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# *Escherichia coli* HGT – a novel high glucose throughput chassis especially designed for typical production conditions in large scale based on comprehensive systems biology studies

*Ralf Takors*

*July 20nd , 2017*

Systems & Synthetic Biology Meeting, Munich

Scaling-up *E. coli*
Modelling Strain Responses
Conclusions for Strain Engineering
Transferring Results

- Joana Simen
- Michael Löffler
- Alexander Niess

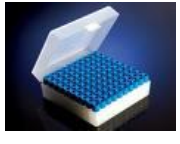
# Scaling Up

The way from the lab to the large-scale production

Takors, R (2015) *J. Biotechnol*

**Master Cell Bank**

**Working Cell Bank**



$10^{-3}$ L

**SK Tests Inoculum**

**Pre-Seed Fermenter**

**Seed Fermenter**

**Production**

Increasing heterogeneity

**PRODUCT**

$10^{5}$ L

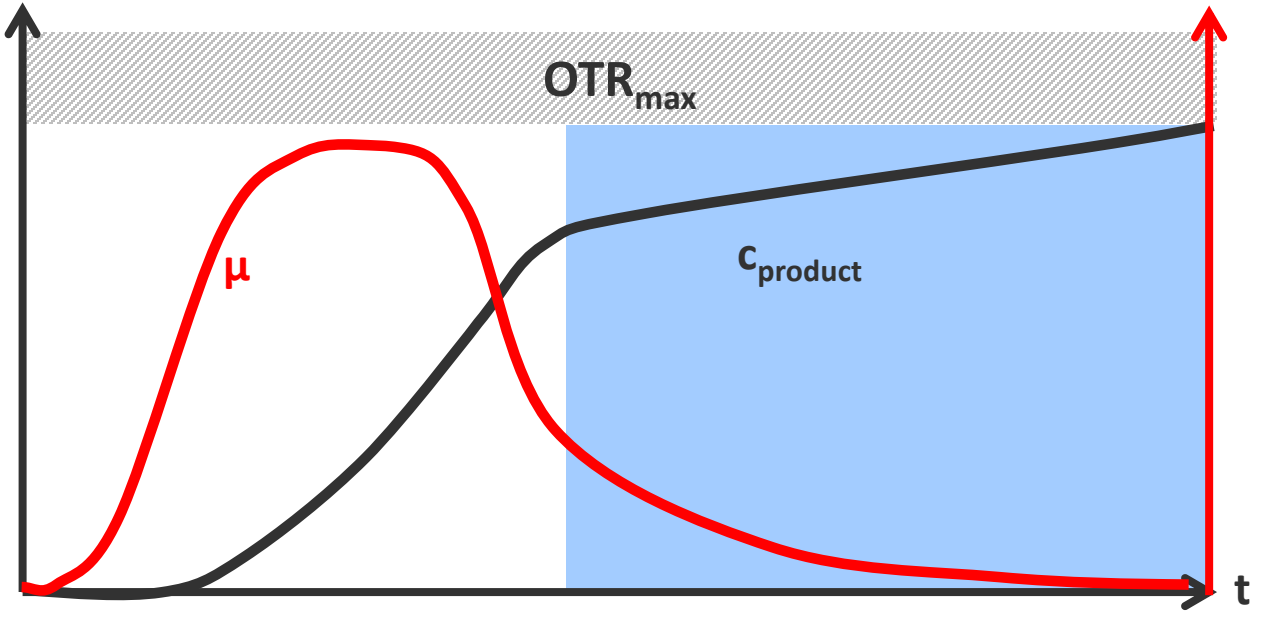
**Scale-up is the process that enables lab-scale performance in large-scale, ensuring economic feasibility.**

# Large Scale Constraints

Michalowski et al, 2017 *Metabolic Engineering*

- Oxygen transfer rate (**OTR) is limited** (~150 – 180 mmol/Lh)
- **Cooling capacities are limited**

But: **productivities (g/$L_{reactor}$h) should be maximized**




**Cells should produce at low growth**

# Ideal Strain for Large-Scale Application

The **ideal producer** should:

- enable high glucose uptake rates, even under resting condition
- be blind with respect to extracellular heterogeneities

**Goal**:

**to create a novel *E. coli* chassis** with fundamentally changed properties.

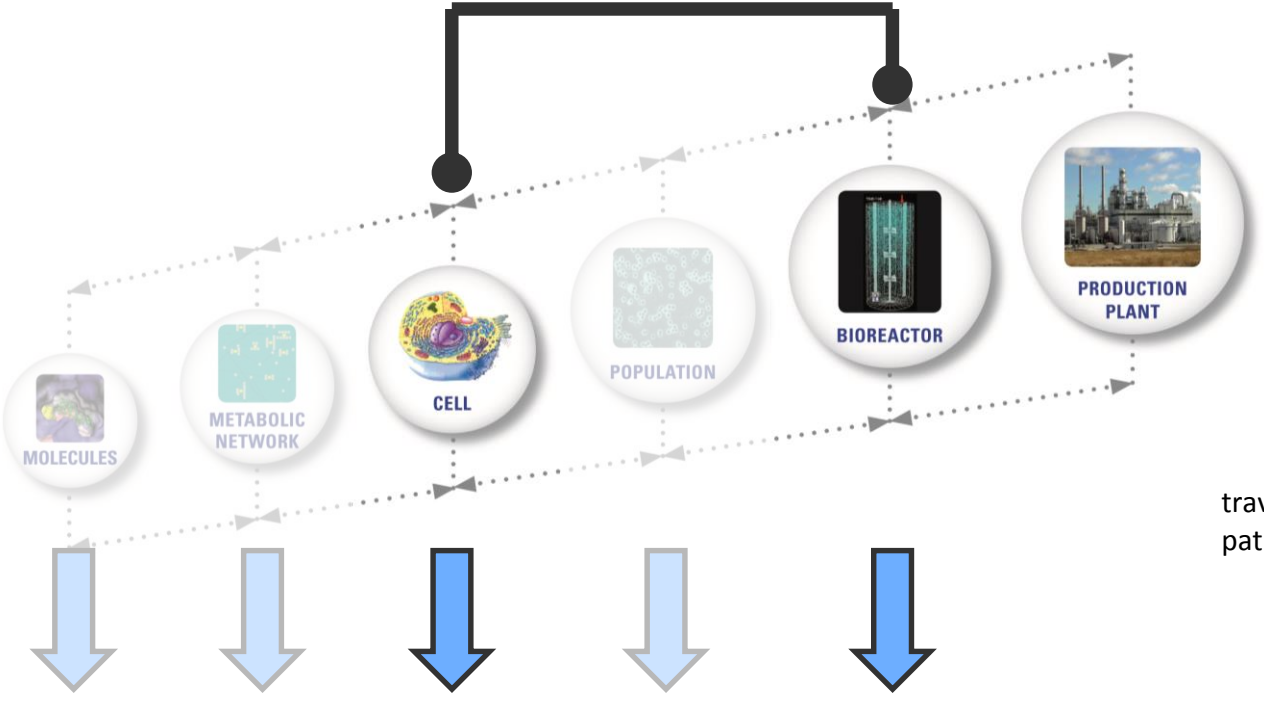
**Drawback**:

**transcriptional responses under large-scale conditions not known**

**Approach**:

To perform systems biology studies on large-scale performance for deriving guidelines for synthetic strain construction.

