CONTIbugs EIB.12.028
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technische universität dortmund

Prof. Schmid / Prof. Bühler
Correlation of population dynamics & productivity

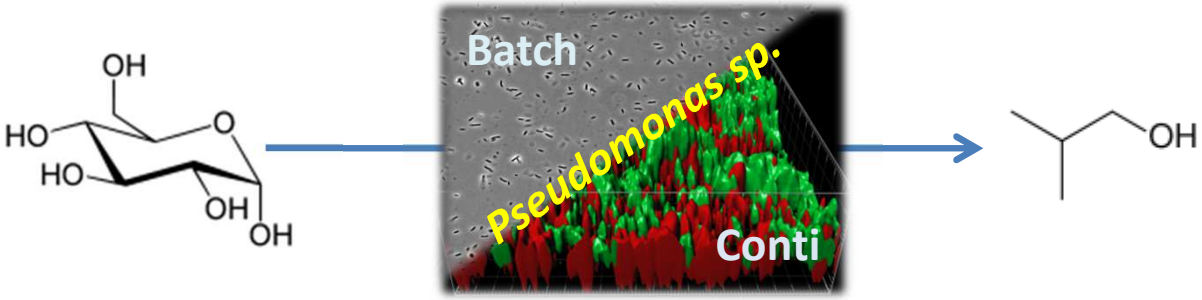
CSIC CNB CENTRO NACIONAL DE BIOTECNOLOGÍA

Prof. de Lorenzo
Tuning genetic circuits towards stable phenotypes

DTU
DTU Systems Biology
Department of Systems Biology

Prof. Molin / Dr. Sternberg
Biofilm specific tool development

Objectives



- Growth
- Geneexpression
- Proteinstability
- Productivity

Cell Heterogeneity

Identify WP 1&2 → Understand WP 3 → Solve WP 4 → CONTIbugs

12 month → 24 month → 36 month

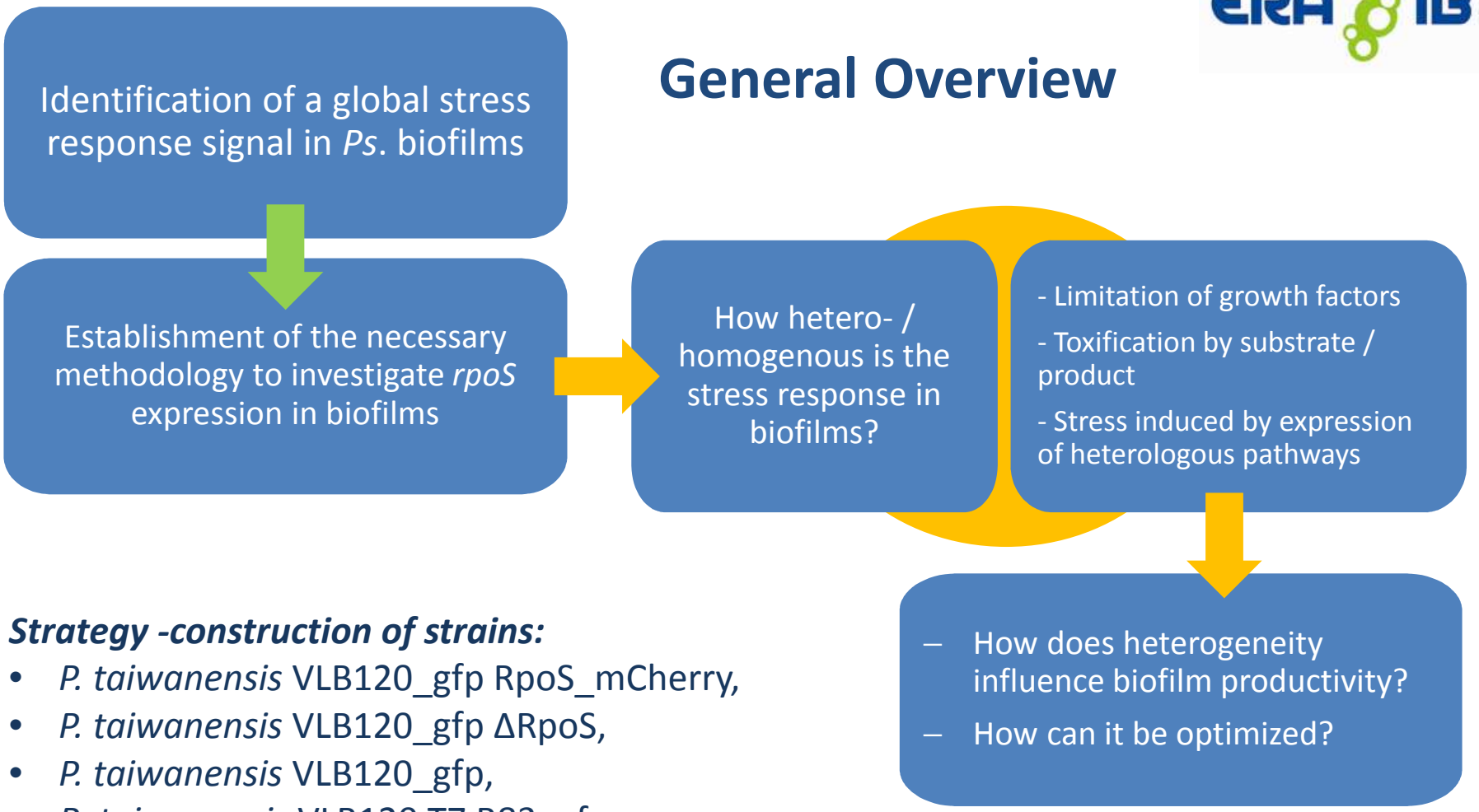
Method establishment

Subpopulation identification / isolation

Implementation of genetic circuits

Design of CONTIbugs

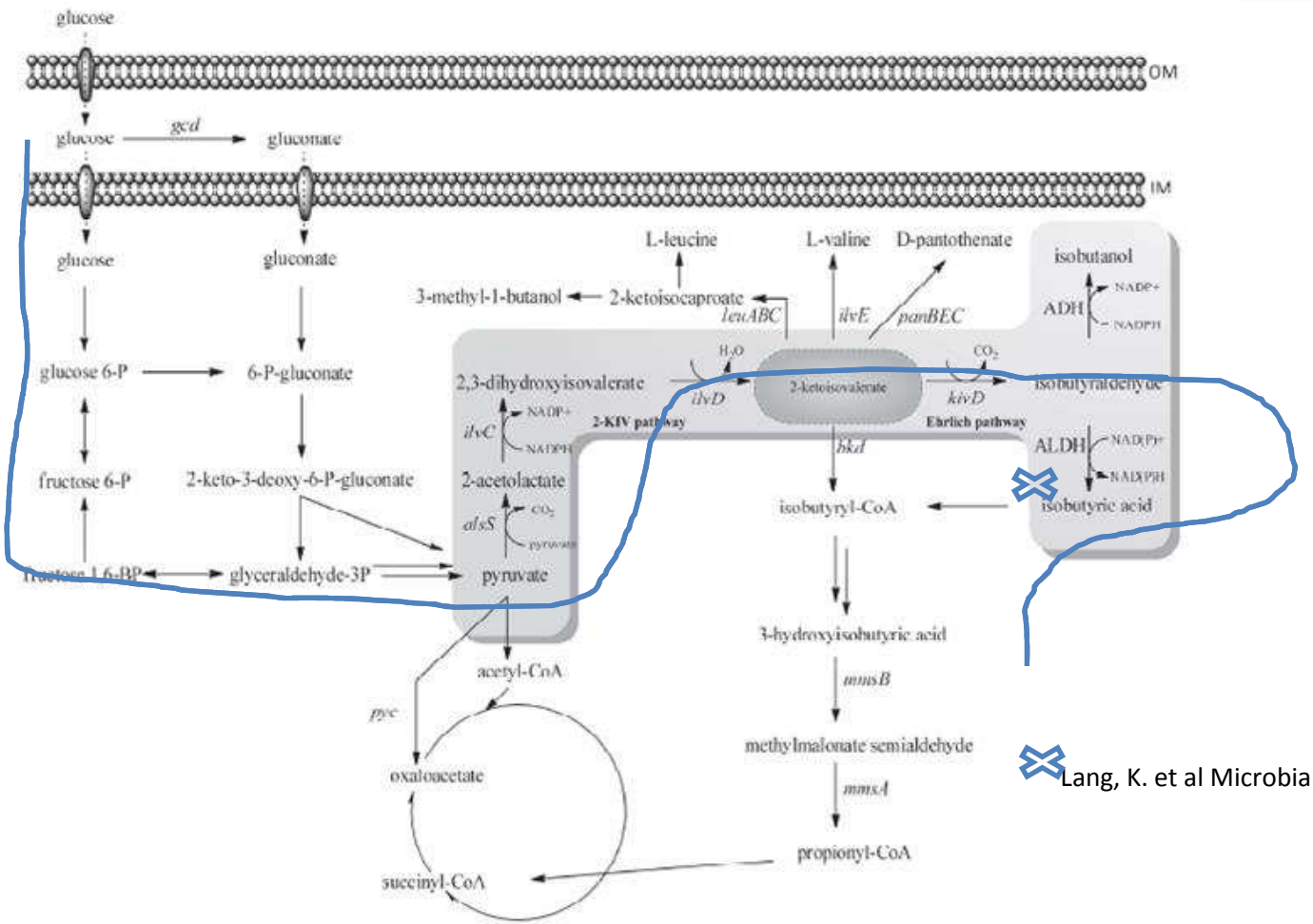
General Overview



Strategy -construction of strains:

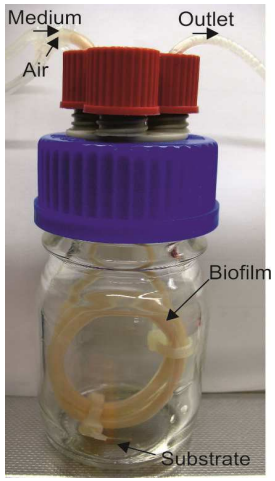
- *P. taiwanensis* VLB120_gfp RpoS_mCherry,
- *P. taiwanensis* VLB120_gfp ΔRpoS,
- *P. taiwanensis* VLB120_gfp,
- *P. taiwanensis* VLB120 T7 B83_gfp
- *P. taiwanensis* VLB120 T7 B83_gfp RpoS_mcherry

