Adaptation and survival of *Burkholderia cepacia* and *Burkholderia contaminans* in saline solutions containing benzalkonium chloride: impact on microbiological quality of pharmaceuticals

Mariana Tavares[1,2] & Isabel Sá-Correia (Supervisor)[1,2]
[1] iBB - Institute for Bioengineering and Biosciences, Instituto Superior Técnico, Universidade de Lisboa, Lisbon, Portugal
[2] Department of Bioengineering, Instituto Superior Técnico, Universidade de Lisboa, Lisbon, Portugal

Abstract: The *Burkholderia cepacia* complex (Bcc) is a group of opportunistic pathogenic bacteria, equipped with an extraordinary phenotypic and genotypic plasticity, allowing their adaptation to diverse hostile conditions, particularly water-based environments, solutions with low nutrient concentrations and the presence of antimicrobial agents. Bcc bacteria are feared contaminants in pharmaceutical industries and the underlying cause of numerous nosocomial outbreaks, posing a serious threat to susceptible individuals, particularly Cystic Fibrosis (CF) patients. In the present study, the survival and adaptation of clonal isolates of *B. cepacia* and *B. contaminans* was investigated after long-term incubation in nutrient depleted saline solutions, and in the presence of two concentrations of benzalkonium chloride (BZK), a biocidal preservative commonly used in pharmaceutical settings. Bacterial strains were previously recovered from contaminated saline solutions for nasal application or from two CF patients under surveillance at a Lisbon hospital, and their clonal nature examined by molecular typing. Viability studies revealed that the bacterial populations persisted for at least 16 months under the three incubation conditions examined, suffering a viability reduction in the range of 90 - 99.9%. Long-term incubation resulted in a marked phenotypic heterogeneity, characterized by a progressive decrease of colony size (with development of small colony variants [SCVs]), loss of pigmentation, rough to smooth morphotype switch and a shift from bacilli to a coccoid-like cellular shape. The development of macroscopically visible cellular aggregates, rich in polysaccharide, was also linked to the presence of BZK. The existence of a metabolic pathway for BZK degradation was confirmed through comparative genomic analysis.

Keywords: *Burkholderia cepacia* complex, pharmaceutical products' contamination, nutrient starvation adaptation, benzalkonium chloride adaptation, small colony variants, comparative genomic analysis.

INTRODUCTION

The *Burkholderia cepacia* complex (Bcc) is a group of Gram-negative non-fermenting β-proteobacteria, which are ubiquitously distributed in the environment, presenting an extraordinary genotypic and phenotypic plasticity, which confers the ability for rapid mutation and adaptation to stressful environmental conditions (1, 2). Bcc bacteria are described as one of the major contaminants of sterile and non-sterile pharmaceutical and personal care products, being the reason behind many sterile and non-sterile product recalls (3). In addition, contamination with Bcc bacteria has caused many nosocomial outbreaks in the last two decades, posing a serious health threat to susceptible individuals, namely immunocompromised patients (HIV and cancer patients) and individuals suffering from chronic granulomatous disease (CGD) or cystic fibrosis (CF) (4), (5).

Many contamination episodes have been associated with the ability of Bcc bacteria to thrive in the presence of antimicrobials and disinfectants, particularly biocides used as preservatives in pharmaceutical products' formulations (3). Contamination of benzalkonium chloride (BZK) solutions with Bcc bacteria has been pointed out as the cause of frequent outbreaks, especially with *B. cepacia*, which has been described as the most prevalent contaminant species (6–9). Bcc bacteria have developed an array of strategies to cope with the presence of BZK, including the active extrusion from the bacterial cell through the action of efflux pumps or its inactivation by catabolic enzymes and further use as carbon source for bacterial growth (10–13). Remarkably, Bcc bacteria also possess the ability to survive and proliferate in water-based environments (water bodies, lakes, rivers, drinking water, pharmaceutical grade water) and under nutrient starvation (14).

In an epidemiological survey performed by our laboratory between 1995 and 2002, which included patients under surveillance at the major Portuguese CF center at Hospital de Santa Maria (HSM), Lisbon, an unusually high representativeness of *B. cepacia* was detected in sputum cultures (in 36% of the patients) (15). From 2002 to 2005, the prevalence of *B. cepacia* continued to increase, affecting 85% of the CF patients receiving treatment at HSM (16). Parallel to these events, a market surveillance performed by INFARMED, the National Authority of Medicines and Health Products, in 2003 and 2006, detected Bcc contamination in several batches of non-sterile saline solutions for nasal application (16). Since those types of products are often administered to CF patients during inhalant therapy, a correlation between these contamination outbreaks and the prevalence of *B. cepacia* was hypothesized. Further analysis by gas chromatography and molecular methods, confirmed that the clinical clones obtained from CF patients and the strains detected in saline solutions were epidemiologically related (16). When this outbreak occurred, all of the clones identified by our laboratory were classified as *B. cepacia*. In 2015, a re-examination of the isolates was conducted, and 20 of them were reclassified as *B. contaminans* (17). Two different Bcc species (*B. cepacia* and *B. contaminans*) were apparently involved in the outbreak, demonstrating that species

which are considered less predominant in CF respiratory infections, which is the case of *B. cepacia* and *B. contaminans*, may also be associated with poor clinical outcomes (17).

The aim of this work was to study the phenotypic alterations that Bcc bacteria undergo during long-term incubation in stressful environmental conditions, including nutrient depletion and the presence of a specific biocidal preservative. Two sets of clonal isolates of *B. cepacia* and *B. contaminans*, recovered from batches of saline solutions inspected by INFARMED in 2003 and 2006, as well as clinical isolates obtained from two CF patients under surveillance at HSM during that contamination outbreak were selected to evaluate the effects of prolonged storage under nutrient scarcity and BZK induced stress. These original isolates were used to induce artificial contamination of saline solutions supplemented with two distinct concentrations of BZK (0.0053% (w/v) and 0.05% (w/v)) and incubated at 23ºC to mimic the typical storage conditions applied for this type of pharmaceutical products.

Long-term incubation in such conditions resulted in a global adaptive response, characterized by the development of sub-populations with different phenotypic characteristics. The evolution of those traits was accompanied during the course of the experiment in regular intervals of time. Cellular viability, colony diameter, morphology and pigmentation changes were evaluated. Intrinsic resistance of the original isolates towards BZK was also assessed. Other adaptation strategies were studied, including the formation of cellular aggregates, which were characterized in terms of polysaccharide and protein contents. Additionally, the comparative genomic analysis performed for two *B. cepacia* saline solutions' isolates confirmed the presence of genes from the BZK degradation pathway, already described in another Bcc strain (10), indicating that this preservative can be used as carbon and energy source by these bacteria. Differences between isolates obtained in 2003 and 2006 from contaminated saline solutions for nasal application were also detected.

MATERIALS AND METHODS

Bacterial Isolates: In the present study, clonal variants of two Bcc strains (*B. cepacia* and *B. contaminans*), obtained from contaminated saline solutions for nasal application and from respiratory secretions of CF patients were selected **(Table 1)**. The clinical isolates were obtained from sputum samples of two chronically infected CF patients (here designated patients AL and V), under surveillance at the major Portuguese CF Center in Hospital de Santa Maria (HSM), Centro Hospitalar Lisboa Norte (CHLN) EPE. Patient AL had been infected with a *B. cepacia* strain correspondent to the *recA* restriction fragment length polymorphism (RFLP) profile D and ribopattern 19 [16], while patient V harboured a *B. contaminans* strain correspondent to a *recA* RFLP profile E and ribopattern 17 [16], [17]. Studies involving these isolates were approved by the CHLN ethics committee and the anonymity of the patients was safeguarded. Informed consent was also obtained from all participants and/or their legal guardians. All the methods were performed according to the relevant guidelines and regulations. The isolates from saline solutions examined in the present study (22 from *B. cepacia* and one from *B. contaminans*) were obtained from batches of intrinsically contaminated non-sterile saline solutions detected during a routine market surveillance performed by the Portuguese Medicines and Health Products Authority (INFARMED) in the end of 2003 and in March 2006 [16]. Bacterial cultures of each isolate are currently stored at -80ºC in glycerol at a proportion of 1:1 (v/v).