# Successful Bioprocess Development Needs to Pass Multiple Scales




cell-free protein synthesis

metabolic analysis

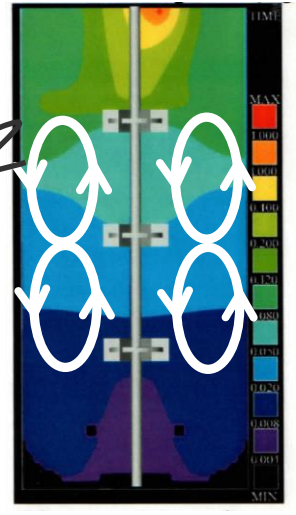
**regulatory networks & strain engineering**

population dynamics

**scale-up analysis**

What is the impact of repeated triggering on cellular performance?

travelling paths of cells



Simulated glucose distribution in 900 L stirred tank reactor
Lapin et al., 2004

# Scaling-up *E. coli*

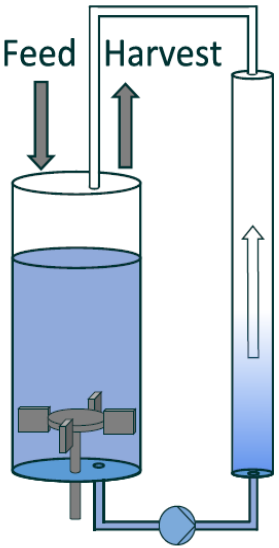
- Impact of Glucose Gradients on Cellular Performance -

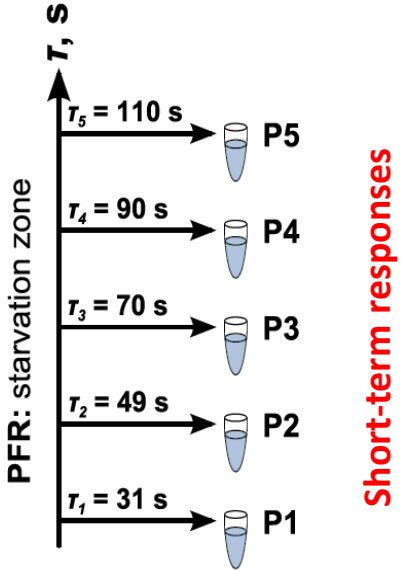
# Plant engineering/construction and validation

Simen, Löffler et al. 2016 *Metabolic Engineering*

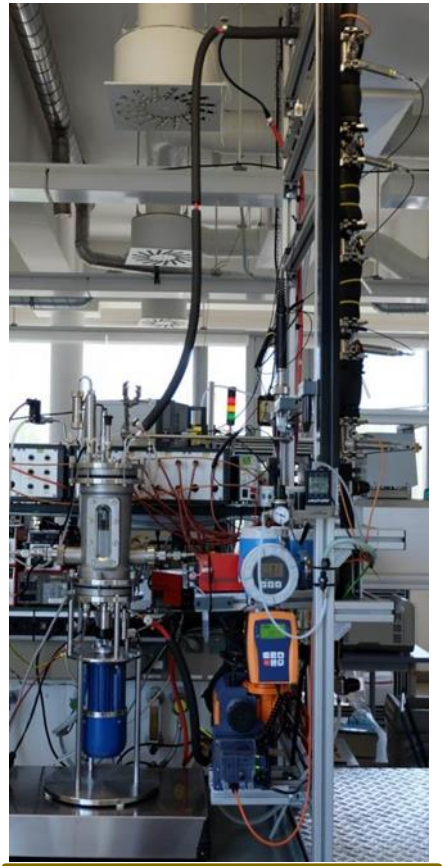
- Development of a STR-PFR system to simulate oscillating gradients

STR
(stirred tank reactor)




- Volume PFR/ total volume: 25 %
- Online: temperature and oxygen
- thermally insulated



**Plug-flow like behavior**
**($\tau_{PFR}$ = 125 s, Bo=83)**

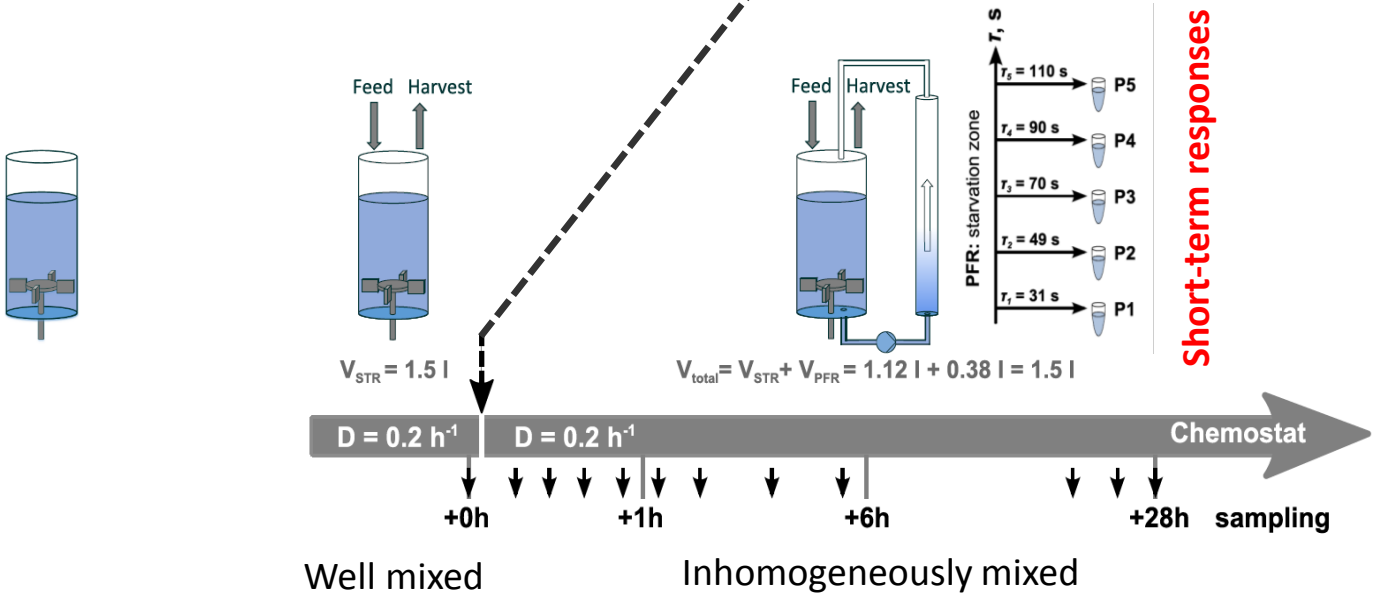
# STR-PFR Experiments With *E. coli: Glucose Limitation*

Simen, Löffler et al. 2016 *Metabolic Engineering*

**I Batch:**
- $\mu=\mu_{max}$
- $V_{total}=V_{STR}=1.5$ l

**II Chemostat ($\mu$ = 0.2 $h^{-1}$)**
- Steady-state (feed)
- $V_{total}=V_{STR}=1.5$ l

**III Chemostat ($\mu$ = 0.2 $h^{-1}$) +PFR**
- PFR: glucose starvation zone
- $V_{total}=V_{STR}+V_{PFR}=1.12$ l + 0.38 l
- Residence time PFR 125 s




**Long-term responses:** 25 min 120 min 28 h

# Glucose:

# Short-Term Responses

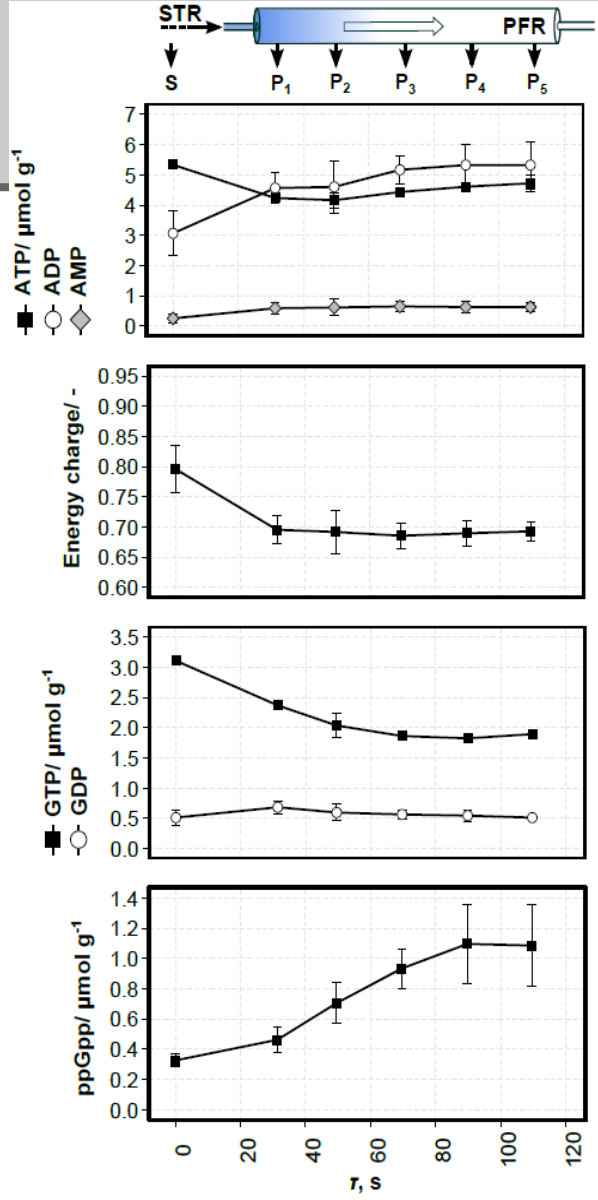
# Intracellular Nucleotides (Carbon Limitation-Starvation)

