

#### Protein synthesis in cell free systems

Olivier Borkowski

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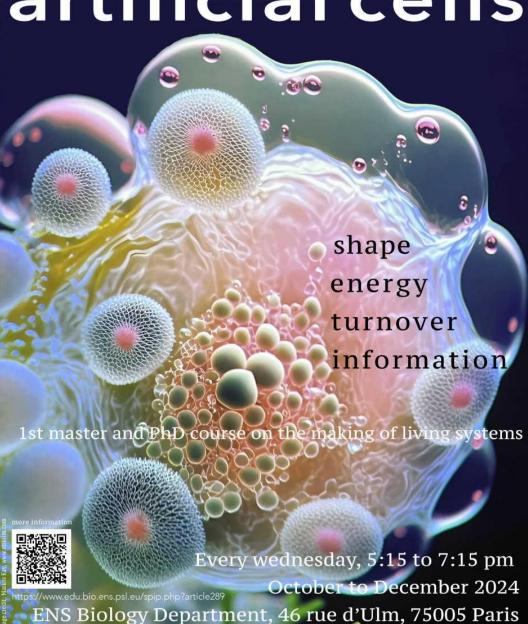
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# artificial cells



Biomolecule synthesis: Part I





## Protein synthesis in cell-free systems

#### Olivier Borkowski, PhD

Permanent researcher in Synthetic Biology Institut Micalis, INRAE - AgroParisTech - Univ. Paris-Saclay, France SyBER team

Email: olivier.borkowski@inrae.fr





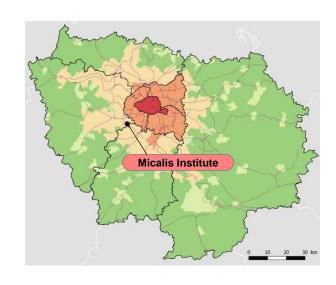






## Micalis Institute (INRAE-AgroParisTech)







#### 21 Research Teams

- 340 people
- 130 scientists and engineers
- 140 PhD students and post-docs 70 scientific and administrative personnel

#### **Themes**

- (1) Emergence, control, and adaptation of pathogenic microorganisms
- (2) Microbial food and intestinal ecosystems(3) Synthetic and systems biology







## **Understand bacterial physiology**

Bacterial physiology is a branch of biology that aims to understand overarching principles of cellular reproduction:

What are the fundamental principles underlying the regulation and coordination of biosynthesis in a given growth environment?

-> Bacterial growth law







## **Understand bacterial physiology**

Bacterial physiology is a branch of biology that aims to understand overarching principles of cellular reproduction:

What are the fundamental principles underlying the regulation and coordination of biosynthesis in a given growth environment?

-> Bacterial growth law

Some major principles studied in bacterial physiology:

- Link between bacterial growth and nutrient concentrations in culture media (Monod, 1942)
- Link between the macromolecular composition and cell growth rate (Schaechter et al., 1958)
- Linear relationships between RNA/DNA, RNA/protein, protein/DNA and growth rate (Bremer & Dennis, 1974)
- Increasing allocation of cellular resources to ribosome synthesis with increasing growth rate (Hwa & Scott, 2010)
- -> What is happening at the gene level?







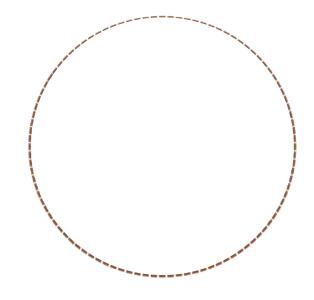
## **Bacterial Physiology**

**Experimental setup** 

**Genomic regulations** 



*E. coli* living cells



More than 4000 genes



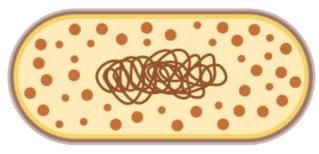




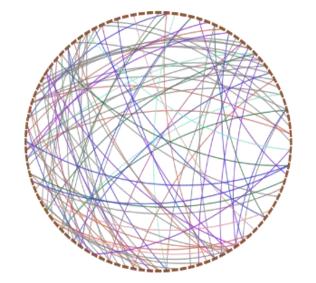
## **Bacterial Physiology**

**Experimental setup** 

**Genomic regulations** 







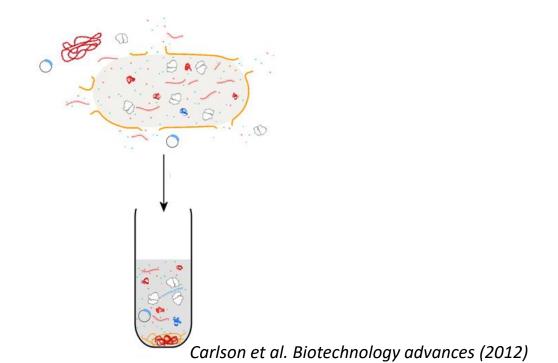
**Intertwined Effects** 

-> New technology to decouple the many effects observed in living cells

#### Cell-free reaction

#### Gene expression in a simpler and open system

A simpler environment to measure direct effect on gene regulation (no cascade of reaction due to living organism adaptation) A direct manipulation of the environment because it is an open system

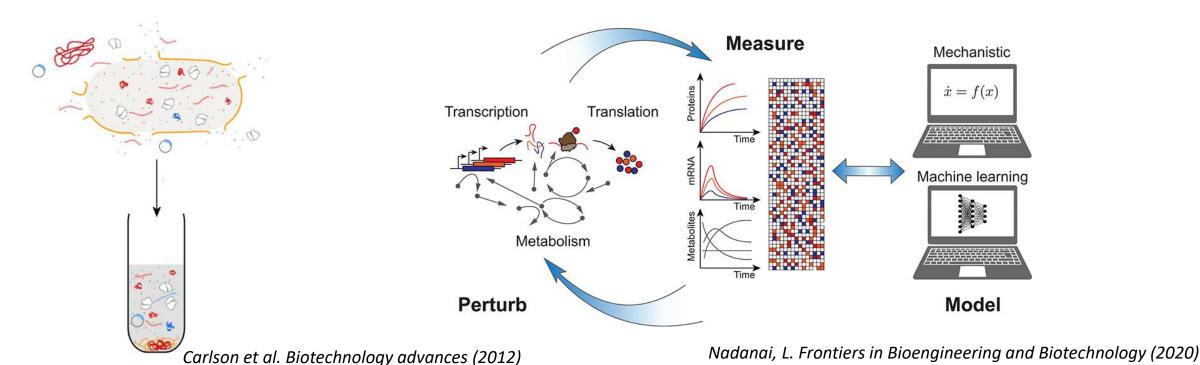


#### Cell-free reaction

#### Gene expression in a simpler and open system

A simpler environment to measure direct effect on gene regulation (no cascade of reaction due to living organism adaptation) A direct manipulation of the environment because it is an open system

- An alternative technology to study central process observed in living cells (replication, transcription, translation, degradation, metabolism)
- A versatile technology for bioengineering (Use in Synthetic Biology): life simulation system









1. Description of Cell-Free Systems: An open system to study biology

2. Understand living organisms: functions preserved in cell-free systems

3. Building artificial cells: **reconstruct functions** defective in cell-free systems

### First major use of cell-free: genetic code

#### Cell-free was first used in the 1960's by Nirenberg and Matthaei to understand the genetic code

Matthaei

Johannes Heinrich



American & German biochemist

Marshall Warren Nirenberg

THE DEPENDENCE OF CELL- FREE PROTEIN SYNTHESIS IN E. COLI UPON NATURALLY OCCURRING OR SYNTHETIC POLYRIBONUCLEOTIDES

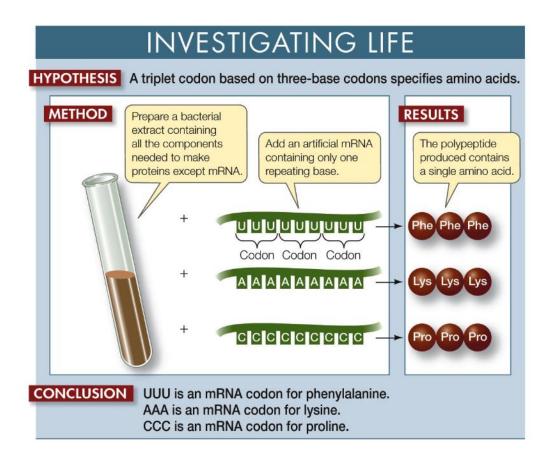
By Marshall W. Nirenberg and J. Heinrich Matthaei\*

NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND

Communicated by Joseph E. Smadel, August 3, 1961

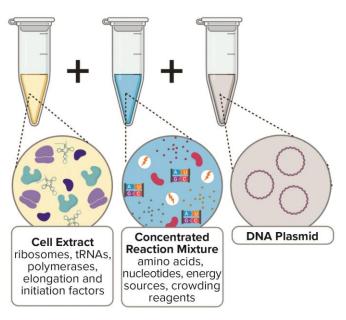
A stable cell-free system has been obtained from E. coli which incorporates C14-valine into protein at a rapid rate. It was shown that this apparent protein synthesis was energy-dependent, was stimulated by a mixture of L-amino acids, and was markedly inhibited by RNAase, puromycin, and chloramphenicol.1 The present communication describes a novel characteristic of the system, that is, a requirement for template RNA, needed for amino acid incorporation even in the

# Nobel prize for the interpretation of the genetic code and its function in protein synthesis



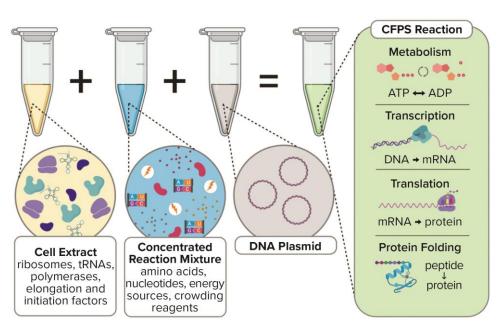
They made a synthetic RNA only with the base uracil (poly U – codon UUU) and tested it in 20 tubes, each one with a different radioactive amino acid.

