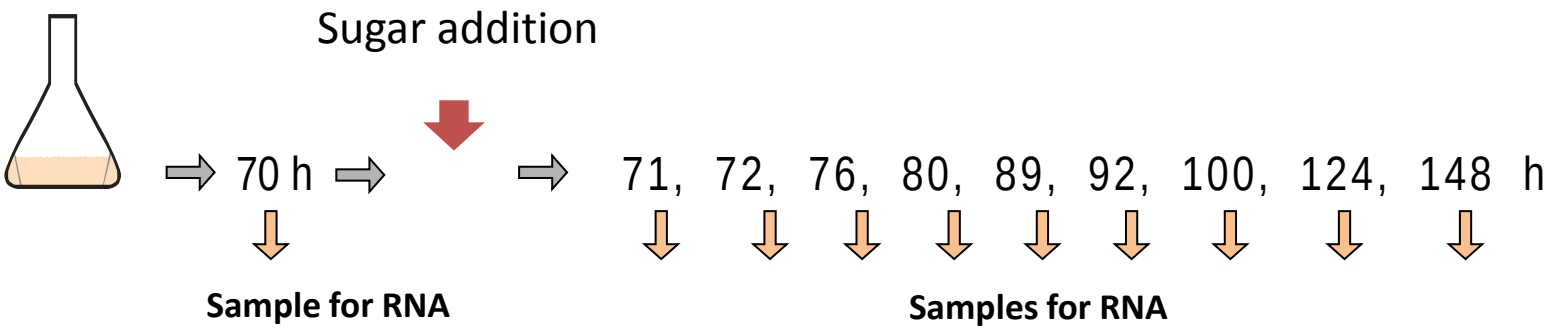


Carbon catabolite regulation at low phosphate concentration

- MG (2.5 mM Pi)
- 10^7 spores/ml, 28ºC 220rpm
- Carbon additions at 70h (3%):

Glucose, Glycerol, Maltose, Sucrose,
- Fructose, Lactose, Manitol and Xylose

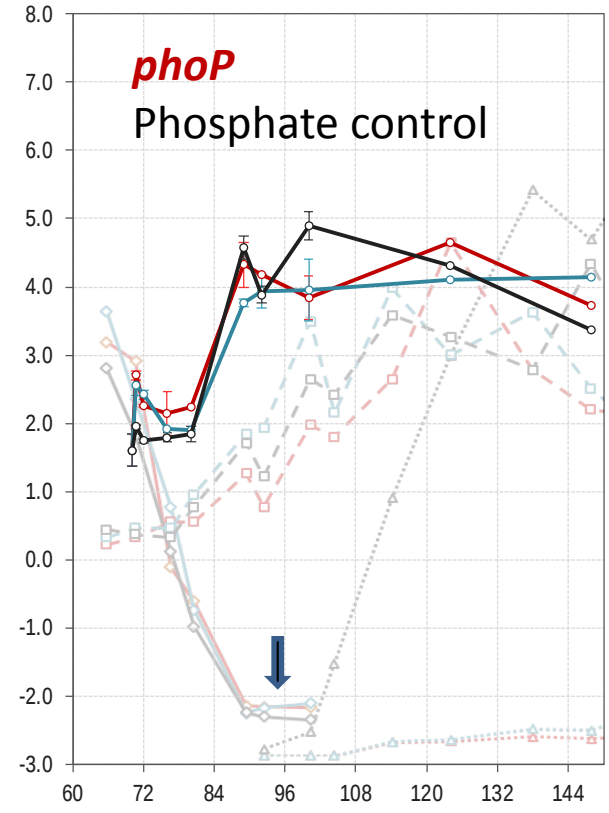
- Bioassay with *Saccharomyces cerevisiae*
- Tacrolimus cuantification by HPLC



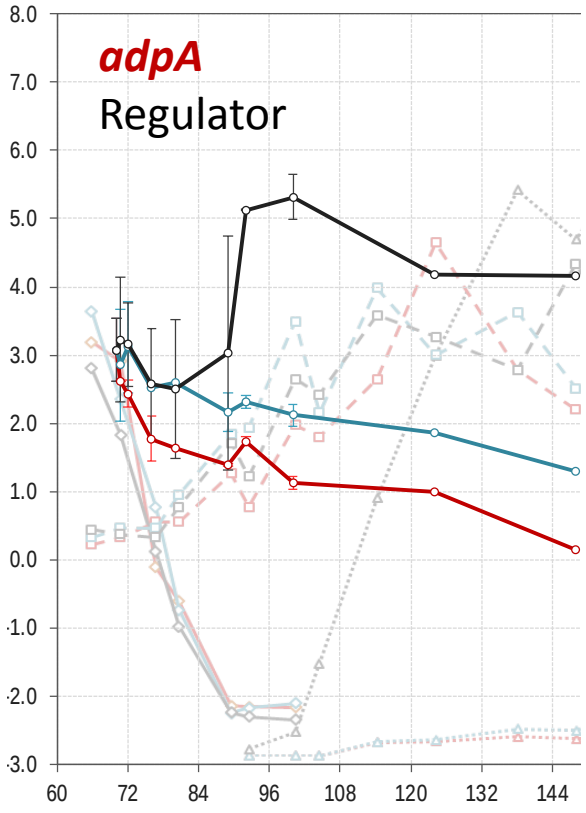
TRANSCRIPTOMIC STUDIES

Custom Agilent microarrays 8x15k

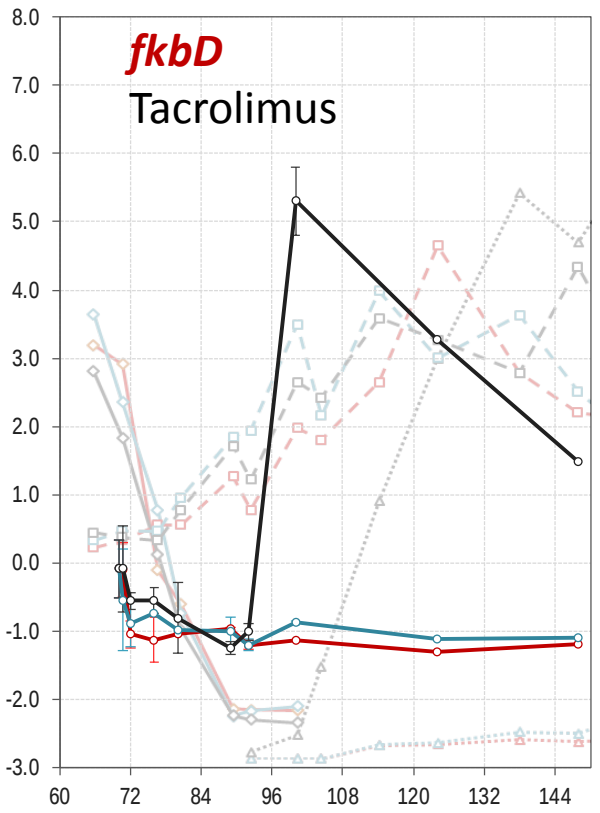
THREE MAIN TYPES OF TRANSCRIPT PATTERN OF GROUPS OF GENES UNDER PHOSPHATE-LIMITING CONDITIONS



STSU_19410, phoP



STSU_23624, adpA



STSU_31980, fkbD

IMMUNOTEC – WP2:

Eberhard-Karls-University of Tübingen, Germany
Institute of Microbiology and Infection Medicine
Depart. Microbiology and Biotechnology

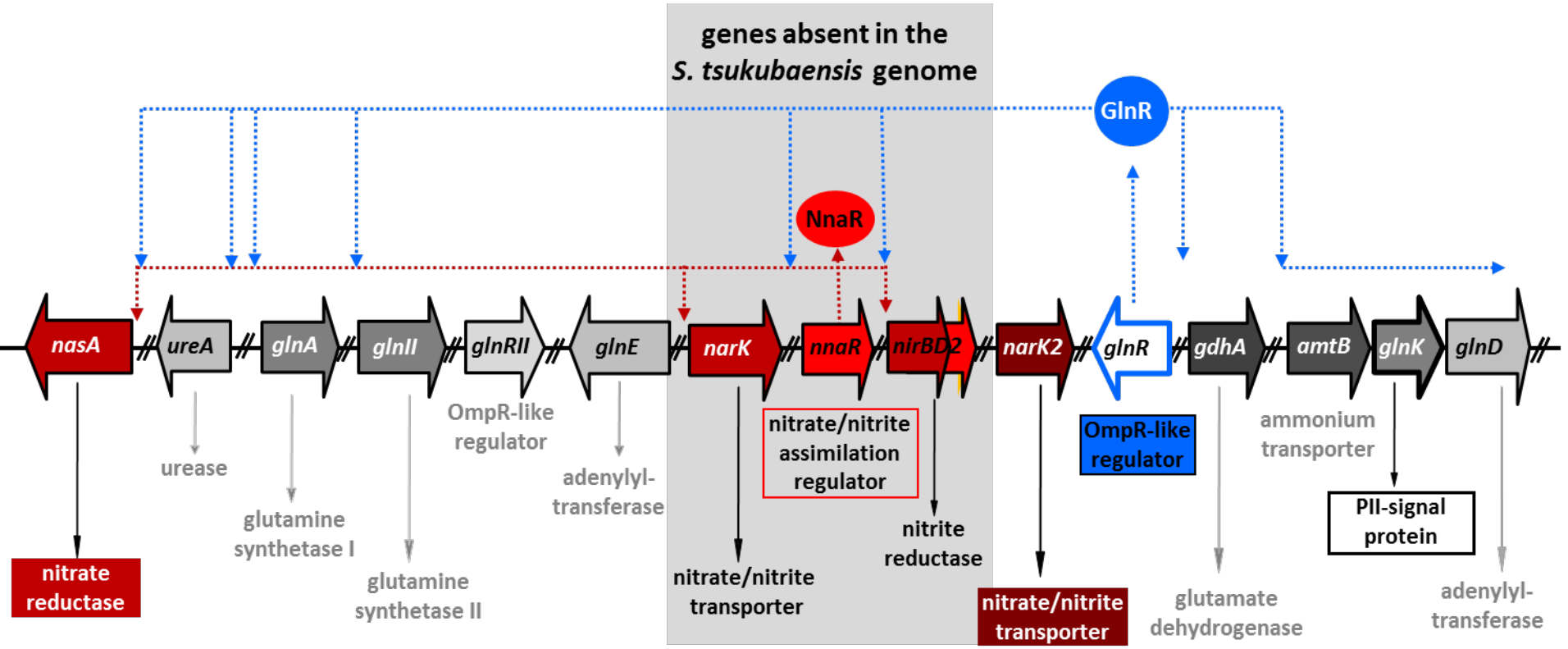
Prof. Wolfgang Wohlleben
Dr. Agnieszka Bera
PhD student Annika Kemeny
PhD student Susann

Nitrogen regulation of primary metabolism and tacrolimus biosynthesis. Mutants altered in nitrogen metabolism.

