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1 **Adaptive response of yeast cells to triggered toxicity of phosphoribulokinase**

2 Catherine Rouzeau^a, Adilya Dagkesamanskaya^a, Krzysztof Langer^b, Jérôme Bibette^b, Jean Baudry^b,
3 Denis Pompon^a and Véronique Anton-Leberre^{a*}

4
5 ^aLISBP, Université de Toulouse, CNRS, INRA, INSA, Toulouse, France

6 ^bLaboratoire Colloïdes et Matériaux Divisés, from the Institute of Chemistry, Biology and Innovation (CBI) - ESPCI ParisTech,
7 CNRS – UMR 8231, PSL* Research University, 10 rue Vauquelin, 75005 Paris, France

8
9 Correspondance to: V. Anton-Leberre, LISBP - INSA Toulouse - 135 avenue de Rangueil - 31077 Toulouse CEDEX 04,
10 France - Email: veronique.leberre@insa-toulouse.fr.

11

12 **Abstract**

13 Adjustment of plasmid copy number resulting from the balance between positive and negative impacts
14 of borne synthetic genes, plays a critical role in the global efficiency of multistep metabolic engineering.
15 Differential expression of co-expressed engineered genes is frequently observed depending on growth
16 phases, metabolic status and triggered adjustments of plasmid copy numbers, constituting a dynamic
17 process contributing to minimize global engineering burden. A yeast model involving plasmid based
18 expression of phosphoribulokinase (PRKp), a key enzyme for the reconstruction of synthetic Calvin
19 cycle, was designed to gain further insights into such a mechanism. A conditional PRK expression
20 cassette was cloned either onto a low (ARS-CEN based) or a high (2-micron origin based) copy
21 number plasmid using complementation of a *trp1* genomic mutation as constant positive selection.
22 Evolution of plasmid copy numbers, PRKp expressions, and cell growth rates were dynamically
23 monitored following gene de-repression through external doxycycline concentration shifts. In the
24 absence of RubisCO encoding gene permitting metabolic recycling, PRKp expression that led to
25 depletion of ribulose phosphate, a critical metabolite for aromatic amino-acids biosynthesis, and
26 accumulation of the dead-end diphosphate product contribute to toxicity. Triggered copy number
27 adjustment was found to be a dynamic process depending both on plasmid types and levels of PRK
28 induction. With the ARS-CEN plasmid, cell growth was abruptly affected only when level PRKp
29 expression exceeded a threshold value. In contrast, a proportional relationship was observed with the
30 2-micron plasmid consistent with large copy number adjustments. Micro-compartment partitioning of
31 bulk cultures by embedding individual cells into inverse culture medium/oil droplets, revealed the
32 presence of slow and fast growing subpopulations that differ in relative proportions for low and high
33 copy number plasmids.

34 **Keywords:** plasmid burden, cell toxicity response, population heterogeneity, metabolic engineering

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39 1. Introduction

40 *Yeast* and *E. coli* are major industry relevant microorganisms used for bulk chemicals or specialized
41 productions (Borodina and Nielsen, 2014) (Chen et al., 2013) (Khatun et al., 2017) (Kwak and Jin, 2017)
42 (Kim et al., 2017) (Wells and Robinson, 2017). ARS-CEN and 2 μ -replicon based *S. cerevisiae* plasmids
43 are commonly used for metabolic engineering (Krivoruchko et al., 2011) (Gnügge and Rudolf, 2017)
44 (Da Silva and Srikrishnan, 2012). In the case of ARS-CEN plasmids, replication and partitioning
45 between daughter cells are synchronous with chromosomal DNA and copy number is maintained at
46 about a single copy per cell (Clarke and Carbon, 1980). In contrast, 2 μ -replicon based plasmids are
47 present with a highly variable copy number (10-40 copies per haploid genome) depending on
48 engineering types and growth conditions (Gerbaud and Guérineau, 1980) (Futcher and Cox, 1984)
49 (Kazemi Seresht et al., 2013) (Gnügge et al., 2016). Plasmid replication has a fitness cost in addition to
50 specific positive or negative effects associated with included expression cassettes (Da Silva and
51 Srikrishnan, 2012) (Futcher and Cox, 1983) (Görgens et al., 2001). The burden associated with copy
52 number was evaluated to affect growth of the yeast cell by ~0.2% per copy for 2 μ -based plasmid
53 (Harrison et al., 2012), independently of expression cassettes. Similar data has been obtained for
54 multicopy plasmid in *E. coli* (Bailey, 1993). In response, cells with high-copy number plasmids are
55 counter-selected resulting into copy number adjustment. Promoter strengths of expressed gene, strain
56 ploidy and selection marker nature can similarly affect cell growth (Kazemi Seresht et al., 2013)
57 (Görgens et al., 2001) (Karim et al., 2013) (Çakar et al., 1999) (Ugolini et al., 2002). For example,
58 auxotrophy complementation markers have been classified in decreasing order, HIS ~TRP > URA >
59 LEU, based on their associated plasmid burden (Karim et al., 2013).

60 Copy number of episomal DNA affects expression of embedded gene(s). This generally results in an
61 advantage with multi-copy plasmids when high level expression of a single heterologous protein is
62 needed. Depending on the host micro-organism considered, expression cassettes involved in multistep
63 engineering can be scattered between genomic and episomal elements. In the case of *S. cerevisiae*,
64 availability of a large range of low and high copy number vectors makes multiple plasmid engineering
65 generally easier and more versatile to implement. Modulation of gene expressions resulting from
66 relative copy number changes in addition to the use of variable promoter strength plays a critical role in
67 tuning multi-step metabolic coupling. Copy number of plasmids, like the 2 μ -based yeast replicon, has a
68 tendency to naturally adjust in response to the balance between positive/negative selections, generally
69 to limit accumulation of potentially toxic metabolite intermediates (Karim et al., 2013).

