Influence of soluble mediators upon the expression of different physiological and virulence hallmarks of bacteria

Received for publication, March 15, 2008
Accepted, March 25, 2008

***MARIANA CARMEN CHIFIRIUC, CRISTINA LARION, CARMEN IORDACHE, MARIANA LIXANDRU, OLGUTA DRACEA, **CORALIA BLEOTU, *MARCELA BUCUR, ANCA MICHAELA ISRAIL**

National Institute for Research in Microbiology and Immunology, Cantacuzino, Spl. Independentei 103, cod 060631, Bucharest Romania
**University of Bucharest, Faculty of Biology, Ale. Portocalelor 1-3, Bucharest Romania*
***Stefan S. Nicolau" Institute of Virology, 285 Mihai Bravu Ave, cod 030304, Bucharest Romania*
Corresponding author email address: <carmen_balotescu@yahoo.com>

Abstract

Traditional treatment of infectious diseases is based upon bactericidal/bacteriostatic compounds, a major concern being the frequent development of bacterial resistance to these substances. The discovery of intra- and intercellular communication systems (quorum sensing systems) regulating bacterial virulence has afforded a new opportunity to control pathogenic bacteria (without interfering with their growth). The purpose of this study was to investigate the influence of soluble mediators present in supernatants of logarithmic phase bacterial cultures upon the growth rate and expression of enzymatic virulence factors, as well as the adherence ability to the cellular and inert substrata of Vibrios, Aeromonas, Pseudomonas and coagulase-positive methicillin resistant staphylococci. Our results demonstrated that bacterial soluble mediators accumulated in logarithmic phase cultures exhibited specific effects on the rate of microbial growth and expression of different virulence factors, i.e.: in case of Vibrio strains, the soluble mediators induced a shortage of the lag and stationary phases, the last one being completely abolished for some strains; the bacterial growth remained unchanged for Aeromonas sp. strains; for Ps. aeruginosa strains, a significant increase of lag phase and a delayed stationary phase were noticed; in case of S. aureus strains the soluble mediators induced either increase or decrease of the growth rate. Whereas the pattern of cell-associated virulence factors of Vibrionaceae strains was not significantly influenced by cell-free supernatants, in case of the most Ps. aeruginosa and S. aureus strains, a significant inhibition of biofilm development on inert surfaces was noticed. The filtrates of log phase cultures induced important changes in adherence patterns to HeLa cells with predominant aggregative aspects in Ps. aeruginosa and S. aureus strains, accompanied by an evident decrease of adherence in the case of the last one. The pattern of soluble enzymatic factors was poorly influenced by the culture filtrates, excepting a constant increase in lipase expression by Vibronaceae and pseudomonades strains; this aspect suggests the inducible feature of this enzymatic virulence factor, which could act as a pore—forming toxin and invasin during the infective process and is demonstrating the implication of the mechanism in the regulation of this virulence factor, dependent on cell density, as well as the possible use of this enzyme as marker in studies concerning quorum-sensing mediated processes. Our results demonstrated that the soluble mediators accumulated in the logarithmic bacterial cultures are capable to change the intracellular communication and the sequential expression of different virulence factors, altering the success of these pathogens in the colonization of a sensitive host and development of an infectious process.

Key words: autoinducer, virulence, growth, intercellular communication, quorum-sensin

Introduction

Most living organisms possess sophisticated cellular signaling networks in which lipid-based modulator is responsible for biological effects such as cell differentiation, reproduction and immune responses. Diffusible signal molecules are used by bacteria for synchronising their behaviours (intercellular communication, metabolic processes dependent on cellular density, plasmidial transfer, competence induction, sporulation, coordinated expression of virulence genes in response to association with eukaryotic host organisms) (2, 17).

The discovery that bacteria synthesize extracellular chemical signals for intercellular communication is signifying a new paradigm for gene regulation process. Bacterial cell-to-cell communication enables the gene transcription by the *quorum sensing* (autoinduction) mechanism, depending of the cellular density and release / sensing of low-molecular weight compounds. In most cases, the concentration of extracellular autoinducers is

increasing simultaneously with the bacterial cell density. When reaching a *critical* concentration of the autoinducer, a signal transduction cascade is triggering the expression of a target gene(s) (2, 17).

Based upon this general mechanism, *quorum sensing* systems have evolved as means to facilitate microbe access to complex nutrients, to environmental niches and to release bacterial defense strategies against other microorganisms or host defense mechanisms (11, 14).

The most studied cell-to cell bacterial communication system is mediated by homoserin-lactones (HL) and their derivatives (used by Gram-negative bacteria) and firstly described in the luminescent marine bacteria (*Vibrio fischeri, Vibrio harveyi, Photobacterium sPs.*) (3, 4, 7, 18). These organisms express genes controlling light emission (the luciferase enzyme) only when associated with their fish or squid symbiotic hosts. When free living in the ocean (i.e. low bacterial densities) luciferase is not expressed. QS system in Gram-positive organisms, such us *Staphylococcus aureus* is based on octapeptides (2).

