

## Supplementary information

### Hypermutability bypasses genetic constraints in SCV phenotypic switching in *Pseudomonas aeruginosa* biofilms.

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## Mutational history in evolutionary lines

### Evolutionary line WT3

The WT3 line adapted over 4 rounds, showing 4 mutations (Table 1, Fig. 1, and Supplementary Fig. 2). Bioinformatic predictions performed (SNPeff and SIFT algorithms) indicated moderate to high effects on protein functions (Table 1). In the SCV1 variant (WT3R1), a T119C substitution in *yfiN* caused a V40A in the conserved N-terminal portion of the PAS domain<sup>1</sup> of YfiN, which interacts with YfiR. This mutation, previously found in CF patients, likely makes YfiN V40A constitutively active<sup>2</sup>. SIFT prediction suggests this mutation tolerated, indicating activation and increased c-di-GMP levels in the WT3R1 clone.

In the next reversion round, WT3R2 acquired a G138A substitution in *yfiN*, causing an R46H in the PAS domain, potentially restoring YfiR, binding and lowering c-di-GMP levels. This mutation has not been previously described in the literature.

WT3R3 showed a  $\Delta$ G289 in *wspF*, causing a frameshift and a premature stop codon at position 101, truncating WspF protein on the methylesterase (ME) domain (Supplementary Fig. 2). This results in WspA hypermethylation and system activation, elevating c-di-GMP levels. Previous reports link *wspF* gene disruption to SCV colony morphology and phenotypic properties in *P. aeruginosa*<sup>3,4</sup>.

In WT3R4, a compensatory A47G mutation in *wspC* (antagonistic to *wspF*) caused a D16G change in the methyltransferase domain (Supplementary Fig. 2). Despite SIFT predicting tolerance, this mutation likely reduces WspA methylation, decreasing Wsp signaling and WspR activity. Deleting *wspC* in *P. aeruginosa*, and in its ortholog in *P. putida*, abolishes signaling in this system<sup>5,6</sup>.

#### **Evolutionary line WT4**

The WT4 line accumulated 4 mutations (Table 1). In the SCV WT4R1 clone, a G1357A substitution in *wspA* resulted in a V453I change in the WspA receptor's methyl-accepting domain (Supplementary Fig. 2). This mutation, while not previously described, likely modifies WspA's methylation status, activating WspR and increasing c-di-GMP levels.

In the next round, a C deletion at position 928 in *wspR*, caused a frameshift and premature stop codon at position 336, truncating the GGDEF domain and decreasing domain-di-GMP levels (Supplementary Fig. 2).

In rounds 3 and 4 of evolution, mutations in the DGC YfiN were observed. WT4R3 had a G231A substitution causing a E87K change in the PAS domain of YfiN (Supplementary Fig. 2), interfering with YfiR binding and leading to increased c-di-GMP synthesis. This mutation has been previously described in hyperadherent *P. aeruginosa* isolates from CF patients<sup>2</sup>. In WT4R4, a 53-nucleotide deletion from position 156 to 209 in *yfiN* caused a frameshift, nullifying the YfiBNR system (Supplementary Fig. 2) and compensating for the previous cycle's mutation, reducing c-di-GMP levels and reverting colony morphology.

#### **Evolutionary line *mutS1* (first four rounds)**

In the round 1 of evolution, a T713C mutation in *wspF* resulted in an L238P substitution in the methylesterase domain of WspF (Table 3), likely causing WspA hypermethylation and increased c-di-GMP levels through constitutive activation of the system. This mutation has not been previously described.

In the next round, the *mutS1*R2 clone acquired a compensatory C289T mutation in WspA, truncating the protein (G97\*) and nullifying the previous mutation's effect (Supplementary Fig. 2).

The *mutS1R3* clone had a G173A mutation in *yfiN*, causing an R58H substitution that impaired YfiR interaction, increasing c-di-GMP levels and inducing SCV morphology. This was compensated by an A1145G mutation in *yfiN* in the next round, resulting in an H382R change in the GGDEF domain, reducing c-di-GMP levels and reversing colony morphology. Both the G173A and A1145G mutations in *yfiN* are new. The T713C mutation in *wspF* was also detected in the *mutS3* line. In *mutS3R2*, this mutation was compensated by a *wspR* mutation (A374G), highlighting the genetic flexibility in modulating c-di-GMP levels.

#### **Evolutionary line *mutS2* (first four rounds)**

The *mutS2* line showed six mutations in the *wsp* and *yfi* genes of both transduction systems (Table 3). The initial T962C mutation in *wspF* caused an L321P substitution in the methylesterase domain (Supplementary Fig. 3), a novel mutation. This was compensated in the *mutS2R2* clone by a C1417T mutation in *wspA*, resulting in a truncated protein (G473\*) that nullified the previous mutation's effect. The *mutS2R3* clone carried an *yfiB* T128C mutation (L43P), previously identified as an activation mutation in YfiB, which promotes SCV conversion by enhancing its ability to form a stable complex with YfiR. This interaction leads to the release and activation of YfiN, driving SCV morphology through elevated c-di-GMP levels<sup>2,7</sup>. Subsequently, this effect was compensated by an *yfiN* G991A mutation (E331K) within the GGDEF domain, which reduces c-di-GMP levels. Notably, this mutation has not been previously characterized.

The *mutS2R5* clone acquired an A1418G mutation in *wspA*, changing the premature stop codon to tryptophan (\*473W), restoring WspA functionality. Finally, the *mutS2R6* clone showed a compensatory C1067T mutation in the histidine kinase domain of WspE (A356V), supporting the reversal of the SCV phenotype through mutations in this domain. The C1067T mutation in *wspE* is also novel.

## 99 **Mathematical framework for study of mutational routes and genomic architecture**

100 In the following paragraphs, we aim to define and outline a set of basic and initial mathematical  
 101 objects and concepts that our mathematical framework provides for the study of mutational  
 102 routes and genomic architecture.

103 First, we define a genomic unit as any set of nucleotides/base pairs chosen for study,  
 104 corresponding to a particular sequence in degenerate nucleotide code. We define the set  $T$  as  
 105 the set of mutation types (e.g., enabling/disabling substitutions, indels),  $M_\alpha \subseteq [0, 1]$  as the set  
 106 of probabilities for a specific mutation type  $\alpha \in T$  at the genomic unit,  $P \subseteq [0,1]$  as the set of  
 107 probabilities that a mutation in a certain genomic unit results in a desired phenotype, and  $n =$   
 108  $|T|$ , referred to as the Mutational Dimensionality (MD). Additionally, we defined  $\Delta$  as the set  
 109 of possible locations of genomic units within the genome,  $\Gamma$  as the set of all possible nucleotide  
 110 compositions of genomic units, and  $\Psi = \{X_0, X_1\}$ , indicating whether the mutation occurs in the  
 111 leading or lagging strand of the genomic unit.

112 With these definitions in place, we then define the Probabilistic Phenotypic Mutational  
 113 Landscape (PPML) of a particular genomic unit as

$$117 \quad f: M_\alpha \times \cdots \times M_\eta \mapsto P$$

114 This function maps a set of mutation probabilities for different types of mutations (e.g.:  
 115 probability of enabling substitutions, disabling indels) to the probability that a mutation at the  
 116 genomic unit (e.g.: gene, signaling pathway) results in the desired phenotype.

