

Biofilm production by multiresistant *Corynebacterium striatum* associated with nosocomial outbreak

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Corynebacterium striatum is a potentially pathogenic microorganism that causes nosocomial outbreaks. However, little is known about its virulence factors that may contribute to healthcare-associated infections (HAIs). We investigated the biofilm production on abiotic surfaces of multidrug-resistant (MDR) and multidrug-susceptible (MDS) strains of *C. striatum* of pulsed-field gel electrophoresis types I-MDR, II-MDR, III-MDS and IV-MDS isolated during a nosocomial outbreak in Rio de Janeiro, Brazil. The results showed that *C. striatum* was able to adhere to hydrophilic and hydrophobic abiotic surfaces. The *C. striatum* 1987/I-MDR strain, predominantly isolated from patients undergoing endotracheal intubation procedures, showed the greatest ability to adhere to all surfaces. *C. striatum* bound fibrinogen to its surface, which contributed to biofilm formation. Scanning electron microscopy showed the production of mature biofilms on polyurethane catheters by all pulsotypes. In conclusion, biofilm production may contribute to the establishment of HAIs caused by *C. striatum*.

Key words: biofilm - *Corynebacterium striatum* - epidemic clone - fibrinogen - multi-resistance - nosocomial outbreak

Corynebacterium striatum is an emerging multidrug-resistant (MDR) potentially pathogenic microorganism that causes nosocomial infection in patients who have experienced long hospital admissions, those who have received several courses of antibiotics (Camello et al. 2003, Otsuka et al. 2006, Renom et al. 2007, Baio et al. 2013), those with acquired immune deficiency syndrome (AIDS) or cancer and those who have received a transplant (Tarr et al. 2003, Martins et al. 2009). Cases of severe infections in both immunocompromised and immunocompetent individuals and nosocomial outbreaks due to MDR *C. striatum* are increasing in both industrialised and developing countries. *C. striatum* has been associated with cases of pulmonary infections, sepsis, endocarditis, meningitis, osteomyelitis, arthritis, sinusitis, skin wounds and intrauterine infections (Rufael & Cohn 1994, Weiss et al. 1996, Fernández-Ayala et al. 2001, Camello et al. 2003, Renom et al. 2007, Scholle 2007, Boltin et al. 2009, Campanile et al. 2009, Martins et al. 2009, Moore et al. 2010, Oliva et al. 2010, Baio et al. 2013).

Genotyping analysis by pulsed-field gel electrophoresis (PFGE) has identified PFGE types associated with nosocomial outbreaks of respiratory origin and with resistance to a broad range of antibiotics (MDR phenotype). In Italy, MDR *C. striatum* isolates have been recovered from hospitalised patients who have undergone surgery or have been admitted to intensive care units (ICUs). These isolates have been responsible for cases of ventilator-associated pneumonia and tracheobronchitis, catheter-related sepsis and wound infections. Infections caused by this species have been strongly associated with devices, including not only tubes and catheters, but also sternal surgical wound wires (Campanile et al. 2009). *C. striatum* has also been isolated from other materials for hospital use, such as endotracheal tubes (Martinez-Martinez et al. 1995). Earlier genotype studies have confirmed that *C. striatum* may be transmitted between patients, from person to person and *via* caretakers (Leonard et al. 1994). Recently, a nosocomial outbreak caused by *C. striatum* was documented in Rio de Janeiro (RJ), Brazil. PFGE analysis indicated the presence of four PFGE profiles, including two related clones of MDR strains (PFGE I and II). The results of these studies demonstrate the predominance of PFGE-type I MDR isolates that are mainly isolated from ICUs and surgical wards. *C. striatum* strains have largely been isolated in pure culture from tracheal aspirates of patients undergoing endotracheal intubation procedures (Baio et al. 2013).