70 However, and while they are regulated by complex mechanisms, multi-copy plasmid replication and
71 their distribution in daughter cells remains in part stochastic (Yen-Ting-Liu et al., 2014). This results into
72 variable balances between coupled activities from cell to cell. Such effects might significantly alter
73 productivity depending on the possibility of diffusion of accumulated metabolic intermediates between
74 cells. In contrast, heterogeneity of gene expression between cells can be an advantage to survive
75 external stress and contribute to robustness (Holland et al., 2014) (Guyot et al., 2015). *Latest*
76 *technological advancements give the possibility to study in more details plasmid encoded gene*
77 *expression in individual cells.* Stochastic fluctuation of gene transcription and translation at the single
78 cell level are additional contributors to phenotypic diversity of clonal cells (Avery, 2006) can be
79 evidenced by flow cytometry or time-lapse microscopy of fluorescently labelled cells (Bódi et al., 2017)
80 (Talia et al., 2007), milli- or micro-fluidic technologies allow monitoring individual cell growth-rate in
81 large populations (Grünberger et al., 2014) (Damodaran et al., 2015) (Grünberger et al., 2017) (Boitard
82 et al., 2012), *while quantification of DNA molecules by droplet digital PCR (ddPCR) combined with cell*
83 *sorting using flow cytometry allows highly precise determination of plasmid copy number* (Jahn et al.,
84 2015) (Jahn et al., 2014).

85 Carbon dioxide capture involving reconstruction of Calvin cycle was reported both in *E.coli* and *S.*
86 *cerevisiae* (Watson et al., 2016, Guadalupe-Medina et al., 2013). A critical step of this engineering is
87 the conversion by the PRKp of the natively present ribulose-5-phosphate into the corresponding 1, 5-
88 diphosphate, the substrate of the RuBisCO carbon dioxide fixing enzyme. When expressed alone,
89 PRKp activity is highly toxic, both for *E.coli* and *S. cerevisiae*, due to the resulting depletion of the
90 ribulose 5-phosphate pool, which is critical for aromatic amino-acid and nucleotide biosynthesis, and to
91 the accumulation of dead-end diphosphate product. Successful reconstruction of a Calvin cycle thus
92 critically depends on the balanced expression level of the PRKp in the absence of the multicomponent
93 biochemical regulations that controls PRKp activity in natural photosynthetic organisms (Zhuang and Li,
94 2013). A synthetic model was built in yeast to evaluate how adjustments to average plasmid copy
95 number occur following conditional promotor controlled changes in PRKp expressing cells.

96

97 **2. Materials and methods**

98

99 **2.1. Yeast strains and plasmids**

100 Plasmids pPRK-CEN (ARS416-CEN4, HA tagged *S. elongatus PRK* gene under the transcriptional
101 control of the TETO7 promoter and TRP1 selection marker) and pPRK-2 μ (2 μ -origin of replication
102 associated to the same functional cassettes) were used to transform *Saccharomyces cerevisiae*
103 CENPK1605 strain (MATa *leu2-3,112 trp1-289 ura3-52*). Resulted strains were named 1605-PRK-CEN
104 and 1605-PRK-2 μ respectively.

105 **2.2. Media, growth conditions and sampling.**

106 The strains were grown at 30°C in YNB-trp liquid or solid (20 g/l of Agar) medium, containing 0.67%
107 (w/v) Yeast Nitrogen Base with ammonium sulfate (Euromedex) and 2% (w/v) glucose and completed
108 with drop-out synthetic mix without tryptophan (USBiological).

109 The frozen cells were spread on a plate containing solid YNB-trp with 2 μ g/ml of doxycycline (Dox,
110 Sigma-Aldrich). After 48 hours incubation at 30°C, cells were collected and washed three times with
111 YNB-trp without Dox and resuspended in the different liquid media supplemented with three different
112 concentrations of Dox: 0, 0.2 or 2 μ g/ml at OD₆₀₀ 0.01. Cell growth over time was monitored based on
113 OD_{600 nm}. The cell concentration was deduced using the equivalence between 1 OD₆₀₀ = 10⁷ cells/ml.
114 Every two hours, a volume of culture was sampled for qPCR and PRKp activity measurements. The
115 samples were centrifuged (5 min, 13000 rpm, 4°C), supernatant removed and cells stored at -80°C.

116 **2.3. Yeast cell lysis and total DNA extraction.**

117 Frozen yeast cells were resuspended in lysis buffer (10 mM Na-phosphate [pH 7.5], 1.2 M sorbitol, 2.5
118 mg/ml Zymolyase 20T Euromedex UZ1000) and incubated for 10 min at 37°C to digest the cell wall.
119 Cell suspension was temperature cycled at 94°C for 15 min, -80°C for 5 min, and 94°C for 15 min
120 (Moriya et al., 2006). Centrifuged supernatant *containing DNA* was stored at -20°C before Q-PCRs
121 analysis.

122 **2.4. Real-time Q-PCR.**

123 The plasmid copy number was estimated by comparing the relative quantity of plasmid encoded *PRK*
124 gene to single copy chromosomal *ALG9* gene. The estimated plasmid copy number was calculated by
125 $2^{\Delta Ct(ALG9-PRK)}$ (Moriya et al., 2006). The DNA levels were analyzed using the MyIQ real-time PCR system
126 from Bio-Rad with the software Bio-Rad iQ5 2.0. The Ct were determined by the software. Each sample
127 was tested in duplicate in a 96-well plate (Bio-Rad, CA). The reaction mix (20 μ l final volume) was
128 composed of 10 μ l of Sso Advanced Universal SYBR Green Supermix (Bio-Rad), 0.4 μ l of each primer

129 (Table S1. *Alg9* sequences used for a described in (Teste et al., 2009)) at 0.2 μ M final concentration,
130 5.2 μ l water, and 4 μ l of a 1:10 dilution of the DNA samples. A blank (no template) and a positive
131 control were also included in each assay. The thermocycling program consisted of one hold at 95°C for
132 30 s followed by 40 cycles of 10 s at 95°C and 45 s at 54°C. After the amplification, a melting curve
133 analysis with a temperature gradient of 0.1°C/s from 70 to 95°C was performed to verify PCR
134 specificity, contamination and the absence of primer dimers.