Strategies to optimize tacrolimus production by the modification of nitrogen metabolism

1. Construction of strains able to use nitrate, a substrate that enhance antibiotic production
2. Optimize the production of pipecolic acid, a tacrolimus precursor
3. Optimize lysine biosynthesis, a precursor of pipecolic acid

Regulation of nitrogen metabolism in *S. coelicolor* and *S. tsukubaensis*

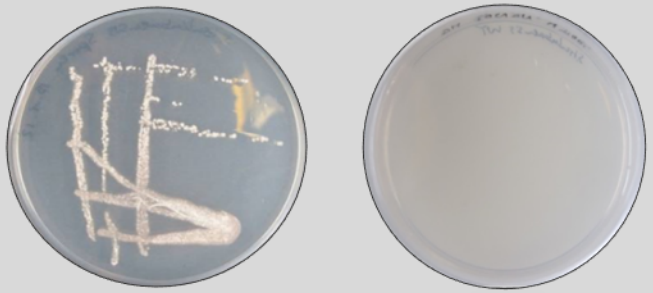


S. tsukubaensis **is unable to grow on nitrate due to impairment of the NO_3/NO_2 assimilation**

Nitrogen sources used by *S. tsukubaensis*:

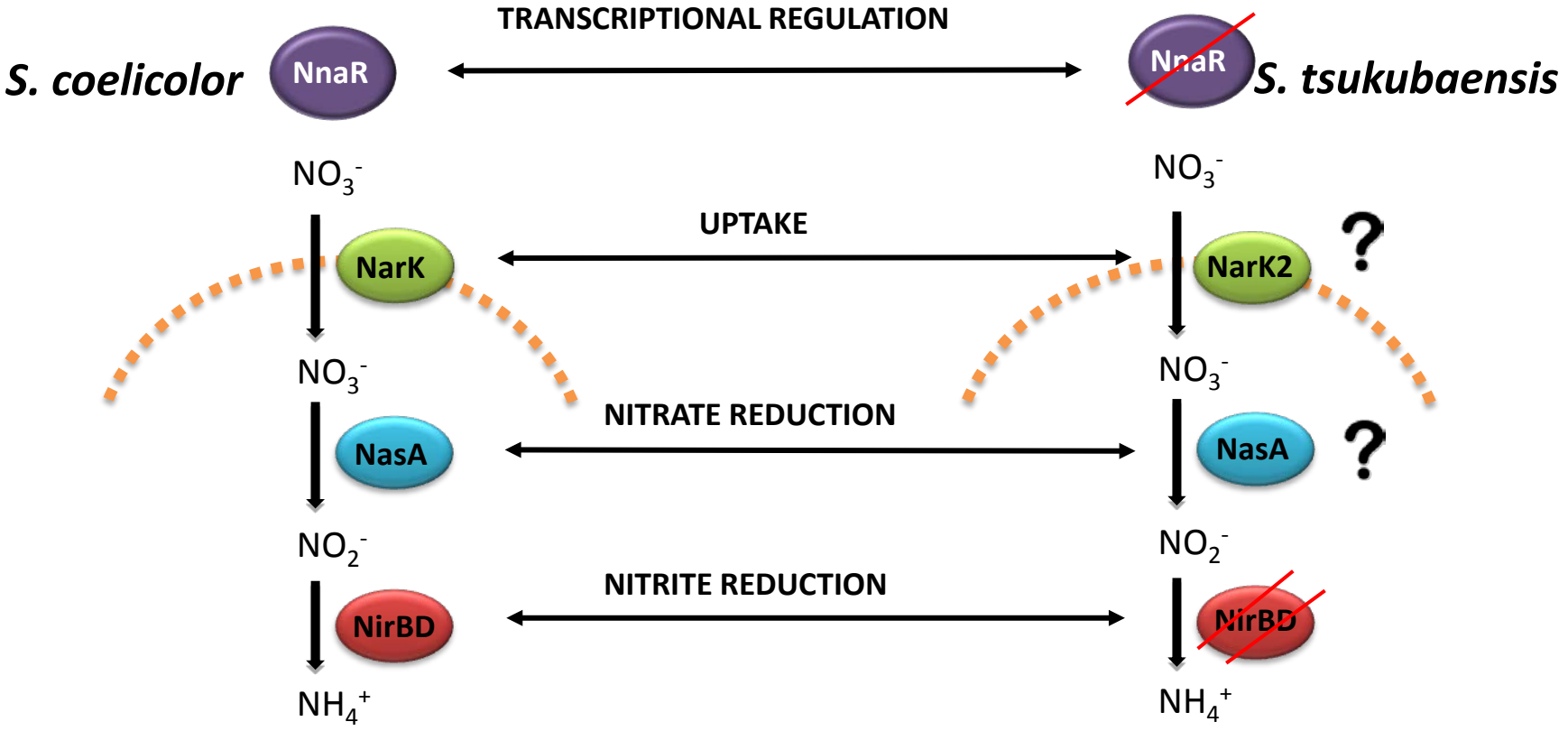
- **ammonium**
- **glutamine**
- **glutamate**
- **arginine**
- **asparagine**

...but no nitrate



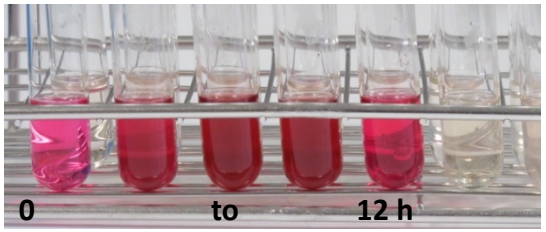
MG suppl. with ammonium **or** **nitrate**

Nitrate/nitrite assimilation pathway



Griess-Ilosvay-Assay: Nitrate to nitrite

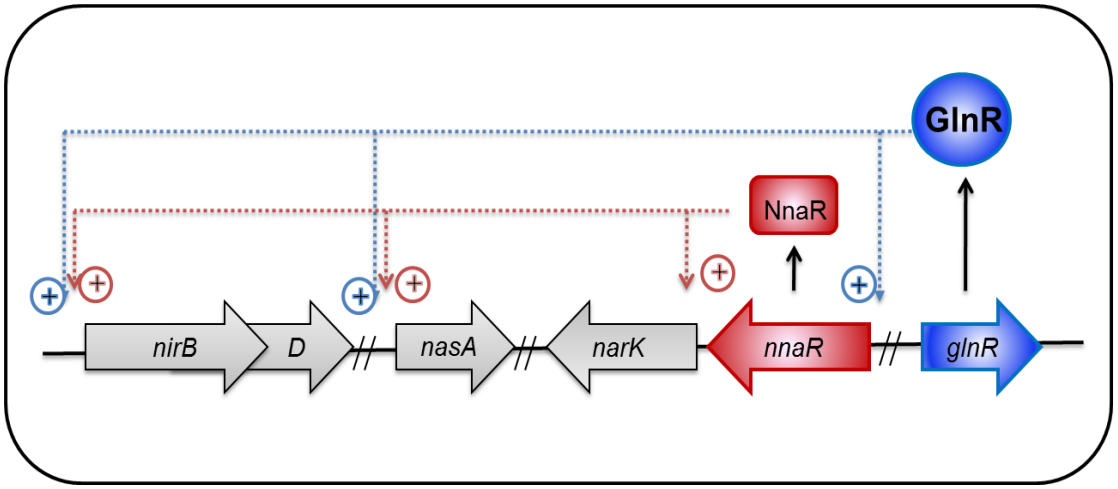
S. coelicolor: *active pathway*



S. tsukubaensis: *inactive pathway*

S. tsukubaensis **is unable to reduce nitrate to nitrite and then nitrite to ammonium**

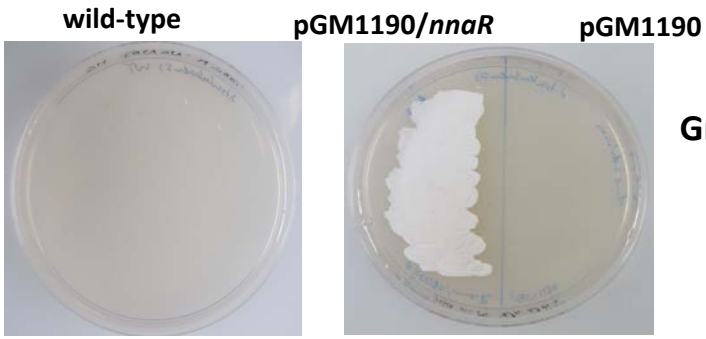
Model for NnaR dependent control of nitrate/nitrite assimilatory genes in *Streptomyces coelicolor*



