Sociedad Argentina de Microbiología General

XIX CONGRESO DE LA SOCIEDAD ARGENTINA DE MICROBIOLOGÍA GENERAL

22 al 25 de octubre del 2024
Centro cultural y Pabellón Argentina de la Universidad Nacional de Córdoba, Córdoba, ARGENTINA.

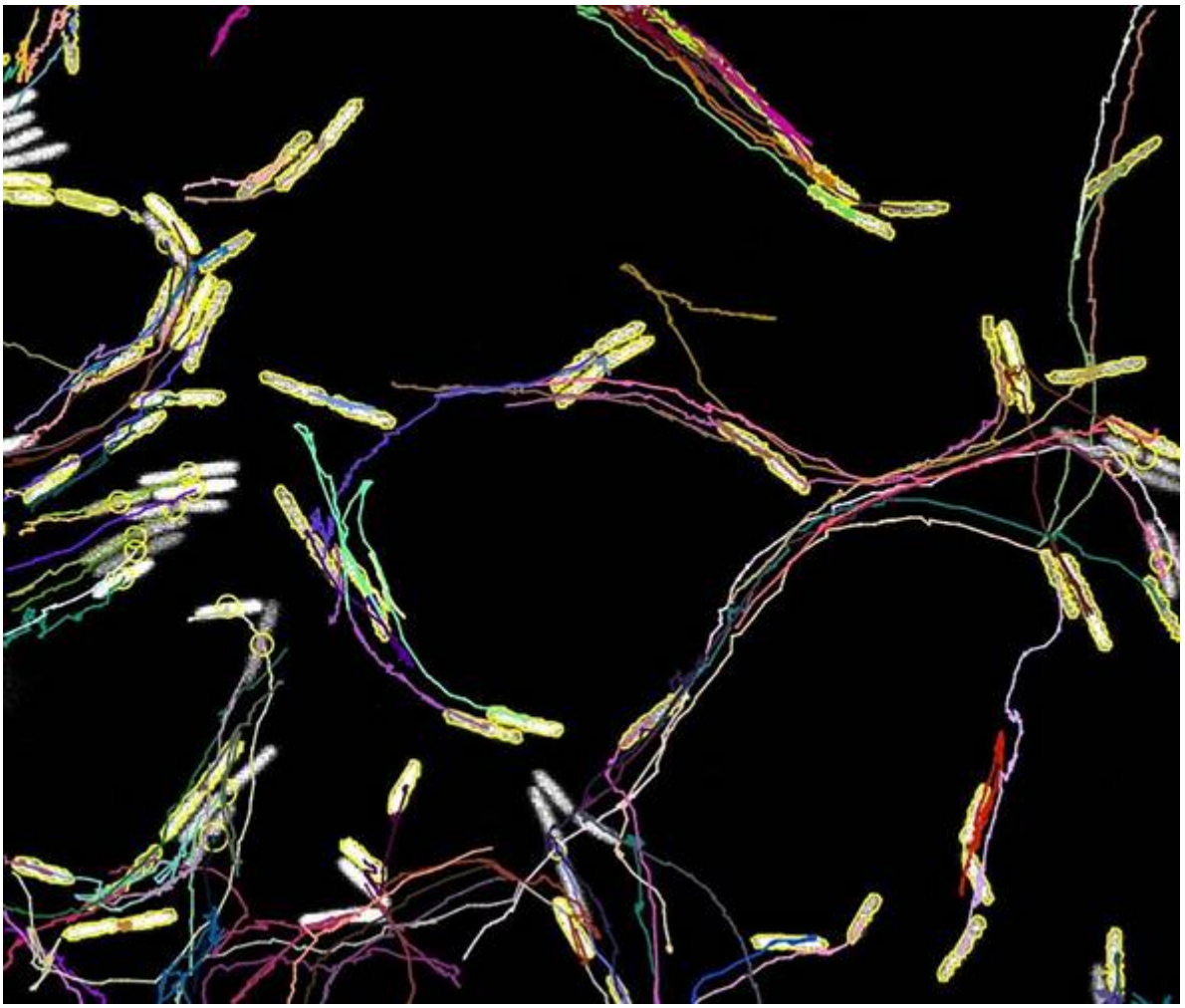

Foto: Se hace camino al andar. Celeste Dea. 1er puesto. Concurso fotográfico SAMIGE 20 años.

