Research Paper

Adaptive response of single and binary *Pseudomonas aeruginosa* and *Escherichia coli* biofilms to benzalkonium chloride

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The main goal of this work was to examine whether the continuous exposure of single and binary *P. aeruginosa* and *E. coli* biofilms to sub-lethal benzalkonium chloride (BC) doses can induce adaptive response of bacteria. Biofilms were formed during 24 h and then put continuously in contact with BC for more 5 days. The six-day-old adapted biofilms were then submitted to BC challenge, characterized and inspected by SEM. Both single and binary adapted biofilms have clearly more biomass, polysaccharides and proteins and less activity even though the number of cells was identical. After BC treatment, adapted biofilms maintained their mass and activity. SEM examination revealed that those adapted biofilms had a slimier and denser matrix that became thicker after BC treatment. Continuous exposure of bacteria to antimicrobials can lead to development of biofilms encompassing more virulent and tolerant bacteria. This adaptive resistance can be the result of a phenotypic adaptation, a genetic acquired resistance or both. Instead of eradicating biofilms and kill microorganisms, the use of a disinfectant can, favour biofilm formation and tolerance. This must be a genuine concern as it can happen in clinical environments, where the use of antimicrobials is unavoidable.

Keywords: Biofilms / Adaptive resistance / Benzalkonium chloride / *Pseudomonas aeruginosa*

Received: March 16, 2011; accepted: May 01, 2011

DOI 10.1002/jobm.201100137

Introducton

Pseudomonas aeruginosa and *Escherichia coli* are two of the most important human associated pathogens and commensal bacteria [1] that can contaminate inanimate surfaces during months, specially when attached and developing biofilms [2]. Both bacteria show an increase in pathogenicity due to their motility structures (type IV pili in *P. aeruginosa* and curli in *E. coli*) and matrix production through extracellular polymeric substances (EPS) secretion. Previous works [3–6] showed that EPS synthesis (alginate for *P. aeruginosa* and colanic acid in *E. coli*) is induced upon attachment of the bacteria to a surface.

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Usually, the procedures for surface cleaning involve the use of detergents followed by application of disinfectants that reduce the viability of microorganisms [7]. Antimicrobials, as quaternary ammonium compounds, have been continuously used in hospitals and healthcare facilities, and have significantly contributed to maintain sanitary conditions and to prevent hospital-acquired infections. Despite their valuable properties, concerns have been raised about the widespread and irrational use of disinfectants that could fail the eradication of the biofilm-associated microorganisms and serve to select disinfectant-insusceptible microbes among hospital-acquired pathogens [8–11].

Physiologic or phenotypic adaptation resulting in "antimicrobial tolerance" has been attributed to biofilms [12, 13]. Biofilm tolerance to disinfectants may result from (i) slow microbial growth rates that are attributable to nutrient depletion within biofilms, (ii) binding of the biocide to EPS, (iii) neutralization or

degradation of the biocide, as well as (iv) the expression of biofilm-specific phenotypes [2, 12, 14]. There are evidences that proteins involved in oxidative stress response, cell envelope synthesis, and EPS synthesis are up-regulated in biofilms, indicating that these altered phenotypes might contribute to cell survival, persistence, and growth in a biofilm community [15, 16].

Moreover, biofilms usually comprise more than one microbial species, and can harbour strains with less ability to develop a biofilm, contributing to their survival and persistence. It has been reported that two bacteria present in an *in vitro* binary biofilm may live synergistically and enhance each other survival to antimicrobial treatments compared with the corresponding single species biofilms [17]. An important aspect of the study of the interactions in multispecies biofilms is to evaluate whether individual species, or the bacterial consortium, gain any fitness advantages compared to single-species biofilms. A fitness advantage in this context is defined as the ability of the organism or biofilm to persist or grow in a given environment or under a particular environmental stress. Multiple species biofilms may be thicker and more stable than the single ones, which may influence their susceptibility to disinfectants [18].

The aim of this work was to examine whether exposure of *P. aeruginosa* and *E. coli* to benzalkonium chloride (BC) during single and binary biofilm formation, could induce an adaptive response in bacteria by evaluating the biofilms behaviour after treatment with the same agent. BC is a well-known quaternary ammonium compound (QAC) that has been widely used as surface disinfectant, antiseptic and preservative in medical arenas. Considering the intense use of BC as an antimicrobial agent, it is important to increase the knowledge about the effects of external chemical pressure and polimicrobial growth on the susceptibility of the sessile target organisms to this QAC.

Materials and methods

Strain and culture conditions

Pseudomonas aeruginosa ATCC 10145 and *Escherichia coli* K12 substrain MG 1655 were preserved in criovials (Nalgene) at −80 ± 2 °C. Prior to each experiment, bacterial cells were grown on Tryptic Soy Agar (TSA, Merck) plates for 24 h at 37 °C.

