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**Spatiotemporal manipulation of the mismatch repair system
of *Pseudomonas putida* accelerates phenotype emergence**

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4 1 **Spatiotemporal manipulation of the mismatch repair system**
5 2 **of *Pseudomonas putida* accelerates phenotype emergence**
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1 ABSTRACT

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Developing complex phenotypes in industrially-relevant bacteria is a major goal of metabolic engineering, which encompasses the implementation of both rational and random approaches. In the latter case, several tools have been developed towards increasing mutation frequencies—yet the precise control of mutagenesis processes in cell factories continues to represent a significant technical challenge. *Pseudomonas* species are endowed with one of the most efficient DNA mismatch repair (MMR) systems found in the bacterial domain. Here, we investigated if the endogenous MMR system could be manipulated as a general strategy to artificially alter mutation rates in *Pseudomonas* species. To bestow a conditional mutator phenotype in the platform bacterium *Pseudomonas putida*, we constructed inducible mutator devices to modulate the expression of the dominant-negative *mutL*^{E36K} allele. Regulatable overexpression of *mutL*^{E36K} in a broad-host-range, easy-to-cure plasmid format resulted in a transitory inhibition of the MMR machinery, leading to a significant increase (up to 438-fold) in DNA mutation frequencies and a heritable fixation of mutations in the genome. Following such accelerated mutagenesis-followed-by selection approach, three phenotypes were successfully evolved: resistance to antibiotics streptomycin and rifampicin (either individually or combined) and reversion of a synthetic uracil auxotrophy. Thus, these mutator devices could be applied to accelerate evolution of metabolic pathways in long-term evolutionary experiments, alternating cycles of (inducible) mutagenesis coupled to selection schemes towards the desired phenotype(s).

1 INTRODUCTION

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Systems metabolic engineering and synthetic biology are used to guide the development of microbial cell factories (MCFs) capable of converting renewable raw materials into value-added compounds¹⁻³. However, low productivities and product yields by most MCFs, even after comprehensive optimization of biosynthetic pathways, continue to make the implementation of economically-viable bioprocesses difficult—especially at an industrial scale⁴. Low product yields are often caused by a decrease in cell viability and genetic instability of MCFs under industrially-relevant production conditions⁵⁻⁶. For instance, the presence of growth inhibitors in renewable raw materials (e.g. crude glycerol and biomass hydrolysates) and the accumulation of toxic compounds during fermentation (including metabolic intermediates and target products) are known to negatively impact cell survival⁷⁻⁹.

Adaptive laboratory evolution (ALE), also known as *evolutionary engineering*, is a valuable tool to improve complex phenotypic traits that can be coupled with microbial growth (e.g. tolerance to inhibitors, substrate utilization and growth temperature)¹⁰⁻¹². At its core, ALE involves the extended propagation of a microbial strain or population, typically over hundreds of generations, in the presence of a desired selective pressure. Mutants that accumulate beneficial mutations will occasionally emerge and expand within the population over time. Selected mutants displaying enhanced phenotypes can be subsequently characterized and sequenced towards reverse engineering¹⁰⁻¹⁵. Unlike purely rational approaches, ALE facilitates the identification of non-intuitive beneficial mutations that occur in a variety of genes in parallel without requiring any knowledge of underlying genetic mechanisms.

Since intrinsic DNA mutation rates are typically very low (ranging in the order of 10^{-9} – 10^{-10} per base pair per generation)¹⁶⁻¹⁷, small and transient increases in mutation frequency can significantly improve the accumulation of beneficial mutations in microbial populations^{12,18}. This rationale has been applied to certain ALE experiments, where the genetic diversity of a microbial population was increased before and/or during growth under restrictive culture conditions^{5,15,19-20}. Chemical and/or physical mutagenesis techniques have been traditionally used due to their simplicity and wide applicability²¹⁻²², but other genome-wide random mutagenesis techniques can be also applied for this purpose⁵. Mutator strains, i.e. bacteria displaying higher mutation rates, frequently have mutations in one or several genes encoding DNA repair or error-avoidance systems²³. Most bacteria control DNA substitution rates through

1 overlapping DNA repair mechanisms, subdivided into three main categories: (i) base selection, (ii)
2 proofreading and (iii) mismatch repair (MMR)²⁴. Base selection encompasses the discrimination between
3 correct and incorrect nucleotides by DNA polymerase, while proofreading is the subsequent editing of the
4 newly incorporated nucleotide by a 3'→5' exonuclease activity that hydrolyzes incorrect bases. Following
5 replication, newly replicated DNA is checked by a MMR system that recognizes and corrects mismatches
6 resulting from replication errors²⁴. Specific mutations in components of MMR (e.g. *mutL* and *mutS*) or in
7 proofreading DNA polymerases (e.g. *dnaQ*), as well as the overexpression of certain dominant-negative
8 mutator alleles of the same genes, have been shown to result in mutator phenotypes^{23,25}. Conditional
9 mutator phenotypes have been applied to the phenotypic optimization of MCFs²⁶⁻³². However, a major
10 problem of these conditional phenotypes is the relatively low control of temporal activity afforded by the
11 cognate devices. A typical drawback of these systems is that the ability of effectively halting DNA
12 mutagenesis is limited, and the cells will continue to mutate even after a desired phenotype is achieved³³.

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14 *Pseudomonas putida* is a ubiquitous Gram-negative bacterium used for biotechnological and
15 bioremediation applications³⁴⁻³⁷. Strain KT2440, for instance, is a promising microbial *chassis* for handling
16 the synthesis of difficult-to-produce chemicals involving harsh reactions and complex biochemistries³⁶⁻⁴⁰.
17 Alas, metabolic engineering of *P. putida* still relies largely on trial-and-error approaches. While advanced
18 genome-wide engineering tools are being constantly developed and optimized⁴¹⁻⁴⁴, complex phenotypes
19 are the result of multi-level regulatory layers that are often difficult to design from first principles. ALE has
20 recently started to be exploited in *P. putida*-based MCFs⁴⁵⁻⁵⁰. On this background, we set out to explore
21 if genome-wide mutation rates in *P. putida* (both wild-type strain and reduced-genome derivatives thereof)
22 could be increased by synthetic control of the well-characterized MMR in this bacterium⁵¹. To this end, in
23 this work we have designed a toolbox to conditionally increase DNA mutation rates by specifically
24 interfering with the endogenous MMR system towards accelerating the evolution of specific phenotypes.
25 Moreover, we focused on the adoption of emerging strategies to easily cure plasmid-born mutator devices
26 from bacterial populations, such that the temporal window of increased mutagenesis rates can be
27 externally controlled. The application of this set of synthetic mutator devices has been systematically
28 validated in evolution experiments targeting both antibiotic resistance and growth phenotypes *via*
29 reversion of a synthetic auxotrophy.

RESULTS AND DISCUSSION

Construction of broad-host-range, plasmid-based mutator devices to increase DNA mutation rates. *Pseudomonas* species have been shown to display one of the highest MMR efficiencies found in bacteria (e.g. as observed in *P. fluorescens*⁵²). Therefore, we hypothesized that manipulating the endogenous MMR system could be a straightforward approach to increase DNA mutation rates in bacterial species of the *Pseudomonas* genus. In order to bestow a conditional mutator phenotype in our model bacterium *P. putida*, we constructed two inducible mutator devices, based on well-characterized expression systems, to tightly modulate the expression of the mutator allele *mutL*^{E36K} from *P. putida*⁵¹ (**Fig. 1** and **Fig. S1** in the Supporting Information). The E36K amino acid change in MutL stems from a 106(G→A) point mutation in the corresponding allele (**Fig. S1**). The overexpression of the homologous, dominant-negative allele *mutL*^{E32K} from *Escherichia coli* has been shown to result in a transitory inhibition of the MMR machinery^{25,53}, which leads to the heritable fixation of mutations in the genome by tampering with the MMR system (**Fig. 1a**). The *mutL*^{E36K} allele, in contrast, has been exploited for genome engineering approaches specifically developed for *P. putida* and related species⁵⁴. In our mutator devices, the expression of *mutL*^{E36K} is driven from two tightly-regulated expression systems, i.e. the thermoinducible *cl857/P_L* expression system from bacteriophage λ and the cyclohexanone-inducible *ChnR/P_{chnB}* system from *Acinetobacter johnsonii*. Both expression vectors have been previously employed for heterologous gene expression in Gram-negative bacteria such as *E. coli* or *P. putida*⁵⁵⁻⁵⁹. Thus, the *mutL*^{E36K} gene was cloned into vectors pSEVA2514 (*cl857/P_L*) and pSEVA2311 (*ChnR/P_{chnB}*) to yield the mutator plasmids pS2514M and pS2311M, respectively (**Fig. 1b**). The subsequent transfer of the mutator devices and plasmids developed herein to various bacterial hosts is facilitated by adopting the rules set in the *Standard European Vector Architecture* (SEVA) platform⁶⁰. Moreover, the implementation of these two expression systems enables the user to decide whether induction of the devices can be done by a temperature shift (to 40°C) or addition of chemicals to the culture medium (cyclohexanone). These two approaches were selected as the first one (*cl857/P_L*) relies on relieving the transcriptional repression mediated by the *cl857* protein when it gets degraded at 40°C, whereas the *ChnR/P_{chnB}* system acts *via* direct activation of the transcriptional response upon addition of a low-cost, small-molecule inducer (**Fig. 1c**). Thermal induction of plasmids pSEVA2514/M was done at 40°C (instead of 42°C, reported to be the most efficient temperature for inactivation of the *cl857* repressor⁶¹), because *P. putida* KT2440 can only survive short periods of exposure at 42°C but it can grow for several

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4 1 hours at 40°C as reported previously⁵⁷. Moreover, thermal induction facilitates alternating cycles of non-
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6 2 induction and induction of DNA mutagenesis by controlling the temperature without the need of removing
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8 3 the inducers from the culture medium—which is oftentimes difficult and time-consuming. The next step
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10 4 was the calibration of these tools as indicated in the sections below.
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6 **Emergence of antibiotic resistance phenotypes in *P. putida* carrying synthetic mutator devices.**

14 7 To investigate the functionality of the mutator devices, the occurrence of antibiotic-resistant mutants was
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16 8 assessed in bacterial cultures grown in liquid medium. Two types of antibiotic resistance were selected
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18 9 to this end, namely, rifampicin (Rif) and streptomycin (Str), and the systems were firstly tested in the wild-
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20 10 type strain KT2440. In these experiments, control strains (i.e. *P. putida* KT2440/pSEVA2514 and
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22 11 KT2440/pSEVA2311) and their derivatives carrying the conditional mutator devices (i.e. *P. putida*
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24 12 KT2440/pS2514M and KT2440/pS2311M) were cultured at 30°C in non-selective M9 minimal medium
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26 13 containing 20 mM glucose, and subjected to a mutagenesis protocol as indicated in **Fig. 2** and *Methods*.
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28 14 In the case of strains carrying vectors with the cl857/P_L expression system, cultures were shifted at 40°C
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30 15 for 15 min for induction; whereas liquid cyclohexanone was added at 1 mM (and not removed afterwards)
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32 16 in cultures of the strains transformed with vectors bearing the ChnR/P_{chnB} system. Cultures were re-
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34 17 incubated at 30°C and were halted at different phases of bacterial growth (i.e. early-exponential, mid-
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36 18 exponential or stationary phase) to assess the appearance of the target phenotypes.
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38 20 The occurrence of mutants developing resistance to either Rif or Str was investigated in the bacterial
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40 21 populations after the treatments indicated above. Resistance to these antibiotics has been widely used
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42 22 for the investigation of spontaneous and induced mutagenesis processes in Gram-negative bacteria⁶²⁻⁶⁴.
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44 23 Rifampicin-resistant (Rif^R) and streptomycin-resistant (Str^R) phenotypes occur due to the appearance of
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46 24 mutations in the *rpoB* and *rpsL* genes, encoding the β-subunit of RNA polymerase and the 30S ribosomal
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48 25 protein S12, respectively⁶²⁻⁶⁵. Mutation frequencies were estimated by assessing the frequency of
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50 26 occurrence of Rif^R or Str^R cells on the total number of viable cells in the bacterial population for each
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52 27 tested experimental condition (**Fig. 3**). In all accelerated mutagenesis experiments, we observed a
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54 28 significantly higher number of Rif^R and Str^R mutants isolated in selective conditions in bacterial clones
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56 29 carrying a mutator device compared to their respective control strains (**Fig. 3**). A visual example of this
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58 30 general trend is presented in **Fig. S2** in the Supporting Information. The number of Rif^R and Str^R colonies
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60 31 present in 5 mL of non-diluted cultures of *P. putida* KT2440/pSEVA2311 (plated after concentrating the