118 Next, we established Genomic Mutation Probability Functions (GMPFs) for a specific  
 119 mutation type  $\alpha \in T$  as

$$120 \quad g_\alpha: \Delta \times \Gamma \times \Psi \mapsto M_\alpha$$

GMPFs delineate the probability of a particular mutation type occurring, with  $g_\alpha$  evaluated for a specific genomic unit using the corresponding information on locations, nucleotide compositions, and strand bias. This comprehensive approach enables the modeling of various genomic mutational biases, encompassing deviations from randomly expected mutational spectra to alterations in mutation rates due to nucleotide context.

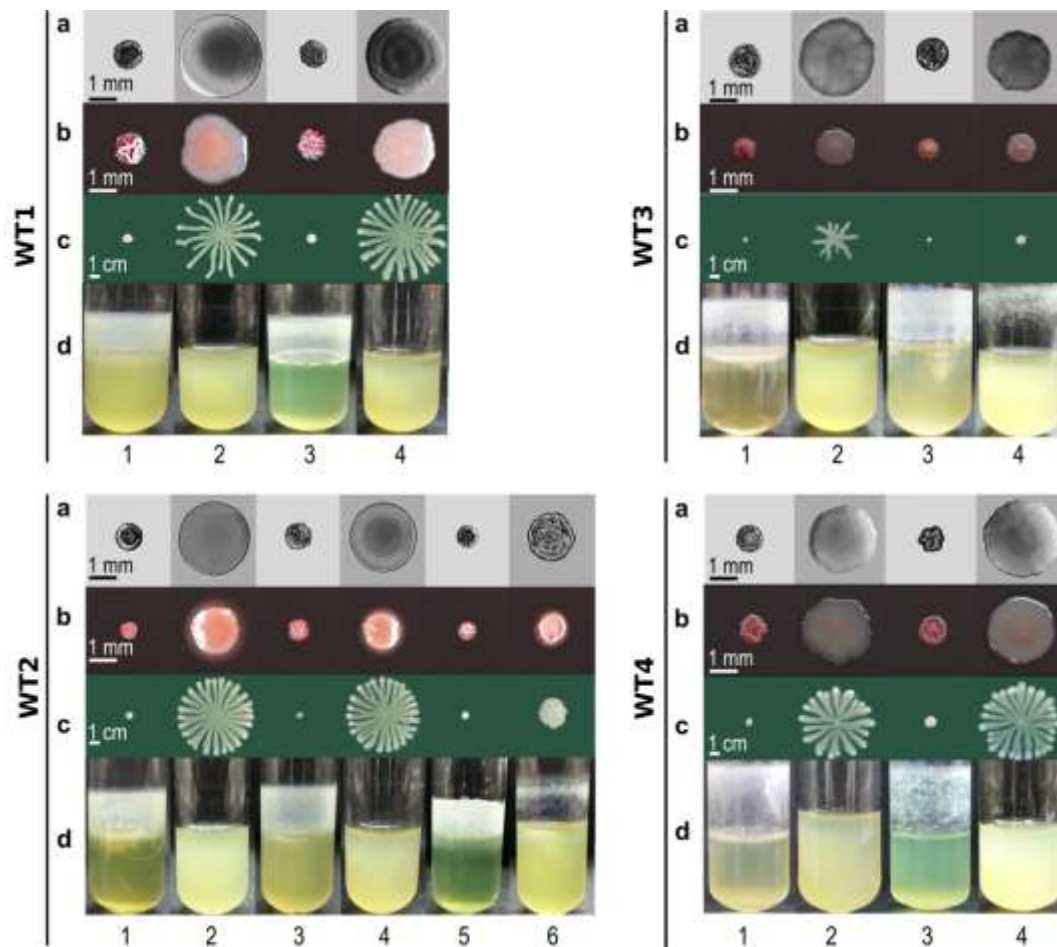
Furthermore, we introduce a concise yet inclusive GMPF classification based on characteristics these functions might model. Firstly, a GMPF is termed a spectral bias if it deviates the mutational spectra from what is expected by chance. Secondly, a rate bias refers to a GMPF that alters mutation rates, deviating from a uniform distribution. Thirdly, a location rate bias denotes a GMPF that influences mutation rates based on genomic position. Finally, a sequence rate bias characterizes a GMPF that alters mutation rates as a function of nucleotide composition or nucleotide context.

With the framework established, we observe that the function mapping genomic information to the probability of mutation into a specific phenotype is:

$$f(g_\alpha, \dots, g_\eta) = f(\vec{g})$$

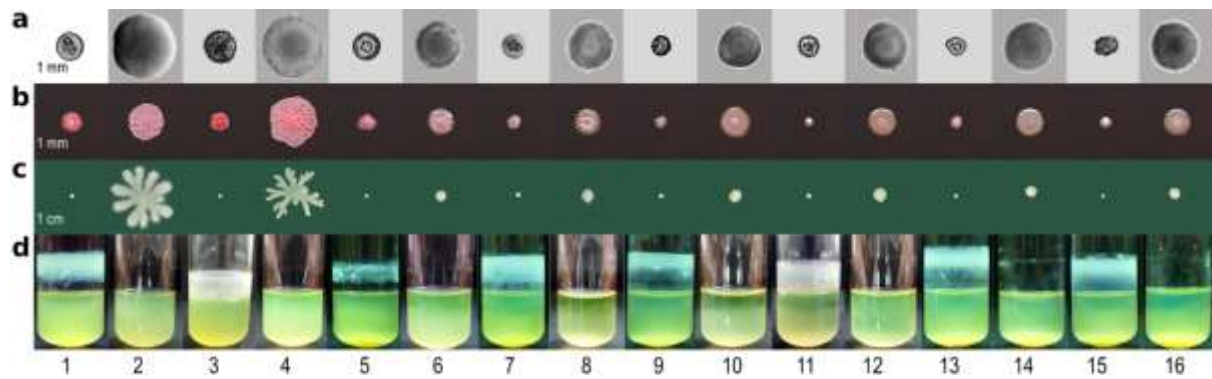
In this manner, with  $Dom(f) \subseteq Ran(\vec{g})$ , a consequential limitation is placed on  $Ran(f)$ , yielding the assertion that GMPFs, apart from molding the PPML, they confine it within defined regions accessible for exploration by the organism during adaptation. Simply put, the likelihood of a specific phenotype resulting from a mutation in a given genomic unit is directly influenced by the presence of genomic mutational biases.