DIFFERENTIAL REGROWTH OF ANTIBIOTIC-TOLERANT SUBPOPULATIONS WITHIN Escherichia coli BIOFILMS AFTER TREATMENT

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Biofilms are bacterial communities formed by an extracellular matrix (ECM). This lifestyle is a significant factor in the persistence of infections, including those caused by *E. coli*. Within biofilms, cell subpopulations survive antibiotic treatments, facilitating the resurgence of infections. Despite extensive research, it is unclear which internal biofilm regions offer better survival conditions for bacteria and how these cells resume growth to rebuild the community after treatment.

Leveraging our understanding of *E. coli* physiology and ECM production in macrocolony biofilms and applying an approach to visually distinguish dead and live cells at the single-cell level, we began to reveal the internal patterns of cell death and survival within aminoglycoside-treated *E. coli* biofilms. By analyzing transverse sections across three regions along the macrocolony radius, we found that in the border region, representing the youngest area with rapidly growing cells, aminoglycosides eradicated all bacteria. In contrast, in the more mature regions towards the microcolony center, we observed clearly defined and interspersed zones of bacterial death and survival, categorized as "susceptibility zones" and "tolerance zones", respectively.

The antibiotic tolerance zones corresponded to the upper part of the upper stratum (tolerance zone I), which represents the top half of the biofilm where nutrient-starved cells enter the stationary phase and produce ECM components, and the inner part of the lower stratum (tolerance zone II). The lower stratum, situated between the upper stratum and the agar, is characterized by suboptimal cell growth and the absence of ECM production.

Based on these findings, we hypothesized that biofilm reconstitution post-treatment would rely on the regrowth of surviving cells from the tolerance zones. To test this, we established a regrowth assay in which aminoglycoside-treated *E. coli* macrocolonies were incubated in growth medium without the antibiotic. We combined this approach with the use of the *E. coli* AR3110 strain harboring a plasmid-encoded *PrrnB1::gfp* fusion to detect cells that resumed growth. This fusion reports ribosomal RNA expression, which directly reflects protein synthesis, thus indicating active cell growth. In parallel, we also used the *E. coli* AR3110 strain harboring another plasmid-encoded *Pind::gfp* fusion, whose expression in macrocolonies was induced during the regrowth stage, reflecting

newly synthesized proteins and thus active growth specifically in that stage. Interestingly, the results revealed that after 48 h of regrowth, cells in tolerance zone II exhibited the highest fluorescence associated with the *PrrnB1::gfp* reporter and exclusive fluorescence of the *Pind::gfp* reporter, indicating that these cells were the only ones that resumed growth at this stage. Since the cells at the border were killed by the treatment, horizontal expansion of the biofilm during regrowth was very limited compared to untreated colonies. Instead, the regrown subpopulation in tolerance zone II drove the expansion of the biofilm primarily in the vertical dimension, increasing its thickness. Regarding the lack of growth of the starved, ECM-encased cells in tolerance zone I, we hypothesize that they are in a deep dormant state that may require more time and/or growth-promoting conditions to activate.

Overall, our study is the first to address the regrowth of *E. coli* macrocolony biofilms following antibiotic treatment, demonstrating that, despite survival, distinct antibiotic-tolerant subpopulations exhibit differential capacities to resume growth. This has significant implications for biofilm repopulation and provides novel insights into how surviving cells persist within biofilms.

Palabras clave: Biofilms – Tolerance - Antibiotics- Regrowth