Phenotypic switching: an opportunity to bacteria thrive

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Bacteria in nature are "plagued" by various unpredictable environmental stresses, being population diversity one of the strategies adopted to survive. Phenotypic switching is one of the evolution processes that causes commutation between phenotypic states. This phenomenon shows up as variation in colony morphology. Alterations in colony morphotype traits may reveal altered cellular basis phenotype which can confer ensured virulence, antimicrobial resistance and persistence. However, the specific correlation between those traits and the biological impact is unknown. Phenotypic switching also occurs during biofilm formation, in that, bacteria have to adapt to this mode of growth expressing different phenotype traits often distinct from those expressed during planktonic growth. Often, after severe stresses, it is observed the survival of a small non-growing population, the persisters cells. The persister-state is fully reversible under growth stimulating conditions and therefore does not depend on genetic alterations. Bacterial persistence has been pointed out as switching between growing and dormant cells. Nevertheless, not only the responsive switching to environmental changes is important for survival of bacteria. Inherent heterogeneity may also be of major importance in environmental adaptation and persistence. Control of stress-response gene expression determines whether bacteria can survive to changing conditions and compete for the resources it needs to proliferate. Reversible phenotypic switching offers considerable advantages over conventional irreversible mutations. This chapter discusses the impact of generating population-level diversity on important clinical issues as resistance, virulence and persistence. It highlights that even though the growing interest and relevance of this phenomenon and its impact on bacterial ecology, the evolutionary origins and adaptive significance remain poorly understood.

Keywords: phenotypic switching, phenotype, colony morphology, morphotypes, variants, stress

1. Introduction

In natural habitats, microorganisms are continuously challenged by external factors, being environmental adaptability essential for their survival. In fact, microorganisms can face many stressful conditions, such as starvation, temperature shocks, pH alterations, hypoxia or anoxia conditions, UV exposure, predation, antimicrobial agents challenge (antibiotics, biocides and disinfectants) and immune host defences [1]. The ability of microorganisms to change according to environmental fluctuations is termed as adaptive response. When environmental conditions alter, microorganisms trigger a set of complex regulatory networks that enable their survival. This microbial adaptation involves physiological, behavioural and genetic changes and can be achieved by different mechanisms, being phenotypic switching one of them.

Phenotypic switching was first described in *Candida albicans* over 20 years ago and later for other fungi as *Crytococcus neoformans* and other *Candida* species [2]. Lately, it was also been observed in bacteria as *Pseudomonas aeruginosa*, *Staphylococcus aureus, Escherichia coli, Salmonella* spp. and *Neisseria* spp. Phenotypic switching is being described for both pathogenic and nonpathogenic species, therefore there are undeniable reasons to examine thoroughly its role in microbial survival, pathogenicity and persistence.

Phenotypic switching or phase variation refers to a reversible switch between two phenotypic states, analogue to a mechanism ON/OFF. It normally occurs in a small fraction of the population at much higher rates than the frequency of spontaneous mutations. Phenotypic switching leads to two subpopulations with different gene expression: one sub-population with altered expression of one or more phase variable genes; and other expressing the full genes profile [3]. An interesting feature of phenotypic switching is the referred mechanism ON/OFF, *i.e.*, microorganisms can enjoy interchange of states. Although microorganisms exhibit one of the phenotypic states, they retain the possibility to switch again, if advantageous, when new environmental stimuli occur, or switch back to the previous phenotype state when the external stressor, that had provoked the switching, vanishes.

It has been long recognized that a genetically identical population (or isogenic population), even in a homogenous environment, can often have two or more phenotypes, being this feature termed as inherent phenotype heterogeneity or individuality. This microbial phenotype heterogeneity is a non-genetic variation, i.e. that does not involve changes in the genome, and it can be due to "noise", *i.e.*, variations result of the stochasticity of chemical reactions, at DNA level, such as random alterations in the rates of protein synthesis and degradation [4, 5]. Inherent phenotype diversity into a genetically identical population becomes evident by heterogeneous responses to stress [6], being this fundamental to microbial fitness and development. Despite phenotypic switching can be, in part, a random event (stochastic switching), several reports demonstrated that it can be modulated by external factors (responsive switching) [3, 7]. The microbial heterogeneity produced by phenotypic switching generates phenotype variants that are "fitter", since this sub-population is better adapted to new environment than the other members of the population [8]. However, the fraction of the

population that preserves its phenotype unaltered ensures the ability of the population to overcome future and new adverse conditions that may occur. According to the ecological model "insurance hypothesis", more biodiversity ensures ecosystems to maintain or enhance their functioning against environmental fluctuations [9]. Several reports about microbial diversity have been supporting this theory [10-12].

Antigenic variation, in part, resembles phenotypic switching because it is also a heritable and reversible mode of switch between two phenotypes. However, antigenic variation involves alternating expression of a set of conserved moieties that are antigenically distinct within an isogenic population [13]. Phenotypic switching can lead to antigenic variation, since, for instances, it can affect the expression of outer lipopolysaccharides (LPS), pili and flagella [14, 15]. Phenotypic switching and antigenic variation can be a mixed strategy adopted by microorganism to face hostile habitats.