One of the most important roles of the QS mechanisms both in Gram-negative and Gram-positive bacteria is to regulate the expression of virulence factors and to prevent the host defense mechanisms during the infectious process (11).

The success of any pathogen in the colonization of a sensitive host and the development of an infectious process is depending on its ability to sense its environment and to modulate the expression of the genes encoding factors required for the colonization of a new habitat.

Ps. aeruginosa is a very important opportunistic agent, resistant to all known antibiotic therapies and the best studied and understood in terms of QS role in pathogenicity. In human *Ps. aeruginosa* infections implicating the bacterial adherence and biofilm development on medical devices, as well as its resistance to biocides are all regulated by QS mechanisms. The bacterial cell-to-cell communication was demonstrated by detecting the presence of HL molecules on catheters from patients with *Ps. aeruginosa* urinary tract infections (12, 15).

The staphylococcal pathogenesis is represented by a large spectrum of infections from minor infected lesions to endocarditis, osteomyelytis and septic shock. Recently, progress has been made in identification and characterization of the regulatory mechanisms of staphylococcal virulence factors encoded by *sar* and *agr* genes. The level of these virulence factors is depending on the extracellular concentration of some octapeptides modulating the expression of *agr* gene. Thus, small amounts of octapeptides are inducing the synthesis of proteins implicated in the bacterial adherence to the extracellular matrix proteins (fibronectin, collagen) during the initial stages of infection, and the accumulation of autoinductors is leading to the release of bacterial exotoxins such as haemolysins, proteases, lipases, generating the tissue damages and necrosis (11).

Bacterial *quorum-sensing* has became a very active area in microbiology and a large group of investigators is working on these fascinating aspects of bacterial biology in the purpose to develop new therapeutic agents for the treatment of associated bacterial infections, especially the chronic and persistent ones (8, 9, 10,16).

The purpose of this study is to investigate the influence of some soluble mediators present in supernatants of logarithmic phase bacterial cultures upon the growth rate, the expression of enzymatic virulence factors as well as the adherence ability to the cellular and inert substrata of some Gram-positive and Gram-negative strains.

Material and Methods

i) Bacterial strains

In this study there were analyzed ten *Vibrio* strains (two *V. metchnikovii,* two *V. alginolyticus,* two *V. fischerii,* two *V. anguillarum,* two *V. parahaemolyticus),* ten*Aeromonas* strains *(*five *A. caviae* and five *A. hydrophila)* isolated from different clinical and environmental sources, 10 coagulase-positive methicillin resistant *Staphylococcus (S.) aureus* strains isolated from central venous catheter-related bacteremia and ten *Pseudomonas (Ps.) aeruginosa* strains isolated from different clinical (tracheo-bronchic secretions, central venous catheters, sputum, urine cultures) specimens. The *Vibrionaceae* strains were identified by help of conventional and API 20 NE biochemical tests (1, 13), while pseudomonads and staphylococci were identified by API microtests and VITEK I automatic system. All strains were preserved in the Microbial Collection of the National Reference Center for *Vibrio* of Cantacuzino Institute.

ii) Methods

The tested strains were cultivated in nutrient broth and the microbial cultures were harvested at different times of incubation (2h, 6h, 24 h, 48h, 72 h) and spotted (10 µl of the adequate dilution) on solid media in order to establish the microbial growth curve and the time of logarithmic growth phase by performing viable cell counts and/or measuring the absorbance of cell cultures density at different intervals. Subsequently the

logarithmic phase cultures were centrifuged and sterilized by 0.22 μm membrane filtration in order to obtain the cell-free cultures, prospected to contain the highest amounts of *quorum-sensing* soluble mediators.

The adhesion and invasion aspect were investigated on HeLa cells by using Cravioto's adapted method) (6). In this purpose, bacterial mid-logarithmic phase cultures of tested strains grown in nutrient broth/cell-free supernatants were centrifuged at 4000 g for 10 minutes, and the pellet washed three times in phosphate buffered saline (PBS) and resuspended in Eagle MEM. Bacterial suspension density was adjusted to 10^7 CFU/ml. HeLa cells were grown in MEM enriched with 10% heat-inactivated (30 min at 56°C) fetal bovine serum (Gibco BRL), 0.1 mM nonessential amino acids (Gibco BRL), and supplemented with 0.5 ml of gentamycin (50 μg/ml) (Gibco BRL) and incubated at 37°C for 24 hrs. HeLa cells monolayers cultivated in 6 multi-well plastic plates were used at 80-100% confluence. For the adherence assay, the HeLa cell monolayers were washed 3 times with PBS; 1 ml of fresh Eagle MEM without antibiotics was added to each well and 1 ml of bacterial suspension was inoculated to each well and the plates incubated for 2 hrs at 37ºC. After incubation, the monolayers were washed 3 times in PBS, briefly fixed in cold methanol (3 min), Giemsa stained and incubated for 30 min. The plates were washed, dried at room temperature, examined microscopically (magnification, ×2500) and photographed with a Contax camera adapted to Zeiss microscope.

