File S1

Supporting Information

Insertions of the chromosomal fluorescent markers

The YFP and CFP genes were inserted at the *rhaA* locus of REL4548 using the one-step inactivation of chromosomal genes technique developed by Datsenko & Wanner (DATSENKO and WANNER 2000). pZA32-YFP and pZE1R-CFP - plasmids kindly provided by Dr Michael Elowitz - were used as templates.

The first step of this construction was to introduce the P_{A1} (bacteriophage λ promoter) upstream of the CFP and YFP genes. Because the YFP and CFP sequences (wild-type codons, developed by University of Washington Yeast Resource Center) only differ by 20 nucleotides (out of 717 bp), the same primers could be used for both PCRs. The primer P-BspHI-promA1-YC/FP for (109 bases) is composed of a 5' TCTC tail (increasing digestion efficiency by BspHI), a BspHI restriction site, the P_{A1} promoter containing a ribosome binding site (rbs) and a 20 bp sequence homologous to the YFP and CFP genes 5' extremity (see supplementary materials for primer sequences and PCR mix). The P-ClaI-YC/FP rev primer is composed of a 5' TCTC tail, a ClaI restriction site and a 20 bp sequence homologous to the YFP and CFP genes 3' extremity. The resulting PCR products were named P_{A1}-CFP and P_{A1}-YFP.

The second step consisted in cloning the P_{A1}-CFP and P_{A1}-YFP in pKD4 (DATSENKO and WANNER 2000). P_{A1}-CFP and P_{A1}-YFP were digested by BspHI and ClaI, and then gel purified. pKD4 was first digested by BspHI and ClaI, and then treated with shrimp alkaline phosphatase (SAP). The SAP catalyzes the release of 5'- and 3'-phosphate groups from DNA, precluding the ligation of the linearized plasmid with the remaining pKD4 BspHI-ClaI fragments. Finally, P_{A1}-CFP and P_{A1}-YFP were cloned in pKD4 by using a T4 ligase. Ligation products called pKD4-CFP and pKD4-YFP were electroporated into DH5-α λ-pir, and plated on LB plates supplemented with 50 µg/mL Kanamycin (LBA-Kan). Fluorescent colonies were purified by streaking on fresh LBA-Kan plates, and the next day, a colony was inoculated in liquid LB-Kan.

After isolation with Qiagen miniprep kit, pKD4-YFP and pKD4-CFP plasmids were used as templates in a second PCR in which the promoter sequence, the fluorescent genes and the Kanamycin cassette carried by pKD4, were amplified (see DATSENKO and WANNER 2000 for

more details). The primers used in this second PCR - p-pKD4-RhaAH1-2863-2888 for (84 bases) and p-pKD4p2H2-1496-1477rev (79 bases) – contained either a BamHI or a SalI restriction site at their 5' extremities (restriction sites not used in this study), fifty bases homologous of the *E. coli rhaA* locus and the 21 or 27 bases homologous to the template at their 3' extremities. The resulting PCR products were electroporated into REL4548 competent cells carrying the thermosensitive plasmid pKD46 (DATSENKO and WANNER 2000) and transformed cells spread on LBA-Kan (incubation at 37°C in order to get rid of pKD46). Fluorescent recombinants were streaked on LBA-Kan for purification, and on LBA-Amp (50 µg/ml Ampicilin) to check for the loss of pKD46. PCRs were carried out on recombinant clones with two sets of primers (with a forward primer p-pKD4-2863-2888 specific to the insert, or p-RhaA-for specific to the *rhaA* gene, and the reverse primer RhaA-1262-1243-rev specific to the *rhaA* gene) to check that the insertion of CFP-Kan and YFP-Kan cassettes occurred at the right locus.

The excision of the kanamycin resistance cassette, corresponding to the last step of this cloning, was performed by the electroporation and induction of the plasmid pCP20 (DATSENKO and WANNER 2000). Transformants were first cultivated at 30°C on LBA-Amp plates. The flipase production (allowing the recombination of the FRT sequences surrounding the Kanamycin cassette) was induced by streaking colonies from the LBA-Amp plates, on LB plates at 42°C. To check for Kanamycin resistance cassette removal and loss of pCP20, colonies were streaked on LBA, LBA-Kan and LBA-Amp plates. PCRs on the *rhaA* locus were performed on 4548 CFP Kan^S Amp^S and 4548 YFP Kan^S Amp^S clones as well as on 4548, 4548 CFP-Kan and 4548 YFP-Kan. Both the Kan^S associated with a fluorescent phenotype and the size of the PCR products confirmed the removal of the kanamycin cassette and the insertion of the fluorescent genes.

