Repeated Phenotypic Evolution by Different Genetic Routes in 
*Pseudomonas fluorescens* SBW25

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**Abstract**

Repeated evolution of functionally similar phenotypes is observed throughout the tree of life. The extent to which the underlying genetics are conserved remains an area of considerable interest. Previously, we reported the evolution of colony switching in two independent lineages of *Pseudomonas fluorescens* SBW25. The phenotypic and genotypic bases of colony switching in the first lineage (Line 1) have been described elsewhere. Here, we deconstruct the evolution of colony switching in the second lineage (Line 6). We show that, as for Line 1, Line 6 colony switching results from an increase in the expression of a colanic acid-like polymer (CAP). At the genetic level, nine mutations occur in Line 6. Only one of these—a nonsynonymous point mutation in the housekeeping sigma factor rpoD—is required for colony switching. In contrast, the genetic basis of colony switching in Line 1 is a mutation in the metabolic gene carB. A molecular model has recently been proposed whereby the carB mutation increases capsulation by redressing the intracellular balance of positive (ribosomes) and negative (RsmAE/CsrA) regulators of a positive feedback loop in capsule expression. We show that Line 6 colony switching is consistent with this model; the rpoD mutation generates an increase in ribosomal gene expression, and ultimately an increase in CAP expression.

**Key words:** evolution, genetics, microbiology.

**Introduction**

The repeated appearance of similar phenotypes is a striking feature amid the diversity of life. Many phenotypes have evolved multiple independent times in different lineages (Conway Morris 1999). Examples include the evolution of analogous wing-like structures for flight in pterosaurs, birds, insects, and bats (Alexander 2015), C4 photosynthetic pathways in plants (Sage et al. 2011), and single-lens camera eyes in vertebrates and molluscs (Ogura et al. 2004). An intriguing aspect of repeated phenotypic evolution is the extent to which the underlying genetics are also conserved. It is commonly thought that the degree of genetic parallelism correlates with the degree to which two organisms are related. The accumulation of genetic data in recent years has shown this assumption to be in need of revision. For example, clonal populations of *Escherichia coli* adapt to thermal stress via different genetic routes (Riehle et al. 2001), whereas pigmentation changes in mice and lizards are both underpinned by mutations in the *Mct1r* gene (Nachman et al. 2003; Rosenblum et al. 2004). The increasing number of examples of disparity between genetic parallelism and degree of relatedness (reviewed in Arendt and Reznick 2008) hints at the underappreciated and poorly understood complexity of biological systems.

An evolution experiment with populations of the model bacterium *Pseudomonas fluorescens* SB25 (Beaumont et al. 2009) has provided an opportunity to characterize a case of repeated phenotypic evolution in unusual detail. Twelve independent populations were subjected to multiple rounds of selection for novel colony morphology. Each round of selection concluded with the isolation of a single colony per population. This colony had a phenotype different from that of the immediate ancestor, and was used to found the subsequent round of selection (fig. 1A). The final result was 12 independent evolutionary lineages, each with a clearly defined history of colony phenotypes and underlying genetic changes. Two lineages (Line 1 and Line 6) converged on a striking capacity to stochastically switch between two different colony morphologies at high frequency.
Colony switching in Line 1 has been extensively investigated (Beaumont et al. 2009; Libby and Rainey 2011; Rainey et al. 2011; Gallie et al. 2015; Remigi et al. 2019). Emergent genotype $B^4$ produces a mixture of opaque and translucent colonies, and a corresponding mixture of capsulated and noncapsulated cells (fig. 1B and C; Beaumont et al. 2009). The capsule consists of a colanic acid-like polymer (CAP), the ON/OFF expression of which leads to colony switching (Gallie et al. 2015). Nine mutational steps occurred during the evolution of $B^4$ (fig. 1B). The first eight occur in genes involved in the production of c-di-GMP, a secondary messenger that affects the expression of an acetylated cellulosic polymer (cellulose for short; Spiers et al. 2003; McDonald et al. 2009, fig. 1B). The final mutation affected the central metabolic gene $carB$ (c2020t, giving amino acid change R674C). This mutation, which is alone sufficient to cause colony switching (fig. 1D), perturbs intracellular pyrimidine pools (Gallie et al. 2015). Pyrimidine deficiency in $B^4$ has recently been shown to generate—by a currently unknown mechanism—an increase in intracellular ribosome concentration (Remigi et al. 2019). This has led to the proposal of a translational control model for capsule switching (Remigi et al. 2019). Briefly, the model proposes that capsule switching results from competition for binding sites on the mRNA of the capsule biosynthetic machinery (e.g., $algC$). CAP biosynthetic machinery (20 genes: $wcAl–wzc$) and CAP regulators ($pflu3655, pflu3656, pflu3657, gacA/gacS$). A direct deletion of the CAP biosynthetic locus from $B^4$ resulted in loss of both cell capsulation and colony bistability (supplementary fig. S1, Supplementary Material online). Together these results demonstrate that the structural basis of the $B^4$ capsule is encoded by the $wcAl–wzc$ locus.

To investigate whether $CAP$ production is controlled at the level of transcription, transcriptional fusions were constructed in $A^4$ and $B^4$; lacZ was transcriptionally fused to $wcAl$ ($pflu3658$), the first gene in the CAP biosynthetic locus (supplementary text S2; see Supplementary Material online for details). Cotranscription of $wcAl$ and $lacZ$ in these strains generates blue colonies (or blue sectors within white colonies) on LB agar supplemented with X-gal. Indeed, $B^4–wcAl–lacZ$ produced a mixture of white and blue colonies (supplementary fig. S1, Supplementary Material online), with high proportions of $CAP^+$ and $Cap^+$ cells, respectively. The same construction in $A^4$—the immediate switch ancestor—resulted in uniform, nonsectored colonies. Together, these results show that CAP expression is at least partially controlled at the level of transcription (later corroborated by RNA-seq data; supplementary tables S3–S5; see Supplementary Material online for details).