Currently, more than half of the infectious diseases that affect mildly immunocompromised patients involve bacterial species that are commonly encountered in the environment or constitute the body's normal flora, including

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TABLE

Origin and pulsed-field gel electrophoresis (PFGE)-types of partially studied *Corynebacterium striatum* strains isolated from patients during a nosocomial outbreak in the metropolitan area, Rio de Janeiro, Brazil used in this study

Strains/year	Gender/ age	Hospital wards	Isolation site	Outcome	Antimicrobial susceptibility patterns	PFGE- types
1987 BR-RJ/09	F/50	Nursery 18	BAL	Death	MDR	I
2369 BR-RJ/09	M/NI	General ICU	TA	Cure	MDR	II
1961 BR-RJ/09	F/37	Infectious diseases	Urine	NI	MDS	III
1954 BR-RJ/09	M/NI	Thoracic MSU	Surgical wound	NI	MDS	IV

BAL: bronchoalveolar lavage; F: female; ICU: intensive care unit; M: male; MDR: multiresistance (≥ 3 types of antimicrobial agents); MDS: multidrug susceptible; MSU: medical surgical unit; NI: not informed; TA: tracheal aspirate;

several corynebacterial species (Martins et al. 2009). Opportunistic pathogens may be endowed with an array of virulence factors that facilitate their ability to survive within host tissues and confer resistance to clearance by host immune mechanisms and antimicrobial killing. The ability to form biofilms may be a prerequisite for the pathogenesis of nosocomial diseases associated (or not) with the use of medical devices (Bonifait et al. 2008). Biofilms have been previously described in *Corynebacterium diphtheriae*, *Corynebacterium pseudotuberculosis*, *Corynebacterium renale*, *Corynebacterium urealyticum* and *Corynebacterium jeikeium* (Mattos-Guaraldi & Formiga 1991, Soriano et al. 1993, Mattos-Guaraldi et al. 1999a, b, Olson et al. 2002, Kwaszewska et al. 2006, Gomes et al. 2009, 2013, Soriano et al. 2009). Thus, the better recognition and understanding of the biology and virulence potential of *C. striatum* strains may help to effectively prevent infections caused by them. Therefore, we investigated the in vitro capacities for biofilm formation of *C. striatum* strains representative of four different PFGE types isolated during a nosocomial outbreak in RJ (Baio et al. 2013).

SUBJECTS, MATERIALS AND METHODS

Bacterial strains - Table shows the epidemiological and microbiological features of the partially studied *C. striatum* strains used in this investigation (Baio et al. 2013). *C. striatum* identification was established by *16S rRNA* and *rpoB* gene sequencing. *C. striatum* pulsotypes I and II exhibited MDR profiles showing susceptibility only to vancomycin, linezolid and tetracycline, while *C. striatum* pulsotypes III and IV showed susceptibility to most of the 21 antimicrobial agents tested and resistance only to fosfomycin and ticarcillin/clavulanate. The *C. diphtheriae* CAT5003748 strain was used as a positive control in all experiments (Gomes et al. 2009).

Biofilm formation on hydrophilic surfaces of glass tubes - Microorganisms were inoculated in glass tubes (13 x 100 mm) containing 4 mL of trypticase soy broth (TSB) and incubated at 37°C for 24 h without shaking. The tubes were gently shaken and supernatants with non-adherent bacterial cells were discarded. TSB (4 mL) was then added and the tubes were reincubated at 37°C for 24 h. This procedure was repeated twice. Glass-adherent

bacteria created a confluent coat of cells on the sides of the tube. Quantitative analysis of viable sessile cells was based on previously described methods (Mattos-Guaraldi & Formiga 1991, Dooley et al. 1996).

Quantitative and semiquantitative analyses of biofilm formation on catheter - Polyurethane 16-gauge percutaneous nephrostomy catheters (Intracath; Deseret Pharmaceutical Co, USA) were used for an evaluation of bacterial adherence and biofilm formation on catheter surfaces. Sterile 4-cm segments of polyurethane catheters were immersed in TSB containing 10^6 colony-forming unit (CFU) mL⁻¹ and incubated at 37°C for 24 h (Gomes et al. 2009). Then, quantitative catheter culturing (Dooley et al. 1996) and a semiquantitative roll-plate technique (Maki et al. 1977) were performed using Columbia agar medium supplemented with 5% sheep blood at 37°C for 24 h.

Scanning electron microscopy (SEM) - Sections of glass coverslips and polyurethane catheters were fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide and dehydrated in a graded series of ethanol. Subsequently, catheter segments were subjected to critical point drying with carbon dioxide, covered with a 10 nm layer of gold palladium and examined with a JEOL JSM 5310 scanning electron microscope. Sterile unused polyurethane catheters were also processed by SEM directly upon removal from commercial packaging (Gomes et al. 2009).