To prepare the bacterial suspension, one colony of each bacterium (*P. aeruginosa* and *E. coli*) were collected from the TSA plates and grown in Tryptic Soy Broth (TSB) for 24 h at 37 °C, in a horizontal shaker (120 rpm). Subsequently, bacteria were washed twice with ultrapure sterilized water (UP). Standardized cell suspensions were prepared in TSB at a cell density of 1×10^7 cfu/ml, unless otherwise stated.

Antibacterial agent

Benzalkonium Chloride (BC), a quaternary ammonium compound, with critical micellar concentration of 5.0 mM was purchased from Calbiochem (Merck Biosciences, UK).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC of BC of *P. aeruginosa* and *E. coli* were determined by the microdilution method according to Clinical and Laboratory Standards Institute (formerly NCCLS) [19].

Briefly, 96-well round-bottom microtiter plates (polystyrene, Orange, USA) with a total well capacity 300 μl were used. In each well, 50 μl of fresh TSB with increasing concentrations of BC were added to 50 μl of each bacterial inoculum (containing approximately 1×10^5 cfu/ml). The culture-plates were incubated at 37 °C for 24 h in an orbital shaker at 120 rpm. The highest concentration of BC that did not promote growth was recorded as the MIC.

MBC determination was performed by transferring 10 μl of culture from each well without visible growth into TSA plates. The lowest antimicrobial concentration that yielded no colony growth after 24 h at 37 °C was documented as the MBC.

Biofilm development and adaptation

The methodology used to grow the bacterial biofilms was based on the microtiter plate test developed by Stepanovic *et al.* [20]. Cell suspensions of *P. aeruginosa*, *E. coli* and binary species suspension (50% of each) were diluted in TSB to obtain a final concentration approximately 1×10^7 cfu/ml. Afterwards, 200 μl/well of the bacterial suspension were transferred to sterile 96-well flat-bottom tissue culture plates (Orange Scientific, Braine-l'Alleud, Belgium). All the plates were incubated aerobically on a horizontal shaker (120 rpm), at 37 °C, during 24 h for biofilm development.

After 24 h of biofilm growth, the supernatant containing planktonic cells and media was removed. The wells were re-filed with fresh TSB or TSB containing BC in a final concentration of 0.9 mM to mimic external BC chemical pressure in order to induce the adaptation of the biofilm-embedded cells. This process of supernatant removal and media filling was repeated for five subsequent days, every 24 h. The six day-old biofilms

formed in TSB supplemented with BC are hereafter referred as adapted biofilms while the biofilms formed only in TSB will be referred as normal biofilms.

Biofilm treatment with BC

After being developed in the absence and presence of BC for 6 d (during the adaptation process), biofilms were subjected subsequently to sudden attack with the same antimicrobial product. For that, the liquid content of each well was removed and washed once with 200 μl of UP sterilized water, being the well-attached biofilms (biofilms formed by *P. aeruginosa* and *E. coli* in the inner surfaces of each well of the microtiter plates) subsequently treated with 200 μl of 1.0 mM of BC for 30 min. This procedure had the major purpose of eliminating more susceptible and outward biofilm-cells. Non-treated wells were filled with 200 μl of UP sterilized water for the same period of time. After that, the content of each well was removed and biofilms were washed twice with 200 μl with UP sterilized water and reserved for subsequent analysis.

Bacterial suspensions of the single and binary *P. aeruginosa* and *E. coli* non- and adapted biofilms to be used in protein and polysaccharide quantification and cfu determination were prepared as described hereafter. Two-hundred microliters of UP sterilized water were added to each well, being the wells-attached biofilms removed by ultrasonic bath in a Sonicor SC-52 (Sonicor Instruments, Copaique, NY, USA) operating at 50 kHz, during 6 min (these parameters were previously optimized in order to promote the complete removal of all the biofilm-attached cells without lysis). Afterwards, the single and mixed bacterial suspensions of each 5 wells per condition were collected, gently vortexed for 2 min [22–24] to disrupt possible cell aggregates, and reserved for later analysis.

Biofilm analyses

Biofilm cell enumeration

In order to determine the number of cfu, the untreated and BC treated axenic and binary biofilm suspensions removed by sonication were serially diluted. After plating the serial dilution on TSA, plates were incubated at 37 °C in an aerobic incubator for 24 h prior to enumeration.