Random Amplified Polymorphic DNA (RAPD) Typing: Three 10-base RAPD primers were selected for this analysis, based on their previously reported ability to produce fingerprinting profiles from *B. cepacia* (18). Primer 270 (5' TGCGCGCGGG 3') was used for a primary typing of the isolates and primers 208 (5' ACGGCCGACC 3') and 272 (5' AGCGGGCCAA 3') were used to confirm the strain types obtained in the first assessment with primer 270. Total genomic DNA was extracted from the 22 *B. cepacia* isolates from saline solutions (described in **Table 1**), which had previously been grown overnight in liquid Luria-Bertani (LB) medium (Difco), at 37ºC and with orbital agitation at 250 rpm. The extraction was performed using the Puregene® DNA Purification Kit (Gentra Systems, Qiagen, Germany), following the manufacturer's specifications. The genomic DNA concentration and purity was assessed using a ND-1000 spectrophotometer (NanoDrop, Thermo Fisher Scientific). RAPD PCR reaction mixtures were prepared, each containing 50 ng of bacterial DNA (50 ng/µL), 1 µL of the respective primer, 2.5U of Speedy Supreme NZYTaq 2X Green Master Mix (NZYTech) and nuclease free water, up to a final volume of 25 µL. Amplification reactions were carried out using a GTC965 Thermal Cycler (Cleaver Scientific Ltd.), with an initial 4-cycle step, consisting of 5 min at 94ºC, 5 min at 36ºC and 5 min at 72ºC; 30 cycles of 1 min at 94ºC for denaturation, 1 min at 36ºC for primer annealing and 2 min at 72ºC for polymerization; one cycle of 10 min at 72ºC for final extension. RAPD PCR products were separated by electrophoresis in 1.5% agarose gels (NZYTech), previously stained with Gel Red (NZYTech), at 90V for 2h30min. Molecular size markers (1 Kb Plus DNA Ladder, Invitrogen) were included in all gels, together with 10X loading buffer (Takara Bio Inc.). Finally, the gels were observed and photographed using the GelDoc™ XR software from Bio-Rad.

Long-Term Incubation of the Bacterial Isolates in Saline Solutions and in the Presence of BZK: Isolates from *B. cepacia* (IST612, IST701, IST4152, IST4168 and IST4222) and *B. contaminans* (IST601, IST4148, IST4241 and IST4224) were selected (refer to **Table 1** for detailed information about the isolates) and used to induce artificial contamination of saline solutions, in an experiment independent from this work (19). Liquid cultures grown until mid-exponential phase in LB medium at 37ºC, 250 rpm were centrifuged for 10 minutes, at 7000 rpm and 20ºC, followed by two washing steps with NaCl 0.9% (w/v). Finally, cells were resuspended in NaCl 0.9% (w/v) and the optical density at 640 nm (OD_{640nm}) was adjusted to 0.2 prior to inoculation into glass flasks, in a final volume of 125 mL of saline solution (NaCl 0.9% (w/vol)). This condition was intended to mimic a nutrient depleted environment. To study bacterial adaptation to the presence of biocides, isolates were inoculated into glass flasks containing NaCl 0.9% (w/vol) supplemented with BZK at two different concentrations: 0.0053% (w/v) and 0.05% (w/v), previously filtered using 0.2 µm pore size filters (Whatman). To simulate the typical storage conditions employed in the pharmaceutical industry for this type of products, the flasks were incubated at 23ºC, in the dark, without agitation, during the entire course of the experiment.

Viability and Morphology Studies: Bacterial populations corresponding to the isolates highlighted in **Table 1** were analysed in terms of cellular viability, using traditional cultivation methods and confocal microscopy.

(i) Traditional cultivation methods: Bacterial samples were harvested from the glass flasks, serially diluted and plated on Tryptic Soy Agar medium (TSA; BD Bacto™ Tryptic Soy Broth [Soybean-Casein Digest Medium], Difco). Samples recovered from saline solutions supplemented with BZK were filtered with 0.2 µm pore size filters (Whatman™, GE Healthcare Life Sciences). The filter disks were placed on TSA plates, incubated for 72h at 30ºC, followed by determination of colony forming units (CFUs).

Table 1 - List of clonal isolates from *B. cepacia* and *B. contaminans* used in this study. The isolates selected to study the effects of long-term incubation in saline solutions supplemented with BZK are indicated in bold and underlined.

Isolate Source	*Species*	*Isolate ID*	*Isolation Date*	*recA RFLP profile*	*Ribopattern*	*Reference*
Patient AL	*B. cepacia*	**IST4152**	October 2003	D	19	Cunha *et al.* (2007) (16, 17)
		IST4168	May 2004	D	19	
		IST4222	December 2005	D	19	
Patient V	*B. contaminans*	**IST4148**	September 2003	E	17	
		IST4241	June 2004	E	17	
		IST4224	December 2005	E	17	
Saline Solutions	*B. contaminans*	**IST601**	2003	-	-	
	B. cepacia	**IST612**	2003	-	-	
		IST621	2003	-	-	
		IST623	2003	-	-	
		IST624	2003	-	-	
		IST625	2003	-	-	
		IST626	2003	-	-	
		IST627	2003	-	-	
		IST628	2003	-	-	
		IST629	2003	-	-	
		IST701	March 2006	-	-	
		IST702	March 2006	-	-	
		IST704	March 2006	-	-	
		IST705	March 2006	-	-	
		IST706	March 2006	-	-	
		IST707	March 2006	-	-	
		IST708	March 2006	-	-	
		IST710	March 2006	-	-	
		IST711	March 2006	-	-	
		IST712	March 2006	-	-	
		IST713	March 2006	-	-	
		IST714	March 2006	-	-	
		IST715	March 2006	-	-	

(ii) Confocal microscopy: A volume of 200 µL of each bacterial suspension was added onto an 8 wells µ-Slide (Ibidi), followed by 0.3 µL of SYTO™ 9 green-fluorescent probe (Life Technologies, ThermoFisher Scientific) and 1 µL of TO-PRO™-3 Iodide red-fluorescent probe (Life Technologies, ThermoFisher Scientific). Sample preparations were incubated at room temperature, in the dark, for 20 minutes and were then visualized with a TCS SP5 Confocal Microscope (Leica), using the water immersion objective, with 63x magnification. Images were captured using the Leica Application Suite, Advanced Fluorescence software.

(iii) Morphology analysis: The global aspect of the colonies obtained after 72h of incubation in solid media was observed with a KL 2500 LCD cold light source (ZEISS) coupled to a microscope camera (ZEISS Axicam 503 color), using the ZEN 2.3 (ZEISS) software. Pictures were taken using the same devices. Around 100 representative colonies of each bacterial isolate were then selected for diameter measurement, which was performed using the ZEN 2 lite (ZEISS) software.

Susceptibility of *B. Cepacia* Isolates to BZK - Minimum Inhibitory Concentration (Mic) Determination: A modified broth minimum inhibitory concentration (MIC) assay was performed using 96-wells microplates (Greiner Bio-One, Germany). A 40 mg/mL BZK stock solution was prepared in double distilled water (ddH_2O), sterilized by filtration with 0.2 µm pore size filters (Whatman) and diluted in sterile ddH_2O to obtain working solutions with concentrations of 16, 32, 64, 128, 192, 256, 384 and 512 µg/mL. Bacterial cultures grown in liquid LB medium until reaching mid-exponential phase were centrifuged for 10 min, at 7000 rpm, 17ºC. The supernatants were discarded, and the pellets were resuspended in Mueller-Hinton broth (MH, Fluka Analytical). Cellular concentrations were adjusted to an OD_{640nm} of 0.011, and 190 µL of each cell culture were added to the 96-well microplates, containing 10 µL of each BZK solution. Duplicate control wells, with either 200 µL of cell culture (positive control) or 200 µL of sterile MH broth (negative control, not inoculated) were also included. The 96-well microplates were incubated in the dark, at 23ºC, for 72h. After incubation, OD_{640nm} was assessed using a microplate spectrophotometer (SPECTROstar^{NANO} - BMG LABTECH; software version 2.10). The lowest BZK concentration leading to bacterial growth inhibition was defined as the MIC value. This test was performed in duplicate, for three biologically independent experiments.

