Mechanisms and experimental models for the assessment of microbial biofilms’ phenotypical resistance /tolerance

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Introduction
The first reports about multicellular prokaryotic communities [1] stated their presence in all natural environments. The description of these microbial communities or biofilms was achieved approximately 30 years later by Costerton and his team, which pointed out the very significant role of adherent microorganisms in human infectious diseases [2]. A biofilm is a sessile microbial community composed of cells embedded in a matrix of extracellular polymeric substances attached to a substratum or interface. The matrix is primarily of microbial origin and the cells embedded in this matrix exhibit a modified phenotype, especially concerning the growth rate and gene transcription [3]. The biofilm may be constituted of a single or many microbial species and is considered as a signifying a survival strategy, this structure being less susceptible to adverse environmental conditions and stress factors. The most important discovery in the last years is that the adherent bacteria are phenotypically very different from their planktonic bacteria [4, 5], the infections with microbial pathogens growing in biofilms being very different when compared to the infections determined by planktonic cells, due to their different behaviour, generation time and susceptibility to antimicrobial agents [4].

Recent public announcements stated that 60% to 85% of all microbial infections involve biofilms developed on natural tissues (skin, mucosa, endothelial epithelia, teeth, bones) or artificial devices (central venous, peritoneal and urinary catheters, dental materials, cardiac valves, intrauterine contraceptive devices, contact lenses, different types of implants). Thus, prosthetic medical devices are risk factors for chronic infections in developed countries and these infections are characterized by slow onset, middle intensity symptoms, chronic evolution and resistance to antibiotic treatment. Biofilm microbes involved in biofilm associated diseases a very large spectrum, from the Gram positive (Staphylococcus epidermidis and S. aureus) to the Gram negative pathogens (Pseudomonas aeruginosa, Escherichia coli) and to different members of the Candida genus (particularly C. albicans, C. parapsilosis).

The pathogenesis of these infections is implicating: resistance to host defense mechanisms; pro-inflammatory effect due to the production of endotoxins (LPS) by Gram negative pathogens leading to a pyogenic reaction; the matrix of biofilm favors the maintenance of infection by nutrients accumulation, simultaneously extending the log phase; detachment of single cells / aggregates is promoting bacterial dissemination (urinary and systemic infections); high resistance or tolerance to antibiotics and other antimicrobials.

In case of biofilm development, a series of genes (40 - 60 % of the prokaryotic genome) are modulated (activated/inhibited) by complex cell to cell signalling mechanisms and the biofilm cells become phenotypically distinct from their counterpart - free cells, being more resistant to stress conditions. Adherence and biofilms formation confer to bacterial cells resistance to host defence mechanisms, both unspecific (i.e. clearance, lysozyme, phagocytes, complement system) and specific (antibodies (1000x ≥)). The human body has defence mechanisms which are efficient against a high number of free bacteria (10^8 cells) in sterile organs (heart, lungs, peritoneum), whereas much less (10^7 sessile or adherent cells) can cause persistent infections in the same organs [6]. The biofilm cells are also resistant to all kind of antimicrobial substances: antibiotics, antiseptics, disinfectants; this kind of resistance, consecutive to biofilm formation is phenotypical, behavioural and, more recently, called TOLERANCE. The biofilm phenotype can reduce antimicrobial susceptibility and increase tolerance up to 1000 - 4000 times, evidently decreasing the antimicrobial efficiency and leading to clinical therapeutical failures. The minimal inhibitory concentration (MIC), determined by standard assay is clearly in contrast with the minimal biofilm eradication concentration (MBEC) [7].

1. Mechanisms of biofilm increased resistance/tolerance
Tolerance is defined as the ability to survive killing by bactericidal factors without necessarily expressing a genetic resistance mechanism. The molecular basis of tolerance is still unknown. Tolerance to antibiotics is especially significant in survival of bacterial biofilms. It has recently been found that “persister cells” are largely responsible for the high tolerance of bacterial biofilms to antimicrobials. In a variety of bacterial species examined, the level of...
“persisters” increased with the density of the culture, reaching ~1% in stationary phase as well as in biofilms of *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* [8, 9].