Biofilm mass determination

Biomass of *P. aeruginosa, E. coli* and binary biofilms was quantified by crystal violet (CV) staining method adapted from Stepanovic *et al.* [20]. For that, the plates containing the normal and adapted biofilms were left to air dry for 30 min, and 200 μl of 98% methanol were transferred to each well in order to fix the remaining attached bacteria, for 15 min. Afterwards, the plates were emptied and left to air dry again. The fixed bacteria were stained with 200 μl of 1% (w/v) CV (Gram colour-staining set for microscopy; Merck) per well, for 5 min. After the staining step, the plates were washed with running tap water and air dried for approximately 20 min and, at last, 200 μl of 33% (v/v) of glacial acetic acid (Merck) were added to each well in order to resolubilise the CV bound to the adherent bacteria. The quantitative analysis of biofilm production was performed through the measurement of optical density at 570 nm (OD_{570}) in each well using a microtiter plate reader being the biofilm mass presented as OD_{570}. Control experiments to avoid false results were also performed in order to determine whether the tested media and the plate material could adsorb CV and interfere with biomass quantification. When the optical density was higher than 1.0 the sample was diluted with 33% (v/v) of glacial acetic acid. For each condition tested, 16 different wells were used to perform biofilm analysis and the experiment was performed with three independent assays.

Biofilm activity determination

Biofilm activity determination was evaluated with 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide sodium salt (XTT) colorimetric method as described by Stevens and Olsen [25], with some modifications.

Bacteria, when metabolically active, reduce XTT to a water-soluble orange formazan that diffuses from the cells and thus does not require solvent extract prior to its quantification. Since the formazan product is water soluble, it is easily quantified by spectrophotometry analysis. Therefore, the quantity of formazan produced is used as a measure of the total respiratory activity of the bacteria. This latter point is important in biofilm research because the XTT assay allows the study of intact biofilms without disruption biofilm structure [26].

After biofilm growth and washing procedures, 200 μl of a combined solution of XTT (Sigma) and phenazine methosulfate (PMS) (Sigma) were added to each well in order to obtain a final concentration of 150 μg/ml of XTT and 10 μg/ml of PMS. After that, plates were incubated at 37 °C for 3 h, at 120 rpm, in the dark. Biofilm activity was determined through the measurement of the optical density at 490 nm (OD_{490}) in each well using a microtiter plate reader, being biofilm activity presented as OD_{490}. Control tests, using culture medium and empty wells, were also carried out in order to avoid

misleading results. For each condition tested, 16 different wells were used to perform biofilm analysis and the experiment was performed with three independent assays.

Proteins and polysaccharide quantification

The total content of proteins of the single and mixed suspensions from normal and adapted biofilms, untreated and treated with BC, was determined using the BCA Protein Assay Kit (Pierce, Bonn, Germany), with bovine serum albumin as a protein standard, and the optical density (OD) values recorded at 740 nm. The total polysaccharides content was determined by the phenol-sulphuric acid method described by Dubois *et al.* [27] with glucose as standard, and the OD values were recorded at 490 nm. This biochemical characterization was performed in three independent experiments.

Scanning electron microscopy (SEM) observations

Prior to SEM observations, the wells-attached washed biofilms were gradually dehydrated in an absolute ethanol (Merck) series (15 min each in 10, 25, 40, 50, 70, 80, 90 and 100% v/v). The wells-attached normal and adapted biofilms were kept in a dessicator until the walls of the wells were cut and coated with gold. The examination of the surface structural conformation of the biofilms was performed with a Leo scanning electron microscope (Cambridge). SEM observations were documented through the acquisition of representative microphotographs.

Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 4.00 software for Macintosh. Normality of data distribution was tested by the Kolmogorov-Smirnov method. Statistical significance values of the groups' means of biofilm mass, biofilm activity, cell number and protein and polysaccharide content were evaluated using a one-way analysis of variance. Subsequent comparisons were performed using Tukey's post-hoc test. The statistical analyses performed were considered significant when $p < 0.05$.

Results

Determination of MIC and MBC

The MIC and MBC of planktonic *P. aeruginosa* and *E. coli* are shown in Table 1. *P. aeruginosa* revealed the highest MIC and MBC values when compared with those obtained for *E. coli*. To promote a selective BC pressure on planktonic growth of both strains, a BC concentration of 0.9 mM was chosen for the biofilm adaptation process.

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of BC against *Pseudomonas aeruginosa* and *Escherichia coli*.

	MIC (mM)	MBC (mM)
P. aeruginosa	0.450	0.900
E. coli	0.225	0.225

Biofilm analyses

Biofilm mass

In order to determine the biofilm formation ability of *P. aeruginosa*, *E. coli* and its binary combination, in the absence (normal biofilms) and in the presence of 0.9 mM of BC (adapted biofilms), the total biomass was determined. Fig. 1A shows that the six day-old biofilms formed by single species and by the combination of both species in TSB have similar biomass values. The posterior treatment with 1.0 mM of BC did not alter the biofilm mass accumulated on the surfaces ($p > 0.05$).