This chapter presents a review about phenotypic switching and its impact on bacteria fitness and persistence, as well as its significance on clinical context. Insights about phenotypic switching may provide valuable inputs into the dynamic of bacterial life.

2. Colony morphology variants: the macroscopic feature of phenotypic switching

The main consequence of phenotypic switching is the generation of heterogeneous and dynamic populations that can overcome stressful challenges. The most visible feature of phenotypic switching is colony morphology variation, termed as "dissociative behavior", firstly described in 1964 by Zierdt *et. al* [16]. Altered colony appearance were demonstrated for several bacteria as *Burkholderia pseudomallei* [17], *Streptococcus pneumonia* [18], *Pseudomonas aeruginosa* [19], *Enterococcus faecalis* [20] and *Haemophilus influenzae* [21]. Any modification in colony morphology, such as color, opacity and texture may be a sign of altered expression of one or more bacterial traits.

Isolation and microbial identification through colony morphology are still methods of first line used by several laboratories and hospitals. The correlation between bacterial features and colony morphologies is just about unknown, however extremely important. Through colonies macroscopic visualization it may be possible to predict about which bacterial traits were probably altered and its relationship with external factors. Furthermore, the inspection of colonies will also help to establish relationships between colony morphology changes, virulence, antimicrobial resistance and persistence, which will be extremely useful on the design of biotechnological and therapeutic approaches.

Several colony mophotypes have been identified and described for several bacteria, being the best studied the small colony variants (SCV) [22, 23], the rugose small colony variants (RSCV) [24], and the mucoid morphotypes [25, 26]. Some of these morphotypes are associated with antimicrobial resistance, altered metabolism and reduced immunogenicity, contributing thus to increased bacterial pathogenicity and persistence. SCV have been implicated in persistent and recurrent human infections, such as cystic fibrosis (CF) [27] and device-related infections [28]. Deziel *et al.* [29] have characterized these variants as hyperpiliated and with increased ability to form biofilms in comparison with the fast growing wild morphotypes. Haussler *et al.* [19] have reported that, beyond hyperpiliated, SCV showed increased twitching motility, increased fitness under stationary growth and autoaggregative traits. Other features as auxotrophy [30], reduced respiration [31], resistance to aminoglycoside antibiotic [32] and ability to revert to normal morphotype [19], not always but often, have been also described. So far, the majority of studies concerning SCV are available for *P. aeruginosa* and *S. aureus*. The largest number of these laboratory-derived morphotypes may result from mutations. D´Argenio *et al* [33] have showed that a mutation in *wspF* of *P. aeruginosa* results in the arising of autoaggregative SCV with defects in swimming, twitching and swarming abilities. Drenkard and Ausubel [34] have reported the emergence of RSCV, a sub-group of SCV, after *P. aeruginosa* PA14 has been subjected to an antibiotic treatment with kanamycin. RSCV showed to be hyperadherent and possibly negatively controlled by the two-component system response regulator homolog PvrR. RSCV are the unique variant of SCV described so far. However, recent work has identified several other subsets of SCV that are not mentioned in literature [35, 36]. Therefore, SCV may encompass a large number of variants possibly with important differences between each other in terms of virulence and resistance [36].

Besides SCV, particular attention has been also addressed to the mucoid phenotype. Mucoidy is associated to the overproduction of exopolysaccharides (EPS) and considered as a strongest virulence factor. *P. aeruginosa* mucoid variants overproduce alginate, a linear copolymer of 1,4-β-linked mannuronic acid and its epimer guluronic acid. Alginate has being ascribed as a significant virulent factor with various functions in the context of the pathogenesis of respiratory infections as in CF disease [26]. *P. aeruginosa* mucoid phenotype is closely associated with chronic infection and destruction of the lungs of patients with CF. During respiratory infection, *P. aeruginosa* converts from non-mucoid form (lower producer of alginate) to mucoid form (higher producer of alginate), being normally mutation in *mucA* responsible of this conversion [37]. The overproduction of EPS protects bacteria from host defences and antibiotic therapy extending the infection for a long time.

Apart from SCV and mucoid morphotype, other morphologies are underestimated, devaluated and poorly studied, being unknown their role and impact on diversity and dynamic of bacterial populations. In fact, there are several other morphologies that arise, for instance, after exposure to antimicrobial stress ensuring the bacterial response. As mentioned previously, stress response genes are triggered whenever bacteria need to adapt to adverse conditions. The *rpoS* and *bolA* genes are examples of those genes. RpoS, an alternative sigma factor of RNA polymerase, is the master

regulator of general stress response in *E. coli* and other proteobacteria. Its expression is susceptible to environmental alterations and is under strict control at transcriptional and post-transcriptional levels [38, 39]. The effect of *rpoS* on bacterial virulence is variable differing between species [40]. *bolA* is a morphogene of *E. coli* that causes round shape of bacteria and increased ability to form biofilms. *bolA* expression is induced by several types of stress factors such as heat shock, acid environment, oxidative and osmotic stress and carbon starvation [41, 42]. Colony morphologies of *E. coli* K12 with *bolA* and *rpoS* mutations were observed (Fig. 1) to assess the impact of these genes on phenotype determination.