## What is the composition of a cell-free reaction?

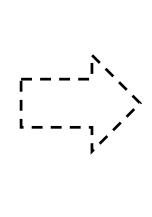


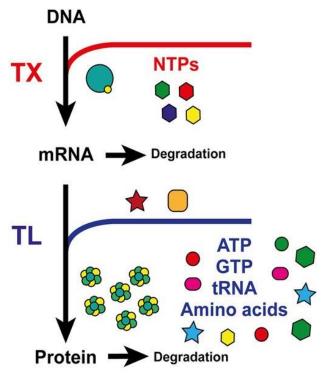
Melinek B, et al. BioProcess International (2020)

## What is the composition of a cell-free reaction?



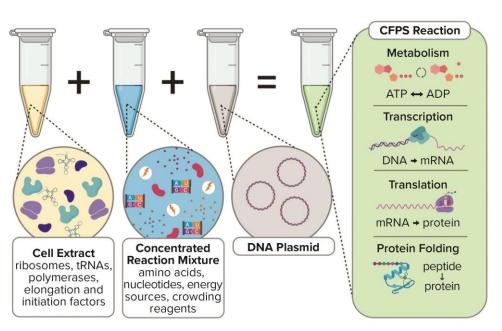
Melinek B, et al. BioProcess International (2020)





Seok Hoon Hong et al. Frontiers in Chemistry 2014

## Cell-extract preparation



Melinek B, et al. BioProcess International (2020)

#### **Physical disruption**

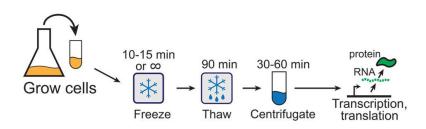


French press

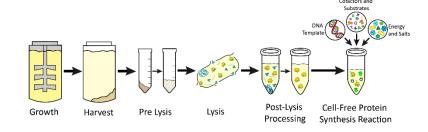


#### **Chemical disruption**

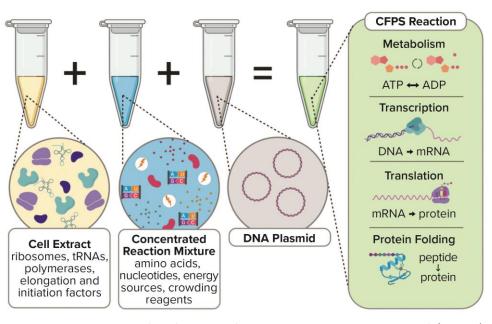
#### **Autolysis**



#### Lysozyme



#### **Buffer composition**



Melinek B, et al. BioProcess International (2020)

**Amino Acid** 

**tRNA** 

Mg-glutamate

K-glutamate

Folinic Acid

Spermidine

Coenzyme A

NAD

3-PGA

**AMPc** 

Nucleoside triphosphate (NTP)

**HEPES** 

Translation

Translation

Ribosome maintenance

Ribosome stability

A precursor for formylmethionine (fMet)

Ribosome activity /RNA stability

Regenerate ATP via glycolysis

Regenerate ATP via glycolysis

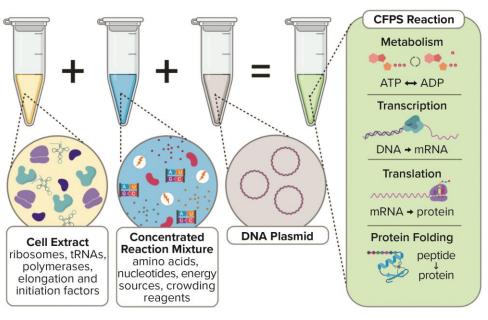
Regenerate ATP

regulating glycolysis

Transcription

PH Buffer

#### Buffer composition



Melinek B, et al. BioProcess International (2020)

Amino Acid Translation

Mg-glutamate Ribosome maintenance

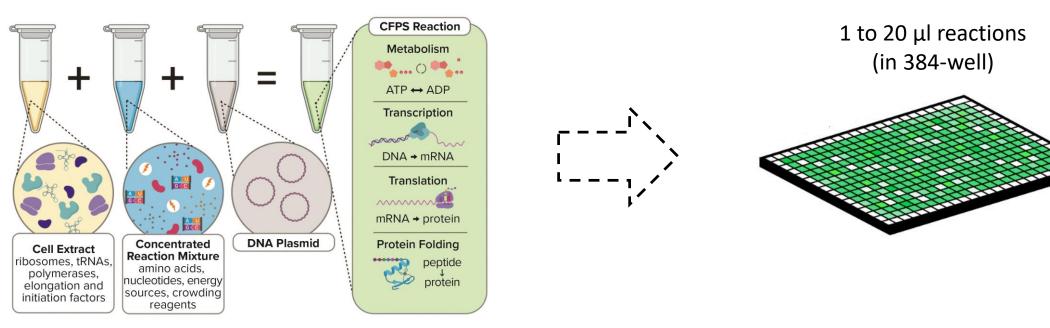
K-glutamate Ribosome stability

Spermidine Ribosome activity /RNA stability

3-PGA Regenerate ATP

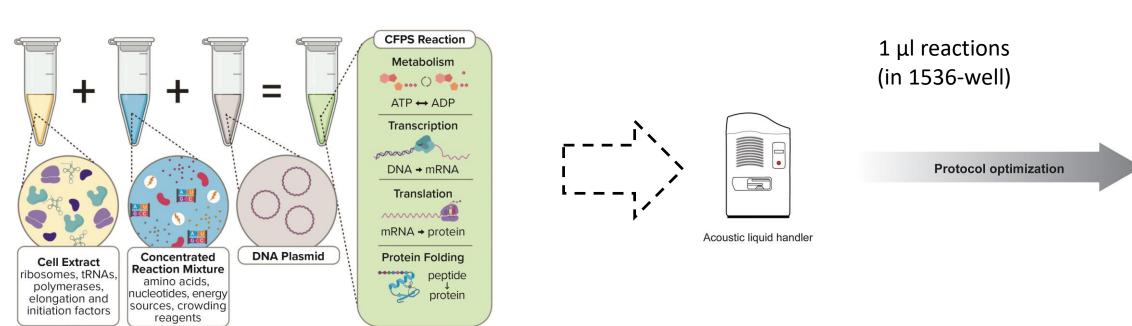
Nucleoside triphosphate (NTP) Transcription HEPES PH Buffer

## Measurements in 384-well plate



Melinek B, et al. BioProcess International (2020)

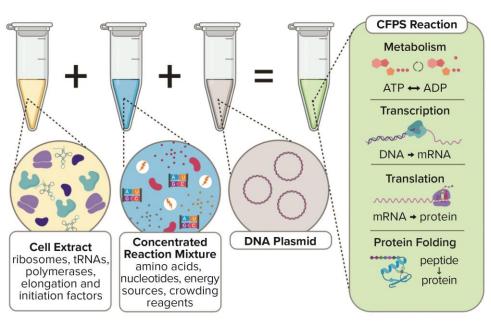
## Measurements in 1536-well plate



Melinek B, et al. BioProcess International (2020)

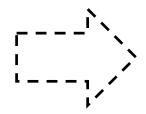
Cell-free assay

## Measurements profils

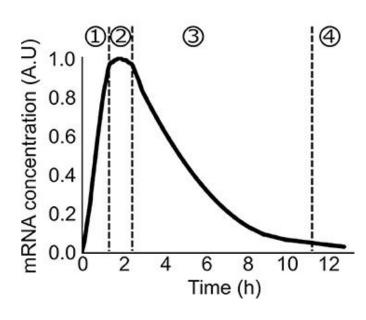


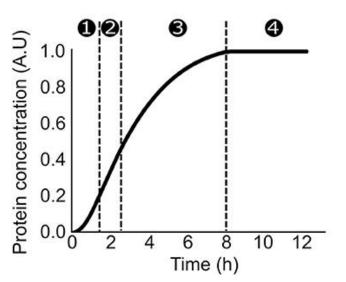
Melinek B, et al. BioProcess International (2020)

#### **Transcription**

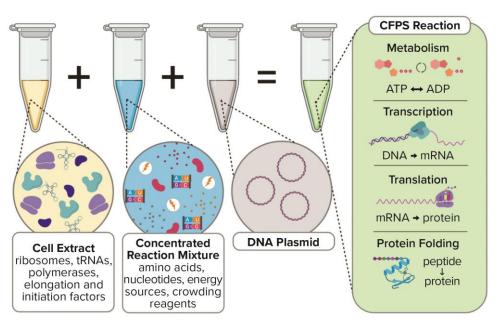


**Translation** 





## Current existing protocol to produce bacterial cell extract



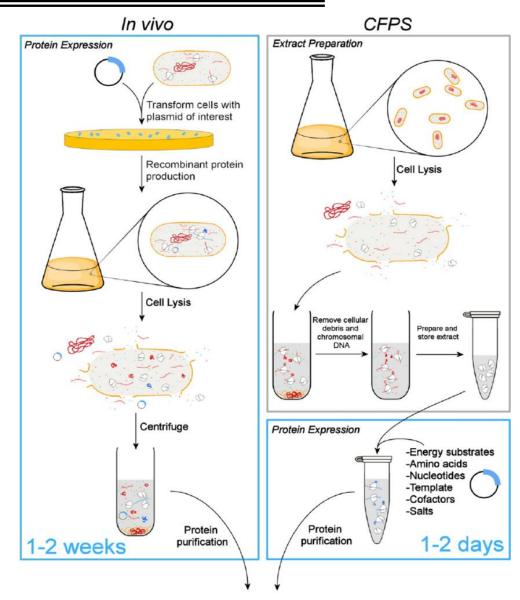
Melinek B, et al. BioProcess International (2020)

Table 1. Current existing protocols to produce cell-free lysates from bacteria cells

Organism	Bacteria Phylum	
E. coli	Proteobacteria	
B. subiltis	Firmicutes	
L. lactis	Firmicutes	
B. megaterium	Firmicutes	
C. autoethanogenum	Firmicutes	
V. natriegens	Proteobacteria	
P. putida	Proteobacteria	
E. fergusoni	Proteobacteria	
S. enterica	Proteobacteria	
K. oxytoca	Proteobacteria	
C. glutamicum	Actinobacteria	
S. venezuelae	Actinobacteria	

## Advantages of producing protein outside of the cell

- Fast reaction to produce protein
- No need for transformation
- No GMO
- No interaction with a living host (No mutation, No burden)
- Decoupling effect
   (e.g. Metabolic burden vs production burden)
- Low volume of reaction
- Higher level of control: Amount of DNA, Composition of the reaction.
- Work with Linear DNA









2. Understand living organisms: functions preserved in cell-free systems







## 2. Understand living organisms: functions preserved in cell-free systems

The central dogma: from DNA to protein

DNA replication

Transcription and transcriptional regulation

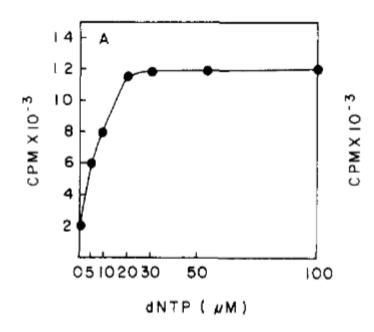
Translation and translational regulation

Metabolism and energy production

Resources utilization

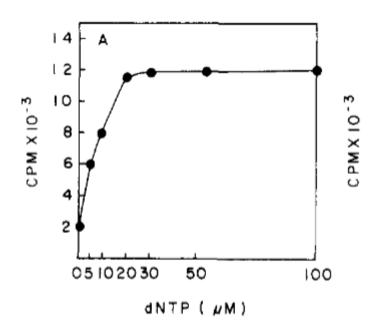
## DNA replication: from the lysate

Cell-free used to understand plasmid replication (measurment using <sup>32</sup>P radioactive isotope)



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Cell-free used to understand plasmid replication (measurment using <sup>32</sup>P radioactive isotope)



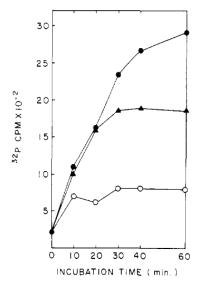
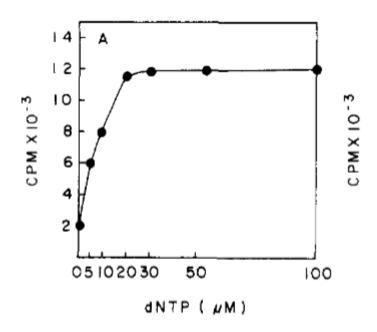


FIGURE 4: Effect of rifampin on R6K DNA synthesis. The reaction conditions were as described in the legend to Figure 2 except that  $[\alpha^{-32}P]dATP$  was used instead of  $[^3H]dTTP$ . Rifampin (20  $\mu g/mL$ ) was either not added ( $\bullet$ ) or added at 0 (O) and 10 min ( $\blacktriangle$ ) after incubation.

"DNA synthesis is dependent on the four deoxyribo- and ribonucleotide triphosphates and is sensitive to rifampin and streptolydigin, inhibitors of DNA-dependent RNA polymerase."