It was also observed that both single and binary six day-old adapted biofilms have clearly more biomass ($p < 0.001$), about five times more, than the normal biofilms formed in the absence of BC. Fig. 1A also reveals that *E. coli* adapted biofilms have more mass than those of *P. aeruginosa* ($p < 0.01$) and than binary biofilms ($p < 0.05$). Furthermore, posterior BC treatment seems to reduce the mass of *P. aeruginosa* adapted biofilms ($p < 0.05$) and to increase the biomass of the binary biofilms ($p < 0.001$).

Biofilm activity

The metabolic activity of the viable biofilm-entrapped cells is presented in Fig. 1B. *P. aeruginosa* cells of normal biofilms revealed an increased activity in comparison with that of adapted biofilm-entrapped bacteria ($p < 0.001$). Data also show that both single and binary adapted biofilms have less activity than those formed in the absence of BC ($p < 0.01$). Among the adapted biofilms, binary biofilms present the highest cell activity and *P. aeruginosa* biofilms the lowest ($p < 0.001$). Concerning biofilm treatment with BC, the activity of the biofilm-associated cells was significantly reduced ($p < 0.001$) in normal biofilms. Furthermore the six-days old *P. aeruginosa* biofilms developed in the presence of BC, when challenged with the same product, revealed even more activity than the biofilms without treatment ($p < 0.001$). Adapted binary biofilms and *E. coli* biofilms did not exhibit major changes in their activity upon QAC treatment ($p > 0.05$).

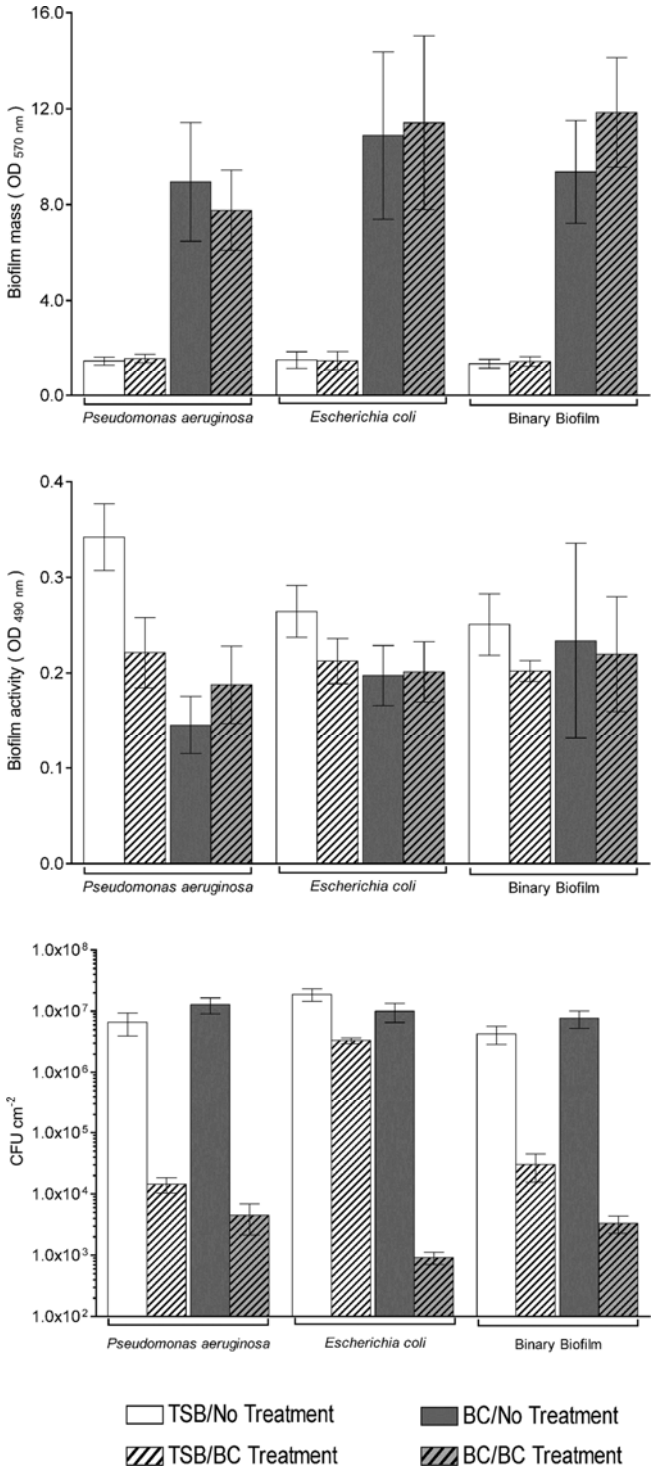

Figure 1. Biomass ($OD_{570\ nm}$) (A), metabolic activity ($OD_{490\ nm}$) (B) and number of cultivable cells (C) of single and binary six day-old *P. aeruginosa* and *E. coli* biofilms developed in TSB (white bars) and TSB supplemented with 0.9 mM of BC (grey bars). Biofilms were after treated with 1.0 mM BC (striped bars), non-treated biofilms (solid bars). Bars represent the average of 3 independent repeats ± SD.