Primer sequences

Name	Sequence	Constructio n step	Reference
P-BspHI-pA1-	TCTC**TCATGA**TTATCAAAAAGAGTA**TTGACT**TAAAGTCT AACCTATAG**GATACT**TACAGCCATCGAGAG*attaaagag*	PCR 1 Introductio	This study

YC/FP for	*gagaaa*GGCGAAATGCGTAAAGGAGAAGAAC (109 bases) In blue : BspHI restriction site In black: P_{A1} In pink: Ribosome binding site In green, CFP/YFP homologous sequence	n of promoter pA1 upstream the CFP or YFP cassette	
P-ClaI-YC/FP rev	TCTCATCGATTTATTTGTATAGTTCATCCATGCC In blue : ClaI restriction site In green, CFP/YFP homologous sequence	PCR 1 Introductio n of promoter pA1 upstream the CFP or YFP cassette	This study
p-pKD4-*rhaA*H1-2863-2888 for	GAGTCGACGGCGCTGCGCCAACTTGATCGTTTACCCGT TTCAATGCACTGCTGGCAGGGAAGCATTTATCAGGGTT ATTGTCTC (84 bases) In blue : SalI restriction site In red: *rhaA* homologous sequence In green: pKD4-CFP (or pKD4-YFP) homologous sequence	PCR 2 Creating a linear fragment for homologou s recombinati on	This study
p-pKD4p2H2-1496-1477rev	ACGGATCCACCACGCGGCAATGCGGTTGATAGAGGCA TCGAAGAAGTCAAGGCCGATATCCTCCTTAGTTCCTATT CCG (79 bases) In blue : BamHI restriction site In red: *rhaA* homologous : e	PCR 2 Creating a linear fragment for	This study

	In green: pKD4-CFP (or pKD4-YFP) homologous sequence	homologous recombination	
p-*rhaA*-for	GACCACTC AACTGGAACAGGCC	PCR 3 Checking for CFP/YFP insertion in the *rhaA* locus	This study
p-pKD4-2863-2888 for	GAAGCATTTATCAGGGTTATTGTCTC	PCR 3 Checking for CFP/YFP insertion in the *rhaA* locus	This study
rhaA-1262-1243-rev	CCACGCTGGCTCAAAATCGC	PCR 3 Checking for CFP/YFP insertion in the *rhaA* locus	This study

PCR reactions:

PCR 1 - Introduction of promoter PA1 upstream the CFP or YFP cassette

Plasmid pZE1R-CFP or pZA32-YFP	1 µL
Primer P-BspHI-pA1-YC/FP for	2 µL
Primer P-ClaI-YC/FP rev	2 µL
Pfu 10X buffer	2 µL
dNTPs	1 µL
Pfu	0.5 µL
H_2O	11.5 µL

Program : Hybridization at 52°C for 1 min, elongation at 72°C for 2min, 25 cycles.

PCR 2 - Creating a linear fragment for homologous recombination

Plasmid pKD4-CFP (or pKD4-YFP)	2 µL
Primer p-pKD4-RhaAH1-2863-2888 for	1 µL
Primer pKD4p2H2-1496-1477rev	1 µL
Phusion 2X mastermix	10 µL
H_2O	6 µL

Program : Hybridization at 60°C for 1 min, elongation at 72°C for 3min, 25 cycles.

PCR 3 - checking CFP and YFP cassette insertions and Kanamycin cassette removal

Colony inoculated in the PCR mix, with a toothpick.

Primer forward	1 µL

Primer reverse	1 µL
Taq 10X buffer	2 µL
$MgCl_2$	1.5 µL
dNTP	1 µL
Taq Goldstar Red	0.2 µL
H_2O	13.3 µL

Program : Hybridization at 60°C for 1 min, elongation at 72°C for 3min 15 sec, 30 cycles.

Effect of "doublets" on coefficient of selection estimates

CY particles (C2), CC and YY doublet particles may pose a problem for accurate analysis of marker proportions. We have found that "singlet" and "doublet" particles can be nicely separated on a FSC TOF (time of flight) - FSC Peak plot. The frequency of YY (C1 region), CY (C2 region), and CC (C4 region) can thus be estimated. If cells were associated randomly in a doublet, when C and Y are initially introduce at a ratio of 1:1, we would expect to have C1 = 0.25, C2 =0.5 and C3 =0.25 within doublets. Most of the time, what we observe is C1 = 0.33, C2 = 0.33 and C3 = 0.33. This means that cells are not associated randomly in a doublet, but that they have more chance to be with a cell of the same color. Our hypothesis for such observation is that when entering stationary phase, a fraction of the daughter cell population might stay attached to each other. In Rang et al 2003, they observed that "*Cultures of the bacteria most affected by GFP exhibited a proportion of elongated cells, which suggests that GFP production could interfere with cell division in these strains*", which is another plausible explanation for our observation.