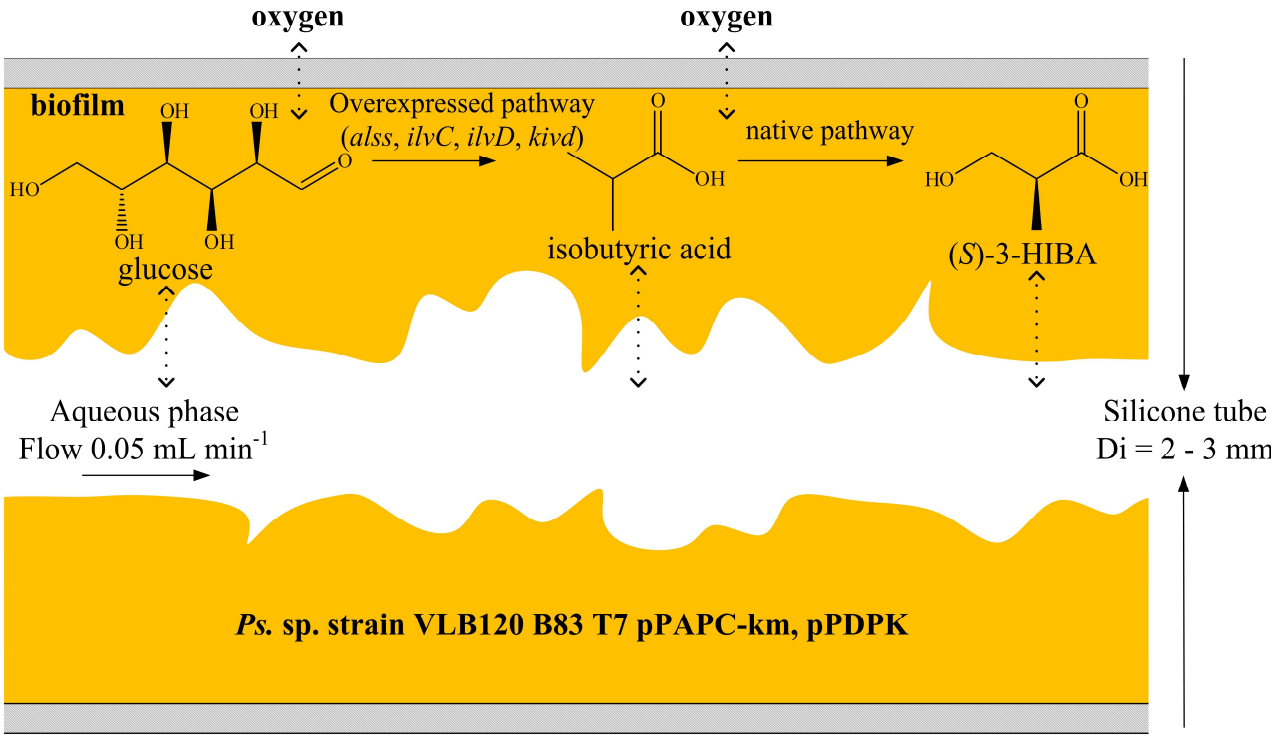
P. taiwanensis VLB120 T7 B83



Lang, K. et al Microbial Cell Factories, 2015

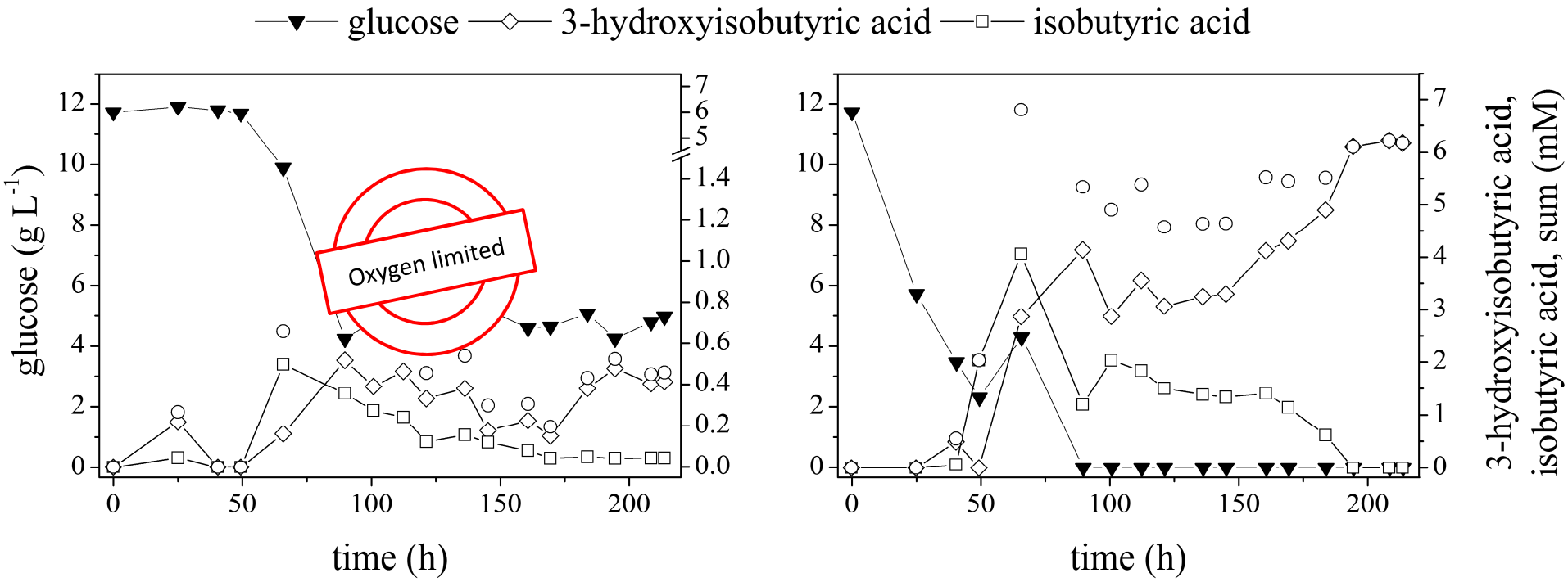
Fermentative production of 3- HIBA using *P. taiwanensis* VLB120 biofilms





- Biofilm-Membrane-Reactor to guarantee continuous (*S*)-3-HIBA production
- Oxygen supply almost exclusively via transmembrane diffusion
- Evaluation of different wall thickness and tube length

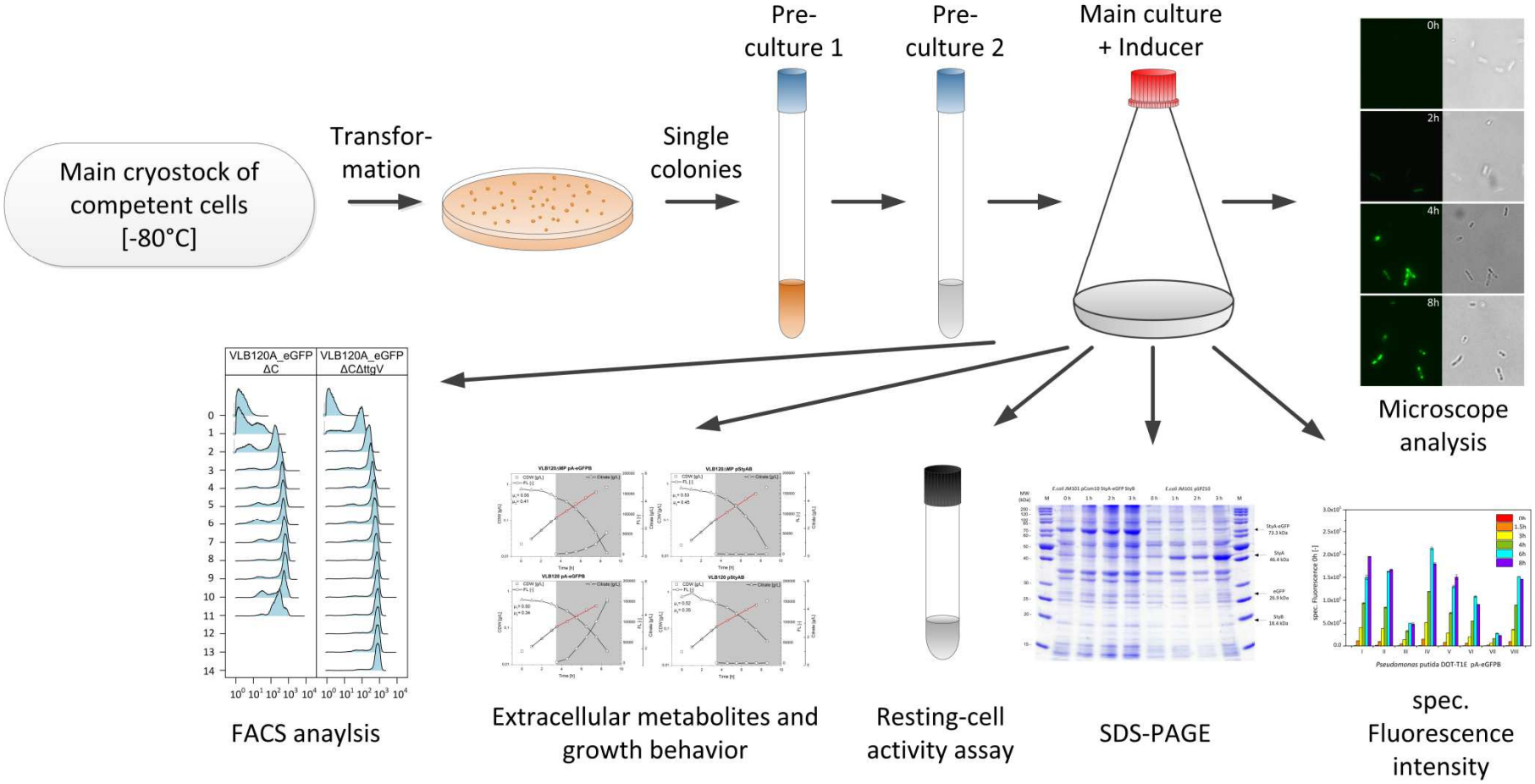
Fermentative production of 3- HIBA using *P. taiwanensis* VLB120biofilms



- Transmembrane oxygen diffusion limits productivity
- Isobutyric acid conversion limits overall reaction rate
- Glucose limitation leads to possitive selection for high producers
- Currently best setup continuously produces up to 6 mM (*S*)-3-HIBA

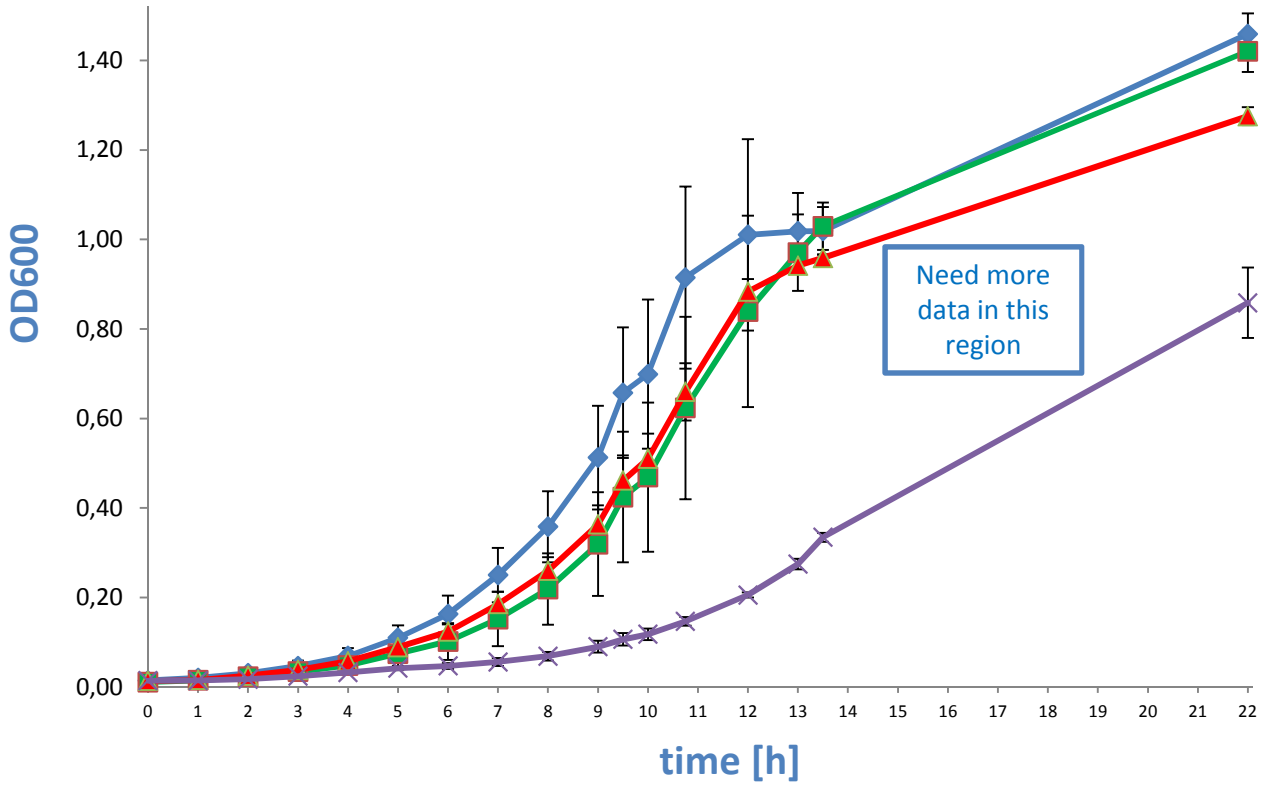
Workflow for clonal heterogeneity / variability investigations

(45 -50 generations)



Starting point for a detailed investigation to identify the reason for clonal heterogeneity