A number of mechanisms accounting for the increased antibiotic resistance / tolerance of biofilms include:

1) failure of the antibiotic agent to penetrate the extracellular matrix (reticular structure: H₂O, minerals, sugars, proteins, DNA) which acts as a diffusion barrier for large molecules (some antibiotics, antibodies, phagocytes) or as an ions exchanging resin;

2) biofilm matrix can accumulate waste products (which enables continuation of the log phase)

3) accumulation pf antibiotic degrading enzymes;

4) a different pattern of gene expression occurs in irreversibly adherent bacterial cells to different substrata (40-60 % of prokaryotic genome being up- or down regulated) [10] ( e.g. under-expression of porin genes as Omp F that mediating the internalization of the majority of hydrophilic antibiotics, over- expression of efflux pumps actively pumping out the antibiotics, the activation of some virulence genes, such as those for toxins or enzymes).

5) the cells experience nutrient limitation in the depth of a mature biofilm and, therefore, a slow-growing or starved state can be present or the non-growing cells are not highly susceptible to antimicrobial agents; this genomic plasticity of biofilm conferring high levels of diversity, so that not any given antibiotic cannot kill all of the cells in the community and certain cells will persist and repopulate the surface –(persisters theory) [11].

6) occurrence of genetic changes such as mutations; cells with a high rate of mutations (hypermutators), were isolated from biofilms, probably selected by different stress conditions, with an adaptive role [12, 13]; and gene transfer (including R and virulence plasmids) facilitated by the proximity of the cellsand leading to an acquired resistance in the initially susceptible bacterial cells of multiclonal or multispecies communities.

“Persisters” were described even since 1944 by Joseph Bigger who noticed that penicillin did not sterilize a culture of *Staphylococcus aureus*. The surviving persister cells, may be small in number, i.e. less than 100 persisters out of 2.5x10⁷ cells after exposure to penicillin. Opposite to resistant mutants, persisters are phenotypic variants of the wild type that after reinoculation are promoting a culture with a similar amount of persister cells. There are data pleading for the fact that persisters are not signifying particular stage in the cell cycle and that they are not produced in response to antibiotics, but they are rare non-growing cells preexisting in a bacterial population (Fig. 1). Bacterial populations produce persister cells that neither grow nor die in the presence of the bactericidal agents, but they are exhibiting multidrug tolerance (MDT) [8].

Identification of MDT genes is an important first step in understanding recalcitrance/tolerance of biofilms to antibiotic therapy and it will probably explain other related and poorly understood phenomena involving a dormant state, such as the latent *Mycobacterium tuberculosis* infection.

A lot of ~ 300 genes was evidently over-expressed in isolated persister cells. Several interesting features of this group are evident. Expression of stress responses (SOS, heat shock, cold shock and phage shock proteins) seems to agree well with proposed survival function of this kind of cells. Of special interest was the presence of messages coding for proteins blocking cellular functions: several TA (toxin-antitoxin) module proteins, RMF (inhibitor of translation), and UmuDC (inhibitor of replication). Persistence is a reversible phenomenon [8].

The persister cells have a lot of interesting properties:

1. after recultivation, persister cells enable repopulation;
2. persisters do not pass their tolerance to their progeny and the progeny do not inherit any greater tendency to become persisters; so that thepersisters do not seem to be genetic variants; a possible explanation is the phenomenon of senescence of same cells, which are splitting functionally asymmetric.
3. persister cells apparently grow slowly or not at all in the presence of antimicrobial agents;
4. persister cells demonstrate tolerance when exposed to multiple antimicrobial agents;
5. bacterial cultures demonstrate biphasic killing patterns in response to antimicrobial challenge;
in continuous culture experiments, persister cells are observed to increase simultaneously with the decreasing of the dilution rates when the persister cells population is growth-phase-dependent, the increase occurring in the later stages of the exponential phase or in the stationary phase.

The gene profiling data support the possibility of persisters to be in a dormant state. A cluster of approximately 600 genes involved in metabolism and flagella synthesis was seen to gradually decreasing in ampicillin treated cells. The same genes showed a further decrease in isolated persisters, suggesting that these might have been down-regulated in persisters prior to the addition of antibiotic. Among these repressed genes were members of the large operons involved in oxidative phosphorylation: NADH dehydrogenase, ATP synthase and cytochrome O- ubiquinole oxidase genes. Mycobacterium tuberculosis resistance to isoniazide is also related to the occurrence of mutations in the primary mycobacterial catalase-peroxidase gene (katG). The altered catalase-peroxidase provides high-level resistance to isoniazide, while retaining a level of oxidative protection, sufficient for the detoxifying activity against host antibacterial radicals [14-17].

Tolerance of both planktonic and biofilm populations to antibiotics has been suggested to be due especially to slow growth rates.