Biofilm-entrapped cells

Concerning the number of biofilm-associated cells, after six days of biofilm growth (Fig. 1C), it was observed that the adaptation to BC, both for single and binary biofilms, seemed not to have influence on the number of total cells. In fact, the total number of cells entrapped in the biofilms was similar (approximately 10^7 cfu/cm^2), whether adapted or not to BC.

On the other hand, BC treatment of single *P. aeruginosa* and binary normal biofilms led to a 3 log reduction ($p < 0.001$) in cell number whereas *E. coli* biofilm-entrapped cells just suffered a reduction of about 1 log.

The effect of BC treatment on adapted biofilms was similar to the one observed in the normal biofilms as it also led to a decrease of about 3 log ($p < 0.001$) in the number of biofilm-entrapped cells.

Biochemical biofilm characterization

As the data showed that adaptation to BC seems to promote an increase in biomass values (Fig. 1A), it was considered pertinent to inspect the biochemical features of all biofilms.

According to Fig. 2, *P. aeruginosa* normal biofilms exhibit more protein content when compared to the other biofilms (*E. coli* and binary) ($p < 0.001$). However, when the biofilms were adapted to BC, *E. coli* biofilms were those that revealed the highest protein content ($p < 0.001$). The presence of BC during biofilm development leads to biofilms characterized by 4-fold higher protein content, for *P. aeruginosa* and binary biofilms, and 8-fold higher for *E. coli* biofilms.

After BC challenge, normal biofilms did not show great variability in their protein content. Concerning adapted biofilms, the treatment with 1.0 mM of BC promoted a reduction of about 50% of the protein content for *P. aeruginosa* and binary biofilms while for *E. coli* biofilms this reduction was just about 20%.

The amount of polysaccharides in normal biofilms was similar for all the strains. The presence of BC during biofilm development led to a significant increase of polysaccharide content, being about 10 times superior in *P. aeruginosa* and binary biofilms, and about 13 times superior in the case of *E. coli*.

The BC treatment promoted an augment in polysaccharide content of *P. aeruginosa* biofilms ($p < 0.01$) while no significant change was observed for *E. coli* and binary biofilms ($p > 0.05$), when developed only in TSB.

The BC challenge after biofilm adaptation to the same product promoted a significant decrease in the polysaccharide content, similar to that obtained for protein content.