Characterization of the Cellular Aggregates' Matrix Composition: The sugar content of the aggregate/biofilm-like structures formed during long-term incubation of *B. cepacia* and *B. contaminans* isolates in the presence of BZK was assessed using the phenol-sulfuric acid method (20). For that purpose, 1 mL samples were collected from each flask and centrifuged for 5 minutes at 10000 rpm, 4ºC. The supernatant was discarded, while the pellet was resuspended in 1 mL of H_2O. The resulting suspension was mixed with 1.5 mL of phenol 0.05% (w/v) and 8 mL of H_2SO_4 96%. After cooling, the OD_{490nm} was measured. The relative sugar content was determined using a previously created glucose standard curve (21). For protein quantification, the Biuret method was applied (22). Samples of 1 mL were collected from the flasks and centrifuged for 10 minutes at 4ºC, 15000 rpm. The supernatant was discarded, and the pellet resuspended in 1 mL of dH_2O. The resultant cell suspension was mixed with 1 mL of dH_2O and 1 mL of NaOH (1mM), heated at 100ºC for 5 min and let to cool at room temperature. Then, 1 mL of 0.025% $CuSO_4.5H_2O$ (w/v) was added, followed by incubation at room temperature for 5 minutes. After centrifugation at 5600g, 20ºC, for 5 min, OD_{550nm} was measured. To estimate the protein content, a standard curve of bovine serum albumin (BSA) was prepared.

Comparative Genomic Analysis:

(i) *De novo* Assembly of *B. cepacia* Isolates: A comparative genomic analysis pipeline was proposed, starting with *de novo* assembly of the genome sequences belonging to the 22 *B. cepacia* isolates recovered from saline solutions, available in our laboratory collection **(Table 1)**, derived from Illumina sequencing and kindly provided by Dr. Vaughn Cooper's group, from University of Pittsburgh. An initial quality control was performed using FastQC (Babraham Bioinformatics). Afterwards,

sequence clean-up for Illumina adapter removal was conducted, using Trimmomatic. The assembly was performed using SPAdes (CAB – Center for Algorithmic Biotechnology) and VelvetOptimiser (Victorian Bioinformatics Consortium) and improved with Pilon (BROAD Institute). A scaffolding step was conducted using SSPACE (BaseClear), followed by its improvement with Pilon. Quality control by QUAST (Algorithmic Biology Lab – St. Petersburg Academic University of the Russian Academy of Sciences) was performed after each assembly and scaffolding improvement step. Finally, Mauve (The Darling Lab) was used for ordering the contigs within the draft genomes.

(ii) *In silico* Multilocus Sequence Typing (MLST) Analysis: The clonal identity of the 22 *B. cepacia* isolates obtained from saline solutions was re-examined through an *in silico* MLST analysis, using the best assembly outputs (whether from SPAdes or VelvetOptimiser). The sequence data corresponding to each bacterial isolate was compared against other known sequences deposited in the *Burkholderia cepacia* complex MLST database (http://pubmlst.org/bcc). The different housekeeping gene sequences existent within each bacterial species were assigned with a different allele and, for each isolate, the combination of alleles attributed to each of the seven loci defined its allelic profile.

(iii) Phylogenetic Tree Construction: The genome sequences resultant from the assembly were aligned by multiple sequence alignment using Mauve (The Darling Lab), and a phylogenetic tree of the 22 *B. cepacia* saline solutions isolates (described in **Table 1**) was constructed, using the Figtree phylogenetic tree builder, with the *B. cepacia* isolate IST612 sequenced by PacBio technology, as the reference genome.

(iv) BLAST ® (Basic Local Alignment Search Tool): The genome sequences of the two *B. cepacia* saline solutions isolates selected for the phenotypic studies (IST612 and IST701), were compared (by BLAST ®) against the sequences of genes reported in the literature as being involved in nutrient starvation tolerance (in *Pseudomonas* PAO1) and in BZK resistance and catabolism (in *B. cenocepacia* AU1054) (10, 23). A total of 178 gene sequences to be screened against the *B. cepacia* isolates' genomes were selected: 15 that encode enzymes reported to be involved in BZK degradation, 117 which encode transporters related to BZK resistance and 46 genes involved in survival under nutrient scarcity.

(v) Comparative Analysis of Genes from the BZK Degradation Pathway Using the Artemis Comparison Tool (ACT): The sequences of the 15 genes involved in BZK biodegradation, described in the literature for *B. cenocepacia* AU1054 (10), were compared against the genome sequences of two selected *B. cepacia* isolates (IST612 and IST701), using the Artemis Comparison Tool (ACT) (24). Information pertaining to the putative gene location (in bp), score and identity percentages, genome inversions and gene order (synteny) was extracted for analysis.

RESULTS

Molecular Typing of the *B. cepacia* Isolates Examined in This Study and Obtained from Saline Solutions. RAPD fingerprinting analysis indicated that the 22 *B. cepacia* isolates obtained from saline solutions **(Table 1)** were clonal variants of the same *B. cepacia* strain. Isolates recovered in 2003 and 2006 presented a very similar RAPD fingerprint **(Figure 1)**, indicating that the *B. cepacia* strain responsible for the contamination event in 2003 was probably the same that caused contamination of the saline solutions inspected by INFARMED three years later. The results of the *in silico* MLST analysis also confirmed the clonal nature of the *B. cepacia* isolates obtained from saline solutions, with exception of IST708, whose allelic profile was searched in the *Burkholderia cepacia* complex MLST database (25), and identified as *Burkholderia fungorum*, prompting its exclusion from further analysis.

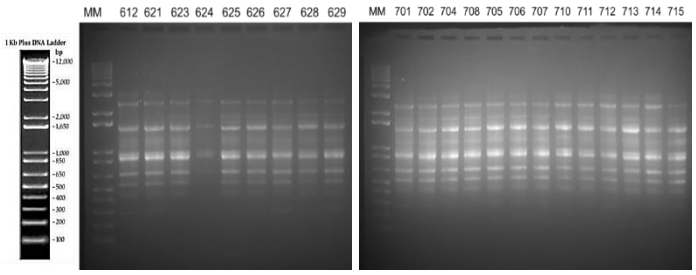


Figure 1. RAPD fingerprint profiles of the *B. cepacia* isolates obtained from contaminated saline solutions in 2003 (left panels) and 2006 (right panels), amplified using the RAPD primer 270. Molecular size markers (MM) were run on the first lane of each gel and their size (in base pairs) is indicated on the leftmost side of the image.

Phylogenetic Analysis of the *B. cepacia* Isolates Obtained from Intrinsically Contaminated Saline Solutions. Phylogenetic analysis of the *B. cepacia* isolates obtained from saline solutions revealed the existence of two main clades, one that included 13 genetically indistinguishable isolates, closely related to the reference genome (clade B), and another clade (A) composed by isolates which are distant from the reference genome and highly heterogeneous, giving rise to two sub-groups, each containing isolates that are essentially different **(Figure 2)**. Given the results, it was not possible to distinguish the group of isolates obtained from contaminated saline solutions in 2003 from those obtained in 2006.

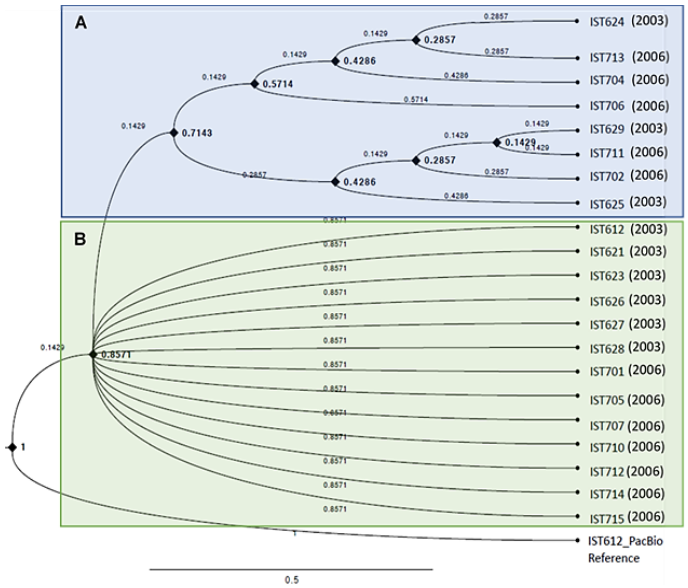


Figure 2 - Phylogenetic tree representing the evolutionary relationships between the *B. cepacia* isolates obtained from saline solutions, examined in this study. Isolation dates are indicated, in brackets, next to each isolate's ID. The numbers next to the tree nodes and above each branch represent node and branch age, respectively. This phylogenetic tree was constructed using the Figtree v1.4.3 software.