#### **DNA** replication

Cell-free used to understand plasmid replication (measurment using <sup>32</sup>P radioactive isotope)



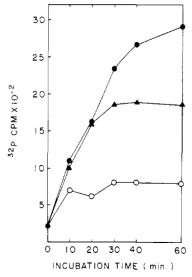
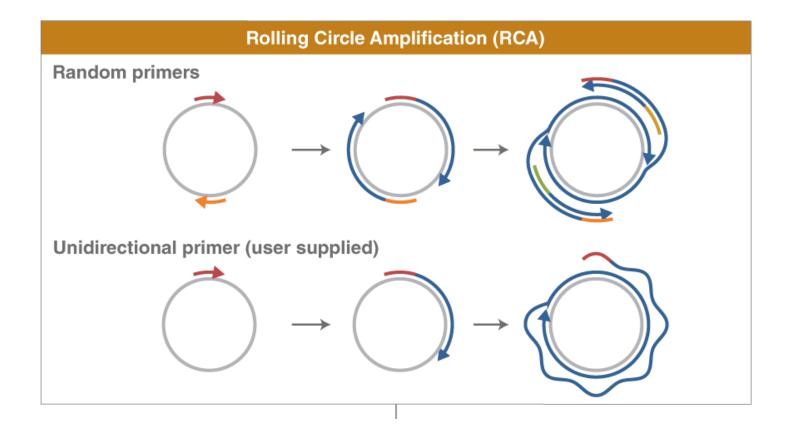


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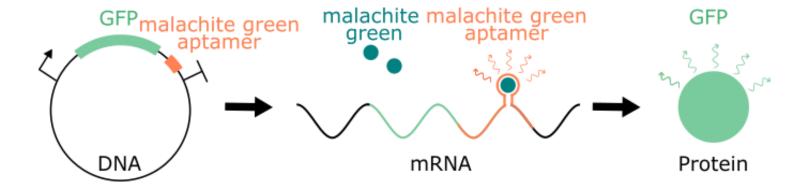
"DNA synthesis is dependent on the four deoxyribo- and ribonucleotide triphosphates and is sensitive to rifampin and streptolydigin, inhibitors of DNA-dependent RNA polymerase."

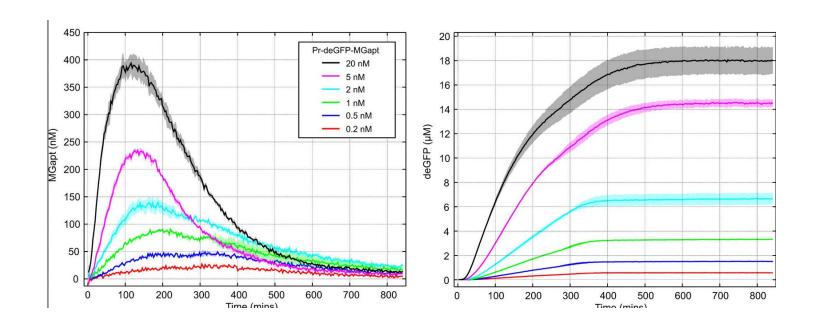
#### DNA replication to amplify DNA

DNA replication system reconstructed by expressing 1 gene of the bacteriophage phi 29 DNA replication .

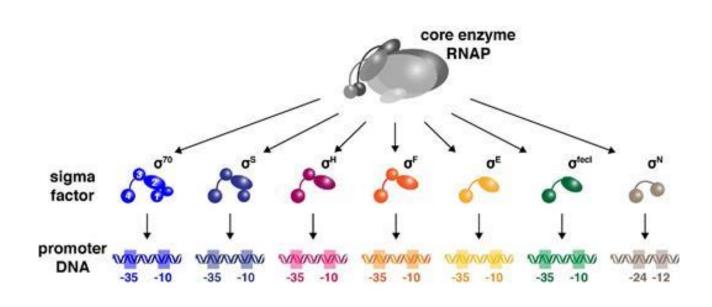


The core RNA polymerase remains present and active in cell-free





Siegal-Gaskins, D. ACS synthetic biology (2014) Wagner L et al. (in progress)

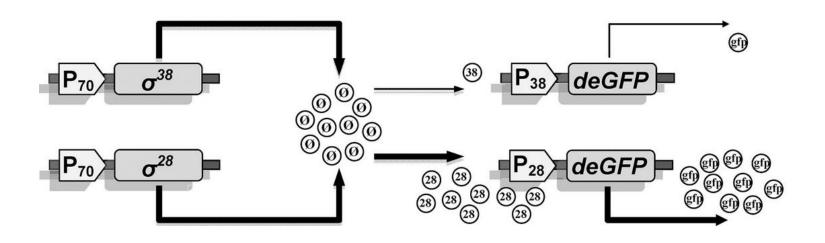


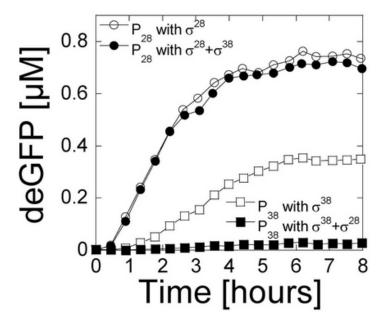
o-factor	Function	Group
$\sigma^{D}/\sigma^{70}$	Housekeeping genes	1
$\sigma^8/\sigma^{38}$	Stationary phase	2
$\sigma^{H}/\sigma^{32}$	Heat shock response	3
σ <sup>F</sup> /σ <sup>28</sup>	Motility genes	3
$\sigma^E/\sigma^{24}$	Extracytoplasmic function	4
Official/O <sup>19</sup>	Extracytoplasmic function	4
$\sigma^{N}/\sigma^{54}$	Nitrogen metabolism	

#### Promoter selection and characterization Orthogonality of *E. coli* sigma factors

Transcription factor expressed									
deGFP [µM]		(σ <sup>70</sup> )*	$\sigma^{19}$	$\sigma^{24}$	$\sigma^{28}$	$\sigma^{32}$	$\sigma^{38}$	T3RNAP	T7RNAP
	P <sub>70</sub>	6.40	5.89	5.45	3.96	4.74	6.01	6.06	5.76
	P <sub>19</sub>	< 0.01	0.89	0.01	0.02	< 0.01	0.01	< 0.01	< 0.01
ည	P <sub>24</sub>	0.01	0.03	0.74	< 0.01	0.01	0.03	0.01	0.01
Promoters	P <sub>28</sub>	< 0.01	0.02	< 0.01	8.06	< 0.01	< 0.01	< 0.01	< 0.01
۱Ę	P <sub>32</sub>	0.20	0.21	0.20	0.12	5.44	0.20	0.20	0.22
٦	P <sub>38</sub>	< 0.01	0.13	0.03	0.01	< 0.01	0.83	0.01	< 0.01
	P <sub>T3</sub>	0.02	0.02	0.03	< 0.01	0.02	0.03	13.44	0.02
	P <sub>T7</sub>	0.02	0.03	0.02	< 0.01	< 0.01	0.02	0.02	17.91
Rate of deGFP		Transcription factor expressed							
synthesis [nM/min]		(σ <sup>70</sup> )*	σ <sup>19</sup>	$\sigma^{24}$	$\sigma^{28}$	$\sigma^{32}$	$\sigma^{38}$	T3RNAP	T7RNAP
	P <sub>70</sub>	23.91	23.53	25.68	20.16	23.08	26.98	23.15	22.98
	P <sub>19</sub>	< 0.01	3.67	0.04	0.01	< 0.01	0.05	< 0.01	< 0.01
ည	P <sub>24</sub>	0.02	0.02	2.35	< 0.01	< 0.01	0.08	0.03	0.03
Promoters	P <sub>28</sub>	< 0.01	< 0.01	< 0.01	35.28	< 0.01	< 0.01	< 0.01	< 0.01
۱Ę	P <sub>32</sub>	0.53	0.55	0.76	0.43	24.30	0.81	0.73	0.78
P.	P <sub>38</sub>	< 0.01	0.27	0.08	0.02	< 0.01	5.38	0.04	< 0.01
	P <sub>T3</sub>	0.07	0.05	0.10	< 0.01	0.18	0.1	75.33	0.16
	P <sub>T7</sub>	0.04	0.06	0.05	< 0.01	< 0.01	0.08	0.83	93.87

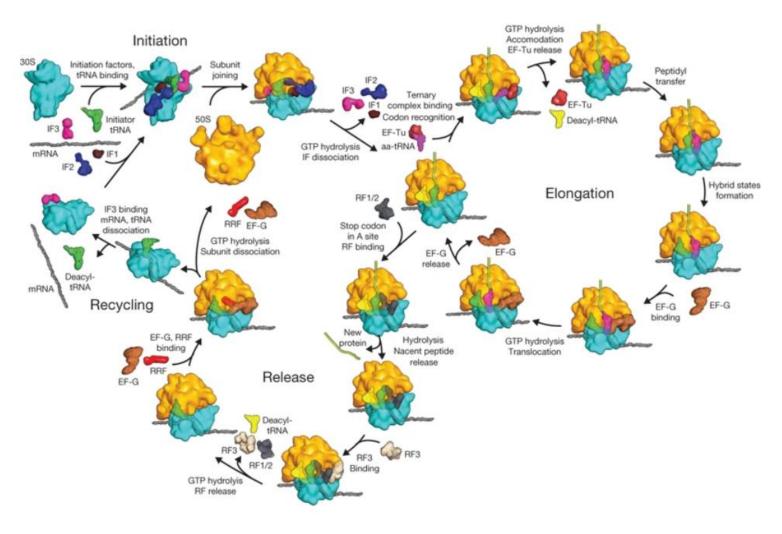
Passive transcription regulation by competition between sigma factors





## Translation and translation regulation

#### Overview of bacterial translation:



T. Martin Schmeing & V. Ramakrishnan Nature 2009

## Translation and translation regulation

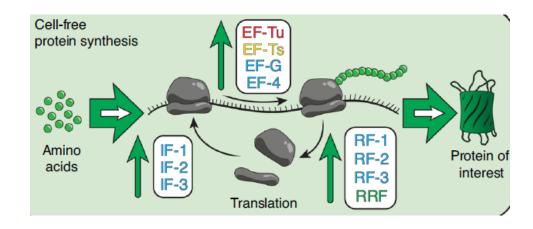
#### All ribosomal proteins + TL factorswere identified by LC/MS in cell-free lysate

#	Identified proteins	Access. No.	MW
Subunit S30			
REF	EF-Tu	WP_000031784.1	43 kDa
1	30S ribosomal protein S1	WP_000140327.1	61 kDa
2	30S ribosomal protein S2	WP_000246882.1	27 kDa
3	30S ribosomal protein S3	WP_000529945.1	26 kDa
4	30S ribosomal protein S5	WP_000940120.1	18 kDa
5	30S ribosomal protein S4	WP_000135224.1	23 kDa
6	30S ribosomal protein S7	WP_001138043.1	18 kDa
7	30S ribosomal protein S11	WP_001029684.1	14 kDa
8	30S ribosomal protein S12	WP_000246815.1	14 kDa
9	30S ribosomal protein S13	WP_000090775.1	13 kDa
10	30S ribosomal protein S19	WP_001138117.1	10 kDa
11	30S ribosomal protein S10	WP_001181004.1	12 kDa
12	30S ribosomal protein S6	WP_001216675.1	15 kDa
13	30S ribosomal protein S9	WP_000829818.1	15 kDa
14	30S ribosomal protein S21	WP_001144069.1	9 kDa
<b>1</b> 5	30S ribosomal protein S15	WP_000059466.1	10 kDa
16	30S ribosomal protein S16	WP_000256450.1	9 kDa
17	30S ribosomal protein S20	WP_001274021.1	10 kDa
18	30S ribosomal protein S8	WP_000062611.1	14 kDa
19	30S ribosomal protein S18	WP_000135199.1	9 kDa
20	30S ribosomal protein S14	WP_001118930.1	12 kDa
21	30S ribosomal protein S17	WP_000130100.1	10 kDa
22	30S ribosomal protein S12 RimO	WP_000049367.1	50 kDa
23	30S ribosomal protein S12 YcaO	WP_001295344.1	66 kDa
TL initiation			
1	Translation initiation factor IF-2	WP_000133044.1	97 kDa
2	Translation initiation factor IF-3	WP_001700733.1	21 kDa
3	Translation initiation factor IF-1	WP_001040187.1	8 kDa
TL elongation			
1	Elongation factor G	WP_000124700.1	78 kDa
2	Elongation factor Ts	WP_000818114.1	30 kDa
3	Elongation factor P	WP_000257278.1	21 kDa
4	Elongation factor 4	WP_000790168.1	67 kDa
5	Elongation factor P-like protein YeiP	WP_001136827.1	22 kDa
TL termination			
1	Peptide chain release factor 3 prfC	WP_000175940.1	60 kDa
2	Peptide chain release factor 1 prfA	WP_000804726.1	41 kDa