Simen, Löffler et al. 2016 *Metabolic Engineering*

- Decreasing intracellular ATP-Concentration
- Increasing ADP-Level
- Fast Energy charge reduction (30 sec)
- Decreasing GTP-Level
- Sigmoidal ppGpp accumulation

**Sequential response along the PFR**
**< 30 sec: Reduced Energy charge**
**< 70 sec: Decreasing GTP-Level**
**30-90 sec: Increasing ppGpp-Level**

**Transcriptional Response**

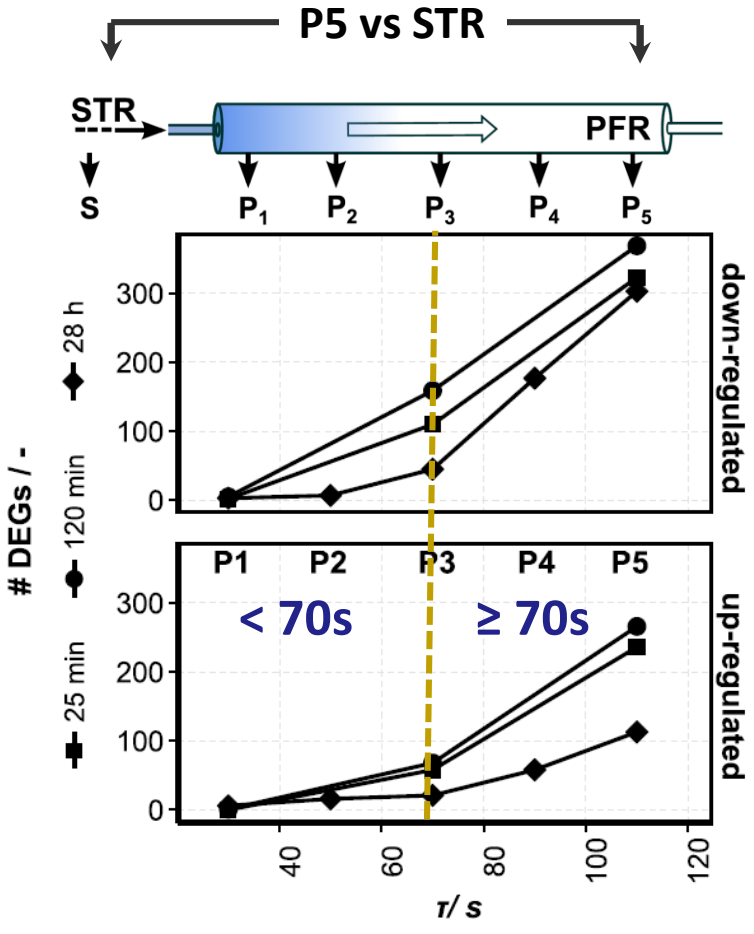


# Short-term Response on Glucose Limitation: Differentially Expressed (DE) Genes

Simen, Löffler et al. 2016 *Metabolic Engineering*

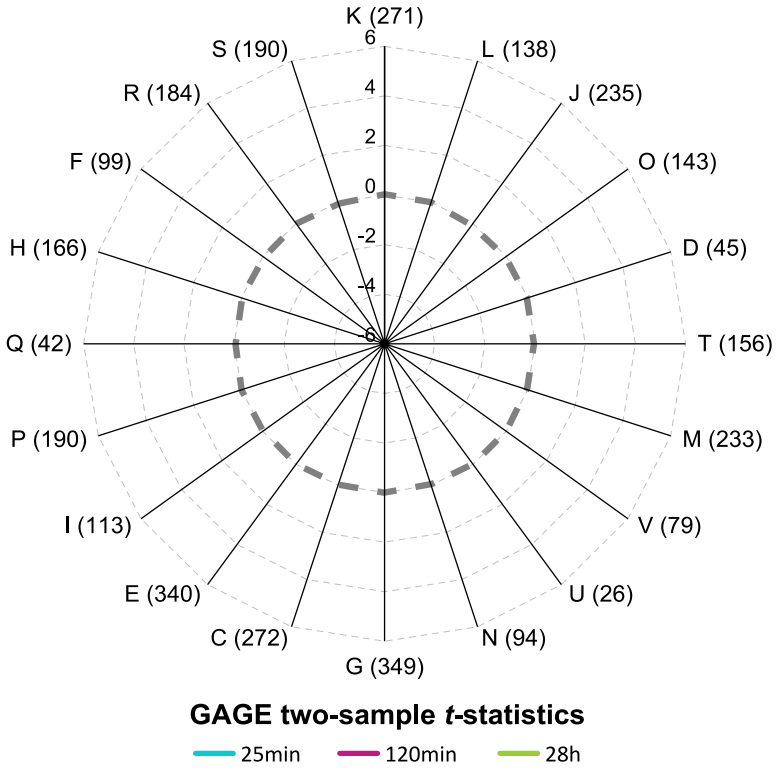
- Increasing limitation inside PFR
- Filter for DE genes
  - 1% false discovery rate
    - P5 down: 836
    - P5 up: 955
  - Fold change >1.5 fold
    - P5 down: 369
    - P5 up 266

→ Immediate DE response after entering PFR
- ‚slow' dynamics < 70 s
- Transcriptional boost > 70 s



# Differentially Expressed Genes (PFR outlet vs STR)

Simen, Löffler et al. 2016 *Metabolic Engineering*




**Information storage and processing**
K Transcription
L Replication, recombination and repair
J Translation, ribosomal structure and biogenesis

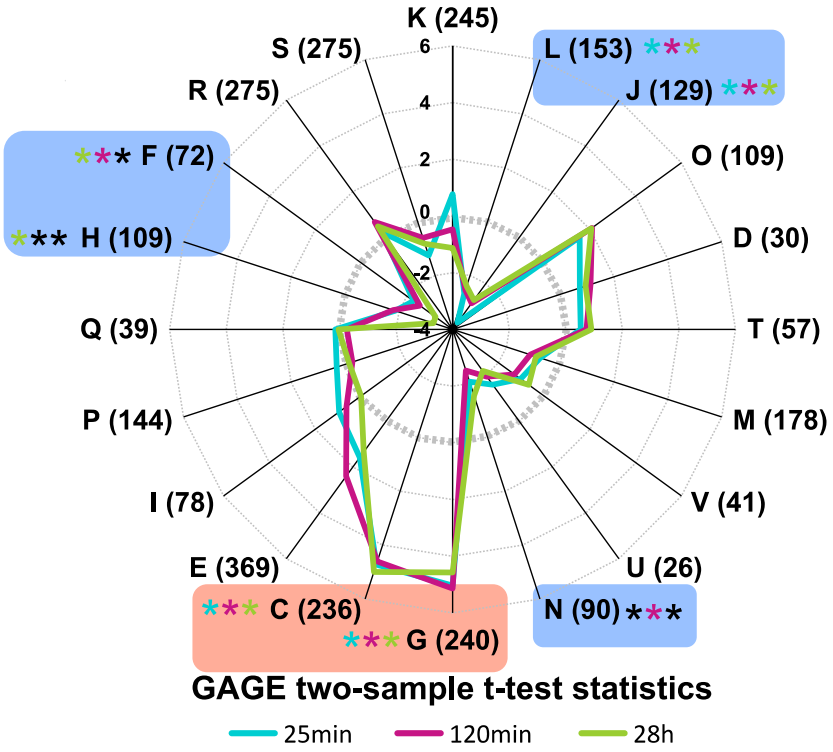
**Cellular processes and signaling**
O Posttranslational modification, protein turnover and chaperones
D Cell cycle control, cell divison and chromosome partitioning
T Signal transduction mechanisms
M Cell wall, membrane, envelope biogenesis
V Defense mechnisms
U Intracellular trafficking, secretion, vesicular transport
N Cell motility

**Metabolism**
G Carbohydrate tranport and metabolism
C Energy production and conversion
E Amino acid transport and metabolism
I Lipids transport and metabolism
P Inorganic ion transport and metabolism
Q Secondary metabolites biosynthesis, transport and metabolism
F Nucleotide transport and metabolism
H Coenzyme transport and metabolism

**Poorly characterized**
R General function prediction only
S Function unknown

# Differentially Expressed Genes (PFR outlet vs STR): 25, 120 min, 28h

Simen, Löffler et al. 2016 *Metabolic Engineering*




INFORMATION STORAGE AND PROCESSING
[K] Transcription
[L] Replication/recombination/repair
[J] Translation/ribosomal structure/biogenesis

