Environmental Stress, Bacterial Cell Differentiation, and Antimicrobial Resistance

Estrés Medioambiental, Diferenciación Celular Bacteriana y Resistencia a Antibióticos

Fernando Baquero[1,2,3,*], Ana Moreno-Blanco[1,2,4], Rosa del Campo[1,2,4]

[1]Servicio de Microbiología, Hospital Universitario Ramón y Cajal, Madrid, Spain.

[2]Instituto Ramón y Cajal de Investigaciones Biomédicas (IRYCIS), Madrid, Spain.

[3]Consorcio de Investigación Biomédica en Red de Epidemiología y Salud Pública (CIBERESP), Madrid, Spain

[4]Consorcio de Investigación Biomédica en Red de Enfermedades Infecciosas (CIBERINFEC), Madrid, Spain.

Correspondence: baquero@bitmailer.net

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Abstract

Environmental stress, either natural or anthropogenic, influences both the form and function of bacterial cells. The general stress adaptive response of bacteria alters the bacterial shape, resulting in functional changes, as the bacterial cell has associated “organules” and molecular interactions that are dependent on the cell’s topology. These changes in form and function are frequently linked to bacterial differentiation, that is, the reversible production of an alternative “type of cells” more tolerant or persistent under stress. The main examples of bacterial cell differentiation are sporulation and conditional filamentation. Both strategies are extremely ancient in the bacterial tree of life, and probably most bacterial cells on Earth adopt one or other, or both of such adaptive responses. However, these phenotypic adaptations (that is, without inheritable genetic changes) can favor the emergence of permanent genetic changes. The main concept is that, because the generalized stress response and cellular differentiation, environmental stress can influence antibiotic resistance, and, conversely, the rise of antibiotic-resistant cells can have consequences in the environmental adaptation of the bacterial organisms. The confluence of both types of stress should therefore be considered as a risk and probably might accelerate the path of bacterial evolution.

Spanish/Español

El estrés medioambiental, natural o antropogénico, influencia las células bacterianas, tanto en su forma como en su función. La respuesta general de las bacterias al estrés frecuentemente conlleva cambios en la forma y estructura celular, ya que las bacterias son “organismos”, con componentes celulares -orgánulos- diferenciados. Estos cambios son de carácter adaptativo para reducir el estrés, y dan lugar a diferenciación celular, esto es, a la emergencia de tipos celulares alternativos

con mayor resistencia. Los mas relevantes son la esporulación y la filamentación. La diferenciación celular parece muy antigua en la historia de la vida. Probablemente todas las bacterias sufren algún tipo de deformación reversible bajo stress. Los cambios de forma afectan a la función, influyendo en la topología de las interacciones entre orgánulos y moléculas endocelulares. El resultado es un mayor grado de persistencia o tolerancia, sin cambio genético. Sin embargo, los cambios mutacionales que permiten una adaptación hereditaria podrían favorecerse en condiciones de persistencia. La resistencia fenotípica a la presencia de antimicrobianos probablemente favorece resistencia a otros cambios medioambientales, y, *viceversa*, los cambios medioambientales, a través de procesos de diferenciación celular, pueden influir en la resistencia a antibióticos. La confluencia de diferentes tipos de estrés, antropogénicos (como la liberación de antimicrobianos, o metales pesados) o naturales (como cambios en la temperatura o la osmolaridad) suponen un riesgo para la resistencia a antibióticos, y también para nuevas adaptaciones a cambios medioambientales, y, en todo caso, podría esperarse una aceleración de los procesos evolutivos en el mundo bacteriano.

Introduction: Microorganisms are organisms

Behind the usual term "microorganism", few microbiologists are aware of the meaning of the core of this expression, implying that a microbe is an "organism". The notion of "organism" describes an individual entity composed of independent but functionally linked physical parts, recalling organs in animals. It was first proposed in 1917 by the neo-Hegelian philosopher John Scott Haldane (1860-1936) (Herring and Radick, 2019; Sturdy, 1988), father of John Burdon Sanderson Haldane (1892-1964), one of the founders of population genetics. He was probably applying to the population level the concept of "parts in the population" that arise from selection, transmission, and random drift. Still, he might also have included the success of the interactive "parts" of the~~Still, he might eventually interact for the success of the~~ common lineage (as a clonal complex or a species). Shortly after John Scott Haldane, the notion of a biological organism as a unity made of interacting parts was applied by Willian Emerson Ritter (1856-1944) to general Biology in 1919 (Ritter, 1919). Note that microorganisms were first described as "animalcules" which implies having organs (Gest, 2004. The word "microorganism" was the Louis Pasteur (1822-1995) preferred term (around 1880), condensing the previous expression "microscopic organisms" used by the French surgeon, Charles Sédillot (1804-1883) (Cavaillon and Legout, 2022).