Subunit SSO         REF         EF-Tu         WP_000031784.1         43 kDa           1         50S ribosomal protein L2         WP_000301864.1         30 kDa           2         50S ribosomal protein L17         WP_001216368.1         14 kDa           3         50S ribosomal protein L9         WP_001196062.1         16 kDa           4         50S ribosomal protein L5         WP_00109600.1         20 kDa           5         50S ribosomal protein L6         WP_000091945.1         19 kDa           6         50S ribosomal protein L1         WP_000729185.1         11 kDa           8         50S ribosomal protein L24         WP_000729185.1         11 kDa           8         50S ribosomal protein L3         WP_000028878.1         12 kDa           9         50S ribosomal protein L28         WP_000028878.1         12 kDa           10         50S ribosomal protein L19         WP_000062523.1         13 kDa           12         50S ribosomal protein L19         WP_000062523.1         13 kDa           13         50S ribosomal protein L10         WP_000424183.1         11 kDa           14         50S ribosomal protein L10         WP_0004247529.1         12 kDa           15         50S ribosomal protein L13         WP_000447529.1         1	#	Identified proteins	Access. No.	MW
1 50S ribosomal protein L2 WP_000301864.1 30 kDa 2 50S ribosomal protein L17 WP_001216368.1 14 kDa 3 50S ribosomal protein L5 WP_001196062.1 16 kDa 4 50S ribosomal protein L5 WP_001096200.1 20 kDa 5 50S ribosomal protein L6 WP_00091945.1 19 kDa 6 50S ribosomal protein L1 WP_001096604.1 25 kDa 7 50S ribosomal protein L1 WP_001096684.1 25 kDa 7 50S ribosomal protein L3 WP_00057983.1 22 kDa 8 50S ribosomal protein L3 WP_00057983.1 12 kDa 10 50S ribosomal protein L7/L12 WP_00005983.1 12 kDa 10 50S ribosomal protein L28 WP_00005983.1 12 kDa 11 50S ribosomal protein L28 WP_000091955.1 9 kDa 11 50S ribosomal protein L25 WP_0000494183.1 11 kDa 13 50S ribosomal protein L10 WP_001207201.1 18 kDa 14 50S ribosomal protein L10 WP_001207201.1 18 kDa 14 50S ribosomal protein L10 WP_001207201.1 18 kDa 15 50S ribosomal protein L13 WP_000494183.1 11 kDa 16 50S ribosomal protein L23 WP_000494183.1 11 kDa 16 50S ribosomal protein L2 WP_000494183.1 11 kDa 16 50S ribosomal protein L2 WP_000494183.1 11 kDa 17 50S ribosomal protein L2 WP_000494193.1 12 kDa 18 50S ribosomal protein L4 WP_000424395.1 22 kDa 18 50S ribosomal protein L4 WP_000424395.1 22 kDa 18 50S ribosomal protein L4 WP_000423951.1 15 kDa 19 50S ribosomal protein L1 WP_000423951.1 15 kDa 19 50S ribosomal protein L1 WP_000433955.1 14 kDa 20 50S ribosomal protein L14 WP_000433955.1 14 kDa 20 50S ribosomal protein L18 WP_000358960.1 13 kDa 22 50S ribosomal protein L18 WP_000358960.1 13 kDa 24 50S ribosomal protein L3 WP_000464741.1 7 kDa 24 50S ribosomal protein L3 WP_000140433.1 7 kDa 25 50S ribosomal protein L20 WP_000464741.1 7 kDa 26 50S ribosomal protein L3 WP_000740797.1 6 kDa 27 50S ribosomal protein L3 WP_000740797.1 6 kDa 50S ribosomal protein L3 WP_00040295.1 15 kDa 50S ribosomal protein L3 W	Subunit S50			
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14         50S ribosomal protein L13         WP_000847559.1         16 kDa           15         50S ribosomal protein L23         WP_000617544.1         11 kDa           16         50S ribosomal protein L22         WP_000447529.1         12 kDa           17         50S ribosomal protein L4         WP_000424395.1         22 kDa           18         50S ribosomal protein L15         WP_001238917.1         15 kDa           19         50S ribosomal protein L14         WP_000613955.1         14 kDa           20         50S ribosomal protein L11         WP_001085926.1         15 kDa           21         50S ribosomal protein L18         WP_000358960.1         13 kDa           22         50S ribosomal protein L21         WP_000271401.1         12 kDa           23         50S ribosomal protein L29         WP_001140433.1         7 kDa           24         50S ribosomal protein L29         WP_00044741.1         7 kDa           25         50S ribosomal protein L20         WP_000124850.1         13 kDa           26         50S ribosomal protein L32         WP_000290727.1         6 kDa           27         50S ribosomal protein L3         WP_000940595.1         9 kDa           30         50S ribosomal protein L3         WP_000710769.1         8 kD	12	50S ribosomal protein L25	WP_000494183.1	11 kDa
15         50S ribosomal protein L23         WP_000617544.1         11 kDa           16         50S ribosomal protein L22         WP_000447529.1         12 kDa           17         50S ribosomal protein L4         WP_000424395.1         22 kDa           18         50S ribosomal protein L15         WP_001238917.1         15 kDa           19         50S ribosomal protein L14         WP_000613955.1         14 kDa           20         50S ribosomal protein L11         WP_001085926.1         15 kDa           21         50S ribosomal protein L18         WP_000258960.1         13 kDa           22         50S ribosomal protein L21         WP_000271401.1         12 kDa           23         50S ribosomal protein L20         WP_001140433.1         7 kDa           24         50S ribosomal protein L29         WP_00044741.1         7 kDa           25         50S ribosomal protein L20         WP_00044741.1         7 kDa           26         50S ribosomal protein L32         WP_000290727.1         6 kDa           27         50S ribosomal protein L3         WP_0009441212.1         15 kDa           28         50S ribosomal protein L3         WP_000940595.1         9 kDa           30         50S ribosomal protein L3         WP_000710769.1         8 kDa<	13	50S ribosomal protein L10	WP_001207201.1	18 kDa
16         50S ribosomal protein L22         WP_000447529.1         12 kDa           17         50S ribosomal protein L4         WP_000424395.1         22 kDa           18         50S ribosomal protein L15         WP_001238917.1         15 kDa           19         50S ribosomal protein L14         WP_000613955.1         14 kDa           20         50S ribosomal protein L11         WP_001085926.1         15 kDa           21         50S ribosomal protein L18         WP_000358960.1         13 kDa           22         50S ribosomal protein L21         WP_000271401.1         12 kDa           23         50S ribosomal protein L30         WP_001140433.1         7 kDa           24         50S ribosomal protein L29         WP_000444741.1         7 kDa           25         50S ribosomal protein L32         WP_000290727.1         6 kDa           26         50S ribosomal protein L32         WP_000290727.1         6 kDa           27         50S ribosomal protein L3         WP_000941212.1         15 kDa           28         50S ribosomal protein L3         WP_000940595.1         9 kDa           30         50S ribosomal protein L31         WP_000710769.1         8 kDa           31         50S ribosomal protein L3 N(5)-GM         WP_0001295704.1         <	14	50S ribosomal protein L13	WP_000847559.1	16 kDa
17       50S ribosomal protein L4       WP_000424395.1       22 kDa         18       50S ribosomal protein L15       WP_001238917.1       15 kDa         19       50S ribosomal protein L14       WP_000613955.1       14 kDa         20       50S ribosomal protein L11       WP_001085926.1       15 kDa         21       50S ribosomal protein L18       WP_000358960.1       13 kDa         22       50S ribosomal protein L21       WP_000271401.1       12 kDa         23       50S ribosomal protein L30       WP_001140433.1       7 kDa         24       50S ribosomal protein L29       WP_00044741.1       7 kDa         25       50S ribosomal protein L32       WP_00024850.1       13 kDa         26       50S ribosomal protein L32       WP_000290727.1       6 kDa         27       50S ribosomal protein L3       WP_000941212.1       15 kDa         28       50S ribosomal protein L3       WP_000940595.1       9 kDa         30       50S ribosomal protein L31       WP_000710769.1       8 kDa         31       50S ribosomal protein L3 N(5)-GM       WP_001295704.1       35 kDa         Other TL proteins         1       Ribosome-associated inhibitor A (YfiA)       WP_000178456.1       13 kDa	15	50S ribosomal protein L23	WP_000617544.1	11 kDa
18         50S ribosomal protein L15         WP_001238917.1         15 kDa           19         50S ribosomal protein L14         WP_000613955.1         14 kDa           20         50S ribosomal protein L11         WP_001085926.1         15 kDa           21         50S ribosomal protein L18         WP_000358960.1         13 kDa           22         50S ribosomal protein L21         WP_000271401.1         12 kDa           23         50S ribosomal protein L30         WP_001140433.1         7 kDa           24         50S ribosomal protein L29         WP_000644741.1         7 kDa           25         50S ribosomal protein L20         WP_000124850.1         13 kDa           26         50S ribosomal protein L32         WP_000290727.1         6 kDa           27         50S ribosomal protein L33         WP_001051798.1         6 kDa           29         50S ribosomal protein L27         WP_000940595.1         9 kDa           30         50S ribosomal protein L31         WP_000710769.1         8 kDa           31         50S ribosomal protein L3 N(5)-GM         WP_001295704.1         35 kDa           Other TL proteins           1         Ribosome-recycling factor (RFF, RF4)         WP_000622418.1         21 kDa           2	16	50S ribosomal protein L22	WP_000447529.1	12 kDa
19         50S ribosomal protein L14         WP_000613955.1         14 kDa           20         50S ribosomal protein L11         WP_001085926.1         15 kDa           21         50S ribosomal protein L18         WP_000358960.1         13 kDa           22         50S ribosomal protein L21         WP_000271401.1         12 kDa           23         50S ribosomal protein L30         WP_001140433.1         7 kDa           24         50S ribosomal protein L29         WP_000644741.1         7 kDa           25         50S ribosomal protein L32         WP_000124850.1         13 kDa           26         50S ribosomal protein L32         WP_000290727.1         6 kDa           27         50S ribosomal protein L16         WP_000941212.1         15 kDa           28         50S ribosomal protein L27         WP_000940595.1         9 kDa           30         50S ribosomal protein L31         WP_000710769.1         8 kDa           31         50S ribosomal protein L3 N(5)-GM         WP_001295704.1         35 kDa           Other TL proteins           1         Ribosome-recycling factor (RFF, RF4)         WP_000622418.1         21 kDa           2         Ribosome-associated inhibitor A (YfiA)         WP_000178456.1         13 kDa           3	17	50S ribosomal protein L4	WP_000424395.1	22 kDa
20	18	50S ribosomal protein L15	WP_001238917.1	15 kDa
21       50S ribosomal protein L18       WP_000358960.1       13 kDa         22       50S ribosomal protein L21       WP_000271401.1       12 kDa         23       50S ribosomal protein L30       WP_001140433.1       7 kDa         24       50S ribosomal protein L29       WP_000644741.1       7 kDa         25       50S ribosomal protein L20       WP_000124850.1       13 kDa         26       50S ribosomal protein L32       WP_000290727.1       6 kDa         27       50S ribosomal protein L33       WP_000941212.1       15 kDa         28       50S ribosomal protein L33       WP_00151798.1       6 kDa         29       50S ribosomal protein L27       WP_000940595.1       9 kDa         30       50S ribosomal protein L31       WP_000710769.1       35 kDa         Other TL proteins         1       Ribosome-recycling factor (RFF, RF4)       WP_000622418.1       21 kDa         2       Ribosome-associated inhibitor A (YfiA)       WP_000178456.1       13 kDa         3       Ribosome-binding factor A       WP_001040205.1       15 kDa         4       Ribosome hibernation promoting factor       WP_001300397.1       17 kDa         5       Ribosome silencing factor RsfS       WP_00116664.1       12 kDa	19	50S ribosomal protein L14	WP_000613955.1	14 kDa
22         50S ribosomal protein L21         WP_000271401.1         12 kDa           23         50S ribosomal protein L30         WP_001140433.1         7 kDa           24         50S ribosomal protein L29         WP_000644741.1         7 kDa           25         50S ribosomal protein L20         WP_000124850.1         13 kDa           26         50S ribosomal protein L32         WP_000290727.1         6 kDa           27         50S ribosomal protein L16         WP_000941212.1         15 kDa           28         50S ribosomal protein L33         WP_001051798.1         6 kDa           29         50S ribosomal protein L27         WP_000940595.1         9 kDa           30         50S ribosomal protein L31         WP_000710769.1         35 kDa           Other TL proteins           1         Ribosome-recycling factor (RFF, RF4)         WP_000622418.1         21 kDa           2         Ribosome-associated inhibitor A (YfiA)         WP_000178456.1         13 kDa           3         Ribosome-binding factor A         WP_001040205.1         15 kDa           4         Ribosome hibernation promoting factor         WP_001300397.1         17 kDa           5         Ribosome silencing factor RsfS         WP_00116664.1         12 kDa	20	50S ribosomal protein L11	WP_001085926.1	15 kDa