1 biomass by centrifugation and resuspension) was roughly similar to that in selective plates seeded with
2 only 100 μ L of an undiluted culture of *P. putida* KT2440/pS2311M. When these differences were
3 quantified, we observed that the frequency of appearance of Rif^R and Str^R mutants in *P. putida*
4 KT2440/pS2311M was 438- and 10-fold higher, respectively, as compared to the control strain when the
5 induction of the expression system was stopped in early-exponential growth phase (**Fig. 3a**). In the same
6 experimental conditions, the frequency of occurrence of Rif^R and Str^R mutants in *P. putida*
7 KT2440/pS2514M was 45- and 14-fold higher compared to the control strain, respectively (**Fig. 3b**).
8 Similar relative mutation frequencies were observed when the cultures of the different recombinant strains
9 were prolonged until reaching mid-exponential and stationary phase (**Fig. 3**). The largest differences in
10 mutation frequencies were observed in actively-growing cells (i.e. during the early- or mid-exponential
11 phase of growth) as compared to bacteria harvested during the stationary phase⁶⁶⁻⁶⁸. We note that the
12 differences detected in the basal level of mutation frequencies between the two control strains (**Fig. 3**)
13 may be related to intrinsic structural properties of each of the plasmids (e.g. different origins of replication,
14 or the level of expression of regulatory genes therein) and the mutagenesis protocol used (either
15 temperature-shift or addition of a chemical inducer to the culture medium). Although the exact nature of
16 the differences is difficult to quantify, these variables could exert some degree of stress in the host cells⁶⁹⁻
17 ⁷⁰. Formation of reactive oxygen species, in turn, could lead to a slight increase in the mutation rates⁷¹⁻⁷².
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19 Taken together, these experimental data demonstrate the functionality of the mutator devices developed
20 in this work to temporarily increase the global mutation rate in *P. putida*. The differences detected in the
21 mutation frequencies as elicited by the two mutator devices may be related to intrinsic properties of each
22 of the plasmids that carry the mutator allele (e.g. origin of replication and promoter used, since this will
23 affect the transcriptional output), as well as to the protocols followed to induce the expression of *mutL*^{E36K}.
24 Moreover, differences in mutation frequencies are known to arise depending on the method used for their
25 estimation (i.e. counting the occurrence of Rif^R or Str^R clones). On the one hand, mutation frequencies
26 and the actual spectrum of mutations have been shown to vary at different chromosomal positions in
27 several bacterial species, including *P. putida*⁷³⁻⁷⁵. Other genetic factors, such as the orientation of the
28 target gene in the replication fork, its level of transcription and/or the immediately flanking nucleotides can
29 also influence the mutation frequency⁷³⁻⁷⁴. On the other hand, the nature of the mutations acquired by
30 *rpoB* (*PP_0447*) and *rpsL* (*PP_0449*) has been demonstrated to lead to distinct levels of resistance to
31 both Rif and Str, which makes it difficult to use these phenotypes for a direct, quantitative estimation of

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4 1 global mutation rates in different bacterial strains. Factors such as the time and temperature of incubation
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6 2 in selective medium (i.e. agar plates supplemented with antibiotic) have been also shown to dramatically
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8 3 affect the estimation of mutation frequencies (e.g. due to the appearance of colonies with uneven sizes)⁶⁴.
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10 4 Therefore, the utilization of alternative phenotypes is highly recommendable for the calibration and
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12 5 validation of our mutator tool. This issue was undertaken as explained in the next section.
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15 7 **Reversion of a uracil auxotrophy in *P. putida* using mutator devices.** To further calibrate the mutator
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17 8 vectors and gain insight into growth phenotypes beyond antibiotic resistance, we investigated the
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19 9 reversion of uracil auxotrophy of the *P. putida pyrF* HM (**Table 1**). This strain is a derivative of reduced-
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21 10 genome *P. putida* EM42 carrying a 163(A→T) mutation in *pyrF*, which results in a Lys55STOP change
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23 11 in the PyrF protein⁷⁶. This change, in turn, leads to abortive translation of the cognate mRNA and the
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25 12 strain thus lacks a functional orotidine 5'-phosphate decarboxylase (i.e. Ura⁻ phenotype), an essential
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27 13 activity for bacterial growth on minimal medium. In these experiments, the control strains, i.e. *P. putida*
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29 14 *pyrF* HM/pSEVA2514 and *pyrF* HM/pSEVA2311, and the conditional mutator strains, i.e. *P. putida pyrF*
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31 15 HM/pS2514M and *pyrF* HM/pS2311M, were cultured at 30°C in non-selective medium (i.e. with uracil
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33 16 supplementation) and subjected to the mutagenesis protocol indicated in **Fig. 2**. After treatment, the
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35 17 cultures were re-incubated at 30°C, and were harvested upon a doubling in the population size (i.e. early-
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37 18 exponential phase). The emergence of uracil prototrophic mutants (Ura⁺) in the evolved bacterial
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39 19 populations was determined by seeding M9 minimal medium agar plates with glucose but without uracil
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41 20 supplementation. Mutation frequencies were estimated by assessing the frequency of occurrence of Ura⁺
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43 21 mutants on the total number of viable cells in the population for each tested experimental condition (**Fig.**
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45 22 **4a**). A significant higher number of Ura⁺ mutants were isolated from the bacterial populations carrying the
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47 23 conditional mutator devices as compared to their respective control strains, again validating the
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49 24 functionality of the mutator tools. In fact, we only isolated a negligible number (0-4) of spontaneous Ura⁺
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51 25 mutants in bacterial populations of control strains under these experimental conditions. Under these
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53 26 conditions, we estimated mutation frequencies with the cyclohexanone-inducible and thermoinducible
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55 27 mutator systems within the same range (i.e. 750 and 860 Ura⁺ mutants per 1×10⁹ viable *P. putida* cells,
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57 28 respectively, **Fig. 4a**). The next objective in this experiment was studying the nature of the mutations
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59 29 acquired by the Ura⁺ clones.
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1 1 **The conditional mutator phenotype favors the emergence of transition mutations in the genome.**

2 2 To investigate the nature of the mutations introduced with the mutator devices, the whole *pyrF* gene
3 3 (*PP_1815*) was amplified by high-fidelity PCR from several Ura⁺ clones and the resulting amplicons were
4 4 sequenced (**Fig. 4b**). All the revertant (i.e. Ura⁺) clones had a very similar growth phenotype when grown
5 5 in M9 minimal medium with glucose as the only carbon source, both when compared to each other or to
6 6 their parental strain EM42 (**Fig. S3a-d** in the Supporting Information). Firstly, we analyzed and catalogued
7 7 the mutations that may occur spontaneously at codon 55 of the *pyrF* gene (**Fig. S3e** in the Supporting
8 8 Information). Next, we isolated multiple Ura⁺ mutants from two independent evolution experiments
9 9 performed with the conditional mutator strains (i.e. *P. putida pyrF* HM/pS2514M and *pyrF* HM/pS2311M).
10 10 The DNA transitions 164(A→G) or 163(T→C), which both eliminate the premature *STOP* codon in the
11 11 *pyrF*^{163(A→T)} variant that leads to uracil auxotrophy, were equally frequent in the mutants analyzed across
12 12 different independent experiments (**Fig. 4b** and **4c**). These observations indicate that the mutator devices
13 13 seem to mediate transition mutations, i.e. changes from a purine nucleotide to another purine (i.e. A:T ↔
14 14 G:C) or a pyrimidine nucleotide to another pyrimidine (C:G ↔ T:A). Note that only these two transitions
15 15 could lead to the disappearance of the premature *STOP* codon and to the production of a potentially
16 16 functional full PyrF protein (**Fig. S3e** in the Supporting Information). When the *pyrF* sequence was
17 17 analyzed in the few Ura⁺ mutants isolated from experiments with the controls strains, we found a
18 18 significant enrichment of transversion mutations, e.g. 163(T→G), 164(A→T) and 164(A→C) (**Fig. 4c**).
19 19 Interestingly, we could not isolate Ura⁺ mutants displaying the wild-type *pyrF* sequence (i.e. with a Lys
20 20 residue at position 55 of PyrF; **Fig. 4b**) in any of these experiments. This finding may be simply due to
21 21 probabilistic reasons (i.e. number of clones isolated and analyzed, or the topology of the gene and access
22 22 to the MMR machinery).

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24 24 In agreement with our results, Long *et al.*⁵² showed that transition mutations are 16 to 82-fold more
25 25 abundant than transversions in bacterial strains lacking a functional MMR system (both *Deinococcus*
26 26 *radiodurans* and *P. fluorescens*), in sharp contrast to the mere < 3-fold found in the wild-type strains (i.e.
27 27 spontaneously occurring). Horst *et al.*²³ also indicated that DNA transitions and frameshift mutations were
28 28 more abundant in *E. coli* cells lacking a functional MMR system. Regardless of the nature of the mutations
29 29 introduced by these tools, these experiments show that the conditional mutator devices can be used to
30 30 accelerate the emergence of different phenotypes. However, a major limitation of this set of plasmid-
31 31 borne devices is the difficulty of curing them from the target cells, even in the absence of selective

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4 1 pressure. This shortcoming was fixed by constructing a new generation of ‘curable’ mutator devices as
5 explained below.
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10 4 **Design and validation of a new generation of plasmid-based, easy-to-cure mutator devices for**
11 **Gram-negative bacteria.** Previous attempts to cure isolated clones from the set of mutator plasmids
12 based on vectors pSEVA2311 and pSEVA2514 proved unsuccessful, even after >10 repeated passages
13 of individual colonies under non-selective conditions (data not shown). This situation not only precludes
14 precise temporal control of the accelerated evolution protocol, but also prevents the precise assessment
15 of the (potential) occurrence of secondary mutations in the genome that do not have a selectable
16 phenotype associated to their emergence. In particular, whole-genome sequencing needs to be
17 performed to study the frequency and nature of mutations arising in conditional mutator strains, as well
18 as the *global* mutation rates—as opposed to the *local* effects in individual genes that confer a macroscopic
19 phenotype. In order to overcome this state of affairs, and due to the tedious work required to cure the
20 mutator and control plasmids in all the strains previously tested, we decided to build a new version of
21 easy-to-cure mutator systems using a technology recently developed in our laboratory. This methodology
22 relies on the target curing of vectors by means of *in vivo* digestion mediated by the I-SceI homing
23 endonuclease⁷⁷. For this purpose, we constructed vectors pS2311SG, pS2514SG, pS2311SGM and
24 pS2514SGM by USER assembly (**Table 1** and **S1**). These standardized vectors, which are all derivatives
25 of pSEVA2311, pSEVA2514, pS2311M and pS2514M, respectively, contain (i) an engineered I-SceI
26 recognition site that can be recognized and cleaved off by the endonuclease I-SceI of *Saccharomyces*
27 *cerevisiae*⁷⁸ and (ii) a module for the constitutive expression of *msfGFP* (i.e. $P_{EM7} \rightarrow msfGFP$, where the
28 gene encoding the monomeric superfolder GFP is placed under control of the synthetic P_{EM7} promoter)
29 (**Fig. 5a**). This last module facilitates the selection of bacterial clones by examination of green-fluorescent
30 colonies under blue light during the plasmid curing protocol. To this end, the accelerated mutagenesis
31 protocol was upgraded by including a plasmid-curing step (presented in **Fig. S4** in the Supporting
32 Information). In this case, isolated clones are transformed with a helper plasmid that carries the gene
33 encoding the I-SceI endonuclease under control of the 3-methylbenzoate (3-*mBz*)–inducible *XylS/Pm*
34 expression system. Loss of the plasmid carrying the mutator device can be easily inspected as the
35 corresponding colonies will also lose green fluorescence. Importantly, the presence of mutator plasmids
36 does not affect the growth profile of the host (**Fig. S5** in the Supporting Information).
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4 1 In these experiments, we firstly subjected the control strains (i.e. *P. putida* KT2440/pS2514SG and
5 2 KT2440/pS2311SG) and the conditional mutator strains carrying the new set of plasmids (i.e. *P. putida*
6 3 KT2440/pS2514SGM and KT2440/pS2311SGM) to the standard mutagenesis protocol to confirm the
7 4 functionality of the easy-to-cure devices (**Fig. S4** in the Supporting Information). We investigated the
8 5 emergence of Str^R mutants after implementing the accelerated mutagenesis protocol, and new induction
9 6 conditions were tested to further characterize the tools. As expected, most of the recombinant strains
10 7 harboring the easy-to-cure plasmids behaved quite similarly to the original strains carrying the first
11 8 generation mutator devices (**Table S2** in the Supporting Information). The mutation frequency mediated
12 9 by the mutator allele under control of the *cl857/P_L* expression system was essentially identical in all
13 10 experiments, irrespective of whether the original or the upgraded set of plasmids was used. We detected
14 11 a lower mutation frequency in strain KT2440/pS2311SGM (ca. 60% lower than the values observed in
15 12 strain KT2440/pS2311M under similar experimental conditions). Such a trait was consistently
16 13 accompanied by loss of green fluorescence in a significant proportion of the bacterial colonies isolated in
17 14 solid medium, i.e. lysogeny broth (LB) agar, with or without Str supplementation. This result could be due
18 15 to multiple factors, e.g. accumulation of loss-of-function mutations in the *msfGFP* gene stimulated by the
19 16 same mutator device or unexpected decay or loss of the mutator plasmid in the absence of selection
20 17 pressure [i.e. plasmid-borne kanamycin (Km) resistance].
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19 To investigate the hypothesis above, we repeated the accelerated mutagenesis protocol with strain
20 KT2440/pS2311SGM while maintaining Km selection on the plates. We observed that, in the presence of
21 the selection pressure borne by the mutator plasmid, all the bacterial colonies maintained green
22 fluorescence and the overall mutagenesis frequencies were significantly higher than in all previous
23 experiments (e.g. 270-fold higher than in the experiments with the same plasmid but omitting Km; **Table**
24 **S2**). Under these experimental conditions, the cyclohexanone-inducible mutator plasmids appear to
25 exhibit leaky expression of the *mutL^{E36K}* allele, which led to similar mutagenesis frequencies in the
26 absence or presence of inducer (**Table S2**). This observation helps explaining why, in the absence of
27 selection pressure, some cells may reduce the copy number of the pS2311SGM plasmid to alleviate the
28 mutagenic effects caused by (semi)constitutive expression of *mutL^{E36K}*—or even force complete plasmid
29 loss in some clones. Most of the colonies analyzed in these experiments showed a significant reduction
30 in the plasmid copy number (and not a total loss of plasmid) accompanied by a dramatic reduction in the
31 fluorescence levels. At the bacterial population level, this phenomenon could further translate into an

1 overall decrease of the *global* mutagenesis frequency. This behavior was not observed in strain
2 KT2440/pS2514SGM, which appears to exhibit a lower—but more tightly-regulated—expression level of
3 the mutator allele than the ChnR/P_{chnB} counterpart (**Table S2**). Actually, extending the thermal induction
4 of the cl857/P_L-based mutator devices from 15 to 30 min did not affect the *global* mutagenesis frequency.
5 In either case, the genetic upgrading of the plasmid toolbox was meant to facilitate the easy curing of the
6 mutator devices, and the results of these experiments are explained in the next section.