## Supplementary Figures and Tables

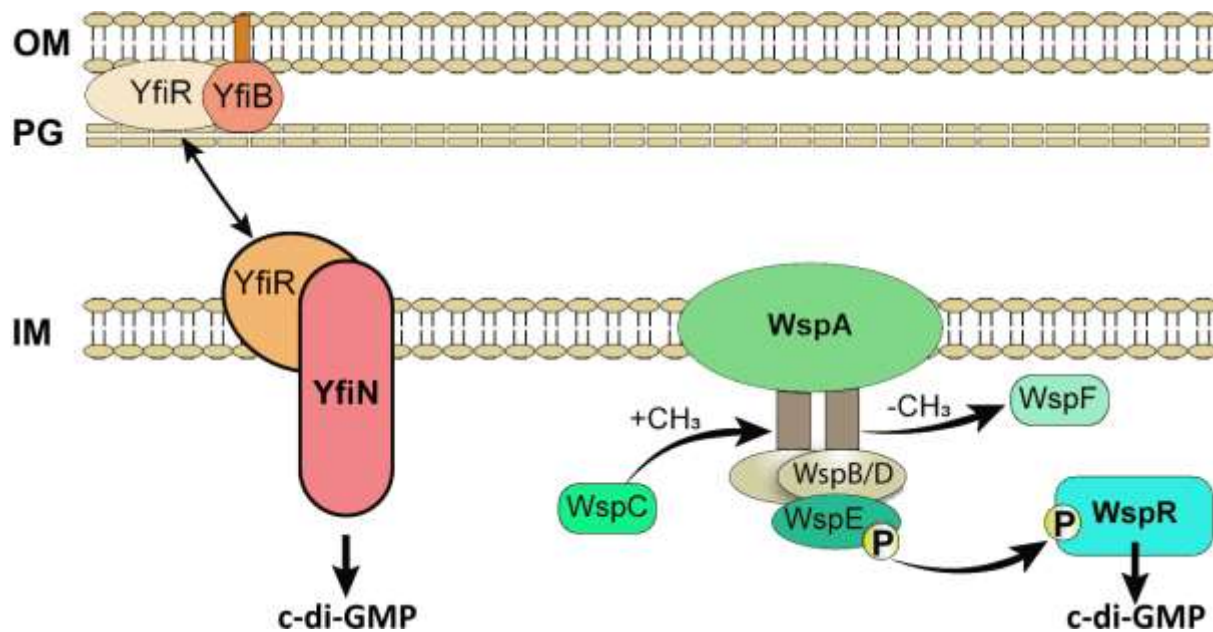


**Supplementary Fig. 1: Phenotypic characterization of *P. aeruginosa* WT lines.**

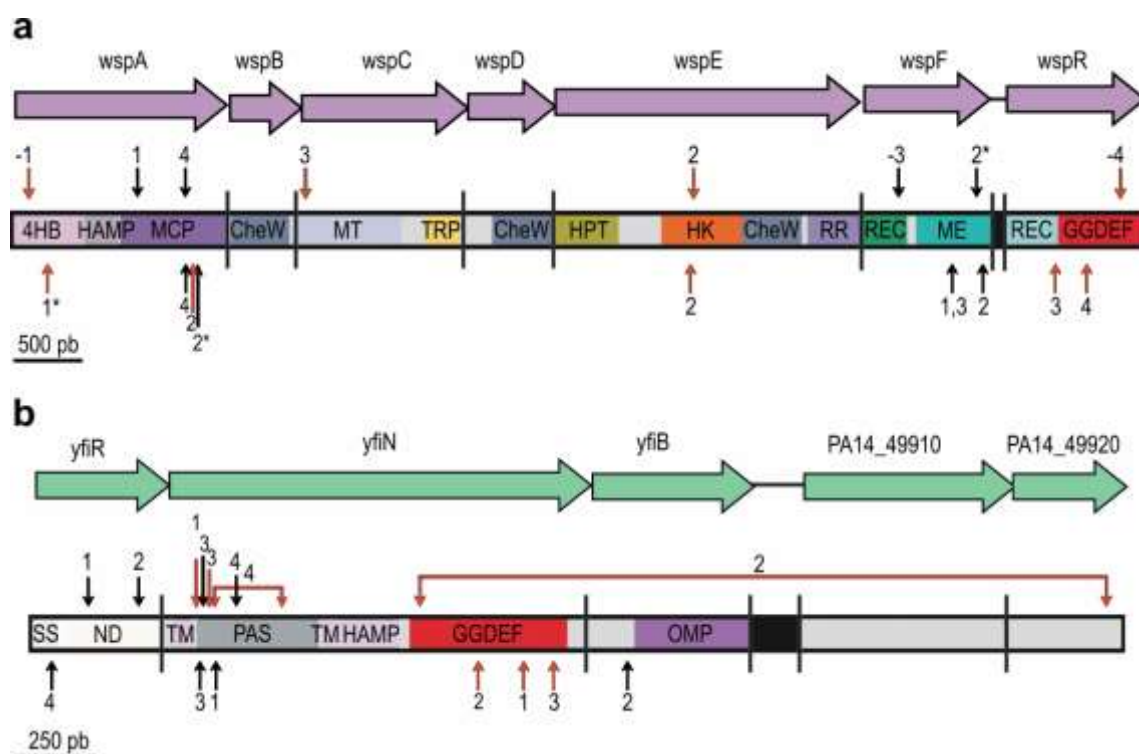
Characterization of SCV and revertant clones of each representative clone from the WT evolutionary history. The phenotypic properties are detailed qualitatively: **a** morphology on LB agar plates, **b** exopolysaccharide production determined by Congo red staining, **c** swarming motility, **d** auto-aggregation (transparency of the culture) and biofilm production (growth ring adhered to the glass). Numbers represent the evolutive round.



**Supplementary Fig. 2: Phenotypic characterization of *P. aeruginosa* *mutS1* evolutionary line.** Each SCV and revertant clones from line *mutS1* were qualitatively characterized as follows: **a** morphology observed on LB agar plates, **b** exopolysaccharide production assessed via Congo red staining, **c** swarming motility, **d** auto-aggregation (transparency of the culture), and biofilm production (presence of growth ring adhered to glass). Numeric values indicate the evolutionary round.



**Supplementary Fig. 3. The *yfi* and *wsp* signal transduction systems of *P. aeruginosa*.** On the left, the interaction model of the YfiBNR complex is shown. The DGC YfiN (PA14\_49890, also known as *tbbB*) is located in the inner membrane (IM) and its activity is controlled by the periplasmic repressor YfiR (PA14\_49880). YfiB (PA14\_49900) is an outer membrane (OM) receptor protein that activates YfiN by recruiting YfiR, causing YfiN to undergo a conformational change and catalyze the synthesis of c-di-GMP. On the right, the interaction model of the WspABCDEF complex is shown. WspA (PA14\_16430) is the receptor protein located in the IM that detects an activation signal associated with cell growth on surfaces. The signal is transduced to WspE (PA14\_16470), which catalyzes the phosphorylation of the response regulator WspR (PA14\_16500), synthesizing the second messenger c-di-GMP. The antagonistic proteins WspC (methyltransferase, PA14\_16450) and WspF (methyl-esterase, PA14\_16480) are involved in signal adaptation, while WspB (PA14\_16440) and WpsD (PA14\_16460) are important scaffold proteins for the function and correct localization of the complex (Modified from Malone et al.<sup>2</sup> and O'Connor et al.<sup>5</sup>). PG, peptidoglycan; +CH<sub>3</sub>, methyl group.



**Supplementary Fig. 4. Compensatory mutation accumulated in *wsp* and *yfi* operons in the**

**first four rounds of evolution.** Genomic location and protein domains organization of the *wsp*

**(a) and *yfi* (b) operons in *Pseudomonas aeruginosa* UCBPP-PA14, according to InterPro**

Database (<http://www.ebi.ac.uk/interpro/>)<sup>8</sup>. Arrows on top of domains indicate the localization

of the mutations found in WT lines. Arrows under the schemes represent mutation found in

mutator lines. Black and red arrow indicate the participation of a mutation during a round of

conversion and reversion, respectively, and numbers represent the respective evolutionary line.