Adhesion to inert substrata and biofilm development was performed by cultivating the microbial cells in 96-well plates, followed by incubation at 37^{o}C for 24 h. After incubation, the plastic wells were discarded, washed three times in PBS, fixed by cold methanol (5 min), dried at room temperature and coloured by violet crystal solution (30 min). After staining, the intensity of blue colour was quantified by reading the absorbance at 490 nm.

The expression of soluble, enzymatic factors (haemolytic activities, lecithinase, lipase, caseinase, gelatinase, amylase, mucinase, DN-ase) was assayed by spot plate method using specific media, as previously described (5).

All tested strains were grown in nutrient broth to which the cell –free logarithmic supernatants were added (vol:vol) and the above-mentioned parameters tested again and compared to the control cultures.

Results and discussion

Our results indicate that bacterial soluble mediators accumulated in logarithmic phase cultures exhibited specific effects on the microbial growth and the expression of different virulence factors, depending on the taxonomic position of the strains.

i) The influence of soluble mediators on bacterial growth

In case of *Vibrio* strains, the soluble mediators induced the shortage of the lag and stationary phases, the last one being completely abolished for some strains (Fig. 1-3).

These results could signify that the modulation of the growth curve by soluble mediators could impair the coordinated expression of the virulence factors depending upon the cell density.

In the present case of *Vibrio* strains, the shortage of the stationary phase induced by the soluble mediators could block or reduce the expression of associated soluble virulence factors (soluble enzymes, toxins), and therefore, of the invasive potential, while the growth of *Aeromonas* strains was not influenced by their homologous supernatants (Fig. 4-5).

Concerning the *S. aureus* strains, even under the influence of the supernatants, by comparison with the corresponding control cultures, the different growth periods of the respective strains remained unchanged, the cell density proved to be higher in the presence of supernatants (Fig. 6).

For *Ps. aeruginosa* strains, a significant increase of the lag phase and delayed entrance in the stationary phase were noticed (Fig. 7).

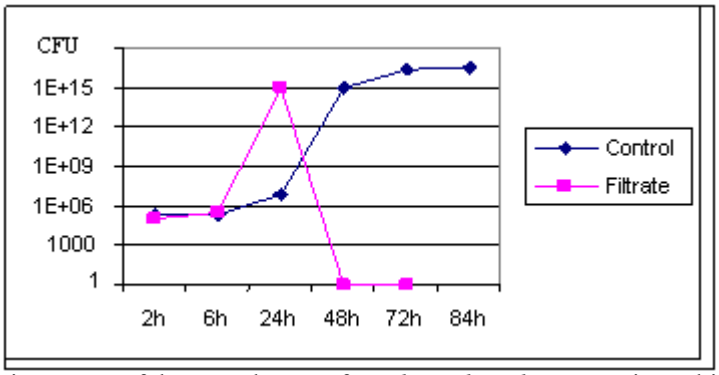

Figure 1. The general comparative aspect of the growth curve for *Vibrio alginolyticus* strains cultivated in nutrient broth and the respective supernatants

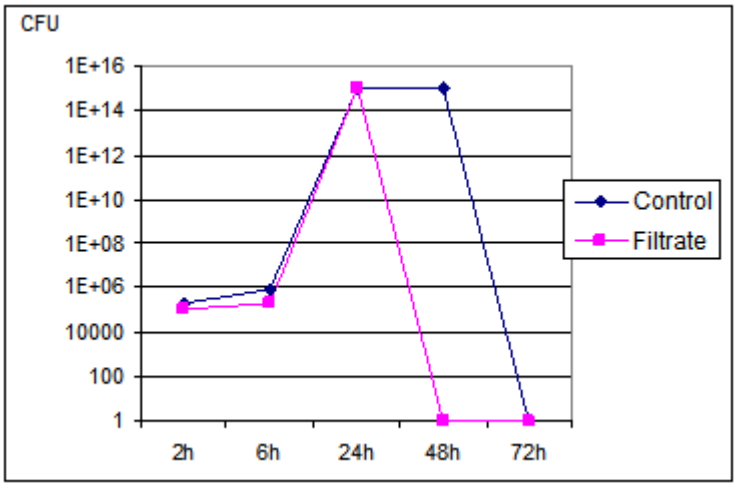

Figure 2. The general comparative aspect of the growth curve for the tested *Vibrio fischerii* strains cultivated in nutrient broth and respective supernatants