7
8 **Easy-to-cure mutator devices enable a tight control of the global mutagenesis and reveal a wide**
9 **landscape of genome modifications upon accelerated evolution.** We decided to sequence the whole
10 genome of several colonies isolated in non-selective medium (i.e. LB agar, 2-5 colonies for each
11 experimental condition) in order to assess the frequency and nature of mutations mediated by the mutator
12 devices. To this end, green-fluorescent colonies were selected after treatment (**Fig. S4** in the Supporting
13 Information) and transformed with the helper pQURE6-L plasmid⁷⁷, a conditionally-replicating vector that
14 requires supplementation of 3-*mBz* to the culture medium to ensure plasmid maintenance (**Fig. S6** in the
15 Supporting Information). As indicated above, plasmid pQURE6-L carries a synthetic module for the 3-
16 *mBz*-inducible expression of the *I-SceI* endonuclease gene (i.e. *XylS/P_m→I-SceI*; **Table 1**) and a second
17 module for the constitutive expression of *mRFP* (i.e. *P_{14g}→mCherry*), which, together, facilitate quick
18 curing of mutator plasmids by positive selection of red-fluorescent colonies (**Fig. S4** in the Supporting
19 Information; see also *Methods* for details on the curing procedure). In all cases, the mutator devices could
20 be easily cured upon introduction of plasmid pQURE6-L. Moreover, this helper plasmid could be typically
21 cured during a simple overnight incubation of individual colonies in LB medium without 3-*mBz* (data not
22 shown), similarly to the observations reported by Volke *et al.*⁷⁷

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24 Multiple colonies were isolated from the accelerated mutagenesis experiments using the upgraded
25 mutator toolbox and, upon curing all plasmids, genomic DNA was extracted and purified prior to next
26 generation sequencing. Whole-genome sequencing of genomic DNA enabled a precise elucidation of the
27 nature of mutations elicited by these devices. In general, whole-genome sequencing data confirmed our
28 previous findings, as the emergence of transitions was a clear signature of clones carrying the *mutL*^{E36K}
29 allele in different configurations (**Fig. 5b** and **Table S3** in the Supporting Information). These single-
30 nucleotide polymorphisms were largely non-synonymous, and transversions were observed to be
31 extremely rare (i.e. 1 transversion per genome in a just a few isolated clones, no different from the

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4 1 frequency of transversions in any of the control strains). Importantly, the mutator devices also promoted
5 2 the emergence of small insertion-deletion mutations (InDel, mostly consisting of 1-2 bp; **Fig. 5b** and **Table**
6 3 **S4** in the Supporting Information). Frameshift insertions were the most abundant type of InDels detected
7 4 in the isolated clones. Taken together, and consistently with the results of experiments reported in the
8 5 previous section, the detailed exploration of mutations elicited by the cyclohexanone-inducible mutator
9 6 devices indicate that this system promotes a nearly-constitutive mutator phenotype. This feature, in turn,
10 7 triggers a relatively high mutagenesis frequency over short induction periods—probably caused by the
11 8 leakiness observed for this system under these conditions.
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13 10 **Evolution of dual antibiotic resistances in *P. putida* with mutator devices.** The results discussed in
14 11 the previous sections indicate that the enrichment of point mutations afforded by the conditional mutator
15 12 systems can be harnessed to select for monogenetic phenotype traits. Next, we asked the question if the
16 13 system can be used to accelerate the emergence of more complex phenotypes. To this end, we adopted
17 14 dual resistance to Rif^R and Str^R as a proxy. The upgraded mutagenesis protocol (**Fig. S4**) was repeated
18 15 by using *P. putida* KT2440 as the host, transformed with either vector pSEVA2311SG (control) or plasmid
19 16 pS2311SGM (where the mutagenic *mutL*^{E36K} allele is controlled by a ChnR/P_{chnB} module). After incubating
20 17 the cultures in M9 minimal medium with 20 mM glucose for 24 h, the emergence of double-resistant
21 18 mutants was estimated by plating the bacterial suspension onto LB agar supplemented with Km, Rif and
22 19 Str. As observed in experiments involving single antibiotic resistances, a significant difference in the
23 20 number of Km^R, Rif^R and Str^R was immediately evident when comparing plates seeded with *P. putida*
24 21 KT2440/pSEVA2311SG or *P. putida* KT2440/pS2311SGM even by the naked eye (**Fig. 6a**). Indeed, the
25 22 emergence of double Rif^R and Str^R *P. putida* mutants was 6-fold higher in cultures of cells carrying the
26 23 mutator device than in control experiments (**Fig. 6b**). These results validate the use of the tools
27 24 constructed herein for the evolution of multigenic traits by the adoption of a simple protocol that can be
28 25 iterated (and even automated) as needed.
29 26

27 28 **CONCLUSION**

29 29 In this work, we have constructed two synthetic biology devices to control the mutation rate in *P. putida*—
30 30 and, due to the nature of the vectors used for these constructs, other Gram-negative bacteria as well—in
31 31 a precise spatiotemporal fashion. Transiently overexpressing the endogenous dominant negative

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4 1 *mutL*^{E36K} allele of *P. putida* interfered with the normal functioning of the endogenous MMR machinery,
5 2 thereby increasing mutation frequencies in multiple strains of *P. putida* by 2- to 438-fold. Following a
6 3 'mutagenesis-followed-by selection' approach, we have successfully evolved three separate phenotypes
7 4 arising from monogenic traits, i.e. resistance to the antibiotics Str and Rif and uracil prototrophy. Within
8 5 this approach, we have firstly increased the genetic diversity in the bacterial population by inducing the
9 6 activity of the synthetic mutator devices and, subsequently, isolated mutants onto a selective solid
10 7 medium. In these experiments, the expression of the mutator *mutL*^{E36K} allele was driven from two inducible
11 8 modules, i.e. the thermoregulated *cl857/P_L* and the cyclohexanone-regulated *ChnR/P_{chnB}* expression
12 9 systems, which have been previously tailored for heterologous gene expression in different Gram-
13 10 negative bacterial species. We observed that the mutation frequencies achieved with the cyclohexanone-
14 11 inducible mutator devices (i.e. vectors pS2311M and pS2311SGM, which represent the first and second
15 12 generation of the tools constructed in this study) were significantly higher than those obtained with the
16 13 thermoinducible mutator counterparts (i.e. vectors pS2514M and pS2514SGM) under most of the
17 14 experimental conditions tested. In agreement with previous studies conducted with *E. coli* and related
18 15 species, we have also observed a higher emergence of transition and frameshift (InDel) mutations in cells
19 16 displaying a temporarily-tampered MMR system²³. We note, however, that this fact should not be
20 17 interpreted as an inherent limitation of the mutator devices developed here, since all of the available
21 18 mutagenesis techniques exhibit mutation biases to different degrees^{21,79}. The preferential enrichment of
22 19 a certain type of mutations is thus unavoidable, irrespective of the system used to induce mutagenesis.
23 20

24 21 Interestingly, the cyclohexanone-triggered mutator devices had a significant level of leaky expression of
25 22 *mutL*^{E36K}, which in turn promoted a nearly-constitutive mutator phenotype that lead to high mutagenesis
26 23 rates. The mutation frequencies achieved with this system were, however, lower than those reported with
27 24 constitutive mutator strains where the mutator phenotype was originated by modifications in components
28 25 of the endogenous MMR system. For example, Kurusu *et al.*⁸⁰ reported that the frequency of occurrence
29 26 of Rif^R mutants in a Δ *mutS* derivative of *P. putida* KT2440 was 1,000-fold higher than that in the wild-type
30 27 strain. Since mutation rates must be precisely controlled to avoid extensive accumulation of deleterious
31 28 mutations and to prevent genomic instability, the overexpression of mutator alleles should be driven from
32 29 tightly-regulated expression systems (which is always challenging, irrespective of the bacterial host⁵⁹) or
33 30 during short periods of time. Thus, the easy-to-cure mutator plasmids developed in this study, which can
34 31 be rapidly removed from isolated clones displaying the phenotype of interest, offer a clear advantage over
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4 1 conventional mutator strains—where the mutator phenotype is elicited by genomic (hence, essentially
5 2 irreversible) modifications, as epitomized by the emergence of mutator phenotypes of *P. aeruginosa* in
6 3 clinically-relevant setups⁸¹⁻⁸². In contrast with the results of the ChnR/P_{chnB}-dependent module, the
7 4 thermoinducible mutator devices allowed for a tightly-regulated expression of *mutL*^{E36K}. This tool may be
8 5 applied to long evolutionary experiments that involves alternating cycles of non-induction and induction
9 6 of mutagenesis coupled to phenotype selection (e.g. growth-coupled approaches). By modifying the
10 7 induction conditions and the number of induction cycles, a landscape of mutation rates could be achieved
11 8 and adapted to the needs of each evolutionary experiment. The control of these parameters might be
12 9 crucial for accelerating the evolution of complex phenotypes in industrial MCFs, since it has been
13 10 previously shown that microbial adaptation to specific stresses is favored with certain mutation rates⁸³.
14 11 Due to its particular metabolic architecture, this would likely be the case for *P. putida* as well⁸⁴.
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16 13 Along the same line, the method described herein suppresses the need of handling chemical or physical
17 14 mutagens—which are typically carcinogenic and, oftentimes, acutely toxic—for phenotype evolution. As
18 15 an example, implementing physical methods for mutagenesis (e.g. UV irradiation) in liquid cultures is not
19 16 only technically difficult but will unavoidably result in an uneven exposure of the cells to the irradiation
20 17 source⁸⁵. UV irradiation results in a single type of mutation in the DNA (i.e. thymine dimer lesions), thereby
21 18 reducing the mutational landscape that can be obtained with such method. Thus, using mutator plasmids
22 19 represents an advantage over the adoption of traditional mutagens, enabling safer operation conditions
23 20 and circumventing the subsequent treatment of contaminated materials. Also, the thermoinducible
24 21 mutator device described herein allows to alternate cycles of non-induction and induction of mutagenesis
25 22 by merely controlling the incubation temperature—altogether suppressing the addition of any chemicals
26 23 to the culture medium.
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28 25 From a more general perspective, it should be noted that the MutL/MutS protein complex of the MMR
29 26 machinery appears to be well-conserved in most bacterial species⁸⁶⁻⁸⁷. For instance, the MutS protein
30 27 from *P. putida* and the MutL protein from *P. aeruginosa* were shown to functionally complement $\Delta mutS$
31 28 and $\Delta mutL$ mutants of *E. coli* and *Bacillus subtilis*, respectively^{80,88}. Therefore, the broad-host-range
32 29 mutator devices developed herein are expected to be functional in other bacterial hosts as well. In addition
33 30 to their application for the accelerated evolution of phenotypes that depend on multiple mutations across
34 31 the bacterial genome, the use of these devices also revealed an important feature of the MMR system
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4 1 relevant for synthetic biology. A number of genome modification approaches rely on specifically interfering
5 2 with the bacterial MMR system to enable strand invasion^{51,53,59,89}. Besides the intended modifications (e.g.
6 3 as encoded in mutagenic oligonucleotides), there are several secondary mutations that could occur due
7 4 to overexpression of mutagenic alleles. The tight spatiotemporal manipulation of this trait, afforded by the
8 5 plasmids reported in this study, could enable a more precise control of genome modifications by restricting
9 6 the mutation landscape to the intended alterations.
10 7