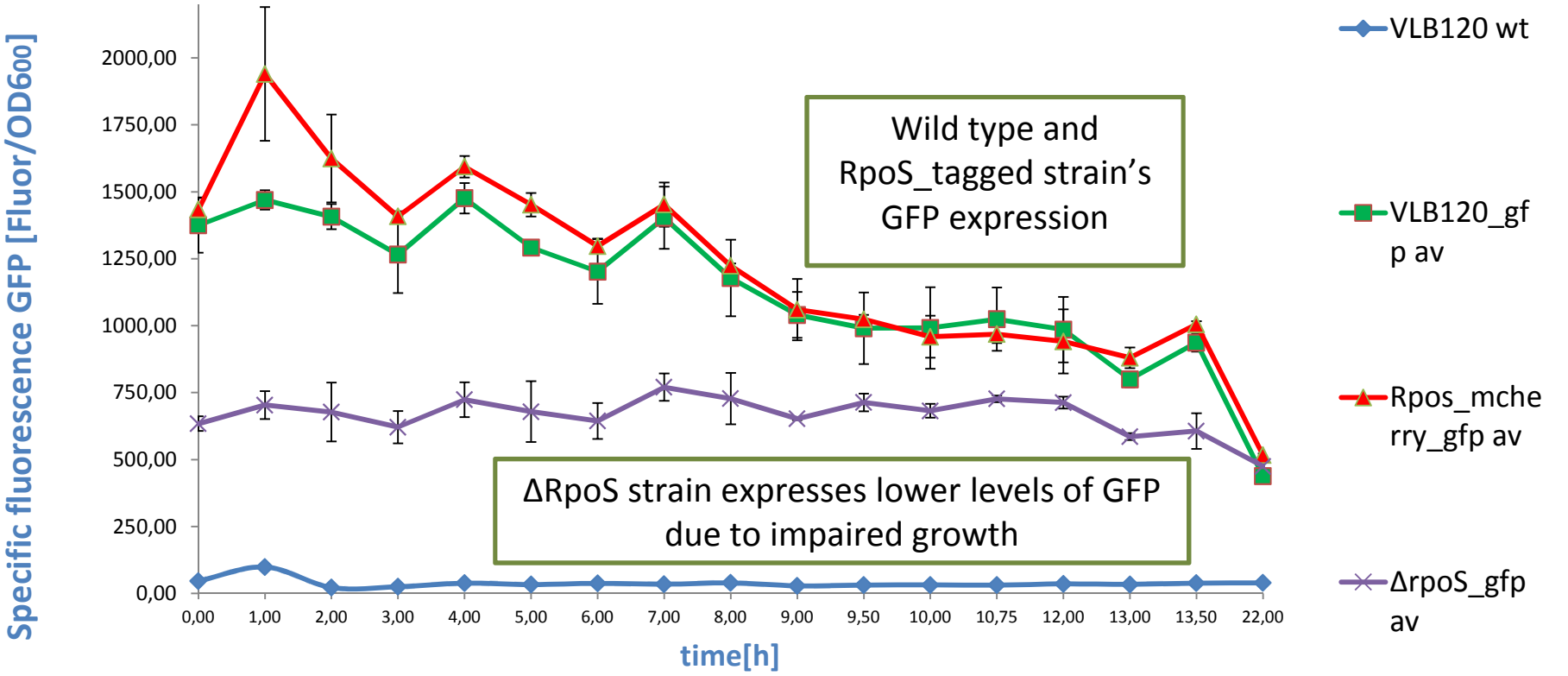
Growth behavior of the various *P. taiwanensis* constructs



Growth rate μ
0,379
0,356
0,379
0,214

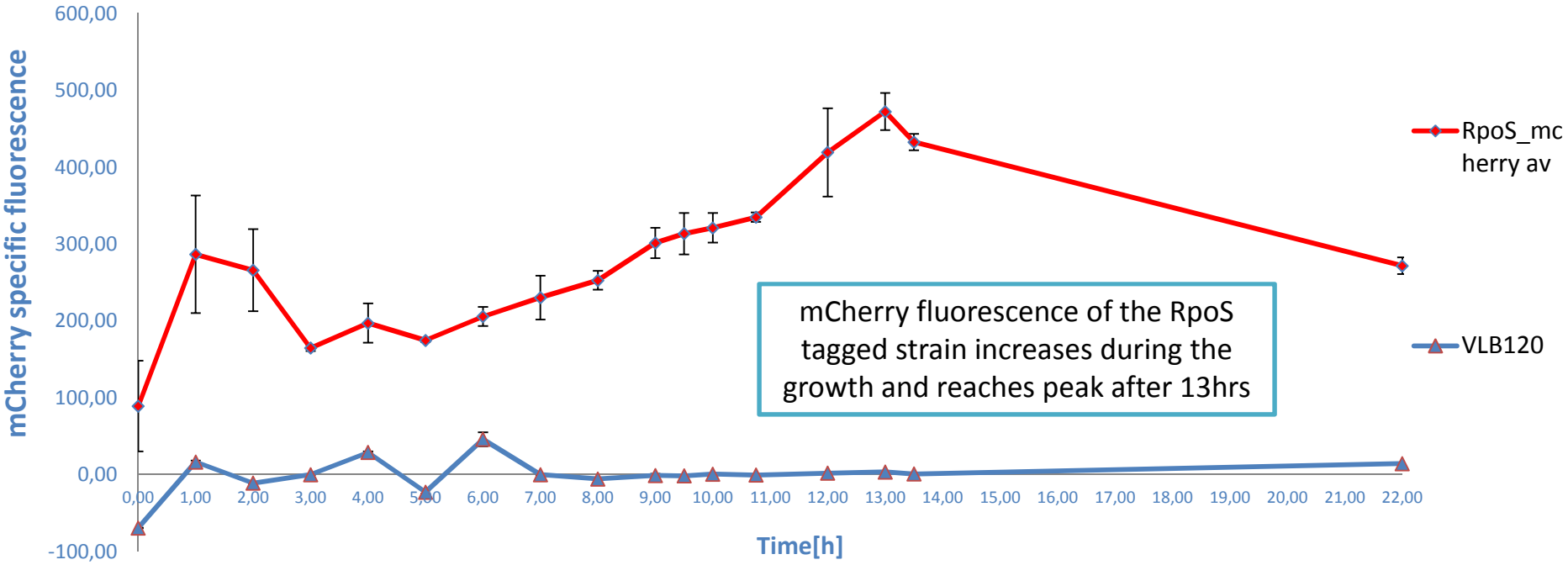
No significant difference in growth behavior between constructs and wild type with an exception of ΔRpoS showing much slower growth

Growth behavior of the various *P. taiwanensis* constructs



Fluorescence profile of *P. taiwanensis* constructs. Experiments performed in M9* media +1% glucose. Measurements of fluorescence were performed with TECAN. Excitation/emission wavelengths: GFP:488/522nm.

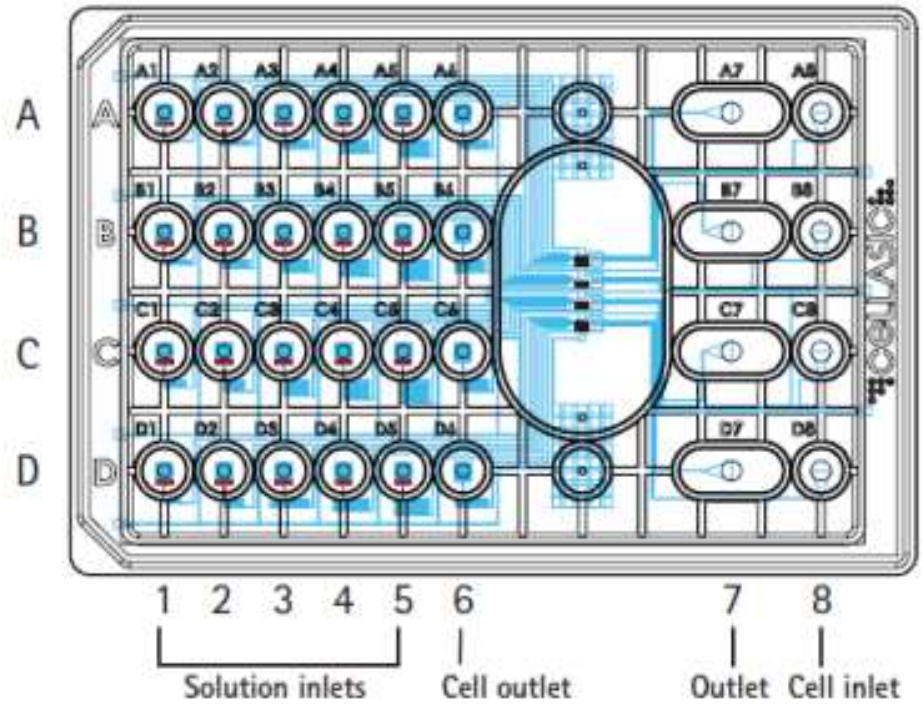
Fluorescence profiles of the various *P. taiwanensis* constructs



Average specific mcherry fluorescence in exponential (E) phase: 279,96
Average specific mcherry fluorescence in stationary (S) phase: 441,47
1.58 fold increase of fluorescence between E and S phases

Advanced flow cell design

CellAsic®ONIX

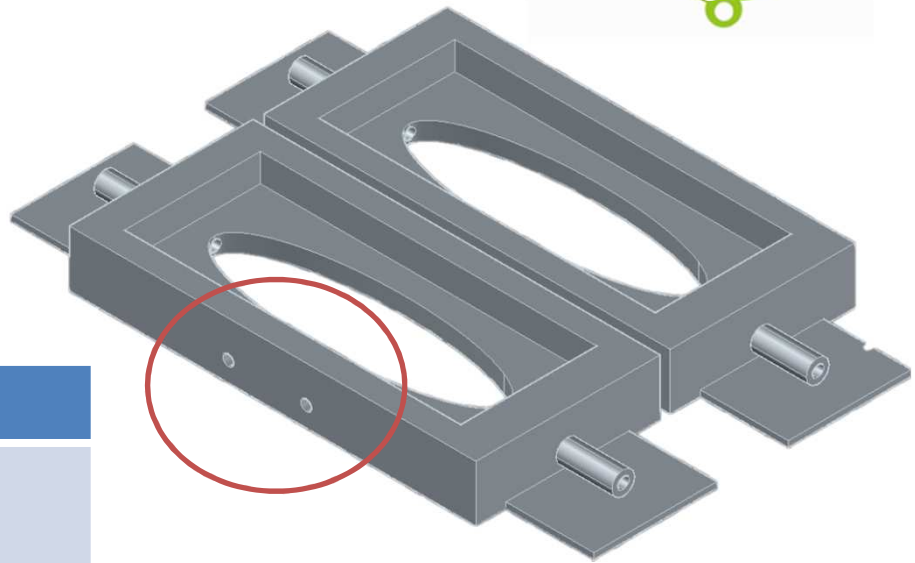


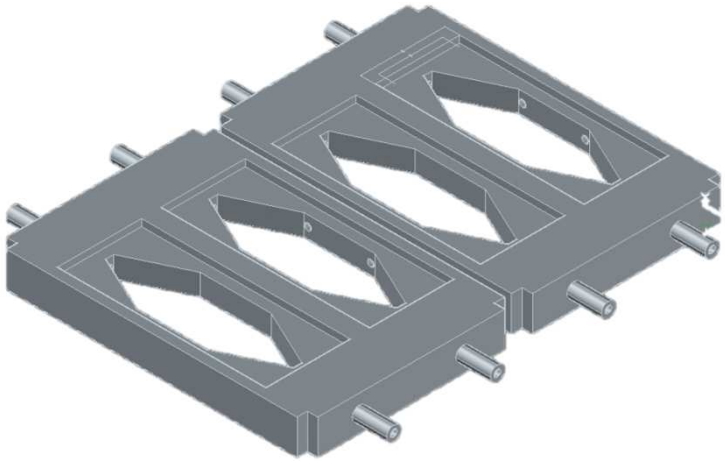
Luer ibiTreat®ONIX

Advanced flow cell design

Custom made 3D printed

Design criteria	
Hydrodynamics	Even flow of the media through the chamber
Working volume of the chamber	Chamber should be optimized to the desired thickness of the biofilm
General geometry	Chamber needs to fit to the inverse microscope
Silicon tube placement	**To create an oxygen gradient**