135 **2.5. HPLC-MS determination of PRK activity.**

136 Cell pellets were resuspended in two volumes of cold lysis buffer (100 mM Tris/HCl buffer [pH7.9], 10
137 mM MgCl₂, 100 mM NaCl), the suspension was put in a FastPrep tube filled with one volume of 500 μ m
138 glass beads (Sigma-Aldrich). The cells were broken using a FastPrep FP120 (MPBiomedicals) with a
139 setting of 6.0 m/s and 6 cycles of 20 s 'on' / 20sec 'off' in ice. The tubes were centrifuged (13 000 rpm,
140 4°C, 5 min) and the supernatant (containing the cell extract) was recovered and stored at - 80°C. Prior
141 to use, the protein extract was centrifuged again (13 000 rpm, 4°C) in order to eliminate aggregates.
142 Total protein content was determined by using the Bradford assay (Sigma Aldrich).

143 The PRKp activity was quantified by monitoring conversion of ribulose-5-phosphate to the 1,5-
144 diphosphate derivative. Protein extracts (12.5 μ l) in a 96-well plate were mixed on ice with 12.5 μ l of 4X
145 assay buffer (400 mM Tris [pH7.9], 40 mM MgCl₂, 400 mM NaCl, 8 mM ATP). Reaction was initiated by
146 25 μ l of 2mM ribulose-5-phosphate (Sigma-Aldrich) in water. The microplate was incubated for 30 min
147 at 30°C and the enzymatic reaction was stopped by heating for 7 min at 90°C. The plate was
148 centrifuged and supernatants diluted two times with water and stored at -80°C before analysis.

149 HPLC analysis was performed on Waters separation module 2790 coupled to Waters micromass
150 ZQ2000. Separation was achieved on a Sheri-5 RP185 column (100–2.1 mm) (PerkinElmer) with
151 mobile phase consisting of tributylamine acetate (TBA) pH5.7 and acetonitrile (ACN). Elution was
152 performed at 1ml/min with 100% TBA for 1 min, a 100:0 to 70:30 TBA:ACN linear gradient for 15 min,
153 and a 70:30 to 0:100 TBA:CAN linear gradient for 18 min. Ions at m/e = 229.0 (ribulose-5-phosphate),
154 309.0 (ribulose-1,5-biphosphate), 426.3 (ADP) and 506.3 (ATP) were monitored. PRK activity was
155 expressed in μ moles of ribulose-1,5-biphosphate formed per min and mg of total proteins.

156 The enzymatic activity measurement was carried out starting from 13 hours of incubation as cell
157 quantities in earlier time points were too low to obtain a detectable activity.

158 **2.6. Observations in reverse emulsion.**

159 Microfluidic devices (droplets generation chip and observation chambers (Fig. S1) were provided by the
160 Laboratory Colloïdes et Matériaux Divisés (LCMD) from Ecole Supérieure de Physique et Chimie
161 Industrielles of Paris (ESPCI). To generate the emulsion, cultures were diluted to OD₆₀₀ = 0.015 in
162 order to encapsulate mostly a single cell per droplet. In such conditions of limit dilution, a large majority
163 of droplets remained empty (Fig.S1D). The emulsion was made by flow-focusing the cell suspension
164 stream with two streams of HFE7500 fluorinated oil (3M) containing 2% (w/w) 008-FluoroSurfactant
165 (RAN Biotechnologies) (Boitard et al., 2012). The droplets were incubated at 30°C in a 1.5 ml tube.
166 After 24 hours of incubation at 30°C, a monolayer of droplets was observed in a custom-made glass
167 chamber with 40- μ m depth using a direct microscope LEICA DM4000B. Snapshots were taken with
168 LEICA DFC300FX camera. Three classes of growth were differentiated: droplets with no or weak
169 growth (1 or 2 cells/droplet), droplets with few cell divisions (3 to 10 cells/droplet) and droplets with
170 many cell divisions (more than 10 cells/ droplet). About a hundred droplets exhibiting each cell growth
171 phenotype were counted.

172

173 3. Results and discussions

174 3.1. Experimental cell model

175 *S. cerevisiae* cells were transformed with a low copy number pPRK-Cen or a high copy number 2-
176 micron origin based pPRK-2 μ plasmid bearing a synthetic cassette encoding a *PRK* synthetic gene
177 under the transcriptional control of a Dox repressed (tet-off) promoter (Dingermann et al., 1992). ARS-
178 CEN plasmids synchronously replicate with chromosomal material and are generally present in yeast at
179 a single copy (in average between 1 and 2 due replication during cell division). In contrast, copy
180 number of 2 μ -plasmids can be highly variable, ranging from single copy when counter-selected, to
181 values than can exceed 80 when they are strongly positively selected. ARS-CEN plasmids are fairly
182 stable but 2 μ -plasmids can be easily lost (typically 5-10% per generation) during cell division due to
183 unequal daughter cell repartition. Their average copy numbers exhibit a large distribution making
184 population of transformed cells heterogeneous. The two plasmid types contained a native *TRP1* gene
185 as positive selection marker complementing a genomic *trp1* mutation. The transcriptional efficiency of
186 the tet-off synthetic promoter driving PRKp expression was controlled by the Dox concentration in a
187 pseudo-log manner due to the presence of multiple enhancer repeats. Dose-response adjustments
188 were performed to select Dox concentrations leading to clear and reproducible differences of growth
189 rates (high, medium, slow) for the strain transformed by the multi-copy plasmid. The same
190 concentrations were subsequently used for experiments involving the ARS-CEN plasmid.

191 Particular attention was drawn to define controlled and reproducible culture conditions. For a given level
192 of PRKp expression, toxicity (as judged by cell growth rate), can depend on initial cell metabolic status.
193 It was thus critical to define reproducible culture conditions and to secure the initial absence of PRKp
194 expression. Growth of frozen cell stock on petri dishes to obtain isolated clones followed by clone
195 pooling, was found to be an optimal starting material. The next step was to define three Dox
196 concentrations causing undetectable, medium, and strong counter-selection during the subsequent
197 liquid culture. For experiments, a controlled shift (lowering) of Dox concentration was required to induce
198 PRKp expression following the initial liquid pre-culture at high Dox (2 μ g/ml) concentration. Conditions
199 minimalizing cell stress associated during this step with external factors like centrifugation, temperature
200 or culture media changes were determined using reproducibility of growth rates and plasmid copy
201 number evolution as criteria. Time series determinations of plasmid copy number and PRKp activities
202 were performed by sampling bulk cultures following Dox concentration changes as illustrated in Fig.1.
203 To evaluate the growth phenotypes of individual cells, culture samples were diluted with fresh culture
204 media to generate water-in-oil emulsion of droplets. The dilution was adjusted so that the majority of
205 droplets were empty or contained a single cell.

