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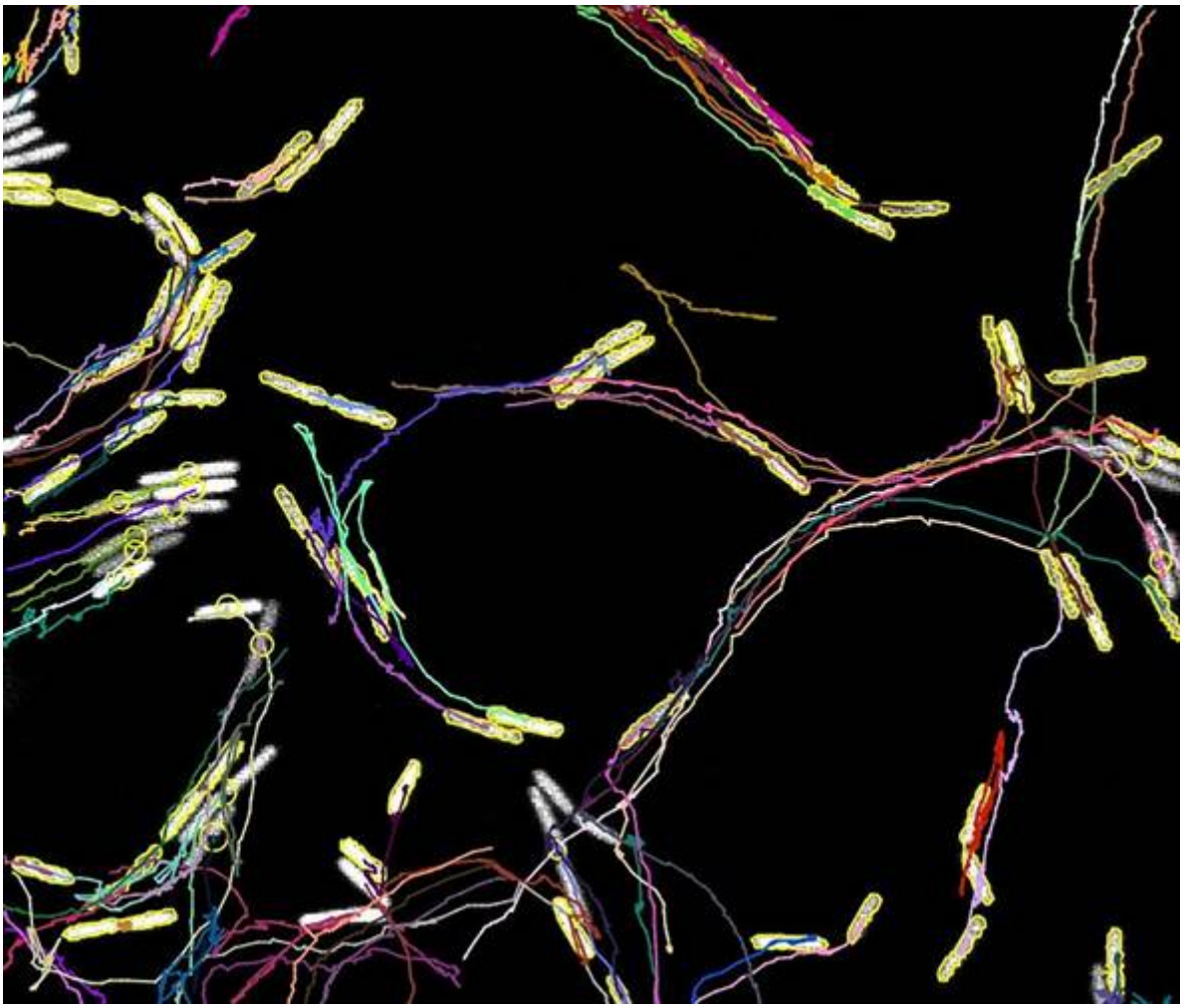

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EXPLORING GENOMIC PATHWAYS FOR BIOFILM FORMATION MECHANISMS IN A Pseudomonas aeruginosa HYPERMUTATOR STRAIN DEPLETED OF DIGUANYLATE CYCLASES

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Biofilms play a vital role in *Pseudomonas aeruginosa* (PA) infections, especially in cystic fibrosis patients, where the bacterium increases morbidity and mortality. Biofilm production correlates with high levels of cyclic-di-GMP (c-di-GMP), a bacterial second messenger that regulates virulence and the transition between planktonic and biofilm lifestyles. PA's genome harbors about 40 genes involved in c-di-GMP synthesis and degradation, highlighting its impact on bacterial behavior. These intracellular levels are controlled by diguanylate cyclases (DGCs) and phosphodiesterases (PDEs), which control its up- and down-regulation. Our previous research unveiled that during biofilm formation and dispersion cycles, PA employs compensatory mutations to progressively inhibit DGCs and PDEs, culminating in genetic constraint. However, mutator strains can circumvent this constraint, suggesting that alternative biofilm formation pathways exist but remain obscured due to the extensive mutational target size of c-di-GMP-related pathways. This led us to ask: What happens if all DGC pathways are disrupted? How do mutator strains adapt when c-di-GMP regulation is nearly halted? To investigate this, we engineered a PA14 strain (PA14?32) with premature stop codons in all 32 genome-encoded DGCs, depleting c-di-GMP-synthesizing proteins and abolishing biofilm formation. We further generated a mutator of this strain by disrupting the mutS gene (PA14?32mutS) and explored PA's capacity to overcome the severe genetic restriction hindering biofilm-producing phenotypes. Would the strain revert the engineered DGC mutations or reveal new, c-di-GMP-independent biofilm pathways? To answer these questions, we used a setup with PA14?32 and PA14?32mutS single-cell lines, conducting parallel evolution experiments in 96-well plates, and incubating them for 96 hours in static broth. We observed that approximately 25% of PA14?32mutS-derived lines produced biofilm, compared to 1.5% in the PA14?32 lines. Remarkably, these lines exhibited small colony variants (SCV) and other colony morphologies associated with heightened biofilm production. Subsequently, we selected 19 SCV-morphotype lines (1 from PA14?32 and 18 from PA14?32mutS) and 3 non-SCV morphotype lines from PA14?32mutS for whole-genome sequencing. Comparative genomics revealed that in 17/19 SCV lines, the engineered stop codon in YfiN—the corresponding DGC of the yfi system—was reverted, which reinstates the yfi pathway functionality. Notably, 3/17 mutations

restored the native DGC protein sequence. The YfiN regulatory mechanism, involving repressor-mediated repression and activation, likely favors single mutations for restoring normal function, which may explain the high mutation rate observed in yfiN. Our results indicate that PA's ability to restore biofilm formation after the complete abrogation of c-di-GMP synthesis pathways relies on the reversion of the engineered mutation of the DGC YfiN.

Palabras clave: Pseudomonas aeruginosa - Biofilm - c-di-GMP - Evolution