2. Experimental models for the assessment of the phenotypical resistance /tolerance

The emergence of antibiotic resistance has been well documented over the last ten years; emergence of the multi-drug resistant (MDR) Gram negative bacilli in the latter part of 2000 and the re-emergence of Gram positive cocci, particularly vancomycin-resistant enterococci (VRE) in 2001. The biofilm embedded cells phenotypically different to their planktonic counterparts and more resistant to stress conditions including antimicrobial substances and the host defence mechanisms have amplified the hole resistance phenomenon. Beside all these aspects, the density/proximity of cells into biofilms is increasing the opportunity for gene transfer, e.g. ESBL genes [18]. Biofilm-associated infections become therefore difficult to treat and it is essential that a new approach be developed to treat such infections.

Since many years laboratories have used standardized methodologies described by NCCLS /CLSI guidelines concerning the evaluation of antibiotic resistance aspects for single species planktonic populations. On the contrary, biofilms exhibit a whole new spectrum of problems for the laboratory diagnostic, none of which having been standardized until now. However, a new parameter called minimum concentration for biofilm eradication (M.C.B.E.) was proposed by Ceri et al. (1999) [19], this one being determined by using monospecies biofilms prepared in a 96-well plate. Biofilm formation of staphylococci on inert substratum can be studied using the slime test, a semi-quantitative 96-well microtiter plate assay: the strains to be studied are inoculated into the microtiter wells in an appropriate growth medium and incubated overnight; then the plates are washed with buffer, the adherent biofilm is fixed and then stained with gentian violet, violet crystal or safranin, and finally the absorbance of light is quantified in a spectrophotometer (Fig. 2).

An original experimental model for the assessment of phenotypical resistance /tolerance [20] is an in vitro model for testing the antimicrobial susceptibility of adherent and biofilm included bacteria developed on an inert material immersed in liquid medium; the advantage of this model is represented by the introduction into the testing plastic wells of different biomaterials to be investigated; example, fine sections of silicon-coated urinary catheters/ central venous catheters/ other materials used in stomatology are incubated in multi-well plates in an appropriate growth medium, with bacterial suspensions and different antibiotic concentrations, simultaneously added or after a preincubation/pre-adherence time; after the incubation period, the catheters samples are washed in sterile PBS, placed in fresh nutrient medium; after a new incubation time, the optical density of the bacterial cultures is measured (O.D. at A_{600} nm), these values being an indirect indicator of the adherence process (Fig. 3, 4).
Another simple model for the study of biofilm susceptibility to antibiotics consists in the inclusion of the bacterial cells in an agarose matrix, mimicking the exopolymeric biofilm matrix [21] (Fig. 5). The model uses Mueller Hinton agar (the culture medium recommended by CLSI-Clinical and Laboratory Standards Institute to determine susceptibility to antibiotics by disk diffusion method) and is generating valid results with superior predictive value for the in vivo effectiveness of antibiotics tested taking into consideration the drastic decrease of adherent bacteria susceptibility to a particular antibiotic to which cells are sensitive in the free, planktonic state.

Fig. 3 Optical density values ($A_{600}$ nm) of bacterial cultures developed in the presence of antibiotic solutions (cloramphenicol) in different concentrations, simultaneously added with catheter pieces and bacterial inoculum.

Fig. 4 Optical density values ($A_{600}$ nm) of bacterial cultures developed in the presence of antibiotic solutions (cloramphenicol) in different concentrations, added after a period of preincubation of catheter pieces with bacterial inoculum.
Conclusions

The recent advances made in highlighting the molecular genetic basis of biofilm development and the deciphering of the inter-species communication based on extracellular led to the development of new ideas for preventive and therapeutic anti-infective strategies. However, only concerted efforts of scientists from a variety of disciplines will be able to explore these complex systems of the microbial world, in order to solve the problem of biofilms associated infections by the development of accurate and realistic experimental models concerning the assessment of biofilms resistance to different antimicrobial substances, the use of combined or new therapeutic methods and searching for new anti-pathogenic strategies based on the inhibition of the intercellular communication and virulence genes expression. Antibiosis is also an expression of ecological relationships among species of a microbial consortium. But QSIs, from the medical point of view, are not growth inhibitors and they are not acting as selection factors for resistance genes, constituting thus an ecological strategy; they are acting as inhibitors of the biofilm formation and of the virulence factors, by disrupting the biofilm’s cells connection rendering the bacterial cells susceptible to usual therapeutic doses of antibiotics.

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References