Arrows connected by a line indicate a DNA deletion, (\*) represent a premature stop codon

(nonsense mutation), and (-) represent the acquisition of 1pb deletion. 4HB, chemotaxis

methyl-accepting receptor HlyB-like; HAMP, domain present in Histidine kinases, Adenyl

cyclases, methyl-accepting proteins, and Phosphatases; MCP, methyl-accepting chemotaxis

protein signaling domain; CheW, CheW-like domain; MT, CheR-type methyltransferase

domain; TPR, Tetratricopeptide-like helical domain; HPT, histidine-containing

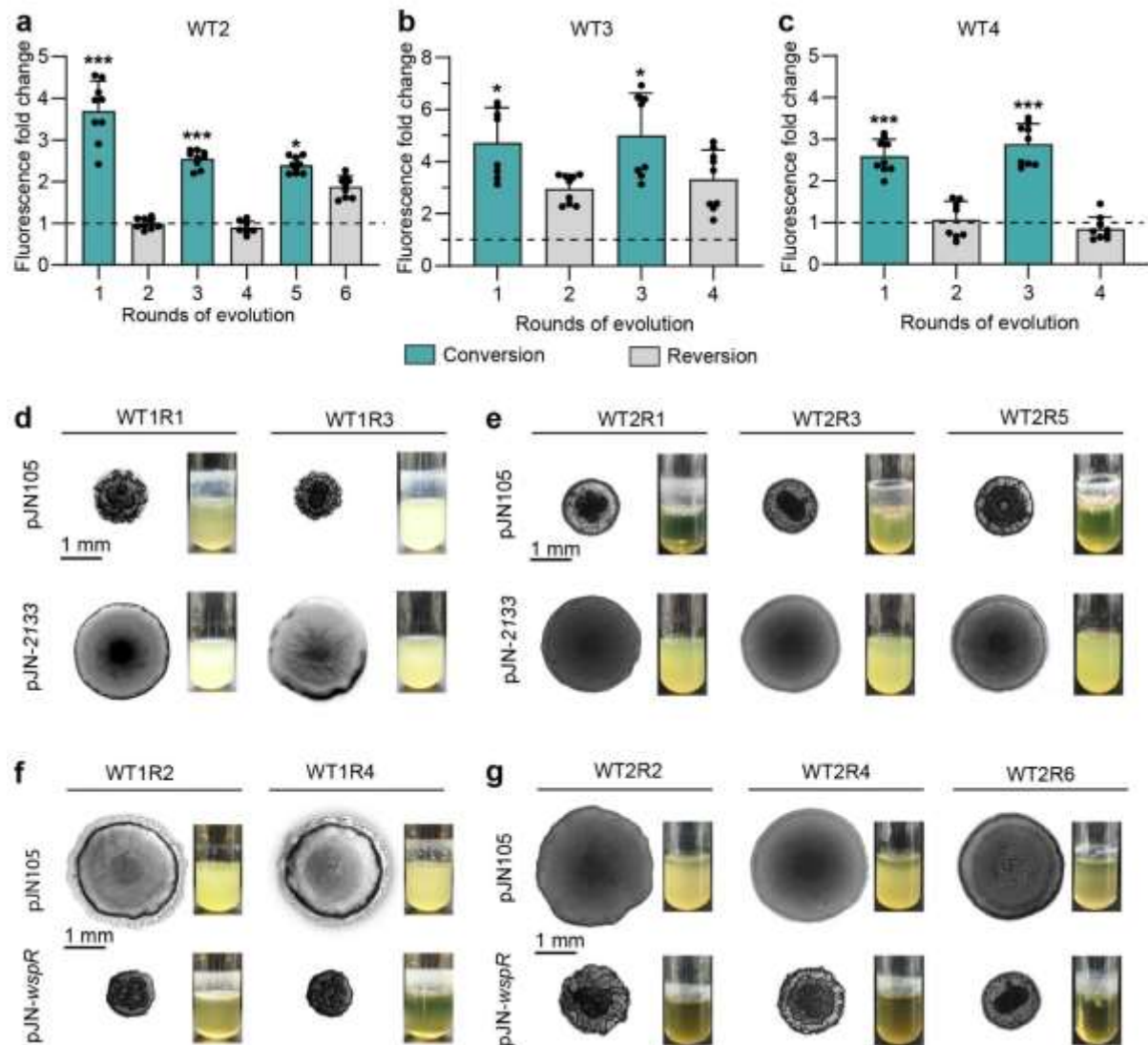
phosphotransfer domain; HK, Histidine kinase domain; REC, response regulatory CheY-like

domain; ME, CheB methylesterase; GGDEF, diguanylate cyclase domain; SS, signal peptide;

194 TM, inner membrane transmembrane domain; PAS, periplasmic sensor CHASE8 domain;

195 OMP, outer membrane OmpA-like domain.

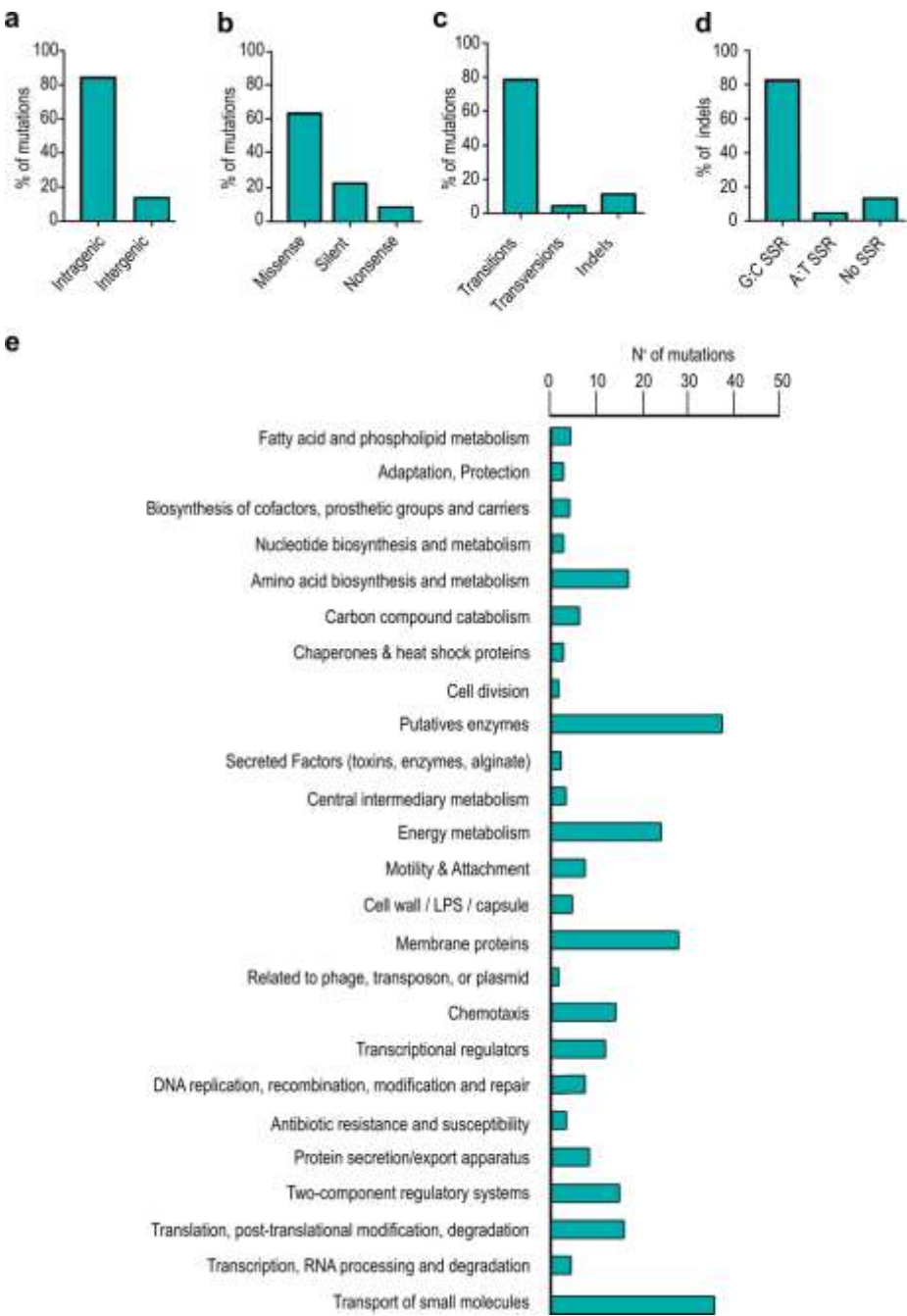
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**Supplementary Fig. 5. *In vivo* c-di-GMP levels in WT strains and overexpression assays.**