Form follows function, and function follows form

Not by chance, the "FFF" motto, "form follows function" (certainly a reversible statement) was originated and disseminated by artists and architects, such as the sculptor Horatio Greenough (1805-1852) and the architect of the Chicago's School, Louis Sullivan (1856-1924) (Greenough, 1947). This classic concept of the linkage between form and function has survived ~~and~~ and remains seminal in ~~vertebrated~~ the sciences of life, not only in anatomy but also in physiology (Saladin,

2021). Bacterial cells are composed of parts (pieces), that is, they have an architectonic and engineering structure (of course, also the intrinsic beauty of all living things) influencing their function, as in the case of antibiotic resistance (Baquero, 2004; Baquero et al., 2023). Around the mid of the last Century, the term "organismic biology" ~~was~~ was conflicted with~~confronted with~~ pure "mechanistic biology" (Nagel, 1961; Elsasser, 1964; Milam, 2010). This controversy is probably futile. Biological mechanisms are causal processes driving a change (frequently responding to an adaptive or developmental need) from start to termination conditions (Machamer et al., 2000). In principle, the organismic view is less causal (the kidney does not have any direct causality on the organization of the brain). However, ~~it is clear that the~~the "organism" is a developmental product of a single original cell. There is a mechanistic ~~biology~~ process in a primary phase, ~~that~~ which is completed by an organismal ~~biology~~ process at a later one. ~~But~~ Every organism is also a "biological individual", ~~as~~ containing organs, cells, and subcellular structures. The bacterial cell is a compartmentalized "organism" (Cornejo et al., 2014).

~~However, it is clear that the "organism" is a developmental product of a single original cell. There is a mechanistic biology in a primary phase, that is completed by an organismal biology at a later one. But an organism is also a "biological individual", as organs, cells, and subcellular structures. The bacterial cell is a compartmentalized "organism" (Cornejo et al., 2014).~~

At a higher organizational level, the human intestinal microbiota, for instance, can be conceived as an organ of the human body (Baquero and Nombela, 2012), influencing other organs. It might influence the brain functioning in the human individual because of the possible similarity between small intestinal microbial peptides and neurotransmitters (Baquero et al., 2024).

The ontological linkage between form and function has evident consequences. Any change in the form should alter the function, and vice versa, any kind of change in function should alter the form.

That means that if both sides of existence do not fit, the results are stress, maladaptation, and possibly extinction. That can be represented using the Frankel's metaphor of the "King and Queen of Hearts," cards that lean against one another, forming a simple card house, strictly dependent on the stability of any of them (Frankel, 1986). There are possible different "equilibriums" to keep the card house out of collapse, but the angle formed by both cards should keep symmetry. However, these equilibriums might differ in stability when the glossy surface on which they rest suffers various directional shakes. This metaphor shows the possibility that a cell can find alternative configurations keeping viability (fitting between form and function) in different o ensure continuous adaptation to environmental variation. Essentially that leads to cellular differentiation.

Environmental stress and cellular differentiation in bacteria

Sporulation is the best bacterial model for cellular differentiation (Freese, 1972). The spore of a *Bacillus* preserves the full identity and potential functions of the vegetative cell, but these "alternative cells", are extremely stable to environmental challenges, and the cellular structure, including size and shape, strongly differs from the vegetative ancestor. Endospores, a dormant type of cells, are formed, under environmental stress conditions, by a "sporulation gene set" present in *Bacillus* and *Clostridium* (Galperin et al., 2022). Other bacteria produce alternative cells resistant to environmental challenges, such as *Myxococcus*, producing myxospores in fruiting bodies (Kaiser and Garza, 2000) or *Streptomyces*, producing chains of spores; in these cases, these alternative cells might maintain some metabolic functions. We cannot discard that spore formation as a cellular differentiation seems to be an extremely old trait in the history of microbes, as sporulation also occurs in Archaea (Tang et al., 2023). It has even been proposed that all current microbial cells could have derived from "ancestor spores", the ones that were able to survive a

~~catastrophic~~ environmental catastrophe, an stringent bottleneck in the early Earth (Tocheva et al., 2016).