CELLULAR PROCESSES AND SIGNALING
[O] Posttranslational modification/protein turnover/chaperones
[D] Cell cycle control/cell division/chromosome partitioning
[T] Signal transduction mechanisms
[M] Cell wall/membrane/envelope biogenesis
[V] Defense mechanisms
[U] Intracellular trafficking/secretion/vesicular transport
[N] Cell motility

METABOLISM
[G] Carbohydrate transport and metabolism
[C] Energy production and conversion
[E] Amino acid transport and metabolism
[I] Lipid transport and metabolism
[P] Inorganic ion transport and metabolism
[Q] Secondary metabolites biosynthesis/transport/catabolism
[F] Nucleotide transport and metabolism
[H] Coenzyme transport and metabolism

POORLY CHARACTERIZED
[R] General function prediction only
[S] Function unknown

**General finding:**
**→induction of carbon import, metabolism and energy generation**
**→repression of energy intensive processes**

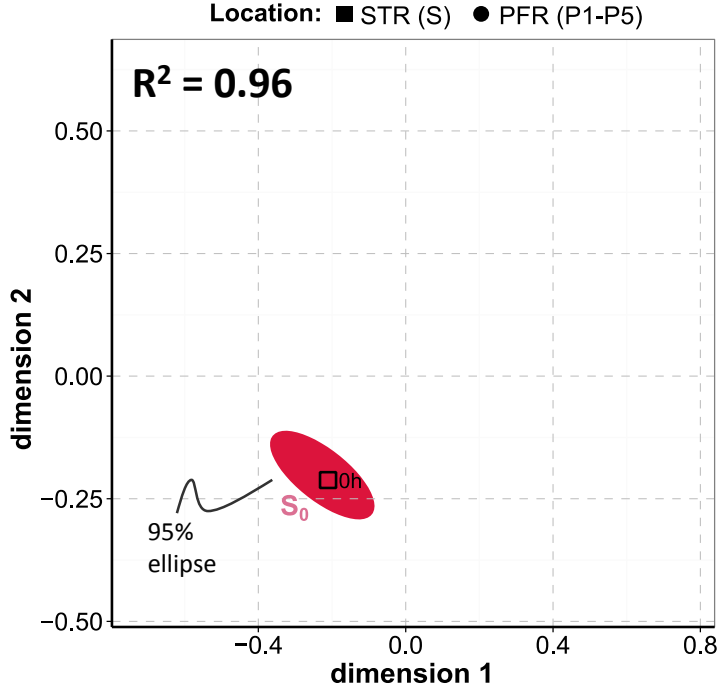
# Glucose:

# Long-Term Responses

# **Long-Term Response on Glucose Gradients** (triplicate results):

Reference – before connection with PFR

Simen, Löffler et al. 2016 *Metabolic Engineering*

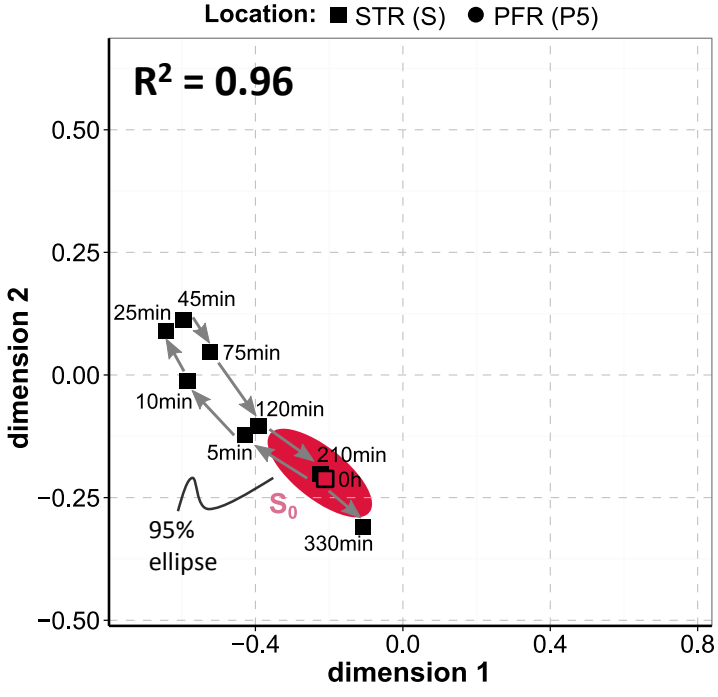



- **distinct initial state $S_0$ in STR**

# **Long-Term Response on Glucose Gradients** (triplicate results):

Phase I after connection with PFR

Simen, Löffler et al. 2016 *Metabolic Engineering*

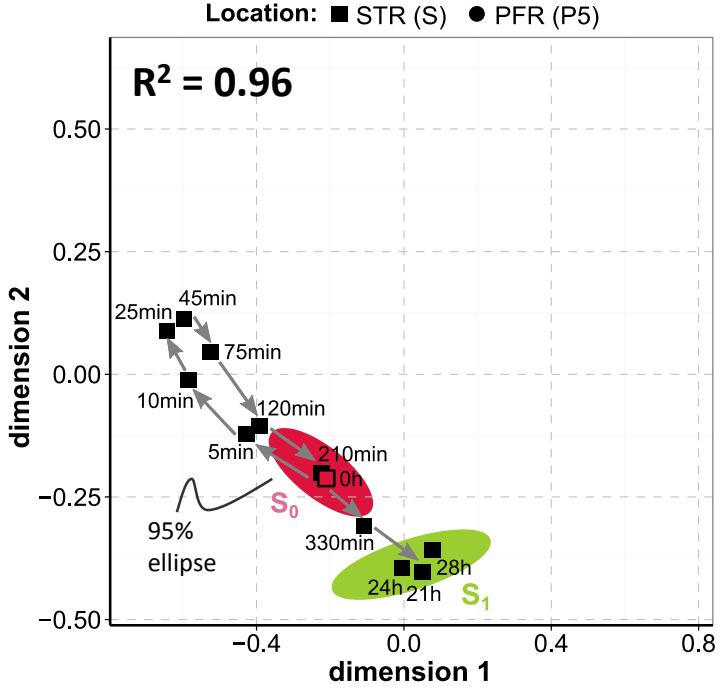



- **distinct initial state $S_0$ in STR**
- **strong transcriptional changes after PFR connection**

# **Long-Term Response on Glucose Gradients** (triplicate results):

Phase II after connection with PFR

Simen, Löffler et al. 2016 *Metabolic Engineering*



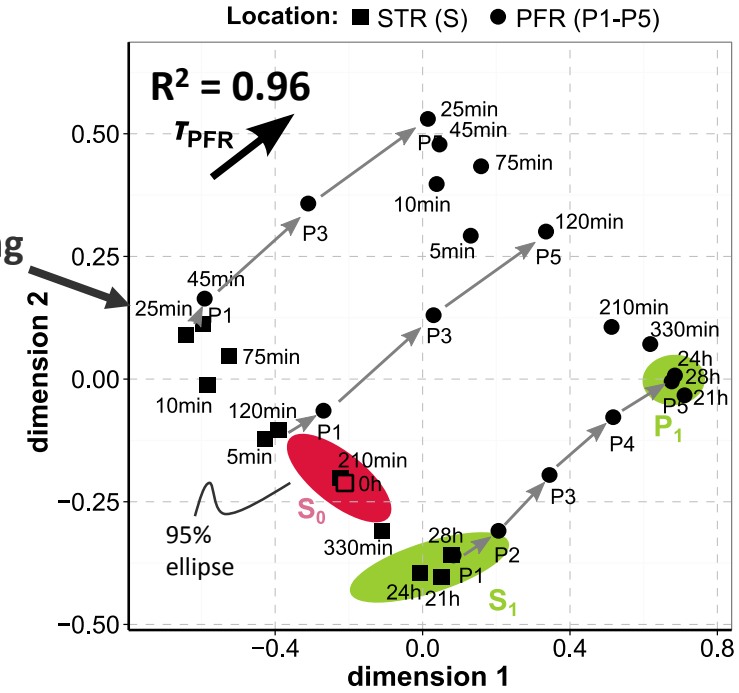

- **distinct initial state $S_0$ in STR**
- **strong transcriptional changes after PFR connection**
- **transcriptional changes converge to a new steady-state**