NnaR dependent expression of the nitrate/nitrite assimilation genes is:

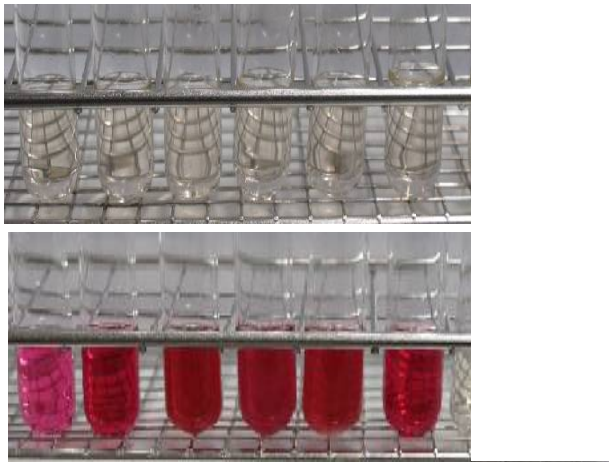
- **activated during general nitrogen limitation**
- **activated in the presence of nitrate**
- **repressed in the presence of high ammonia concentrations**

HETEROLOGOUS EXPRESSION OF *nnaR*



S. tsukubaensis:
Growth in MG + 100 mM Nitrate

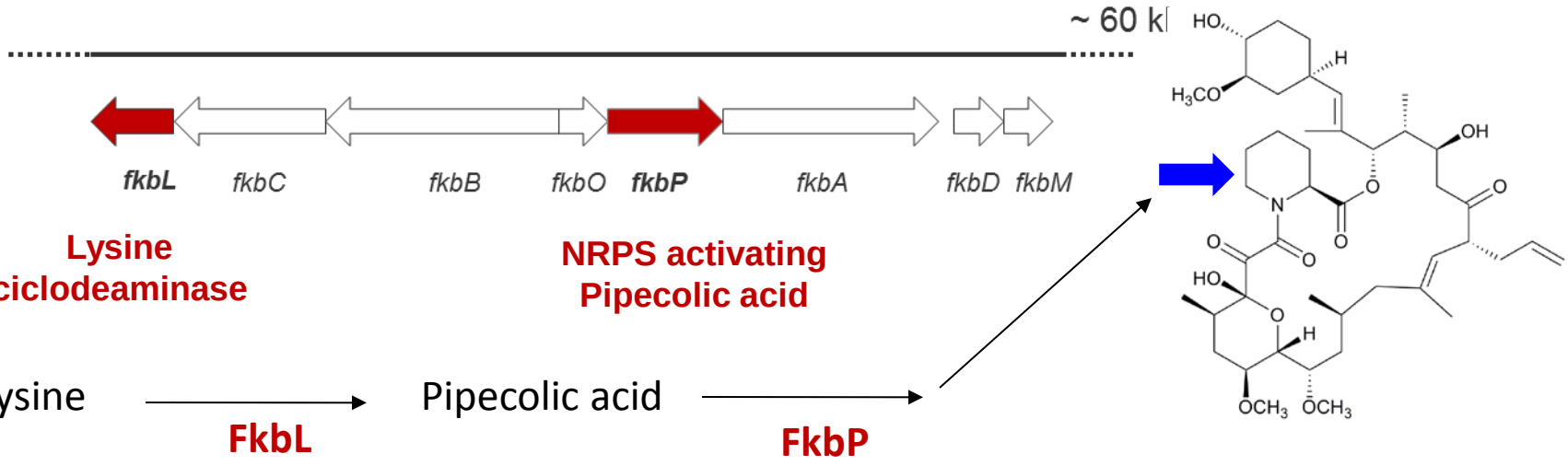
***Streptomyces tsukubaensis* pGM1190**



Streptomyces tsukubaensis* pGM1190/*nnaR

Heterologous expression of NnaR initiated an activation of the NO_3/NO_2 assimilation pathway

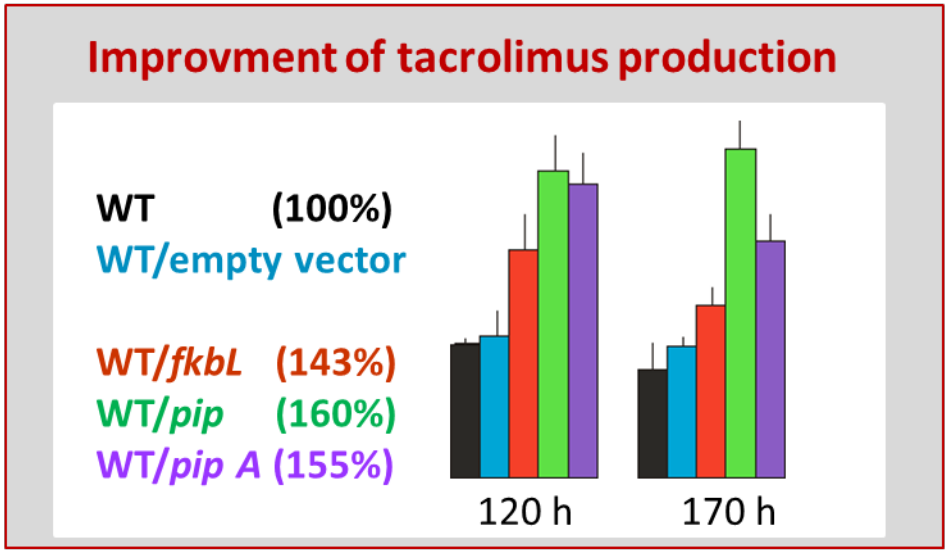
II. Strategies to optimize the pipecolic acid precursor



1. **Overexpression of the *fkbL* or *fkbL-fkbP* increased tacrolimus production by 45%.**

2. **Heterologous expression of *pip* genes from:**
   - ***Streptomyces pristiniaspiralis (pipA)***
   - ***Actinoplanes friuliensis (pip)***

resulted in significant increase of the tacrolimus producton by over 60%.



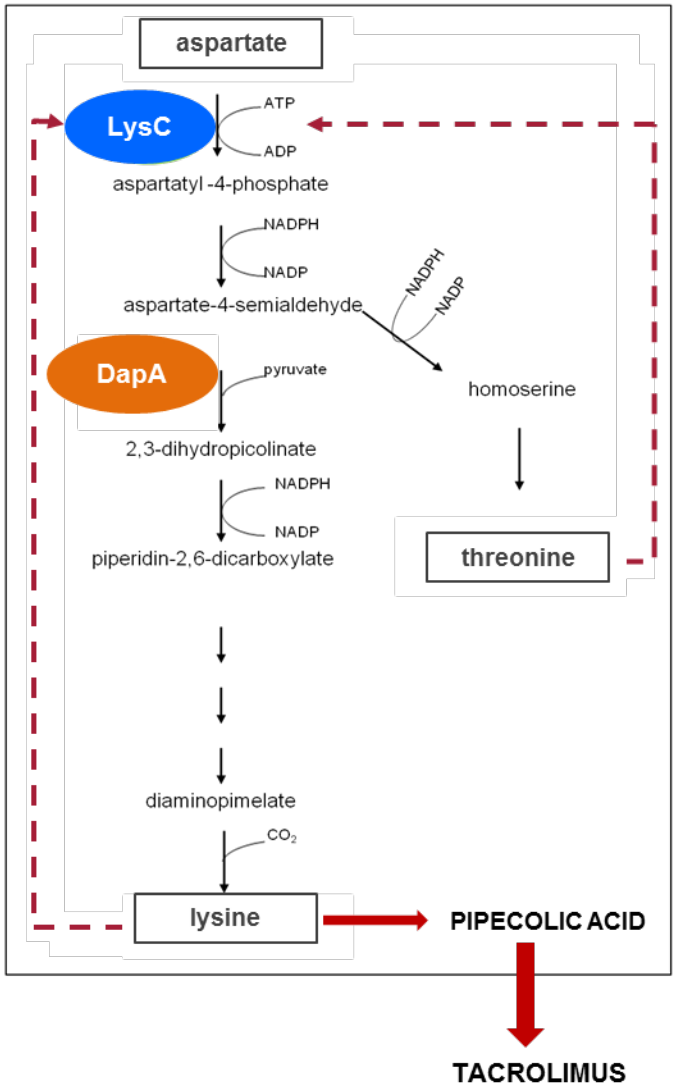
Optimization of the lysine biosynthesis in *S. tsukubaensis*

Homologs of *lysC* and *dapA* in *S. tsukubaensis* identified

Approaches:

- ***lysC**** (STSU_3111): site-directed mutagenesis (Ser→Tyr) in *lysC*$_{ß}$ region to get a feedback inhibition resistant aspartate kinase.
- Overexpression of ***lysC**** (STSU_3111) and ***dapA*** (STSU_1603) resulted in slight increase of the tacrolimus production.