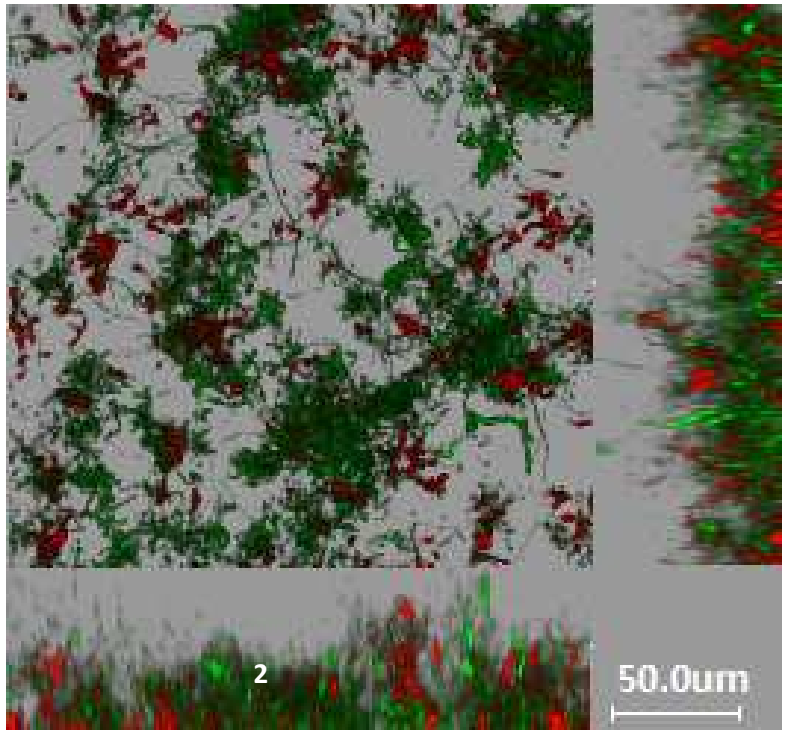


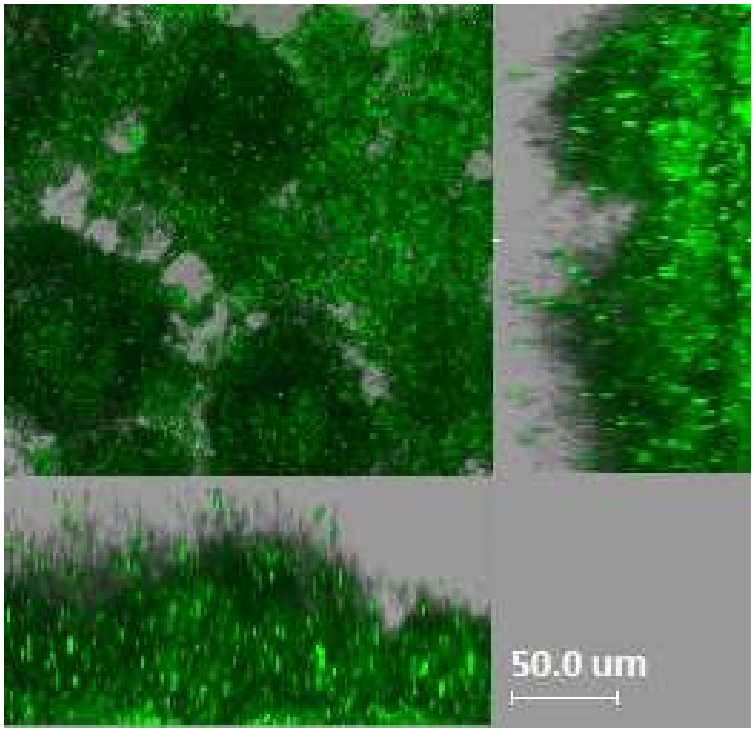
Advanced flow cell design

	3D Flow cell	CellAsic	µ-Slide
Channel Height	6mm	undefined	0,80mm
Channel Volume	4,8ml	undefined	0,2ml
Flow rate	100µl/min	0.0083 µL/min	50µl/min
Flow velocity	1,042 mm/min	undefined	12,5 mm/min
Temperature control	Not possible*	Possible	Not possible*
Application for biofilm studies	++	+	+++

*Possible only with heating jacket

RpoS knock-out mutant develops thicker biofilms





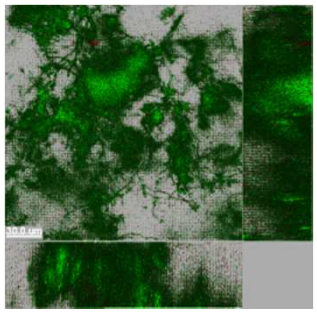
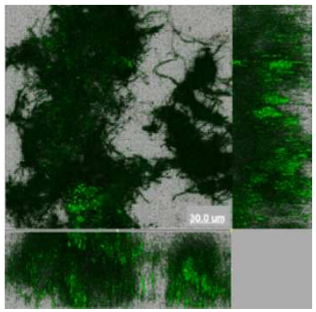
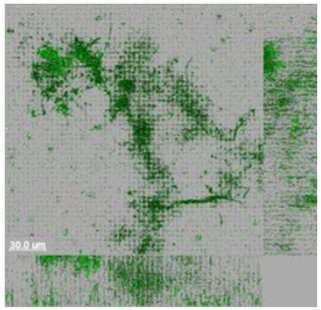
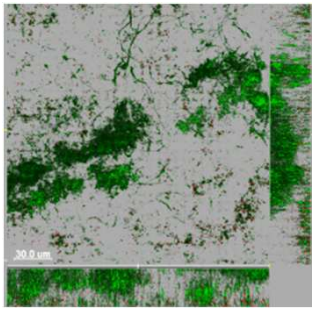
CLSM pictures of a 7-day old *P. taiwanensis* VLB120 rpoS_mCherry (1), *P. taiwanensis* VLB120 ΔrpoS (2) biofilm grown in flow-chambers, Medium: FB + 0.3mM glucose, stained with Syto9. Experiment performed in Copenhagen.

Influence of Oxygen

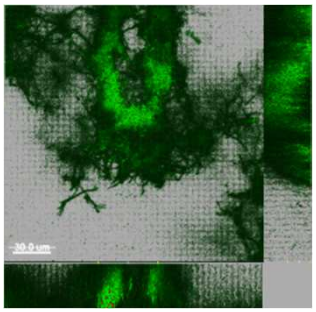
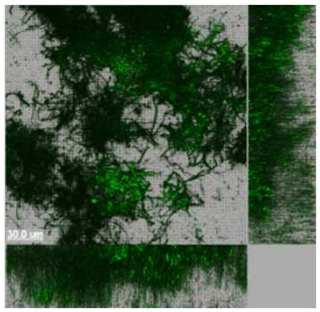
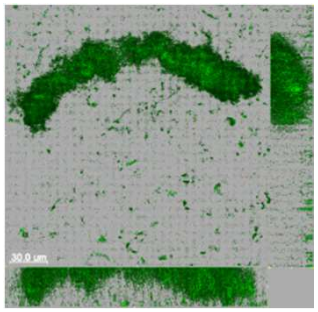
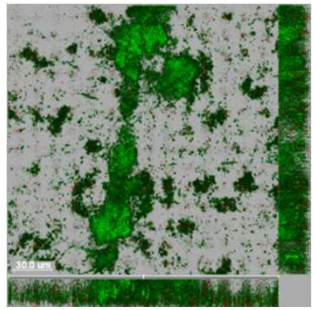
Additional O2 supply via silicon membrane

No additional O2 supply

Central

Peripheral

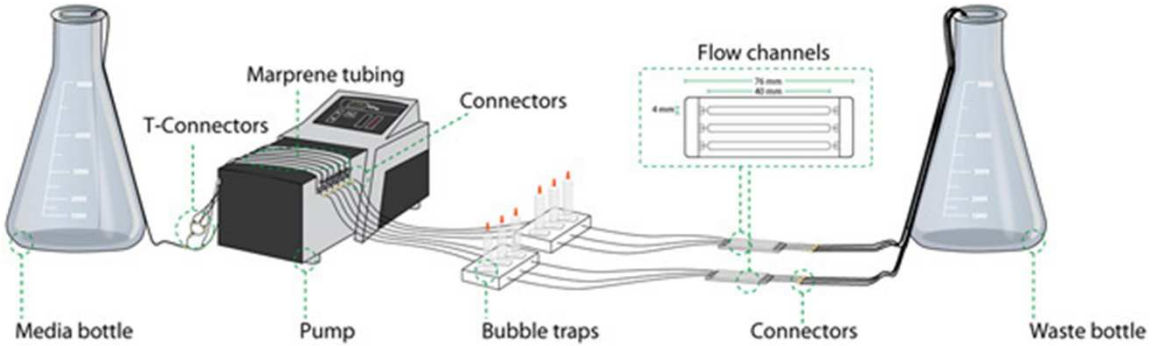





Increased biomass formation;
big macrocolonies;
bead like structures

less beads structures
thick carpet of single cells in upper layers

Carbon sources influence heterogeneity in *P. putida* KT2440

- Objective:
  - Investigate the impact of different carbon sources on *P. putida* KT2440 biofilm three-dimensional structure and population heterogeneity
- Methodology:
  - Dynamic biofilm flow chamber system
  - Confocal scanning laser microscopy of biofilm during 7 days
  - Plate mature biofilm (day 7) and look for differences in morphology



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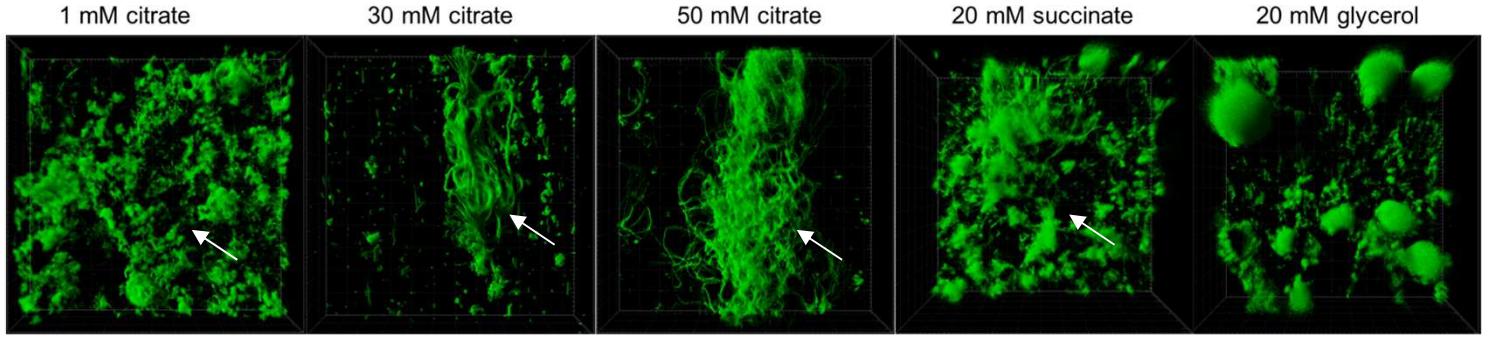
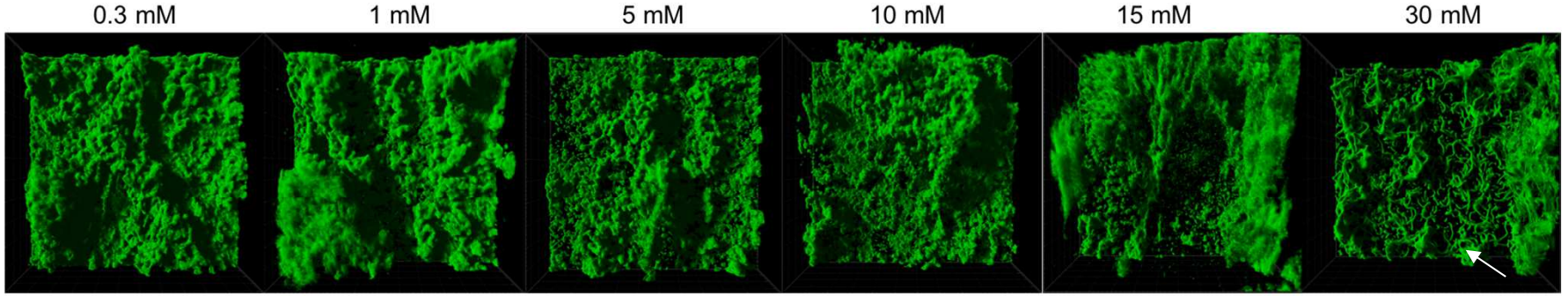
Carbon sources influence heterogeneity in *P. putida* KT2440

Citrate, succinate and glycerol:



	1 mM citrate	30 mM citrate	50 mM citrate	20 mM succinate	20 mM glycerol
Filaments	Yes	Yes	Yes	Yes	No
Morphotypes	Wrinkled,small irregular	Wrinkled, small irregular	Wrinkled, small irregular	None	SCV

Glucose dose response:



	0.3 mM	1 mM	5 mM	10 mM	15 mM	30 mM
Filaments	No	No	No	No	Yes	Yes
Morphotypes	None	None	None	Large colonies	Large colonies	Wrinkled and