**a** *In vivo* c-di-GMP levels were quantified in WT lines 2, 3 and 4 by flow cytometry. Quantification was performed on *P. aeruginosa* WT parental strain and all the colonies selected from WT lines evolved in the first four rounds of evolution. A single copy of the c-di-GMP reporter *PpelA-BCD2-msf●gfp* was integrate in the genome of all clones<sup>9</sup>. Cells were grown ON and a minimum of 10.000 cells were analyzed per sample. Relative fluorescent output values were calculated by normalizing the average fluorescence level of the wild-type parental strain (dashed lines). Bars represent the values of the *msf●gfp* fluorescence as a fluorescent fold change  $\pm$  standard deviation of quintuplicate measurements from at least two independent

experiments. Blue bars represent the relative fluorescence of the SCV morphotype and light grey bars represent the relative fluorescence of the reverted morphotype. Values were analyzed by one-way ANOVA and Tukey post hoc test (\* $P < 0.05$ , \*\*\* $P < 0.001$ ). For the overexpression experiment, a moderate-copy-number vector, pJN105, which harbors the P<sub>BAD</sub> promoter to control expression of cloned genes, was used. Colony morphology on LB agar and behavior in liquid cultures of the SCV and revertant variants from evolutionary lines WT1 (**b**) and WT2 (**c**) were analyzed. SCV colonies were transformed with the vector pJN105 carrying the PDE *PA2133* gene (pJN-2133), resulting in the reversion to a smooth morphotype. Similarly, revertant colonies were transformed with the vector pJN105 carrying the DGC *wspR* gene (pJN-*wspR*), resulting in the conversion to a rough morphotype corresponding to the SCV. In all cases, the original clone was transformed with the empty vector pJN105 as a control.



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221 **Supplementary Fig. 6. Mutational spectra and functional characterization of mutations**

222 **found in mutator lines.** Classification of mutations found in four mutator lines based on their

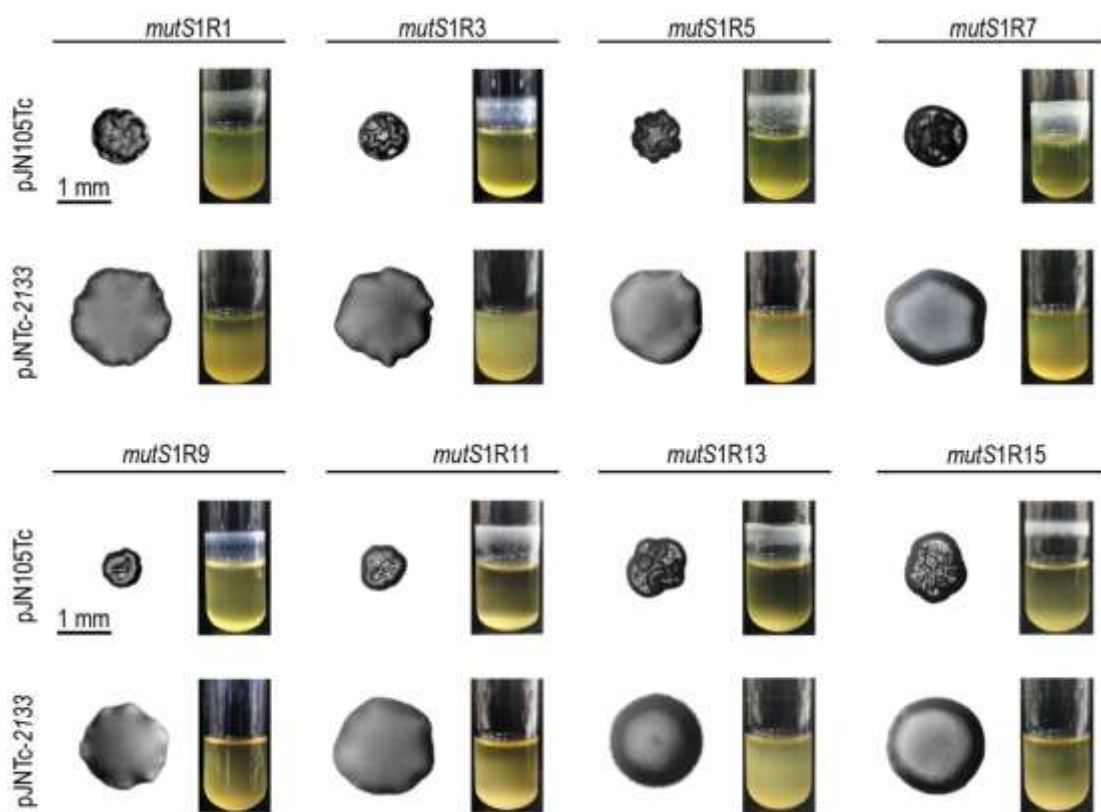
223 localization in the genome (**a**), the predicted effect on protein translation (**b**), type of mutation

224 based on percentage of substitutions and indels (**c**) and localization of indels inside or outside

225 single sequence repeats (SSR) (**d**). Bars represent the number of mutations in each category. **e**