- Heterologous expression of ***lysC**** from *C. glutamicum* – on going
- Simultaneous overexpression of ***pip*** and ***lysC*/dapA*** *in S. tsukubaensis* – on going



IBMC

SCIENCE FOR THE FUTURE

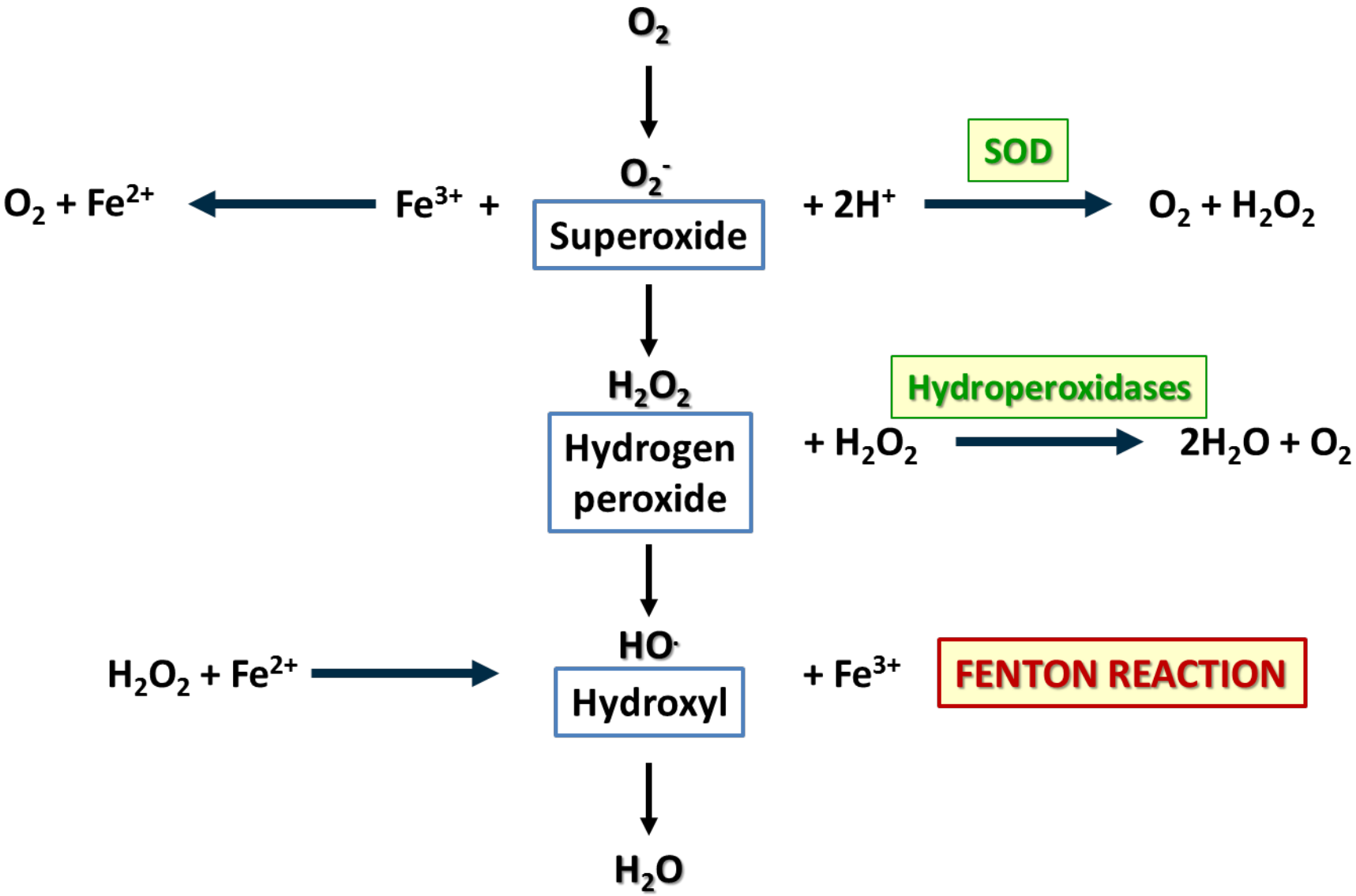
WP3. MODULATION OF OXIDATIVE STRESS

Partner 3:

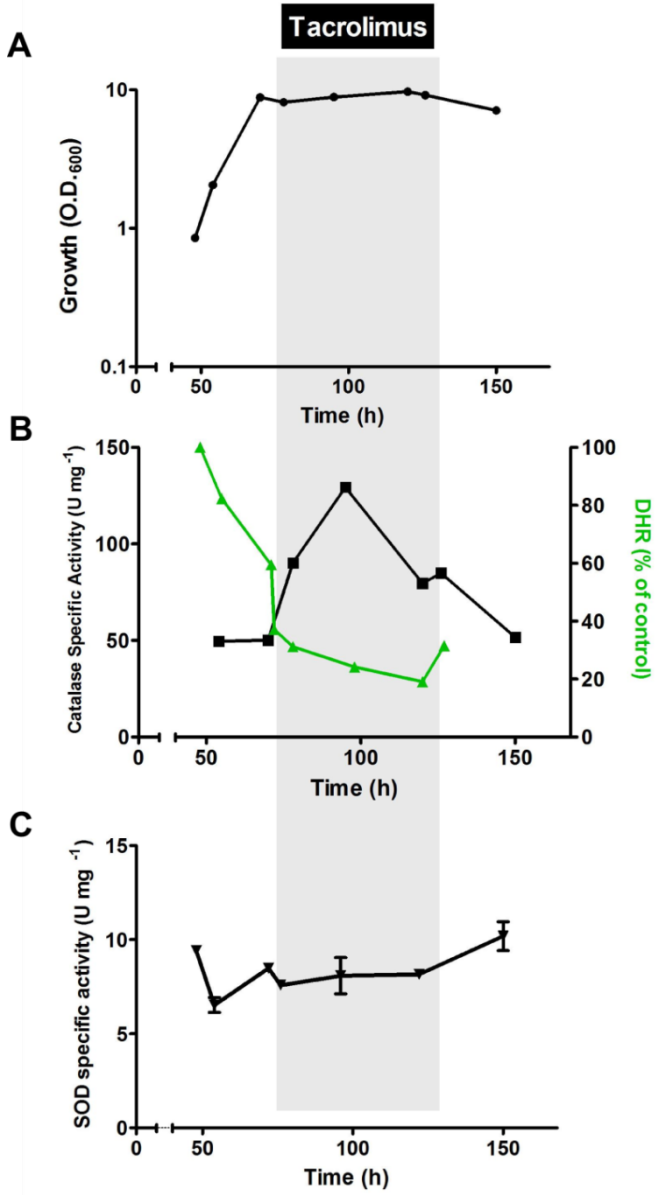
Marta V. Mendes - PI
Sílvia Pires - PhD student
Rute Oliveira - Research fellow

PORTO (PORTUGAL)

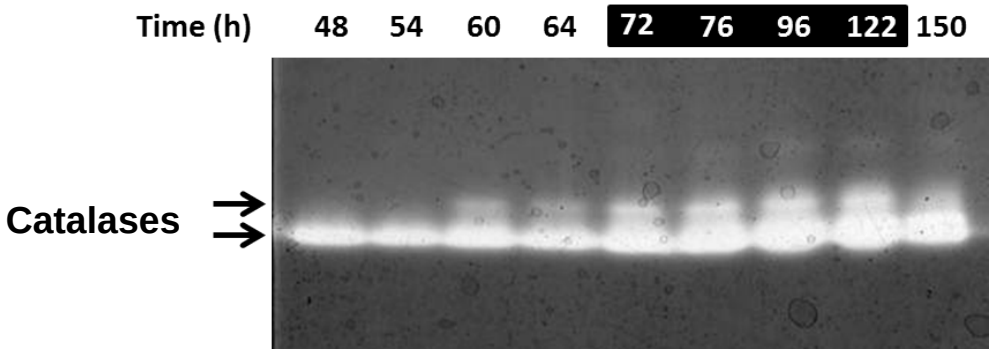
WP3. REACTIVE OXYGEN SPECIES (ROS) AND DETOXIFICANT ENZYMES



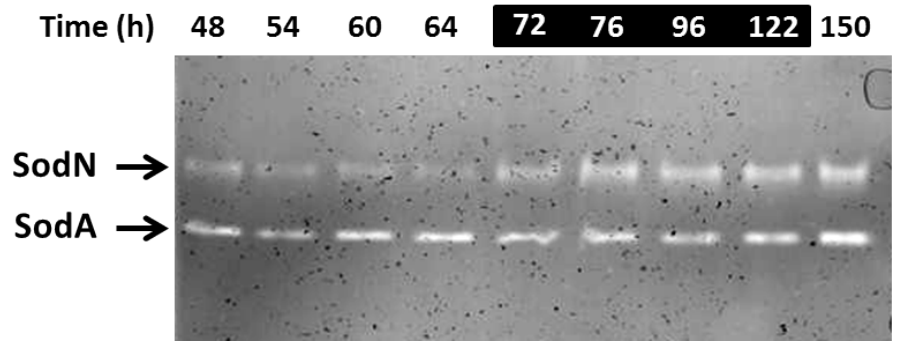
WP3. Antioxidant defences in *S. tsukubaensis* (MGm medium)