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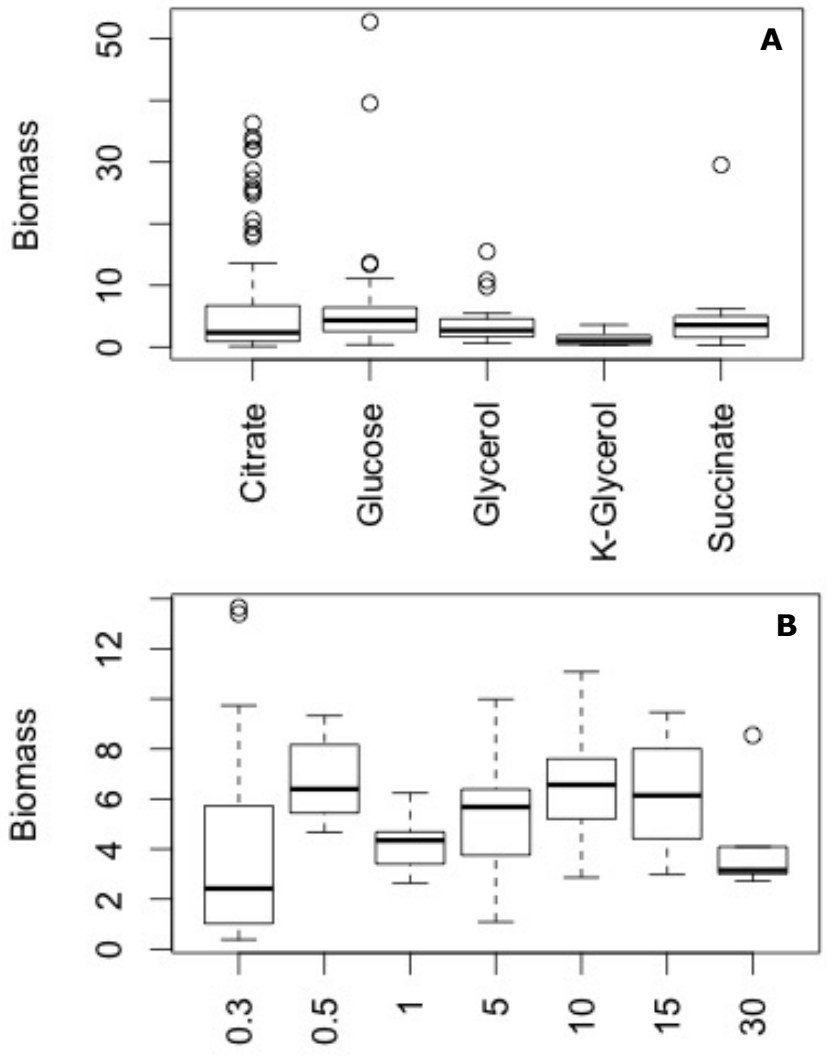
Carbon sources influence heterogeneity in *P. putida* KT2440 – COMSTAT2



Biomass µm³/µm² after 7 days of cultivation of the following carbon sources:

- 1 mM citrate,
- 0.3 mM glucose,
- 20 mM glycerol and
- 20 mM succinate,
- 2 % glycerol in K10T-1 medium

- Glucose dose response [mM]

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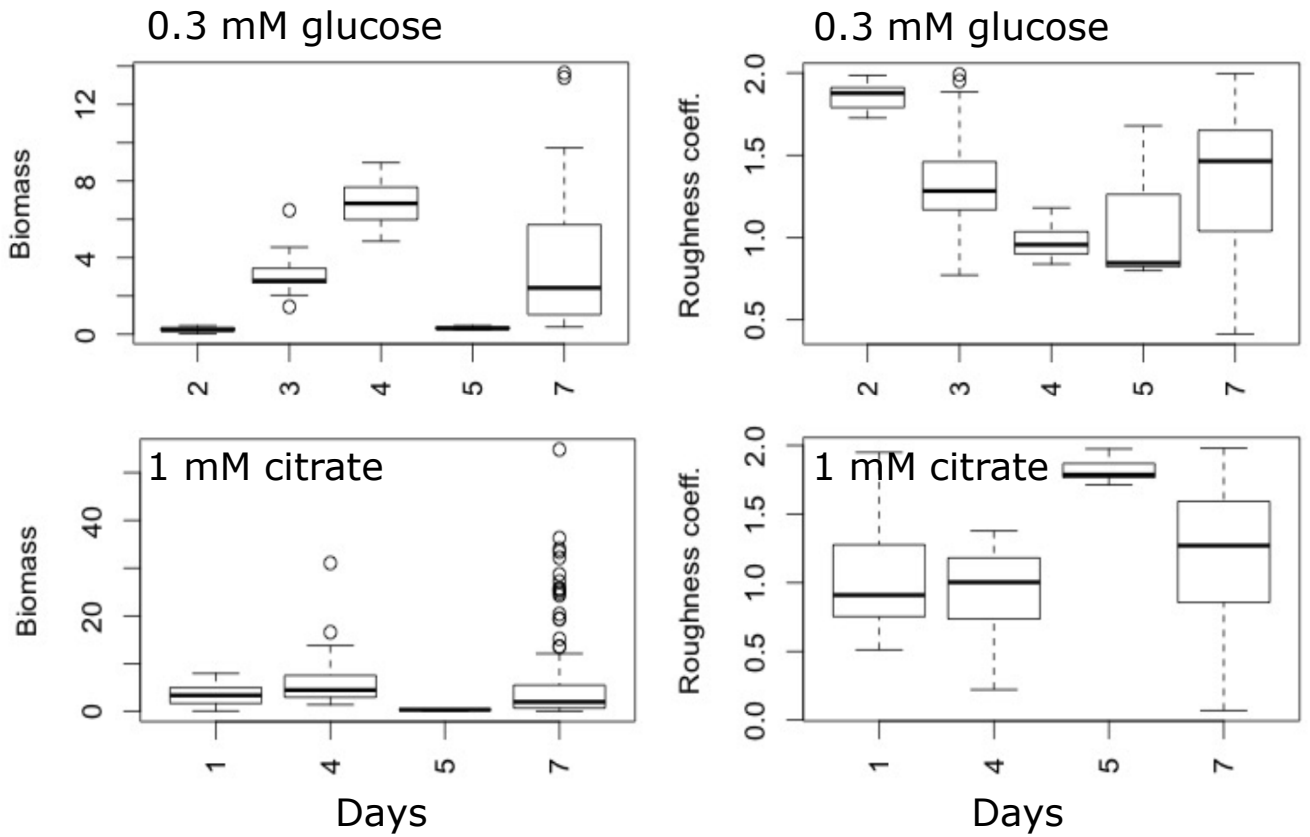
Carbon sources influence heterogeneity in *P. putida* KT2440 – COMSTAT2

Biomass ($\mu m^3/\mu m^2$) and roughness coefficient over time:



- Loss of biomass over time
- Citrate: larger variation of biomass
- roughness remains constant over time
- Glucose and citrate: Equally amount of biomass at day 7
- Glucose: roughness decreases over time

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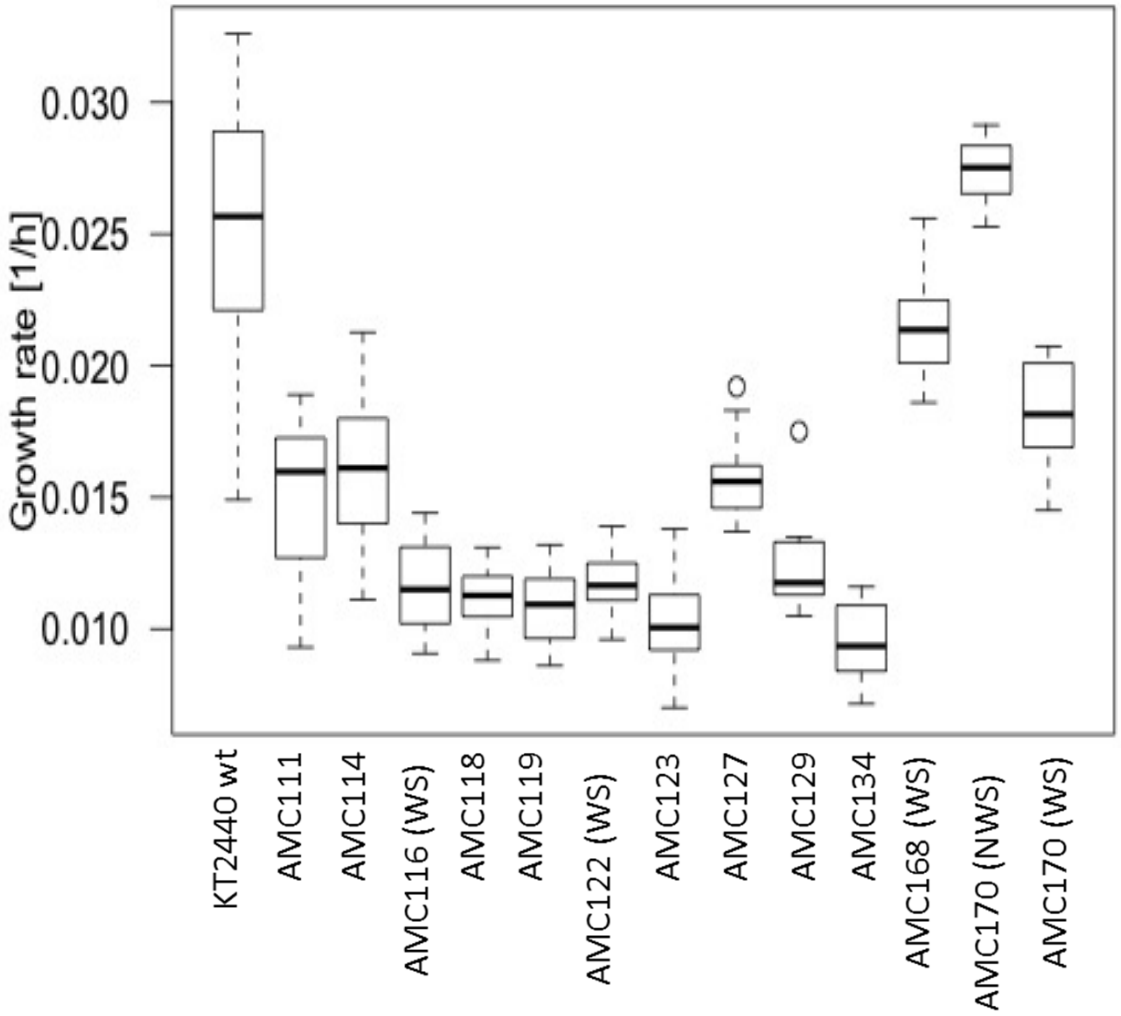
Citrate induces c-di-GMP alterations in *P.putida* KT2440 biofilms

- Objective:
  - Study differences of *P. putida* KT2440 variants obtained from 7 day-old biofilm grown on citrate, in order to investigate the impact of citrate on cell heterogeneity both genotypic and phenotypic.
- Methodology:
  - Phenotypic analysis on variants e.g. motility, biofilm capability and growth
  - Whole genome sequencing of selected variants
  - Biofilm flow chamber experiments on variants

Citrate induces c-di-GMP alterations in *P.putida* KT2440 biofilms - Phenotypes



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Citrate induces c-di-GMP alterations in *P.putida* KT2440 biofilms - Phenotypes

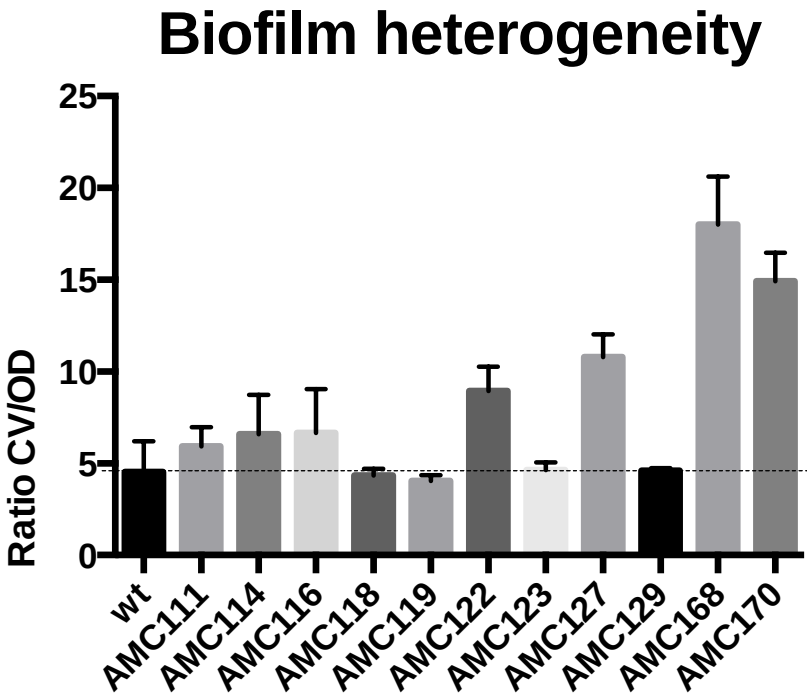
Swimming motility

Relative to wt

	24 h	48 h
KT2440 wt	**1,0**	**1,0**
AMC111	0,4	0,5
AMC114	0,6	0,7
AMC116(WS)	0,2	0,3
AMC118	0,1	0,0
AMC119	0,0	0,0
AMC122(WS)	0,2	0,3
AMC123	0,1	0,0
AMC127	0,6	0,7
AMC129	0,0	0,0
AMC134	0,1	0,2
AMC168(WS)	1,0	1,0
AMC170(WS)	0,4	0,6

