Supplementary Note 1: Evolution of bet-hedging genotypes

Bet-hedging genotypes evolved in replicate selection lines one ($1B^4$) and six ($6B^4$) in the ninth selection round, after a total of 16 and 14 propagations, respectively. A propagation involved 72 hours of growth in batch culture (equivalent to approximately 10 generations). The number of propagations required for the evolution of a genotype with a new colony morphology in a selection round varied between 1 and 4, and did not correlate with round number or selection environment. After bet-hedging had evolved, new colony types were present after one propagation without exception.

The bet-hedging genotypes were selected, as were all other genotypes, on the basis of having a different morphology. However, the fact that they produced additional colony variants upon re-streaking (See Methods) prompted a more detailed examination, which indicated that they randomly switched colony morphology.

Supplementary Note 2: Extinction of a bet-hedging genotype under a modified regime

Rapid stochastic colony morphology switching facilitated persistence under our selection regime. To examine the mechanism behind this ecological success, we re-imposed this regime on $1B^4$ in static and shaken microcosms, but ignored the colony variants that this genotype generated through rapid colony switching. To this end, six replicate selection lines were founded with $1B^4$ in both static and shaken microcosms. New genotypes with different colony morphologies emerged in all replicates after approximately 8 propagations. Some of these genotypes no longer switched (3 and 4 of the 6 replicates in static and shaken microcosms, respectively), while the others switched between colony types distinguishable from those of $1B^4$. Under the rules of our selection regime, these new genotypes would pass on to the next round of selection, and thus drive $1B^4$ to extinction. These observations show that rapid colony switching persisted by acting as a bet-hedging strategy, facilitating evasion of the fluctuating selection for colony innovation before the evolution of new genotypes with novel colony morphologies by mutation and selection.

Supplementary Method 1: Genetic analysis of the capsule phenotype

To determine the genetic architecture of the capsule phenotype, the bet-hedging genotype ($1B^4$) was subjected to transposon mutagenesis using a derivative of Tn5 (ref. 19). A screen of 68,743 transposon mutants identified 183 mutants of which the colonies were 'locked' in either the translucent or opaque state. The genomic location of the transposon was determined in 154 of these mutants by sequencing of the DNA flanking the transposon, and locating this sequence in the *Pseudomonas fluorescens* SBW25 genome sequence[23]. This identified 33 mutants in which the transposon had inserted in a gene involved in the synthesis of colanic acid – a previously characterised capsule polysaccharide[20] – or a precursor thereof (Supplementary Table 1). Mutants locked in the translucent state contained a severely diminished proportion of Cap^+ cells, while transposon insertions in *galU* completely abolished the formation of Cap^+ cells. Together, these findings implicate that the capsules consist of colanic acid, and corroborate the link between the morphology of colonies and cells.

We screened a large library of transposon mutants incapable of rapid switching, but failed to identify any insertions in regions of DNA resembling contingency loci such as specific unstable DNA sequences (*e.g.* short repeats), genes encoding methylases, or invertase genes, which have been shown to underlie rapid stochastic phenotype switching in bacteria. Thus, these data also support the notion that switching in $1B^4$ does not involve such contingency loci.

Supplementary Table 1: Transposon mutants of 1B[4] in colanic acid genes

Gene	Putative protein function[a]	Metabolic pathway[a]	Hits[b]
wcaJ	UDP-glucose lipid carrier transferase	Colanic acid	3
Pflu3659	–	Colanic acid[c]	1
wza	Capsular polysaccharide translocation	Colanic acid	2
Pflu3663	–	Colanic acid[c]	1
wcaI	Glycosyl transferase	Colanic acid	1
waaE	Heptose kinase/adenyltransferase	Colanic acid	1
wcaF	Acetyl transferase	Colanic acid	1
Pflu3671	Glycosyl transferase	Colanic acid	1
Pflu3673	Glycosyl transferase	Colanic acid	2
Pflu3674	Acetyl transferase	Colanic acid	1
wzx	O-antigen flippase	Colanic acid	1
ugd	UDP-glucose-6-dehyrogenase	Colanic acid	1
wzc	Tyrosine protein kinase	Colanic acid	2
galU	UTP-glucose-1-phosphate uridylyltransferase	Colanic acid precursor	4
pgi	Glucose-6-phosphate isomerase	Colanic acid precursor	9
algC	Phosphomannomutase/phosphoglucomutase	Colanic acid precursor	2