Biofilm formation on hydrophobic polystyrene surfaces - Biofilm formation on negatively charged polystyrene surfaces was determined quantitatively in 96-well flat-bottomed microtitre plates according to previously described methods (Stepanovic et al. 2000, Gomes et al. 2009). Aliquots of 200 μ L of bacterial suspensions [0.2 optical density (OD) at $\lambda=570$ nm] were added to the microplate wells. After incubation at 37°C for 24 h, the contents of each well were aspirated and washed three times with 200 μ L phosphate-buffered saline (0.01 M, pH 7.2). The remaining attached bacteria were fixed with 200 μ L of 99% methanol and stained with 2% crystal violet. The negative controls contained TSB only. The bound dye was then solubilised with 160 μ L of 33% glacial acetic acid and the OD of the solution was measured at $\lambda = 570$ nm using an enzyme immunosorbent assay reader (BioRad,

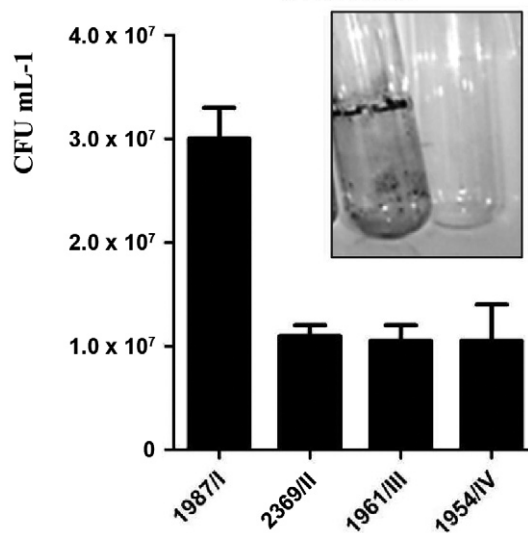


Fig. 1: *Corynebacterium striatum* adherence to glass surfaces (24 h incubation) evaluated by quantitative and semiquantitative tests. In detail, glass tubes with 1987/I strain exhibiting confluent coat of sessile forms on the surface of glass tube wall and with negative control (trypticase soy broth medium without bacteria). CFU: colony-forming unit.

model 550). The cut-off OD (OD_c) was defined as the mean OD of the negative control. All strains were classified into the following categories based on the ODs of the bacterial films: nonadherent (0: $OD \leq OD_c$) or weakly (+: $OD_c < OD \leq 2 \times OD_c$), moderately (++: $2 \times OD_c < OD \leq 4 \times OD_c$) or strongly (+++: $4 \times OD_c \leq OD$) adherent.

Influence of fibrinogen (Fbg) on biofilm formation

- Biofilm formation (24 h) was determined in 96-well flat-bottom polystyrene microtitre plates as described above, with some modifications. In these experiments, the wells of the microplates were pre-treated (or not) with human plasmatic Fbg (Sigma Chemical Co, USA) at a concentration of $50 \mu\text{g mL}^{-1}$ overnight at 4°C . Fbg-coated wells containing 200 μL of TSB medium without bacteria were used as negative controls (Lembke et al. 2006, Gomes et al. 2009).

Statistical analysis - Each experiment was carried out in triplicate and repeated three times. The biofilm formation by each representative pulsotype strain was compared by ANOVA with Tukey's post test. Student's *t* test was used to compare the means of biofilm formation (OD) in the presence of Fbg for each pulsotype and a $p < 0.05$ was considered to be statistically significant. Statistical analyses were performed using GraphPad Prism v.6 (USA).