Long-Term Survival of *B. cepacia* and *B. contaminans* Cellular Populations Under Nutrient Starvation and BZK Induced Stress – Analysis of Cellular Viability. To study the effects of prolonged exposure to nutrient starvation and to the presence of distinct BZK concentrations (0.0053% (w/v) and 0.05% (w/v)), five *B. cepacia* isolates (IST612, IST701, IST4152, IST4168 and IST4222) and four *B. contaminans* isolates (IST601, IST4148, IST4241 and IST4224) from our laboratory collection were selected **(Table 1)**. This long-term incubation experiment was initiated by a former MSc student, who analysed these bacterial populations during the first 10 months of incubation (19). Despite the apparent decrease in cellular viability, both *B. cepacia* and *B. contaminans* cell

populations survived for at least 16 months, under the stress conditions tested. The decrease of cellular viability was dose-dependent, being more pronounced for 0.05% BZK in comparison with 0.0053% (Figure 3A; B). In the absence of BZK, cellular viability declined after an initial phase where relatively stable CFU counts were registered, whereas incubation with 0.0053% and 0.05% BZK led, respectively, to a steady decrease of cellular viability throughout the incubation period, and to an abrupt decrease in the CFU counts during the first month of incubation, followed by a stabilization period, presumably due to the development of a persistent bacterial population (Figure 3A; B). Since cellular viability was assessed in terms of CFUs/mL, the decline registered for all the studied conditions is not necessarily related with loss of cellular viability and might be due to intrinsic limitations of cultivation methods.

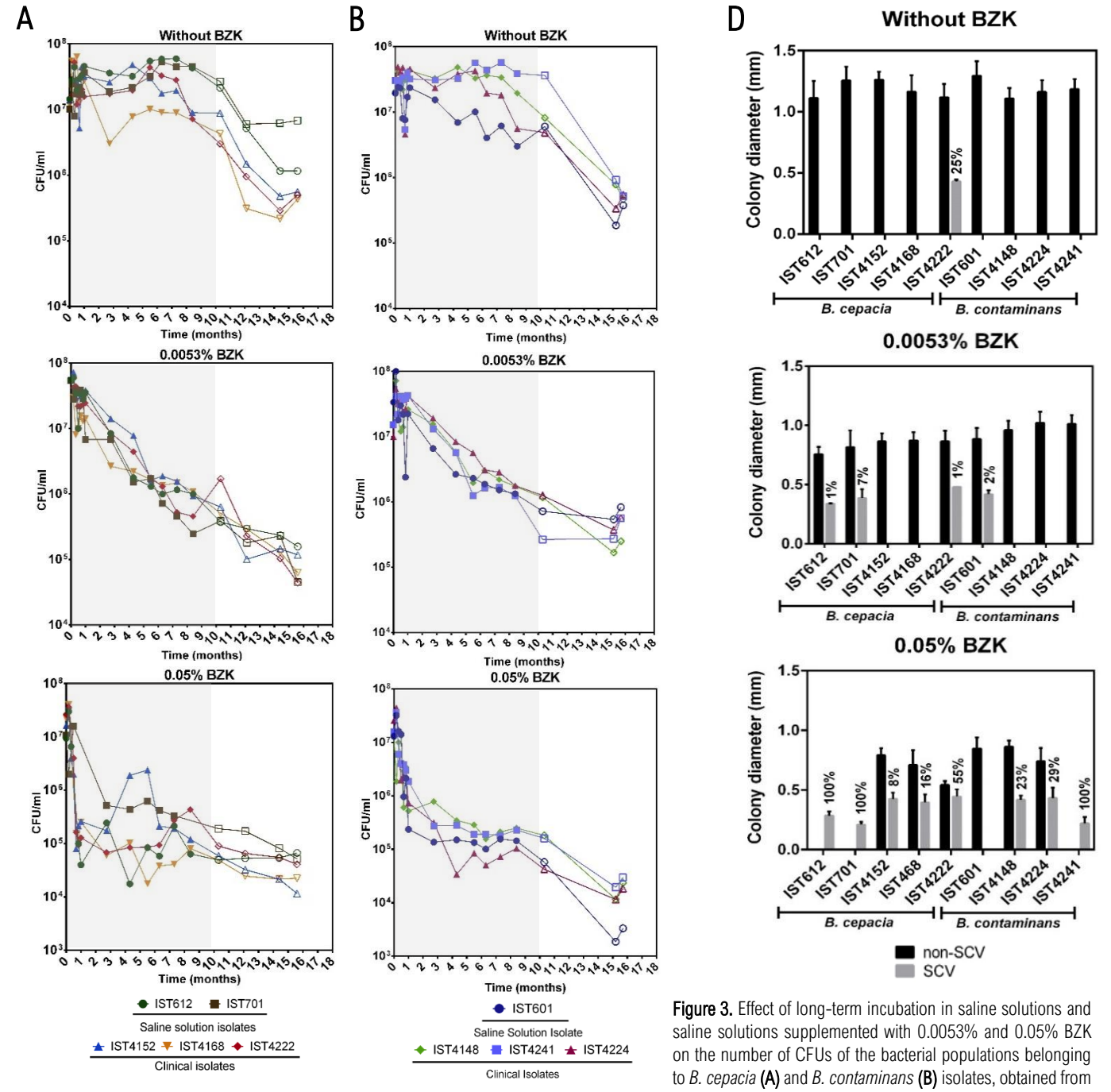
Reduction of colony size and morphology alterations after 16 months of incubation in nutrient deprived conditions and under BZK induced stress. Long-term incubation in different conditions resulted in the development of phenotypically heterogeneous bacterial populations, characterized by a diversification of colony morphotypes and a clear decrease in terms of colony diameter. After 16 months of incubation in saline solutions without BZK, both *B. cepacia* and *B. contaminans* cellular populations displayed pigmented colonies with an essentially rough morphology. In saline solutions supplemented with 0.0053% BZK, rough colonies with yellow pigmentation, as well as smooth and less pigmented colonies were observed. Incubation with 0.05% BZK resulted in the development of a smooth phenotype, with generally unpigmented colonies (Figure 3C). Long-term incubation in a nutrient depleted environment and the additional stress imposed by BZK resulted in the development of a sub-population of smaller sized colonies (with diameters inferior to 0.5 mm), here termed small colony variants (SCVs). The mean colony diameters were inversely proportional to the BZK concentration and, consequently, the percentage of SCVs increased for higher biocide concentrations. While SCVs were rarely observed in the absence of BZK and very low percentages were registered in the presence of 0.0053%, for the highest BZK concentration (0.05%) all of the isolates except IST601 exhibited SCVs, which in some cases represented 100% of the population (Figure 3D).

Susceptibility of the *B. cepacia* Isolates to BZK – MIC Assays. Original isolates IST612 and IST701, recovered from contaminated saline solutions in 2003 and 2006, respectively, and the first isolate recovered from patient AL, IST4152, were selected to evaluate the level of intrinsic resistance to BZK. From the three isolates in study, IST612 appears to be the most resistant (MIC value between 256/384 µg/mL), followed by the clinical isolate IST4152, with a susceptible concentration for BZK between 192/256 µg/mL. IST701 seems to be the most susceptible of the group (MIC value of 192 µg/mL). These results are apparently not consistent with what was observed in the viability assay, where IST701 attains the highest CFU counts during long-term exposure to 0.05% BZK, indicating that it is more tolerant to BZK. Nonetheless, the MIC results concern only to short-term exposure to the chemical (3 days), while the viability assay evaluates the effects of long-term exposure, which might explain the verified discrepancies. Moreover, in this experiment, cells are grown in nutrient rich medium, which eliminates the problem of nutrient scarcity and lack of energy sources, verified in the viability assay.