23       50S ribosomal protein L30       WP_001140433.1       7 kDa         24       50S ribosomal protein L29       WP_000644741.1       7 kDa         25       50S ribosomal protein L20       WP_000124850.1       13 kDa         26       50S ribosomal protein L32       WP_000290727.1       6 kDa         27       50S ribosomal protein L16       WP_000941212.1       15 kDa         28       50S ribosomal protein L33       WP_001051798.1       6 kDa         29       50S ribosomal protein L27       WP_000940595.1       9 kDa         30       50S ribosomal protein L31       WP_000710769.1       8 kDa         31       50S ribosomal protein L3 N(5)-GM       WP_001295704.1       35 kDa         Other TL proteins         1       Ribosome-recycling factor (RFF, RF4)       WP_000622418.1       21 kDa         2       Ribosome-associated inhibitor A (YfiA)       WP_000178456.1       13 kDa         3       Ribosome-binding factor A       WP_001040205.1       15 kDa         4       Ribosome hibernation promoting factor       WP_001300397.1       17 kDa         5       Ribosome silencing factor RsfS       WP_001161664.1       12 kDa         7       Ribosome assembly protein YhbY       WP_001054420.1       1	21	50S ribosomal protein L18	WP_000358960.1	13 kDa
24         50S ribosomal protein L29         WP_000644741.1         7 kDa           25         50S ribosomal protein L20         WP_000124850.1         13 kDa           26         50S ribosomal protein L32         WP_000290727.1         6 kDa           27         50S ribosomal protein L16         WP_000941212.1         15 kDa           28         50S ribosomal protein L33         WP_001051798.1         6 kDa           29         50S ribosomal protein L27         WP_000940595.1         9 kDa           30         50S ribosomal protein L31         WP_000710769.1         8 kDa           31         50S ribosomal protein L3 N(5)-GM         WP_001295704.1         35 kDa           Other TL proteins           1         Ribosome-recycling factor (RFF, RF4)         WP_000622418.1         21 kDa           2         Ribosome-associated inhibitor A (YfiA)         WP_000178456.1         13 kDa           3         Ribosome-binding factor A         WP_00140205.1         15 kDa           4         Ribosome hibernation promoting factor         WP_001300397.1         17 kDa           5         Ribosome silencing factor RsfS         WP_001161664.1         12 kDa           7         Ribosome assembly protein YhbY         WP_001054420.1         11 kDa <td>22</td> <td>50S ribosomal protein L21</td> <td>WP_000271401.1</td> <td>12 kDa</td>	22	50S ribosomal protein L21	WP_000271401.1	12 kDa
25       50S ribosomal protein L20       WP_000124850.1       13 kDa         26       50S ribosomal protein L32       WP_000290727.1       6 kDa         27       50S ribosomal protein L16       WP_000941212.1       15 kDa         28       50S ribosomal protein L33       WP_001051798.1       6 kDa         29       50S ribosomal protein L27       WP_000940595.1       9 kDa         30       50S ribosomal protein L31       WP_000710769.1       8 kDa         31       50S ribosomal protein L3 N(5)-GM       WP_001295704.1       35 kDa         Other TL proteins         1       Ribosome-recycling factor (RFF, RF4)       WP_000622418.1       21 kDa         2       Ribosome-associated inhibitor A (YfiA)       WP_000178456.1       13 kDa         3       Ribosome-binding factor A       WP_001040205.1       15 kDa         4       Ribosome hibernation promoting factor       WP_001176599.1       11 kDa         5       Ribosome maturation factor       WP_001300397.1       17 kDa         6       Ribosome silencing factor RsfS       WP_001161664.1       12 kDa         7       Ribosome assembly protein YhbY       WP_001054420.1       11 kDa	23	50S ribosomal protein L30	WP_001140433.1	7 kDa
26         50S ribosomal protein L32         WP_000290727.1         6 kDa           27         50S ribosomal protein L16         WP_000941212.1         15 kDa           28         50S ribosomal protein L33         WP_001051798.1         6 kDa           29         50S ribosomal protein L27         WP_000940595.1         9 kDa           30         50S ribosomal protein L31         WP_000710769.1         8 kDa           31         50S ribosomal protein L3 N(5)-GM         WP_001295704.1         35 kDa           Other TL proteins           1         Ribosome-recycling factor (RFF, RF4)         WP_000622418.1         21 kDa           2         Ribosome-associated inhibitor A (YfiA)         WP_000178456.1         13 kDa           3         Ribosome-binding factor A         WP_00140205.1         15 kDa           4         Ribosome hibernation promoting factor         WP_001176599.1         11 kDa           5         Ribosome maturation factor         WP_001300397.1         17 kDa           6         Ribosome silencing factor RsfS         WP_001161664.1         12 kDa           7         Ribosome assembly protein YhbY         WP_001054420.1         11 kDa	24	50S ribosomal protein L29	WP_000644741.1	7 kDa
27         50S ribosomal protein L16         WP_000941212.1         15 kDa           28         50S ribosomal protein L33         WP_001051798.1         6 kDa           29         50S ribosomal protein L27         WP_000940595.1         9 kDa           30         50S ribosomal protein L31         WP_000710769.1         8 kDa           31         50S ribosomal protein L3 N(5)-GM         WP_001295704.1         35 kDa           Other TL proteins           1         Ribosome-recycling factor (RFF, RF4)         WP_000622418.1         21 kDa           2         Ribosome-associated inhibitor A (YfiA)         WP_000178456.1         13 kDa           3         Ribosome-binding factor A         WP_001040205.1         15 kDa           4         Ribosome hibernation promoting factor         WP_001176599.1         11 kDa           5         Ribosome maturation factor         WP_001300397.1         17 kDa           6         Ribosome silencing factor RsfS         WP_001161664.1         12 kDa           7         Ribosome assembly protein YhbY         WP_001054420.1         11 kDa	25	50S ribosomal protein L20	WP_000124850.1	13 kDa
28         50S ribosomal protein L33         WP_001051798.1         6 kDa           29         50S ribosomal protein L27         WP_000940595.1         9 kDa           30         50S ribosomal protein L31         WP_000710769.1         8 kDa           31         50S ribosomal protein L3 N(5)-GM         WP_001295704.1         35 kDa           Other TL proteins           1         Ribosome-recycling factor (RFF, RF4)         WP_000622418.1         21 kDa           2         Ribosome-associated inhibitor A (YfiA)         WP_000178456.1         13 kDa           3         Ribosome-binding factor A         WP_001040205.1         15 kDa           4         Ribosome hibernation promoting factor         WP_001176599.1         11 kDa           5         Ribosome maturation factor         WP_001300397.1         17 kDa           6         Ribosome silencing factor RsfS         WP_001161664.1         12 kDa           7         Ribosome assembly protein YhbY         WP_001054420.1         11 kDa	26	50S ribosomal protein L32	WP_000290727.1	6 kDa
29 50S ribosomal protein L27 WP_000940595.1 9 kDa 30 50S ribosomal protein L31 WP_000710769.1 8 kDa 31 50S ribosomal protein L3 N(5)-GM WP_001295704.1 35 kDa  Other TL proteins  1 Ribosome-recycling factor (RFF, RF4) WP_000622418.1 21 kDa 2 Ribosome-associated inhibitor A (YfiA) WP_000178456.1 13 kDa 3 Ribosome-binding factor A WP_001040205.1 15 kDa 4 Ribosome hibernation promoting factor WP_001176599.1 11 kDa 5 Ribosome maturation factor WP_001300397.1 17 kDa 6 Ribosome silencing factor RsfS WP_001161664.1 12 kDa 7 Ribosome assembly protein YhbY WP_001054420.1 11 kDa	27	50S ribosomal protein L16	WP_000941212.1	15 kDa
30 50S ribosomal protein L31 WP_000710769.1 8 kDa 31 50S ribosomal protein L3 N(5)-GM WP_001295704.1 35 kDa  Other TL proteins  1 Ribosome-recycling factor (RFF, RF4) WP_000622418.1 21 kDa 2 Ribosome-associated inhibitor A (YfiA) WP_000178456.1 13 kDa 3 Ribosome-binding factor A WP_001040205.1 15 kDa 4 Ribosome hibernation promoting factor WP_001176599.1 11 kDa 5 Ribosome maturation factor WP_001300397.1 17 kDa 6 Ribosome silencing factor RsfS WP_001161664.1 12 kDa 7 Ribosome assembly protein YhbY WP_001054420.1 11 kDa	28	50S ribosomal protein L33	WP_001051798.1	6 kDa
31         50S ribosomal protein L3 N(5)-GM         WP_001295704.1         35 kDa           Other TL proteins           1         Ribosome-recycling factor (RFF, RF4)         WP_000622418.1         21 kDa           2         Ribosome-associated inhibitor A (YfiA)         WP_000178456.1         13 kDa           3         Ribosome-binding factor A         WP_001040205.1         15 kDa           4         Ribosome hibernation promoting factor         WP_001176599.1         11 kDa           5         Ribosome maturation factor         WP_001300397.1         17 kDa           6         Ribosome silencing factor RsfS         WP_001161664.1         12 kDa           7         Ribosome assembly protein YhbY         WP_001054420.1         11 kDa	29	50S ribosomal protein L27	WP_000940595.1	9 kDa
Other TL proteins           1         Ribosome-recycling factor (RFF, RF4)         WP_000622418.1         21 kDa           2         Ribosome-associated inhibitor A (YfiA)         WP_000178456.1         13 kDa           3         Ribosome-binding factor A         WP_001040205.1         15 kDa           4         Ribosome hibernation promoting factor         WP_001176599.1         11 kDa           5         Ribosome maturation factor         WP_001300397.1         17 kDa           6         Ribosome silencing factor RsfS         WP_001161664.1         12 kDa           7         Ribosome assembly protein YhbY         WP_001054420.1         11 kDa	30	50S ribosomal protein L31	WP_000710769.1	8 kDa
1         Ribosome-recycling factor (RFF, RF4)         WP_000622418.1         21 kDa           2         Ribosome-associated inhibitor A (YfiA)         WP_000178456.1         13 kDa           3         Ribosome-binding factor A         WP_001040205.1         15 kDa           4         Ribosome hibernation promoting factor         WP_001176599.1         11 kDa           5         Ribosome maturation factor         WP_001300397.1         17 kDa           6         Ribosome silencing factor RsfS         WP_001161664.1         12 kDa           7         Ribosome assembly protein YhbY         WP_001054420.1         11 kDa	31	50S ribosomal protein L3 N(5)-GM	WP_001295704.1	35 kDa
2       Ribosome-associated inhibitor A (YfiA)       WP_000178456.1       13 kDa         3       Ribosome-binding factor A       WP_001040205.1       15 kDa         4       Ribosome hibernation promoting factor       WP_001176599.1       11 kDa         5       Ribosome maturation factor       WP_001300397.1       17 kDa         6       Ribosome silencing factor RsfS       WP_001161664.1       12 kDa         7       Ribosome assembly protein YhbY       WP_001054420.1       11 kDa	Other TL pro	teins		
3         Ribosome-binding factor A         WP_001040205.1         15 kDa           4         Ribosome hibernation promoting factor         WP_001176599.1         11 kDa           5         Ribosome maturation factor         WP_001300397.1         17 kDa           6         Ribosome silencing factor RsfS         WP_001161664.1         12 kDa           7         Ribosome assembly protein YhbY         WP_001054420.1         11 kDa	1	Ribosome-recycling factor (RFF, RF4)	WP_000622418.1	21 kDa
4 Ribosome hibernation promoting factor WP_001176599.1 11 kDa 5 Ribosome maturation factor WP_001300397.1 17 kDa 6 Ribosome silencing factor RsfS WP_001161664.1 12 kDa 7 Ribosome assembly protein YhbY WP_001054420.1 11 kDa	2	Ribosome-associated inhibitor A (YfiA)	WP_000178456.1	13 kDa
5         Ribosome maturation factor         WP_001300397.1         17 kDa           6         Ribosome silencing factor RsfS         WP_001161664.1         12 kDa           7         Ribosome assembly protein YhbY         WP_001054420.1         11 kDa	3	Ribosome-binding factor A	-	15 kDa
6 Ribosome silencing factor RsfS WP_001161664.1 12 kDa 7 Ribosome assembly protein YhbY WP_001054420.1 11 kDa	4	Ribosome hibernation promoting factor	WP_001176599.1	11 kDa
7 Ribosome assembly protein YhbY WP_001054420.1 11 kDa	5	Ribosome maturation factor	WP_001300397.1	17 kDa
	6	Ribosome silencing factor RsfS	WP_001161664.1	12 kDa
8 Ribosome maturation factor RimM WP_000043335.1 21 kDa	7	Ribosome assembly protein YhbY	WP_001054420.1	11 kDa
	8	Ribosome maturation factor RimM	WP_000043335.1	21 kDa