16 8 **METHODS**

10 **Bacterial strains and growth conditions.** The bacterial strains used in this work are listed in **Table 1**.
11 *E. coli* DH5 α was used for cloning and plasmid maintenance. *E. coli* and *P. putida* strains were routinely
12 grown in lysogeny broth (LB) medium (10 g L⁻¹ tryptone, 5 g L⁻¹ yeast extract and 5 g L⁻¹ NaCl) at 37°C
13 and 30°C, respectively, in an orbital shaker at 150 rpm. For mutagenesis experiments, *P. putida* was
14 grown in M9 minimal medium supplemented with 20 mM glucose as the sole carbon source—as typically
15 done when cultivating this species on sugars for physiological studies⁸⁴. Cyclohexanone was added at 1
16 mM to cultures for induction of *mutL*^{E36K} expression as necessary. When appropriate, antibiotics were
17 also added at the following concentrations ($\mu\text{g mL}^{-1}$): gentamicin, 10; Km, 50; Str, 100; and Rif, 50.
18 Supplementation of 20 $\mu\text{g mL}^{-1}$ uracil was implemented to support bacterial growth of uracil-auxotrophic
19 strains. Bacterial growth was estimated by measuring the optical density at 630 nm (OD₆₃₀).
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21 **General DNA manipulations and sequencing.** Molecular biology techniques were performed
22 essentially as described in standard protocols⁹⁰. Oligonucleotides were purchased from Integrated DNA
23 Technologies (IDT; Leuven, Belgium) and their sequences are provided in **Table S1** in the Supporting
24 Information. DNA amplification was performed on a C1000 TouchTM Thermal Cycler (Bio-Rad Corp.,
25 Hercules, CA, USA) using Phusion *U* Hot Start DNA Polymerase or Phusion Hot Start II DNA Polymerase
26 from Thermo Fisher Scientific (Waltham, MA, USA). DNA fragments were purified with a NucleoSpinTM
27 Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). Restriction enzymes and T4 DNA ligase
28 were obtained from Thermo Fisher Scientific and were used according to the supplier's specifications.
29 USER assembly was performed essentially as described by Nour-Eldin *et al.*⁹¹ with the commercial USER
30 enzyme from New England BioLabs (NEB, Ipswich, MA, USA). Plasmid DNA was prepared with a
31 NucleoSpinTM Plasmid EasyPure kit (Macherey-Nagel). *E. coli* chemical competent cells were prepared
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4 1 using the *Mix & Go E. coli* Transformation Kit from Zymo Research (Irvine, CA, USA). DNA amplification
5 2 from a single colony (i.e. colony PCR) was performed with One *Taq* 2× Master Mix (NEB).
6 3 Electrocompetent *P. putida* cells were prepared by washing twice an overnight culture of *P. putida* with
7 4 300 mM sucrose⁹². All cloned inserts and DNA fragments were confirmed by DNA sequencing (Eurofins
8 5 Genomics, Ebersberg, Germany).
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7 **Construction of broad-host range mutator expression vectors.** Plasmid pSEVA2514-*rec2-*
8 *mutL*_{E36K}^{PP}, described by Aparicio *et al.*⁵⁴, was double-digested with *Xba*I and *Hind*III to obtain a 1.9-kb
9 DNA fragment corresponding to the dominant-negative mutator allele *mutL*^{E36K} of *P. putida* KT2440. The
10 purified DNA fragment was subsequently ligated with the pSEVA2541 and pSEVA2311 vectors,
11 previously digested with the same restriction enzymes, to generate plasmids pS2514M and pS2311M,
12 respectively. The easy-to-cure plasmids pS2514SG, pS2514SGM, pS2311SG and pS2311SGM, were
13 subsequently constructed by USER assembly with the primers indicated in **Table S1**. These vectors
14 contain an engineered *I-Sce*I recognition site and an *msfGFP* gene under the control of the constitutive
15 *P*_{EM7} promoter (**Fig. S4** in the Supporting Information), that make them compatible with the plasmid
16 curation approach recently developed by Volke *et al.*⁷⁷.
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18 **Accelerated evolution experiments with *P. putida* recombinant strains carrying mutator plasmids.**

19 Overnight pre-cultures of the conditional mutator strains [e.g. *P. putida* KT2440/pS2514(SG)M and
20 KT2440/pS2311(SG)M], as well as of their respective control strains [e.g. *P. putida* KT2440/pS2514(SG)
21 and KT2440/pS2311(SG)], were used to inoculate 25 mL of non-selective M9 minimal medium at an initial
22 OD₆₀₀ of 0.075. After 5 h of incubation in an orbital shaker at 30°C (OD₆₀₀ = 0.3), the expression systems
23 were induced thermally (by incubation at 40°C for 15 min in a water bath) or chemically (with addition of
24 1 mM cyclohexanone). The cultures were subsequently re-incubated at 30°C with shaking and stopped
25 after 1.5 h (early-exponential phase, OD₆₀₀ = 0.6), 2 h (mid-exponential phase, OD₆₀₀ = 1) or 24 h
26 (stationary phase, OD₆₀₀ = 3). Several aliquots of bacterial cultures were plated on selective solid medium
27 (e.g. LB agar supplemented with 100 μg mL⁻¹ Str or with 50 μg mL⁻¹ Rif as appropriate) to determine the
28 appearance of mutant cells (e.g. Rif^R or Str^R) in the bacterial population. The total number of viable cells
29 in the bacterial population was also estimated by plating dilutions of the cultures on non-selective medium
30 (e.g. LB agar plates). After 32 h of incubation at 30°C, the number of colony forming units (CFUs) in the
31 different culture conditions was estimated by visual inspection of the plates (see **Fig. S2** in the Supporting
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4 1 Information for an example). At least two biological replicates and two technical replicates were performed
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6 2 for each bacterial strain and selective culture condition, respectively.
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9 4 **Vector curing procedure for easy-to-cure plasmids carrying mutator devices.** Overnight pre-cultures
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11 5 of green-fluorescent colonies isolated from evolution experiments were transformed by electroporation
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13 6 with plasmid pQURE6-L (Table 1 and Fig. S6). Transformed cells were recovered in LB medium
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15 7 supplemented with 2 mM 3-*mBz* during 2 h. Dilutions were then plated on LB agar supplemented with 10
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17 8 $\mu\text{g mL}^{-1}$ Gm and 1 mM 3-*mBz*. Red-fluorescent colonies that had lost the mutator plasmids were easily
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19 9 isolated after 24-48 h of incubation at 30°C. For curing the helper pQURE6-L plasmid, overnight pre-
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21 10 cultures of red-fluorescent colonies were grown and dilutions were plated on non-selective medium (e.g.
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23 11 LB agar). Non-fluorescent colonies were selected after 24 h of incubation and were stored for further
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25 12 analysis. Loss of both plasmids in the selected colonies was further confirmed by Gm and Km sensitivity
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27 13 (Fig. S4).
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30 15 **Genomic DNA purification, library construction, and whole genome sequencing (WGS).** DNA was
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32 16 purified using the PureLink™ Genomic DNA purification kit (Invitrogen, Waltham, MA, USA) from 2 mL of
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34 17 overnight LB cultures inoculated from cryostocks prepared after curing the plasmids from the strains. The
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36 18 genomic DNA of each sample was randomly sheared into short fragments of about 350 bp using ultrasonic
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38 19 interruption. Short and large DNA fragments were removed using magnetic bead size selection and
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40 20 subsequently verified by capillary gel electrophoresis. The obtained DNA fragments were subjected to
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42 21 library construction using the NEBNext™ DNA Library Prep Kit (NEB), following the supplier's
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44 22 specifications. Libraries quality control was performed with a Qubit® 2.0 fluorometer and an Agilent™
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46 23 2100 BioAnalyzer. Subsequent sequencing was performed using the Illumina NovaSeq™ 6000 PE150
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48 24 platform. The original sequencing data acquired by high-throughput sequencing platforms recorded in
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50 25 image files were firstly transformed to sequence reads by base calling with the Illumina's CASAVA
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52 26 software. The sequences and corresponding sequencing quality information were stored in a FASTQ file.
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54 27 For quality-control purposes, paired reads were discarded when: (i) either read contains adapter
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56 28 contamination, (ii) uncertain nucleotides (N) constitute >10% of either read, or (iii) low quality nucleotides
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58 29 (base quality less than 5, $Q \leq 5$) constitute >50% of either read. The effective sequencing data were
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60 30 aligned with the reference sequence (NCBI Reference Sequence: NC_002947.4) through Burrows-
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32 31 Wheeler Aligner (BWA) software⁹³, and the mapping rate and coverage were counted according to the

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4 1 alignment results. The duplicates were removed by means of the SAMtools package⁹⁴. Single nucleotide
5 2 polymorphisms (SNPs) and InDels were detected using SAMtools and followed by annotation using
6 3 ANNOVAR⁹⁵. Libraries construction, sequencing and subsequent data quality control was performed by
7 4 Novogene Co. Ltd. (Cambridge, United Kingdom).
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6 SUPPORTING INFORMATION

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16 8 **Table S1.** Oligonucleotides used in this work.

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18 9 **Table S2.** Mutation frequencies estimated with the different versions of mutator plasmids created in this
19 10 work.

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21 11 **Table S3.** Distribution of single nucleotide polymorphisms (SNP) in evolved populations of *P. putida*.

22 12 **Table S4.** Distribution of small insertion-deletion (InDel) mutations in evolved populations of *P. putida*.

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24 13 **Figure S1.** Protein sequence alignment of the NH₂-terminal region of MutL proteins from different
25 14 bacteria.

26 15 **Figure S2.** Appearance of rifampicin- and streptomycin-resistant mutants in populations of *P. putida*
27 16 KT2440 carrying a mutator device.

28 17 **Figure S3.** Growth profile and characteristics of selected *P. putida* Ura⁺ mutants isolated in mutagenesis
29 18 experiments.

30 19 **Figure S4.** Upgraded protocol for accelerated evolution of phenotypes using the new generation of easy-
31 20 to-cure mutator devices.

32 21 **Figure S5.** Growth profile of engineered *P. putida* strains carrying mutator plasmids.

33 22 **Figure S6.** Physical map of the helper pQURE6-L plasmid.
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AUTHOR CONTRIBUTIONS

L.F.C. and P.I.N. designed the experimental plan and the overall research setup. L.F.C. and A.C. carried the experimental work and drafted the figures and the manuscript, with further contributions by P.I.N. All authors discussed the results and interpreted the experimental data.

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CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

REFERENCES

1. Calero, P., and Nikel, P. I. (2019) Chasing bacterial *chassis* for metabolic engineering: A perspective review from classical to non-traditional microorganisms. *Microb. Biotechnol.* 12 (1), 98-124.
2. Chen, Y., Banerjee, D., Mukhopadhyay, A., and Petzold, C. J. (2020) Systems and synthetic biology tools for advanced bioproduction hosts. *Curr. Opin. Biotechnol.* 64, 101-109.
3. Choi, K. R., Jang, W. D., Yang, D., Cho, J. S., Park, D., and Lee, S. Y. (2019) Systems metabolic engineering strategies: Integrating systems and synthetic biology with metabolic engineering. *Trends Biotechnol.* 37 (8), 817-837.

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4 1 4. Ko, Y. S., Kim, J. W., Lee, J. A., Han, T., Kim, G. B., Park, J. E., and Lee, S. Y. (2020) Tools and
5
6 2 strategies of systems metabolic engineering for the development of microbial cell factories for
7
8 3 chemical production. *Chem. Soc. Rev.* 49 (14), 4615-4636.
- 9
10 4 5. Fernández-Cabezón, L., Cros, A., and Nikel, P. I. (2019) Evolutionary approaches for engineering
11
12 5 industrially-relevant phenotypes in bacterial cell factories. *Biotechnol. J.* 14 (9), 1800439.
- 13
14 6 6. Gong, Z., Nielsen, J., and Zhou, Y. J. (2017) Engineering robustness of microbial cell factories.
15
16 7 *Biotechnol. J.* 12 (10), 1700014.
- 17
18 8 7. Amoah, J., Kahar, P., Ogino, C., and Kondo, A. (2019) Bioenergy and biorefinery: Feedstock,
19
20 9 biotechnological conversion, and products. *Biotechnol. J.* 14 (6), e1800494.
- 21
22 10 8. Poblete-Castro, I., Wittmann, C., and Nikel, P. I. (2020) Biochemistry, genetics, and
23
24 11 biotechnology of glycerol utilization in *Pseudomonas* species. *Microb. Biotechnol.* 13 (1), 32-53.
- 25
26 12 9. Becker, J., and Wittmann, C. (2019) A field of dreams: Lignin valorization into chemicals,
27
28 13 materials, fuels, and health-care products. *Biotechnol. Adv.* 37 (6), 107360.
- 29
30 14 10. Portnoy, V. A., Bezdán, D., and Zengler, K. (2011) Adaptive laboratory evolution—Harnessing
31
32 15 the power of biology for metabolic engineering. *Curr. Opin. Biotechnol.* 22 (4), 590-594.
- 33
34 16 11. Dragosits, M., and Mattanovich, D. (2013) Adaptive laboratory evolution—Principles and
35
36 17 applications for biotechnology. *Microb. Cell Fact.* 12, 64.
- 37
38 18 12. Winkler, J. D., and Kao, K. C. (2014) Recent advances in the evolutionary engineering of
39
40 19 industrial biocatalysts. *Genomics* 104 (6), 406-411.
- 41
42 20 13. Shepelin, D., Hansen, A. S. L., Lennen, R. M., Luo, H., and Herrgård, M. J. (2018) Selecting the
43
44 21 best: Evolutionary engineering of chemical production in microbes. *Genes* 9 (5), 249.
- 45
46 22 14. Jang, S., Kim, M., Hwang, J., and Jung, G. Y. (2019) Tools and systems for evolutionary
47
48 23 engineering of biomolecules and microorganisms. *J. Ind. Microbiol. Biotechnol.* 46 (9-10), 1313-
49
50 24 1326.
- 51
52 25 15. Sandberg, T. E., Salazar, M. J., Weng, L. L., Palsson, B. Ø., and Feist, A. M. (2019) The
53
54 26 emergence of adaptive laboratory evolution as an efficient tool for biological discovery and
55
56 27 industrial biotechnology. *Metab. Eng.* 56, 1-16.
- 57
58 28 16. Drake, J. W. (1991) A constant rate of spontaneous mutation in DNA-based microbes. *Proc. Natl.*
59
60 29 *Acad. Sci. USA* 88 (16), 7160-7164.
- 30
31 17. Lang, G. I., and Murray, A. W. (2008) Estimating the per-base-pair mutation rate in the yeast
Saccharomyces cerevisiae. *Genetics* 178 (1), 67-82.