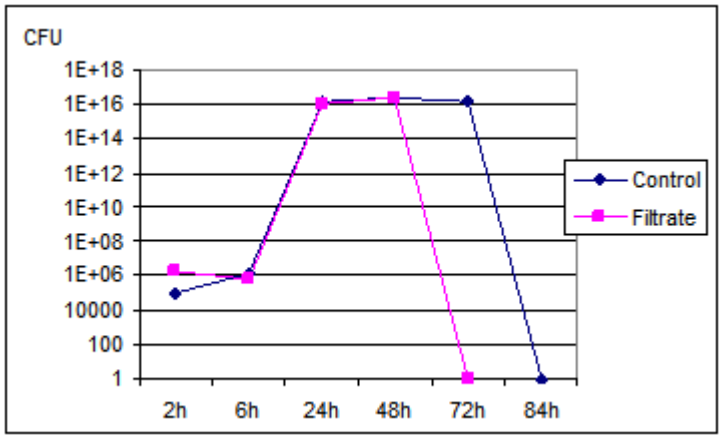

Figure 3. The general comparative aspect of the growth curve for the tested *Vibrio metschnikovii* strains cultivated in nutrient broth and respective supernatants

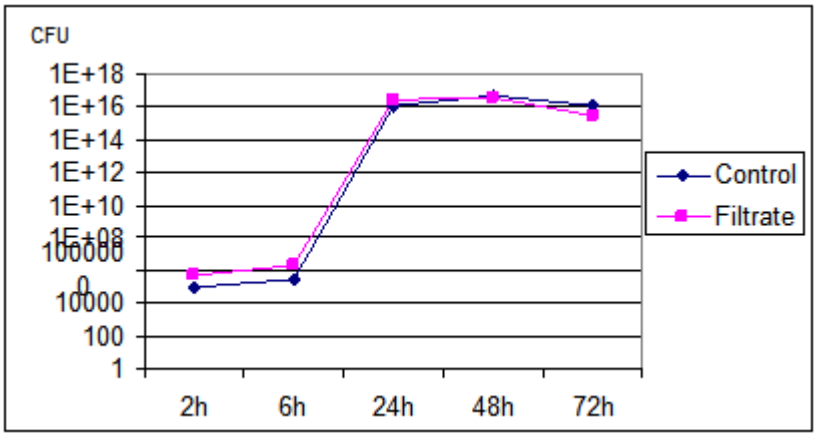

Figure 4. The general comparative aspect of the growth curve for the tested *Aeromonas caviae* strains cultivated in nutrient broth and respective supernatants

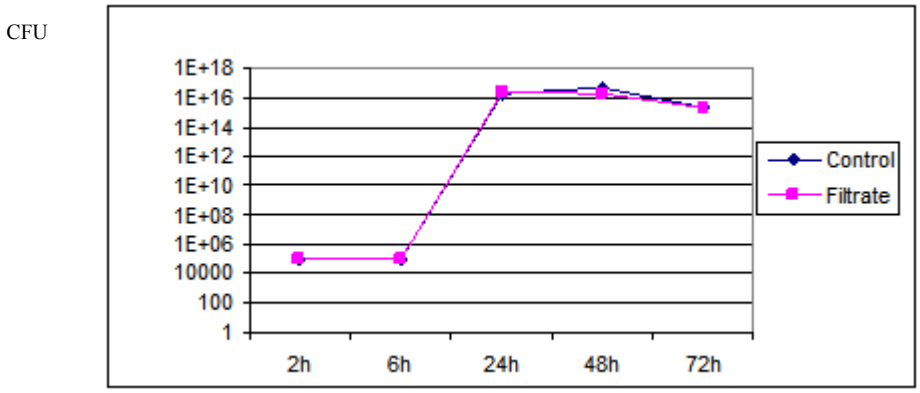

Figure 5. The general comparative aspect of the growth curve for the tested *Aeromonas hydrophila* strains cultivated in nutrient broth and respective supernatants

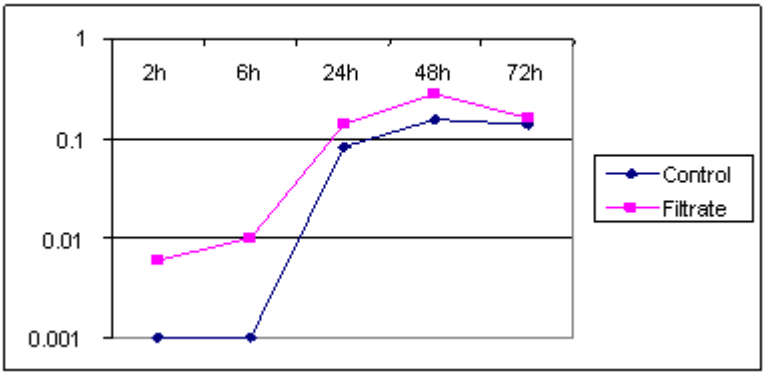

Figure 6. The comparative aspect of the growth curve for the tested *S. aureus* 10936 strain cultivated in nutrient broth and respective supernatants, quantified by measuring the absorbance at 600 nm.