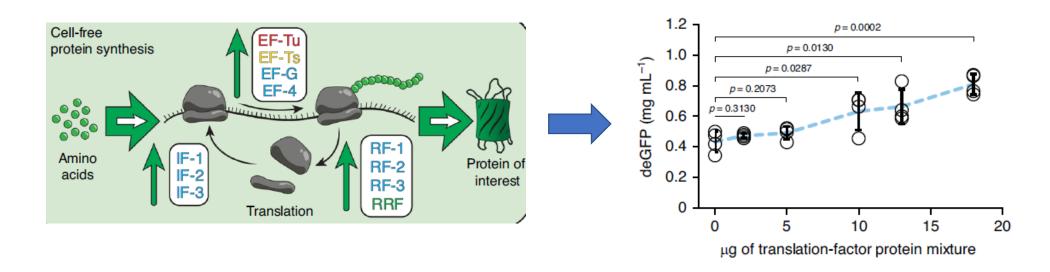
Garenne D et al. Rapid Commun Mass Spectrom. 2019

## Translation and translation regulation



Add purified proteins of the translation machinery

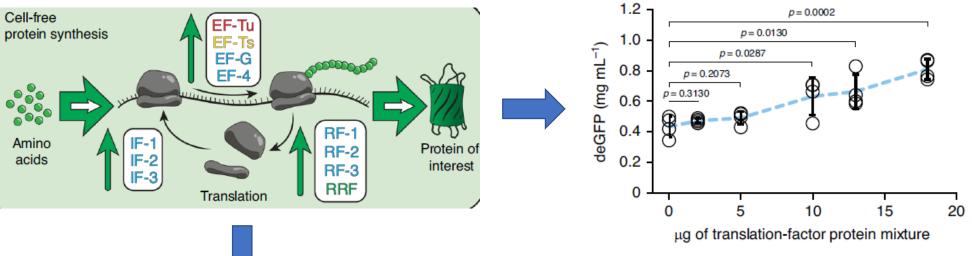
#### Translation and translation regulation

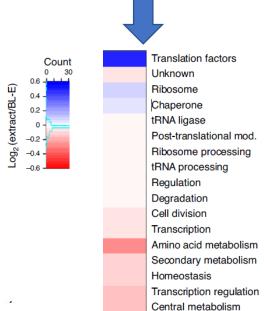


Increase protein production with purified translation machinery

#### Translation and translation regulation



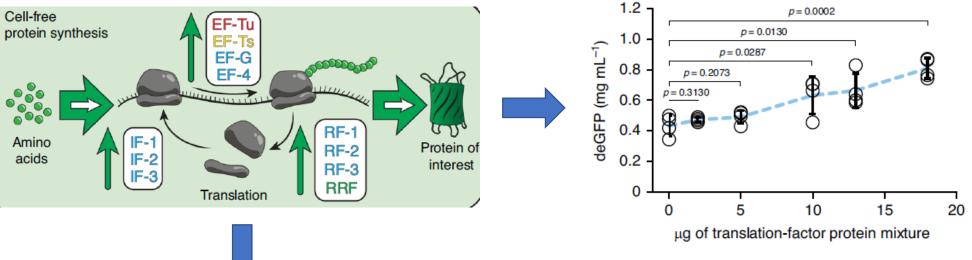


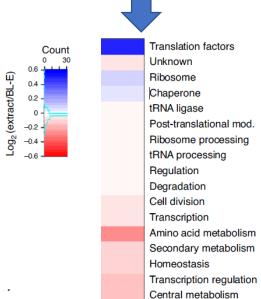


Proteome reprogramming: Mimic some bacterial law in living cell

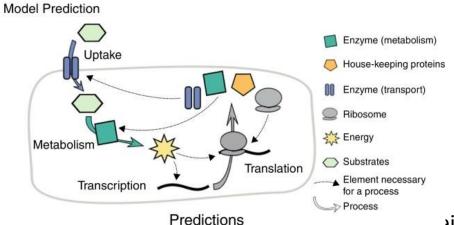
#### Translation and translation regulation







#### Proteome reprogramming: Mimic some bacterial law in living cell



vvoiße *et al. PNAS (2015)* 







2. Understand living organisms: functions preserved in cell-free systems

Metabolism and energy production

#### Metabolism: ATP regeneration system

Overview of bacterial central metabolism: glucose NADPH **←** fructose-6P glycerol CO<sub>2</sub> glycerol-3P fructose-1,6BP erythrose-4P sedoheptulose-7P ribose-5P NADPH\* glycerate-3P 2-keto-3-deoxy-gluconate-P glycerate-2P lactate phosphoenolpyruvate malate FHD MAD+ succinate > ATP, CoA CO<sub>2</sub> oxaloacetate succinyl-CoA glyoxylate formate CoA 2-ketoglutarate NADP+  $CO_2$ non-oxidative pentose phosphate pathway TCA cycle and glyoxylate shunt

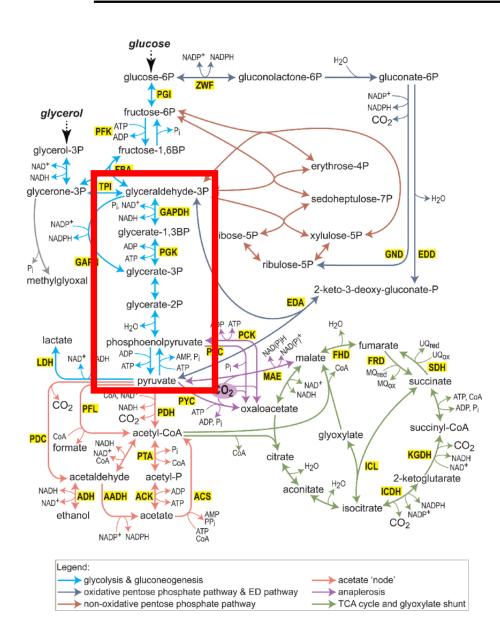
Aslan S et al. Biochemical journal 2017

**500–800 proteins** were identified by LC/MS in cell-free lysate

Garenne D et al. Rapid Commun Mass Spectrom. 2019

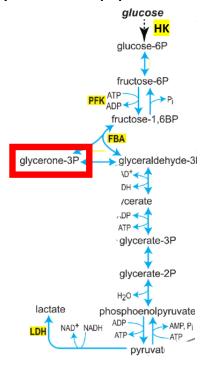
#### Metabolism: ATP regeneration system

ATP regeneration: 3-PGA



#### Metabolism: ATP regeneration system

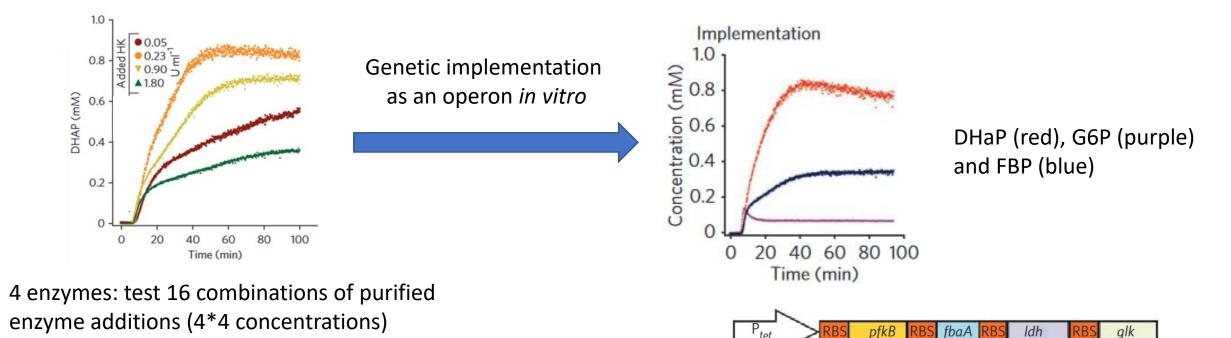
Pathway optimization for glycerone-3p production



#### Metabolism: Optimization of in vitro glycolysis

Pathway optimization for glycerone-3p production

In cell-free the system properties can be monitored directly for real-time analysis



<mark>LDH</mark>

HK

**PFK** 



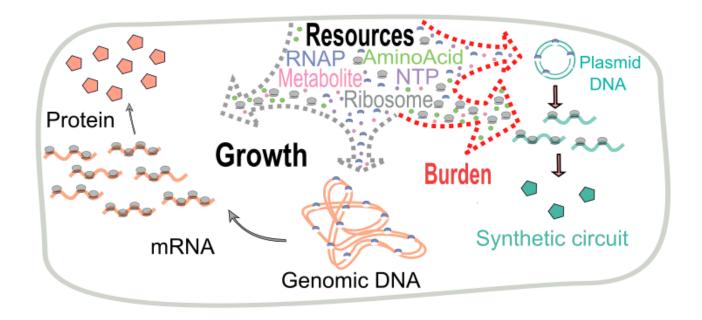




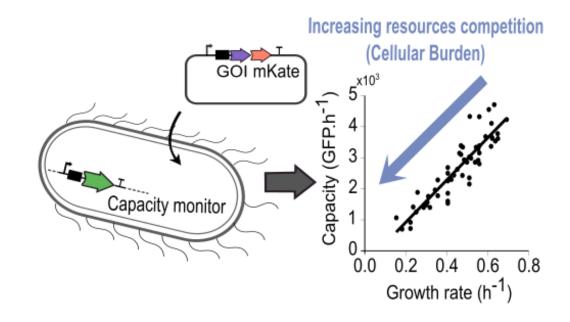
2. Understand living organisms: functions preserved in cell-free systems