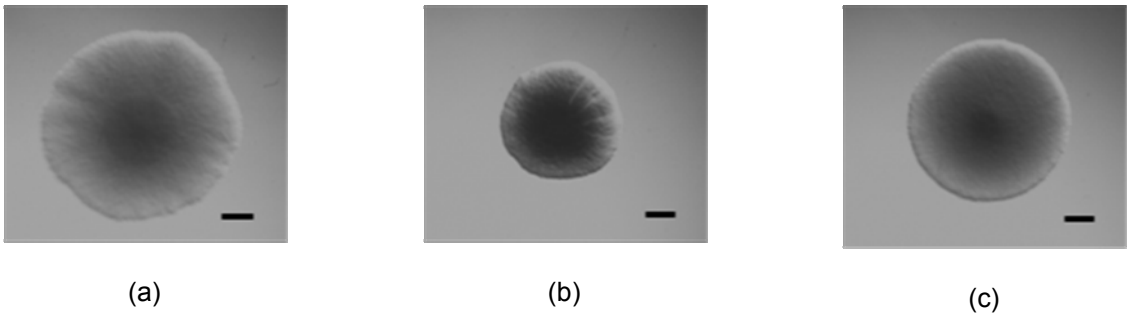

Fig.1 Colony morphology of *E. coli* (a) wild-type, (b) *rpoS* mutant and (c) *bolA* mutant on TSA plates. Black bars = 1 mm.

The observation of Fig. 1 allowed to denote important differences between the colony morphologies adopted by the three *E. coli* strains, confirming the influence of *bolA* and *rpoS* on morphology variation. *rpoS* mutation reduced significantly the size of the colonies and altered its texture. *bolA* mutation also altered the colony texture and induce a well defined circular shape. The impact of these genes on colony morphology when *E. coli* is exposed to antimicrobial stressors is under investigation. Preliminary results revealed that colony morphology variation between stressed mutants is even greater than that observed with un-stressed *E.coli*.

3. Biofilm phenotypic switching

In "real world" environments, bacteria prevail in organized communities entrapped in an exopolysaccharide matrix called biofilm [43]. Biofilm formation is an efficient microbial strategy to persist in unfavourable environments. Bacteria in biofilm benefit from various advantages over their planktonic counterparts, such as increased antimicrobial resistance and protection against predation, dehydration and phagocytose [44]. Biofilms are initiated when free-living bacteria attach irreversibly to an abiotic or biotic surface. Furthermore, bacteria grow and divide forming a mature biofilm. During biofilm growth, bacteria adopt a biofilm-specific phenotype, radically different from that expressed in the corresponding planktonic cells.

To switch from planktonic to biofilm mode of growth, bacteria undergo a number of complex physiological, metabolic and phenotypic differentiations [45]. Biofilm-growing bacteria undertake specific changes in physiological and protein regulation, especially those related with proteins involved in resistance to oxidative damage, exopolysaccharide production, phospholipids synthesis and membrane transport [46]. This switch to biofilm-specific phenotype can trigger mechanisms responsible for antimicrobial resistance, enhanced virulence and persistence [47]. Some genes are differentially expressed in biofilm mode of growth, such as those implicated in pili and flagella synthesis, signifying that these elements are no longer required in mature biofilms [45, 46].

Within biofilms there are a range of microniches with specific biological activities that may somewhat translate the well-known biofilm heterogeneity. This biofilm diversity can generate several distinct colony morphologies because of those sub-populations. The morphotypes resulting from clinical-isolated and wild-type *P. aeruginosa* biofilms (Fig 2) emphasize the diversity of colony morphologies presented by biofilm-growing cells.