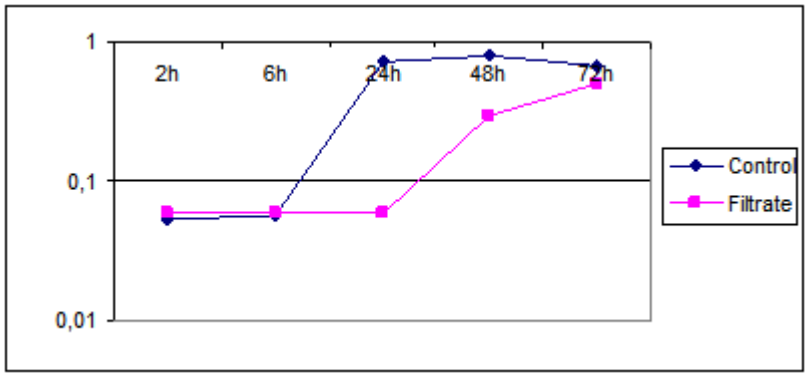

Figure 7. The general comparative aspect of the growth curve for the tested *Ps. aeruginosa* strains cultivated in nutrient broth and respective supernatants quantified by measuring the absorbance at 600 nm.

ii) The influence of the soluble mediators on the expression of virulence factors

Whereas the pattern of cell-associated virulence factors of *Vibrionaceae* strains was not significantly affected by the respective supernatants, in case of the most tested *Staphylococcus* strains significant changes in the expression of virulence factors were noticed. All supernatants, irrespective to the growth phase strongly inhibited the biofilm development of the tested strains on inert surfaces (Fig. 8).

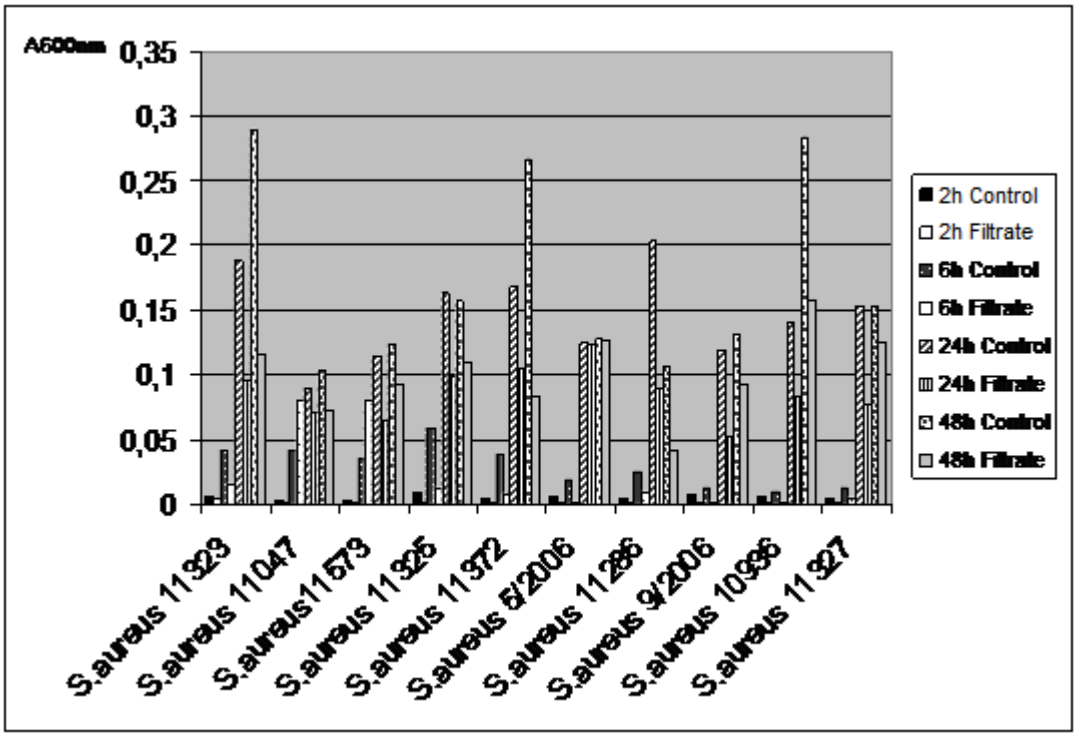

Figure 8. The comparative level of the adherence ability of the *S. aureus* strains to the inert substrate represented by plastic wells, cultivated in nutrient broth and in respective supernatants, quantified by measuring the absorbance at 600 nm.

For *Ps. aeruginosa* strains, an inhibitory effect on biofilm development was observed for 6 and 24 hours culture supernatants; these results could be explained by the increase of lag phase induced by the respective supernatants and the subsequent delay in the adhesins expression, no more correlated with the logarithmic growth.

