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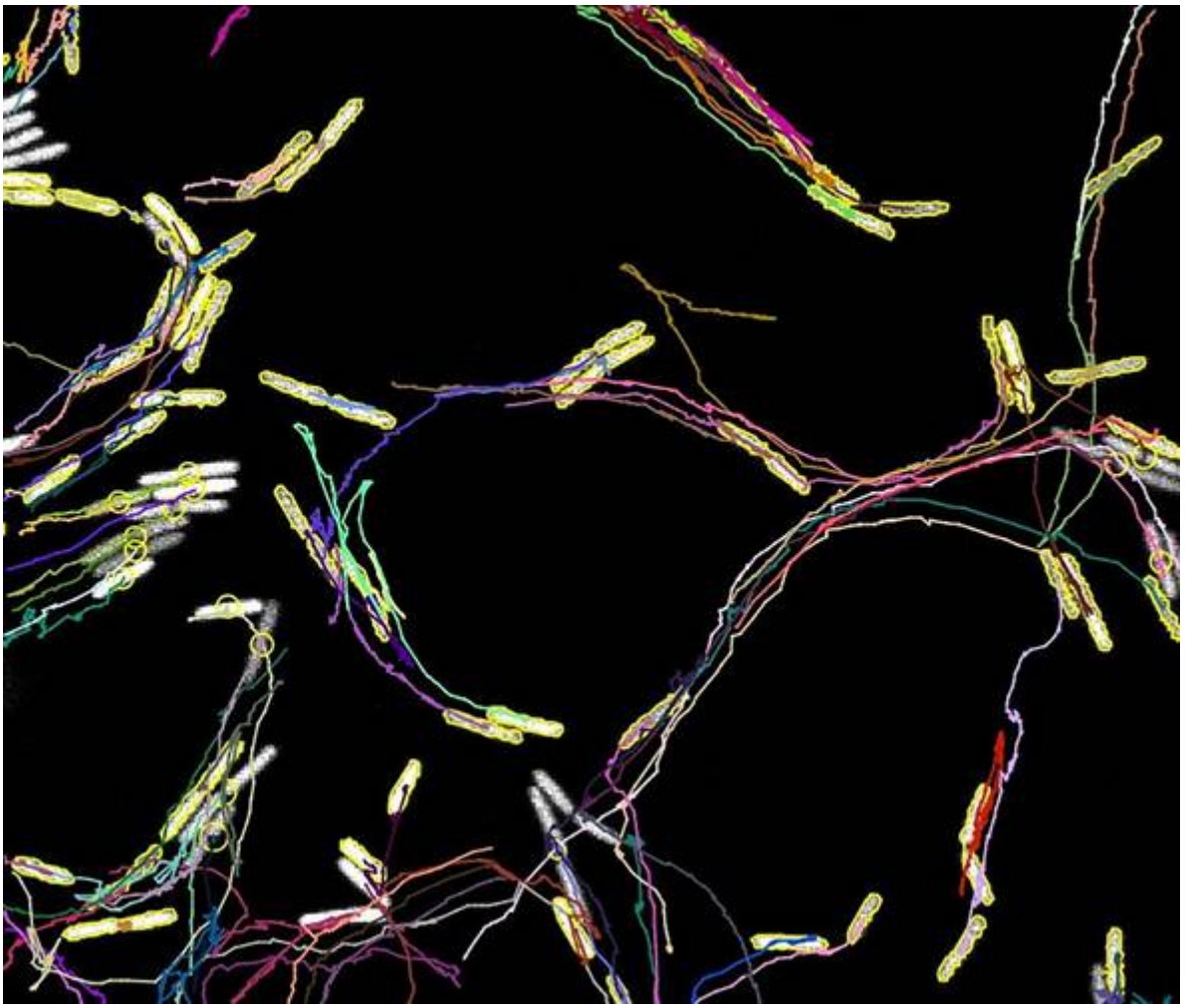

Foto: Se hace camino al andar. Celeste Dea. 1er puesto. Concurso fotográfico SAMIGE 20 años.

INTERPLAY BETWEEN C-DI-GMP AND THE EXTRACELLULAR MATRIX IN SHAPING SPATIAL PATTERNS OF ANTIBIOTIC TOLERANCE IN ESCHERICHIA COLI BIOFILMS

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Biofilms are bacterial communities structurally supported by an extracellular matrix (ECM). In *E. coli*, the distribution and organization of ECM components, such as amyloid curli and cellulose, have been well characterized using agar-grown macrocolony biofilms as a model system. In these macrocolonies, the interplay of opposing nutrient and oxygen gradients drives physiological differentiation in *E. coli* cells, resulting in ECM production being confined to the upper half of the biofilm, known as the upper stratum, while the lower half, adjacent to the agar, remains ECM-free. Although ECM production is known to depend on the second messenger c-di-GMP, how *E. coli* cells regulate c-di-GMP levels within biofilms remains unclear. Given the asymmetric distribution of ECM within the biofilm, we hypothesized that c-di-GMP levels might vary accordingly across the different biofilm strata. Furthermore, we hypothesized that this heterogeneity in ECM distribution or the associated c-di-GMP levels could contribute to a differential spatial pattern of antibiotic tolerance within *E. coli* biofilms. To test the first hypothesis, we utilized a plasmid-encoded c-di-GMP biosensor (pRib-cdiG) based on a c-di-GMP-activated triple-tandem riboswitch that drives the transcription of a fluorescent reporter to assess cellular c-di-GMP levels. Macrocolonies of *E. coli* AR3110 wild type and derivative mutants deficient in ECM components and/or key diguanylate cyclases and phosphodiesterases, which synthesize and degrade c-di-GMP and are involved in ECM synthesis, were analyzed both macro- and microscopically in thin sections to evaluate c-di-GMP levels. For each macrocolony, transverse sections across three regions along the macrocolony radius were examined. We found that in the outermost region (region 1) of wild-type and all mutant macrocolonies analyzed, no fluorescence signal was detected, indicating that c-di-GMP levels in those cells were below the biosensor's detection limit. This is consistent with the observation that the border is typically occupied by rapidly growing cells that exhibit flagella. In contrast, in region 2, the transition area between the border and the macrocolony center, and in region 3, the central region, fluorescence reflecting c-di-GMP levels was observed exclusively in the upper stratum of all macrocolonies analyzed. However, the intensity of fluorescence, i.e., the c-di-GMP levels, varied depending on the absence of specific c-di-GMP-metabolizing enzymes and showed an unexpected increase in the absence of ECM components. When testing the second hypothesis by analyzing aminoglycoside-

treated macrocolonies of *E. coli* AR3110 wild-type and mutant strains deficient in ECM production and/or exhibiting altered c-di-GMP levels, we found that the absence of ECM, combined with elevated c-di-GMP levels, rendered *E. coli* cells in the top zone of the upper macrocolony stratum, particularly in region 3, highly susceptible to the antibiotic. This contrasts sharply with the high survival rates observed in cells within the same zone/region of antibiotic-treated wild-type macrocolonies. In summary, our studies reveal, for the first time, the spatial pattern of c-di-GMP levels in relation to ECM distribution within *E. coli* macrocolony biofilms. Moreover, our findings underscore a striking inverse relationship between ECM and c-di-GMP in promoting antibiotic tolerance within a specific subzone of the biofilms. This subzone can act as a discrete "hot spot" for surviving cells, including persisters.

Palabras clave: biofilms- c-di-GMP- extracellular matrix (ECM)- riboswitch- antibiotic tolerance.