And, what about Gram-negative bacteria? Some Gram-negative bacteria (having outer membrane lipopolysaccharide), the *Negativicutes*, belong~~sing~~ to the phylum Bacillota, classically grouping ~~of~~ Gram-positive bacteria. ~~,~~ T~~t~~his is typically the case of the anaerobe *Acidaminococcus* (D'Auria et al., 2011). Endospores have been found in closely related *Veillonellaceae* (also Gram-negative Bacillota), as is the case of *Acetonema longum* (Tocheva et al., 2011). It has been proposed that diderm cell envelope architecture (inner and outer membranes) is an ancestral character in the Bacillota, and that the classical monoderm phenotype in this phylum arose ~~by~~ from the loss of the outer membrane (Megrian et al., 2020). Other organisms, such as the Gram-negative Alfaproteobacteria *Caulobacter,* divide asymmetrically giving rise to functionally and structurally different swarmer and stalked cells. This dimorphism provides a bimodal response to stress (Lawarée et al., 2016).

These approaches suggest that there might be a widespread ancestral sporulation-like strategy in the microbial world, evolving in different ways, but many of them are based on asymmetrical cell division when the microbial populations confront environmental challenges. In clinically relevant bacteria, such as *Staphylococcus* or *Enterococcus*, environmental stress induces (via the SOS response) small colony quasi-dormant variants (SCVs) (Painter et al., 2015). These are a "different type" of cells (Bui et al., 2015) with aberrant shapes, probably resulting from asymmetric, branched, and multiple cross walls without obvious cell separation (Wellinghausen et al., 2009). G~~G~~ram-negative bacteria, such as *Escherichia coli*, also produce almost-dormant SCVs cells linked to stress response. Interestingly, *E. coli* has proteins with peptidoglycan-bound SPOR domains, localized to septal rings, altering its cellular structure and protecting this organism from

158 bile (and might be from other stressful molecules). The SPOR founding member is a sporulation
159 gene in *Bacillus subtilis* (Arends et al., 2010; López-Garrido and Casadesús, 2010).
160
161 **Conditional bacterial filamentation** is also a major cellular differentiation process resulting from
162 cell division inhibition in response to environmental stresses, including temperature changes, low
163 water availability, high osmolarity, chemicals, including antimicrobials, or UV exposure (Karasz
164 et al., 2022). Filamentation provides a compromise between cell multiplication and inhibition of
165 cell division; individual cells inside the filament persist as small cells, separated by the
166 cytoskeleton (Wagstaff and Löwe, 2018). Filamentation seems to be a very quick response to
167 stress; for instance, even bacteria containing enzymes detoxifying antibiotics (as beta-lactamases)
168 can form filaments (Kjedsen et al., 2015).
169 Why does filamentation increase survival under stress? We have previously mentioned the increase
170 in surface/volume ratio so that the uptake of nutrients and the excretion of catabolites might
171 improve fitness. Another possible advantage is cellular robustness, as cell ruptures by stressors are
172 more frequent during division (Zahir et al., 2020). Possibly a multi-cell filament might reduce
173 energy expenses in ATP-expensive constructions of membrane lipoproteins and ribosomes. One of
174 the key consequences of stress, that might coincide with filamentation, is the release of superoxides
175 (Zhao and Drlica, 2014), also increasing the mutation rate (Pribis et al., 2022). Filaments are
176 polyploid cells with cytoplasmic contiguity, so the loss of function of a mutated copy of a gene in
177 one of the cells composing the filament can be replaced by the function of another intact gene in
178 the filament, assuring phenotypic delay of the deleterious mutation (Sun et al., 2018). Similarly,
179 polyploidy facilitates DNA mutational repair by homologous recombination, or CRISP-Cas
180 mediated adaptive immunity (Bos et al. 2015, Wang et al. 2019). Also, filamentation favors