In case of 6 strains, the 48 hours supernatants induced the highest level of biofilm development and also a delayed entrance in logarithmic phase with a prolonged expression of adhesins, whereas, at this moment the control cultures were already entering the stationary or even the decline phase of growth (Fig. 9).

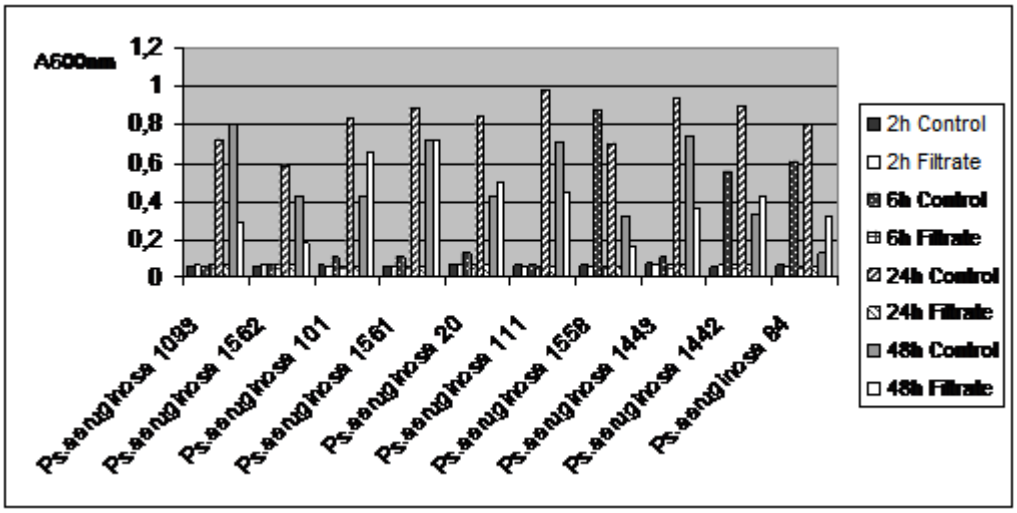

Figure 9. The comparative level of the adherence ability of the *PS. aeruginosa* strains to the inert substrate represented by plastic wells, cultivated in nutrient broth and in respective supernatants, quantified by measuring the absorbance at 600 nm.

The log phase supernatants induced also important changes concerning the adherence pattern to HeLa substrate, from a predominantly aggregative to a diffuse one (Fig. 10); probably due to the decreased synthesis of the extracellular polysaccharide specific to *Ps. aeruginosa*, usually implicated in the aggregative adherence ability, but lost during the cultivation of *Ps. aeruginosa* in the presence of homologous supernatants (Fig. 11). In the case of *S. aureus* strains, we noticed a drastic decrease in the adherence ability to the cellular substrate, accompanied by changes in the adherence patterns (Fig. 12).

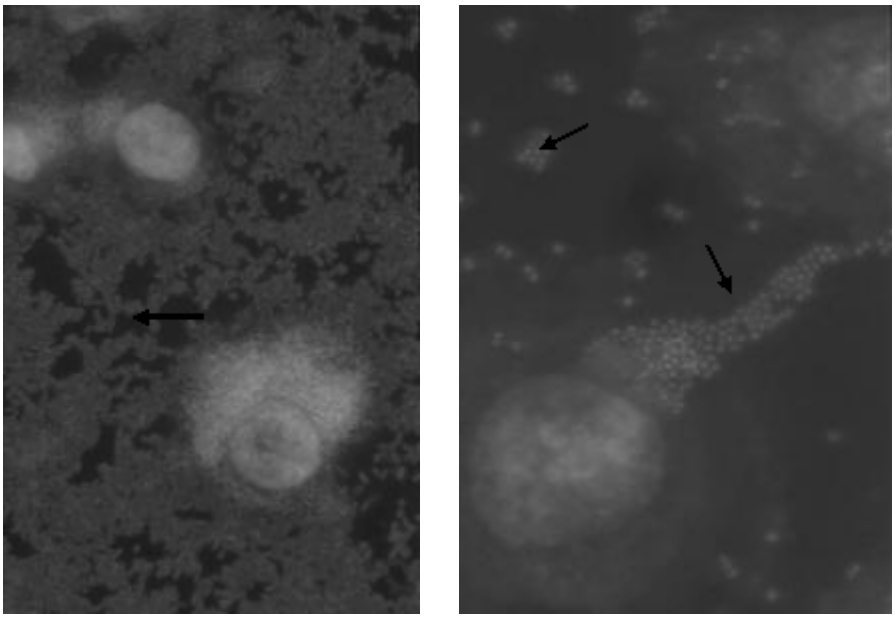

Figure 10. Propidium iodide fluorescent staining of HeLa cells infected with *Ps. aeruginosa* (left) and *S. aureus* (right) with predominant aggregative adherence patterns (2500X)