206 3.2. Bulk cell growths as a function of plasmid type and induction.

207 1605-PRK-CEN and 1605-PRK-2 μ strains were cultivated at the three Dox concentrations previously
208 determined leading to repressed, intermediate and fully induced PRKp expressions (Fig. 2). In the case
209 of the 1605-PRK-CEN strain, similar growth curves were observed for the repressed (Dox=2 μ g/ml) and
210 intermediate (Dox=0.2 μ g/ml) expression states (Fig. 2A). In contrast, cells 1605-PRK-2 μ exhibited
211 three clearly distinct growth behaviors depending on Dox concentrations (Fig2.B). In the conditions of
212 full induction, growth rate was significantly faster for cells transformed by the pPRK-CEN plasmid in
213 comparison to the 2 μ -plasmid case. However, a delay of about 7 hours of culture, (3-4 cell doubling)
214 was needed to establish this difference. This delay could correspond to the duration required for *PRK*
215 gene induction and corresponding protein accumulation.

216
217 The above observations established that for a low copy number plasmid, intermediate Dox level does
218 not allow PRKp to reach expression levels that affect cell growth. Such a situation was in contrast to the
219 full induction of the pPRK-CEN plasmid or for full and intermediate induction levels of the multi-copy

220 plasmid. In the latter case, growth inhibition was stronger with the multi-copy plasmid. This experiment
221 demonstrated that the experimental model allowed us to define at least four types of PRKp expression/
222 growth phenotype relationships (none, intermediate, strong and maximal effects). Consequently, for
223 similar expression cassettes and induction levels, observed effects were much more marked with 2 μ -
224 plasmids. Careful examination of growth curves evidenced that for the intermediate induction level and
225 the 2 μ -plasmid, inhibition of growth decreases with time. After 15h of partial induction, the growth rate
226 for the 1605-PRK-2 μ cells reached similar value to the one observed in the fully repressed condition.
227 This suggested that cells transformed with the 2 μ -plasmid might be able to adjust the level of toxicity by
228 reducing copy number to reach a situation similar to the one observed with the ARS-CEN-based
229 plasmid. At this point, it is important to note that a threshold of PRKp expression level was required to
230 affect cell growth and that plasmid copy number adjustment might in some way compensate for initial
231 difference of PRK expression. These findings will be further confirmed by direct analysis of plasmid
232 copy number and PRKp activity changes during time courses.
233

234 **3.3. Plasmid copy number and PRK activity relationships with cell growth phenotypes**

235 Time courses of plasmid copy numbers and PRKp activities were monitored during the same cultures
236 as the growth curves previously described (Fig.3). Plasmid copy number quantifications were
237 normalized using the single chromosomal copy of the *ALG9* gene in order to correct for DNA recovery
238 yields (Fig.3 A&B). Concerning the 1605-PRK-CEN strain, plasmid copy number remained in the 0.8 to
239 1.6 copy/cell range independently of PRKp expression levels and culture durations. This confirmed that
240 the ARS-CEN copy number remained under the control of mechanisms similarly regulating
241 chromosomal DNA replication. Cells transformed with the 2 μ -plasmid exhibited a higher copy number
242 (12-14 copies per cell) in the Dox repressed state. This level remained constant within a margin of error
243 during the pre-culture and the subsequent 20h of culture at 2 μ g/ml Dox. Such a high copy number is
244 standard for plasmids involving the 2 μ -origin in the absence of strong positive or negative selection
245 (Jordan et al., 1996). Dox concentration shifts (from 2 to 0.2 μ g/ml or absence) induced partial or full
246 enzyme expression and caused a clear and progressive decrease (more than 2 fold in 15h) of plasmid
247 copy number with a similar time dependence and that extended to the two conditions. However, the
248 final copy number (~5-6 copies/cell) remained significantly higher than the one observed for ARS-CEN
249 plasmids. Observation of similar copy number values for the partial and full induction contrasted with
250 the differential impact previously illustrated on growth curves. However, this finding has to be mitigated
251 considering that expressed PRK activities and metabolic disturbance might only partially reflect gene
252 copy numbers (see latter).

253 To be able to better interpret results, PRKp activities were quantified into cell extracts obtained from the
254 same experiments (Fig.3 C & D). While copy numbers for ARS-CEN-constructs were identical
255 regardless of Dox concentrations and induction durations, associated PRKp activities markedly differed.
256 Activities appeared fairly independent of culture duration for the full and partial induction conditions but
257 exhibited some increase at the shorter times for the lower PRK expression level. These results also
258 show that in the fully repressed state, some residual PRKp expression was still present while being
259 100-fold lower than ones observed at maximal induction. From the ensemble of these results and the
260 growth observations, the threshold for PRKp activities able to impact cell growth was evaluated to be
261 ~10 nmoles/min/mg of total proteins. PRKp activity levels for the 2 μ -construct followed similar time
262 courses in experiments involving partial and full induction, consistent with similar copy number
263 evolutions. However, for technical reasons (low cell density) the initial time course of PRKp activities
264 cannot be accurately determined during the first 12 hours of incubation (arbitrary dotted on the figure)
265 following DOX concentration shifts. During this initial period, a simple relationship between copy
266 number and expressed activity is not expected. This transient phase results from time required for
267 transcriptional de-repression and protein accumulation in the growing population. Final protein level is

268 rarely expected to be proportional to transcription and copy numbers (McManus et al., 2015) (Barrett
269 et al., 2012) (Wethmar et al., 2010) (Tang and Amon, 2013).