Formation of Cellular Aggregates by Bcc Isolates in Response to Long-Term Incubation in Nutrient Limited Conditions and BZK Induced Stress. Throughout the incubation period, alterations in the general aspect of the bacterial suspensions inoculated in the glass flasks were also detected. While the flasks containing only saline solution or saline solution supplemented with 0.0053% BZK remained clear or presented just slight turbidity after homogenization, respectively, it was possible to observe the formation of a white material that deposited on the bottom of the flasks containing 0.05% BZK. In general, the *B. cepacia* isolates incubated in 0.05% BZK exhibited a homogeneously turbid appearance, while the *B. contaminans* isolates formed visible floccular structures.

(i) Observation of cellular aggregates by confocal microscopy. Confocal microscopy observation of cellular suspensions belonging to saline solutions isolates IST612 (*B. cepacia*) and IST601 (*B. contaminans*), after 16 months of incubation in the three stress conditions was performed. Cellular viability was assessed by co-staining of bacterial samples with SYTO™ 9 green-fluorescent probe and TO-PRO™-3 Iodide red-fluorescent probe. Incubation in saline solutions without BZK led to the development of a dispersed cellular population, essentially composed of dead single cells, in the case of *B. contaminans* isolate IST601, and live single cells for the *B. cepacia* isolate IST612 (Figure 4A). *B. contaminans* bacterial populations, incubated in the presence of 0.0053% BZK, were generally composed by cellular aggregates of intermediate size, with mostly dead or membrane compromised cells. The *B. cepacia* cellular population, incubated in the same conditions, did not display any aggregates of considerable size, but the majority of the cells were also dead/membrane compromised (Figure 4A). For the highest BZK concentration (0.05%), the bacterial populations showed a clear tendency to form denser and bigger aggregate structures, composed by live, dead and membrane compromised cells (Figure 4A). The observation of a significant number of live cells within the biofilm structure contrasts with the lower CFU counts registered for isolates incubated in the presence of 0.05% BZK, confirming that aggregate formation might lead to an underestimation of the actual viability by traditional cultivation methods. Interestingly, after 16 months of incubation in stressful conditions, cells from both species did not display the typical Gram-negative bacilli shape, but a morphology closer to coccus/coccobacilli.

(ii) Characterization of the cellular aggregates produced by *B. cepacia* and *B. contaminans* bacterial populations. To assess the composition of the aggregates/biofilm-like structures produced by the Bcc isolates in study, the ratio between polysaccharide and protein concentration was determined after initial inoculation ("day zero") and after 1 and 18 months of incubation in the three stress conditions analysed. The results indicate that the composition of the cellular aggregate's matrix changes over time, becoming richer in polysaccharides, especially for higher BZK concentrations. (Figure 4B). These observations suggest that cellular populations adapt to the presence of higher BZK concentrations by producing aggregates rich in polysaccharides, which is also corroborated by the macroscopic observation of these structures.



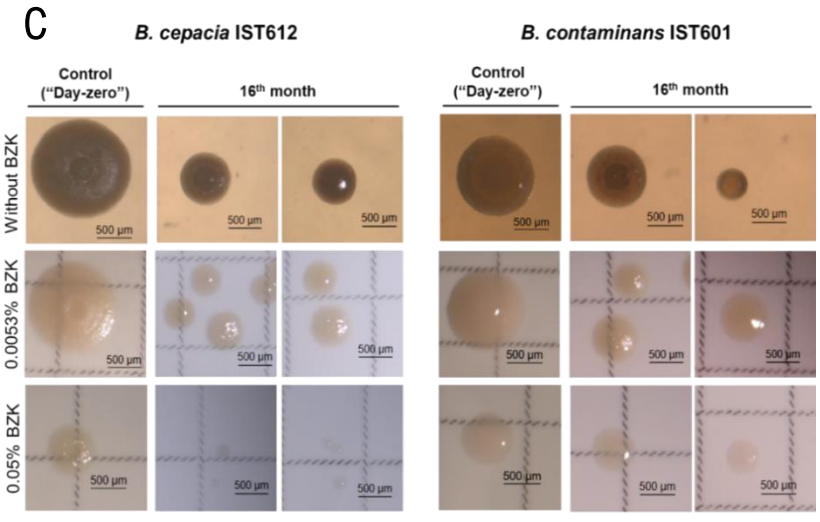


Figure 3. Effect of long-term incubation in saline solutions and saline solutions supplemented with 0.0053% and 0.05% BZK on the number of CFUs of the bacterial populations belonging to *B. cepacia* **(A)** and *B. contaminans* **(B)** isolates, obtained from contaminated batches of saline solutions and from the sputum of two CF patients. Cell viability was measured in terms of CFU's/mL, obtained through colony counts from three separate plates, correspondent to a range of three different serial dilutions. The datapoints correspondent to an incubation period of 10 months were obtained during a previous MSc project (indicated by a grey shade) (19). **(C)** Different colony morphotypes exhibited by the *B. cepacia* IST612 and *B. contaminans* IST601 cellular populations incubated in saline solutions without BZK and in saline solutions supplemented with 0.0053% and 0.05% BZK, after 16 months of incubation. Colony morphologies were compared with those obtained for the same bacterial isolates at initial inoculation ("day-zero") in the same conditions. It is possible to observe differences in terms of colony size, morphology and pigmentation. **(D)** Comparison of the mean diameters of a group of ~100 colonies, representative of each *B. cepacia* and *B. contaminans* cellular populations (■ non-SCV ■ SCV), incubated for 16 months in saline solutions without BZK and saline solutions supplemented with 0.0053% and 0.05% BZK. Colonies with an average diameter inferior to 0.5 mm were termed SCVs and their respective percentages are indicated above the grey bars.

Genomic Analysis of the *B. cepacia* Saline Solutions' Isolates IST612 and IST701: Focus on BZK and Nutrient Starvation Tolerance Genes.

(i) Identification of genes related with tolerance to nutrient starvation and BZK catabolism in the genomes of *B. cepacia* IST612 and IST701. *B. cenocepacia* AU1054 is the best studied Bcc strain in terms of BZK resistance, for which several mechanisms have been described, including active extrusion of the biocide through efflux pumps and its biodegradation by catabolic enzymes (10). Considering tolerance to nutrient scarcity, Bcc bacteria have not yet been extensively studied and *P. aeruginosa* PAO1 is the closest bacterial strain for which these mechanisms have been described (23). To identify the presence of genes that could explain the apparent inherent resistance of the original *B. cepacia* isolates IST612 and IST701 to both nutrient starvation and the presence of BZK, their genomes were compared against 178 gene sequences reported in the literature, using the BLASTN tool. These included 117 genes encoding transporters of various categories and 15 genes encoding BZK degradation enzymes, all from *B. cenocepacia* AU1054, as well as 46 genes described for *P. aeruginosa* PAO1 as being involved in the process of survival under nutrient starvation. The BLASTN analysis indicated that 127 of the *B. cenocepacia* AU1054 genes, including all of those encoding BZK degradative enzymes, 112 transporter-encoding genes and 7 of the 46 genes related to nutrient starvation had corresponding homologues in the *B. cepacia* IST612 and IST701 genomes. It was possible to validate the existence of a degradation pathway for BZK and that it might be used as a carbon and energy source.

(ii) Identification of BZK catabolism genes in *B. cepacia* IST612 and IST701. The 15 genes encoding BZK degradation enzymes, reported for *B. cenocepacia* AU1054, were compared against the