6B$^4$ Capsule Expression Is Due to Transcriptional Regulation of $wcAl–wzc$

To identify the genetic basis of the $6B^4$ capsule, $6B^4$ was subjected to transposon mutagenesis. In a screen of $\sim$10,000 transposon mutants, 55 with altered levels of capsule were identified, and the transposon insertion site determined for each (supplementary table S1, Supplementary Material online). Microscopic screening of cells showed capsule production to be eliminated in 43 genotypes, and severely reduced in a further nine genotypes. Three genotypes showed an increase in capsule production.

Of the genotypes with eliminated or reduced capsule production, 41 (75%) contained insertions in genes required for the production of a CAP, a polymer previously described as the structural basis of the $B^4$ capsule (Gallie et al. 2015). These include insertions in genes predicted to encode CAP precursor biosynthetic machinery (e.g., $algC$), CAP biosynthetic machinery (20 genes: $wcAl–wzc$) and CAP regulators ($pflu3655, pflu3656, pflu3657, gacA/gacS$). A direct deletion of the CAP biosynthetic locus from $B^4$ resulted in loss of both cell capsulation and colony bistability (supplementary fig. S1, Supplementary Material online). Together these results demonstrate that the structural basis of the $B^4$ capsule is encoded by the $wcAl–wzc$ locus.

6B$^4$ Shows Colony and Capsule Instability

The evolutionary history of $6B^4$ includes ten colony phenotypes, with translucent–opaque colony instability emerging after nine rounds of selection (fig. 2A). $6B^4$ colonies comprised a mixture of capsulated and noncapsulated cells, and $6B^4$ populations contain a significantly higher proportion of capsulated cells than those of the immediate ancestor, $6A^4$ (fig. 2B and C; Welch two-sample $t$-test $P = 1.3 \times 10^{-10}$). Single $6B^4$ cells of either type give rise to mixed $Cap^+/Cap^-$ populations (supplementary text S1, Supplementary Material online). We conclude that colony switching in $6B^4$ has the same underlying phenotypic basis as in $B^4$: the ON/OFF switching of capsule biosynthesis. However, under the conditions tested, the proportion of capsulated cells is significantly higher in $6B^4$ than in $B^4$ populations (fig. 2C; Welch two-sample $t$-test $1.9 \times 10^{-6}$). In addition, on KB agar $6B^4$ capsules are 1.26–1.83 times larger than those in $B^4$ (two sample $t$-test for no difference in capsule area $P = 9.602 \times 10^{-18}$), despite no difference in cell size (two sample $t$-test for no difference in cell area $P = 0.5236$; see supplementary text S1, Supplementary Material online).

The Structural Basis of the 6B$^4$ Capsule Is CAP

To directly investigate composition of the $6B^4$ capsule, extracellular polysaccharide (EPS) was extracted from SBW25, $6A^4$, $6B^4$, $1A^4$, and $1B^4$, and the component sugars from each strain analyzed by chromatography (results for SBW25, $1A^4$, $1B^4$ reported previously in Gallie et al. 2015; supplementary fig. S1 and table S2, Supplementary Material online). The analysis shows differences in the expression of several components: in $6B^4$ relative to $6A^4$, the expression of $\alpha$-fucose (Fuc), $\alpha$-glucuronic acid (GlcA), $\beta$-galacturonic acid (GalA), and two
unknowns are increased. Each of these is also increased in 1B4 relative to 1A4, indicating that the 1B4 and 6B4 capsule polymers are very similar.

Thus far, the transposon mutagenesis, strain constructions and structural analysis of the capsule polymers (and later, RNA-seq data) point to the same phenotype for 1B4 and 6B4: switching between opaque and translucent colonies caused, at the single cell level, by ON/OFF expression of CAP. The only difference observed between the two genotypes lies in the frequency of capsulation and size of capsules (both increased in 6B4 relative to 1B4).

The Mutational History of 6B4

Next, the genetic basis of 6B4 capsule switching was investigated. Whole genome sequencing of 6B4 identified seven mutations. This was surprising, as at least nine mutations were expected - one per round of reverse evolution experiment (REE) selection (see fig. 1A). Sanger sequencing across the evolutionary series revealed two gaps: SBW25—6B4 (selection round 1) and 6B4—6A1 (selection round 2; table 1, fig. 2A). Extensive previous knowledge suggested that these two genotypes almost certainly carried mutations in one of three loci (wsp, aws, mwsR; McDonald et al. 2009). Sanger
sequencing of wspF revealed a point deletion in 6B0 (Δt475) that was reversed in 6A1, rendering 6A1 isogenic to the wild type SBW25 (fig. 2A). The reversal event was not repeated among 20 independent replicates of a single round of REE from 6B0, meaning that 6A1 is the result of either a rare mutational event, or recovery of SBW25 from previous rounds.

The Line 6 mutations occur in a modular, paired fashion. The first six mutations occur in previously identified c-di-GMP producing loci (awsX/awsR, wspF/wssB); mutations in these loci are known to cause the gain and loss of cellulose production and wrinkly spreader colony morphology (Beaumont et al. 2009; McDonald et al. 2009; Gallie et al. 2015; Lind et al. 2015, 2017; Lind PA, Libby E, Herzog J, Rainey PB, 2019). The sixth mutation—an in-frame, six bp deletion in the cellulose biosynthetic gene wssB—completely abolishes cellulose production (fig. 2A). Accordingly, the next pair of mutations occur in an unrelated locus: nlpD (pflu1301), which encodes a lipoprotein predicted to have a function in cell wall formation and cell separation in a range of bacteria (Stohl et al. 2016; Lind et al. 2017; Tsang et al. 2017; Yang et al. 2017). The first of these, mutation seven, generates a nonsense mutation in nlpD resulting in the production of cell chains and round colonies in 6B3 (fig. 2A). This mutation has previously been reported to generate a cell chain phenotype in SBW25 (Lind et al. 2017), and similar mutations have been reported in E. coli (Uehara et al. 2010), Vibrio cholerae (Möll et al. 2014), and Yersinia pestis (Tidhar et al. 2009).