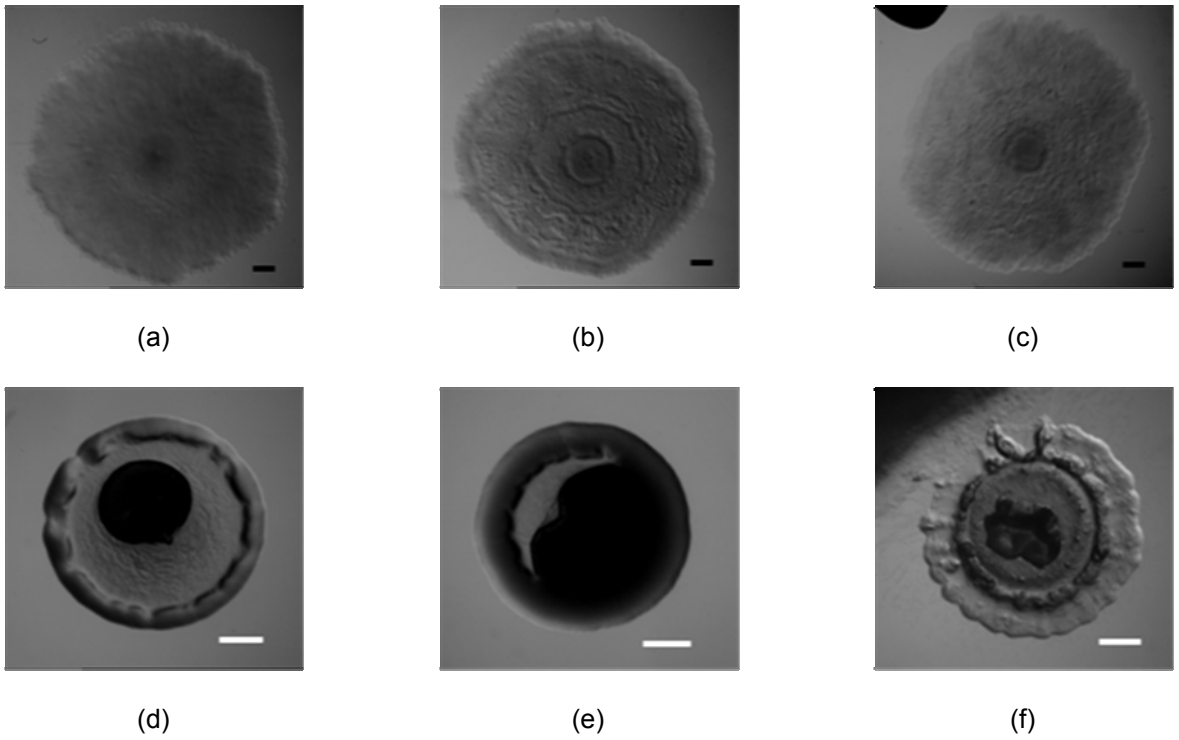

Fig. 2 Colony morphology diversity of 24-hour-old *P. aeruginosa* biofilms formed on TSB by ATCC10145 strain (top row) and by clinical isolate (down row). Black bars = 1 mm, white bars = 0,5 mm

P. aeruginosa ATCC10145 biofilms generated three different colony morphotypes (Fig 2). This population variability can be the evidence of the well-described heterogeneity within biofilms imposed by chemical gradients, such as nutrients, oxygen and metabolic waste products. As Stewart and Franklin [48] reported, in a mature biofilm, at least three distinct physiological states can be anticipated: cells near the biofilm-bulk-fluid or in the more superficial layer, presenting similarities with planktonic cells; cells in the middle zone; and cells in the deeper zone. The three colony variants showed on the top row of Fig. 2 can be the macroscopic expression of the phenotypes of these different zones. In fact, it was verified that the colony variant represented by Fig 2a is similar to the morphotype commonly observed with *P. aeruginosa* planktonic cultures [35].

The biofilms formed by the isolated *P. aeruginosa* strain gave rise to a morphotype diversity almost 4-fold higher than the observed with *P. aeruginosa* wild-type (only few morphotypes are displayed in Fig. 2). The clinical isolate demonstrated to have a huge ability to generate new colony morphotypes, especially, in stressful conditions. Furthermore, the features of the colony variants were clearly different from those of the reference strain - ATCC10145 - (Fig 2 d, e and f), probably due to their different genetic backgrounds [35]. It was evident the prevalence of SCV resulting from the clinical-isolated *P. aeruginosa* biofilms. This occurrence has been associated with a better response of bacteria to environmental challenges. For instance, the antimicrobial resistance often exhibited by biofilms has been, in part, attributed to the biofilm-specific phenotype, where SCV play a key role [18, 19, 22, 34]. However, it was demonstrated that the SCV phenotype may not be exclusive of biofilms [49]. Therefore, it remains unclear the existence or not of a colony morphology that could represent exclusively a biofilm-specific phenotype.

Biofilm-colony diversity is also influenced by the stage of biofilm development [46]. Boles *et. al* [10] showed that biofilms over time were differently composed by "mini" and wrinkled colony variants. The different proportion of each variant during biofilm formation and, especially, after biofilm exposure to environmental stresses, suggests that colony variants have specialized functions in biofilm establishment and in its survival strategy that ensure the increased biofilm tolerance to stressors [12, 50, 51].

The phenotypic heterogeneity within biofilms is considered one of major reasons of biofilm recalcitrance. Many different colony morphologies can be isolated from biological samples from chronic infections such as CF sputum, in which bacteria reside in biofilms [52]. However, the study regarding biofilm phenotypic switching is recent and several questions still remain to be answered. Despite the progress obtained in this topic, several issues related, for instance, with polymicrobial biofilms are still an unexplored research area. In nature, biofilms are rarely composed by unique specie. In fact, biofilms are consortia of several species including bacteria, fungi and virus, being thus essential the characterization and study of the relationships and interplays between species and their adaptive responses.