[a] Based on amino acid similarity.
[b] Independent transposon insertions.
[c] Based on location in putative colanic acid biosynthesis gene cluster.

Supplementary Method 2: Testing for reversible switching

We observed a relationship between the morphology of colonies and cells: opaque colonies contained a higher proportion of Cap^+ cells than translucent colonies and, *vice versa*, Cap^+ cells were more likely to form opaque colonies. This led us to hypothesise that stochastic colony morphology switching was caused by reversible stochastic switching between the Cap^+ and Cap^- states at the level of the cell. Alternatively, stochastic colony switching could involve two distinct Cap^+ and Cap^- genotypes that do not switch, but rather form colonies with different morphologies due to plating of cell aggregates that contain both cell types, or via chance co-localisation of Cap^+ and Cap^--cells upon plating.

To distinguish between these hypotheses, we performed an experiment that examined the capacity of cells of $1B^4$ to switch reversibly between the Cap^+ and Cap^- states within a growing cell lineage (Supplementary Fig. 1a). In this experiment, ten replicate cell-lineages were generated by passing populations through two sequential single-cell bottlenecks (plating to single colonies, and continuing with cells from a single colony), allowing the populations to grow after each bottleneck (Supplementary Fig. 1b). We measured the number of replicate cell-lineages in which both Cap^+ and Cap^- cells were present in the population after each of the bottlenecks.

The logic behind this experiment is that the number of replicate lineages in which both Cap^+ and Cap^- cells are present after each of the two bottlenecks (hereafter positive lineages) depends on whether cells are capable of reversible switching. If the cells do not switch reversibly, positive lineages can only occur as a consequence of co-localisation and mixed cell-type aggregates – that is, failure of bottlenecking by plating to single colonies (Supplementary Figure 1b). We estimated the expected number of positive lineages due to these mechanisms on the basis of the proportions of Cap^+ cells, and the proportion of Cap^+ and Cap^--cell aggregates, which both were measured before each bottleneck. Using statistics, we compared the expected number of positive lineages under the null hypothesis of no reversible switching to that observed in the experiment. Rejection would lead us to except the alternative hypothesis of reversible switching (this analysis is explained in Supplementary Note 3).

Because we were specifically interested in detecting reversible switching, we enriched for Cap^+ and Cap^- cells before the first and second bottleneck, respectively. This increased the probability that the cells passing these bottlenecks were of the opposite type, and thus the power of our experiment to detect reversible switching. Enrichment was achieved using a serendipitously discovered method based on centrifugation (13,000 r.p.m., 1 min), which caused Cap^- cells to form a pellet, while Cap^+ cells formed a viscous aggregate that remained in the supernatant.

Ten replicate lineages were founded from independent colonies that had been obtained by plating of a Cap^+ enriched population of which the Cap^+ ratio had been measured (the first bottleneck). Subsequently, cells from each of these colonies were used to found liquid cultures

that were grown for 24 h. Next, these populations were enriched for Cap^- cells, analysed to determine the Cap^+ proportion, and plated to single colonies (the second bottleneck). After plating, a randomly selected colony from each of the ten replicates was screened for the presence of both Cap^+ and Cap^- cells. Positive lineages were those that contained both cell types after each bottleneck.