- Native-PAGE stained for catalase activity

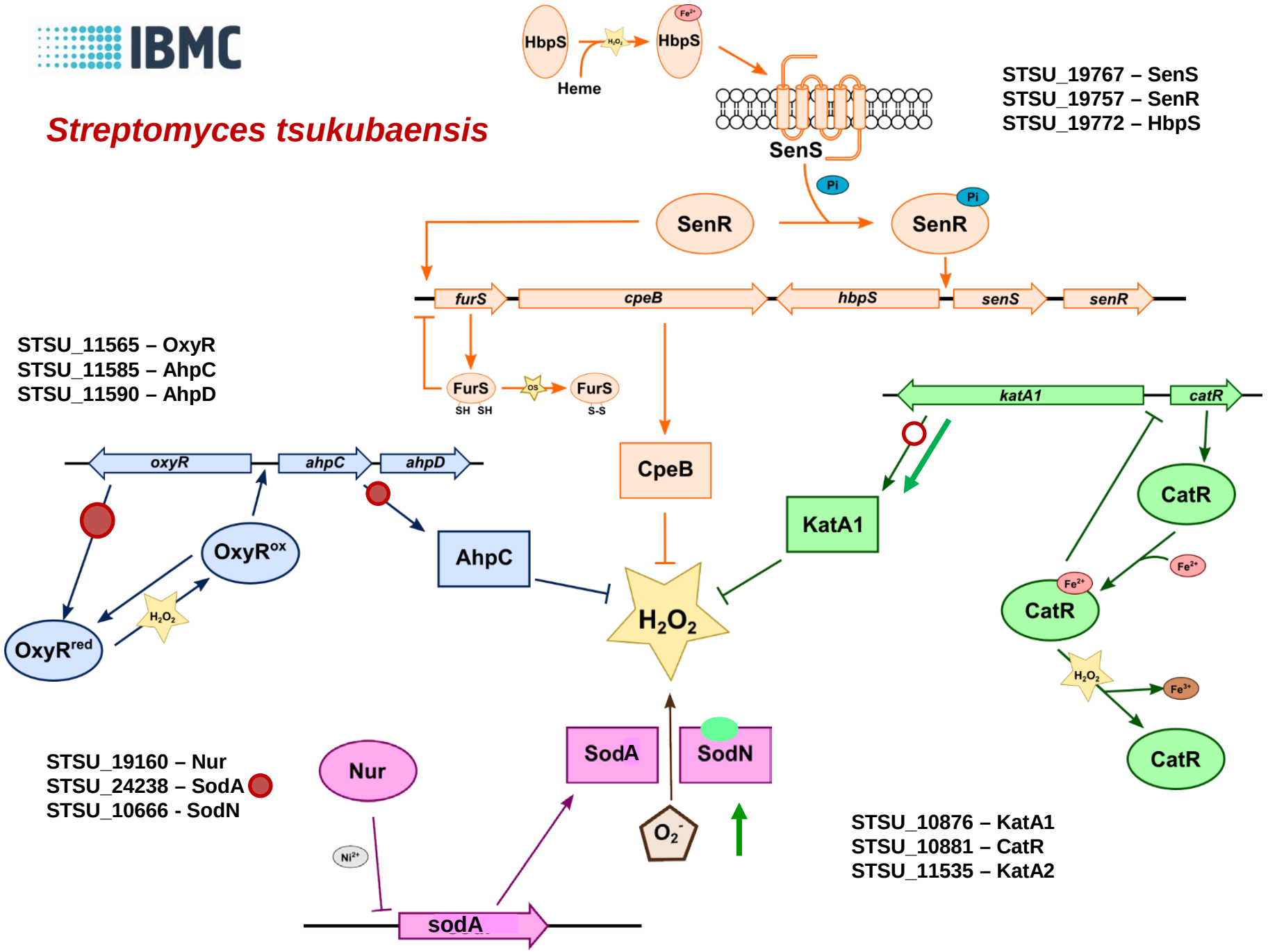


- Native-PAGE stained for SOD activity

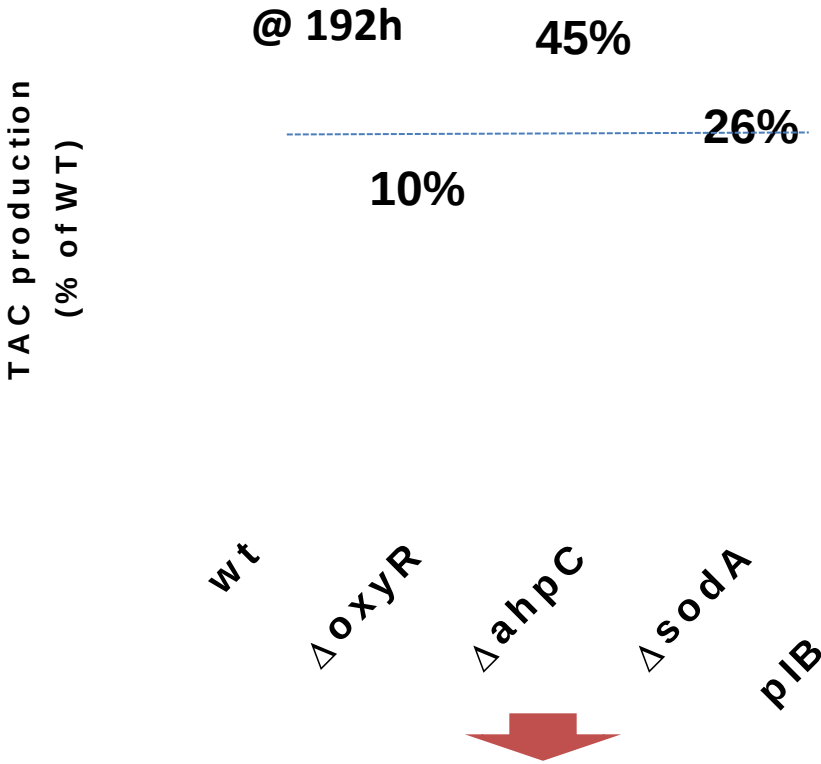


IBMC

Streptomyces tsukubaensis



Tacrolimus production by the mutants



1 - Ascorbic acid (50mM)

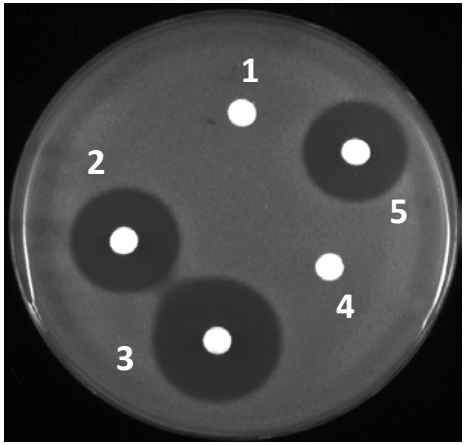
2 - Ascorbic acid (50mM) + H_2O_2 (1M)

3 - H_2O_2 (1M)

4 - FK-506 (0,25µg/µL)

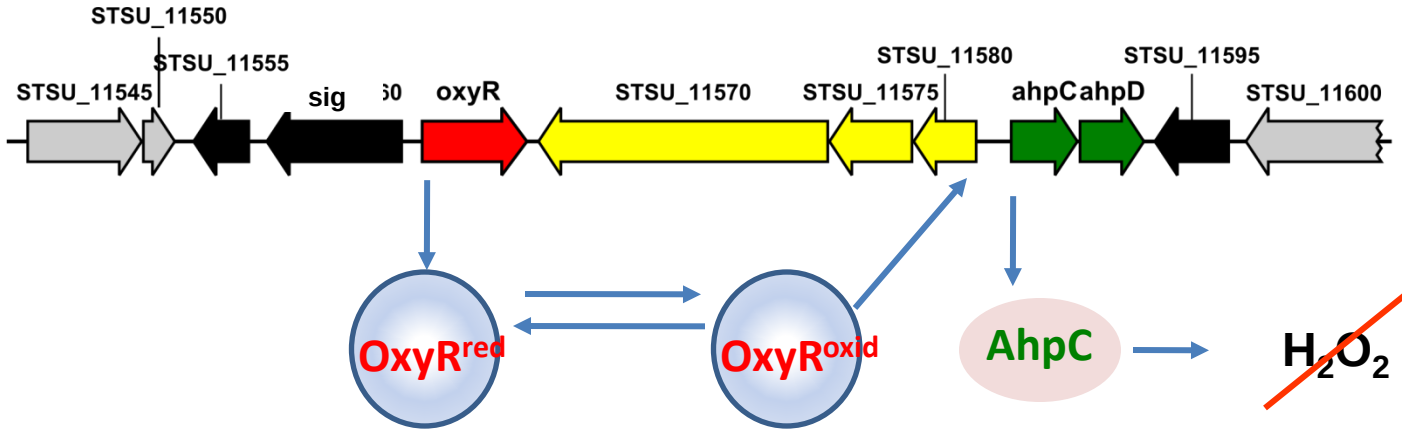
5 - FK-506 (0,25µg/µL) + H_2O_2 (1M)

Expression by PCR in the Δ*sodA* mutant
of the tacrolimus genes
oxidative stress genes
Iron uptake metabolism
Phosphate metabolism

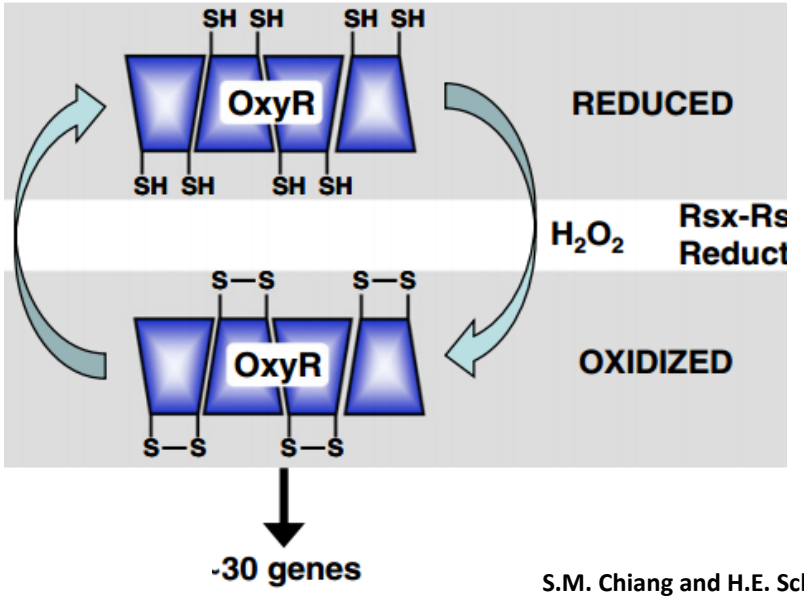


[*Saccharomyces cerevisae* BY4741]