270 PRKp activities reached similar levels for partial or full induction of the 2 μ -construct and induction times
271 ranging from 12 to 20h, when effects on cell growth rates markedly differed (Fig. 2 and 3). This lack of
272 correlation was not observed for the ARS-CEN construct that featured stable copy number during
273 PRKp induction course. It is thus tempting to associate these contrasting behaviors to some adaptation
274 associated with plasmid properties. Growth curves (Fig. 2) upon full induction of PRKp expression can
275 be accurately simulated by a model (data not shown) involving a mix of two subpopulations, one
276 corresponding to dying cells expressing PRKp above a lethal threshold and the other corresponding to
277 still efficiently growing cells expressing lower PRKp levels. Depending on parameters (dying rate and
278 cell division time) and proportions of the two subpopulations, growth arrest after few generations or only
279 reduced apparent growth rates are predicted. Development of such heterogeneity within an initially
280 clonal population might appear surprising. In fact, it could be easily explained by the initial distribution of
281 copy number for the 2 μ -constructs that is absent with ARS-CEN plasmids. In such conditions, PRKp
282 expression in cells initially featuring high plasmid copy number will rapidly be lethal. In contrast, a non-
283 lethal effect is expected for cells initially featuring a lower copy number, preserving potentiality for cell
284 division and allowing further lowering of copy number through unequal plasmid repartition. Such
285 mechanism is not expected with ARS-CEN construct and appeared globally fully consistent with
286 observations.

287 **3.4. Micro-compartment based analysis of toxicity adaptation mechanisms**

288 At this point, and while reconciliation of growth phenotypes, copy number evolution and PRKp
289 expression could appeared generally good enough, potential impact of the copy number heterogeneity
290 associated with the 2 μ -construct has to be confirmed. Assuming a population of cells in the bulk culture
291 containing heterogeneous copy numbers of the 2 μ -construct, different phenotypes must be evidenced
292 by isolating and monitoring growth of individual cells into micro-compartments. A microfluidic device,
293 provided by the LCMD (Fig.S1), consisting of a water in oil emulsion forming unit and an observation
294 chamber was used for such purpose following micro-compartmentation of induced or repressed bulk
295 cultures. Samples withdrawn after 10h liquid culture at different Dox concentration were diluted (same
296 culture media and Dox concentrations) in order to generate droplets containing mostly no cell or single
297 cells. The droplets were observed after 24h of additional incubation at 30°C and the number of cells per
298 droplet were counted to evaluate growth potentiality. Counts were roughly classified into three groups:
299 no growth (dead cells), 4-10 cells and more than 10 cells/droplet (Fig. 4A).

300 Control experiments with cells transformed with plasmids lacking the PRKp expression cassette led to
301 droplets containing only fast growing cells indicating that encapsulation did not induce growth
302 heterogeneity in the absence of PRK expression. Similarly, for cells with the fully repressed PRKp
303 expression, a large majority (70% for the 2 μ -based and 83% for ARS-CEN-based constructs) of
304 droplets contained more than 10 cells following incubation (Fig. 4A and B, Table S2). In contrast, a near
305 absence of growth (1 to 3 cells per droplet) or some limited growth (4 to 10 cells per droplet) was
306 observed for the majority of droplets containing cells encapsulated from a fully induced (no DOX) bulk
307 culture. Partial induction of 2 μ -construct containing cells in bulk culture resulted after micro-
308 compartmentation in a more balanced distribution of the three types of growth. In the same conditions
309 (repressed and partial induction), the fast-growing subpopulation was always predominant with ARS-
310 CEN-constructs. The small proportion of droplets containing non-growing cells observed in the
311 repressed state both with the 2 μ - and the ARS-CEN constructs, likely resulted from a low proportion of
312 plasmid-free cells having accumulated during the bulk culture. This was confirmed by the observation of
313 a 98% survival rate for repressed cells transformed by the 2 μ -construct when further cultivated on a
314 medium supplemented with tryptophan.

315 This work suggested that growth inhibition phenotypes observed upon controlled PRKp expression
316 mostly resulted from population heterogeneity preexisting in bulk cultures. Comparison of growth
317 behaviors in bulk (Fig 2) and in droplets (Fig 4B) illustrated that the micro-fluidic approach was more
318 prone to detect moderate toxicity of gene expression than analysis of growth rates in bulk cultures. For
319 the multicopy plasmid construction, a large majority (63 \pm 6%) of droplets featuring no growing cells
320 were counted in the absence of Dox, when 70 \pm 7% of droplets accumulated more than 10 cells in the
321 fully repressed condition (Dox= 2 μ g/ml). Approximately the same proportions of droplets containing
322 no growing, 4-10 cells/droplet or more than 10 cells/droplet were observed with the 2 μ -constructs and
323 an intermediate (0.2 μ g/ml) Dox concentration. These results are in accordance with the growth curves
324 shown in Fig 2 and Table 1 and illustrated three clearly distinct growth behaviors depending on Dox
325 concentrations. Concerning the ARS-CEN-construct, 83 \pm 13% and 61 \pm 16% of droplets contained
326 more than 10 cells were counted for the fully repressed and intermediate Dox conditions respectively.
327 In contrast, only 19 \pm 8% of droplets contained 10 cells or more were observed for fully induced
328 condition (no Dox). These results are consistent with the similarity of growth curves in the repressed
329 and intermediate expression states. Thus differential growth in droplets encapsulating cells from a bulk
330 liquid culture likely resulted mostly from the preexisting copy number heterogeneity in the population
331 with an additional contribution of cells having lost their plasmid during the bulk culture.