# **Long-Term Response on Glucose Gradients** (triplicate results):

Repeated Transcriptional Shifts from STR to PFR

Simen, Löffler et al. 2016 *Metabolic Engineering*

Start of dominating strategic response




- distinct initial state $S_0$ in STR
- strong transcriptional changes after PFR connection
- transcriptional changes converge to a new steady-state
- gradients in PFR cause trackable trans-criptional changes leading to a stable distribution of transcriptional patterns

**Gradients cause massive, periodic on-/off-switching of genes finally leading to stable transcriptional patterns coexisting next to each other**

# What can we learn from modelling?

# What can we learn from modelling?

## Predicting the Transcriptional Dynamics

# Agent-Based Modelling For Predicting Transcript Dynamics

Niess et al. .2017. *Frontiers in Microbiology*

**Agent** (single cell)

- polymerase movement: constant elongation rate
  - constraint: minimum distance between ribosomes
- attenuation modelling neglected for simplification
  - Simplification: start only valid under nitrogen limitation
  - Transcription and translation are closely coupled
- Number of ribosomes per gene is variable and specific for the gene
- mRNA degradation considered with constant degradation elongation rate, initiated at start codon of transcription
- No protein degradation, only dilution by growth

**Population** (STR-PFR operating in continuous mode)

- STR = ideally mixed; PFR = plugflow reactor
- Population balances:
  - (1) PFR entering
  - (2) drained off by efflux
  - (3) cell division, no initiation of transcription
- Fate of 10.000 cells was tracked

**Number of likely events per time:**

$$\alpha_1 = N_{STR}\frac{\dot{V}_{PFR}}{V_{STR}}$$

$$\alpha_2 = N_{STR}\frac{\dot{V}_{Feed}}{V_{STR}}$$

$$\alpha_3 = N^0_{STR}\,D$$

**Gillespie algorithm:**

$$\tau = \frac{1}{\sum \alpha_i}\ln\left(\frac{1}{r_1}\right)$$

$$\sum_{j=1}^{i-1}\alpha_j \le r_2 \sum_{j=1}^{3}\alpha_j \le \sum_{j=1}^{i}\alpha_j$$

:016

# Simulation Parameters – No Regression

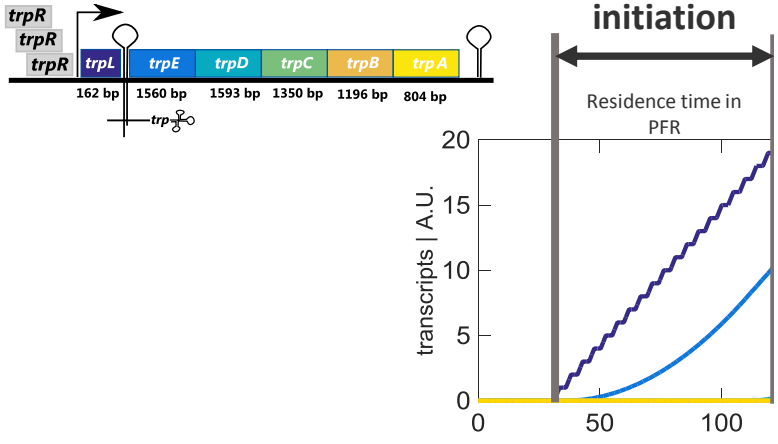
Niess et al. .2017. *Frontiers in Microbiology*

| Parameter | Value | Unit | |
|---|---|---|---|
| $\nu_{elo}^{RNAP}$ | 21 | Nucleotides per second | Elongation rate |
| $\nu_{elo}^{Ribosome}$ | 21 | Nucleotides per second | Ribosome movement on mRNA |
| $\nu_{elo}^{RNase}$ | 21 | Nucleotides per second | RNAse degragation rate |
| $\Delta x$ | 100 | Nucleotides | Minimum distance between two RNAPs |
| $\Delta y$ | 100 | Nucleotides | Minimum distance between ribosomes |
| $\Delta z$ | 100 | Nucleotides | Closest distance of RNAse to ribosome |
| $t_{ind}$ | [30 125] | Seconds | Induction period |
| $\dot{V}_{PFR}$ | 180 | mL $min^{-1}$ | Flow through PFR |
| $\dot{V}_{Feed}$ | 5 | mL $min^{-1}$ | Feed |
| $V_{STR}$ | 1,120 | mL | STR volume |
| $D$ | 0.2 | $h^{-1}$ | Dilution rate of the system |
| $N_{STR}^{0}$ | 10,000 | cells | Total number of tracked cells |

# Once Initiated, Transcriptional (TC) Stimulus Propagates From PFR into STR Followed by Translation (TL): Example TRP Operon

Induction of *trp* operon during N-limitation

Niess et al. .2017. *Frontiers in Microbiology*



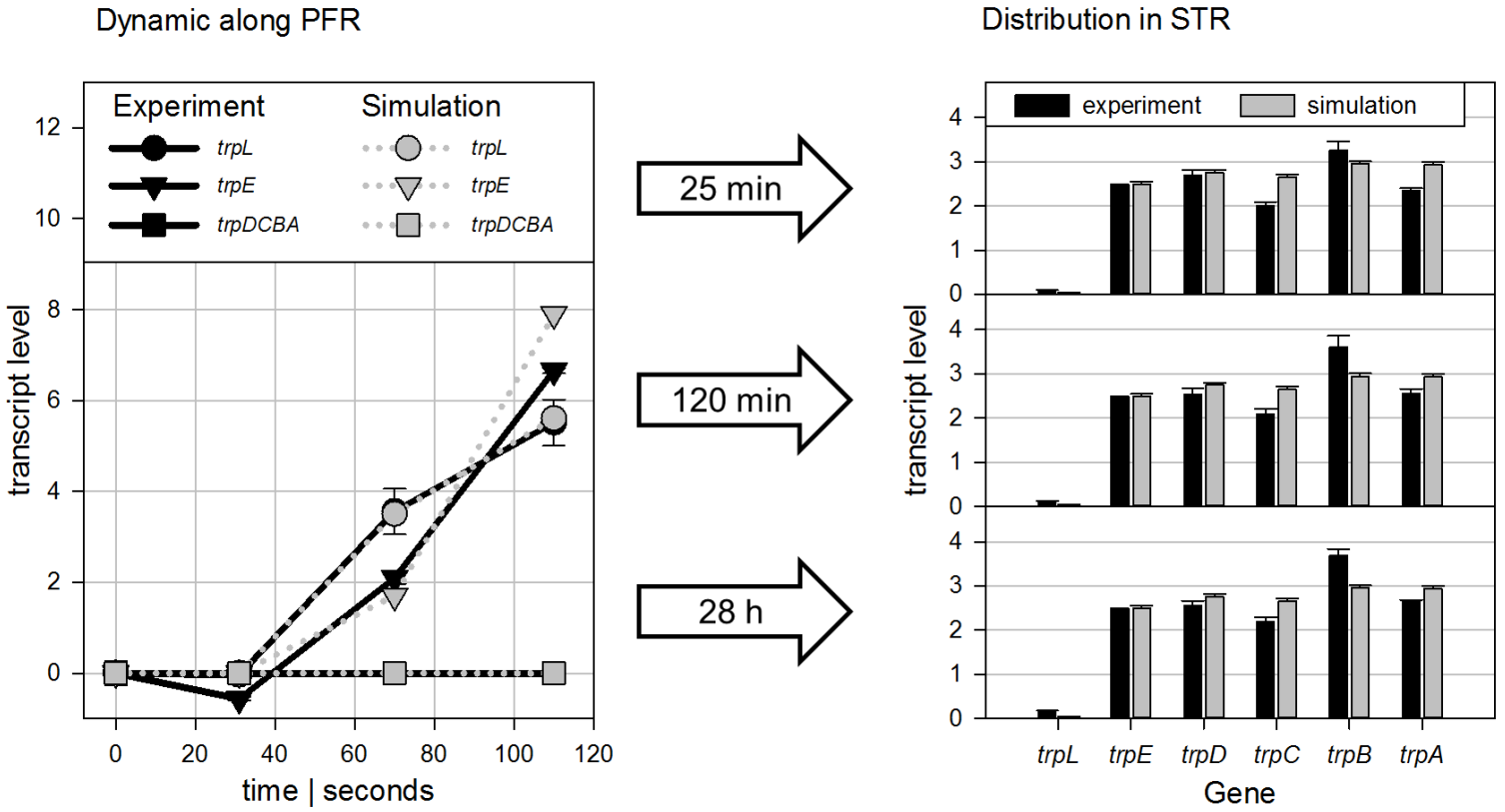

## Key findings:

- Once initiated in PFR, transcription and translation continues in STR
- Mayor burden is located in STR
- Cells with different transcriptional patterns co-exist next to each other: population heterogeneity