From the evolutionary standpoint, biofilms represent an undeniable advantage to persist in hostile environments. The relationship between biofilms and biofilm-specific colony morphotypes is unclear. Hence, it is necessary to elucidate

the role of colony variants of the biofilm-associated bacteria for understanding hot issues related with the biofilm mode of growth, like chronic infections, ecological processes and antimicrobial resistance.

4. Virulent phenotypic variants

Phenotypic switching is traditionally considered as a mechanism to evade host immune defences because it has been associated with many human pathogens. In fact, phenotypic switching assists bacteria to escape to innate and acquired immune mechanisms during colonization and infection, by affecting the host-pathogen interactions [3]. The basis of this process is that the subpopulations generated by phenotypic switching exhibit different sets of antigens and the immune system only react against those antigens that recognize. Therefore, colony variants become a problem when the host immune system does not operate effectively in their elimination [53]. This concept of enhanced virulence is supported by antigenic variation principle. However, if bacteria switch OFF the expression of an antigen, it may means that they lose the biological function associated with this antigen. Hence, functional redundancy is required for bacteria not mislay the biological function of that antigen. The frequency by which antigenic variation occurs is unclear mainly due to limited number of studies about it [3].

In a study related to systemic infection in animal model, Kim *et. al* [54] have reported that opaque colony variants of *Streptococcus pneumoniae* exhibited higher virulence than translucent variants in each three strains examined. This difference can be explained by the 6-fold amount of capsular polysaccharide and 2-fold less teichoic acid synthesis of opaque variants. Similar trends were observed for *Helicobacter pylori* [55], *Salmonella enterica* [56] and *Pseudomonas fluorescens* [7]. So far, it is unclear what prompts bacteria to switch despite some authors proposed that the host environment may exert bacterial selection pressure [57].

For some chronic infections, the switch of colony morphotype is one of the crucial stages. As previously mentioned, in CF lungs, *P. aeruginosa* colony morphology undergoes a conversion to mucoid form by overproduction of alginate. Alginate is thought to have a protective function in a relatively harsh environment in which the bacteria are continually subjected to oxidative stress and attacked by the immune system. Therefore, in the mucoid phenotype, bacteria become more difficult to eradicate [58], being able to survive for years with the most surface and virulence factors downregulated [59, 60]. The frustrated immunologic response, characterized by an influx of neutrophils to the lung, causes tissue damage over time, due to the indiscriminate release of reactive oxygen species during oxidative burst, resulting in significant morbidity and mortality [61, 62]. The conversion to mucoidy, together with biofilm formation, are both important virulence aspects of CF pathogenesis, however the relationship between them is at the present unclear.

Colony variants as SCV exhibit several particular characteristics that make them excellent opponents of the host defence agents. Besides the features abovementioned, SCV have better ability to develop biofilms and to adhere to epithelia, being sometimes more cytotoxic to macrophages [19, 34, 50]. The SCV emergence, for instance in CF lungs, may be caused by host environment selective pressure, namely by the presence of antibiotics, the action of host immune agents or by the synergy of the two factors [57]. The hyper-biofilm ability of any colony variant is an important virulence factor. Biofilms demonstrated reduced activation of the complement system and, as biofilm-growing bacteria are entrapped within the EPS matrix, they are less susceptible to phagocytosis. In some cases, biofilms are resistant even to humoral immune response [61].

5. Phenotypic variants resistant to antimicrobial agents

Phenotypic switching does not only help bacteria to evade the host immune defences, but also have impact on other biological features. As aforesaid, for some microorganisms the relationship between phenotypic switching and microbial virulence are already established, however, it is urgent to re-examine phenotypic switching as a phenomenon with impact on other biological processes, as antimicrobial resistance.

Nowadays, disinfection procedures and antimicrobial therapeutic treatments are usually noneffective. It is well-known that the use and over-use of antimicrobial agents have amplified the emergence of resistant bacteria [63]. Antimicrobial research has been focused essentially on antibiotics because the huge impact of recalcitrant infections caused by antibiotic-resistant strains on humans. The several resistance mechanisms adopted by bacteria include modification of the target site, degradation of the antibiotic molecule and reduction of effective intracellular antibiotic concentration through decreased permeability and efflux pumps [64, 65]. Antibiotic resistance is generally associated with fitness costs for bacteria, however, Massey *et al.* [66] demonstrated a mechanism that contradicts those bacterial costs, the arising of SCV. According to the latter authors, *S. aureus* exposure to gentamicin both in vitro and in vivo resulted in the emergence of SCV. In the absence of antibiotic pressure, the SCV reverts to normal phenotype, avoiding thus the disadvantageous fitness costs associated with irreversible resistance mutation. The typical characteristics of SCV, such as small size, slow growth rate, pigmentation and higher resistance to antibiotics and cell-wall inhibitors, are due to defects in electron transport chain [28, 67] caused by genetic mutations as *menD*, *hemB* and *ctaA* [28].