Motile

Non-motile



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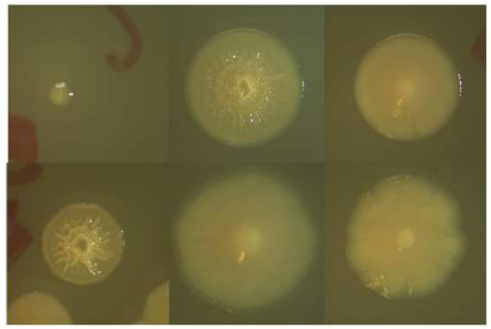
Citrate induces c-di-GMP alterations in *P.putida* KT2440 biofilms - WGS



Illumnia MiSeq
- 50x coverage
- 150bp paired end

Strain #	Locus	Mutation Function	Morphology	Motility Swim	Growth	Biofilm CV stain	Air-liquid LB medium	Remarks
AMC72 wt				+++	+++	0	+	
AMC111	PP4943	fs glycosyl transferase	small	++	++	+	+	
AMC116	PP0129	SNP Diguanylate cyclase	wrinkled	+	+	+++	++	In operon with *dsbA*
AMC119	PP5129	SNP predicted phosphatase		-	+	0	-	
AMC122	PP4671	SNP unknown	wrinkled	+	+	++	+++	In operon with a diguanylate cyclase
AMC124		SNP Intergenic region		+	?	+++		In region with flagella related genes
AMC127		SNP Intergenic region		+++	++	++	++	
AMJ168	PP4959	SNP Response regulator c-di-GMP	wrinkled	+++	+++	+++	+++	
AMC170	PP0129	SNP Diguanylate cyclase	wrinkled	++	++	+++	+	In operon with *dsbA*

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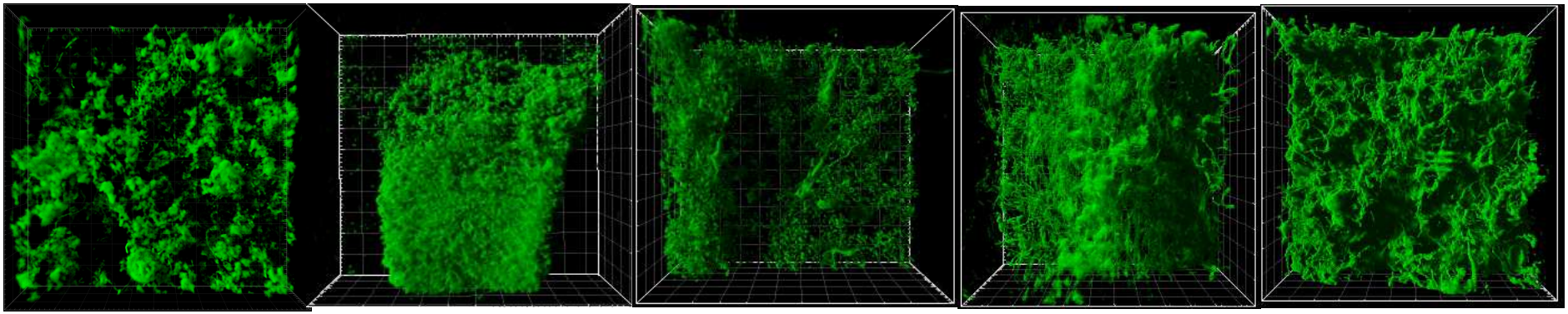
Citrate induces c-di-GMP alterations in *P.putida* KT2440 biofilms - biofilm flow chambers



KT2440 wt **AMC111 (small)** **AMC116 (w)** **AMC168 (w)** **AMC170 (w)**

- Low filamentation in AMC111 and AMC116
- Same SNP in variant AMC116 and AMC170 but different biofilm capability
- Variant AMC170 high biofilm capability-> can filaments be reduced when grown on glucose?

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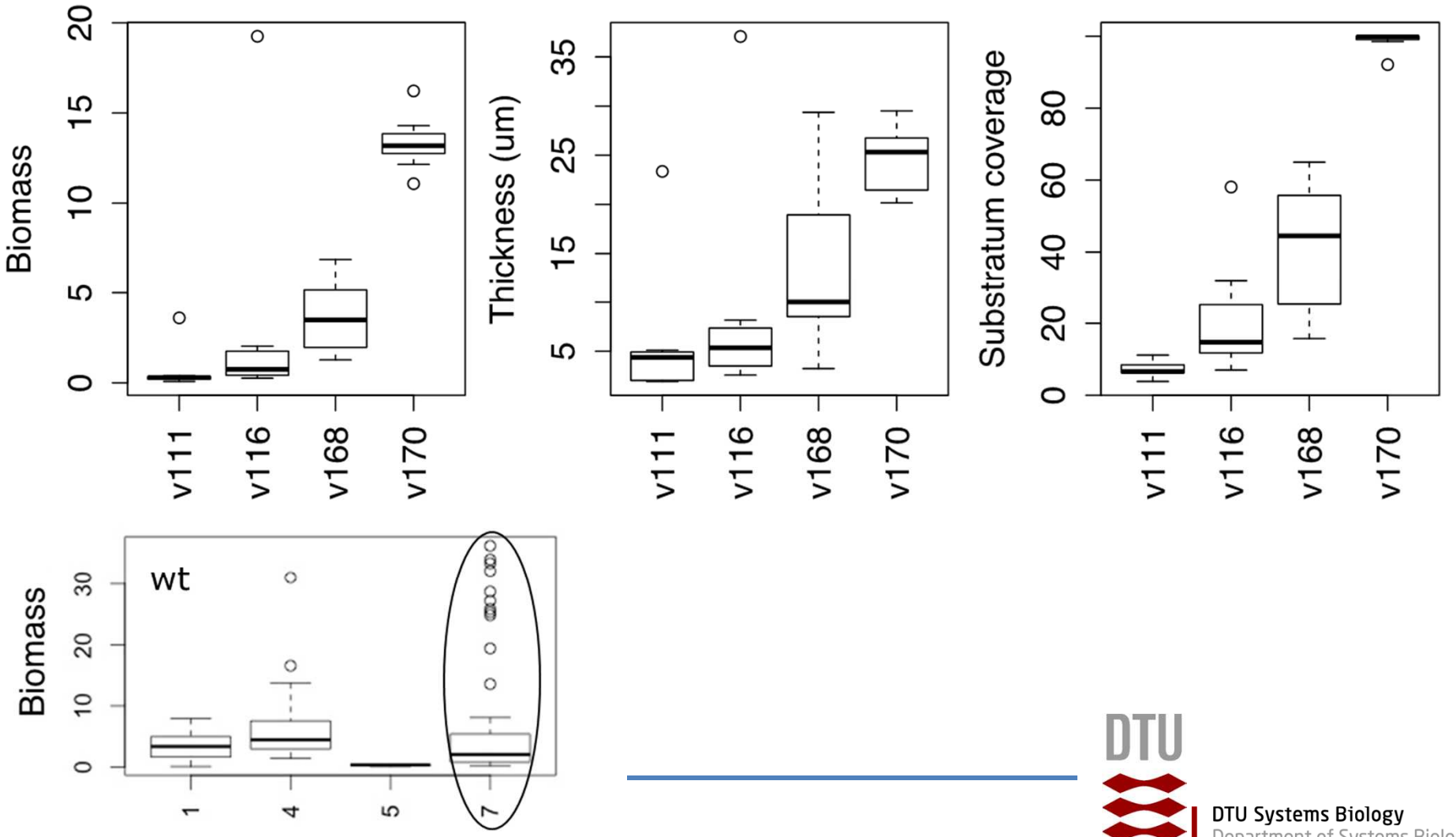
Citrate induces c-di-GMP alterations in *P.putida* KT2440 biofilms - COMSTAT2

After 7 days of cultivation on citrate



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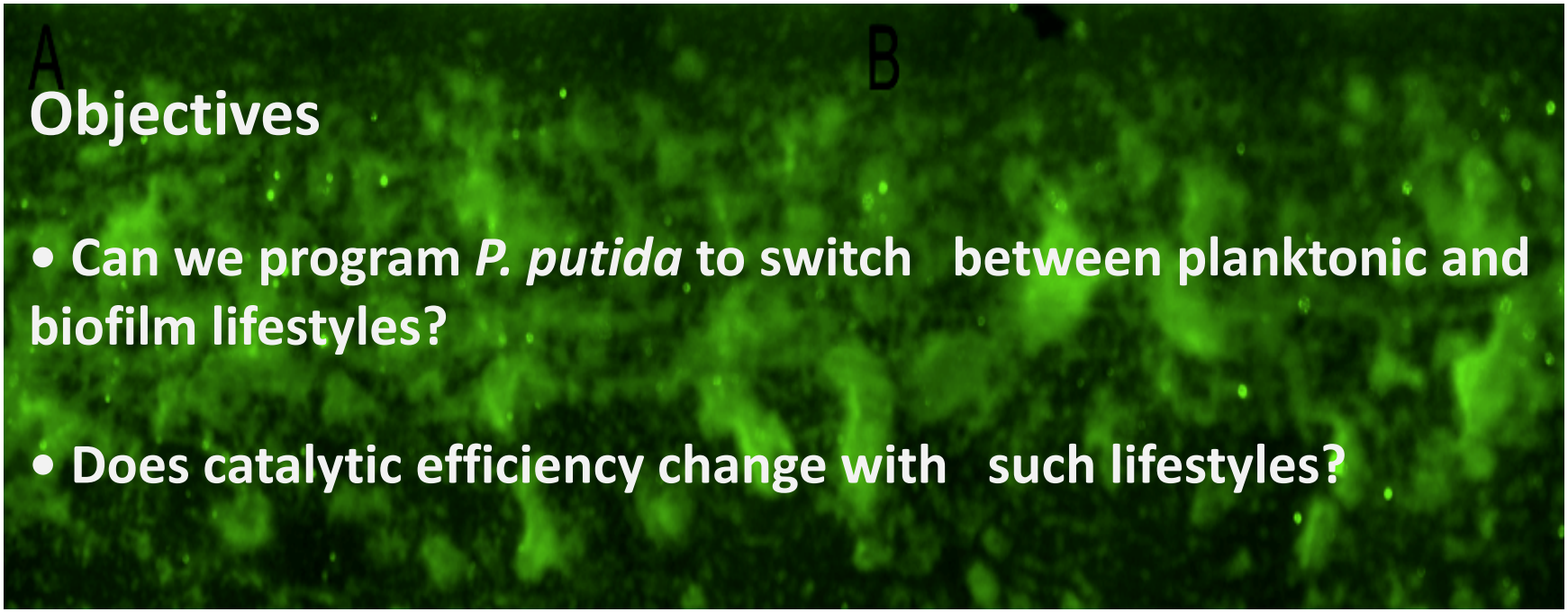
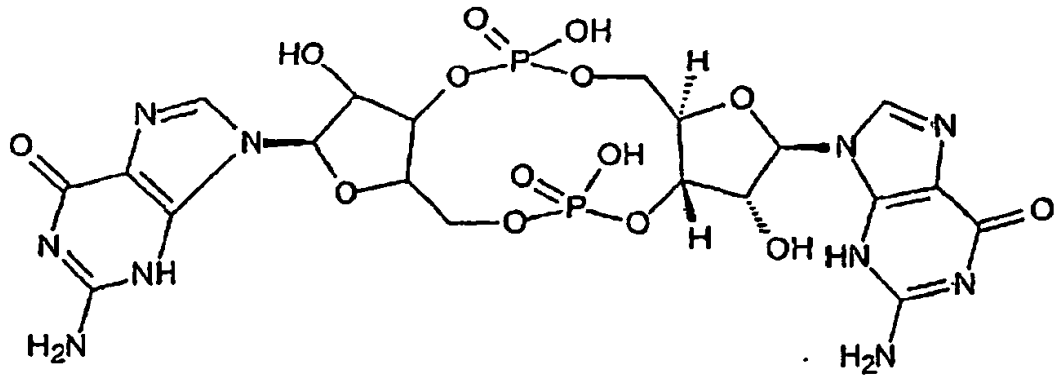
Reprogramming lifestyle and catalytic efficiency of *P. putida*



Benedetti I, de Lorenzo V, Nikel PI. (2016) Genetic programming of catalytic *Pseudomonas putida* biofilms for boosting biodegradation of haloalkanes. *Metab Eng*. **33**:109-18.