S. tsukubaensis



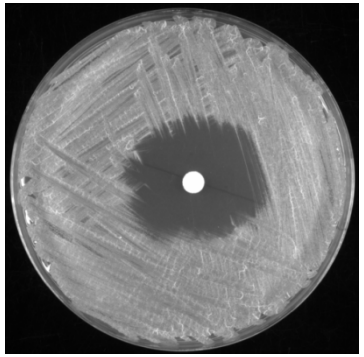
OxyR redox activation (LysR-type)



S.M. Chiang and H.E. Schellhorn (2012)

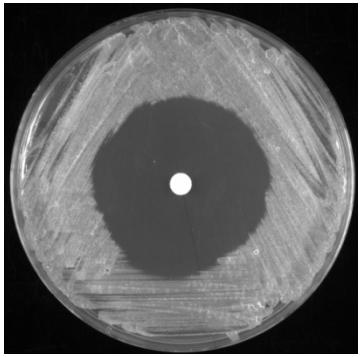
H_2O_2 sensitivity

WT



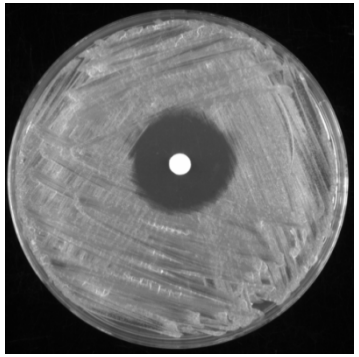
3.47 cm ± 0.229

ΔoxyR



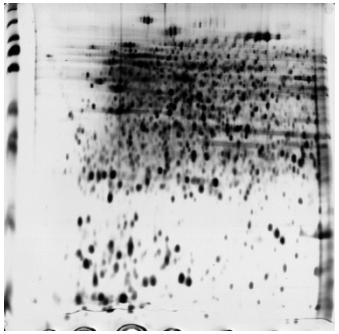
4.65 cm ± 0.069

ΔahpC

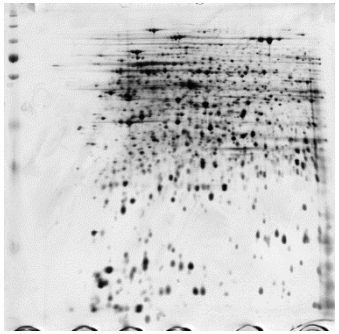


2.73 cm ± 0.068

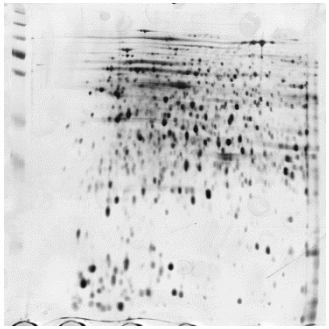
2% YED Medium
9 M H_2O_2



wt_vs_Δ*oxyR* : 47 differences



wt_vs_Δ*ahpC* : 66 differences

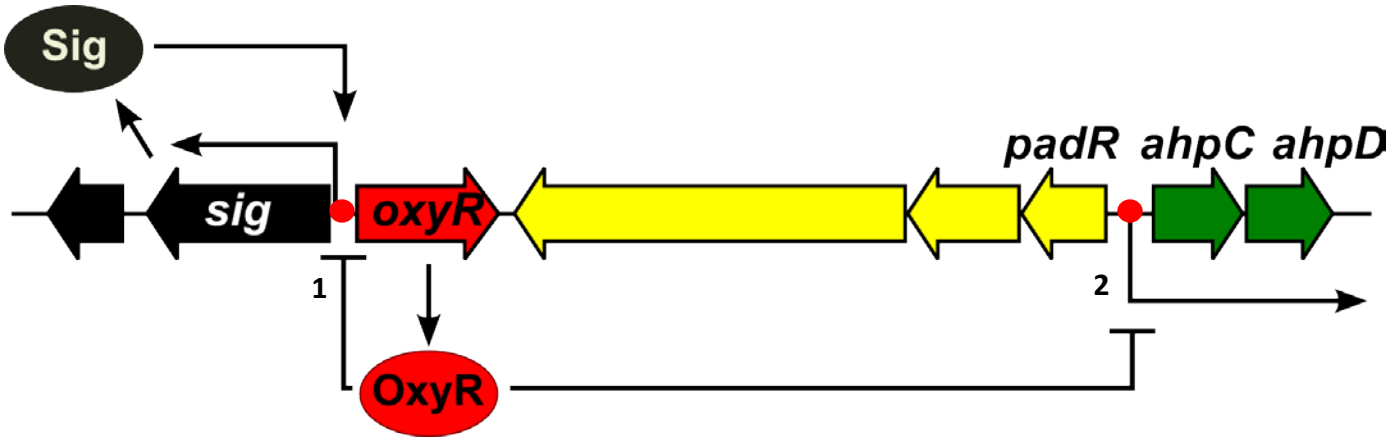


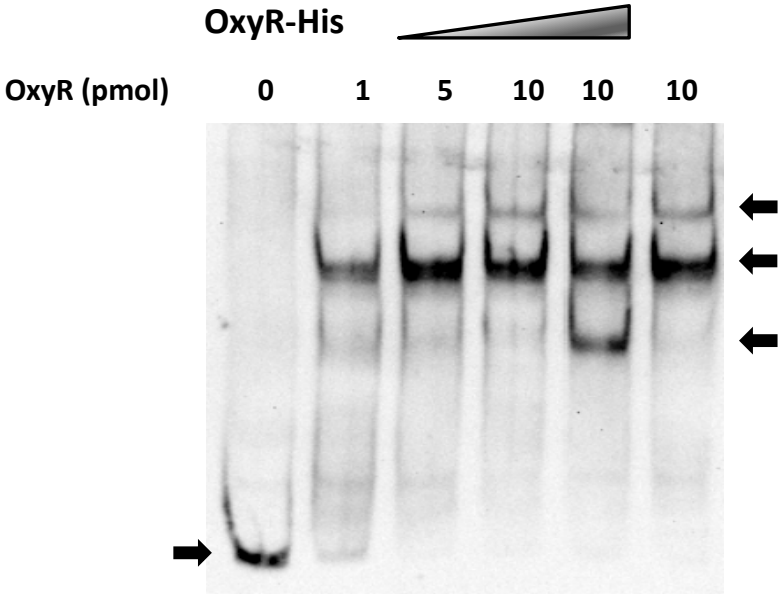
- Δ*oxyR* _vs_ Δ*ahpC* : 46 differences

Positive regulation: 22 genes,
e.g *aphC*

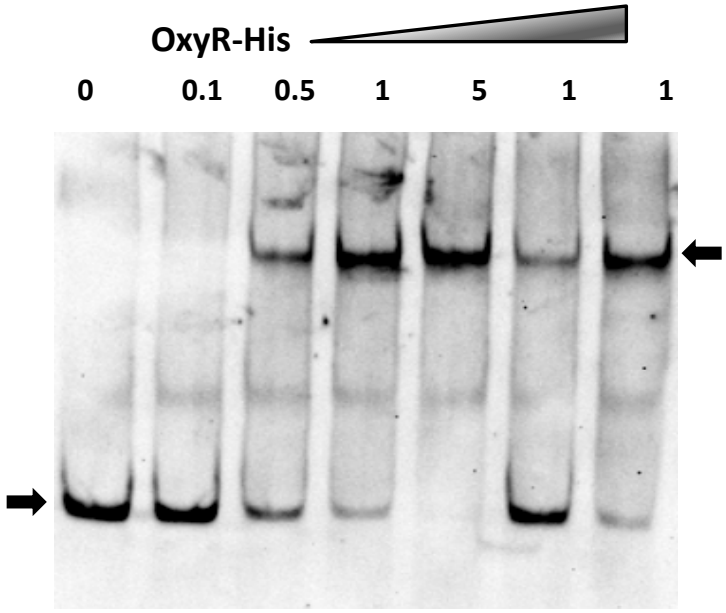
Negative regulation: 10 genes,
e.g *oxyR*

The oxyR regulon





1-*sig-oxyR* promoter region



2-*padR-ahpC* promoter region

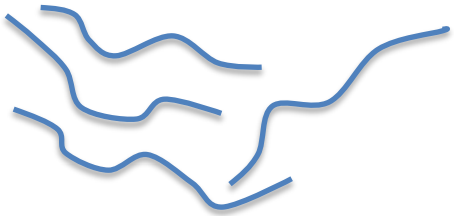
WP5. HETEROLOGUS EXPRESSION OF THE TACROLIMUS BIOSYNTHETIC GENE CLUSTER IN MODIFIED *Streptomyces* STRAINS