The majority of SCV are isolated from biofilms either under stresse or not. Actually, biofilms have been increasingly recognized as an important issue in human disease due to their notoriously resistance, achieving 10 to 1000 fold higher tolerance to antimicrobial agents than corresponding planktonic bacteria. This resistance has multifactorial nature, *i.e.*, results from the combination of several mechanisms including restricted penetration of antimicrobials through the exopolysacharide matrix, slow growth of bacteria within biofilms caused by nutrient and oxygen restriction and accumulated metabolic wastes and quorum-sensing molecules that leads to biofilm recalcitrance [61].

In the field of antimicrobial resistance, research related with bacterial resistance to disinfectants and biocides has been undervalued. Clinical settings are a particular environment that shelters a lot of pathogens mainly in abiotic environmental surfaces, air and water. Surface disinfection is one of the most important clinical procedures that can effectively reduce the number of healthcare-associated infections [68]. This kind of infections is somewhat caused by the spreading of pathogens through surfaces surrounding patients and healthcare professionals. Unfortunately, disinfection procedures are not always performed in an accurate way. In fact, inadequate agents and doses are frequently used which may contribute to the development of resistant bacteria [63]. Although there is much concern regarding the risks of antibiotic resistance, there is a lack of studies evaluating the performance of disinfectants and biocides against planktonic and sessile bacteria and the tolerance induced by the use of those agents. Studies that relate the phenotypic switching and the development of tolerance by biofilm-growing bacteria towards disinfectants and biocides are even less. Recent work showed that disinfectant exposure altered biofilm population diversity [35, 36]. In order to attempt comprehending the relationship between disinfectant stress and biofilm diversity, 24-hour-old biofilms were exposed to several different disinfectants of clinical use and bacterial colony diversity was assessed. Data demonstrated that biofilm diversity was altered for all disinfectants tested but with different proportions of colony variants. Given the variety of phenotypes observed in the unstressed and stressed biofilms, it is likely that those variants an important play a role in the biofilm adaptive response and community survival.

6. Phenotypic persisters

One the most remarkable feature of bacteria is their ability to persist after severe stressful situations. When an isogenic population is exposed to lethal conditions, such as a bactericidal dose, most of the cells are killed. However, a small fraction of the bacterial population, called as persister cells, can survive. Persisters are not resistant cells and do not have genetically acquired antimicrobial resistance. They are dormant cells, *i.e*., they remain metabolically inactive in stressful conditions, in a “non-dividing state” [69]. After removal of the lethal conditions, they can resort to normal growth rates and generate a new population that is equally susceptible. It has been proposed that the persister cells ability to revert to original population is a sign of phenotypic switching.

Despite the discovery of persister cells over 60 years by Bigger *et. al* [70], the knowledge about them is still too young mainly due to technical difficulties of working with a such small amount of cells, normally range from 10^{-6} to 10^{-4} of the entire population. The fact of such important phenotype be expressed only temporarily may also account for the lack of studies related with persister cells. Therefore, the mechanisms underlying bacterial persistence remain a puzzle and the knowledge about the traits of colonies formed by persisters is unknown.

Currently, there are in literature various hypotheses explaining persisters formation. The most important is related to the assumption that persistence is a phenotypic state [71]. In previous works developed by Lewis [72, 73], it is suggested that stochastic switching or “noise” in toxin-antitoxin balance could be responsible for the switch to persister state. Prokaryotes have in its genome and plasmids genetic elements designed as toxin-antitoxin modules (TA). TA are generally two genes operons: one encoding a stable toxin that inhibits some important cellular functions; and another one encoding an unstable antitoxin that neutralizes the toxin and also acts as an autoregulator of expression [74, 75]. The role of TA in programmed cell death is controversy. The most recent hypothesis proposed that toxins target essential biological processes such as DNA replication and protein translation promoting by this way, bacteriostasis. Some studies have been proposed that stochastic fluctuations in TA ratios can lead to transient growth states and antibiotic resistance. Thus, evolution seems to have favoured stochastic switching rather than responsive switching that, in some stressful situations, may not occur convicting the whole population to death [76]. In addition, if the population were diverse or “insure”, the probability of bacteria survive further increase with the diversity degree.

Persisters have been greatly associated with antibiotic resistance because antibiotics failure to kill this residual fraction of cells. In general, non-growing or slow-growing cells are not, or at least are less, susceptible to antibiotic action. Thus, persisters can prevent clearance of bacteria, especially when bacteria are within biofilms. The occurrence of persisters cells within a biofilm population has been pointed out as a mechanism of biofilm resistance towards antimicrobials In fact, the enhanced antimicrobial resistance of biofilms has been associated not only to SCV phenotype but also to the presence of the persister phenotype [77, 78]. Singh *et. al* [22] reported that biofilms harbour a increased number of persisters cells when compared with planktonic counterparts and those persisters were more difficult to eradicate due to the exopolysaccharide matrix protection. So far, only the operon *hipAB* is known to affect the development of persisters [79].