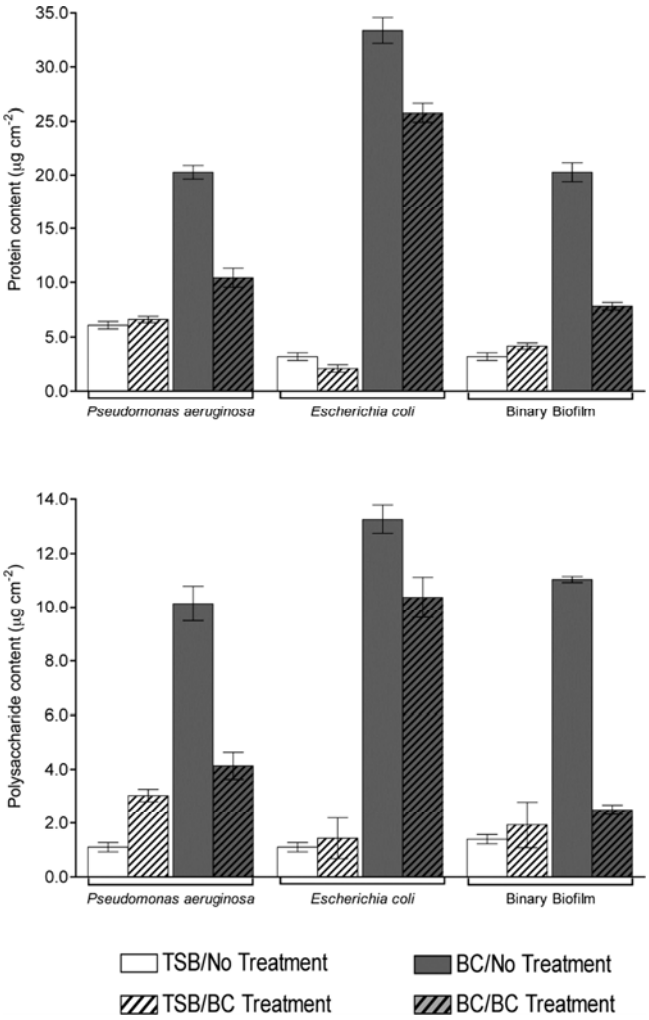

Figure 2. Biochemical biofilm characterization. Values of protein (A) and polysaccharide (B) content for of single and binary six day-old *P. aeruginosa* and *E. coli* biofilms developed in TSB (white bars) and TSB supplemented with 0.9 mM of BC (grey bars). Biofilms were after treated with 1.0 mM BC (striped bars), non-treated biofilms (solid bars). Bars represent the average of 3 independent repeats ± SD.

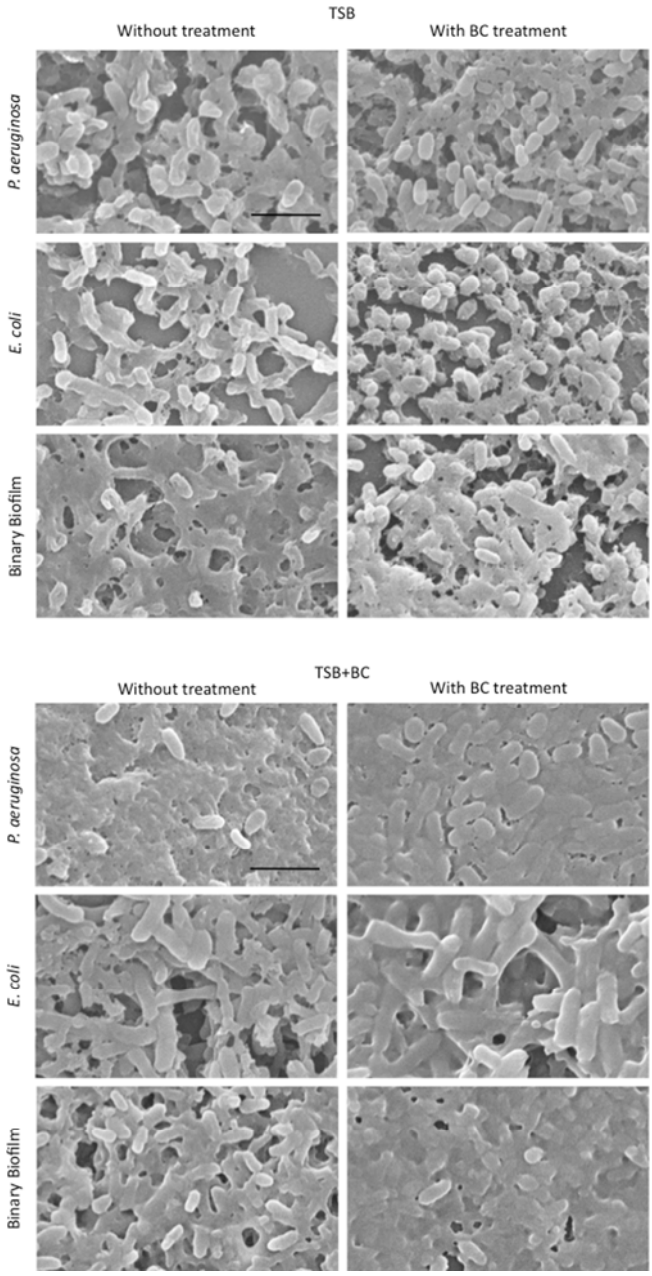

Figure 3. Scanning electron microscopy of single and binary six day-old *P. aeruginosa* and *E. coli* biofilms developed in TSB and TSB supplemented with 0.9 mM of BC (TSB + BC) without and with 1.0 mM BC treatment. X 10 000 magnification, bar = 2.0 μm.

Scanning electron microscopy observations

The influence of BC, on biofilm superficial structure and morphology was assessed by SEM inspection (Fig. 3).

SEM observations revealed a morphological alteration induced by the presence of BC during biofilm formation. The images of the adapted biofilms surface seem to indicate a larger amount of EPS, especially for *P. aeruginosa* and *E. coli* single-species biofilm.

The BC treatment after biofilm formation has different effects depending on the biofilms being adapted to BC or not. For biofilms formed in TSB, BC treatment appears to cause some damages in the EPS matrix protecting biofilms. This matrix disruption is well noticed in *E. coli* and binary biofilms. For adapted biofilms, the images suggest that the subsequent BC challenge strengthens the existing matrix, giving the biofilm-entrapped cells an interconnected structure.