- 1
2
3
4 1 18. Swings, T., van den Bergh, B., Wuyts, S., Oeyen, E., Voordeckers, K., Verstrepen, K. J., Fauvart,
5 M., Verstraeten, N., and Michiels, J. (2017) Adaptive tuning of mutation rates allows fast response
6 2 to lethal stress in *Escherichia coli*. *eLife* 6, e22939.
7 3
8
9 4 19. Kang, M., Kim, K., Choe, D., Cho, S., Kim, S. C., Palsson, B. Ø., and Cho, B. K. (2019)
10 5 Inactivation of a mismatch-repair system diversifies genotypic landscape of *Escherichia coli*
11 6 during adaptive laboratory evolution. *Front. Microbiol.* 10, 1845.
12
13
14 7 20. Wang, X., Li, Q., Sun, C., Cai, Z., Zheng, X., Guo, X., Ni, X., Zhou, W., Guo, Y., Zheng, P., Chen,
15 8 N., Sun, J., Li, Y., and Ma, Y. (2019) GREACE-assisted adaptive laboratory evolution in endpoint
16 9 fermentation broth enhances lysine production by *Escherichia coli*. *Microb. Cell Fact.* 18 (1), 106.
17
18
19 10 21. Foster, P. L. (1991) *In vivo* mutagenesis. *Methods Enzymol.* 204, 114-125.
20
21
22 11 22. Kodym, A., and Afza, R. (2003) Physical and chemical mutagenesis. *Methods Mol. Biol.* 236,
23 12 189-204.
24
25
26 13 23. Horst, J. P., Wu, T. H., and Marinus, M. G. (1999) *Escherichia coli* mutator genes. *Trends*
27 14 *Microbiol.* 7 (1), 29-36.
28
29
30 15 24. Schaaper, R. M. (1993) Base selection, proofreading, and mismatch repair during DNA
31 16 replication in *Escherichia coli*. *J. Biol. Chem.* 268 (32), 23762-23765.
32
33
34 17 25. Aronshtam, A., and Marinus, M. G. (1996) Dominant negative mutator mutations in the *mutL* gene
35 18 of *Escherichia coli*. *Nucleic Acids Res.* 24 (13), 2498-2504.
36
37
38 19 26. Selifonova, O., Valle, F., and Schellenberger, V. (2001) Rapid evolution of novel traits in
39 20 microorganisms. *Appl. Environ. Microbiol.* 67 (8), 3645-3649.
40
41
42 21 27. Emlyn-Jones, D., Price, G. D., and Andrews, T. J. (2003) Nitrogen-regulated hypermutator strain
43 22 of *Synechococcus* sp. for use in *in vivo* artificial evolution. *Appl. Environ. Microbiol.* 69 (11), 6427-
44 23 6433.
45
46
47 24 28. Endo, A., Sasaki, M., Maruyama, A., and Kurusu, Y. (2006) Temperature adaptation of *Bacillus*
48 25 *subtilis* by chromosomal *groEL* replacement. *Biosci. Biotechnol. Biochem.* 70 (10), 2357-2362.
49
50
51 26 29. Shimoda, C., Itadani, A., Sugino, A., and Furusawa, M. (2006) Isolation of thermotolerant mutants
52 27 by using proofreading-deficient DNA polymerase delta as an effective mutator in *Saccharomyces*
53 28 *cerevisiae*. *Genes Genet. Syst.* 81 (6), 391-397.
54
55
56 29 30. Luan, G., Cai, Z., Gong, F., Dong, H., Lin, Z., Zhang, Y., and Li, Y. (2013) Developing controllable
57 30 hypermutable *Clostridium* cells through manipulating its methyl-directed mismatch repair system.
58 31 *Protein Cell* 4 (11), 854-862.
59
60

- 1
2
3
4 1 31. Overbeck, T. J., Welker, D. L., Hughes, J. E., Steele, J. L., and Broadbent, J. R. (2017) Transient
5 2 MutS-based hypermutation system for adaptive evolution of *Lactobacillus casei* to low pH. *Appl.*
6 3 *Environ. Microbiol.* 83 (20), e01120-01117.
7
8
9 4 32. Laviña, W. A., Sakurai, S. S. M., Pontrelli, S., Putri, S. P., and Fukusaki, E. (2020) Metabolomics
10 5 analysis reveals global metabolic changes in the evolved *E. coli* strain with improved growth and
11 6 1-butanol production in minimal medium. *Metabolites* 10 (5), 192.
12
13
14 7 33. Kim, S. G., Noh, M. H., Lim, H. G., Jang, S., Jang, S., Koffas, M. A. G., and Jung, G. Y. (2018)
15 8 Molecular parts and genetic circuits for metabolic engineering of microorganisms. *FEMS*
16 9 *Microbiol. Lett.* 365 (17).
17
18
19 10 34. Nickel, P. I., and de Lorenzo, V. (2018) *Pseudomonas putida* as a functional chassis for industrial
20 11 biocatalysis: From native biochemistry to *trans*-metabolism. *Metab. Eng.* 50, 142-155.
21
22
23 12 35. Poblete-Castro, I., Becker, J., Dohnt, K., Martins dos Santos, V. A. P., and Wittmann, C. (2012)
24 13 Industrial biotechnology of *Pseudomonas putida* and related species. *Appl. Microbiol. Biotechnol.*
25 14 93 (6), 2279-2290.
26
27
28 15 36. Kivisaar, M. (2020) Narrative of a versatile and adept species *Pseudomonas putida*. *J. Med.*
29 16 *Microbiol.* 69 (3), 324-338.
30
31
32 17 37. Martínez-García, E., and de Lorenzo, V. (2019) *Pseudomonas putida* in the quest of
33 18 programmable chemistry. *Curr. Opin. Biotechnol.* 59, 111-121.
34
35
36 19 38. Calero, P., Volke, D. C., Lowe, P. T., Gottfredsen, C. H., O'Hagan, D., and Nickel, P. I. (2020) A
37 20 fluoride-responsive genetic circuit enables *in vivo* biofluorination in engineered *Pseudomonas*
38 21 *putida*. *Nat. Commun.* 11 (1), 5045.
39
40
41 22 39. Sánchez-Pascuala, A., Fernández-Cabezón, L., de Lorenzo, V., and Nickel, P. I. (2019) Functional
42 23 implementation of a linear glycolysis for sugar catabolism in *Pseudomonas putida*. *Metab. Eng.*
43 24 54, 200-211.
44
45
46 25 40. Sánchez-Pascuala, A., de Lorenzo, V., and Nickel, P. I. (2017) Refactoring the Embden-Meyerhof-
47 26 Parnas pathway as a whole of portable *GlucoBricks* for implantation of glycolytic modules in
48 27 Gram-negative bacteria. *ACS Synth. Biol.* 6 (5), 793-805.
49
50
51 28 41. Martínez-García, E., Aparicio, T., de Lorenzo, V., and Nickel, P. I. (2014) New transposon tools
52 29 tailored for metabolic engineering of Gram-negative microbial cell factories. *Front. Bioeng.*
53 30 *Biotechnol.* 2, 46.
54
55
56
57
58
59
60

- 1
2
3
4 1 42. Volke, D. C., Turlin, J., Mol, V., and Nikel, P. I. (2020) Physical decoupling of XylS/*Pm* regulatory
5 2 elements and conditional proteolysis enable precise control of gene expression in *Pseudomonas*
6 3 *putida*. *Microb. Biotechnol.* 13 (1), 222-232.
7
8
9 4 43. Wirth, N. T., Kozaeva, E., and Nikel, P. I. (2020) Accelerated genome engineering of
10 5 *Pseudomonas putida* by I-SceI—mediated recombination and CRISPR-Cas9 counterselection.
11 6 *Microb. Biotechnol.* 13, 233-249.
12
13
14 7 44. Durante-Rodríguez, G., de Lorenzo, V., and Nikel, P. I. (2018) A post-translational metabolic
15 8 switch enables complete decoupling of bacterial growth from biopolymer production in
16 9 engineered *Escherichia coli*. *ACS Synth. Biol.* 7, 2686-2697.
17
18
19 10 45. Kuepper, J., Otto, M., Dickler, J., Behnken, S., Magnus, J., Jäger, G., Blank, L. M., and Wierckx,
20 11 N. (2020) Adaptive laboratory evolution of *Pseudomonas putida* and *Corynebacterium*
21 12 *glutamicum* to enhance anthranilate tolerance. *Microbiology* 166 (11), 1025-1037.
22
23
24 13 46. Mohamed, E. T., Werner, A. Z., Salvachúa, D., Singer, C. A., Szostkiewicz, K., Jiménez-Díaz, R.
25 14 M., Eng, T., Radi, M. S., Simmons, B. A., Mukhopadhyay, A., Herrgård, M. J., Singer, S. W.,
26 15 Beckham, G. T., and Feist, A. M. (2020) Adaptive laboratory evolution of *Pseudomonas putida*
27 16 KT2440 improves *p*-coumaric and ferulic acid catabolism and tolerance. *Metab. Eng. Commun.*
28 17 11, e00143.
29
30
31 18 47. Espeso, D. R., Dvořák, P., Aparicio, T., and de Lorenzo, V. (2020) An automated DIY framework
32 19 for experimental evolution of *Pseudomonas putida*. *Microb. Biotechnol.*, DOI: 10.1111/1751-
33 20 7915.13678.
34
35
36 21 48. Li, W. J., Narancic, T., Kenny, S. T., Niehoff, P. J., O'Connor, K., Blank, L. M., and Wierckx, N.
37 22 (2020) Unraveling 1,4-butanediol metabolism in *Pseudomonas putida* KT2440. *Front. Microbiol.*
38 23 11, 382.
39
40
41 24 49. Li, W. J., Jayakody, L. N., Franden, M. A., Wehrmann, M., Daun, T., Hauer, B., Blank, L. M.,
42 25 Beckham, G. T., Klebensberger, J., and Wierckx, N. (2019) Laboratory evolution reveals the
43 26 metabolic and regulatory basis of ethylene glycol metabolism by *Pseudomonas putida* KT2440.
44 27 *Environ. Microbiol.* 21 (10), 3669-3682.
45
46
47 28 50. Bator, I., Wittgens, A., Rosenau, F., Tiso, T., and Blank, L. M. (2019) Comparison of three xylose
48 29 pathways in *Pseudomonas putida* KT2440 for the synthesis of valuable products. *Front. Bioeng.*
49 30 *Biotechnol.* 7, 480.
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3
4 1 51. Aparicio, T., Nyerges, A., Nagy, I., Pál, C., Martínez-García, E., and de Lorenzo, V. (2020)
5 2 Mismatch repair hierarchy of *Pseudomonas putida* revealed by mutagenic ssDNA
6 3 recombineering of the *pyrF* gene. *Environ. Microbiol.* 22 (1), 45-58.
7
8
9 4 52. Long, H., Miller, S. F., Williams, E., and Lynch, M. (2018) Specificity of the DNA mismatch repair
10 5 system (MMR) and mutagenesis bias in bacteria. *Mol. Biol. Evol.* 35 (10), 2414-2421.
11
12 6 53. Nyerges, Á., Csörgő, B., Nagy, I., Bálint, B., Bihari, P., Lázár, V., Apjok, G., Umenhoffer, K.,
13 7 Bogos, B., Pósfai, G., and Pál, C. (2016) A highly precise and portable genome engineering
14 8 method allows comparison of mutational effects across bacterial species. *Proc. Natl. Acad. Sci.*
15 9 *USA* 113 (9), 2502-2507.
16
17 10 54. Aparicio, T., Nyerges, Á., Martínez-García, E., and de Lorenzo, V. (2020) High-efficiency multi-
18 11 site genomic editing (*HEMSE*) of *Pseudomonas putida* through thermoinducible ssDNA
19 12 recombineering. *iScience* 23 (3), 100946.
20
21 13 55. Benedetti, I., Nikel, P. I., and de Lorenzo, V. (2016) Data on the standardization of a
22 14 cyclohexanone-responsive expression system for Gram-negative bacteria. *Data in Brief* 6, 738-
23 15 744.
24
25 16 56. Akkaya, Ö., Pérez-Pantoja, D., Calles, B., Nikel, P. I., and de Lorenzo, V. (2018) The metabolic
26 17 redox regime of *Pseudomonas putida* tunes its evolvability toward novel xenobiotic substrates.
27 18 *mBio* 9 (4), e01512-18.
28
29 19 57. Aparicio, T., de Lorenzo, V., and Martínez-García, E. (2019) Improved thermotolerance of
30 20 genome-reduced *Pseudomonas putida* EM42 enables effective functioning of the $P_{l}Cl857$
31 21 system. *Biotechnol. J.* 14 (1), e1800483.
32
33 22 58. Benedetti, I., de Lorenzo, V., and Nikel, P. I. (2016) Genetic programming of catalytic
34 23 *Pseudomonas putida* biofilms for boosting biodegradation of haloalkanes. *Metab. Eng.* 33, 109-
35 24 118.
36
37 25 59. Lammens, E. M., Nikel, P. I., and Lavigne, R. (2020) Exploring the synthetic biology potential of
38 26 bacteriophages for engineering non-model bacteria. *Nat. Commun.* 11, 5294.
39
40 27 60. Martínez-García, E., Goñi-Moreno, A., Bartley, B., McLaughlin, J., Sánchez-Sampedro, L.,
41 28 Pascual del Pozo, H., Prieto Hernández, C., Marletta, A. S., de Lucrezia, D., Sánchez-Fernández,
42 29 G., Fraile, S., and de Lorenzo, V. (2019) *SEVA 3.0*: An update of the Standard European Vector
43 30 Architecture for enabling portability of genetic constructs among diverse bacterial hosts. *Nucleic*
44 31 *Acids Res* 48 (D1), D1164–D1170.
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3
4 1 61. Villaverde, A., Benito, A., Viaplana, E., and Cubarsi, R. (1993) Fine regulation of cl857-controlled
5 2 gene expression in continuous culture of recombinant *Escherichia coli* by temperature. *Appl.*
6 3 *Environ. Microbiol.* 59 (10), 3485-3487.
7
8
9 4 62. Hosokawa, K., Park, N. H., Inaoka, T., Itoh, Y., and Ochi, K. (2002) Streptomycin-resistant (*rpsL*)
10 5 or rifampicin-resistant (*rpoB*) mutation in *Pseudomonas putida* KH146-2 confers enhanced
11 6 tolerance to organic chemicals. *Environ. Microbiol.* 4 (11), 703-712.
12
13
14 7 63. Baltz, R. H. (2014) Spontaneous and induced mutations to rifampicin, streptomycin and
15 8 spectinomycin resistances in actinomycetes: Mutagenic mechanisms and applications for strain
16 9 improvement. *J. Antibiot.* 67 (9), 619-624.
17
18
19 10 64. Jatsenko, T., Tover, A., Tegova, R., and Kivisaar, M. (2010) Molecular characterization of Rif^R
20 11 mutations in *Pseudomonas aeruginosa* and *Pseudomonas putida*. *Mutat. Res.* 683 (1-2), 106-
21 12 114.
22
23
24 13 65. Miskinyte, M., and Gordo, I. (2013) Increased survival of antibiotic-resistant *Escherichia coli*
25 14 inside macrophages. *Antimicrob. Agents Chemother.* 57 (1), 189-195.
26
27
28 15 66. Ryall, B., Eydallin, G., and Ferenci, T. (2012) Culture history and population heterogeneity as
29 16 determinants of bacterial adaptation: The adaptomics of a single environmental transition.
30 17 *Microbiol. Mol. Biol. Rev.* 76 (3), 597-625.
31
32
33 18 67. Galhardo, R. S., Hastings, P. J., and Rosenberg, S. M. (2007) Mutation as a stress response and
34 19 the regulation of evolvability. *Crit. Rev. Biochem. Mol. Biol.* 42 (5), 399-435.
35
36
37 20 68. Kivisaar, M. (2010) Mechanisms of stationary-phase mutagenesis in bacteria: Mutational
38 21 processes in pseudomonads. *FEMS Microbiol. Lett.* 312 (1), 1-14.
39
40
41 22 69. Dvořák, P., Chrást, L., Nikel, P. I., Fedr, R., Soucek, K., Sedlacková, M., Chaloupková, R., de
42 23 Lorenzo, V., Prokop, Z., and Damborský, J. (2015) Exacerbation of substrate toxicity by IPTG in
43 24 *Escherichia coli* BL21(DE3) carrying a synthetic metabolic pathway. *Microb. Cell Fact.* 14, 201.
44
45
46 25 70. Silva, F., Queiroz, J. A., and Domingues, F. C. (2012) Evaluating metabolic stress and plasmid
47 26 stability in plasmid DNA production by *Escherichia coli*. *Biotechnol. Adv.* 30 (3), 691-708.
48
49
50 27 71. Foster, P. L. (2007) Stress-induced mutagenesis in bacteria. *Crit. Rev. Biochem. Mol. Biol.* 42
51 28 (5), 373-397.
52
53
54 29 72. Węgrzyn, G., and Węgrzyn, A. (2002) Stress responses and replication of plasmids in bacterial
55 30 cells. *Microb. Cell Fact.* 1 (1), 2.
56
57
58
59
60