7. Phenotype plasticity: mechanisms of phenotypic switching

Despite the increasing number of studies about phenotypic switching, the molecular mechanisms underlying this phenomenon are poorly understood. In some bacterial species, specific phenotypic switching mechanisms with genetic basis seem to be more prevalent and, in other species, mechanisms with no genetic basis, as epigenetic regulation, have been identified [6, 13].

Bacteria can respond to environmental stresses by modulating gene expression through sigma factors, proteolysis and other mechanisms [80]. Nevertheless, bacteria can experience many, in number and variety, unpredictable stimuli that gene regulation system cannot foresee. Thus, in absence of any pre-determined mechanism, the classical genetic view of a sensor-effect regulatory system, as bacteria strategy to respond to environmental stresses, seems to be inadequate [14, 80]. In addition, classical genetics does not explain the phenotype heterogeneity into an isogenic population.

The best approach to bacteria achieve adaptation by genetic strategies is through mutations and/or horizontal gene transfer. However, adaptation through spontaneous mutations is difficult, although not impossible, since these mutations occur at very low frequency. Increased mutation rates, known as hypermutability, could be an efficient approach to acquired genetic diversity [80, 81]. Hypermutability could be beneficial or adverse since bacteria, in the long run, will face a high risk of accumulation of unwanted and deleterious mutations or antagonistic pleiotropy or both. The mechanisms of hypermutability have exhaustively reviewed by Jayaraman [82].

As previously mentioned, phenotypic switching is an efficient strategy of generate a diverse population, able to survive in hostile environments, by high frequency and reversible switch (ON/OFF) of the expression of one or more genes. This class of genes that are expressed in a phase variable manner is called as “phase variable genes” or, more often, as “contingency genes”. Contingency genes are genomic regions able to reversibly alter between two forms at high frequency. Most of the contingency genes are involved in the synthesis or modification of surface-associated structures and enzymes. Thus, mutations in those genes cause modifications in protein regulation and in bacterial phenotype [13-15], being the majority of them located in coding sequence of virulent factors. The reversible switch of phenotypes has been explained by a mechanism called “slipped strand mispairing” (SSM) [8, 83]. SSM has been identified in several bacterial species [83]. This process can occur during DNA synthesis, as replication, repair and recombination. Short sequence repeats (SSRs), homopolymeric tracts (e.g TTTTTTTTTTTTT) or multimeric repeats (e.g. TAATAATAATAATAATAA), associated with SSM regulate gene expression at the translational or transcriptional level. SSR associated with a single locus, located in the promoter region or in coding region, can then alter gene expression and lead to phenotypic switching [80, 81, 83]. SSM occurs when a repeat sequence pair out of the original complementary part of the opposing strand (template), but with some other area containing the complementary base. A loop is formed in the template or nascent DNA, as the mismatching occurs, if it is a forward or a backward slipping, resulting in a contraction or expansion of the length of repeats, respectively. The altered DNA repeat length can result in altered translation of an encoded protein or different transcriptional activity of a determined gene.

SSM is the most common mechanism underlying phenotypic switching, but not the only one. Other mechanisms, including homologous recombination, site specific recombination, transposon insertion/excision and differential DNA methylation, may cause phenotypic switching [80]. In this review, only a brief description of the features of those mechanisms will be presented. Additional information can be obtained in literature [8, 81, 84].

The switch of phenotype determines the presence or absence of several surface structures. At simplest outlook, homologous recombination is the breaking out of two DNA molecules at a single point and the crossing between each other. The result is two DNA molecules, called recombinant DNA, with a part of parental molecule. This mechanism requires some homology between sequences to happen. An example of phenotypic switching by homologous recombination is the transcriptionally interchange between active gene *pilE* and silent of *pilS* gene. In contrast, site-specific recombination requires little or no homology between molecules since it works with specific sequences. In site-specific recombination occurs the inversion (flipping) of short DNA segments encompassing the promoter of a phase variable gene which is recognized by the corresponding recombinase. The activity of the recombinase may be influenced by environmental factors. The switch of phenotypes occurs when the inversion changes the transcription or translation of a gene. In one orientation the gene is expressed (ON state) and in the other not (OFF state) [8, 80, 84]. The best characterized example of site-specific recombination is the phase variable expression of *E. coli* type 1 fimbria expressed by the *fimA* gene. Phenotypic switching can be the result of insertion and excision of transposable elements. Transposons are mobile genetic elements able to move (transpose) from one DNA site to another. Some transposons target site seems to be more or less random and others seem have target specificity. For instance, the insertion of IS2561transposon causes phase variable expression of the *cca* gene in *S. epidermidis* [13]. DNA methylation is a pseudo-genetic mechanism because is a process of regulation of gene expression but, as it acts directly on DNA, it can be considered on the set of genetic mechanisms. Phenotypic switching by this mechanism results from differential methylation patterns carried out by deoxyadenosine methyolase (Dam) which methylates 5’-GATC sequences. The competition between Dam and transcriptional regulatory proteins for DNA binding sites cause changes in methylation and, consequently, they can be used by bacteria to regulate the expression of certain genes in a phase-variable manner. The type of the binding regulator determines the ON and OFF state [8, 83]. The phase variable expression of the *E. coli* pap pilin is a well-known example of the Dam-mediated phenotype variation.