# Prediction of population transcript levels in STR

Agent based modeling tracking the fate of 10.000 cells

Niess et al. .2017. *Frontiers in Microbiology*




High prediction quality of short – and long-term transcriptional dynamics

# What can we learn from modelling?

## Deriving Constraints for Creating Robust Strains For Large-Scale Application

# Modeling Assumptions for Estimating Cellular Efforts Periodically Switching Genes ON/OFF

Simen, Löffler et al. 2016 *Metabolic Engineering*

- Select transcripts with **FDR < 1%**
- Group in **up**- and **downregulated** genes
- *De novo* precursor synthesis:
  - calculate individual ATP needs for G, A, T, C formation from metabolic precursors
  - calculate ATP needs for amino acid production assuming average protein composition

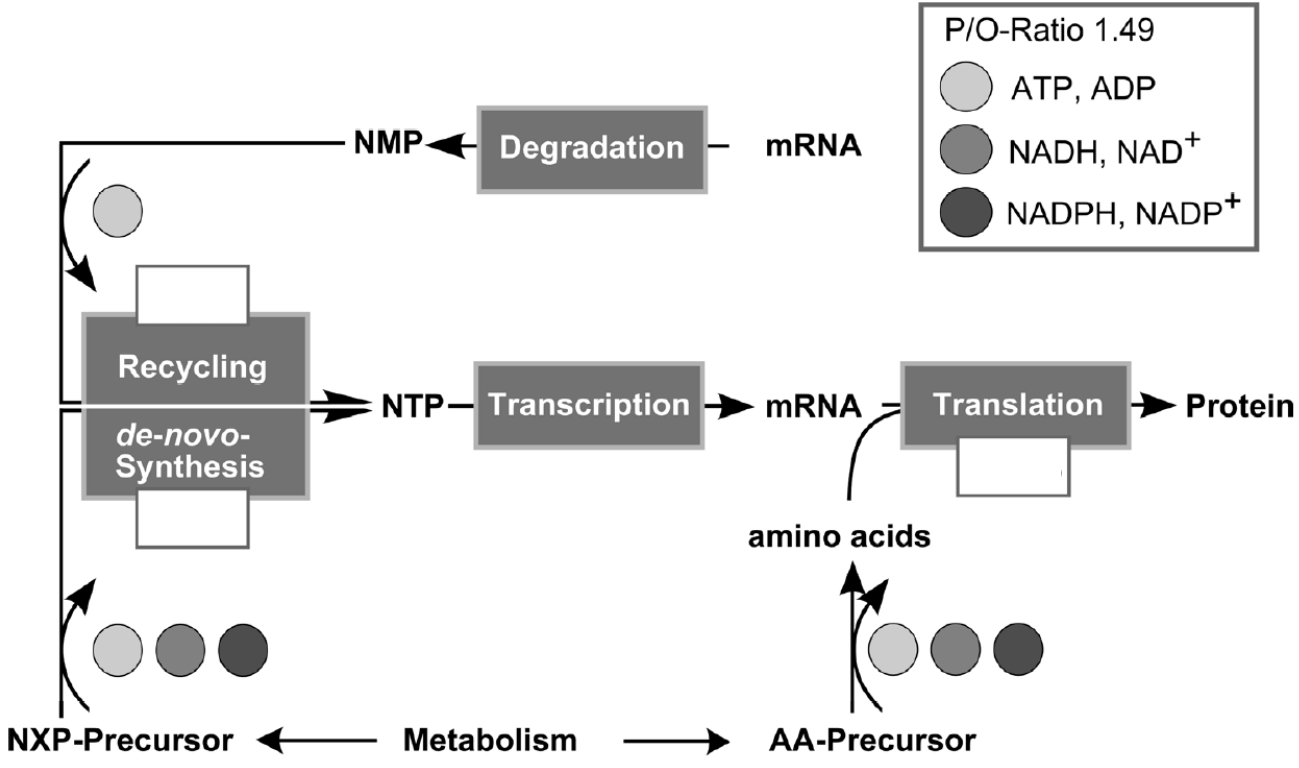
- **Transcription**: For each mRNA with individual G, A, T, C content: 2 ATP/nucleotide
- **Translation**: 4 ATP/amino acid
- Considering mRNA dynamics and PFR residence time: **11 ribosomes per mRNA**
- **Completing translation** after recycling in STR

# Modeling Additional ATP Costs for Transcription and Translation

Individual ATP balances for each gene, reference: native maintenance

Simen, Löffler et al. 2016 *Metabolic Engineering*

ATP cost reference: *E. coli* maintenance (Taymaz-Nikerel et al. 2010)




**Maintenance demands are increased by 40 – 50 % in large-scale!**
**Represents missing energy for hyperproducers.**

# Exploiting the Results: Identifiers for Smart Genome Reduction

## Top 20 Gene Targets for Deletion/Modulation Saving ATP

Simen, Löffler et al. 2016 *Metabolic Engineering*

| *Gene*[†] | Percentage increasing the growth-independent maintenance, % | | | COG | Function |
|---|---|---|---|---|---|
| | ***De novo* mRNA synthesis** | **Translation** | ∑ | | |
| *fliC* | 2.70 | 0.40 | 3.10 | N | flagellar biosynthesis; flagellin, filament structural protein |
| *aroF* | 0.67 | 0.10 | 0.77 | E | 2-dehydro-3-deoxyphosphoheptonate aldolase (DAHP synthase) |
| *aldA* | 0.48 | 0.07 | 0.55 | C | aldehyde dehydrogenase A, NAD-linked |
| *cstA* | 0.35 | 0.05 | 0.40 | T | peptide transporter induced by carbon starvation |
| *aceA* | 0.34 | 0.05 | 0.39 | C | isocitrate lyase monomer |
| *cspD* | 0.31 | 0.05 | 0.36 | K | DNA replication inhibitor |
| *aceB* | 0.27 | 0.04 | 0.31 | C | malate synthase A |
| *trg* | 0.27 | 0.04 | 0.31 | N | methyl accepting chemotaxis protein – ribose/galactose/glucose sensing |
| *groL* | 0.26 | 0.04 | 0.30 | O | GroEL chaperonin, peptide-dependent ATPase, heat shock protein |
| *dnaK* | 0.24 | 0.03 | 0.27 | O | chaperone protein DnaK |
| *yfiA* | 0.18 | 0.03 | 0.21 | J | stationary phase translation inhibitor and ribosome stability factor |
| *gatC* | 0.17 | 0.03 | 0.20 | G | galactitol PTS permease - GatC subunit |
| *flgL* | 0.16 | 0.02 | 0.19 | N | flagellar biosynthesis; hook-filament junction protein |
| *flgK* | 0.13 | 0.02 | 0.15 | N | flagellar biosynthesis, hook-filament junction protein 1 |
| *acs* | 0.13 | 0.02 | 0.14 | I | acetyl-CoA synthetase (AMP-forming) |
| *mdh* | 0.12 | 0.02 | 0.14 | C | malate dehydrogenase |
| *kgtP* | 0.11 | 0.02 | 0.12 | E | α-ketoglutarate: $H^+$ symporter |
| *fliA* | 0.11 | 0.02 | 0.12 | K | RNA polymerase, sigma 28 (sigma F) factor |
| *glnH* | 0.10 | 0.02 | 0.12 | E | glutamine ABC transporter - periplasmic binding protein |
| *yjdA* | 0.10 | 0.01 | 0.11 | n.a. | clamp-binding sister replication fork colocalization protein |

[†]Only genes which expression was always significantly changed between STR and PFR P5 (FDR < 0.01) were selected for the calculations (core genes).