- 1
2
3
4 1 73. Juurik, T., Ilves, H., Teras, R., Ilmjärv, T., Tavita, K., Ukkivi, K., Teppo, A., Mikkel, K., and Kivisaar,
5 2 M. (2012) Mutation frequency and spectrum of mutations vary at different chromosomal positions
6 3 of *Pseudomonas putida*. *PLoS One* 7 (10), e48511.
7
8
9 4 74. Long, H., Sung, W., Miller, S. F., Ackerman, M. S., Doak, T. G., and Lynch, M. (2014) Mutation
10 5 rate, spectrum, topology, and context-dependency in the DNA mismatch repair-deficient
11 6 *Pseudomonas fluorescens* ATCC948. *Genome Biol. Evol.* 7 (1), 262-271.
12
13
14 7 75. Kivisaar, M. (2020) Mutation and recombination rates vary across bacterial chromosome.
15 8 *Microorganisms* 8 (1), 25.
16
17
18 9 76. Aparicio, T., Jensen, S. I., Nielsen, A. T., de Lorenzo, V., and Martínez-García, E. (2016) The Ssr
19 10 protein (*T1E_1405*) from *Pseudomonas putida* DOT-T1E enables oligonucleotide-based
20 11 recombineering in platform strain *P. putida* EM42. *Biotechnol. J.* 11, 1309-1319.
21
22
23 12 77. Volke, D. C., Friis, L., Wirth, N. T., Turlin, J., and Nikel, P. I. (2020) Synthetic control of plasmid
24 13 replication enables target- and self-curing of vectors and expedites genome engineering of
25 14 *Pseudomonas putida*. *Metab. Eng. Commun.* 10, e00126.
26
27
28 15 78. Watabe, H., Iino, T., Kaneko, T., Shibata, T., and Ando, T. (1983) A new class of site-specific
29 16 endodeoxyribonucleases. Endo.Scel isolated from a eukaryote, *Saccharomyces cerevisiae*. *J.*
30 17 *Biol. Chem.* 258 (8), 4663-4665.
31
32
33 18 79. Badran, A. H., and Liu, D. R. (2015) Development of potent *in vivo* mutagenesis plasmids with
34 19 broad mutational spectra. *Nat. Commun.* 6 (1), 8425.
35
36
37 20 80. Kurusu, Y., Narita, T., Suzuki, M., and Watanabe, T. (2000) Genetic analysis of an incomplete
38 21 *mutS* gene from *Pseudomonas putida*. *J. Bacteriol.* 182 (18), 5278-5279.
39
40
41 22 81. Luján, A. M., Maciá, M. D., Yang, L., Molin, S., Oliver, A., and Smania, A. M. (2011) Evolution
42 23 and adaptation in *Pseudomonas aeruginosa* biofilms driven by mismatch repair system-deficient
43 24 mutators. *PLoS One* 6 (11), e27842.
44
45
46 25 82. Colque, C. A., Albarracín Orió, A. G., Feliziani, S., Marvig, R. L., Tobares, A. R., Johansen, H.
47 26 K., Molin, S., and Smania, A. M. (2020) Hypermutator *Pseudomonas aeruginosa* exploits multiple
48 27 genetic pathways to develop multidrug resistance during long-term infections in the airways of
49 28 cystic fibrosis patients. *Antimicrob. Agents Chemother.* 64 (5), e02142-02119.
50
51
52 29 83. Luan, G., Bao, G., Lin, Z., Li, Y., Chen, Z., Li, Y., and Cai, Z. (2015) Comparative genome analysis
53 30 of a thermotolerant *Escherichia coli* obtained by genome replication engineering assisted
54
55
56
57
58
59
60

- 1
2
3
4 1 continuous evolution (*GREACE*) and its parent strain provides new understanding of microbial
5 heat tolerance. *New Biotechnol.* 32 (6), 732-738.
6 2
7 3 84. Nikel, P. I., Fuhrer, T., Chavarría, M., Sánchez-Pascuala, A., Sauer, U., and de Lorenzo, V.
8 (2021) Reconfiguration of metabolic fluxes in *Pseudomonas putida* as a response to sub-lethal
9 oxidative stress. *ISME J.*, In press, DOI: 10.1038/s41396-41020-00884-41399.
10 4
11 5
12 6 85. Shibai, A., Takahashi, Y., Ishizawa, Y., Motooka, D., Nakamura, S., Ying, B. W., and Tsuru, S.
13 (2017) Mutation accumulation under UV radiation in *Escherichia coli*. *Sci. Rep.* 7 (1), 14531.
14 7
15 8 86. Castañeda-García, A., Prieto, A. I., Rodríguez-Beltrán, J., Alonso, N., Cantillon, D., Costas, C.,
16 Pérez-Lago, L., Zegeye, E. D., Herranz, M., Płociński, P., Tonjum, T., García de Viedma, D.,
17 Paget, M., Waddell, S. J., Rojas, A. M., Doherty, A. J., and Blázquez, J. (2017) A non-canonical
18 mismatch repair pathway in prokaryotes. *Nat. Commun.* 8, 14246.
19 9
20 10
21 11
22 12 87. Groothuizen, F. S., and Sixma, T. K. (2016) The conserved molecular machinery in DNA
23 mismatch repair enzyme structures. *DNA Repair* 38, 14-23.
24 13
25 14 88. Jacquelin, D. K., Filiberti, A., Argaraña, C. E., and Barra, J. L. (2005) *Pseudomonas aeruginosa*
26 MutL protein functions in *Escherichia coli*. *Biochem. J.* 388 (3), 879-887.
27 15
28 16 89. Nyerges, Á., Csorgó, B., Nagy, I., Latinovics, D., Szamecz, B., Pósfai, G., and Pál, C. (2014)
29 Conditional DNA repair mutants enable highly precise genome engineering. *Nucleic Acids Res.*
30 42 (8), e62.
31 17
32 18 90. Green, M. R., and Sambrook, J., *Molecular cloning: a laboratory manual*. 4th ed.; Cold Spring
33 Harbor Laboratory Press: Cold Spring Harbor, NY, 2012.
34 19
35 20 91. Nour-Eldin, H. H., Geu-Flores, F., and Halkier, B. A. (2010) *USER* cloning and *USER* fusion: The
36 ideal cloning techniques for small and big laboratories. *Methods Mol. Biol.* 643, 185-200.
37 21
38 22 92. Choi, K. H., Kumar, A., and Schweizer, H. P. (2006) A 10-min method for preparation of highly
39 electrocompetent *Pseudomonas aeruginosa* cells: Application for DNA fragment transfer
40 between chromosomes and plasmid transformation. *J. Microbiol. Methods* 64, 391-397.
41 23
42 24 93. Li, H., and Durbin, R. (2009) Fast and accurate short read alignment with Burrows-Wheeler
43 transform. *Bioinformatics* 25 (14), 1754-1760.
44 25
45 26 94. Danecek, P., Bonfield, J. K., Liddle, J., Marshall, J., Ohan, V., Pollard, M. O., Whitwham, A.,
46 Keane, T., McCarthy, S. A., Davies, R. M., and Li, H. (2021) Twelve years of SAMtools and
47 BCFtools. *GigaScience* 10 (2).
48 27
49 28
50 29
51 30
52
53
54
55
56
57
58
59
60