226 Functional classification of mutations found in four evolutionary lines was performed

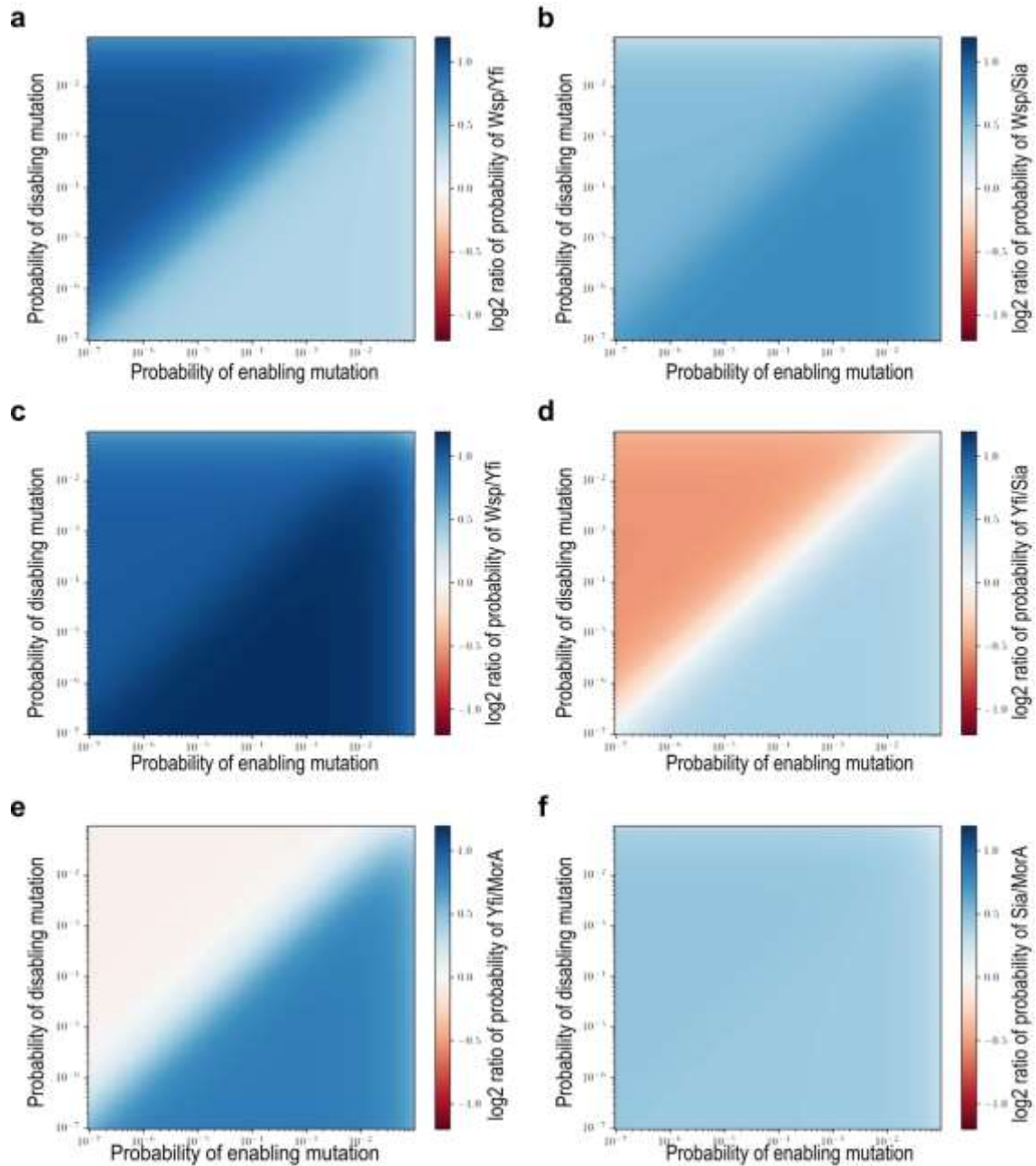
227 according to the functional analysis of proteins and prediction domains described in  
228 the InterPro database. Bars represent the number of mutations in each category. Only non-  
229 synonymous mutations were evaluated. The category of mutations labeled "Hypothetical,  
230 unclassified, unknown," which represents 21 % of mutations, was not included in the analysis.  
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234 **Supplementary Fig. 7. Overexpression of PDE PA2133 in *mutS4* evolutionary line.** For the  
 235 overexpression experiment in the *mutS4* line, the moderate-copy-number vector pJN105 was  
 236 modified with a Tc cassette to obtain pJN105Tc and introduced by electroporation. Colony  
 237 morphology on LB agar and behavior in liquid cultures of the SCV variants transformed with  
 238 the vector pJN105Tc carrying a wild-type copy of the PDE *PA2133* gene (pJNTc-2133) were  
 239 analyzed. In all cases, a reversion to a smooth morphotype was observed. The original clone  
 240 was transformed with the empty pJN105Tc vector as a control.

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**Supplementary Fig. 8. Observed genomic architecture is predicted by mechanistic model proposed in previous literature (Lind *et al*, 2015)<sup>10</sup>.** Pairwise ratio of probabilities for all four pathways of interest (Wsp, Yfi, Sia and MorA) are plotted as a function of probability of enabling mutations and probability of disabling mutations. **a** Probability of Wsp relative to Yfi. **b** Probability of Wsp relative to Sia. **c** Probability of Wsp relative to MorA. **d** Probability of Yfi relative to Sia. **e**) Probability of Yfi relative to MorA. **f**) Probability of Sia relative to MorA.

251 **Supplementary Table 1.** Mutation frequencies of ancestral and evolved WT and mutator strains.

| Strain                        | Mutation Frequency            | Antibiotics |
|-------------------------------|-------------------------------|-------------|
| Ancestor WT                   | $3.1 \pm 1.9 \times 10^{-8}$  | a           |
| Ancestor <i>mutS</i>          | $2.4 \pm 0.7 \times 10^{-6}$  | a           |
| WTR4                          | $3.2 \pm 1.4 \times 10^{-8}$  | a           |
| WT4R4 <i>mutS</i> *           | $2.4 \pm 1.2 \times 10^{-6}$  | a           |
| Ancestor WT-p3                | $4.3 \pm 0.6 \times 10^{-10}$ | b           |
| Ancestor <i>mutS</i> -p3      | $2.4 \pm 1.2 \times 10^{-7}$  | b           |
| <i>mutSR4</i> p3- <i>mutS</i> | $0.8 \pm 0.2 \times 10^{-10}$ | b           |

252 Mutation frequency was measured as the occurrence of spontaneous resistance to streptomycin  
253 (500 µg/ml) (a), with tetracycline (30 µg/ml) additionally included to maintain the p3 plasmid  
254 (b).  
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263 **Supplementary Table 2.** SCV count in WT evolutionary lines in rounds 5 and 7.

| Evolutionary lines | SCV count in round 5                | SCV count in round 7          |
|--------------------|-------------------------------------|-------------------------------|
| WT1                | 0 SCVs out of 18,700 CFUs screened  | -                             |
| WT2                | 764 SCVs out of 5,000 CFUs screened | 0 out of 15,000 CFUs screened |
| WT3                | 0 SCVs out of 13,000 CFUs screened  | -                             |
| WT4                | 0 SCVs out of 12,000 CFUs screened  | -                             |

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268 **Supplementary Table 3.** Accumulated mutations in *mutS* evolutionary line 1 beyond round  
269 four in genes related to c-di-GMP metabolism and biofilm formation.

| Locus number | Gene name    | Mutation <sup>1</sup> | SNeff <sup>2</sup> | SIFT <sup>3</sup> | Protein function                                                 |
|--------------|--------------|-----------------------|--------------------|-------------------|------------------------------------------------------------------|
| PA14_05190   | <i>pilU</i>  | T359C (L120P)         | Moderate           | Tolerated         | Twitching motility                                               |
| PA14_31290   | <i>Pa1L</i>  | A261G (L120P)         |                    |                   |                                                                  |
| PA14_23380   | <i>orfH</i>  | C478T (R160*)         | Moderate           | Tolerated         |                                                                  |
| PA14_23410   | <i>orfJ</i>  | ΔG549                 | High               |                   |                                                                  |
| PA14_24490   | <i>pelB</i>  | A2872G (S958G)        | Moderate           | Tolerated         | Biofilm formation                                                |
| PA14_31290   | <i>plsM</i>  | G1210A (G404S)        | Moderate           | Tolerated         |                                                                  |
| PA14_45960   | <i>lasR</i>  | A577G (T93A)          | Moderate           | Tolerated         | Quorum sensing                                                   |
| PA14_50110   | <i>fliH</i>  | 137C>T (P46L)         | Moderate           | Tolerated         | Flagellar Assembly protein                                       |
| PA14_50140   | <i>fliF</i>  | A1444G (F482L)        | Moderate           | Affected          | Flagellar assembly, bacterial adherence                          |
| PA14_53140   | <i>rbdA</i>  | A1666G (W556R)        | Moderate           | Tolerated         | PDE activity. Biofilm dispersal, EPS production and motility     |
| PA14_59290   | <i>pilQ2</i> | ΔG612                 | High               |                   | Twitching motility, biofilm formation, and adherence to surfaces |
| PA14_59800   | <i>pvrS</i>  | ins1C1697             | High               |                   | Biofilm development. Form part of the operon of the PDE pvrR     |
| PA14_60300   | <i>pilX</i>  | T80C (V27A)           | Moderate           | Affected          | Twitching motility                                               |
| PA14_60870   | <i>morA</i>  | ins C 270             | High               |                   |                                                                  |
| PA14_60870   | <i>morA</i>  | T2840C (V947A)        | Moderate           | Affected          |                                                                  |
| PA14_69760   | PA14_69760   | ΔG667                 | High               |                   | Motility and attachment                                          |

270 <sup>1</sup>Aminoacid change is shown in parentheses. The \* symbol indicates a premature stop codon; Ins  
271 indicates an insertion and Δ indicates a deletion. <sup>2</sup>Bioinformatic analysis of the effect of a mutation on  
272 gene expression using the SNPeff algorithm<sup>11</sup>. <sup>3</sup>Bioinformatics analysis of the effect of a mutation on  
273 protein function using the SIFT algorithm<sup>12</sup>.