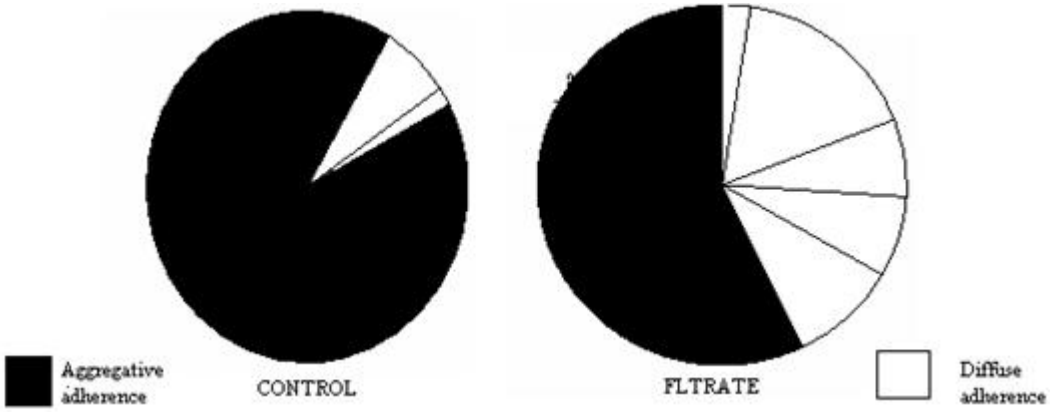

Figure 11. Predominance of aggregative and diffuse adherence patterns of *Ps. aeruginosa* control cultures comparison with cultures grown in the presence of log phase supernatants

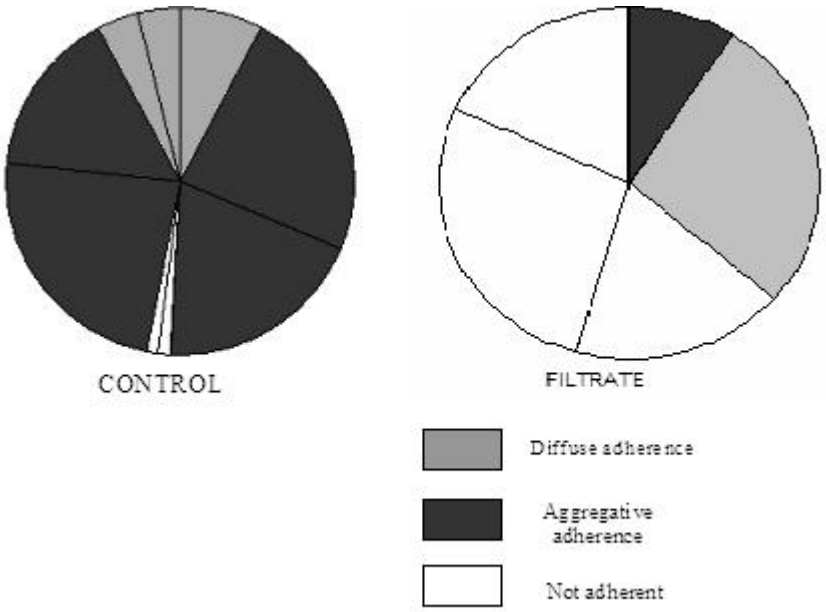

Figure 12. Predominant aggregative and diffuse adherence patterns of *S. aureus* control cultures by comparison with cultures grown in the presence of log phase supernatants

The pattern of soluble enzymatic factors was poorly influenced by the homologous culture supernatants, excepting a constant increase in lipase expression in *Vibronaceae* and pseudomonades (Fig. 13), and a decreased expression of haemolytic properties in some *Vibrionaceae* strains.