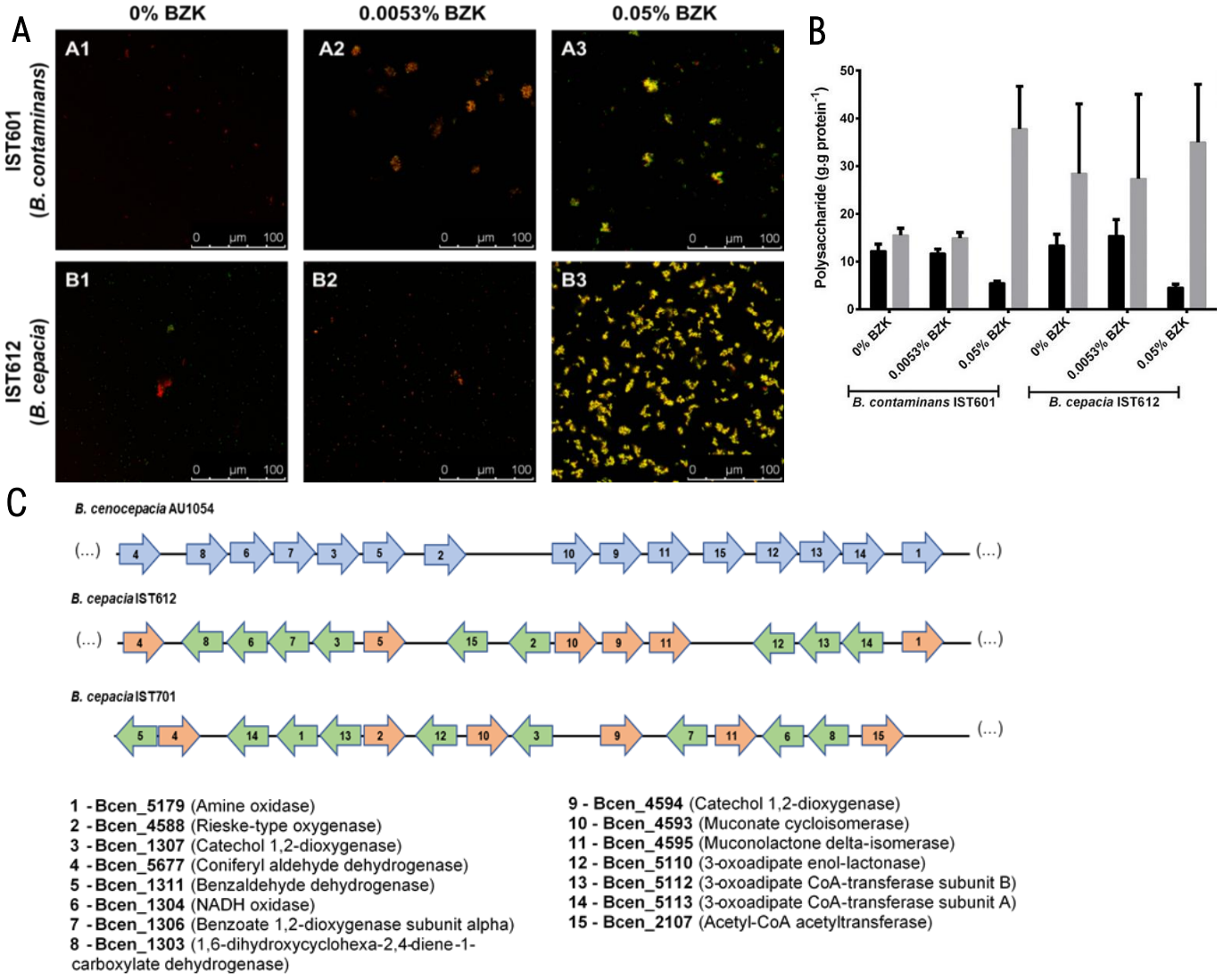


Figure 4. (A) Microscopic observation of the structures formed by *B. cepacia* IST612 and *B. contaminans* IST601, after 16 months of exposure to saline solutions supplemented with increasing concentrations of BZK. (A1; B1) Control, with only saline solution (NaCl 0.9% (w/v)); (A2; B2) Cells exposed to 0.0053% (w/v) of BZK; (A3; B3) Cells exposed to 0.05% (w/v) of BZK. Cellular viability was assessed through co-staining of cells with SYTO 9 (green) and TO-PRO-3 iodide (red). Images were acquired using a confocal microscope (the same settings were applied for each image) and are equally scaled to allow direct comparison. **(B)** Ratio of polysaccharides/proteins present within the aggregate structures produced by *B. cepacia* IST612 and the *B. contaminans* IST601, assessed by the phenol-sulphuric acid method and expressed in grams of total sugars per grams of protein (g.g protein-1), after 1 and 18 months of incubation in saline solutions and saline solutions supplemented with 0.0053% or 0.05% BZK. The results are means of two independent experiments with three replicates each. The ratios of polysaccharide/protein at initial inoculation ("Time = 0") are not represented, due to the lack of polysaccharides registered at that time. **(C)** Schematic diagram of the genomic organization of the 15 homologues of the *B. cenocepacia* AU1054 BZK catabolism genes, identified within the genomes of *B. cepacia* IST612 and IST701. *B. cenocepacia* AU1054 was used as a reference genome. Genes are represented by labelled arrows and their respective locus-tag and products are listed below the diagram. Blue arrows – reference genes; Green arrows – genes inverted with respect to the reference genome; Orange arrows – genes that are in the same orientation with respect to the reference genome. The real distances between the genes were modified to assist visual interpretation. Image not to scale.

genome sequences of IST612 and IST701, using the ACT Artemins software (24). In terms of genomic organization, *B. cepacia* IST612 (isolated in 2003) is the most similar to the reference genome, sharing the same pattern of gene distribution (synteny) for 13 of the 15 genes analysed **(Figure 4C)**. Nevertheless, more than half of the genes were inverted with respect to the reference strain, indicating the occurrence of genomic rearrangements. IST701, originally isolated in 2006, displayed major differences in comparison to the reference genome and to IST612, with several genomic inversions and very low synteny **(Figure 4C)**. These observations suggest that, in this particular region, the isolates recovered in 2006 have evolved differently in comparison with those recovered in 2003, despite being phylogenetically related. The differences registered at the genome level, might explain why the *B. cepacia* isolate IST701 (isolated in 2006) appears to be more tolerant to the presence of BZK, in comparison to IST612 (isolated in 2003), which registered a faster and more significant viability loss.

DISCUSSION

The present work provides insights into the adaptation mechanisms developed by different clonal isolates of *B. cepacia* and *B. contaminans*, obtained from batches of contaminated saline solutions for nasal application, detected during a market surveillance performed by INFARMED in 2003 and in March 2006, or from the sputum of two CF patients under surveillance at Hospital de Santa Maria (HSM), when subjected to long-term incubation in saline solutions and in saline solutions supplemented with different concentrations of benzalkonium chloride (BZK), mimicking the effects of nutrient starvation and the presence of this preservative in pharmaceutical products' formulations.

RAPD analysis revealed that the 22 *B. cepacia* isolates obtained from intrinsically contaminated saline solutions in 2003 and 2006 were clonal variants of the same *B. cepacia* strain. However, by *in silico* MLST analysis, isolate IST708 presented an allelic profile corresponding to *B. fungorum*, which is not a member of the Bcc. The discrepancies registered between the two methods might mean that RAPD does not provide enough discriminatory power to detect certain differences among the bacterial isolates, or that the original sample collected from saline solutions could have contained a mixed population of *B. cepacia* and *B. fungorum*. Nevertheless, the fact that clonal variants of the isolates recovered in 2003 were detected again in 2006 indicates that any preventive measures eventually applied by the manufacturers to control *B. cepacia*-related contamination were not effective.

Phylogenetic analysis of the same 22 *B. cepacia* isolates, based on their genome sequences, identified two main groups (clades): one was homogeneous, composed by isolates that were phylogenetically indistinguishable, while the other clade was considerably heterogeneous and phylogenetically distant from the reference genome. The identities of the manufacturers that produced contaminated saline solutions are not known and cannot be disclosed due to confidentiality constrains. However, it is known that the products in question were produced by two different manufacturers – manufacturer A, which produced only one brand of contaminated saline solutions (K), detected in 2003, and manufacturer B, which produced 3 brands of contaminated saline solutions (brand X, where *B. cepacia* was detected in 2003 and 2006, as well as brands Y and Z, identified to be contaminated in 2006) (16). Given that, isolates from clade B, which were phylogenetically very close, probably correspond to the same manufacturer and brand (B[X], since it is the only one that contains isolates recovered in both years). Due to its heterogeneity, clade A most probably contains isolates from both manufacturers (A and B) and different brands within the same manufacturer.