- 1
2
3
4 1 95. Wang, K., Li, M., and Hakonarson, H. (2010) *ANNOVAR*: Functional annotation of genetic
5 variants from high-throughput sequencing data. *Nucleic Acids Res.* 38 (16), e164.
6 2
7 3 96. Meselson, M., and Yuan, R. (1968) DNA restriction enzyme from *E. coli*. *Nature* 217 (5134), 1110-
8 1114.
9 4
10
11 5 97. Bagdasarian, M., Lurz, R., Rückert, B., Franklin, F. C. H., Bagdasarian, M. M., Frey, J., and
12 Timmis, K. N. (1981) Specific purpose plasmid cloning vectors. II. Broad host range, high copy
13 number, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*.
14 7
15 8
16 9 98. Martínez-García, E., Nickel, P. I., Aparicio, T., and de Lorenzo, V. (2014) *Pseudomonas* 2.0:
17 10 Genetic upgrading of *P. putida* KT2440 as an enhanced host for heterologous gene expression.
18 11
19 12 99. Silva-Rocha, R., Martínez-García, E., Calles, B., Chavarría, M., Arce-Rodríguez, A., de las Heras,
20 A., Páez-Espino, A. D., Durante-Rodríguez, G., Kim, J., Nickel, P. I., Platero, R., and de Lorenzo,
21 V. (2013) The Standard European Vector Architecture (SEVA): A coherent platform for the
22 13 analysis and deployment of complex prokaryotic phenotypes. *Nucleic Acids Res.* 41 (D1), D666-
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4 **1 Table 1. Bacterial strains and plasmids used in this work.**
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Bacterial strain	Relevant characteristics ^a	Reference
<i>Escherichia coli</i>		
DH5 α	Cloning host; F ⁻ λ^- <i>endA1 glnX44(AS) thiE1 recA1 relA1 spoT1 gyrA96(Nal^R) rfbC1 deoR nupG Φ80(lacZΔM15) Δ(argF-lac)U169 hsdR17 rK7(<i>r_K</i>⁻ <i>m⁺_K</i>)</i>	Meselson and Yuan ⁹⁶
<i>Pseudomonas putida</i>		
KT2440	Wild-type strain; derivative of <i>P. putida</i> mt-2 cured of the TOL plasmid pWW0	Bagdasarian <i>et al.</i> ⁹⁷
EM42	Derivative of <i>P. putida</i> KT2440; Δ prophage1 Δ prophage2 Δ prophage3 Δ prophage4 Δ Tn7 Δ endA-1 Δ endA-2 Δ hsdRMS Δ flagellum Δ Tn4652	Martínez-García <i>et al.</i> ⁹⁸
<i>pyrF</i> HM	Derivative of <i>P. putida</i> EM42; <i>pyrF</i> ^{Lys55STOP} (A163T)	Aparicio <i>et al.</i> ⁷⁶
Plasmids		
pSEVA2311	Standard cyclohexanone-responsive expression vector; Km ^R ; <i>oriV</i> (pBBR1), <i>chnRIP_{chnB}</i>	Benedetti <i>et al.</i> ⁵⁵
pSEVA2514	Standard thermo-inducible expression vector; Km ^R ; <i>oriV</i> (RSF1010), <i>cl857/P_L</i>	Aparicio <i>et al.</i> ⁵⁷
pSEVA2514- <i>rec2-mutL^{E36K}</i> ^{PP}	Derivative of vector pSEVA2514 carrying the <i>rec2</i> recombinase and the dominant-negative mutator <i>mutL^{E36K}</i> allele from <i>P. putida</i>	Aparicio <i>et al.</i> ⁵¹
pQUIRE6-L	Conditionally-replicating vector; derivative of vector pJBSD1 carrying <i>XylIS/Pm</i> \rightarrow <i>I-SceI</i> and <i>P_{14g}</i> \rightarrow <i>mCherry</i> ; Gm ^R	Volke <i>et al.</i> ⁷⁷
pS2311SG	Derivative of vector pSEVA2311 with an engineered <i>I-SceI</i> recognition site for easy vector curing and <i>P_{EM7}</i> \rightarrow <i>msfGFP</i>	This work
pS2514SG	Derivative of vector pSEVA2514 with an engineered <i>I-SceI</i> recognition site for easy vector curing and <i>P_{EM7}</i> \rightarrow <i>msfGFP</i>	This work
pS2311M	Derivative of vector pSEVA2311 carrying the dominant-negative mutator <i>mutL^{E36K}</i> allele from <i>P. putida</i>	This work
pS2514M	Derivative of vector pSEVA2514 carrying the dominant-negative mutator <i>mutL^{E36K}</i> allele from <i>P. putida</i>	This work

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pS2311SGM	Derivative of vector pS2311SG carrying the dominant-negative mutator <i>mutL</i> ^{E36K} allele from <i>P. putida</i>	This work
pS2514SGM	Derivative of vector pS2514SG carrying the dominant-negative mutator <i>mutL</i> ^{E36K} allele from <i>P. putida</i>	This work

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2 ^a Antibiotic markers: Km, kanamycin; Gm, gentamicin; Nal, nalidixic acid.

FIGURE LEGENDS

Figure 1. Broad-host-range mutator devices to conditionally increase mutation rates. (a) The bacterial DNA mismatch repair (MMR) system recognizes and fixes mutations that arise during DNA replication and recombination. MutS recognizes genomic DNA mismatches and recruits MutL. The MutL/MutS complex activates the MutH endonuclease, which cleaves the newly synthesized, unmethylated daughter strand at the nearest hemimethylated d(GATC) site, and thereby marks it for a removal and a repair–synthesis process that involves a variety of other proteins. Overexpression of the dominant-negative mutator allele *mutL*^{E32K} from *E. coli* increases mutation rates²⁵. **(b)** Structure of the two mutator devices used in this work. Plasmids pS2514M and pS2311M, based on the *Standard European Vector Architecture*⁹⁹, were designed for thermo-inducible or cyclohexanone-inducible expression of the mutator allele *mutL*^{E36K} from *P. putida*, respectively. Functional elements in the plasmids not drawn to scale; *Km*^R, kanamycin-resistance marker. **(c)** Two strategies for tampering with the MMR system of *P. putida*. When using plasmid pS2514M, the temperature-sensitive repressor *cl857* is constitutively produced at 28–32°C and specifically binds to the *P_L* promoter, mediating transcriptional repression of the gene cloned downstream (i.e. *mutL*^{E36K}). By shifting the temperature above 37°C (e.g. 40°C), the expression of *mutL*^{E36K} can take place due to denaturation of *cl857*. When using plasmid pS2311M, the ChnR transcriptional regulator is constitutively synthesized and binds to the *P_{chnB}* promoter in the presence of its inducer (cyclohexanone), thus causing the expression of the gene cloned downstream (i.e. *mutL*^{E36K}).

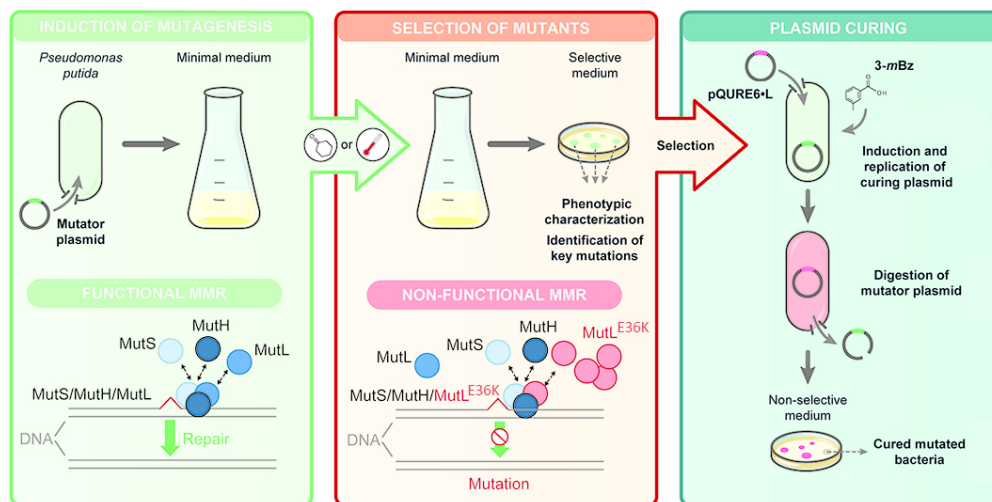
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4 **1 Figure 2. Experimental setup for evolution of *P. putida* strains carrying mutator plasmids.** Control
5 (ctrl.) strains (i.e. *P. putida* KT2440/pSEVA2311 and KT2440/pSEVA2514, bearing empty vectors) and
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7 3 conditional-mutator strains (i.e. *P. putida* KT2440/pS2311M and KT2440/pS2514M) were incubated in
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9 4 shaken-flask cultures in a non-selective medium (i.e. M9 minimal medium containing 20 mM glucose).
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11 5 After 5 h, when cultures reached an optical density at 600 nm (OD_{600}) = 0.3, the expression systems were
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13 6 induced either thermally (incubating the flasks in a water bath) or chemically (adding liquid cyclohexanone
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15 7 to the medium). The cultures were re-incubated at 30°C with shaking and the process was stopped after
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17 8 1.5 h (early exponential phase, OD_{600} = 0.6), 2 h (mid-exponential phase, OD_{600} = 1) or 24 h (stationary
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19 9 phase). In the case of cultures of *P. putida* KT2440 carrying either vector pSEVA2311 or plasmid
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21 10 pS2311M, the biomass was washed twice with phosphate buffer to remove any traces of cyclohexanone.
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23 11 Several aliquots were plated onto a selective solid medium [e.g. LB agar supplemented with streptomycin
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25 12 (Str) or rifampicin (Rif)] to assess the appearance of mutants in the bacterial population [e.g. rifampicin-
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27 13 (Rif^R) or streptomycin-resistant (Str^R) mutants]. The total number of viable cells in the bacterial cultures
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29 14 was estimated by plating dilutions of the cultures on non-selective solid medium (e.g. LB agar). *CFU*,
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31 15 colony-forming unit.
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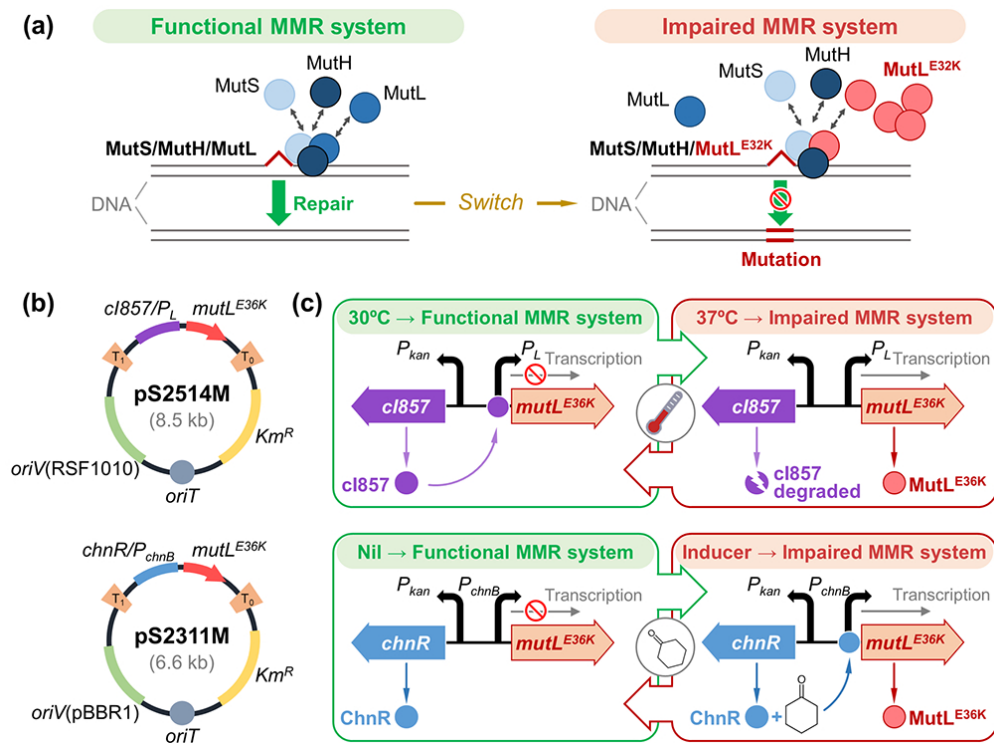
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4 **1 Figure 3. Evolution of antibiotic resistance in *P. putida* using conditional mutator devices.** *P. putida*
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6 2 strains carrying the systems inducible by cyclohexanone (a) and temperature shifts (b) were evolved by
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8 3 following the mutagenesis protocol described in Fig. 2. Culture aliquots were plated onto selective
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10 4 medium [i.e. LB agar supplemented with 100 $\mu\text{g mL}^{-1}$ streptomycin (Str) or 50 $\mu\text{g mL}^{-1}$ rifampicin (Rif)]
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12 5 to determine the appearance of Rif- (Rif^R) or Str-resistant (Str^R) mutants. The total number of viable cells
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14 6 was estimated by plating dilutions of each of the cultures onto LB agar plates. Two technical replicates
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16 7 and several dilutions for replicate were performed for each bacterial strain and per each selective culture
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18 8 condition. Columns represent mean values of mutation frequencies (MF, expressed as the number of
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20 9 mutant cells per 1×10^9 viable *P. putida* cells) from at least two independent experiments \pm standard
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22 10 deviation. *Relative mutation frequencies* were obtained by comparing the mutation frequency of the
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24 11 conditional mutator strain with the respective control (ctrl.) strain in the same experimental setup (i.e.
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26 12 expressed as fold-change).