**Ongoing research**

# Transferring Results:

# *E. coli* HGT

# Ideal Strain for Large-Scale Application

Michalowski et al, 2017 *Metabolic Engineering*

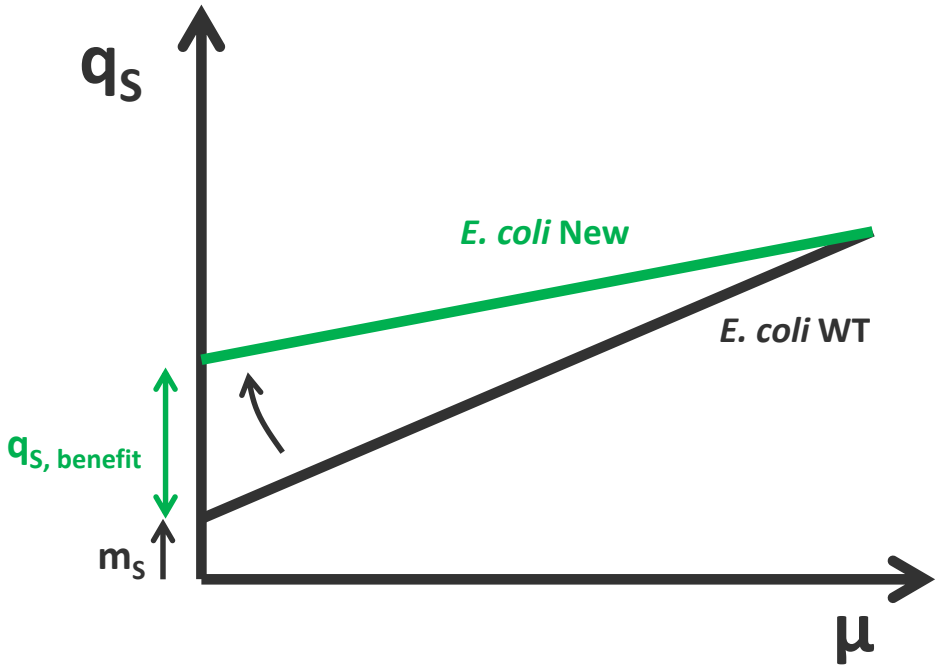
The **ideal producer** should:

- be blind with respect of extracellular heterogeneities
- enable high glucose uptake rates, even under resting condition

Goal:

**to create a novel *E. coli* chassis** with fundamentally changed properties.

**$q_S$ is increased but $m_s$ stays constant**

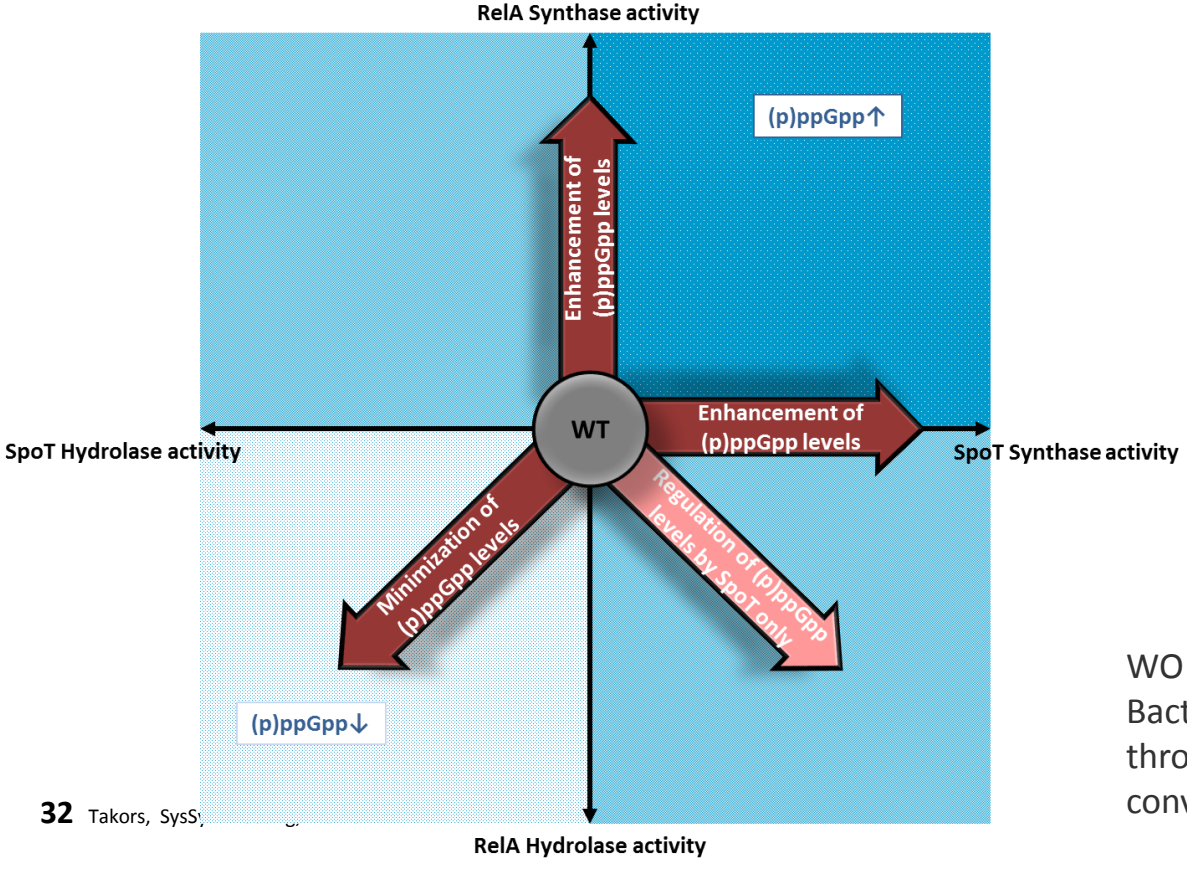


# Engineering *E. coli* HGT Following Two Guidelines

HGT = high glucose throughput

Michalowski et al, 2017 *Metabolic Engineering*

## Modulating stringent response to make *E. coli* blind



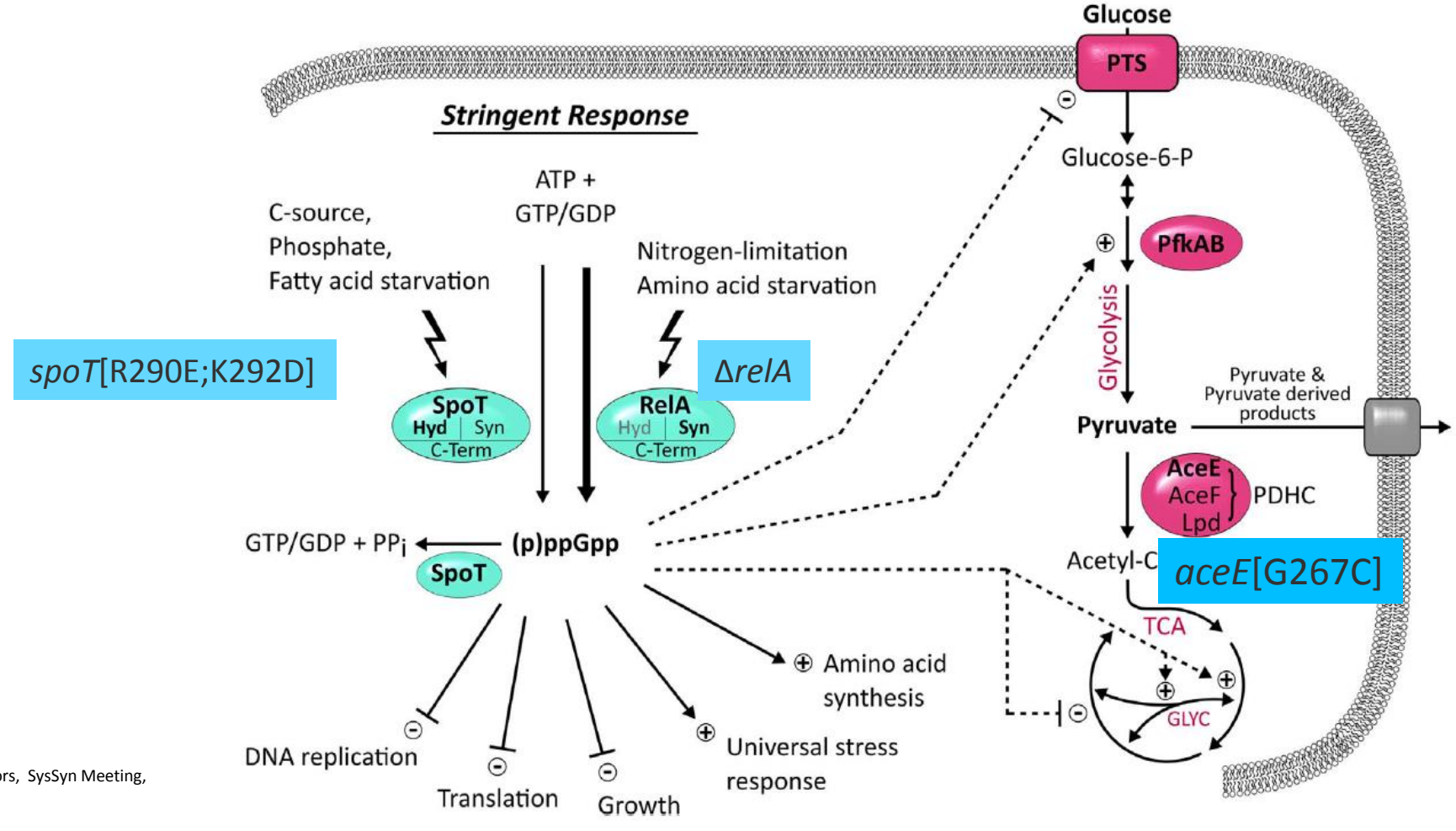

## Modulating central metabolism

Integrating *aceE** (pyruvate dehydrogenase subunit)