Discussion

P. aeruginosa and *E. coli* are two human pathogens and commensal organisms often related with foreign body infections, mainly due to their ability to form biofilms. It is widely held that a biofilm represents a highly selective micro-niche where pathogens may also have high mutation rates or easily exchange genetic material, thus facilitating the development of antimicrobial drug resistance [36].

The continuous and, sometimes, inappropriate use of antimicrobials in several settings (households, hospitals and other institutions), together with the increased use of antibiotics to cure everyday diseases has been on the basis of selection of resistant bacteria. Moreover, the well-known biofilm microniche properties and their intrinsic resistance to antimicrobial products due to their specific features, namely, EPS matrix protective barrier, nutrient and oxygen gradients, cell-cell signalling, persister cells and genetic diversity, play a key-role in this easily acquired resistance.

The planktonic bacterial adaptation to BC is a well documented subject [37–40] and, recently, there have been some studies reporting biofilm adaptation to BC sub-MIC [16, 41–44]. From the analyses of the latter studies, it was observed that the BC concentrations used for biofilm adaptation are not always the same, albeit being always lower than the MIC concentration determined. The adaptation method used to induce an adaptive tolerance of the biofilm-entrapped cells is not standardized, varying among authors, microorganisms and studies.

In the present work, the adaptation of the *P. aeruginosa* and *E. coli* bacteria embedded in sessile life-style was attained at a BC concentration of 0.9 mM. It was not our objective to characterize which phenomenon (recalcitrance or resistance) was involved, but to study the biofilm phenotype after exposure to chemical stress conditions. The BC concentration used is higher than the ones used to adapt *Salmonela* (0.29 mM) [16, 41] within biofilms and *Listeria* planktonic cells (0.012 mM) [44]. Despite being the highest MBC determined for the most resistant strain (*P. aeruginosa*), the concentration of 0.9 mM was used to promote the BC pressure. Results showed that the presence of this BC concentration in the liquid environment surrounding biofilms did not impair their growth or caused cell death within the biofilms, since no reduction of the absorbance related with biomass (Fig. 2A) or decrease in cell number (Fig. 2C) was observed. These data are not surprising since it is widely accepted that cells entrapped in biofilms easily gain tolerance to antimicrobials. Normally, this insusceptibility is overcome with the use of supra inhibitory concentrations (supra-MIC) to ensure sanitation. The unexpected result of this study is the significant increase of biofilm mass due to BC pressure (5-fold higher) regardless the strain or the number of strains that generate the biofilm (Fig. 1A). These results show how the inappropriate use of an antimicrobial can favour bacteria attachment and biofilm formation, instead of eradicating biofilms and kill microorganisms. In fact, in real situations, this can be a genuine concern as it can happen in clinical settings or apparatus, where the use of antimicrobials is unavoidable, as reported by Lee *et al.* [45] in liquid reservoirs where antimicrobials are kept.

The adapted biofilms showed an increase in biofilm biomass (Fig. 1) that was corroborated by its biochemical characterization (Fig. 2) since the biofilms formed in TSB supplemented with BC revealed a protein and polysaccharide content higher than the normal biofilms. Since extracellular polysaccharides and proteins are two of the major matrix components [46, 47], our data suggest that adapted biofilms present a well-defined and strong EPS matrix.

SEM pictures (Fig. 3) confirmed this feature as it was well noticed a denser outward EPS matrix evolving bacteria, being this especially evident in *P. aeruginosa* and *E. coli* single biofilms. Based on these characteristics, it can be speculated that the presence of BC during biofilm formation may give rise to adapted biofilms with a stronger and cohesive structural conformation. EPS are a complex and extremely important component of biofilms, providing architectural structure and mechanical stability to the attached bacteria [48]. From the point of view of disinfection, this feature can represent an additional nuisance since biofilm matrix acts as shield to external stresses, limiting, for instance, the diffusion of antimicrobials. According to Branda *et al.* [49], small changes in the environmental conditions may promote dramatic changes in biofilm architectures being the environment where biofilms are developed responsible for the matrix composition. So, it can be concluded that the presence of BC in the liquid interface surrounding the single and binary biofilms during its establishment can be the environmental factor that changes biofilm surface structure, making the biofilm-associated bacteria possibly more tolerant to antimicrobials.

In attempting to expand the understanding of biofilm cell physiology, the response of *P. aeruginosa* and *E. coli* entrapped in single and binary biofilms to BC treatment was examined. The BC concentration used for the subsequent attack of the established 6-days-old

biofilms was similar to that used for biofilm adaptation due to the fact 0.9 mM is already a high concentration when compared with the normally in-use BC concentration in cleaning products [1.0 % (w/v)] [21].