bacterial adhesion to biological or inert surfaces (Möller et al., 2013); perhaps, adhesion is required for effective filamentation (Jin et al., 2020), including microbiotic particles where different organisms coalesce (Baquero et al., 2022), where nutrients also accumulate. On surfaces, filamentation facilitates biofilm formation (Anbumani et al., 2021; Yoon et al, 2011), and probably functions associated with quorum sensing (Chuang et al., 2019). Filamentation is probably a driver of the post-antibiotic effect, the time required for a bacterial population to re-grow after antibiotic exposure (Gould and MacKenzie, 1997). From an ecological perspective, filamentation could be beneficial for the rapid re-colonization of a niche after a stressful period, as the resolution of the filaments liberates many cells (Bos et al., 2015), favoring the original population to be re-established against competitors. Re-establishment of the original population, h~~H~~owever, ~~that~~ should occur before any damage in the filament cell wall; in that case, the whole filament lyses with loss of their cellular components (Rolinson et al., 1980).

Antibiotic modes of action and reaction drive cellular differentiation

As stated in the previous paragraph, there is a link between bacterial stress and cellular differentiation. Antimicrobial agents exert their effects by altering bacterial functions and cellular structures, which leads to cellular stress and altered bacterial forms (Lorian and Atkinson, 1975). At subinhibitory or MIC concentrations, the **mode of action of antibiotics** frequently results in changes in cell shapes. It is well known that the subinhibitory action of various beta-lactams, targeting different penicillin-binding proteins and thus altering in different ways the peptidoglycan topology, results in gamma-proteobacteria cell filamentation, or cellular rounding, remembering spheroplasts. There is a kind of antibiotic-induced cellular reversible differentiation resulting in altered-shaped cells that might present a “phenotypically resistant” phenotype, resembling

antibiotic-persisters or antibiotic-tolerant slow-growing (bacteria are not killed or are killed at a slow rate, respectively), this phenotype being unrelated to changes in the minimal bactericidal concentration (Balaban et al., 2019, Kaldalu~~, N.~~ et al., 2020). The term "filamentous persisters" has been used for ampicillin-tolerant filamentous *E. coli* variants, resulting from altered inner membrane protein composition, and active oxidative stress response with decreased ROS levels (Sulaiman et al., 2020). As other "persister cells", the altered bacteria revert to the normal susceptible cells in the absence of antibiotic exposure (Cross et al., 2019), in an apparent stochastic dynamics (Sulaiman et al., 2020).

Filaments seem to occur in an antibiotic concentration window, variable for different antibiotics; in general, the lower the MIC, the fewer filaments are produced. However, for most combinations, filament induction starts at sub-MIC or MIC levels but may extend to concentrations far above the MIC (Buijs et al., 2008, Gould and McKenzie, 1997). A ~~If the polyploid filaments a~~ beneficial mutation in a gene involved in antibiotic resistance in one of the filament chromosomes could disseminate by recombination with other homologous genes (something like gene conversion) so that when the filament split, a bunch of resistant cells might emerge. We cannot exclude the possibility that a beneficial mutation in one of the chromosomes of the filament, particularly influencing diffusible enzymes (for instance de-repression of an AmpC beta-lactamase) might protect the whole filament in the presence of an antibiotic (in this example a beta-lactam). As reviewed before, filaments might increase the mutation rate, and thus the number of antibiotic-resistant mutations. Also stated in the previous section, cellular stress frequently induces bacterial elongation, providing potential adaptive features to improve cellular viability. The mode of action of ribosome-targeting antibiotics also contributes to modifying cellular size and shape. ~~Reduction in the number of functional ribosomes is followed by a compensatory over-synthesis of these~~

~~particles~~A compensatory over-synthesis of these particles follows the reduction in the number of functional ribosomes. and the cells invest in growth rather than in duplication, which results in round cells. Round cells reduce the surface-to-volume ratio so that bacteria can reduce the intracellular antibiotic concentration by decreasing antibiotic influx (Ojkic et al., 2022). Filaments and round cells can be detected directly on clinical specimens (Gould and McKenzie, 1997).