WO patent filed:
Bacterial strain and method for high throughput of sugar in the microbial conversion into biosynthetic products

# *E. coli* HGT

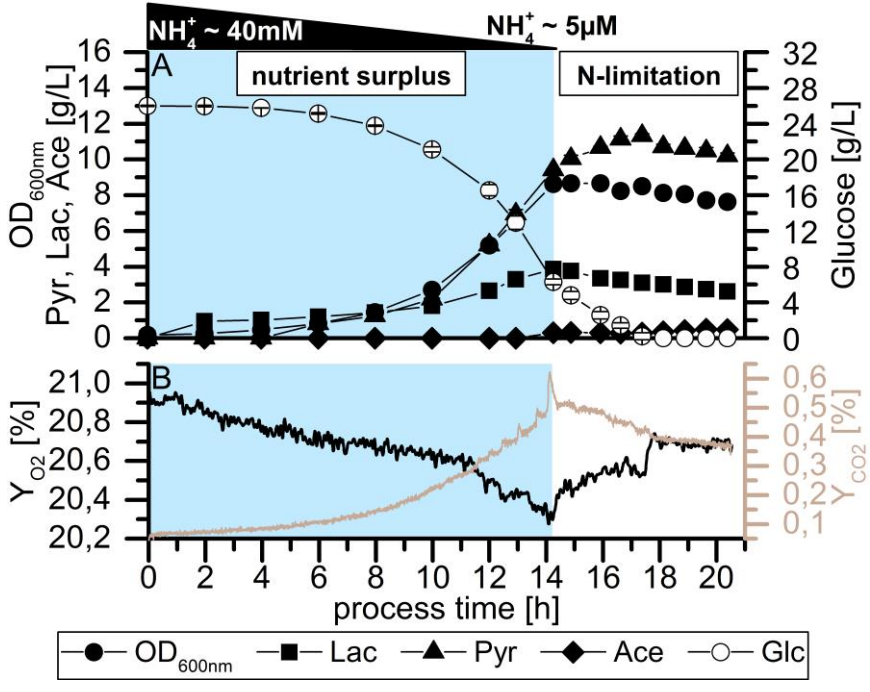
Michalowski et al, 2017 *Metabolic Engineering*



# Experimental Setup

Michalowski et al, 2017 *Metabolic Engineering*

Installing ammonia limitation under glucose saturated conditions to test engineered strains

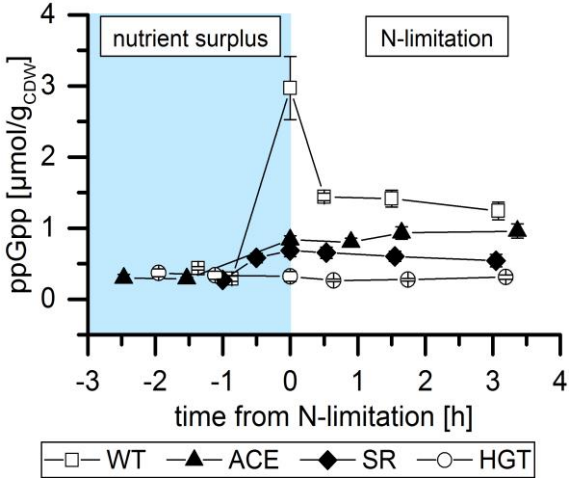


# *E. coli* HGT

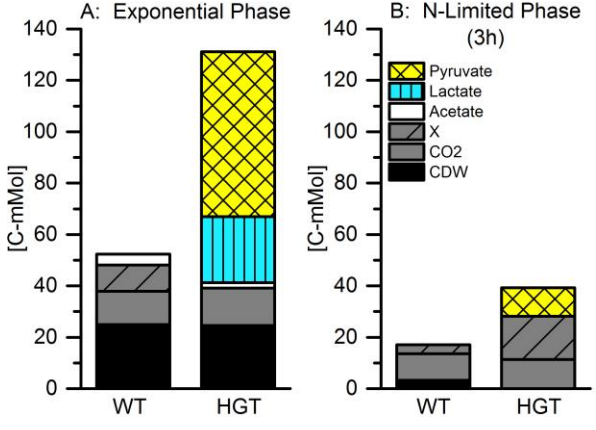
Includes *ace*E* + changes in *rel*A and *spo*T

Michalowski et al, 2017 *Metabolic Engineering*

*E. coli* HGT shows no ppGpp changes under ammonia limited conditions, i.e. no stringent response initiated



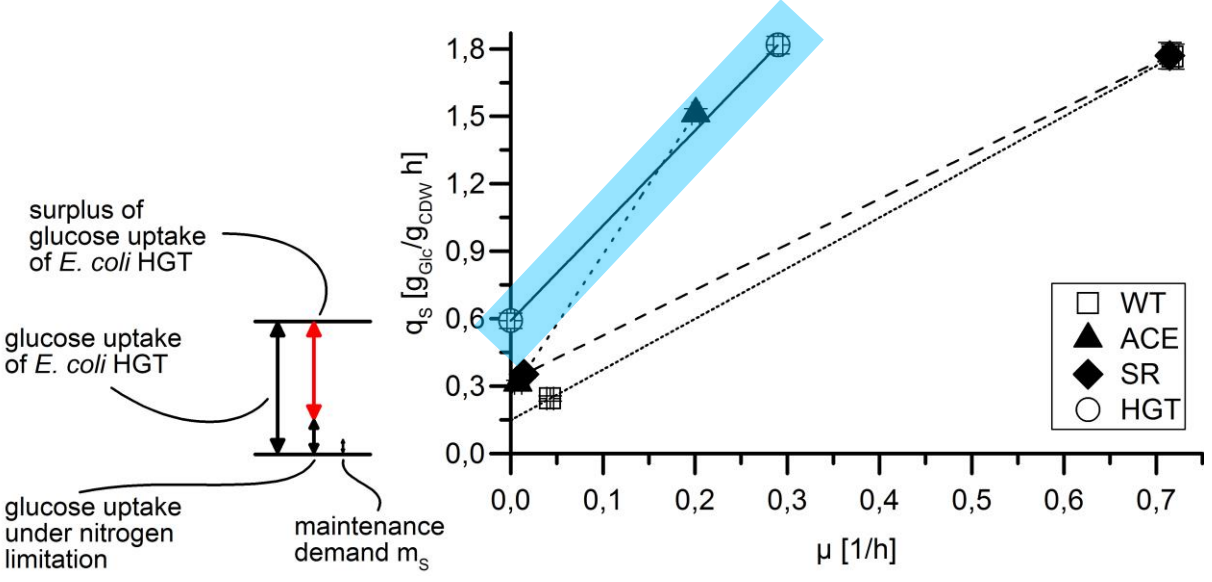

*E. coli* HGT provides a surplus of pyruvate for downstream use.



# *E. coli* HGT – glucose uptake kinetics

Michalowski et al, 2017 *Metabolic Engineering*

*E. coli* HGT shows about 10 fold higher glucose uptake for non-growing conditions than needed for maintenance demands and reaches maximum uptake with 0.3 1/h. The surplus of carbon is available as pyruvate predominately.



# Summary & Thanks

- Mixing times of max 70 s should be installed preventing massive transcriptional responses
- Massive transcriptional dynamics are induced by substrate heterogeneity causing maintenance increase (1.4-1.5 fold).
- Maintenance dynamics and transcriptional adaptation can be well modelled/predicted.
- Large-scale performance can be simulated.
- Novel chassis *E. coli* HGT created.

**Thanks to:** **Joana Simen, Michael Löffler, Annette Michalowski, Alexander Niess**

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# Different Time Scales of Response

Niess et al. .2017. *Frontiers in Microbiology*