Phenotype variation can also be due to stochastic variation or "noise" at transcriptional and translational level [5, 6]. Although all population members have the same genome, noise can wrest differently the information in each individual cells. Some biological reactions are, in fact, inherently noisy because mainly the reduced number of participating molecules. For instance, transcription and translation are processes involving low-abundance molecules in comparison with protein-protein interactions [4, 5]. Noise with amplification, *i.e.*, in the presence of positive feedback, can originate at population level the switch-like behaviour, resulting in two or more different sub-populations. This process is called bistability, if there are two stable genetic states, or multistationarity, if there are multiple stable genetic states [4]. However, not always noise can be translated in phenotype variation. In some cases, the biochemical network, in which the gene product is inserted compensates the fluctuations without alter the phenotype [5].

Currently, there are still a lot of gaps about molecular mechanisms related with phenotypic switching. New experimental approaches to study the phase variable systems are being developed and promising data will soon arise.

8. Concluding Remarks

To successful colonized new environments or simply to survive to stressors, bacteria need to recur to evolution and diversification. Phenotypic switching provides a source of microbial diversity fundamental to bacterial fitness and development. Through phenotypic switching, bacteria have the potential to better survive in hostile environments due to the creation of several variants equipped to persist to stresses. In addition, bacteria have not the costs of an irreversible state, as mutations, allowing, if more advantageous, to revert to the previous phenotype. There are numerous phenotypic variants that have been recovered after in vitro stresses or even isolated from patients with different infections. This huge diversity is the evidence of the significance of phenotypic switching.

Phenotypic switching has been shown to influence bacterial virulence and also antimicrobial resistance. SCV are the best example of how phenotypic switching can alter the antimicrobial resistance profile and virulence ability. SCV has been isolated from several types of disease, as respiratory, urinary and device-related infections demonstrating their great adaptability in such different environments.

Biofilm development is an important strategy to bacterial survival. To form biofilms, bacteria have to switch to the biofilm-specific phenotype. The study of phenotypic switching in the scope of biofilms is still limited. It is essential understanding how phenotype conversion contributes to better form biofilms and the impact of this conversion on infectious disease, antimicrobial resistance and persistence. Biofilm studies are not simple because the multifaceted nature and adaptive plasticity exhibited by bacteria in response to external factors. Simulating real conditions with collection and clinical microbial specimens will be useful towards the understanding of the evolved concepts in biofilms.

The concept of on-off switching of phenotypic states is a quite simple view of a complex process. Phenotypic diversity can be mediated by specific genetic alterations or by epigenetic mechanisms. Hence, it has been difficult to demonstrate whether the mechanisms attributed to phenotypic switching in vitro are the same at play in vivo. The majority of experimental reports regarding phenotypic variation have studied new and constant environmental changes. However, in real situations changes occur instantaneously and are unlikely to remain constant over the time for the fixation of the mutations. It starts now to be evident some differences between in vitro and in vivo emergence of microbial colony variants. Because of this discrepancy between laboratory studies and real situations, the knowledge about phenotypic variation is delayed. Therefore, it is necessary to perform in vivo studies to test hypotheses raised by the in vitro experiences and to draw correlations between morphotypes generated in vitro and in vivo.

Apart from phenotypic switching, it is evident another important phenomenon, the bacterial individuality or inherent phenotypic variation. The cell-to-cell phenotypic variability within an isogenic population also provides a dynamic source of diversity. It has received attention from the scientific community due to their implications on the treatment of chronic infections. Inherent heterogeneity is often masked in experimental conventional, since they generally used data average population-level phenotypic responses from thousand or even millions of bacteria in a single sample. Bacterial individuality has been proposed to be owed to "genetic noise", i.e., cell-to-cell fluctuation in gene expression. The development of diverse phenotypes independently of environmental factors is a relevant issue from the evolutionary and adaptation standpoints.

Taking into account all the information available so far, it is difficult to argue against the great importance of phenotypic switching. However, there is still a lack of knowledge about phenotypic varying systems. The understanding of the phenotypic switching processes and the response of the bacterial population to environmental stresses is fundamental to develop adequate disinfection protocols and therapeutic treatments. The knowledge of the determinants of phenotypic switching can be of greater significance to solve real problems, such as multi-antimicrobial resistance, to design therapeutic approaches and vaccine development for some clinical diseases.

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