RESULTS

Bacterial adherence to hydrophilic surface of glass tube - Successful bacteria may survive in the hospital environment due to their ability to adhere to different substrates. To determine whether *C. striatum* is able to adhere to glass, we quantified the amount of viable sessile forms of bacteria associated with glass. Viable sessile bacterial cells were observed on glass surfaces at 48 h post-incubation with *C. striatum* strains of PFGE types

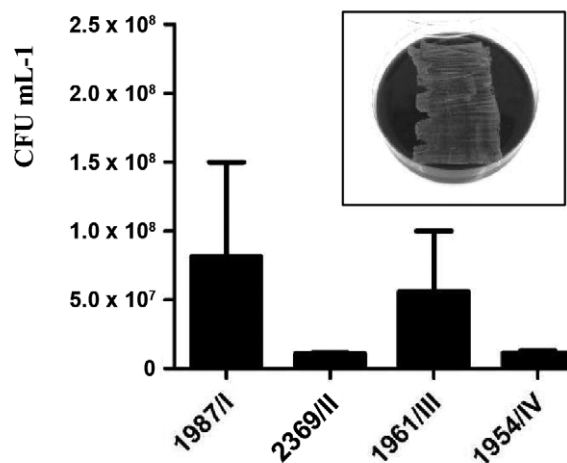


Fig. 2: *Corynebacterium striatum* adherence to polyurethane surfaces evaluated by quantitative and semiquantitative tests. In detail, 1987/I strain infected catheter segment cultured on blood agar plates (roll plate technique). CFU: colony-forming unit.

I, II, III and IV at different levels (Fig. 1). In Fig. 1, a representative side figure illustrates sessile bacteria stained with crystal violet, indicating the formation of a positive slime/biofilm on the glass surface. All strains were able to strongly adhere to the glass surface, however the *C. striatum* 1987/I-MDR strain showed the highest ability to adhere to this hydrophilic abiotic surface ($p < 0.05$).

Biofilm formation on polyurethane catheter surface

- The ability to adhere to catheter materials for intravenous use and medical devices inserted into the body are important characteristics of bacteria associated with healthcare-associated infections. To determine whether *C. striatum* is able to adhere and form biofilms on catheter surfaces, segments of polyurethane catheters were colonised in vitro by *C. striatum* 1987/I, 2369/II, 1961/III and 1954/IV strains. The evaluation of the adherence and viability of microorganisms on the polyurethane catheter segments by the semiquantitative roll plate method (> 15 CFU) and by quantitative catheter culture assays ($> 1.5 \times 10^6$ CFU) showed that viable *C. striatum* cells were extensively adherent to and multiplied on the polyurethane catheter surface (Fig. 2). In Fig. 2A, the representative side figure illustrates bacterial growth on an agar plate after catheter colonisation, as assessed by the roll-plate technique. Although all strains were able to adhere to the catheter surface, the *C. striatum* 1987/I-MDR strain again exhibited significantly greater adherence ($p < 0.05$) to this abiotic hydrophobic surface compared with the representative strains of pulsotypes II, III and IV.

Morphological aspects of biofilm formation on the surface of polyurethane medical device as evaluated by SEM - After determining that *C. striatum* was able to adhere to glass and catheter surfaces, forming a visible biofilm, the morphological aspects of the biofilm were inves-