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278 **Supplementary Table 4.** Bacterial strains and plasmids used in this study.  
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| Strain                               | Relevant characteristics <sup>a</sup>                                                                                                                                                                                                              | Reference or source |
|--------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------|
| <b><i>Escherichia coli</i></b>       |                                                                                                                                                                                                                                                    |                     |
| DH5α $\lambda$ pir                   | Cloning host; F <sup>-</sup> $\lambda^-$ <i>endA1 glnX44(AS) thiE1 recA1 relA1 spoT1 gyrA96(Nal<sup>R</sup>) rfbC1 deoR nupG</i> $\Phi$ 80( <i>lacZ</i> Δ <i>M15</i> ) Δ( <i>argF-lac</i> )U169 <i>hsdR17(r_K^- m_K^+)</i> , $\lambda$ pir lysogen | 13                  |
| HB101                                | Helper strain; F <sup>-</sup> $\lambda^-$ <i>hsdS20(r_B^- m_B^-) recA13 leuB6(Am) araC14</i> Δ( <i>gpt-proA</i> )62 <i>lacY1 galK2(Oc) xyl-5 mtl-1 thiE1 rpsL20(Sm<sup>R</sup>) glnX44(AS)</i>                                                     | 14                  |
| PIR2                                 | <i>F-</i> Δ <i>lac169 rpoS(Am) robA1 creC510 hsdR514 endA reacA1 uidA(ΔMlui)::pir</i>                                                                                                                                                              | Life Technologies   |
| <b><i>Pseudomonas aeruginosa</i></b> |                                                                                                                                                                                                                                                    |                     |
| PA14 WT                              | Wild type, UCBPP-PA14, human isolate                                                                                                                                                                                                               | 15                  |
| PA14 <i>mutS</i>                     | Δ <i>mutS</i> ; Gm <sup>r</sup>                                                                                                                                                                                                                    | 16                  |
| PA14 WTR4 <i>mutS</i> *              | C463T (Q155*)                                                                                                                                                                                                                                      | This study          |
| Plasmid                              | Relevant characteristics <sup>a</sup>                                                                                                                                                                                                              | Reference or source |
| p3                                   | pBBR1MCS-3, Tc <sup>R</sup>                                                                                                                                                                                                                        | 17                  |
| p3- <i>mutS</i>                      | p3 derivative vector carrying a copy of <i>mutS</i> gene, Tc <sup>R</sup>                                                                                                                                                                          | This study          |
| pJN105                               | Arabinose-inducible expression plasmid; Gm <sup>R</sup>                                                                                                                                                                                            | 18                  |
| pJN-2133                             | pJN105 derivative vector carrying a copy of PA2133 locus gene, Gm <sup>R</sup>                                                                                                                                                                     | This study          |
| pJN-wspR                             | pJN105 derivative vector carrying a copy of PA14_16500 locus gene, Gm <sup>R</sup>                                                                                                                                                                 | This study          |
| pJN105Tc                             | pJN105 derivative vector carrying Tc resistance cassette, Gm <sup>R</sup> Tc <sup>R</sup>                                                                                                                                                          | This study          |
| pJNTc-2133                           | pJN2133 derivative carrying Tm resistance cassette, Gm <sup>R</sup> and Tc <sup>R</sup>                                                                                                                                                            | This study          |
| pJNTc-wspR                           | pJNwspR derivative carrying Tm resistance cassette, Gm <sup>R</sup> and Tc <sup>R</sup>                                                                                                                                                            | This study          |
| pRK600                               | Cm <sup>R</sup> , <i>ori ColE1</i> , <i>tra<sup>+</sup>mob<sup>+</sup></i> of RK2                                                                                                                                                                  | 19                  |
| pTnS-1                               | Ap <sup>R</sup> , <i>ori R6K</i> , <i>TnSABC<sup>+</sup>D operon</i>                                                                                                                                                                               | 20                  |
| pBG:: <i>pelA-Gm</i>                 | <i>pBG</i> carrying the P <sub>pelA</sub> promoter as a P <sub>pelA</sub> -BDC2- <i>msfGFP</i> transcriptional fusion Km <sup>R</sup> Gm <sup>R</sup>                                                                                              | 9                   |
| pBG:: <i>pelA-Sm</i>                 | pBG- <i>pelA</i> derivative vector carrying Sm resistance cassette. Km <sup>R</sup> Sm <sup>R</sup>                                                                                                                                                | This study          |
| pMBEC6                               | Cytosine base editing plasmid; GFP; UGI; <i>oriV</i> (pRO1600/ColE1); <i>sacB</i> ; Gm <sup>R</sup>                                                                                                                                                | 21                  |
| pMBEC6- <i>mutS</i>                  | pBEC6 derivative vector carrying the PA14_17500 spacer, Gm <sup>R</sup>                                                                                                                                                                            | This study          |

280 <sup>a</sup> Antibiotic marker: Km, kanamycin; Nal, nalidixic acid; Gm gentamicin; Tc tetracycline and  
281 Sm streptomycin