Upon incubation in saline solutions without BZK (mimicking a nutrient depleted environment), the bacterial populations of both *B. cepacia* and *B. contaminans* remained viable, with stable colony forming units (CFU) counts for ~10 months. However, in the last six months of incubation, there was a tendency towards a faster decline in the bacterial populations' cellular viability, reaching even higher specific death rates in comparison with cells incubated in the presence of 0.0053% BZK, indicating that after long-term incubation in the absence of nutrients, part of the cell population entered a death phase, while cells incubated with the lower BZK concentration have apparently adapted to the incubation conditions. Upon incubation in saline solutions supplemented with 0.0053% BZK, the initial phase observed in the absence of the biocide was replaced by a continuous decline of cellular viability, characterized by steady death rates throughout the entire incubation period. Long-term incubation in saline solutions supplemented with 0.05% BZK had more pronounced effects in terms of viability loss, evidenced by the lack of an initial stable phase, as already observed for 0.0053% BZK, and by the occurrence of an exponential death period (with a duration of approximately one month), when cells were suddenly exposed to saline solutions containing this high biocide concentration. During the rest of the incubation period, a stabilization of the bacterial populations incubated with 0.05% BZK was verified, reaching very low specific death rates, which might coincide with the development of bacterial persistence. In fact, this kind of pattern corresponds to a biphasic killing curve, characteristic of persister cells, where initially the majority of the cells die in an exponential manner, followed by a period of slower killing rate (26–28).

Despite the significant viability decrease registered in all situations, viable cells persisted and were still observed for the bacterial populations of both saline solutions and clinical isolates, during the course of the experiment, upon plating on TSA solid medium. The bacterial populations incubated in saline solutions without BZK suffered a decline of viable population of around 90%, but the highest decrease was verified in the presence of BZK (~99.9% for both of the concentrations tested). However, for 0.05% BZK, this decrease occurred within the first month of incubation, after which the percentage of viable population remained broadly unchanged (~0.1%). Contrastingly, the viability loss verified for the bacterial populations incubated with 0.0053% BZK was continuous throughout the incubation period and, even after 16 months, the cellular populations had not yet stabilized.

These results suggest that even in the absence of any external carbon and energy sources, the *B. cepacia* and *B. contaminans* bacterial populations were able to survive, implying that, at some point, a strategy for nutrient obtention must have been developed. This could be achieved through a "sacrifice-for-survival" mechanism, in which intracellular components released by dead

cells might be used to maintain a smaller viable population. Despite of BZK's deleterious effects, after the bacterial populations are adapted to its presence, the biocide might also be used as carbon and energy source to maintain cellular viability. Early studies have shown that a *B. cepacia* strain incubated in ammonium acetate solutions supplemented with 0.05% BZK was able to use both compounds as substrates for growth, allowing bacterial survival for 14 years in such conditions (7).

Traditional MIC assays revealed that the *B. cepacia* isolates examined were intrinsically resistant to high BZK concentrations, with susceptible concentrations ranging from 192 to 384 µg/mL. However, viable cells could be recovered from flasks containing 0.05% BZK, even after 16 months of incubation, indicating that the bacterial populations became even more resistant after exposure to the biocide, suggesting that this higher concentration would also be ineffective in preventing contamination with Bcc microorganisms. In Bcc bacteria, the mechanisms of BZK tolerance may involve its extrusion from the cell by chromosomally encoded efflux pumps and the production of metabolic enzymes capable of degrading the biocide (10). It was also reported that Bcc bacteria have the ability to generate acetyl-CoA from BZK, which can be used in the central carbon metabolism, helping cells to remain viable, besides being an effective way of degrading BZK without accumulation of intermediates or toxic metabolites (10).

A gradual decrease in colony size during long-term incubation in saline solutions was observed. This pattern was observed to be further enhanced in the presence of BZK, especially for higher concentrations, suggesting that it might exert a selective pressure towards the formation of smaller colonies, in a dose dependent manner. Moreover, long-term incubation resulted in the alteration of cell shape, from typical Gram-negative bacilli to a coccoid/coccobacilli-like shape. One of the most commonly observed manifestations of colony size reduction was the development of small colony variants (SCVs), which are slow-growing bacterial subpopulations characterized by being one-tenth the size of the wild-type bacteria (29, 30). This particular phenotype was observed to arise in all of the conditions tested, but much more significantly for higher BZK concentrations (0.05%). SCVs usually have deficiencies in the electron transport chain, which translate into lower ATP production and reduced metabolic state (29, 30). Since ATP is essential for cell wall and membrane biosynthesis, as well as for pigment production, the "dormancy" state that characterizes SCVs might explain their reduced size and general lack of pigmentation (29, 30). The capacity of conversion into a state of low metabolic activity (dormancy) has been reported to contribute for bacterial persistence and tolerance to the presence of antimicrobial agents (26, 31). Alteration of cellular shape has also been reported to occur in response to nutrient starvation, in *P. aeruginosa*, *P. putida* and *B. pseudomallei* (23, 32, 33). The selection of smaller colonies induced by the presence of BZK might constitute an adaptive strategy for energy conservation, contributing to bacterial survival under nutrient depleted conditions or in the presence of BZK.

Long-term incubation in different stress conditions resulted in the appearance of distinct colony morphologies. Cells exposed only to nutrient starvation presented an overall rough morphology, with essentially nonmucoid colonies. In the presence of BZK, cells transitioned to a smooth and mucoid phenotype, suggesting that BZK might induce the switch from nonmucoid to mucoid morphotype. For Bcc bacteria, the transition from a mucoid to a nonmucoid phenotype is more common in the context of CF infections, where the nonmucoid phenotype is associated with increased virulence, leading to a faster lung function decline (34). Contrastingly, the mucoid phenotype is associated with persistence and decreased virulence, which might be more favourable in stressful environmental conditions, such as nutrient starvation and/or the presence of BZK.

One of the most striking observations was the formation of macroscopically visible cellular aggregates/biofilm-like structures, in the presence of 0.05% BZK. Microscopic observation of bacterial samples also confirmed the development of cellular aggregates, suggesting that BZK might have induced this trait. Characterization of the aggregates' matrix revealed that these structures were mainly composed by polysaccharides. Moreover, the polysaccharide content significantly increased during long-term incubation, a tendency that was accompanied by the development of denser cellular aggregates, especially for higher biocide concentrations. This biomass production suggests that BZK might be catabolized within the cells and further used as carbon and energy source, possibly even providing carbon backbones for polysaccharide production. Considering the results of the viability assessment and the fact that the protein concentration remained broadly unchanged, cell growth (division) is likely not occurring. The increase in the protein concentration after one month of incubation with 0.05% BZK is probably not a result of cell division, since the majority of the cellular population had already died at that stage. Proteins might have leaked out from the cells, becoming trapped within the aggregates' matrix. During long-term incubation, the protein existent within the matrix was probably degraded, explaining the decrease of protein concentration from the first to the 18th month of incubation. Since polysaccharide and biofilm production implies a significant carbon and energy expense, the lower concentration displayed by the bacterial populations incubated in the absence of BZK is consistent with the carbon and energy starvation expected in a nutrient depleted environment. Overall, polysaccharide production might constitute a long-term adaptation trait, allowing the consolidation of the aggregates' structure, and increasing even more the chances of bacterial survival in the presence of BZK.

The presence of genes involved in the BZK degradation pathway, as well as genes encoding efflux pumps, described in the literature for *B. cenocepacia* AU1054 (10), was also verified for the *B. cepacia* isolates IST612 and IST701, confirming that BZK can be catabolized by the *B. cepacia* strains examined. Considering the putative BZK catabolism genes, isolate IST612 presented a high degree of synteny with respect to the reference genome (*B. cenocepacia* AU1054), while isolate IST701 (isolated in 2006) differed significantly from both the reference genome and IST612 (isolated in 2003), suggesting the occurrence of evolutionary events between the two isolation dates, which might explain why the bacterial population corresponding to isolate IST701 appears to have a higher tolerance to the presence of BZK.

The present study constitutes further evidence for the role of Bcc bacteria as emergent opportunistic pathogens and the implications

of Bcc related contaminations in the pharmaceutical industry, reinforcing the urgent need to re-evaluate the application of biocides in pharmaceutical products' formulations and the concentrations in which they are used, in order to prevent contamination and subsequent infection outbreaks.

ACKNOWLEDGEMENT

This document was written and made publicly available as an institutional academic requirement and as a part of the evaluation of the MSc thesis in Biotechnology of the author at Instituto Superior Técnico. The work described herein was performed at the Institute for Bioengineering and Biosciences of Instituto superior Técnico (Lisbon, Portugal), during the period September 2018 – September 2019, under the supervision of Prof. Dr. Isabel Maria de Sá Correia Leite de Almeida. Dr. Vaughn Cooper's group (for providing the genomic sequences of the *B. cepacia* isolates here analysed), Dr. Sandra Pinto (for the help provided with the confocal microscopy) and the former MSc students Alexandra Balola and Mariya Kozak are also acknowledged.