In short, NlpD is an activator of cell division protein AmiC; inactivation of NlpD leads to incomplete cell division. Mutation eight converts the nlpD nonsense mutation to a frameshift at the start of the encoded protein, resulting in a truncated protein product that is unlikely to function. This results in the gain of wrinkly spreading colony morphology in 6B4 (fig. 2A). The ninth mutation, in rpoD (t1682c), results in capsulated and noncapsulated cell counts and colony morphologies (fig. 2A). The tenth mutation, in pflu1312, results in the production of translucent (Tr) and opaque (Op) colonies in 6B4 (fig. 2A).

**FIG. 2.** Emergence of colony switching in REE Line 6. (A) Phenotypes and genotypes of Line 6: colony morphology on KB agar (row 1), morphology of cells grown in shaken KB microcosms and stained with India ink (row 2), ability of cells grown on KB agar with calcofluor to produce cellulose (row 3), and mutations (shown at point of occurrence; bottom). (B) Colony and cell morphologies of 6B4 and its nonswitching immediate ancestor, 6A4. 6B4 gives rise to translucent (Tr) and opaque (Op) colonies, plus capsulated and noncapsulated cells. (C) The proportion of capsulated cells in various populations during stationary phase. Each bar is the mean of five replicate populations grown overnight in KB microcosms. Error bars are one standard error and stars denote statistical significance (**P < 0.001). Contrast and/or exposure of some images altered in Preview.
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Table 1. Mutations in the Line 6 Evolutionary Series.

| Strain       | Gene | Nucleotide Change | Amino Acid Change | Morph* |
|--------------|------|------------------|-------------------|--------|
| SBW25        | —    | —                | —                 | Smooth |
| 6B           | 1224 | wspF              | Δt1682c           | Smooth |
| 6A           | 1224 | wspF              | Δt1682c           | Smooth |
| 6B           | 5211 | awx               | Δc229–261         | Wrinkly|
| 6B           | 5210 | arsR              | c691t             | Wrinkly|
| 6B           | 1224 | wspF              | Δc151–165         | Wrinkly|
| 6B           | 1301 | nlpD              | Δc1720–1725       | Smooth |
| 6B           | 1301 | nlpD              | Δc565t            | Smooth |
| 6B           | 1301 | nlpD              | Δc566g            | Smooth |
| 6B           | 5592 | rpoD              | t1682c            | Wrinkly|

*pΔt1682c(40) indicates a frame shift caused by a base deletion; number of new residues before a stop codon is in parentheses.

**pΔt1682c(178) indicates a frame shift by a base insertion; number of new residues prior to a stop codon is in parenthesis.

*Morph indicates phenotype on the basis of colony morphology on KB agar and cellulose production.

into a tryptophan residue, reversing the cellular and colony phenotypes (fig. 2A). The final mutation, with which colony switching emerges, is in rpoD (t1682c, resulting in amino acid change V561A). This gene encodes the housekeeping sigma factor (σ70) that controls transcription of many genes involved in cell growth and division (Schulz et al. 2015).

There are two notable points of similarity and contrast between the evolutionary histories of 6B* (fig. 2A) and 1B* (fig. 1B). First, both lineages begin in a similar fashion with mutations affecting cellulose production and wrinkly spreader colony morphology. In Line 6, mutational routes to the wrinkly spreader phenotype are presumably rendered inaccessible by the sixth mutation (in wssB), providing an opening for a pair of mutations in nlpD. Contrastingly, cellulose production is not abolished in Line 1, with 1B* staining positive for cellulose (Gallie et al. 2015, fig. 1B). Accordingly, Line 1 mutations are in cellulose-affecting loci up until the final, switch-causing mutation. Second, the final mutation in each lineage—that with which colony switching emerges—is a nonsynonymous point mutation in different and, at first glance, functionally unrelated housekeeping genes.

The rpoD t1682c Mutation Alone Generates an Increase in Capsulation

To confirm that the final mutation causes colony switching in the presence of the prior mutations, t1682c rpoD was engineered into the immediate ancestor, giving 6A*rpoD* (supplementary text S2, Supplementary Material online). 6A*rpoD* gives rise to high-level colony and CAP switching, showing the same proportion of capsulated cells as 6B* (two sample t-test \( P = 1.6 \times 10^{-5} \); fig. 3B). Therefore, whereas the rpoD mutation does cause CAP switching, one or more of the prior mutations—or additional mutations that were not identified by analysis of the genome sequencing data—contribute(s) quantitatively to 6B* capsule switching. This is in contrast to the c2020t carB mutation in Line 1, which alone accounts for 1B* capsule switching (Gallie et al. 2015).

Repeated Evolution of Switcher Genotypes Reveals Additional rpoD Mutations

To identify additional mutations able to cause capsule switching in 6A*, new switcher genotypes were evolved from 6A*. Each of 56 independent microcosms was inoculated with 6A* and put through a single round of the REE (Beaumont et al. 2009). Nine new switcher genotypes were isolated from nine independent microcosms (genotypes Re1–Re9; supplementary text S1 and S2, Supplementary Material online). Sequencing of rpoD revealed a single, nonsynonymous point mutation in each; eight of the new switchers (Re1–Re8) contain mutation a1723c leading to amino acid change T575P, whereas one (Re9) carries a1745c causing amino acid change Q231STOP. All three rpoD mutations (t1682c, a1723c, a1745c) are located in the H–T–H motif that interacts with the −35 consensus sequence of σ70-dependent promoters (Hu and Gross 1988; Siegele et al. 1989, fig. 4A). Interestingly, mutation a1723c leads to a significantly higher capsulation rate than the others (two sample t-tests \( P < 0.001 \); fig. 4B and C).