Reprogramming lifestyle and catalytic efficiency of *P. putida*



C-di-GMP ↑ **Biofilm**

C-di-GMP ↓ **Planktonic**

The key for lifestyle decision is intracellular levels of cd-GMP

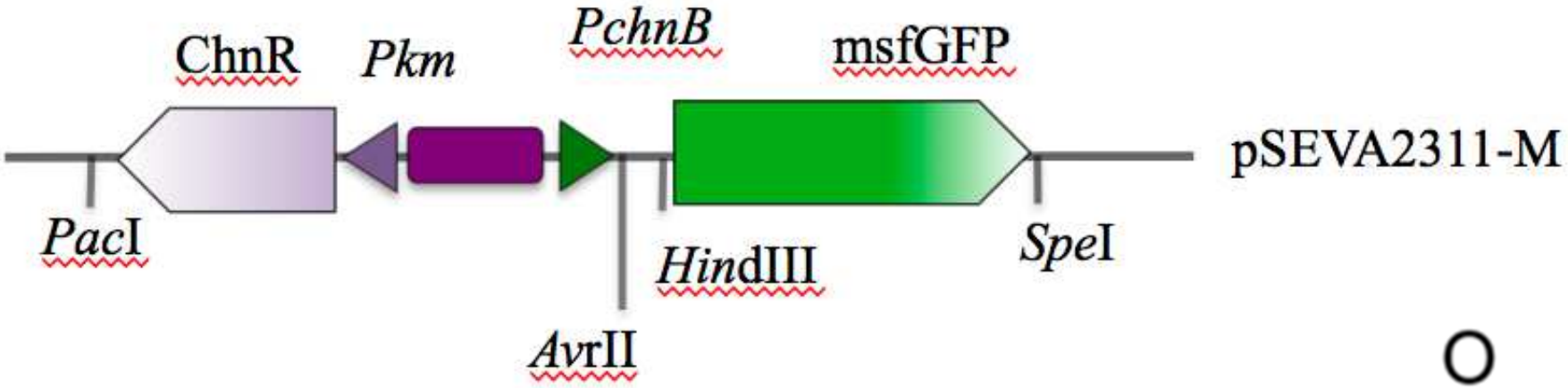
Taking c-diGMP to its extremes

Levels determined by interplay of { GGDEF domains; EAL & HD-GYP domains

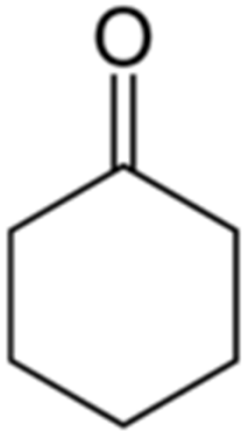
YedQ ➔ cdGMP cyclase

YhjH ➔ cdGMP phosphodiesterase

Engineering an inducible switch

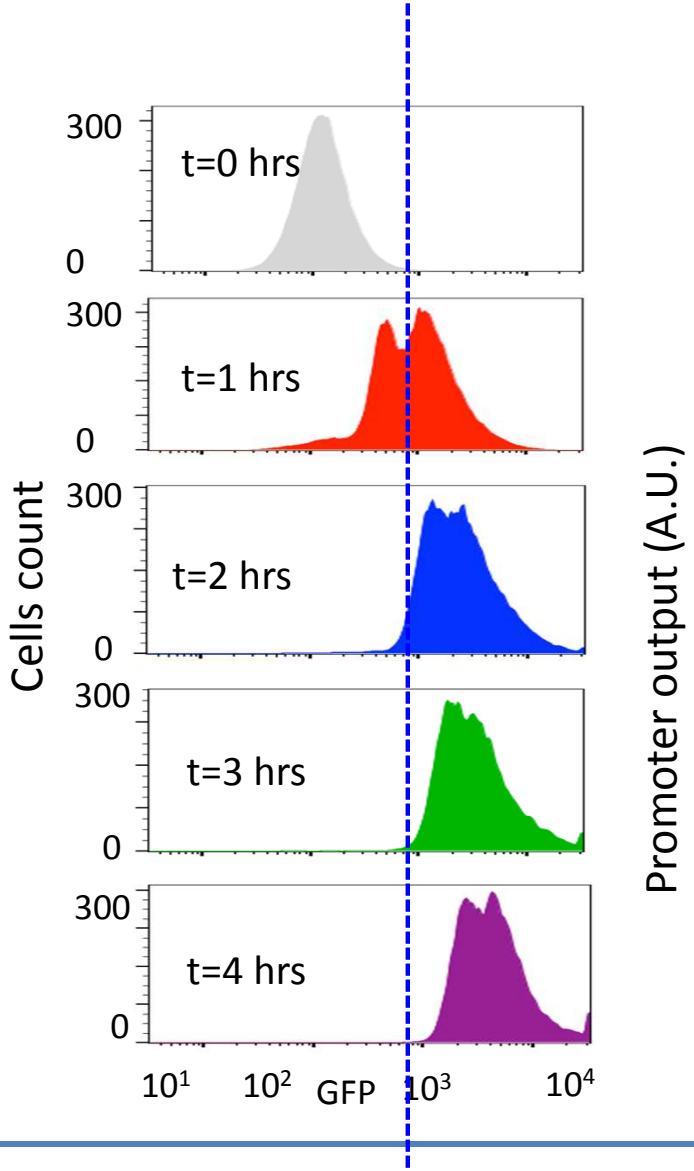


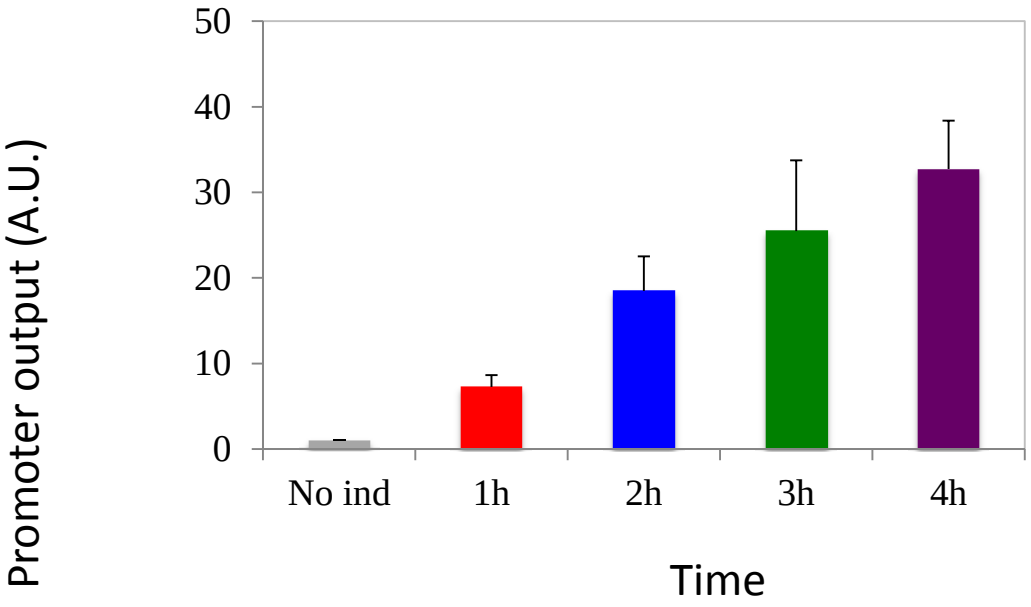
Orthogonal inducer:
cyclohexanone



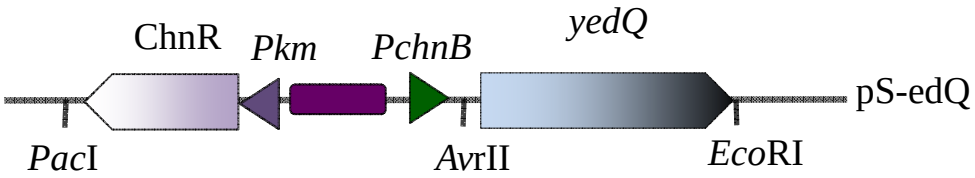
Parts from *Acinetobacter*
(Steigedal & Valla, 2008)