| Oligonucleotide         | Sequence (5'→3')          | Reference     | Use          |
|-------------------------|---------------------------|---------------|--------------|
| <i>utS</i> -sp-for      | GTGGCGTCCAGGAGATAAAAGGCT  | This study    | Base editing |
| <i>mutS</i> -sp-rev     | AAACAGCCTTTTATCTCCTGGACG  | This study    | Base editing |
| <i>mutS</i> -seq-for    | GGGTCAGGTTCTGGCAGCCG      | This study    | Sequencing   |
| <i>mutS</i> -seq-rev    | TGGCGATCTGCGAGCAGATC      | This study    | Sequencing   |
| <i>PTn7R</i>            | CACAGCATAACTGGACTGATTTC   | <sup>22</sup> | Cloning      |
| <i>PglmS</i> -down      | GCACATCGGCGACGTGCTCTC     | <sup>22</sup> | Cloning      |
| <i>wspR</i> - EcoRI For | GATGAATTCATGCACAACCCCTCAT | This study    | Cloning      |
| <i>wspR</i> -XbaI Rev   | TCTCTAGAGAGGTCCTTGATGCTG  | This study    | Cloning      |
| <i>wspA</i> 1406876 For | CGGCAACATCAACCAGGTG       | This study    | Sequencing   |
| <i>wspA</i> 1406876 Rev | CCTCGGAGAACTTGTCCAT       | This study    | Sequencing   |
| <i>wspA</i> F1          | TTGTGGCGGCAGGAACGAATC     | This study    | Sequencing   |
| <i>wspA</i> R1          | GGCGATGTCCTTGTTTCAG       | This study    | Sequencing   |
| <i>wspA</i> F2          | CTGAACAAGGACATCGCC        | This study    | Sequencing   |
| <i>wspA</i> R2          | GCCTTCTCGTTGAGGATCGC      | This study    | Sequencing   |
| <i>wspA</i> F3          | CGGCAACATCAACCAGGTG       | This study    | Sequencing   |
| <i>wspA</i> R3          | AGCGGGACACGGTCACA         | This study    | Sequencing   |
| <i>wspR</i> F2          | CTCCAACCGTCGTCAC          | This study    | Sequencing   |
| <i>wspR</i> R2          | CGCCACTCAGCCCGCC          | This study    | Sequencing   |
| <i>wspR</i> F1          | CATGATCGGAGAGGCTGTGC      | This study    | Sequencing   |
| <i>wspR</i> R1          | CGAAGTGACGACGGTTGGAG      | This study    | Sequencing   |
| <i>wspF</i> F1          | ATCGGAATCGTCAATGA         | This study    | Sequencing   |
| <i>wspF</i> R1          | CCTTCGGCGGCACCACT         | This study    | Sequencing   |
| <i>wspF</i> F2          | AGCGTCTGGTGGCGAT          | This study    | Sequencing   |
| <i>wspF</i> R2          | GCGATGGTGAGGAAGC          | This study    | Sequencing   |
| <i>wspF</i> F3          | CCAGCGAATCGAAGCTGCCG      | This study    | Sequencing   |
| <i>wspF</i> R3          | GTTCCACGCACCGCCACG        | This study    | Sequencing   |
| <i>wspC</i> F1          | ATTGCTGCCCCGACGAAGT       | This study    | Sequencing   |
| <i>wspC</i> R1          | GCGAAACCCCAAGTTCA         | This study    | Sequencing   |
| <i>wspD</i> F2          | TCGCCTGCCTTTCCT           | This study    | Sequencing   |
| <i>wspD</i> R2          | CAATCCTCCATGACGTGC        | This study    | Sequencing   |
| <i>wspR</i> 1413318 For | GGTGCGTGGAACAGCATTCAA     | This study    | Sequencing   |
| <i>wspR</i> 1413318 Rev | TCGGCTCTTCCTTGGTGCG       | This study    | Sequencing   |
| <i>wspE</i> 1410052 For | ATGGAGGATTGCCTGGTGG       | This study    | Sequencing   |
| <i>wspE</i> 1410052 Rev | CTCGTCGTTGTCGTCGCT        | This study    | Sequencing   |
| <i>morA</i> For         | GCTACATCGTCCAGGTCAAC      | This study    | Sequencing   |
| <i>morA</i> Rev         | AGGAACATCAGCACCACCCAC     | This study    | Sequencing   |
| <i>morA</i> 5431831 For | CTTCGTCTGCTTCTTCAG        | This study    | Sequencing   |
| <i>morA</i> 5431831 Rev | AGGTGCTTTCCAGTTCC         | This study    | Sequencing   |
| <i>morA</i> 5430523 For | GAAGGGGTCGGCGTGG          | This study    | Sequencing   |
| <i>morA</i> 5430523 Rev | GTTCTTCCTGGTTGTAGCCC      | This study    | Sequencing   |
| <i>yfiN</i> F1          | TGATGAACCGTCGTCGTCGC      | This study    | Sequencing   |
| <i>yfiN</i> R1          | CACCGAACCGATCTTCTGGC      | This study    | Sequencing   |
| <i>yfiN</i> F2          | TCGGTGGAGGTGAAAGGCT       | This study    | Sequencing   |
| <i>yfiN</i> R2          | GGCGGTGCTTGATCTCCTTG      | This study    | Sequencing   |

|                          |                               |            |            |
|--------------------------|-------------------------------|------------|------------|
| <i>yfi</i> N F3          | AAGGAGATCAACGACCGCC           | This study | Sequencing |
| <i>yfi</i> N R3          | CTACTTGGGTGGAGCCTCTG          | This study | Sequencing |
| <i>yfi</i> B 4436837 For | AGGAGATAGACAGTGCTACC          | This study | Sequencing |
| <i>yfi</i> B 4436837 Rev | ATCTTCGTCAGGGTGTTGCG          | This study | Sequencing |
| <i>nfx</i> B F2          | GACAGCAGAAAGGCGTGT            | This study | Sequencing |
| <i>nfx</i> B R2          | TCAGGGACAGGCGGTCGT            | This study | Sequencing |
| <i>yfi</i> R F           | GCACACCGACTACACTGGC           | This study | Sequencing |
| <i>yfi</i> R R           | GCAGCGATGGATTGGAACC           | This study | Sequencing |
| <i>yfi</i> B F           | AGGAGATAGACAGTGCTACC          | This study | Sequencing |
| <i>yfi</i> B R           | GCAGGATTACTCGGCGGGA           | This study | Sequencing |
| <i>yfi</i> B F2          | TGGCGGAACCTCAACGACC           | This study | Sequencing |
| <i>yfi</i> B R2          | GCAGGATTACTCGGCGGGA           | This study | Sequencing |
| Tc-NcoI For              | GCGCCATGGATAAAAATAGGCGTATCACG | This study | Cloning    |
| Tc-ApaI Rev              | ATAGGGCCCGCTCACTGCCCGCTTT     | This study | Cloning    |
| PA14_49930 REV           | GAGGTTTCCGCTCAGGGCT           | This study | Sequencing |

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**Supplementary Table 6.** Whole genome sequencing (BioProject ID PRJNA1099955)

| Clone name           | Sequencing platform | Read length | Library preparation kit          | Accession number |
|----------------------|---------------------|-------------|----------------------------------|------------------|
| Ancestor WT          | Illumina Hiseq 2500 | 2X150pb     | Truseq Nano DNA Library prep kit | SAMN40962354     |
| WT3R4                | Illumina Hiseq 1500 | 2X100pb     | TruSeq DNA Sample Prep Kit       | SAMN40962356     |
| Pool WT1R5           | Illumina Miseq      | 2X150pb     | Nextera XT DNA Library Prep      | SAMN40962355     |
| Pool WT4R5           | Illumina Miseq      | 2X150pb     | Nextera XT DNA Library Prep      | SAMN40962357     |
| Ancestor <i>mutS</i> | Illumina Hiseq 2500 | 2X150pb     | Truseq Nano DNA Library prep kit | SAMN40962358     |
| <i>mutS1R3</i>       | Illumina Hiseq 1500 | 2X100pb     | TruSeq DNA Sample Prep Kit       | SAMN40962359     |
| <i>mutS2R3</i>       | Illumina Hiseq 1500 | 2X100pb     | TruSeq DNA Sample Prep Kit       | SAMN40962362     |
| <i>mutS3R3</i>       | Illumina Hiseq 1500 | 2X100pb     | TruSeq DNA Sample Prep Kit       | SAMN40962363     |
| <i>mutS4R3</i>       | Illumina Hiseq 1500 | 2X100pb     | TruSeq DNA Sample Prep Kit       | SAMN40962364     |
| <i>mutS1R5</i>       | Illumina Miseq      | 2X150pb     | Nextera XT DNA Library Prep      | SAMN40962360     |
| <i>mutS1R16</i>      | Illumina Miseq      | 2X150pb     | Nextera XT DNA Library Prep      | SAMN40962361     |

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