Changes in a gene as central as rpoD are expected to have major effects on cell growth. Indeed, the growth profiles of each rpoD mutant differ from those of the ancestral strains (fig. 4D). The rpoD mutations increase growth rate and final density—at the cost of a longer lag phase—in comparison with 6A* (fig. 4D–F). These results are consistent with the rpoD mutations affecting σ70 activity during exponential growth.

Epistatic Interactions in Line 6 and Line 1

Nine independent switchers isolated from 6A* each carried a point mutation in rpoD (see above and fig. 4A). Six
independent switchers isolated from 1A4 each carried a point mutation in a pyrimidine biosynthetic gene (five in carB, one in pyrH; Gallie et al. 2015). To investigate this striking degree of lineage-dependent repeated evolution, the rpoD and carB mutations were swapped into the opposing backgrounds. That is, t1682c rpoD* (from 6B4) was engineered into 1A4 (giving 1A4-rpoD*) and c2020t carB (from 1B4) was engineered into 6A4 (giving 6A4-carB*; supplementary text S2, Supplementary Material online). The engineered genotypes showed characteristic switcher colony morphologies (fig. 5A) and an increase in capsulation (fig. 5B; Wilcoxon rank sum tests $P = 0.02857$), demonstrating that the rpoD and carB mutations cause switching in both genetic backgrounds. Notably the rpoD mutation causes a significantly higher capsulation rate than the carB mutation in both Line 6 and Line 1 (fig. 5B; one-sided Wilcoxon rank sum test $P = 0.01429$ and one-sided two sample t-test $P = 9.7 \times 10^{-4}$, respectively).

Next a competition experiment was performed under REE conditions between the evolved and engineered types from each Line (i.e., 6B4 vs. 6A4-rpoD* and 1A4 vs. 1B4 c2020t carB). In each case, the evolved genotype outcompeted the engineered genotype—the rpoD mutation is fitter than the carB mutation in 6A4, and vice versa in 1A4 (fig. 5C). These results demonstrate epistatic interactions between switch-causing and prior mutation(s) in each Line.

**Gene Expression Differences in the Presence of the t1682c rpoD Mutation (RNA-seq)**

Changes in the $\sigma^{70}$-promoter recognition and binding domain are expected to affect expression from $\sigma^{70}$-dependent promoters (or a subset thereof). Thus, the effect of the t1682c rpoD mutation on intracellular mRNA pools was investigated. Total mRNA was isolated from three biological replicates of exponentially growing 6A4, 6B4-Cap, and 6B4-Cap*. RNA-seq was performed on the mRNA fraction, and three comparative analyses were generated: (A) 6A4 versus 6B4-Cap, (B) 6A4 versus 6B4-Cap*, and (C) 6B4-Cap versus 6B4-Cap*.

A list of genes with detectable expression levels ($\sim$98% of all predicted genes in the SBW25 genome [Silby et al. 2009]) was generated for each comparison, and the three lists were then further split into genes with and without statistically significantly different expression levels (supplementary tables S3–S5, Supplementary Material online).

The greatest number of genes showing statistically significantly different expression was found in comparison B, 6A4 versus 6B4-Cap*, indicating that these are the two most physiologically distinct morphotypes. Of the 1,438 genes identified, 612 were more highly expressed in the ancestral 6A4 (including 33 flagella biosynthetic genes), and 826 were more highly expressed in 6B4-Cap* (including 24 CAP and seven alginate biosynthetic genes). Comparison A, 6A4 versus 6B4-Cap*, identified 495 differentially expressed genes with statistical significance, 427 (86%) of which are shared with comparison B. Comparison of 6B4-Cap versus 6B4-Cap* identified 945 differentially expressed genes with statistical significance, 427 (86%) of which are shared with comparison B. Comparison of 6B4-Cap* and 6B4-Cap+ identified 82 significantly differently expressed genes, 52 of which are more highly expressed in 6B4-Cap+ (including 33 flagella biosynthetic genes) and 30 in 6B4-Cap+ (including 12 CAP genes). Notably, mutant rpoD was found to be $\sim$1.74 times more highly expressed in 6B4-Cap+ than the wild type rpoD counterpart in 6A4 (adjusted $P = 0.0324$) indicating that the...
t1682c rpoD mutation leads to activation of rpoD transcription and/or inhibition of mRNA degradation (supplementary table S4, Supplementary Material online). Levels of rpoD mRNA in 6B4-Cap− are intermediate between those in 6A4 and 6B4-Cap+, as no significant difference in rpoD mRNA levels was detected between 6B4-Cap− and either of the other two types (supplementary tables S3 and S5, Supplementary Material online). A further five putative sigma factors are more highly expressed in 6B4-Cap+ than in 6A4 (rspl, pflu2609, pflu2725, pflu3898, pflu4613), indicating a general shift in gene expression.