Supplementary Figure 1

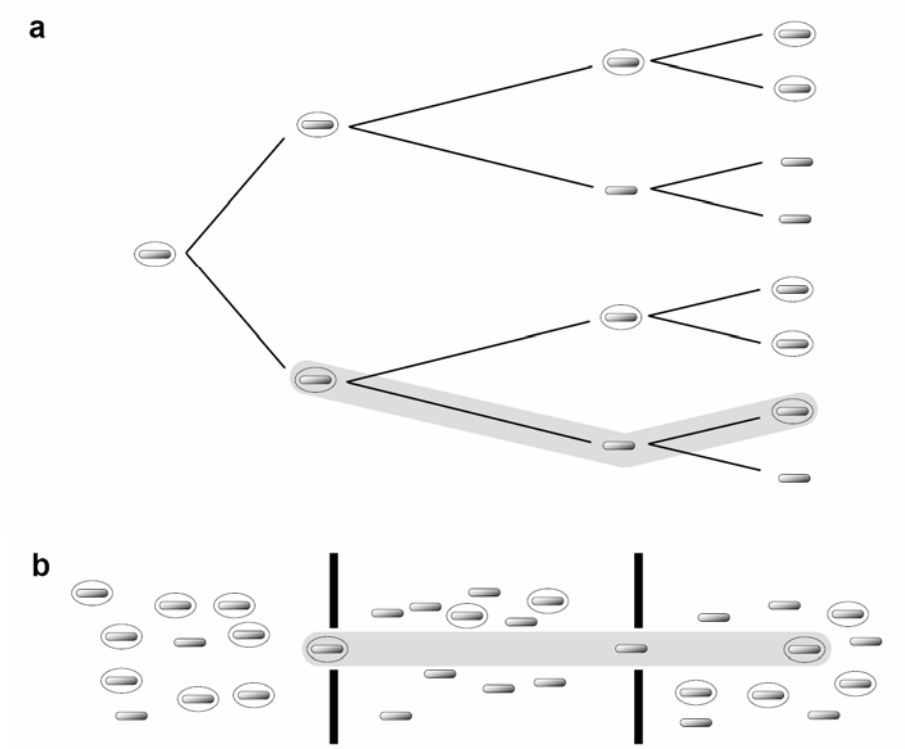

Figure S1. Testing for reversible stochastic on-off switching of capsule expression. a, Stochastic capsule switching occasionally causes cell-lineages to switch between Cap^+ and Cap^- during growth (highlighted lineage). **b,** Demonstrating reversible switching of capsule expression in a cell-lineage using two single-cell bottlenecks. A Cap^+ enriched population (left) was passed through a single-cell bottleneck (black lines), and allowed to grow. The resulting population was subsequently enriched for Cap^- cells (middle), passed through a second single-cell bottleneck, and again allowed to grow (right). When Cap^+ and Cap^- cells passed through the first and second bottlenecks, respectively, then any Cap^+ cells in the population after the second bottleneck (right) are derived from a lineage that switched back and forth between Cap^+ and Cap^- (highlighted).

Supplementary Note 3: Results and analysis of test for reversible switching

We found that all ten replicate lineages contained both Cap^+ and Cap^- cells after each of the two bottlenecks. To test the null hypothesis of no switching against this observation, we estimated the number of positive lineages expected under this hypothesis. This required estimation of the likelihood of colonies being founded by both Cap^+ and Cap^- cells, rather than by a single cell.

To this end, we first estimated the probability of a colony being founded by a cell aggregate containing both cell types. This was done by determining the proportion of such aggregates in the cell suspensions used in the experiment. We examined 888 particles and found 0 aggregates containing both Cap^+ and Cap^- cells. Based on this, we calculated a conservative estimate (with respect to rejection of the null hypothesis) of the true proportion of mixed cell-type aggregates. This involved determining the 95% binomial confidence interval, and taking the upper confidence limit as the proportion of cell aggregates present in any population before each bottleneck. Thus we estimated that the probability of a colony being formed by a cell aggregate containing Cap^+ and Cap^- cells was 0.004.