Burden and resources competition

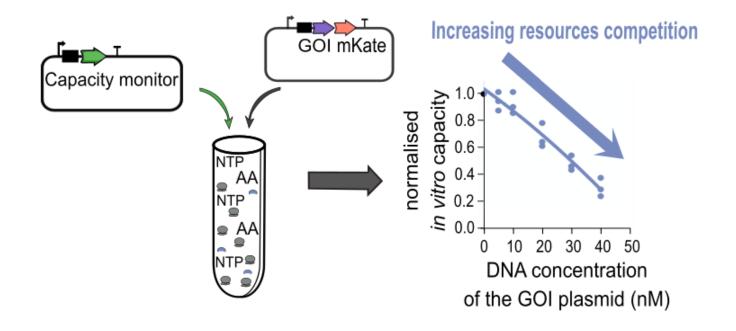
#### **Exploring Resource Competition**



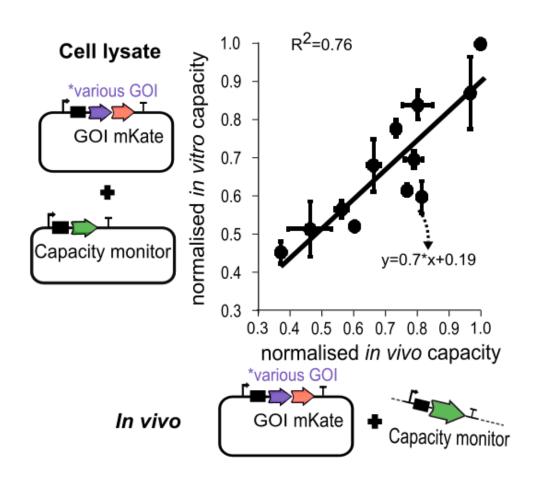
#### **Exploring Resource Competition**

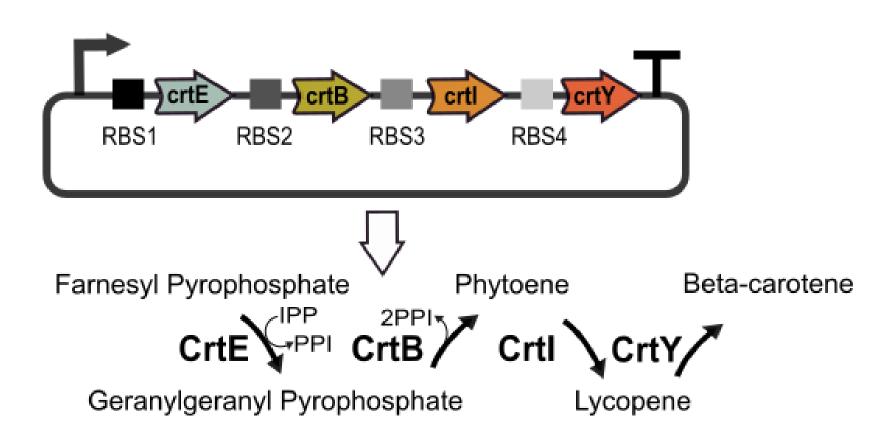


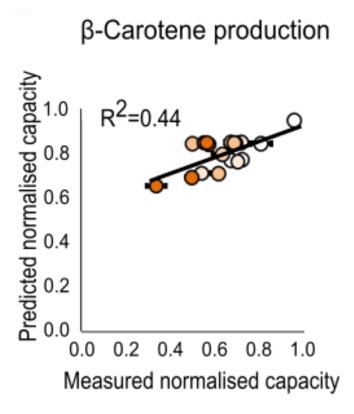
#### **Exploring Resource Competition in cell-free**

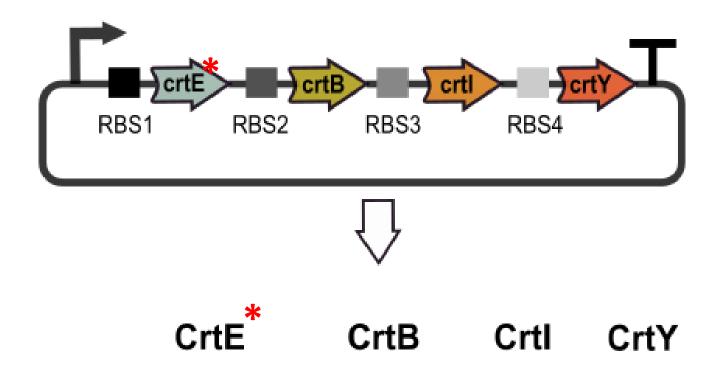


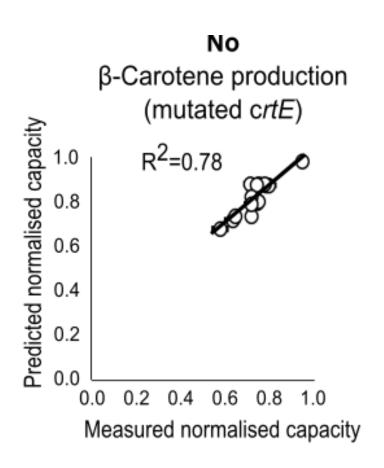
### **Exploring Resource Competition in cell-free**



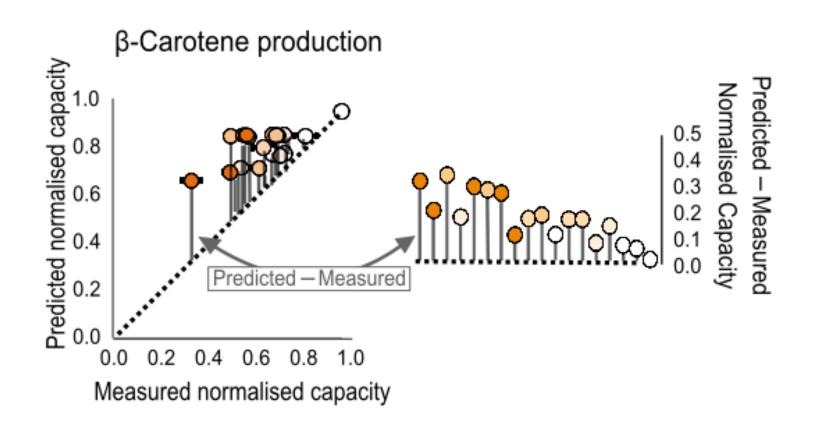








Decouple production and metabolic burden



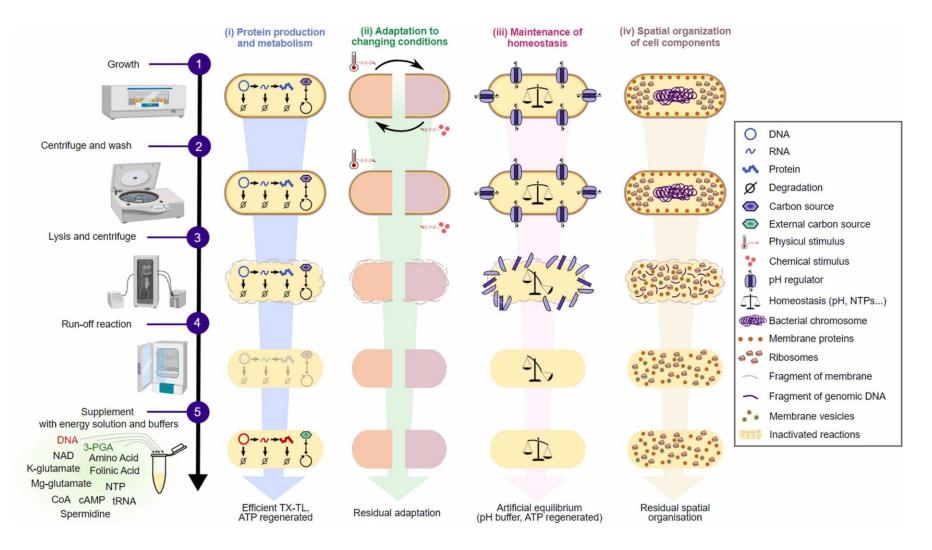






3. Building artificial cells: **reconstruct functions** defective in cell-free systems

#### What remains from living cells in bacterial lysate-based cell-free systems



Wagner, L, et al. Computational and Structural Biotechnology Journal, 2023







# 3. Building artificial cells: **reconstruct functions** defective in cell-free systems

Spatial organization

Homeostasis & Adaptation to changing conditions

**Cell Division** 

#### Liposome (phospholipid bilayers ) encapsulation

Cell-free reaction + mineral oil with dissolved phospholipids



extract-oil emulsion

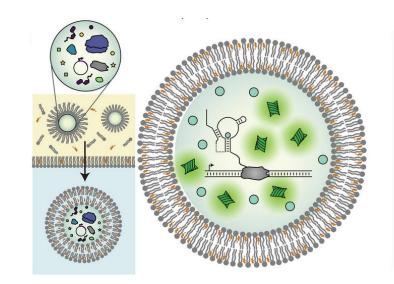


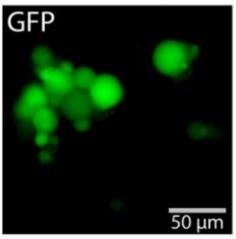
Wait a few minutes

Microdroplets stabilized by a monolayer of phospholipid

Transfer in feeding solution (Amino acid + ribonucleotides) + centrifugation

Vesicule







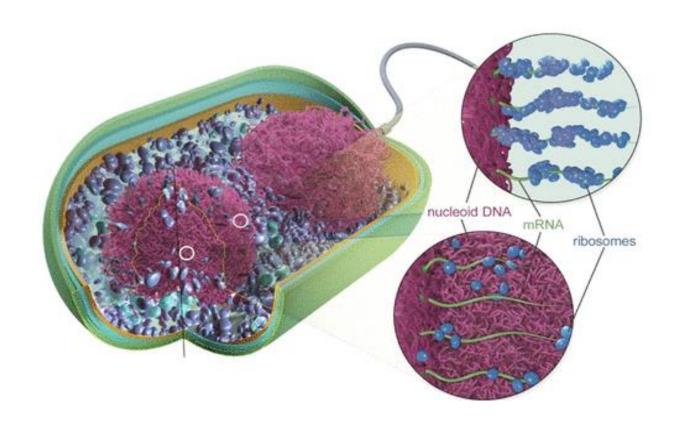




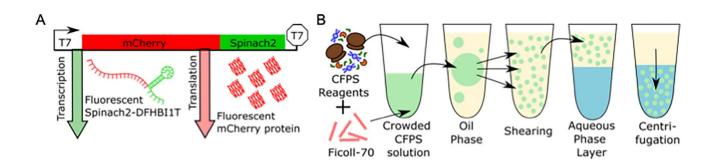
# 3. Building artificial cells: **reconstruct functions** defective in cell-free systems