A direct comparison of the changes in gene expression resulting from the rpoD (Line 6) and carB (Line 1) mutations may provide insight into molecular similarities between the strains. The equivalents of the above comparisons have been previously published for Line 1 (GEO GSE48900; Gallie et al. 2015). Although the numbers of differentially expressed genes are much higher in the Line 1 comparisons—most likely
attributable to there being only a single biological replicate for each Line 1 morphotype—the overall pattern remains; the highest number of differentially expressed genes is between 1A4 and 1A4-Cap⁺, and the lowest between 1B4-Cap⁻/C0 and 1B4-Cap⁺. A “comparison of comparisons” was performed, whereby each of comparisons A, B, and C for Line 6 was equated to the Line 1 counterpart. Lists of shared and unique genes for comparisons A, B, and C for Line 6 was supplemented with Table S6, Supplementary Material online. For comparison C, B4-Cap⁻ versus B4-Cap⁺, 26 genes are common between Line 6 and Line 1; nine of these are more highly expressed in Cap⁻ forms compared with the Cap⁺, and include four flagella genes and five genes of unknown function. The remaining 17 genes are more highly expressed in the Cap⁺ forms than in the Cap⁻, and include seven CAP genes, a transcriptional regulator, an inorganic ion transport gene and eight genes of unknown function. Together, the results corroborate the finding that capsules and flagella are mutually exclusive. A similar finding has recently been reported in Cronobacter sakazakii, in which induction of colanic acid

**Fig. 5.** The fitness effect of switch-causing rpoD and carB mutations is lineage dependent. (A) The t1682c rpoD and c2020t carB mutations both cause colony switching in the 6A4 and 1A4 backgrounds (seen as two colony types and/or sectors on KB agar, 72 h). (B) A capsule counting assay in stationary phase shows that the rpoD and carB mutations cause an increase in capsulation relative to the immediate ancestor in both lineages (Wilcoxon rank sum tests \( P = 0.02857 ^* \)). Bars = mean of four replicates, error bars are one SE. (C) Box plots of the fitness of the evolved type versus the engineered type in Line 6 and Line 1. Competition assays (1:1) were performed under the REE conditions in which the evolved types were originally isolated (72-h static microcosms). Values greater than 1 indicate a higher relative fitness of the first competitor (evolved types). Both competitions show a significant deviation from 1 (one-sided one sample t-test \(* P < 0.05, \*** P < 0.001\)).
biosynthesis is accompanied by a reduction in flagella gene expression (Chen et al. 2018).

Genes Encoding Ribosomal Proteins Are Overexpressed in 6B^4

The recently proposed ribosome–RsmAE model of 1B^4 capsule switching postulates that capsulation is controlled by the combined intracellular pool of ribosomes, RNA-binding proteins RsmA/RsmE and pflu3655–pflu3657 mRNA (Remigi et al. 2019, fig. 6A and B). According to the model, ribosomes and RsmAE compete for binding sites in pflu3655–pflu3657 mRNA; ribosome binding results in translation of transcriptional activators Pflu3655, Pflu3656, and Pflu3657, the downstream targets of which include pflu3655–pflu3657 and capsule biosynthetic genes. Thus, transcription of pflu3655–pflu3657 activates a positive feedback loop that triggers capsulation. RsmAE binding results in inhibition of pflu3655–pflu3657 mRNA translation, and thus promotes the noncapsulated state. RsmA and RsmE function (discussed in Vakulskas et al. 2015; Remigi et al. 2019) is under the immediate control of two small, noncoding RNAs: rsmY and rsmZ. These are each predicted to contain multiple RsmAE binding sites, allowing them to act as molecular sponges that remove RsmAE from the pool. Finally, rsmYZ expression is itself under the control of at least two master regulators: positive control by the GacA/GacS phosphorelay system (pflu2189/pflu3777; Lapouge et al. 2007) and repression by the transcriptional regulator MvaT (pflu4939; Breneci et al. 2009).

The above model predicts that genotypes with increased capsulation (such as 6B^4) contain higher levels of pflu3655–3657 mRNA as a result of a net increase in ribosomes. Consistent with the model, pflu3655, pflu3656, and pflu3657 mRNA levels are significantly higher in 6B^4-Cap^+ and 6B^4-Cap^- than in 6A^4. Indeed pflu3655 is the most highly differentially expressed gene in all three RNA-seq comparisons; it is expressed 572-fold more highly in 6B^4-Cap^- than in 6A^4, 31-fold more highly in 6B^4-Cap^- versus 6A^4, and 19-fold more highly in 6B^4-Cap^+ versus 6B^4-Cap^- (supplementary tables S3–S5, Supplementary Material online). Furthermore, the rpoD mutation leads to an increase in the mRNA of genes encoding ribosomal proteins (fig. 6A): 40 and 43 (of 53) genes encoding ribosomal protein show increased expression in 6B^4-Cap^- compared with 6A^4 and 6B^4-Cap^-, respectively (supplementary table S7, Supplementary Material online). Although only three of these show statistical significance (rpmG, rpmB, and rpsT are more highly expressed in 6B^4-Cap^- than in 6A^4; supplementary text S3, Supplementary Material online), there is a clear pattern of higher expression in the capsulated form. Further binomial tests provide strong evidence for increased expression of genes encoding ribosomal proteins in 6B^4-Cap^- versus 6A^4 and 6B^4-Cap^- (P < 0.001; supplementary text S3, Supplementary Material online). The second major constituent of mature ribosomes is ribosomal RNA, encoded by the 23S, 16S, and 5S rRNA genes. The RNA-seq data do not provide quantifiable differences in the expression of rRNA genes. This is because rRNA is highly abundant in total RNA preparations (~80% to 90% in exponential growth; Tissieres and Watson 1958), and so rRNA was removed from the preparation to quantify mRNA.

Taken together, the observed increases in pflu3655–pflu3657 and ribosomal mRNAs are consistent with the rpoD mutation generating an increase in ribosome expression and thus increasing the probability of capsulation.