REFERENCES

1. Depoorter E, Bull MJ, Peeters C, Coenye T, Vandamme P, Mahenthiralingam E. 2016. *Burkholderia*: an update on taxonomy and biotechnological potential as antibiotic producers. Appl Microbiol Biotechnol 100:5215–5229.
2. Lessie TG, Hendrickson W, Manning BD, Devereux R. 1996. Genomic complexity and plasticity of *Burkholderia cepacia*. FEMS Microbiol Lett 144:117–128.
3. Jimenez L, Kulko E, Barron E, Flannery T. 2015. *Burkholderia cepacia*: a problem that does not go away! EC Microbiol 2:205–210.
4. Sutton S, Jimenez L. 2012. A Review of Reported Recalls Involving Microbiological Control 2004-2011 with Emphasis on FDA Considerations of "Objectionable Organisms." Am Pharm Rev 15:42–57.
5. Sousa SA, Ramos CG, Leitao JH. 2010. *Burkholderia cepacia* complex: emerging multihost pathogens equipped with a wide range of virulence factors and determinants. Int J Microbiol 2011:607575.
6. Weber DJ, Rutala WA, Sickbert-Bennett EE. 2007. Outbreaks associated with contaminated antiseptics and disinfectants. Antimicrob Agents Chemother 51:4217–4224.
7. Geftic SG, Heymann H, Adair FW. 1979. Fourteen-year survival of *Pseudomonas cepacia* in a salts solution preserved with benzalkonium chloride. Appl Environ Microbiol 37:505–510.
8. Ahn Y, Kim JM, Lee Y-J, LiPuma J, Hussong D, Marasa B, Cerniglia C. 2017. Effects of extended storage of chlorhexidine gluconate and benzalkonium chloride solutions on the viability of *Burkholderia cenocepacia*. J Microbiol Biotechnol 27:2211–2220.
9. Kim JM, Ahn Y, LiPuma JJ, Hussong D, Cerniglia CE. 2015. Survival and susceptibility of *Burkholderia cepacia* complex in chlorhexidine gluconate and benzalkonium chloride. J Ind Microbiol Biotechnol 42:905–913.
10. Ahn Y, Kim JM, Kweon O, Kim S-J, Jones RC, Woodling K, Da Costa GG, LiPuma JJ, Hussong D, Marasa BS, Cerniglia CE. 2016. Intrinsic resistance of *Burkholderia cepacia* complex to benzalkonium chloride. MBio 7:e01716-16.
11. Kampf G. 2018. Adaptive microbial response to low level benzalkonium chloride exposure. J Hosp Infect 100:e1–e22.
12. Knapp L, Rushton L, Stapleton H, Sass A, Stewart S, Amezquita A, McClure P, Mahenthiralingam E, Maillard J. 2013. The effect of cationic microbicide exposure against *Burkholderia cepacia* complex (Bcc); the use of *Burkholderia lata* strain 383 as a model bacterium. J Appl Microbiol 115:1117–1126.
13. Guglierame P, Pasca MR, De Rossi E, Buroni S, Arrigo P, Manina G, Riccardi G. 2006. Efflux pump genes of the resistance-nodulation-division family in *Burkholderia cenocepacia* genome. BMC Microbiol 6:66.
14. Ahn Y, Kim JM, Ahn H, Lee Y-J, LiPuma JJ, Hussong D, Cerniglia CE. 2014. Evaluation of liquid and solid culture media for the recovery and enrichment of *Burkholderia cenocepacia* from distilled water. J Ind Microbiol Biotechnol 41:1109–1118.
15. Cunha M V, Leitao JH, Mahenthiralingam E, Vandamme P, Lito L, Barreto C, Salgado MJ, Sá-Correia I. 2003. Molecular analysis of *Burkholderia cepacia* complex isolates from a Portuguese cystic fibrosis center: a 7-year study. J Clin Microbiol 41:4113–4120.
16. Cunha M V, Pinto-de-Oliveira A, Meirinhos-Soares L, Salgado MJ, Melo-Cristino J, Correia S, Barreto C, Sá-Correia I. 2007. Exceptionally high representation of *Burkholderia cepacia* among *B. cepacia* complex isolates recovered from the major Portuguese cystic fibrosis center. J Clin Microbiol 45:1628–1633.
17. Coutinho CP, Barreto C, Pereira L, Lito L, Cristino JM, Sá-Correia I. 2015. Incidence of *Burkholderia contaminans* at a cystic fibrosis centre with an unusually high representation of *Burkholderia cepacia* during 15 years of epidemiological surveillance. J Med Microbiol 64:927–935.
18. Mahenthiralingam E, Campbell ME, Foster J, Lam JS, Speert DP. 1996. Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. J Clin Microbiol 34:1129–1135.
19. Kozak M. 2018. Long-term survival and phenotypic variation of *Burkholderia cepacia* complex bacteria in saline solutions with or without benzalkonium and direct molecular detection of these bacteria. Instituto Superior Técnico.
20. Dubois M, Gilles KA, Hamilton JK, Rebers PA t, Smith F. 1956. Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356.
21. Coutinho CP, de Carvalho CCCR, Madeira A, Pinto-de-Oliveira A, Sá-Correia I. 2011. *Burkholderia cenocepacia* phenotypic clonal variation during a 3.5-year colonization in the lungs of a cystic fibrosis patient. Infect Immun 79:2950–2960.
22. Herbert D, Phipps PJ, Strange RE. 1971. Chapter III chemical analysis of microbial cells. Methods Microbiol 5:209–344.
23. Lewenza S, Abboud J, Poon K, Kobryn M, Humplik I, Bell JR, Mardan L, Reckseidler-Zenteno S. 2018. *Pseudomonas aeruginosa* displays a dormancy phenotype during long-term survival in water. PLoS One 13:e0198384.
24. Carver TJ, Rutherford KM, Berriman M, Rajandream M-A, Barrell BG, Parkhill J. 2005. ACT: the Artemis comparison tool. Bioinformatics 21:3422–3423.
25. Public databases for molecular typing and microbial genome diversity (PubMLST). 2019. *Burkholderia cepacia* complex MLST Databases. https://pubmlst.org/bcc/. Accessed 16 August 2019.
26. Maisonneuve E, Gerdes K. 2014. Molecular mechanisms underlying bacterial persisters. Cell 157:539–548.
27. Van den Bergh B, Fauvart M, Michiels J. 2017. Formation, physiology, ecology, evolution and clinical importance of bacterial persisters. FEMS Microbiol Rev 41:219–251.
28. Fauvart M, De Groote VN, Michiels J. 2011. Role of persister cells in chronic infections: clinical relevance and perspectives on anti-persister therapies. J Med Microbiol 60:699–709.
29. Johns BE, Purdy KJ, Tucker NP, Maddocks SE. 2015. Phenotypic and genotypic characteristics of small colony variants and their role in chronic infection. Microbiol insights 8:15–23.
30. Proctor RA, Von Eiff C, Kahl BC, Becker K, McNamara P, Herrmann M, Peters G. 2006. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat Rev Microbiol 4:295–305.
31. Fisher RA, Gollan B, Helaine S. 2017. Persistent bacterial infections and persister cells. Nat Rev Microbiol 15:453–464.
32. Pumpuang A, Chantratita N, Wikraiphat C, Saiprom N, Day NPJ, Peacock SJ, Wuthiekanun V. 2011. Survival of *Burkholderia pseudomallei* in distilled water for 16 years. Trans R Soc Trop Med Hyg 105:598–600.
33. Givskov M, Eberl L, Møller S, Poulsen LK, Molin S. 1994. Responses to nutrient starvation in *Pseudomonas putida* KT2442: analysis of general cross-protection, cell shape, and macromolecular content. J Bacteriol 176:7–14.
34. Zlosnik JEA, Costa PS, Brant R, Mori PYB, Hird TJ, Fraenkel MC, Wilcox PG, Davidson AGF, Speert DP. 2011. Mucoid and nonmucoid *Burkholderia cepacia* complex bacteria in cystic fibrosis infections. Am J Respir Crit Care Med 183:67–72.