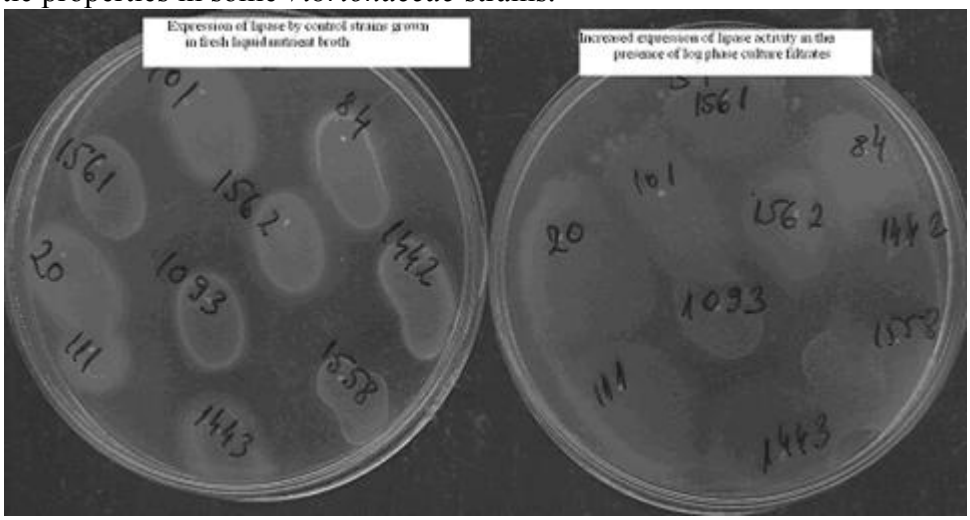

Figure 13. Aspect of lipase positive strains on selective medium

The modulation of lipase expression, in the presence of soluble mediators accumulated in the log phase cultures suggests the inducible feature of this enzymatic virulence factor, which could act as a pore—forming toxin and invasin during the infective process and is demonstrating the implication of a mechanism dependent upon the cell density in the regulation of lipase expression, as well as the possible use of this enzyme as marker in studies concerning *quorum-sensing* mediated processes.

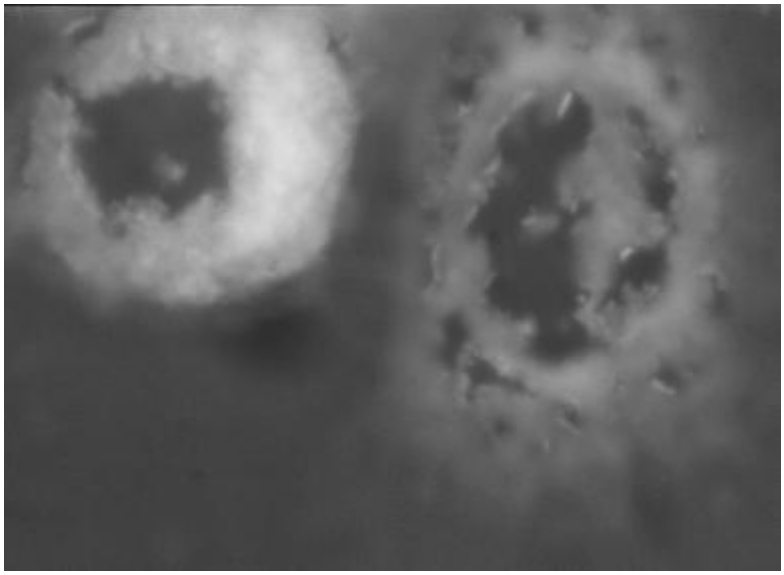

Figure 14. DAPI fluorescent staining of HeLa cells infected with *Ps. aeruginosa* showing the invasive potential of the tested strain and the apoptotic aspect of the infected cells (picnotic nucleus and the peripheral distribution of heterochromatin) (2500X)

The changes in the expression of soluble virulence factors in the presence of log phase supernatants (soluble mediators) are demonstrated by the interference of these molecules with the *quorum sensing* mechanisms implicated (in Gram-negative and Gram-positive bacteria) in regulation of virulence factors expression during the infectious process. Concerning bacterial virulence, a coordinated expression of certain factors is required for bacterial survival in the host organism, under stress conditions (absence of nutrients, the presence of immune antibacterial factors), with the purpose to find a proper habitat, proliferation and development of the infectious process, dissemination and turnover of infectious cycle in new locations. Thus, the positive / negative switchover concerning the bacterial virulence expression is playing an important role in the relationship host-infectious agents and can shed light upon the pathogenic mechanisms of infectious processes. In opportunistic pathogens, the bacterial ability to detect the population density and to react as function of this parameter, can assure the coordination of the expression of virulence factors implicated in the neutralization of the host immune defense system. For example, the production of a high amount of exoenzymes, only after reaching a threshold density will allow the rapid destruction of the host tissues before the occurrence of a defense reaction. Probably, if bacteria would start the production of virulence factors at low densities in the host, these factors, due to their immunogenicity, will trigger an immune response, able to eliminate the infection. On the contrary, the immune response occurred at high densities of the infective agent could not be able to eliminate the infection and the infectious process could go on.

Our results demonstrated that the soluble mediators accumulated in the logarithmic bacterial cultures are able to influence the intracellular communication and the sequential expression of different virulence factors, altering the effect of these pathogens, by decreasing the colonization of a sensitive host and development of an infectious process. The identification of the chemical structure of the soluble substances responsible of these effects, could represent the start point for implementing a new anti-infective strategy, with great therapeutic and preventive role in the biomedical field (especially in the treatment of chronic infections determined by multiresistant and biofilm forming microorganisms), but also in the management of the environmental quality, agriculture and industry, by reducing the chemical pollution burden delivered in the external medium and preventing the surfaces colonization with microorganisms and the development of natural biofilms.

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