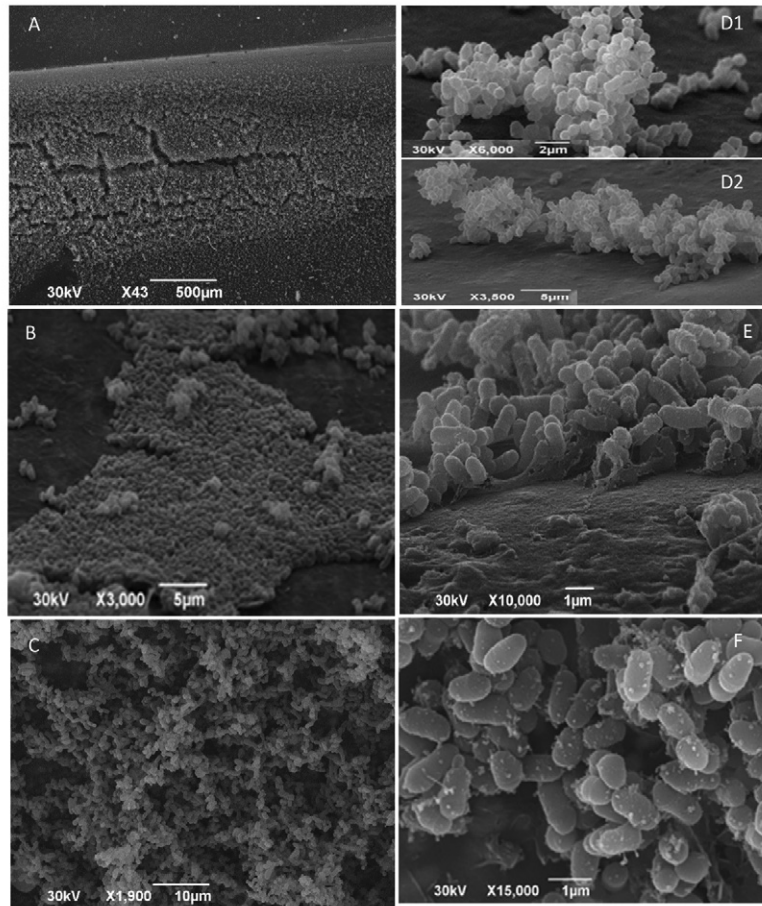


Fig. 3: scanning electron micrographs illustrating biofilm formation on polyurethane catheter surfaces after 24 h incubation with different *Corynebacterium striatum* strains. Pulsed-field gel electrophoresis (PFGE)-types I-IV: A, C, F: 1987/I; B, E: 2369/II; D1: 1961/III; D2: 1954/IV; A-C: a large amount of biofilm material exhibiting; B, D: bacterial microcolonies; E: amorphous material on the catheter surface is evident; C, F: presence of hollow voids indicative of mature biofilm formation on surfaces of polyurethane catheters.

tigated by SEM. SEM assay confirmed that all *C. striatum* strains were able to colonise the polyurethane catheter and form mature biofilms (Fig. 3). Fig. 3A-C shows the large amount of biofilm material that was observed. Bacterial microcolonies were present (Fig. 3B, D) and amorphous material on the catheter surface was evident (Fig. 3E). In addition, hollow voids were present (Fig. 3C, F). The presence of autoaggregative microcolonies, extracellular slime and hollow voids on the polyurethane surface were all indicative of mature biofilm structure.

Bacterial adherence to hydrophobic polystyrene surface and influence of Fbg on biofilm formation - In addition to glass and catheter surfaces, we also investigated the ability of *C. striatum* to adhere to plastic using negatively charged polystyrene 96-well flat-bottom microtitre plates. The quantification of biofilm revealed that all *C. striatum* strains of PFGE types I, II, III and IV were able to adhere to the negatively charged plastic (polystyrene) surface at different intensities (Fig. 4). Again, the *C. striatum* 1987/I-MDR strain exhibited a significantly ($p < 0.05$) greater level of adherence to

this abiotic hydrophobic surface compared with the *C. striatum* 2369/II-MDR, 1961/III-multidrug-susceptible (MDS) and 1954/IV-MDS strains.

Several microorganisms use extracellular matrix proteins, such as Fbg, to increase their ability to interact with different cells and also with abiotic surfaces. To investigate the influence of Fbg on the ability of *C. striatum* to adhere to plastic microtitre plates, plates were pre-treated with human plasmatic Fbg prior to bacterial colonisation. As shown in Fig. 4, human Fbg enhanced biofilm formation on the polystyrene surfaces by the *C. striatum* 1987/I-MDR ($p = 0.00362$), 2369/II-MDR ($p = 0.0022$) and 1961/III-MDS ($p = 0.0105$) strains, suggesting that this matrix protein may contribute to the interaction of different clones of this human pathogen with abiotic hydrophobic surfaces.

DISCUSSION

Bacterial biofilms form in association with many human activities, including food processing, transportation, public infrastructure and, most importantly, healthcare (Rzhphishevskaya et al. 2013). Biofilms have been found on

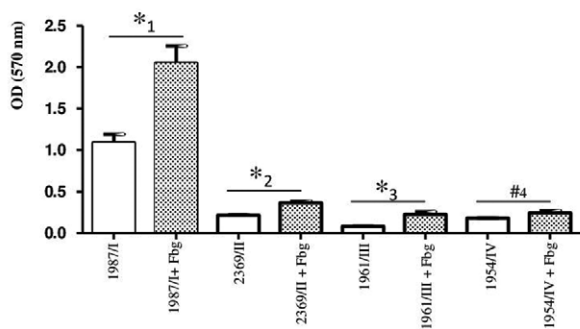


Fig. 4: biofilm formation (24 h incubation) on polystyrene surface by *Corynebacterium striatum* strains evaluated by a semiquantitative microplate assay. Effect of human plasma fibrinogen (Fbg) on biofilm formation by representative strains of pulsed-field gel electrophoresis (PFGE)-types I-IV. Experiments were performed in triplicate and the means standard deviations of three independent experiments. *: $p < 0.05$; #: $p > 0.05$.