Manipulating Components of the Model Generates Changes in 6B^4 Capsule Switching

To test whether the ribosome–RsmAE model (fig. 6A and B) underpins switching in 6B^4, components of the model were manipulated to bias the switch in favor of ribosomes (Cap^+) or RsmA/RsmE (Cap^-). The switch was tipped in favor of ribosomes by decreasing RsmA/E activity in two ways. First, rsmA1 (pflu4746) and rsmE (pflu4165) were individually deleted from 6B^4, giving 6B^4^-ΔrsmA1 and 6B^4^-ΔrsmE (supplementary text S2, Supplementary Material online). A capsule counting assay revealed increases in capsulation (fig. 6C); in particular, deletion of rsmA1 resulted in significantly higher levels of capsulation (one-sided t-test P = 0.001112), bringing the percentage of capsulated cells to almost 100%. The difference in capsulation between 6B^4^-ΔrsmA1 and 6B^4^-ΔrsmE may be partially explained by different expression profiles: in P. fluorescens CHA0, RsmA expression has been shown to be relatively constant, whereas RsmE is expressed at very low levels during exponential growth (Reimann et al. 2005).

Second, the model predicts RsmAE activity to be reduced by deletion of mvaT, which encodes a transcriptional repressor of rsmZ—itself a negative regulator of RsmAE—in Pseudomonas aeruginosa (Brenci et al. 2009). Deletion of mvaT (pflu4939) from 6B^4 or 1B^4 results in a significant increase in capsulation (P < 0.01; fig. 6D, supplementary text S2, Supplementary Material online).

In contrast to the above, any bias of the switch machinery in favor of RsmAE is expected to inhibit translation of pflu3655–pflu3657 mRNA, and thus reduce capsulation. Support for this side of the model comes from the transposon mutagenesis screen (supplementary table S1, Supplementary Material online). First, three capsule-reducing insertions were obtained in the GacA/GacS two-component sensory system, which is a negative regulator of RsmAE in γ-proteobacteria (Lapouge et al. 2007, fig. 6A). Inactivation of these genes is expected to increase RsmAE expression and decrease capsulation. Indeed, 6B^4-TnCre-gacA and 6B^4-TnCre-gacS (Cre-deleted forms of the transposon mutants, see “Materials and Methods” section and supplementary text S2, Supplementary Material online) showed a complete absence of capsulation (fig. 6E). Second, four transposon insertions were identified in genes involved in the production of mature tRNAs: two in gidA/mnmG (pflu6129), one in truA (pflu4189), and one in thiL (pflu0349; reviewed in Yacoubi et al. 2012). Each of these insertions resulted in a reduction in capsulation (fig. 6F). Although not lethal, disruption of each tRNA modification gene is expected to reduce translational speed (Yacoubi et al. 2012; reviewed in Shepherd and Ibba 2015), suggesting a role for efficient translation in capsulation.
The ability to increase and decrease 6B^4 capsules by manipulating components of the 1B^4 ribosome–RsmAE circuitry (as predicted by the model) demonstrates that the same intracellular architecture underpins switching in both genotypes.

**Discussion**

In this work 6B^4 has been extensively characterized. Its phenotype and genotype have been compared with those previously reported for 1B^4—a strain evolved in parallel to, but independently of, 6B^4 (Beaumont et al. 2009; Gallie et al. ...
6B\textsuperscript{a} and 1B\textsuperscript{a} populations show elevated levels of CAP-based capsule expression and emergent colony switching (figs. 1B–E and 2A). The phenotype is realized by two distinct genetic routes, culminating in a mutation in either rpoD (Line 6) or carB (Line 1). Both mutations promote increased expression of mRNA encoding positive regulators of the CAP biosynthetic machinery. These regulators also activate their own transcription, forming a positive feedback loop that results in bistable capsule expression (outlined in Remigi et al. 2019, fig. 6A and B).

Line 6 and Line 1 were derived from a single clonal ancestor (\textit{P. fluorescens} SBW25). This means that the genotypes of interest, 6B\textsuperscript{a} and 1B\textsuperscript{a}, share an evolutionary history of many millions of years followed by a comparatively minuscule period of several weeks of independent evolution in experimental microcosms. Given the extensive shared history, it is not surprising that the same phenotype emerged in both lineages. It is surprising, however, that such different genetic routes generate the same phenotype.

Repeated phenotypic evolution has been documented many times in both the laboratory (Riehle et al. 2001; Cooper et al. 2003; Fong et al. 2005; Ostrowski et al. 2005; Bantinaki et al. 2007; Meyer et al. 2012; Lindsey et al. 2013) and natural populations (Nachman et al. 2003; Rosenblum et al. 2004; Stern and Frankel 2013; Riveron et al. 2014). In many of these examples, repeated phenotypic evolution is determined by changes in the same gene or molecular pathway (Meyer et al. 2012; Lindsey et al. 2013; Riveron et al. 2014; Bertels et al., 2018). The fact that colony switching in Line 6 and Line 1 arise by different genetic pathways—despite extreme shared ancestry—is surprising. At first glance, rpoD and carB seem functionally unrelated and, as such, it is natural to assign them to separate functional compartments. However, this work shows that the two genes are connected at the level of their effects on the ribosome—RsmAE pool: both mutations increase expression of ribosomal genes (supplementary table S7, Supplementary Material online; Gallie et al. 2015; Remigi et al. 2019) tipping the switch in favor of CAP mRNA translation (fig. 6A and B).

The precise molecular mechanisms by which rpoD and carB mutations alter ribosomal gene expression remain to be elucidated. However, it is conceivable that the rpoD mutation directly increases the transcription of one or more ribosomal genes. A point mutation in \textit{Salmonella typhimurium} rpoD has recently been shown to increase transcription from rpsT (Knöppel et al. 2016). In the case of the carB mutation, which perturbs intracellular pyrimidine pools (Gallie et al. 2015), the reported influence of nucleotide triphosphate concentrations on \textit{rnm} promoters may play a mechanistic role (Gaal et al. 1997; Schneider et al. 2002, 2003; Murray et al. 2003; Schneider and Gourse 2003). If cellular components show a high degree of connectivity, it follows that many other factors could also affect the switch circuitry. Possible candidates include those affecting capsule expression and identified via the transposon mutagenesis screens (e.g., hslO, saHA, ndk; supplementary table S1, Supplementary Material online; Gallie et al. 2015).