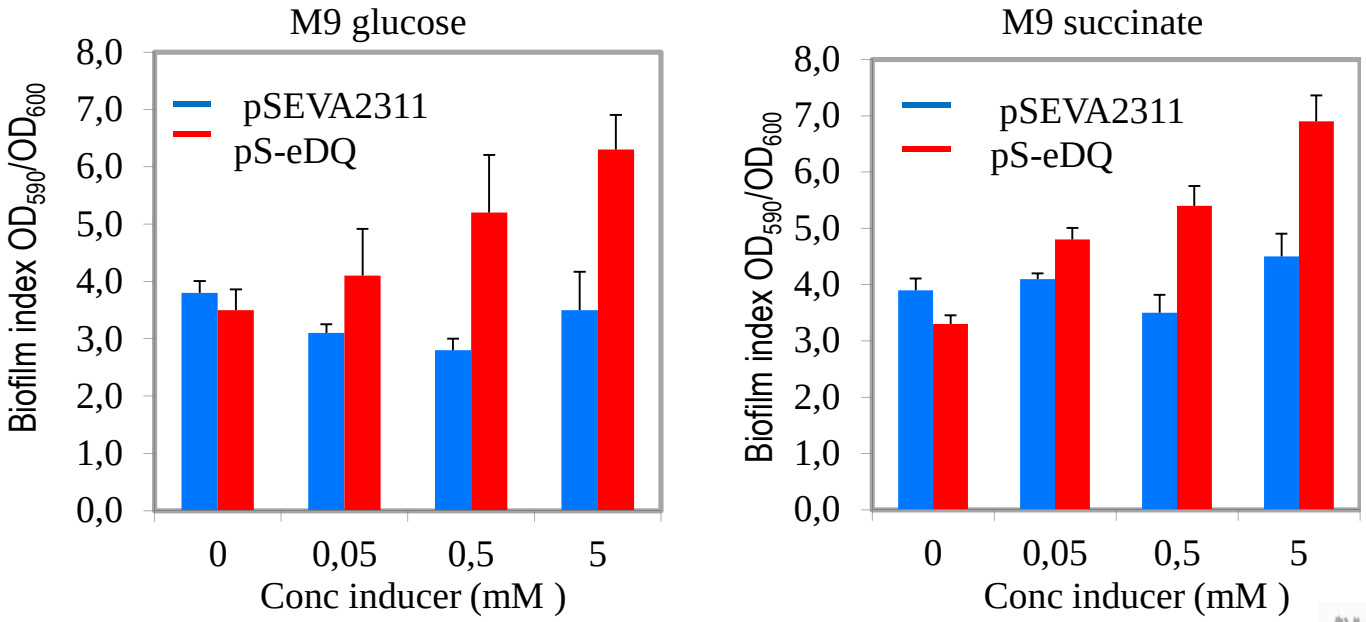
Engineering an inducible switch



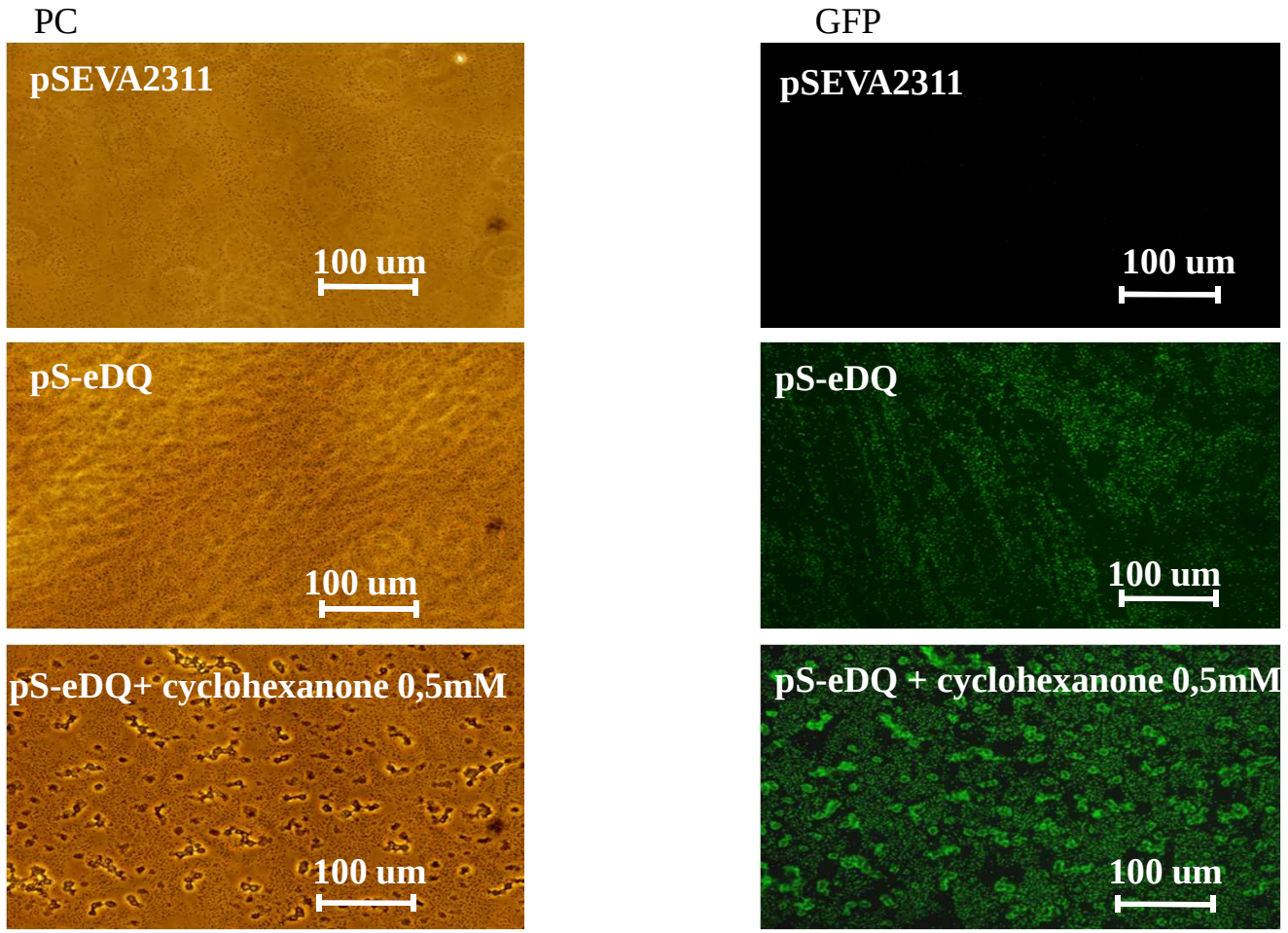


Cyclohexanone-dependent Biofilm formation

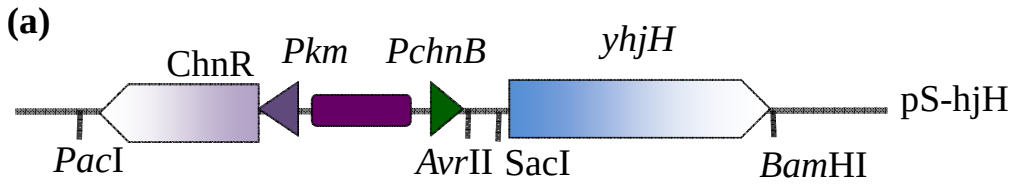


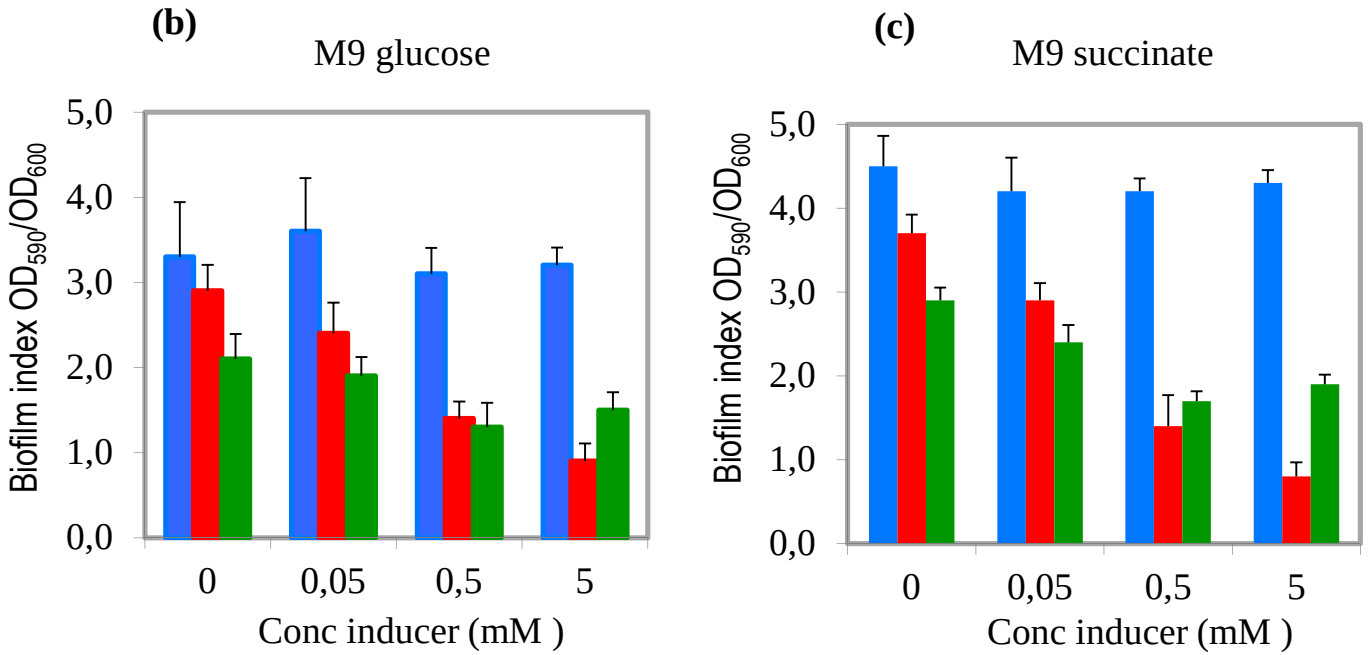


Cyclohexanone-dependent Biofilm formation



Cyclohexanone-dependent Biofilm formation

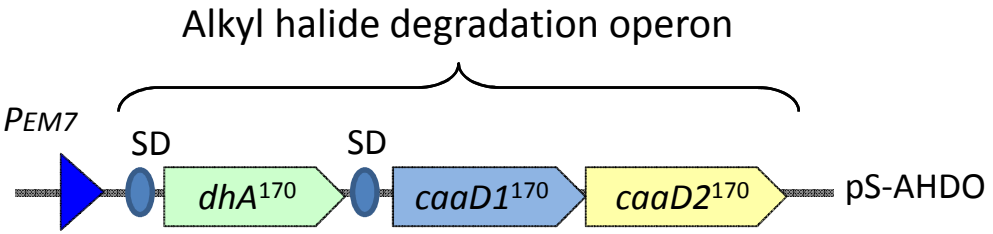


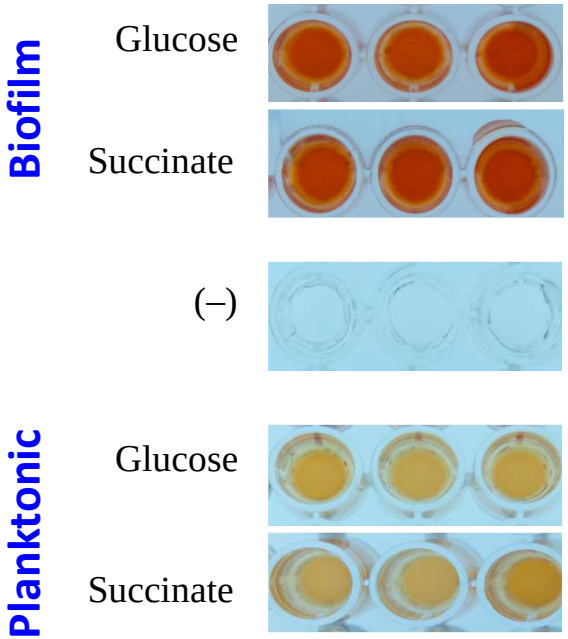


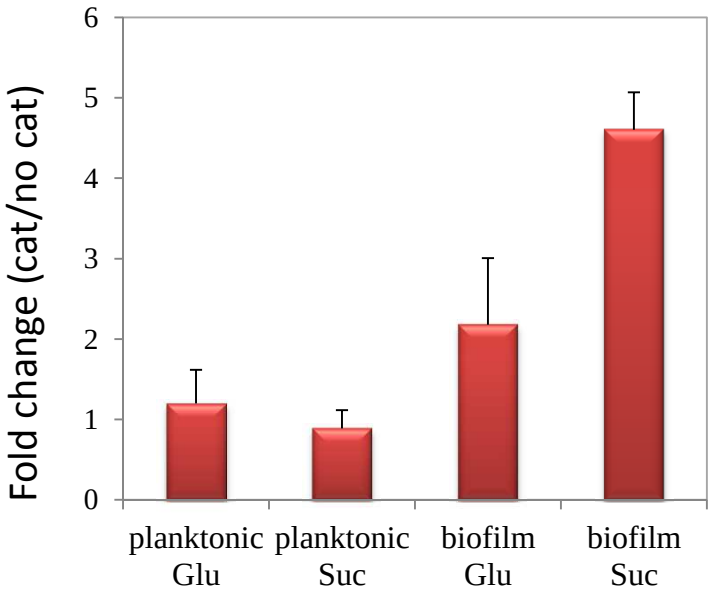
pSEVA2311
pS-hjH
pS-lacH

pSEVA2311
pS-hjH
pS-lacH

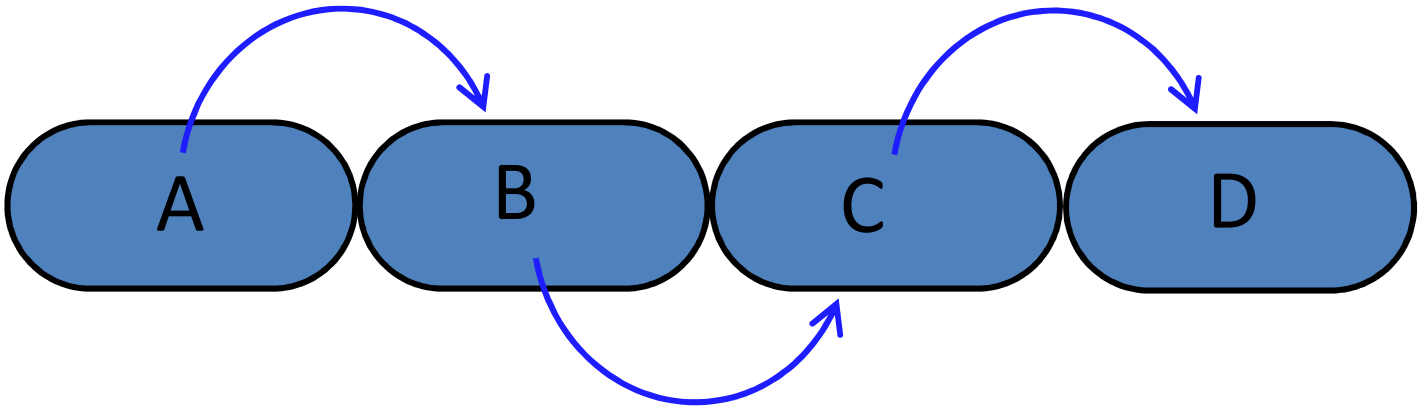
1-chlorobutane degradation: biofilm vs planktonic







Why catalysis is more efficient in a biofilm?



Summary & Outlook

- Various experimental set-ups established for cultivation and subpopulation identification in planktonic as well as biofilm cultures
- RpoS based detection system is working
- Subpopulations / conditions identfied in biofilms
- In planktonic cultures significant differences in activities between different organisms observed
- Inducible genetic switches developed and established

Future Work:

- Transferring methodology to 3-HIBA producing strain
- Establishing a FACS protocol for biofilm growing organisms
- Biofilm analysis of RpoS tagged strains

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