Spatial organization

### **Spatial organization**

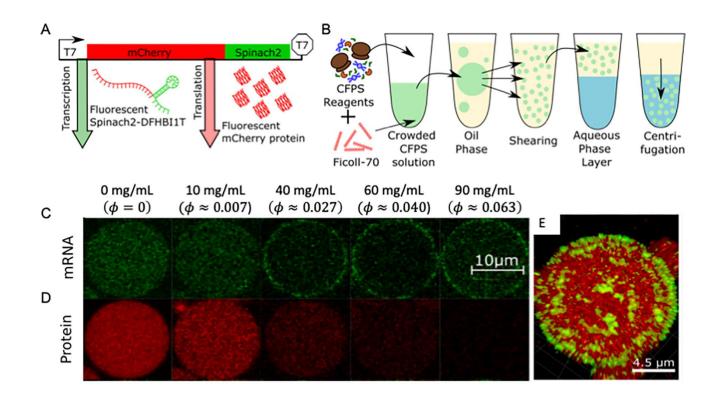


### **Spatial organization**

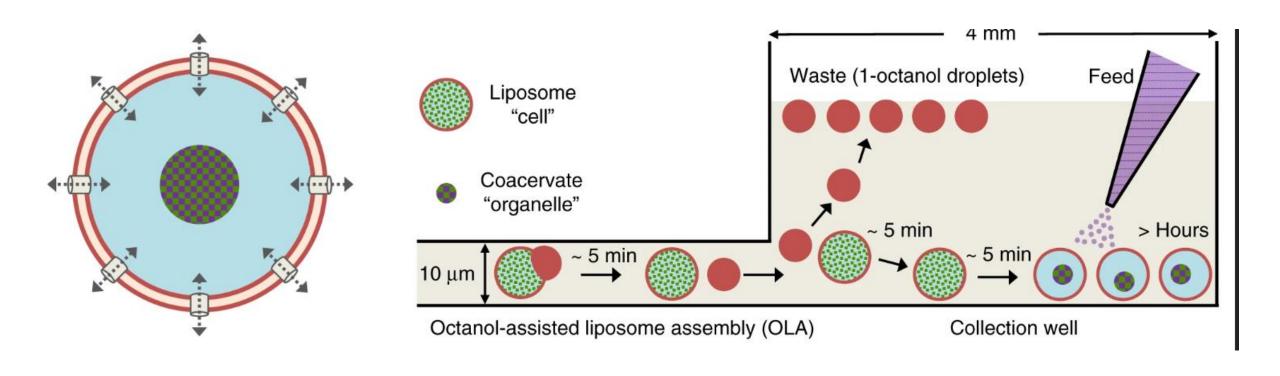


Ficoll-70

#### **Spatial organization**

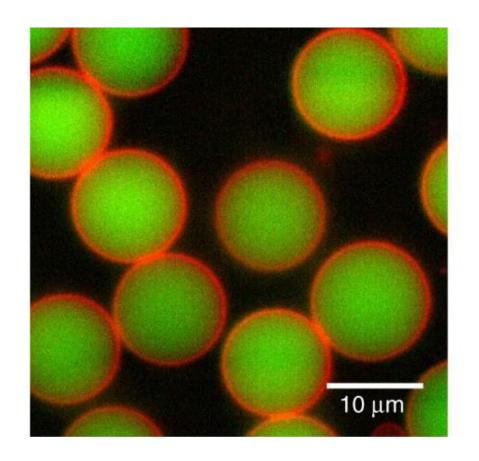


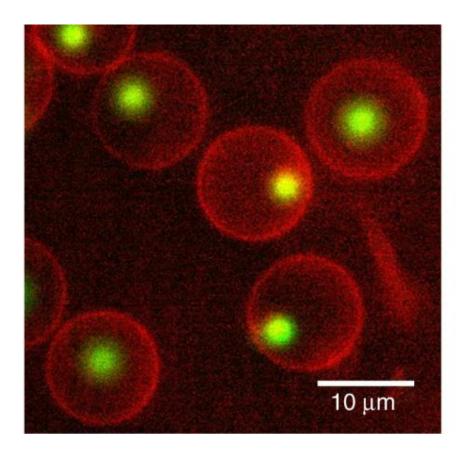
#### **Spatial organization: compartement**



Deshpande, S et al. Nature communications (2019)

### **Spatial organization: compartement**





Deshpande, S et al. Nature communications (2019)







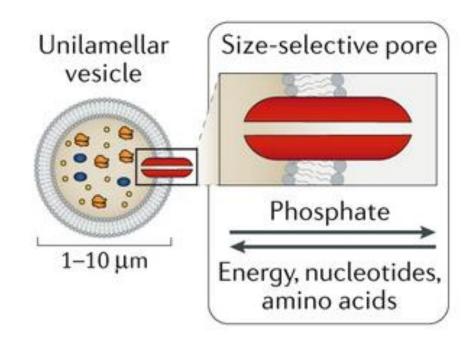
# 3. Building artificial cells: **reconstruct functions** defective in cell-free systems

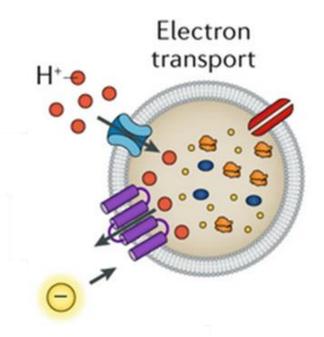
Homeostasis & Adaptation to changing conditions

#### Homeostasie & Adaptation to changing conditions

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) is a commonly used buffer in CFS with a pKa of 7.5 and useful pH range of 6.8–8.2

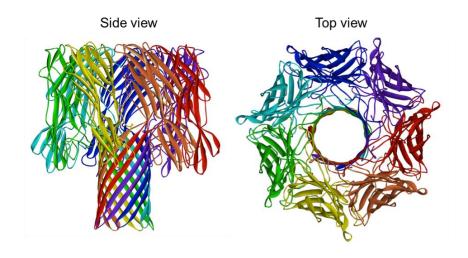
Exchange with the environment





#### Homeostasie & Adaptation to changing conditions

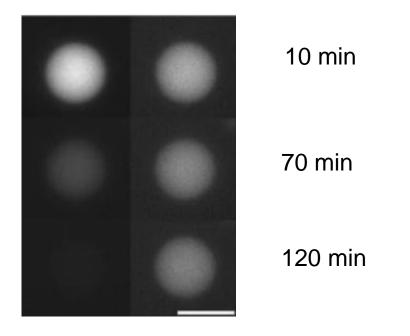
#### haemolysin protein pores



The extract with the plasmid (Pasmid pIVEX2.3d a hemolysin ) and the fluorescent probes (BSA-rhodamine and fluorescein-UTP) are encapsulated in the vesicles

Ribonucleotide UTP labeled with fluorescein

BSA protein labeled with rhodamine



Ribonucleotides and amino acids can diffuse in and out while the proteins are kept inside the vesicle





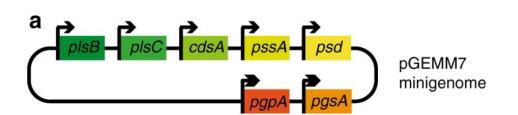


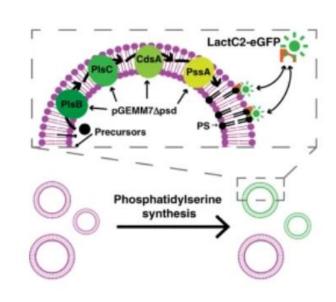
# 3. Building artificial cells: **reconstruct functions** defective in cell-free systems

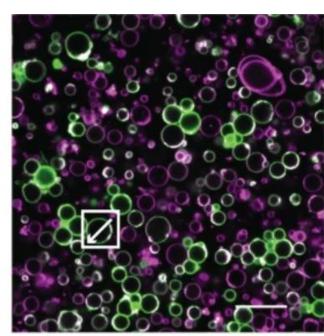
**Cell Division** 

#### **Cell Division**

#### De novo synthesis phospholipids

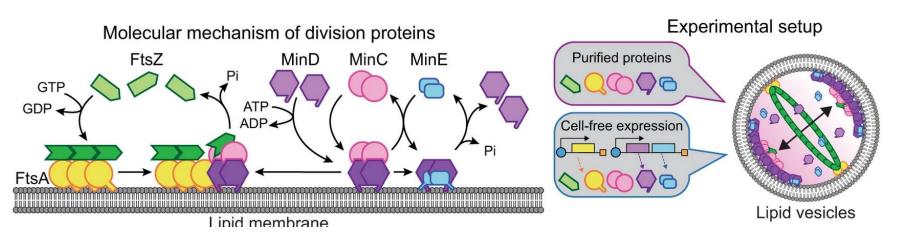


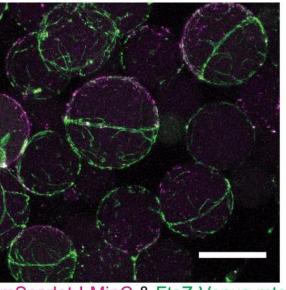




#### **Cell Division**

#### **Reconstitution of proto-ring**



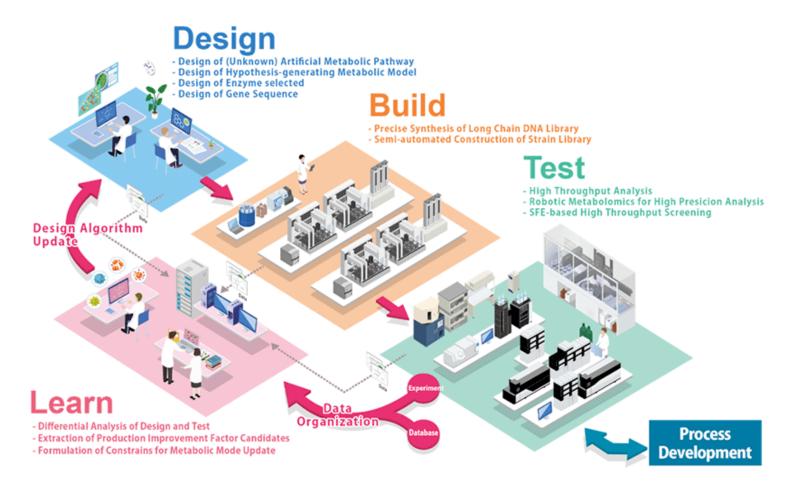


mScarlet-I-MinC & FtsZ-Venus-mts

#### Cell-free Biofoundry at INRAE jouy-en-josas

#### Part of the **Bioconvergence Paris Biofoundry**:

INRAE/Paris-Saclay Université + Sorbonne Université + Institut Curie + Genopole Evry











## universite PARIS-SACLAY

## Acknowledgements



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London

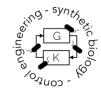


Matthieu Jules Léa Wagner Olivier Delumeau



Jean-Loup Faulon

Joan Hersisson An Hoang



TOM ELLIS LAB

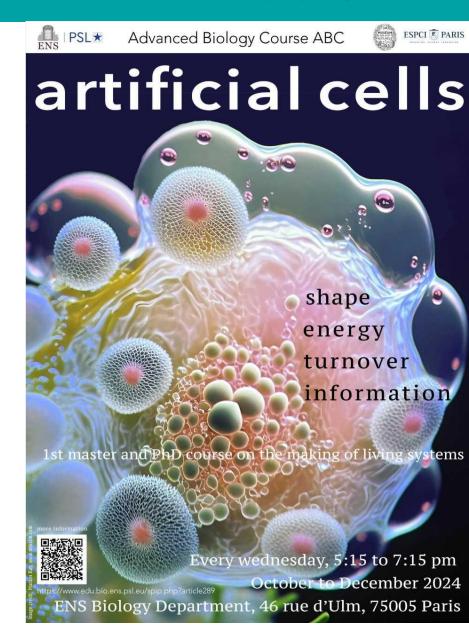
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**Fabrice Agou** Agnès Zettor



### Communication between Cell-free reaction

https://doi.org/10.1101/2024.01.10.575012 (Ricouvier et al 2024, biorxiv)