numerous medical devices (e.g., urinary catheters, central venous catheters and endoscopes). Their presence can have serious implications for immunocompromised patients and those with indwelling medical devices (Brown & Williams 1985, Russell & Russell 1995, Rutala et al. 2008, Al Akhrass et al. 2012). The acquisition of the ability to form biofilms may represent a good strategy for a microorganism to acquire enhanced survival under conditions of stress, e.g., during host invasion or following antibiotic treatment, because cells growing in biofilms are highly resistant to components of the human immune system and to numerous types of antimicrobial agents. In addition, the ability of bacterial cells to transfer genes horizontally is enhanced within biofilm communities, thereby facilitating the spread of antibiotic resistance (Stewart & Costerton 2001, Lee et al. 2008).

Our results reveal the capacities of diverse *C. striatum* isolates to adhere to various abiotic surfaces and to form biofilms in an in vitro catheter model. The data revealed variations among the capacities of diverse clones of MDR and MDS *C. striatum* strains identified during a nosocomial outbreak in RJ to adhere to and survive on positively and negatively charged abiotic surfaces. Notably, we identified an association of increased biofilm formation, antimicrobial multiresistance and clonality of the *C. striatum* strains. In the present study, *C. striatum* MDR PFGE types I and II were predominantly isolated during the nosocomial outbreak from in-patients undergoing endotracheal intubation procedures in the ICU or in surgical wards. The clinical isolates of these PFGE types expressed a high capacity to form biofilms on hydrophilic (glass; positively charged) and hydrophobic (polystyrene; negatively charged) abiotic surfaces, including polyurethane (positively charged) catheter surfaces.

The *C. striatum* strains showed properties similar to other pathogenic biofilm producers. The results of the semiquantitative roll-plate method and quantitative catheter culture assays showed that viable bacterial cells

extensively adhered to and multiplied on the surfaces of polyurethane catheters. SEM revealed a large amount of biofilm on polyurethane catheter surfaces produced by all *C. striatum* strains tested.

The developmental biology of biofilm formation can be characterised into three stages: initial attachment, development of microcolony formation and detachment (O'Toole et al. 2000). Similar to *C. diphtheriae* (Gomes et al. 2013), *Acinetobacter baumannii* (Rao et al. 2008) and other nondiphtherial *Corynebacterium* species (Soriano et al. 1993, Gomes et al. 2009), autoaggregative *C. striatum* strains were able to attach to and form microcolonies (a hallmark of biofilm formation) on abiotic surfaces. *C. striatum* also formed matrix-enclosed microcolonies on in vitro colonised polyurethane surfaces. Hollow voids indicative of mature biofilm formation on the surfaces of polyurethane catheters were also observed. The formation of hollow voids seems to be involved in the dispersion of sessile bacterial cells during the final stage of biofilm formation, which can increase bacterial virulence (Rice et al. 2009).

Some bacterial properties are associated with biofilm production, including the increased synthesis of exopolysaccharides, hydrophobic properties and the development of antibiotic resistance (Olson et al. 2002, Costerton et al. 2003, Rao et al. 2008). A previous study has addressed the prevention of biofilms and has shown that the surface charge of an abiotic substrate may influence the morphology and physiology of a biofilm (Rzhepishevskaya et al. 2013). In the present study, the MDR and MDS *C. striatum* strains were able to adhere at different levels to negatively charged plastic (polystyrene) and positively charged (glass) surfaces, as previously observed with *C. diphtheriae* and/or *C. urealyticum* (Mattos-Guaraldi & Formiga 1991, Mattos-Guaraldi et al. 1999a, b, Soriano et al. 2009, Gomes et al. 2013). Polyurethane implanted subcutaneously into mice led to an infiltration of erythrocytes and subsequent haemolysis, possibly due to the attraction of this positively charged plastic to negatively charged cells (Rigdon 1970). In accordance with previous observations of *C. diphtheriae* (Mattos-Guaraldi & Formiga 1991, Mattos-Guaraldi et al. 1999a, b, Gomes et al. 2013), the negatively charged cell surfaces of *C. striatum* strains and their adherence to polyurethane may be partially explained by the positive electric charge associated with this polymer. Moreover, the amorphous deposited substances or glycocalyx noted surrounding *C. striatum* microcolonies on the surfaces of the polyurethane catheters suggest that this bacteria may produce or attract substances that strengthen their attachment to inert surfaces in vitro.