Lindsay *et al.* referred that the growth of *Pseudomonas* spp with other strains might be beneficial as the EPS produced by *Pseudomonas* spp may confer some protection to the binary biofilm and thus protect it against sanitizer treatment [17, 50]. In the present study, the data did not corroborate that study, as the binary growth of biofilm does not seem to give any advantage or disadvantage concerning the response to the presence of BC during biofilm development or the BC challenge.

The *E. coli* biofilms formed in normal conditions, i.e. in the absence of BC, seem to show less sensitivity to BC attack (Fig. 1C) presenting also more unwavering protein and polysaccharide content (Fig. 2). Actually, a careful observation of the values obtained with the biochemical characterization of *E. coli* biofilms reveals that after BC treatment there is just a small reduction in these contents. In this case, the EPS matrix seems to be a crucial structural parameter for *E. coli* biofilm stability and architecture providing a refuge for bacterial community [49]. The limited effectiveness of antimicrobials to eradicate the microorganisms of biofilm infections may be related to non-mutational or physiological conditions that allow survival, like those related to stationary-phase physiology, low oxygen and nutrient penetration, low penetration of antimicrobials or antibiotics and slow growth. This particular phenotype confers biofilms tolerance or recalcitrance [31–33].

P. aeruginosa is known to produce mainly alginate during EPS excretion [48, 51] and there are studies referring alginate overproduction during nitrogen limitation [52] and after *P. aeruginosa* membrane perturbation induced by ethanol [53]. *E. coli* matrix is composed mainly of colanic acid [5, 6, 54–57]. Pringent-Combaret *et al.* [57] reported that the copious amount of appendages present at the cell surface (such as curli in *E. coli* and type IV pili in *P. aeruginosa*) and the various exopolymers excreted by bacteria can also concentrate ionic molecules from the biofilm phase as the biofilm develops. These different cell appendages and matrix main components together with the possible different response of each strain to BC stress can be the reason for the different biofilm behaviour observed and it can also explain the ability of BC treatment to disrupt matrices.

The maintenance of the number of entrapped-cells of adapted biofilms (Fig. 1C) can be due to the protective effect of the cohesive matrix observed in SEM (Fig. 3). In this study, *E. coli* biofilm-entrapped cells probably have undergone some kind of phenotypic change due to the fact of being part of a biofilm. In fact, the entrapped-cells of adapted biofilms appeared to be more sensitive to BC than the cells of normal biofilms. It can also be due to the difficulty of detecting persister cells by culturability methods [31]. According to a study of Mangalappalli-Illathu [41], the regrowth of *Salmonella enterica* serovar Enteritidis after BC exposure was due to a small percentage of survivor cells that, after BC stress relief, were able to recover and multiply, being these survivors the sign of the so called persistence and regrowth of biofilms.

When considering the antimicrobial effect of disinfectants, it is not merely the killing of bacteria that is of interest. Removal of biofilm from the surfaces is also important as residual sessile cells may facilitate rapid regrowth of new biofilms [58]. So, in conclusion, it appears that *E. coli* and *P. aeruginosa* entrapped in single and mixed biofilms may develop adaptive resistance to BC, noticeable by the over secretion of matrix components during biofilm development in presence of that antimicrobial product. Overall results clearly point out that BC had poor antimicrobial efficacy against *E. coli* and *P. aeruginosa* biofilms development and control.

Knowing that the amount of matrix is one of the most important contributes to the virulence of biofilms, the use of BC as an antimicrobial agent should be avoided and special care should be taken when dealing with inappropriate doses of this antibacterial agent. The suitable concentration of antimicrobial should always be supported by experimental assays. Otherwise, biofilm establishment and bacteria virulence might be favoured, contributing, in this way, to the increased prevalence of surface contamination and biofilm-associated infections. The data of this study did not allow the determination of which phenomenon (phenotypic adaptation, genetic acquired resistance or both) was responsible for the adaptation of biofilms to sublethal QAC exposure. Therefore, future work will be required to unravel the mechanism(s) by which the biofilm-entrapped bacteria respond to environmental pressures. This can be achieved by the determination of genetic and proteomic regulation of EPS production under BC stress.

Acknowledgements

The financial support from IBB-CEB and Fundação para a Ciência e Tecnologia (FCT) and European Community fund FEDER, through Program COMPETE, in the ambit

of the Project PTDC/SAUESA/64609/2006/FCOMP-01-0124-FEDER-00702 and Idalina Machado PhD Grant (SFRH/BD/31065/2006) and Susana Lopes PhD Grant (SFRH/BD/47613/2008) are gratefully acknowledged.

Conflict of Interest Statement

We declare that we have no proprietary, financial, professional or any other personal interest of any nature or any kind in any service, product or company that could influence opinions and positions presented in the manuscript.

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