Now, the question is to evaluate the bias introduced by ignoring doublets. We can introduce some notation to be specific. There are several unknowns: the fraction of doublets (either CC, YY or CY, only the latter being measured in the C2 region). Let's denote it ϕ. Then there is the frequency of C bacteria (*p*). Last, we can consider that doublets may not form by the random association of two bacteria (e.g. two C bacteria may be more likely to form a

doublets than a pair of C and Y bacteria, as explained above). We can introduce a departure from random association *F* (akin to a departure from Hardy Weinberg proportion). It is then straightforward to compute the frequency of each type of particle (C, Y, CC, YY, CY). We measure frequency by the ratio (C+CC)/(C+CC+Y+YY), which introduces a bias from the true *p* value equal to

$$-(1-F)(1-p)p(1-2p)\phi + 0(\phi)^2.$$

This bias can be expressed in terms of ε, the proportion of the C2 population (defined as CY/(C+CC+CY+YY+Y). It is simply

$$-\epsilon(p-1/2)$$

which is very small, especially when *p* is close to ½. In our case, average initial frequency is 0.493±0.015 and ϵ < 1%, which corresponds to a bias close to 10^{-4}. The bias made on the frequency change, Δp, during the competition is different and it is this bias that is most relevant to estimating the strength of selection. Following the same approach and assuming F and ϕ are approximately constant during the competition, it is

$$\frac{1}{2}\Delta p(1-F)\phi$$

In other words the bias on the frequency change is proportional to itself. Expressing this in terms of ϵ and the intensity of selection per generation (with $\Delta p = s\ g\ pq$), we obtain $gs\epsilon pq$. The bias on selection coefficient is therefore equal to $s\epsilon$. Thus, it is always very small compared to *s*, provided the fraction of C2 population remains <<1. Correcting for doublets would be probably necessary when ϵ is greater than a few % (which, in our experiments, it is not). Under those circumstances, it would be important to have a clear experimental understanding of the origin of doublets particles (and measures of F and ϕ as we did). Last, it is important to underline that these slight biases do not introduce errors, so that estimations of variances remain unaltered.

Impact of de novo beneficial mutations during the assay

Beneficial mutations should have an implausibly high selection coefficient to impact fitness measures made in our experiments. The computation is different if we consider between week variance or within week (between replicates) variance.

Computation between weeks

The pre-cultures start from a frozen glycerol stock with a sample size of N_{gly} around 10^5. Importantly, all replicates within a week use the same pre-culture and only experiments performed in different weeks use different pre-cultures (*i.e.* different subsamples of the same glycerol stock). Seventeen generations later (around 10 generations during pre-culture, and seven more during a second round of pre-culture removing any trace of glycerol), the competition starts for 6.6 more generations. During the competition, the presence of a beneficial mutation with selection coefficient s_e may distort the frequency change measured in our experiment. If competitions always start with this beneficial mutation, little variance among competitions will be generated. On the contrary, variance in *s* among weeks will occur if the mutation is only present in some weeks. Hence, the worst case situation occurs when there is around ½ chances to sample the beneficial mutation from the glycerol. Computing this probability from a Poisson distribution, we find that the worst case is a frequency in the glycerol of around

$$p_{gly}=\text{Log}[2]/\ N_{gly}.$$

Starting from this initial frequency, the beneficial mutation will end up at the end of the preculture after 17 generations at a frequency

$$p_o = p_{gly}\ \text{Exp}[s_e\ 17]$$

(from logistic growth). Let's assume that the beneficial mutation occurred in a YFP cell. In our experiments, p_{yfp} –the frequency of the YFP cells—is always close to ½. During the

competition itself, the beneficial mutation will cause an extra change Δp_e in frequency of the YFP background

$$\Delta p_e = s_e p_o (1\text{-}p_{yfp}).$$

The bias per generation in the estimated selection coefficient is then

$$s_{bias} = \Delta p_e \,/\, (p_{yfp}(1\text{-}p_{yfp})\ 6.6).$$

Solving for the selection coefficient s_e causing a bias in selection at least as large as our precision (*i.e.* of the order of $s_{bias} = 2.10^{-4}$), we obtain $s_e = 0.33$. We are unaware of adaptive changes as high as this in the ecological situation of the growth environments we used. In particular, none has been found in the Lenski experiment. Moreover, our strain has already evolved 10,000 generations, which is the period where the strongest adaptive changes have already been fixed (Barrick et al., 2009). Indeed, the fitness of the relevant population increased by less than 30% in 20,000 generations following the time point at which REL4548 was isolated (Barrick et al., 2009).

Computation within weeks

Each replicated competition within a week starts from the same preculture and a large inoculum (10^6 cells). The worst case is when the beneficial mutation occurs early in half of the competitions. Since we sample $N_{start} = 5.10^5$ cells of a given competitor, it means that

$$p_{start}=\text{Log}[2]/\ N_{start}$$

Using the exact same computation as above, except that the beneficial has only 6.6 generation to change in frequency, we obtain s_e = 476, which is clearly unrealistic. Importantly, we observe inflated var(*s*) within weeks of the same order of magnitude as between weeks, so that it is quite clear that adaptive changes are unable to explain our results.