Eberhard Karls Universitat Tübingen

Overexpression of the tacrolimus cluster. Heterologous systems. Superhosts

Partner 4:
Prof. Lutz Heide
Dr. Adam Jones
Dr. Bertolt Gust
Dr. Christian Appel

I. Cloning of FK506 (tacrolimus) gene cluster into P1-derived phage artificial chromosome (PAC)



Streptomyces tsukubaensis gDNA

1. digest with BamHI

2. resolve with pulse field gel electrophoresis

100-150 kb DNA fragments

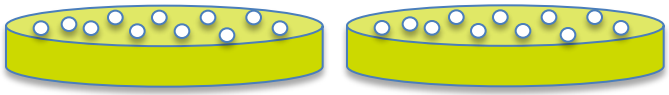
fragmented DNA

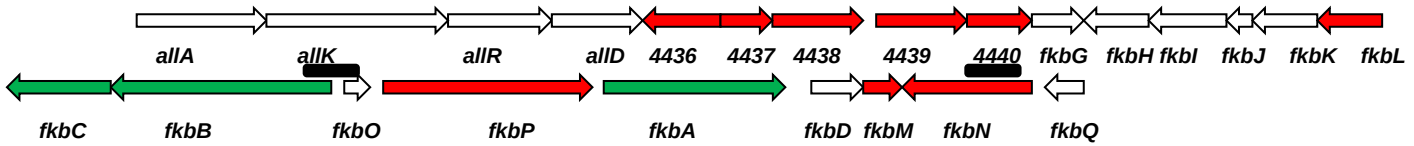
pESAC-13
23 kb

PAC vector digested with BamHI

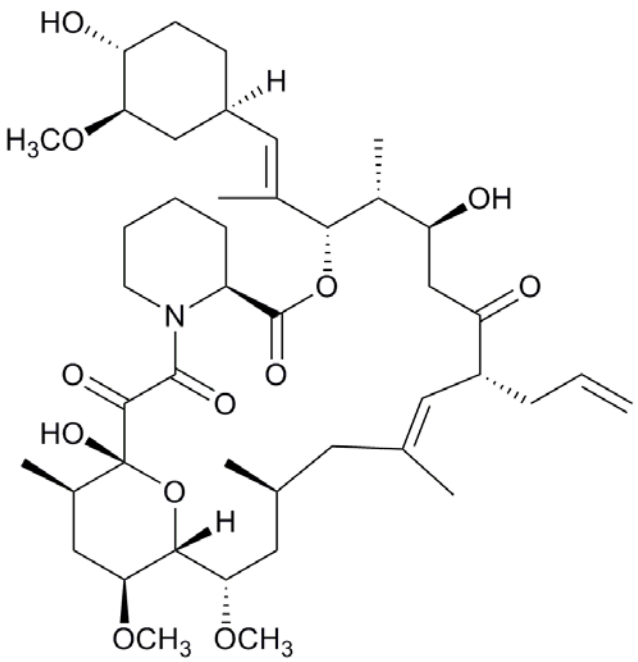
ligation for PAC library

screen library for FK506 gene cluster

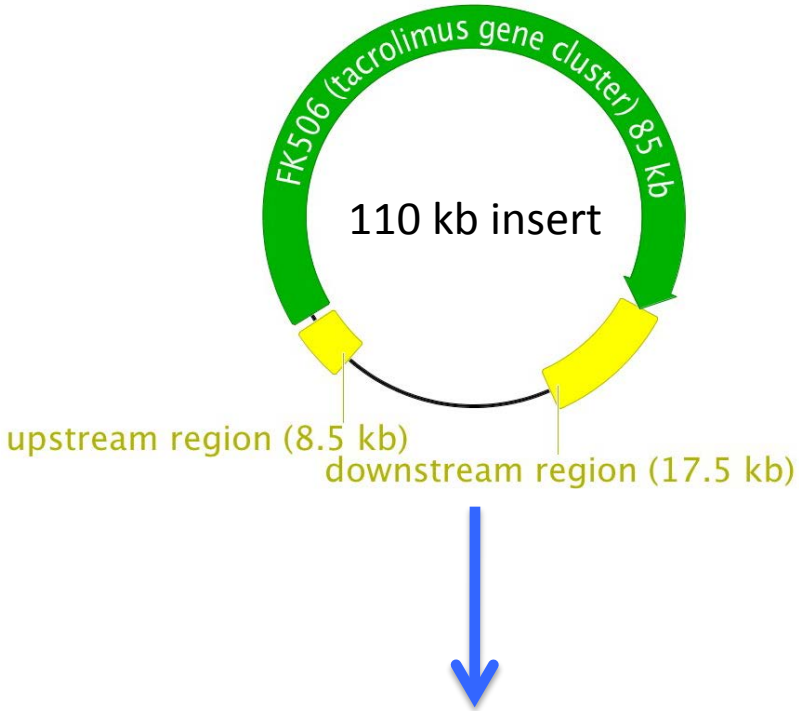




Cloning of FK506 (tacrolimus) gene cluster into phage artificial chromosome (PAC)

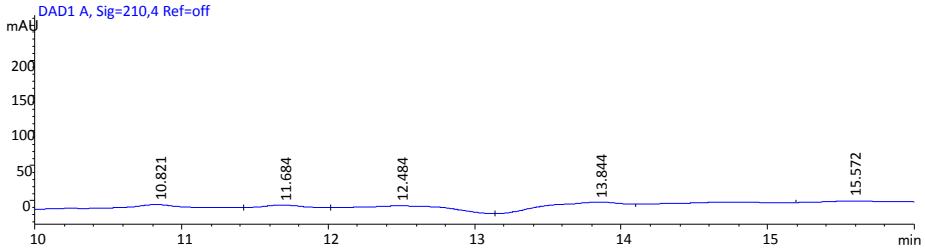


FK506

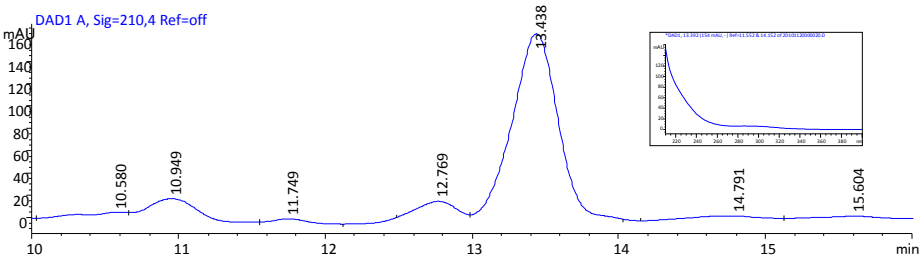


Introduce PAC into *Streptomyces coelicolor*
Screen for FK506 production

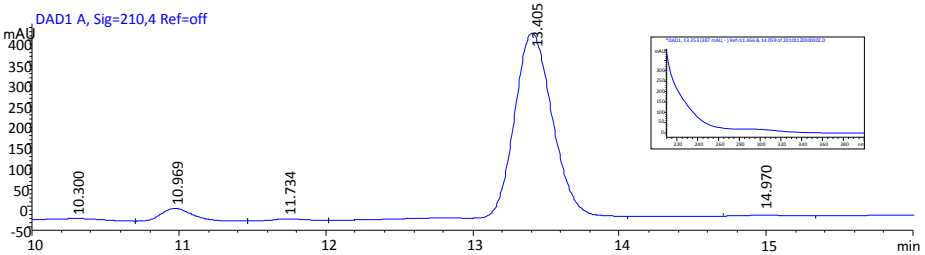
FK506 production in *Streptomyces coelicolor*