332 **Conclusions**

333 PRKp activity is toxic by interfering with critical metabolic branches involved in amino-acid and
334 nucleotide biosynthesis. This resulted in a clear-cut threshold value for toxicity of PRKp expression that
335 plays the role of amplifier for the functional impact of the natural scattering of 2 μ -construct copy
336 number. Our model allowed us to address how yeast cells transformed with plasmids involving ARS-
337 CEN- and 2 μ -origin and carrying a conditional PRKp expression cassette responded to the conditional
338 protein expression through adjustment of plasmid copy number, gene expression, and finally cell
339 growth rates. The PRKp expression model is of particular interest in constituting a key step for the
340 reconstruction of synthetic microorganisms able to capture (or recapture) carbon dioxide through an
341 artificial Calvin cycle. However, the model has a more general interest for tuning strategies of
342 engineering involving sequence of critically balanced enzymatic steps prone to accumulate toxic
343 intermediates. Control of plasmid copy number is potentially a powerful approach for engineering
344 optimization. Natural unequal plasmid partition over cell divisions progressively create a distribution of
345 copy numbers counteracted by a large range of regulation mechanisms acting at level of plasmid
346 replication. However, these mechanisms can be disrupted in a dynamic manner by strong positive or
347 negative selection pressures associated with plasmid encoded functional cassettes. Playing with such
348 mechanisms is of interest to optimize the production of biomolecule and cause a significant metabolic
349 load. Maintaining multi-copy plasmid in the off-state during the growth phase before activation, once
350 high cell density is reached and cell division arrested, is a classical approach to optimize expression.
351 However, our work illustrated that copy number distribution can led to the formation of subpopulations
352 with markedly distinct phenotypes.

353 **Acknowledgements**

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355 France.

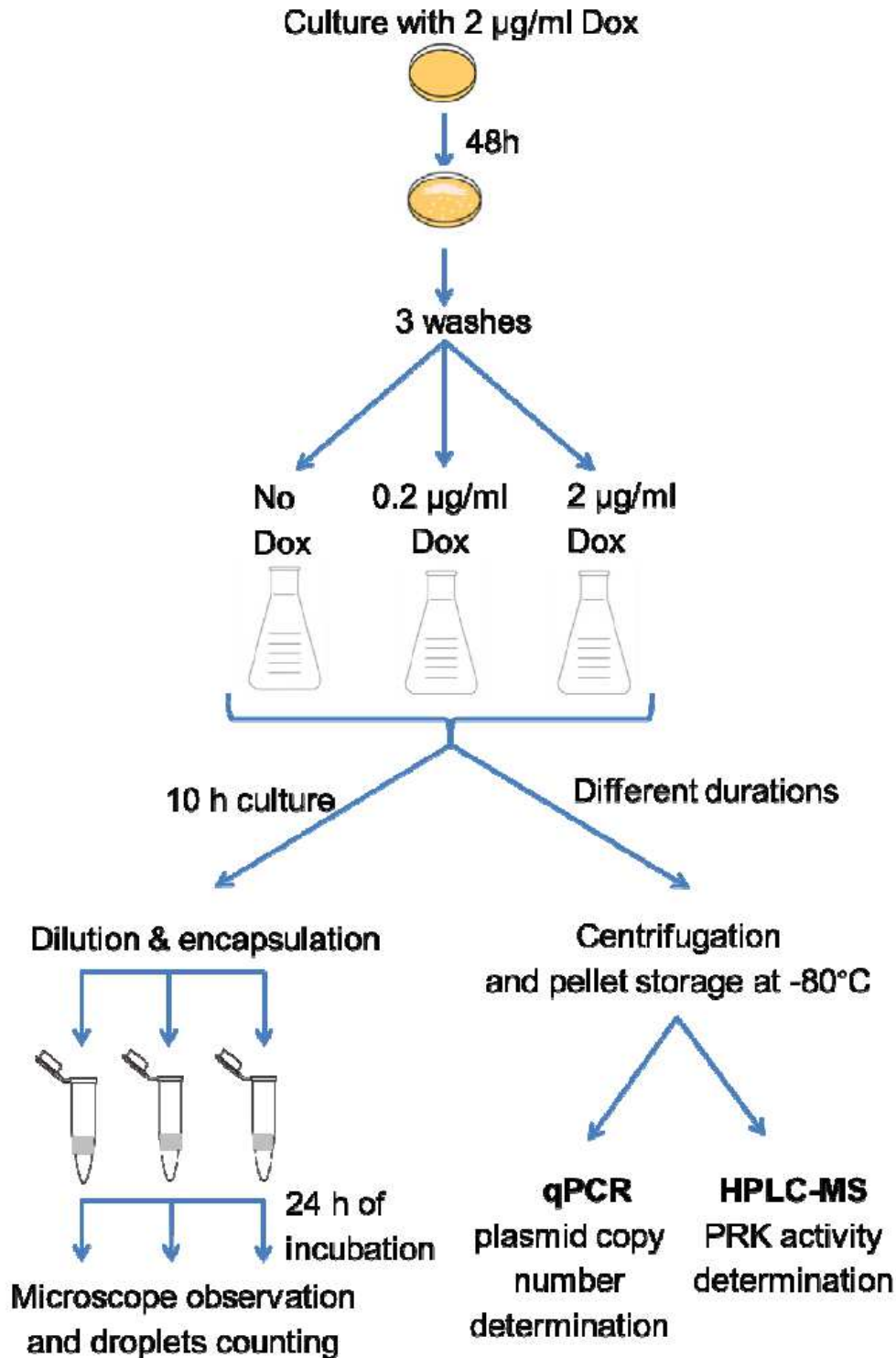
356 We thank Roxanne Diaz (English natives speaker) for language improvement of the manuscript.