In stark contrast to the disparate molecular evolution of 6B\textsuperscript{a} and 1B\textsuperscript{a}, repeated bouts of evolution from the same immediate ancestor of the 6B\textsuperscript{a} switching genotype, namely, 6A\textsuperscript{a}, resulted in re-evolution of the switching genotype by mutations solely in rpoD (fig. 4A). Similar repeated bouts of evolution from 1A\textsuperscript{a} (the immediate ancestor of the Line 1 switching genotype) resulted in switching types with mutations in genes encoding the determinants of pyrimidine biosynthesis (five in \textit{carB}, one in \textit{pyrH}; Gallie et al. 2015). In other words, the comparatively tiny portion of evolutionary history for which Line 6 and Line 1 diverged—several weeks compared with millions of years of common history—has a significant impact on molecular evolution.

The distinct classes of switcher mutations in Line 6 and Line 1 result from positive epistatic interactions: whereas both types of switch-causing mutations presumably arise in both backgrounds, rpoD mutations outcompete carB mutations in 6A\textsuperscript{a}, and vice versa in 1A\textsuperscript{a} (fig. 5C). Precisely which of the preswitcher mutations in each evolutionary series contribute to the observed epistatic effects remains to be tested. In the case of Line 6, the two \textit{nlpD} mutations immediately preceding the rpoD mutation are prime candidates for two reasons. First, \textit{nlpD} is the only locus that is mutated in Line 6 but not Line 1 (figs. 1B and 2A). Second, \textit{nlpD} is immediately upstream of rpoS (\textit{pflu1302}), which encodes the stationary phase sigma factor RpoS (\textit{\sigma}\textsuperscript{B}). RpoS and RpoD (together with other sigma factors) compete for binding of core RNA polymerase (Ishihama 2000; Mauri and Klumpp 2014), and so their relative intracellular concentration affects the expression level of their respective regulons (Gross et al. 1998; Mauri and Klumpp 2014). It is possible that the \textit{nlpD} mutations, in addition to altering colony morphology via a reduction of NlpD/AmIC activity, also alter the expression of rpoS. Indeed, a promoter for \textit{rpoS} has previously been reported within \textit{E. coli} and \textit{P. aeruginosa} \textit{nlpD} (Lange and Hengge-Aronis 1994; Takayanagi et al. 1994; Kojic and Venturi 2001). A change in RpoS concentration could conceivably set the stage for compensatory mutations in RpoD.

Understanding the molecular bases of adaptive phenotypes continues to present significant challenges even when aided by high-throughput genomic technologies. As shown here and elsewhere (Larsen et al. 2008; Bershtein et al. 2015; Gallie et al. 2015; Grenga et al. 2017; Carvalho et al. 2018), mutations—particularly those in central metabolism—can have complex effects that extend well beyond the immediate neighborhood of gene function. The point mutations in two seemingly unrelated genes (\textit{rpoD} and \textit{carB}) can generate stochastic capsule switching draws attention to the interconnectedness of cell physiology and highlights the extensive mutational opportunities available to evolution.

**Materials and Methods**

**Bacterial Strains, Plasmids, and Media**

Details of bacterial strains and plasmids used are provided in supplementary text S2, Supplementary Material online. Unless otherwise stated, \textit{P. fluorescens} strains were grown...
for 24 h at 28 °C in shaken microcosms with 6 ml King's Medium B (KB; Ward et al. 1954). Strains were assayed for cellulose production by overnight growth on KB agar containing 200 μg ml⁻¹ calcofluor (Fluorescent Brightener 28). Where indicated, 2 mM uracil (Sigma-Aldrich, U1128) was added to KB agar. Antibiotics were used at the following concentrations: tetracycline (12.5 μg ml⁻¹; Tc); kanamycin (100 μg ml⁻¹; Km); nitrofurantoin (100 μg ml⁻¹; NF); d-cycloserine (800 μg ml⁻¹).

Microscopy

Cell microscopy was performed using a Zeiss Axiostar Plus bright field microscope, coupled with fluorescence lighting for calcofluor visualization. Microscopy images were cropped and processed in Preview as indicated in figure legends.

Capsule Counting Assay

Capsule staining and the counting assay were performed as previously described in Gallie et al. (2015). Briefly, for each strain to be assayed, three to five single colonies were grown to stationary phase in KB cultures. Cultures were transferred to fresh KB and grown to mid-exponential or early stationary phase as indicated. Cells were stained with 1:8 diluted India ink (Pébeo) and photographed under bright field 60× magnification. Capsule expression was recorded manually for 500 cells per replicate (∼100 cells per photograph).

Gene Deletions and Mutation Construction

Gene deletions were constructed by pUIC3-mediated two step allelic exchange as described elsewhere (Zhang and Rainey 2007). For details of genetic constructs see Supplementary Material online for details.

Transposon Mutagenesis

6B4 was subjected to random mutagenesis as described in Giddens et al. (2007). Approximately 10,000 transposon mutants from 11 independent conjugations were screened on LB+Km agar plates, on which 6B4 mutants typically form opaque colonies after ~72 h. Translucent or otherwise different colonies were selected and screened microscopically for obvious alterations in capsule expression. Mutants of interest were purified and insertion sites determined by AP-PCR. In selected strains, the bulk of the transposon was deleted leaving 189 bp at the insertion site ("TnCre"-genotypes) and eliminating polar effects.