Similarly, alterations in bacterial cellular shape can be promoted not by the primary antibiotic action, but by the secondary **mode of reaction** of the bacterial cell. Bacterial killing by bactericidal antibiotics occurs as a consequence of loss of spatial individuality (damage of cellular envelopes) and genetic individuality (DNA degradation) (Baquero and Levin, 2021). Before killing, DNA-targeting antibiotics, and, in general, bactericidal antibiotics induce an SOS response, upregulating DNA damage, and mutagenesis, but also tolerance and repair and involving filamentation (Phillips et al., 1987; Chatterjee, 2017). The timing of DNA damage responses is critical to persistence (Mok et al., 2018). However, these adaptive responses are frequently insufficient to avoid cellular death, except for cells that have obtained hereditary mutations during the "filamentous persistence" stage (Barrett et al., 2019). On the contrary, bacteriostatic antibiotics are bacteriostatic because they promote "an alternative type of cells" with high resistance to killing. The bacterial cell differentiation mechanism has been compared with the induction of sporulation and frequently gives rise to "small colony variants", composed of "variant types of cells" both in Gram-positive and Gram-negative bacteria. Instead of "bacteriostatic antibiotics" we could, more appropriately, use the term "bacteriostatic cells" (Baquero et al., 2023; Gil-Gil et al., 2023).

Form and function in cellular differentiation following generalized stress responses.

Bacterial organisms are blind to most causes of stress. That is, they react similarly to a variety of stress. Generalized stress responses follow different challenges as osmotic stress, envelope stress, cold stress, acid shock, nutritional stress, stationary growth phase, adhesion and colonization stress, or stress by exposure to natural or anthropogenic antimicrobial agents. Induction of the generalized stress response by any type of stress produces cross-protection against other stresses (Ron, 2012). Cellular differentiation, following generalized stress responses, creates "variant types of cells" able to persist during the stressed period. Changes in the form of differentiated cells condition their function, resulting from a new interactive network between the intracellular "organs" and biomolecules. This topic has been recently reviewed (Baquero et al, 2023b). Such a network of "new interactions" and "loss of interactions" affects phenotypes and cellular fitness under various (often combined) sources of stress. The term "structural epistasis" was coined to reflect the establishment of new molecular interactions between molecules located at particular spaces inside the bacterial cell in the emergence of novel phenotypes. For instance, the architecture of gram-negative bacteria essentially consists of concentrical layers of organized membranes, organ-like particles (as ribosomes), and molecules with differing configurations and densities. Environmental changes determine stress, as well as antibiotic exposure (and also the expression of antibiotic resistance!) altering the cell's internal molecular topology, resulting in unexpected interactions among biomolecules (architectural epistasis). Any environmental change modifying cell's form and function. In contrast, changes in shape and size might alter antibiotic action. The mechanisms of antibiotic resistance (and their vectors, as mobile genetic elements) also influence molecular connectivity in the bacterial cell and can produce unexpected phenotypes, influencing the action of other antimicrobial agents, and other environmental stressors.

A critical field for future research is how climate changes and other anthropogenic-driven effects on Earth might influence antibiotic resistance (Tiedje et al., 2022). As an example, the adaptation to warming in the Antarctic gamma proteobacteria *Shewanella frigidimarina*, belonging to the *Shewanella* genus, including pathogenic bacteria, is a precondition for human and warm animal colonization eventually leading to emerging pathogenicity. The adaptation of this organism to a new, stressful environment is mainly driven by the composition of chaperone interaction networks (García-Descalzo et al., 2014). In particular, one of the proteins exceptionally induced by high temperature was the aryl hydrocarbon receptor (AHR). This protein protects against peroxides in *E. coli* and osmotic stress in *Staphylococcus aureus* (thus probably protecting from bactericidal activities). The *mcr* gene, encoding colistin resistance, has been found in *S. frigidimarina* (Zhang et al., 2019).

Final perspective

Antimicrobial pollution of wild Earth environments, and the ever-variable conditions of the biosphere, submitted to unexpected changes, are sources of stress for bacterial microorganisms, which react by generalized adaptive modifications in cellular shape and function. Antibiotic resistance might modify environmental adaptation and vice versa, environmental changes might modify antimicrobial resistance in bacterial organisms. During this trade-off, new networks of molecular interactions take place in the cell, generally contingent on the stressful conditions. However, we should be aware that microbial evolution is not only dependent on mutational changes able to be selected. An adaptive configuration of a given ensemble of objects (as molecules, organelles, in microbial organisms), ephemeral in cellular differentiation events, could be selected according to the “assembly theory” (Sharma et al. 2023), influencing microbial

evolution at large. Environmental-driven changes in the form and function of cells should influence both bacterial phylogenesis and ontogenesis (Smit, 1968). We, humans, should be aware of our causal role in the acceleration of microbial evolution, with unpredictable global consequences.

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