357

358 **Figure legends and tables**

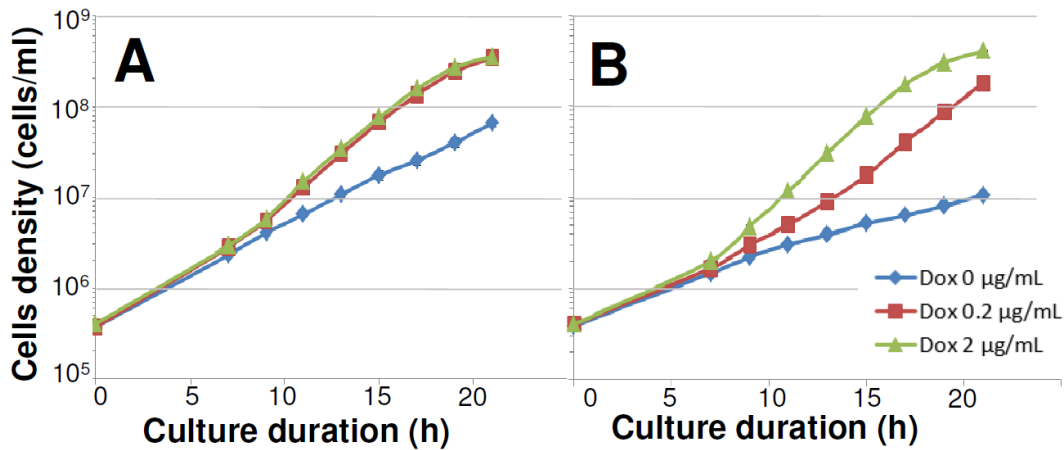
359

360 **Figure 1.** Experiment flow chart. Plate incubation was performed in solid YNB-trp supplemented with 2
361 $\mu\text{g/ml}$ Dox at 30°C . Washes were performed with YNB-trp without Dox and culture started at
362 $\text{OD}_{600}=0.01$. Liquid cultures were performed in the same media containing various Dox concentrations
363 at 30°C with 200 rpm shaking. Samples were either pelleted for qPCR and HPLC-MS analysis at 0, 7,
364 9, 11, 13, 15, 17, 19 and 21h of culture or diluted to $\text{OD}_{600}=0.015$ after 10h culture for encapsulation.
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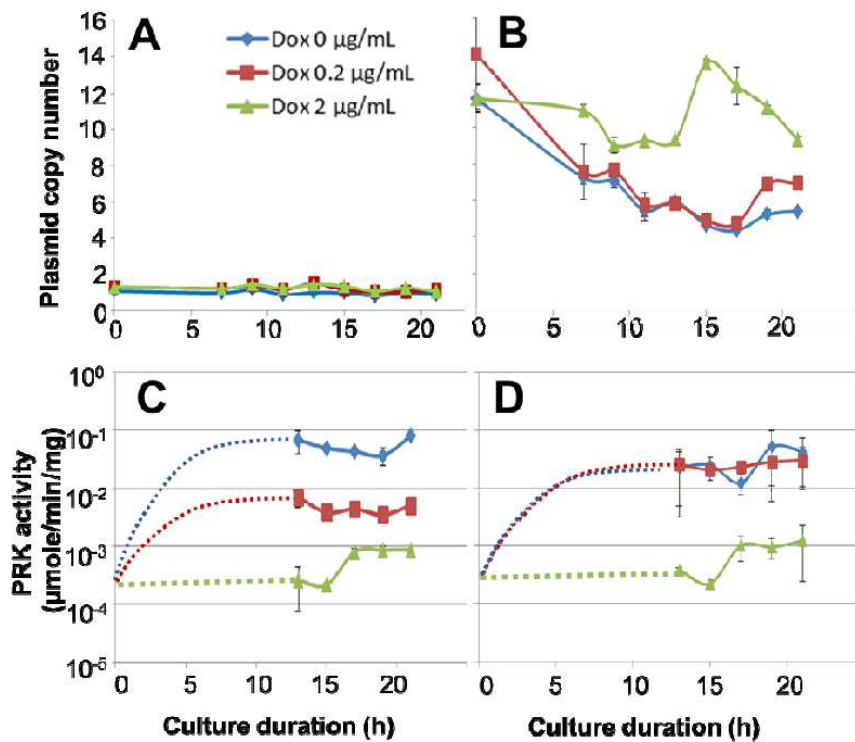
368 **Figure 2.** Growth curves of yeast cells at different Dox concentrations (— 0 $\mu\text{g/ml}$, — 0.2 $\mu\text{g/ml}$,
 369 — 2 $\mu\text{g/ml}$) for cells bearing centromeric (A) or multi-copy plasmids (B).
 370



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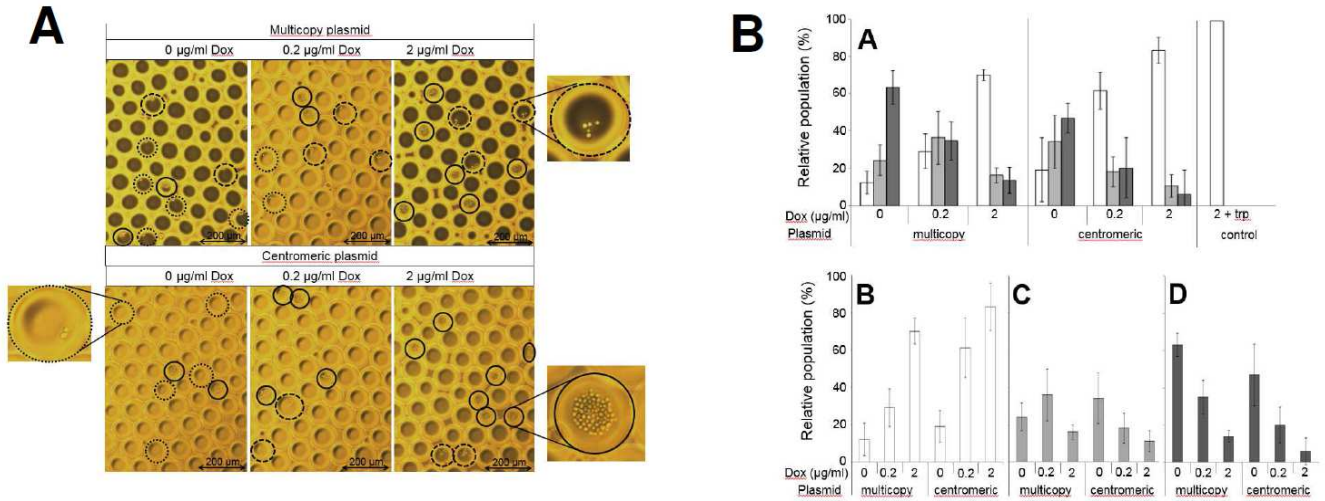
373 **Figure 3.** Time dependence of relative plasmid copy number as function of Dox concentration in culture
 374 media (— 0 $\mu\text{g/ml}$, — 0.2 $\mu\text{g/ml}$, — 2 $\mu\text{g/ml}$) for centromeric (A) or multi-copy (B) plasmids. Time
 375 dependence of PRKp activity for cells with centromeric (C) or multi-copy (D) plasmids. Dotted lines are
 376 arbitrary drawn and given only as help to visualize time course.