Isolation and Analysis of EPS

EPS was isolated and processed from 6A4 and 6B4 in parallel with that from SBW25, 1A4, and 1B4 previously reported in Gallie et al. (2015). EPS analysis was performed by The Callaghan Research Institute (New Zealand).

Genome Sequencing of 6B4

A colony of 6B4 was grown in a microcosm. Cap⁺ and Cap⁻ fractions were separated by centrifugation and genomic DNA isolated from each fraction using the cetyl trimethyl ammonium bromide (CTAB) method. Equal quantities of Cap⁺/⁻ DNA were mixed, and whole genome resequencing was performed (Illumina; Massey University, New Zealand). Point mutations were identified by aligning ~4.8 million 36 bp reads to the SBW25 genome (Silby et al. 2009) via SOAP2 (Li et al. 2009). Reads with more than two mismatches and/or that could not be uniquely aligned to the genome were discarded. A total of 4,028,678 reads aligned, giving a mean coverage of 21.57. 98.8% of the genome was covered by at least four nucleotides. Within this 98.8%, all positions with a minimum variation frequency of 0.8 were considered candidate point mutations; candidate insertions and deletions were identified by analyzing genomic regions with unusual coverage and BLAST analyses of discarded sequences. All candidate loci were checked by PCR amplification and Sanger sequencing from 6B4 and, where confirmed, were chronologically ordered by Sanger sequencing across Line 6. Genome sequence and analysis files are available on request.

Re-Evolution of Switchers from 6A4

Nine independent switcher genotypes were isolated from 6A4 in static microcosms, according to the REE protocol (Beaumont et al. 2009). Each switcher was purified, and the rpoD gene sequenced. Three strains, one carrying each of the rpoD mutations (6B4, Re1, Re9), were checked for bidirectional capsule switching (supplementary text S1, Supplementary Material online).

Growth Curves and Analysis

Eight colonies per strain were grown independently in 200 μl KB (26 °C, 200 rpm). Two microliters of each were grown in 198 μl fresh KB at 26 °C (BioTek Epoch 2 plate reader; OD₆₀₀ measured at 5 min intervals, 5 s of 3 mm orbital shaking preceding each read). Mean and SE of all wells per strain were used to draw figure 4D. Vₚmax (maximum growth rate) and lag time were calculated using a sliding window of six time points during exponential growth (between 1 and 24 h, based on observation of growth curves) using Gen5 Software version 3.00.19.

RNA-seq Analysis

For each of 6A4 and 6B4, three single colonies were grown in KB, diluted 1:1,000 into 20 ml KB in 250 ml flasks and grown to mid-exponential phase (~OD₆₀₀ of 0.4–0.6). Total RNA was harvested; for 6A4, 0.5 ml of culture were pelleted and resuspended in 1 ml of RNAlater (Ambion). For 6B4, Cap⁺ cells were harvested from larger culture volumes by centrifugation and resuspended in RNAlater to achieve a similar cell density to that of the Cap⁺ aliquot. All mRNA extractions proceeded using a RiboPure Bacteria Kit (Ambion). Specific depletion of rRNA (Ribo-Zero rRNA Removal Kit [Bacteria], Illumina), normalized mRNA-seq library preparation (TruSeq Stranded total RNA kit, Illumina) and 100 bp paired-end Illumina HiSeq 2500 sequencing was performed by New Zealand Genomics Limited (University of Otago, New Zealand; GEO submission number GSE116490). The data were analyzed with Bowtie2 (Langmead and Salzberg 2012), HTSeq (Anders et al. 2015), and R package DESeq2 (Love et al. 2014). First, RNA-seq data sets were mapped to the SBW25 genome (annotation file: GenBank NC_012660) via Bowtie2.
with default settings. The coverage per gene was determined with HTSeq. Differentially expressed genes were identified by applying DESeq2. The standard workflow in https://bioconductor.org/packages/release/bioc/manuals/DESeq2/man/DESeq2.pdf (last accessed 8 March 2019) was used, except that the alpha parameter was set to 0.3 to reduce the number of genes falsely classified as not significantly differentially expressed. Three comparisons were made: 6A4 versus 6B4-Cap+, 6A4 versus 6B4-Cap−, and 6B4-Cap+ versus 6B4-Cap− (supplementary tables S3–S5, Supplementary Material online). The corresponding comparisons for Line 1 are available elsewhere (Gallie et al. 2015).

Fitness Assays
Four (Line 6) or eight (Line 1) single colonies of each competitor were grown independently in shaken KB (28 °C). Four (Line 6) or eight (Line 1) competition microcosms were inoculated with ~5 × 10^6 cells of each competitor and incubated statically at 28 °C for 72 h. Competitor frequencies were determined by plating on KB agar at 0 and 72 h. Competing genotypes were readily distinguished by their distinctive morphologies and differing response to uracil (adding 2 mM uracil to KB agar visibly reduces switching caused by carB mutations, while having no effect on rpoD mutant switching). Relative fitness was expressed as the ratio of Malthusian parameters (Lensi et al. 1991). Deviation of relative fitness from 1 was determined by one-sample t-tests.

Statistical Analyses
To detect differences in capsule levels or nucleotide concentrations between two strains, two-sample t-tests (parametric or Welch) or, where normality assumptions were violated, Wilcoxon rank sum tests were applied. Kruskal–Wallis tests were used to detect differences in capsule levels across the three rpoD mutant strains. Exact binomial tests were used to detect differences in ribosomal gene expression between morphotypes in the RNA-seq data (see also supplementary text S3, Supplementary Material online). All analyses were performed in R version 3.3.3. On graphs: *0.05 < P < 0.01, **0.01 < P < 0.001, ***P < 0.001.

Supplementary Material
Supplementary data are available at Molecular Biology and Evolution online.

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