Hydrophobicity has been significantly associated with biofilm formation of lipophilic skin corynebacteria on solid surfaces (Kwaszewska et al. 2006). For *C. diphtheriae* strains, bacterial autoaggregation and hydrophobicity are mainly related to biofilm formation on polystyrene surfaces (Mattos-Guaraldi et al. 1999a). The cell surface hydrophobicity of *C. striatum* strains was demonstrated by their ability to adhere to polystyrene surfaces. Therefore, *C. striatum* strains should be included among bacterial species that have a natural tendency to adhere to available biotic and/or abiotic surfaces and to

form biofilm (Olson et al. 2002, Kwaszewska et al. 2006, Soriano et al. 2009) and that are also capable of rapid physiological responses following exposure to surfaces with varying physicochemical characteristics, enabling some bacterial colonisation on negatively charged surfaces (Kwaszewska et al. 2006).

In natural environments, bacteria typically adhere to the layer of adsorbed molecules that coats inert surfaces, the so-called “conditioning film” and not directly to the substratum. In vivo, any material surface is rapidly covered by plasma and matrix proteins, to which bacteria may display specific adhesins. The stimulation of bacterial biofilm formation by exogenous mammalian proteins has been reported for many human pathogens (Bonifait et al. 2008, Gomes et al. 2009, 2013). Fbg is a major protein in human plasma and is primarily involved in the coagulation cascade system through its conversion to insoluble fibrin. Fbg synthesis is dramatically upregulated during inflammation or under stress conditions, such as systemic infections. Fbg and fibrin play overlapping roles in blood clotting, fibrinolysis, the inflammatory response, cellular and matrix interactions and wound healing (Mosesson 2005). The Fbg binding properties of *Staphylococcus aureus* (O’Neill et al. 2008), *Streptococcus suis* (Bonifait et al. 2008) and *C. diphtheriae* (Gomes et al. 2009, Sabbadini et al. 2010) allow them to attach to each other through Fbg-mediated cross-bridging, contributing to biofilm production. The ability of *C. striatum* strains to bind to Fbg was also demonstrated in the present study. In addition to the ability to form biofilms directly on hydrophilic and hydrophobic abiotic surfaces, *C. striatum* also produced biofilms on Fbg-associated “conditioning films”. Compared with the formation of biofilms on the uncoated polystyrene surfaces, the Fbg-coated surfaces showed enhanced biofilm formation by the *C. striatum* 1987/I-MDR strain, which was responsible for a previous nosocomial outbreak. The enhancement occurred at a typical in vivo concentration of Fbg in blood plasma of approximately 2.5 mg/mL. Therefore, the expression of Fbg-binding adhesins at different levels may be implicated in biofilm formation on “conditioning films” by *C. striatum* strains, as has been previously reported for *S. suis* (Bonifait et al. 2008).

In conclusion, *C. striatum* may form biofilms in vivo by an adherent biofilm mode of growth in vitro, as was demonstrated on hydrophilic and hydrophobic abiotic surfaces, including polyurethane catheters. The affinity of *C. striatum* for human Fbg was determined to be an additional potential virulence trait of this organism. In addition to its multi-resistance to antimicrobial agents used in therapy, the ability to produce a “conditioning film” may contribute to the establishment and dissemination of nosocomial infections caused by this organism, including those in patients with indwelling medical devices.

Thus, *C. striatum* strains capable of forming biofilms may be selected under antibiotic pressure, or conversely, *C. striatum* may acquire resistance to multiple drugs within biofilm communities. In either event, the high colonising capacity of *C. striatum* combined with its resistance to multiple drugs will contribute to the survival and further dissemination of this organism in the hospital setting.

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