Next, we estimated the probability of mixed cell-type colonies due to chance co-localisation of Cap^+ and Cap^--cells upon plating. This was done on the basis of measurements of the proportion of Cap^+ cells before each bottleneck (388 cells examined before the first bottleneck, and 50 cells examined per replicate before the second bottleneck). We assumed that 150 cells were plated per plate. Using the measured Cap^+-cell ratios, we calculated the fraction of the total plate area that would be covered by Cap^+-cell founded colonies if (hypothetically) the cells of this type had been plated first. We then calculated the number of Cap^- cells in the sample that, if these Cap^--cells had been plated next (hypothetically), would localise on the plate such that their colonies would overlap with those founded by the Cap^+ cells. Overlapping colonies would appear as single colonies containing cells of both types. Based on this number, we calculated the fraction of colonies that was founded by cells of different types. The estimate obtained in this way is conservative with respect to rejection of the null hypothesis. The probability of a colony being founded by cells of two types due to colocalization was 0.01 for the first bottleneck, and between 0 and 0.02 for the second bottleneck.

On the basis of these probabilities, we calculated the number of positive lineages expected to occur if cells did not switch. The mean probability of observing Cap^+ and Cap^- cells after each of the two bottlenecks in a lineage due to aggregates and co-localisation was 6.45×10^{-5}. Accordingly, the expected number of positive replicates under the hypothesis of no switching was zero. It is highly unlikely that this mechanism accounts for the experimental observation of ten positive lineages from ten replicates (one-tailed Fisher's exact test, $P < 0.0001$). Thus, we rejected the null hypothesis. The addition of unidirectional switching to these mechanisms also did not explain the data (one-tailed fisher's Exact Test, $P = 0.0004$), leading us to accept the

alternative hypothesis that cells of $1B^4$ are capable of reversible switching between the Cap^+ and Cap^- states.

We estimated that seven of the ten replicate cell-lineages passed through a Cap^+ and a Cap^- single-cell bottleneck, and thus switched reversibly between Cap^+ and Cap^- during the experiment (estimated 95 % binomial confidence interval: 4 – 9).

Supplementary Note 4: ANCOVA for interaction between the *carB* mutation and the genetic background

Direct statistical testing for an interaction between the *carB* mutation and the genetic backgrounds of $1A^0$ and $1A^4$ required statistical comparison of the relative fitness data from the $1A^0$ vs. $1A^0$*mut* and $1A^4$ vs. $1A^4$*mut* competitions (Fig. 4). A conventional one-way ANOVA did not reveal a significant interaction ($P > 0.05$). However, the capacity to detect such an interaction was limited by the large variation in the $1A^0$ vs. $1A^0$*mut* data (Fig. 4). A likely explanation for this variation was the evolution of new niche specialist genotypes, which occurred during the course of the $1A^0$ fitness assays. To examine if the emergence of these new genotypes could account for the variation, we compared the effect of the *carB* mutation in the two genetic backgrounds (by comparing the relative fitness of $1A^0$ vs. $1A^0$*mut* to that of $1A^4$ vs. $1A^4$*mut*) using ANCOVA with the fraction of new genotypes evolved as a covariant. The fraction of new genotypes had a significant positive effect on fitness (analysis of deviance, $F(1,16) = 11.636$, $P = 0.0036$), and the ANCOVA revealed a significant interaction between the effect of the mutation and the genetic background (analysis of deviance, $F(1,16) = 8.536$, $P = 0.01$). Thus, controlling for the variance caused by the stochastic emergence of the new types exposed a significant epistatic interaction for fitness between the *carB* mutation and the genetic background. Moreover, the estimated mean fitness effects of the *carB* mutation at covariate value of 0 for $1A^0$ was 0.88 , while for $1A^4$ this was 1.13 (95% confidence intervals 0.73 – 1.03 and 1.05 – 1.24, respectively). These estimates are significantly different (*t*-test, $P < 0.01$), and indicate opposing fitness effects of the *carB* mutation in the two genetic backgrounds.