***S. coelicolor* M1146**
empty pESAC-13

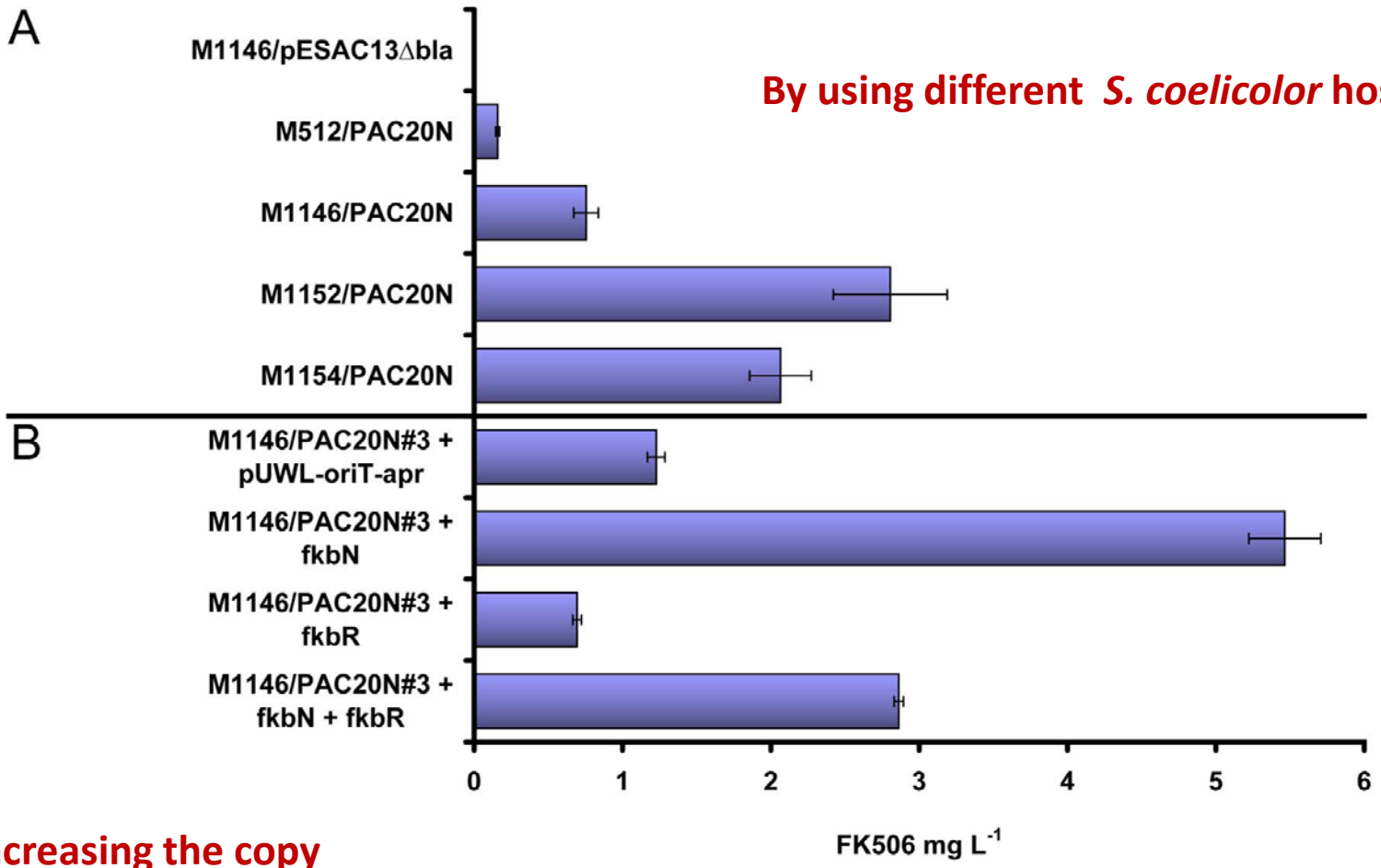


***S. coelicolor* M1146**
with FK506 PAC



FK506 standard

Optimization of tacrolimus production in *S. coelicolor*



By using different *S. coelicolor* hosts

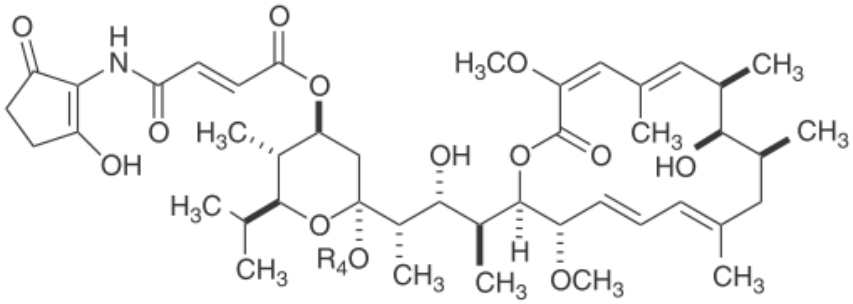
By increasing the copy number of the regulators

Increased production by 80%

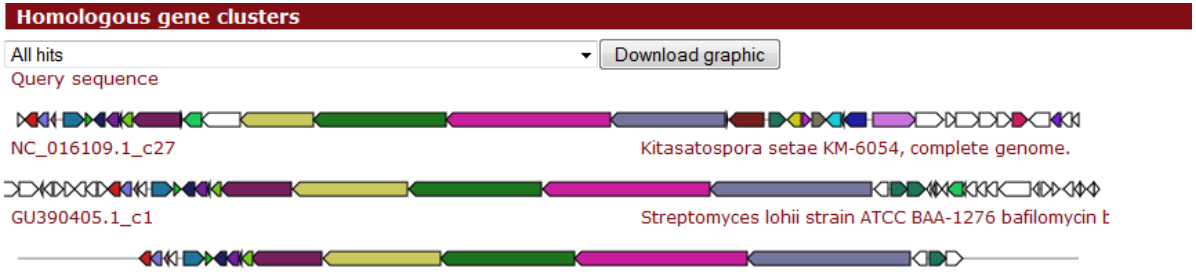
Heterologous expression of the bafilomycin-like gene cluster from *S. tsukubaensis*

- **A family of 16-membered ring macrolide antibiotics.**
- **Potent vacuolar H+-ATPase inhibitors**
  - **antifungal, immunosuppressant, antitumor and antiparasitic (Yu *et al*, 2011)**
  - **Employed to study ATPase**
- **Produced by various actinomycetes**
  - ***S. griseus* for bafilomycins A1, A2, B1, B2, C1 and C2**
  - ***Kitasatospora setae* for bafilomycin B1**
  - ***Streptomyces lohii***

Bafilomycins



Bafilomycin B1 (**6**, $R_4 = H$)
Bafilomycin B2 (**7** $R_4 = CH_3$)



CONCLUSSIONS

S. tsukubaensis **genome has been sequenced and information provided to all the partners**

The *bul* DNA region for butyrolactone biosynthesis and receptors and their binding to specific promoter sequences has been analyzed

The *pho* DNA region has been analyzed and transcriptomic analysis on the CCR response in low phosphate concentration, favoruable for tacrolimus production, has been studied

The genes for nitrogen asimilation and their regulators have been analyzed. *S. tsukubaensis* transformants, carrying the *S. coelicolor nnaR* gene, able to grow on nitrate have been obtained

The genes involved in oxidative stress regulation have been studied and related to tacrolimus production

Tacrolimus overproducer strains have been obtained by transformation with the *fkbN* gene, the *pipA* gene o by knock out of the *aphC* gene. This results in 40 to 60% increase production in each case.

The tacrolimus gene cluster of *S. tsukubaensis* has been expressed in *S. coelicolor* and the heterologous production has been optimized 80% over the original production

PUBLICATIONS : 4 published or in press, others submitted

Martínez-Castro, M., Salehi-Najafabadi Z., Romero, F., Pérez-Sanchis, R., Fernández-Chimeno R.I., Martín, J.F., Barreiro, C. (2013). Taxonomy and chemically semi-defined media for the analysis of the tacrolimus producer *Streptomyces tsukubaensis*. Applied Microbiology and Biotechnology 97:2139-2152. **WP1**

Goranovič D, Blažič M, Magdevska V, Horvat J, Kuščer E, Polak T, Santos-Aberturas J, Martínez-Castro M, Barreiro C, Mrak P, Kopitar G, Kosec G, Fujs S, Martín JF, Petković H. (2012) FK506 biosynthesis is regulated by two positive reglatory elements in *Streptomyces tsukubaensis*. BMC Microbiol. 12:238. **WP1**

Jones AC, Gust B, Kulik A, Heide L, Buttner MJ, Bibb MJ (2013) Phage P1-derived artificial chromosomes facilitate heterologous expression of the FK506 gene cluster. PLoS One 8: e69319. **WP5.**

Salehi-Najafabadi, Z., Barreiro C, A. Rodríguez-García A, A. Cruz A, López GR, Martín JF (2014) The γ-butyrolactone receptors BulR1 and BulR2 of *Streptomyces tsukubaensis* control the butyrolactone synthetases and the production of tacrolimus: Characterization of BulR1 DNA-binding sequences. In press. **WP1**

Kocadinc S, Wohlleben W. and A. Bera. (2014) Optimization of the N-containing precursor supply by genetic engineering of *Streptomyces tsukubaensis* for FK506 production improvement. Manuscript in preparation. **WP2**

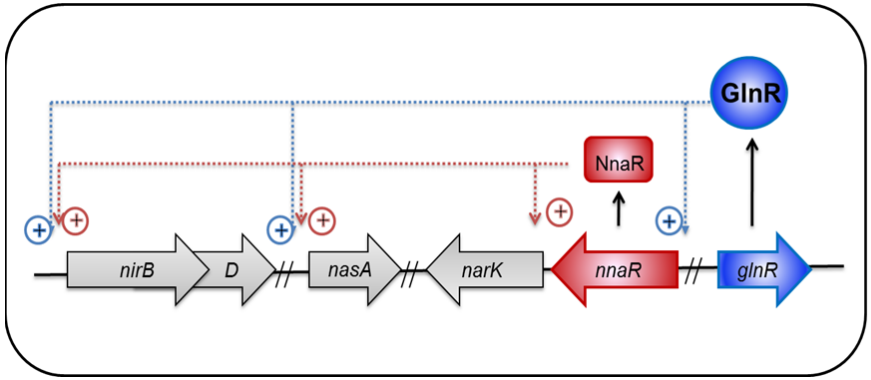
Pires S, R. Oliveira, T. Beites, P. Moradas-Ferreira and M.V. Mendes (2014) The OxyR-dependent regulatory mechanisms of oxidative stress response and iron metabolism interplaywith tacrolimus production in *Streptomyces tsukubaensis.* Send for publication. **WP3**

Jones A. C., Flinspach K., Herbig A., Apel A. K., Nieselt K. and Heide L. (2014) RNA-seq transcriptional analysis of the FK506 biosynthetic gene cluster in *Streptomyces tsukubaensis* NRRL118488. International Microbiology. Submited, under revisión. **WP5**

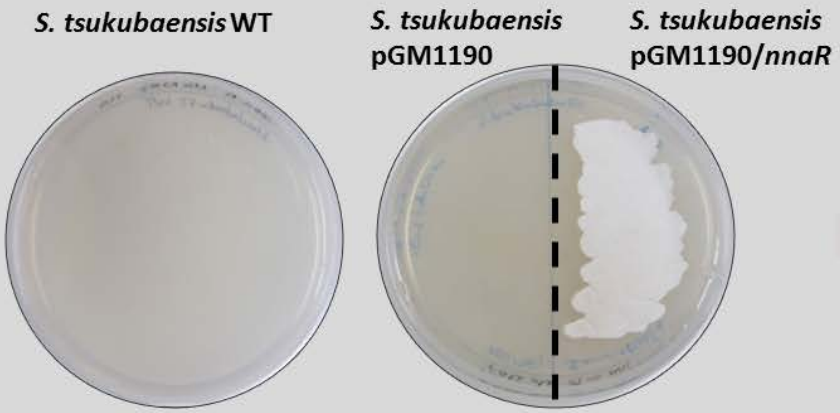
Heterologous expression of *S. coelicolor nnaR* in *S. tsukubaensis*

Expression of nitrate/nitrite assimilatory genes is:

- activated during general nitrogen limitation
- activated in the presence of nitrate
- repressed in the presence of high ammonium conc.



Heterologous expression of *nnaR* restored nitrate assimilation in *S. tsukubaensis*



S. tsukubaensis growth on MG suppl. with 100mM nitrate

Heterologous expression of NnaR initiated an activation of the NO_3/NO_2 assimilation pathway