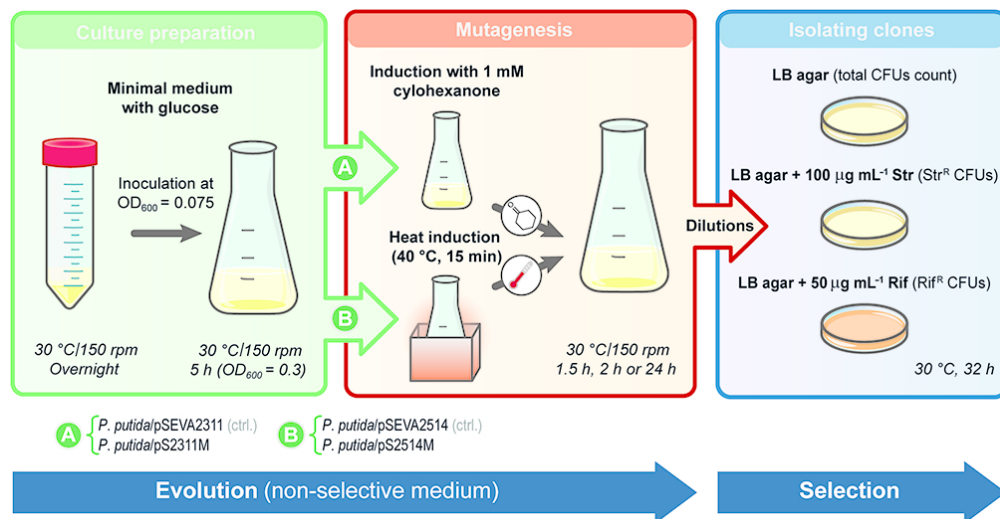
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4 **1 Figure 4. Reversion of a synthetic uracil auxotrophy in *P. putida* using mutator devices. (a) *P.***
5 **2 *putida pyrF* HM, carrying the *pyrF*^{FLys55STOP} allele that confers uracil auxotrophy (Ura⁻ phenotype), was**
6 **3 transformed with the two conditional mutator systems (i.e. inducible by cyclohexanone or temperature**
7 **4 shifts) or the corresponding control (ctrl.) vectors, and evolved by following the mutagenesis protocol**
8 **5 described in Fig. 2. Several aliquots of these bacterial cultures were plated on selective solid medium**
9 **6 (i.e. M9 minimal medium containing glucose as the only carbon source) to estimate the appearance of**
10 **7 uracil prototrophic mutants (Ura⁺) in the population. The total number of viable cells was estimated by**
11 **8 plating dilutions of the same cultures onto M9 minimal medium plates supplemented with 20 mM glucose**
12 **9 and 20 μg mL⁻¹ uracil. Two technical replicates and several dilutions for replicate were performed for**
13 **10 each bacterial strain and per each selective culture condition. Columns represent mean values of mutation**
14 **11 frequencies (expressed as the number of mutant cells per 1×10⁹ viable *P. putida pyrF* HM cells) from at**
15 **12 least two independent experiments ± standard deviation. (b) Mutations found in the *pyrF* gene (*PP_1815*)**
16 **13 in the Ura⁺ mutants isolated from two independent experiments. A total of eight Ura⁺ mutants for each**
17 **14 mutator/control strain were analyzed. (c) Frequency of mutation occurrence in a control (ctrl.) strain and**
18 **15 in the strain carrying the conditional mutator devices. Stop codons are indicated with the abbreviation**
19 **16 STOP.**

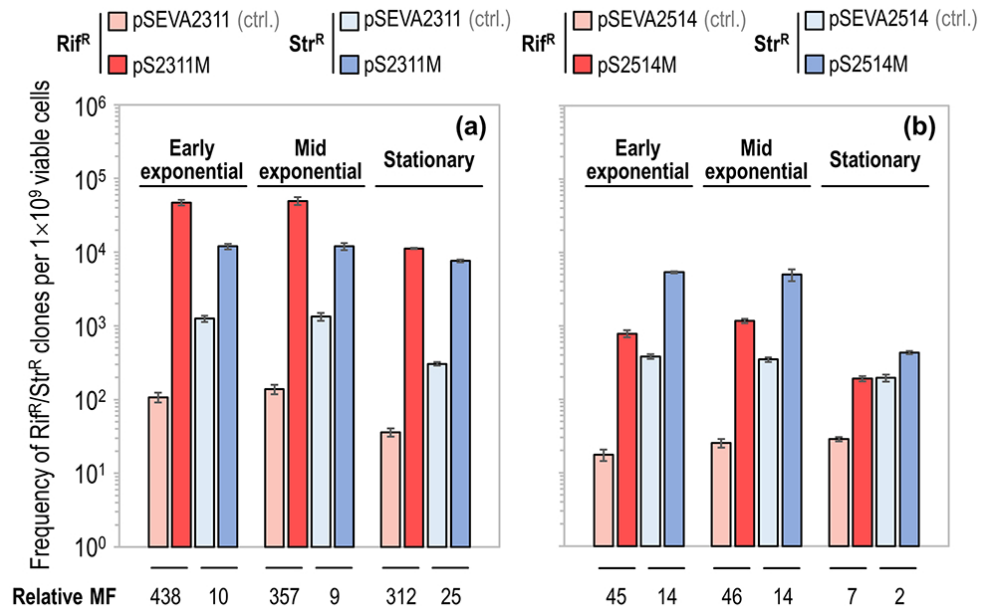
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4 **1 Figure 5. Design and implementation of a new generation of plasmid-based, easy-to-cure mutator**
5 **2 devices for Gram-negative bacteria. (a)** Plasmids pS2311SG, pS2514SG, pS2311SGM and
6 **3** pS2514SGM, derivatives of vectors pSEVA2311, pSEVA2514, pS2311M and pS2514M, respectively,
7 **4** were engineered with a I-SceI recognition site (indicated at the bottom of the figure) to render them
8 **5** compatible with the plasmid curing system based on targeted degradation mediated by the I-SceI
9 **6** endonuclease⁷⁷. These conditional mutator plasmids also carry a synthetic module for the constitutive
10 **7** expression of *msfGFP* (i.e. $P_{EM7} \rightarrow msfGFP$) that facilitate the selection of green fluorescent clones by
11 **8** examination of colonies under blue light. Functional elements in the plasmids not drawn to scale; Km^R ,
12 **9** kanamycin-resistance marker; MCS, standard multiple cloning site. **(b)** Mutation spectra caused by the
13 **10** mutator devices classified in functional categories. Control strains [i.e. *P. putida* KT2440/pS2514SG (TC)
14 **11** and KT2440/pS2311SG (CC)] and the conditional mutator strains [i.e. *P. putida* KT2440/pS2514SGM
15 **12** (TM) and KT2440/pS2311SGM (CM)] were incubated in shaken-flasks in a non-selective culture medium.
16 **13** After 5 h ($OD_{600} = 0.3$), expression systems were induced thermally (in a water bath at 40°C for 15 min)
17 **14** or chemically (addition of 1 mM cyclohexanone). All cultures (induced and non-induced) were re-
18 **15** incubated at 30°C until reaching an $OD_{600} = 0.6$. Several dilutions of the cultures were plated on LB agar
19 **16** for isolation of individual colonies. After curing off the mutator and control (i.e. empty) plasmids from the
20 **17** respective clones, genomic DNA was isolated, sequenced and the readings assembled. The emergence
21 **18** of transition (A:T→G:C; G:C→A:T), transversion (A:T→T:A; G:C→T:A; A:T→C:G; G:C→C:G) and
22 **19** small insertion-deletion (InDel) events (< 50 bp) mutations was analyzed in the individual strains. Clones
23 **20** 1-5 and clones 6-10 were obtained from induced and non-induced cultures, respectively.
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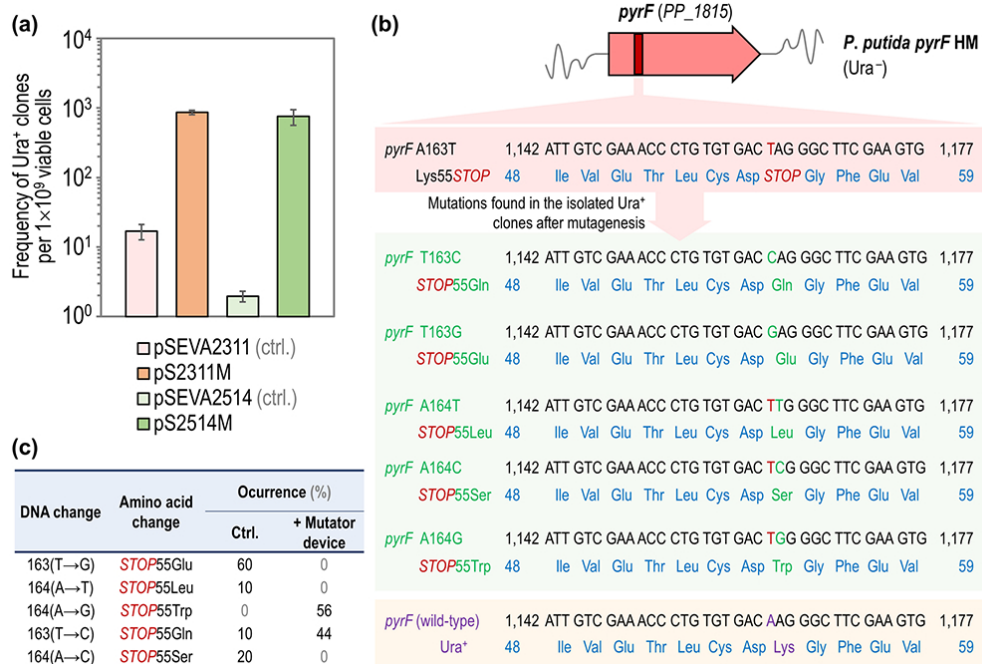
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4 **1 Figure 6. Evolution of a dual antibiotic resistance in *P. putida* using conditional mutator devices.**
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6 2 The control strain *P. putida* KT2440/pSEVA2311SG and the conditional-mutator strain *P. putida*
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8 3 KT2440/pS2311SGM were incubated in shaken-flask cultures in a non-selective medium (i.e. M9 minimal
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10 4 medium containing 20 mM glucose) for 24 h (stationary phase). Several aliquots cultures were plated
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12 5 onto a selective solid medium [i.e. LB agar supplemented with 50 $\mu\text{g mL}^{-1}$ kanamycin (Km), 50 $\mu\text{g mL}^{-1}$
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14 6 rifampicin (Rif) and 100 $\mu\text{g mL}^{-1}$ streptomycin (Str)] to assess the appearance of mutants [e.g. rifampicin-
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16 7 (Rif^R) and streptomycin-resistant (Str^R) mutants]. The total number of viable cells in the bacterial cultures
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18 8 was estimated by plating dilutions of the cultures on non-selective solid medium (e.g. LB agar
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20 9 supplemented with Km). Two replicates and several dilutions for replicate were performed for each
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22 10 bacterial strain and per each selective culture condition. **(a)** Representative Petri dishes from these
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24 11 experiments were photographed for the sake of a rough comparison of the number of colonies observed
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26 12 for each culture under selective culture conditions. **(b)** Quantification of mutation frequencies. Columns
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28 13 represent mean values of the actual mutation frequencies (expressed as the number of mutant cells per
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30 14 1×10^9 viable *P. putida* cells) from two independent experiments \pm standard deviation.
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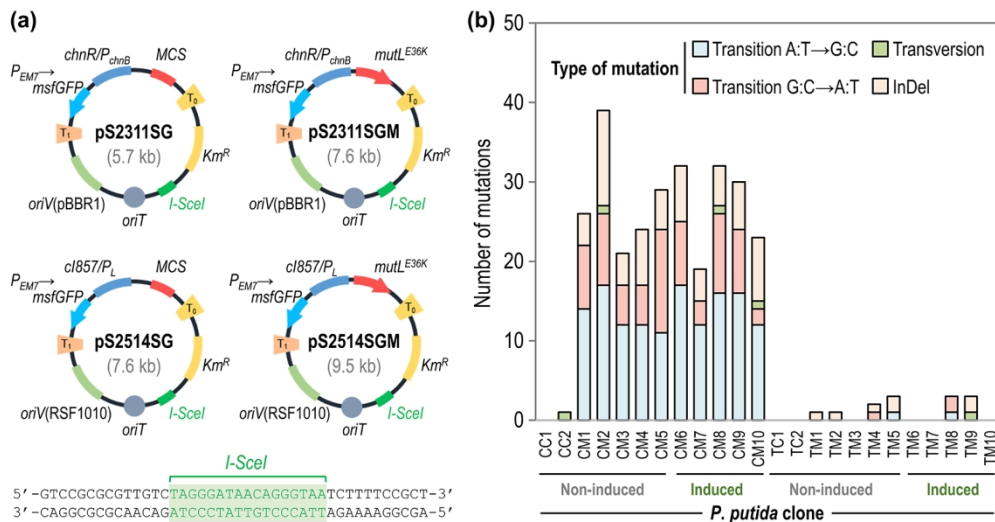


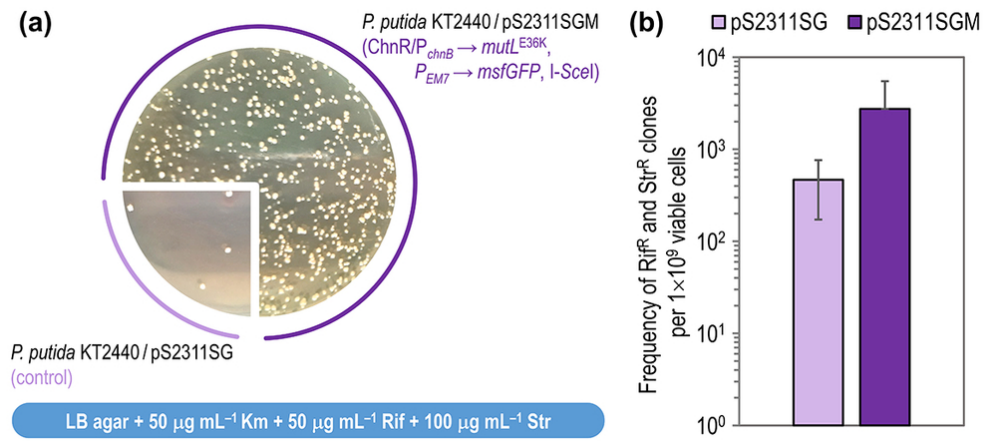












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