377

378

379 **Figure 4.** (4A) Microscopy observation of droplets for different Dox concentrations and plasmid types.
 380 High (full circles), medium (dashed circles) and low (dotted circles) cell densities in droplets. (4B) Cell
 381 growth analysis in droplets after 24h incubation. Classified by (A) Dox concentrations or (B-D) growth
 382 level in the droplet. 1 to 3 cells per droplet (black boxes), 4 to 10 cells per droplet (grey boxes), and
 383 more than 10 cells per droplet (white boxes). A control was realized with cells transformed with multi-
 384 copy plasmid lacking the PRKp expression cassette, in presence of 2 $\mu\text{g/ml}$ of Doxycycline and
 385 tryptophan supplementation (2 + trp). Color codes are identical for upper and lower panels. Values are
 386 averages of microfluidic experiments triplicates.



387

388 **Table 1.** Results summary. Growth rate (μ) calculated between 13 and 15h of culture. Relative plasmid
 389 copy numbers measured at start of culture (0h) and after 21h and fold changes. PRKp activities after
 390 21h of incubation. Microfluidic observations in droplets of the relative populations of fast and slow
 391 growing cells.

392

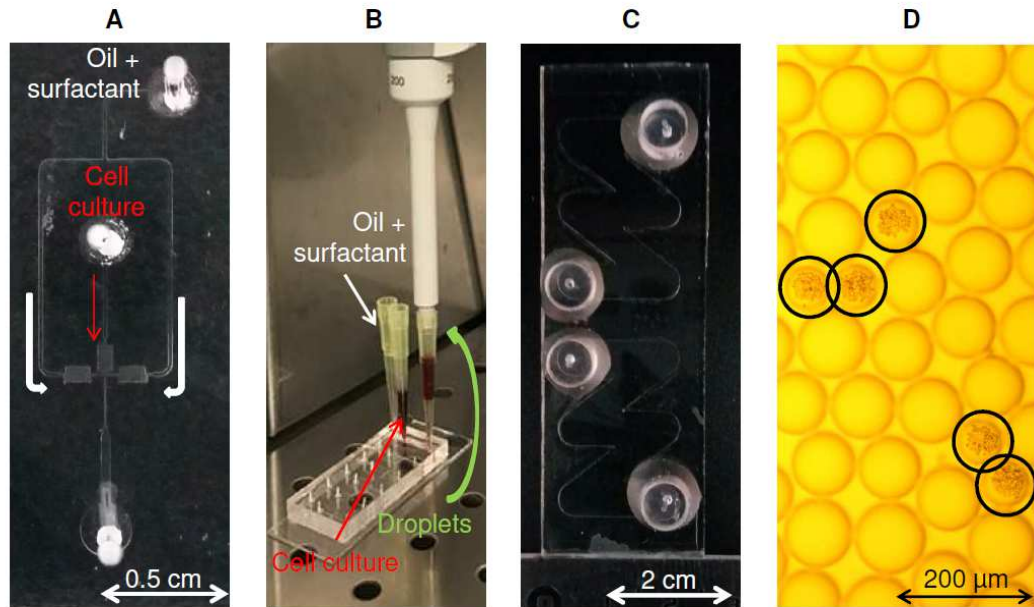
Plasmid type	Dox ($\mu\text{g/ml}$)	Growth rate (μ) 13 - 15h (h^{-1})	Relative copy numbers		PRK activity 21 h ($\mu\text{moles/min/mg}$)	Microfluidic	
			Value 0h - 21h	Fold change T21/T0		% cells Slow growing	% cells Fast growing
multicopy	0	0.06	$12 \pm 0.7 - 5 \pm 0.0$	0.42	$4.10^{-2} \pm 3.10^{-2}$	63 ± 8	12 ± 9
	0.2	0.14	$14 \pm 3.0 - 7 \pm 0.1$	0.50	$3.10^{-2} \pm 2.10^{-2}$	35 ± 14	29 ± 10
	2	0.20	$12 \pm 0.8 - 9 \pm 0.2$	0.75	$1.10^{-3} \pm 1.10^{-3}$	14 ± 4	70 ± 7
centromeric	0	0.11	$1.1 \pm 0.2 - 0.7 \pm 0.2$	0.64	$8.10^{-2} \pm 4.10^{-3}$	47 ± 14	19 ± 8
	0.2	0.18	$1.3 \pm 0.1 - 1.0 \pm 0.1$	0.77	$5.10^{-3} \pm 1.10^{-4}$	20 ± 8	61 ± 16
	2	0.17	$1.3 \pm 0.1 - 1.1 \pm 0.1$	0.85	$9.10^{-4} \pm 2.10^{-4}$	6 ± 6	83 ± 13

393

394 **Supplementary material**

395

396 **Figure S1.** Microfluidic system for cell encapsulation in droplets and observation of growth. (A) Flow-
 397 focusing geometry: cells in culture media (vertical arrow), Oil and surfactant (arrows). (B) Multi-line
 398 droplet generating system driven by suction.



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402 **Table S1.** QPCR primer list.

Gene		5' – 3' sequence
ALG9	Forward	CACGGATAGTGGCTTTGGTGAACAATTAC
	Reverse	TATGATTATCTGGCAGCAGGAAAGAACTTGGG
PRK6	Forward	ACACGCAAACACAAATAC
	Reverse	TACCACAACCAGAATCAC

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405

406 **Table S2.** Relative numbers of droplets featuring low, medium and high number of cells following
 407 incubation of initially single cell transformed with centromeric and multi-copy plasmids in medium
 408 containing various Dox levels.

Plasmide type	Dox ($\mu\text{g/ml}$)	1-3 cells/droplet	4-10 cells/droplet	>10 cells/droplet
multicopy	0	63% \pm 6%	24% \pm 8%	12% \pm 9%
	0.2	35% \pm 9%	36% \pm 14%	29% \pm 10%
	2	14% \pm 3%	16% \pm 4%	70% \pm 7%
centromeric	0	47% \pm 17%	34% \pm 14%	19% \pm 8%
	0.2	20% \pm 10%	18% \pm 8%	61% \pm 16%
	2	6% \pm 7%	11% \pm 6%	83% \pm 13%

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