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MICROBIOLOGY

Investigation of factors related to biofilm formation in *Providencia stuartii*

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Abstract: *Providencia stuartii* is one of the *Enterobacteriaceae* species of medical importance commonly associated with urinary infections, which can also cause other ones, including uncommon ones, such as liver abscess and septic vasculitis. This bacterium stands out in the expression of intrinsic and acquired resistance to antimicrobials. Besides, it uses mechanisms such as biofilm for its persistence in biotic and abiotic environments. This study investigated the cellular hydrophobicity profile of clinical isolates of *P. stuartii*. It also analyzed genes related to the fimbrial adhesin in this species comparing with other reports described for other bacteria from *Enterobacteriaceae* family. The investigated isolates to form biofilm and had a practically hydrophilic cell surface profile. However, *fimH* and *mrkD* genes were not found in *P. stuartii*, unlike observed in other species of *Enterobacteriaceae*. These results show that *P. stuartii* has specificities regarding its potential for biofilm formation, which makes it difficult to destabilize the infectious process and increases the permanence of this pathogen in hospital units.

Key words: *Enterobacteriaceae*, *fimH, mrkD*, virulence.

INTRODUCTION

Providencia is one of the genera that make up the *Enterobacteriaceae* family. The species *P. stuartii* being one of the most frequent and widely associated with urinary tract infections, especially in patients undergoing long periods of catheterization (Armbruster et al. 2014, Kurmasheva et al. 2018). As it is an opportunistic pathogen, this bacterium can cause other infections: septicemia (Aires et al. 2016), diarrhea (Shima et al. 2016), pneumonia (Abdallah & Balshi 2018), infections in burns and open wounds (Pirii et al. 2018, Libertucci et al. 2019), even those uncommon for this species, such as conjunctivitis (Crane et al. 2016), liver abscesso (Lin et al. 2017), rectal abscesso (Lee et al. 2017), and septic vasculitis (George et al. 2020).

Providencia stuartii corresponds to one of the β-lactamase-producing enterobacteria of the chromosomal AmpC type. Therefore, it has a natural resistance to most β-lactam antimicrobials, including penicillins, cephalosporins, and combinations with β-lactamase inhibitors lactamases (Magiorakos et al. 2012, Santiago et al. 2016). It is also intrinsically resistant to aminoglycosides (except amikacin) and antimicrobials of last therapeutic choice, such as tigecycline, colistin, and polymyxin B (CLSI 2018). Additionally, it can acquire resistance genes that encode different types of enzymes such as aminoglycoside-modifying enzymes (AMEs), β-lactamases of Extended Spectrum (ESBLs), and carbapenemases (Miró et al. 2013, Oikonomou et al. 2016, Mao et al. 2018, Molnár et al. 2019).

The pathogenicity of this species is further amplified by its ability to form biofilms (El-Khatib et al. 2017, 2018). The biofilm corresponds to a matrix composed mainly of exopolysaccharides (EPS), which allows the passage of nutrients through pores and channels. In most cases, there found bacteria living in communities or adhered to the surfaces of biotic or abiotic materials, such as human body tissues and clinical devices (Pelling et al. 2019).

The formation and structure of biofilms depend on factor varieties such as the type of microorganism, the type of surface, and environmental conditions such as pH and temperature. Physico-chemical characteristics such as the forces of electrostatic and hydrophobic attraction, interactions of var der Waals, hydrogen bonds, and covalent bonds are relevant to the formation of biofilm. Besides, genetic factors for bacterial adhesion are considered, such as the expression of flagella, polymers, and adhesion fimbriae (Flemming et al. 2016).

Although these data are relevant, the literature provides little information about the factors that contribute to the formation of biofilms in *P. stuartii*. In the case of urinary infections, there observed that to colonize the urinary tract, this microorganism can express resistance to calcium and magnesium, tolerates high concentrations of urea and pH variations (El-Khatib et al. 2017). But little is known about the cell adhesion processes of this species on biotic or abiotic surfaces. Thus, this study aimed to investigate cell hydrophobicity and genes encoding fimbrial adhesins in biofilm-forming *P. stuartii* isolates and compare with data found in other representatives of the *Enterobacteriaceae* family.

MATERIALS AND METHODS

Biological material and growing conditions

There investigated *P. stuartii* isolates (n = 28) from a public hospital in Recife-PE, Brazil. The samples were collected between June 2017 and April 2018 from a variety of infection sites and sectors of the hospital (Silva et al. 2021), which were stored in the Brain Heart Infusion (BHI) medium with glycerol (15%) in a Deep Freezer at -80 °C and with mineral oil at room temperature. Subsequently, they were reactivated and incubated at 37 °C for 24 hours. This study was approved by the Committee for Ethics in Research (CEP) of the Universidade Federal de Pernambuco (UFPE), and has Certificate of Presentation of Ethical Appreciation (CAAE) (number: 84509218.3.0000.5208).

Biofilm formation

Biofilm formation was carried out in polystyrene microplates by the violet crystal method described by Stepanović et al. (2007), with different culture media: BHI, Luria Bertani (LB), and Tryptose Soy Broth (TSB). After incubation (37 ºC/24h), the inoculum (1.5×10^6 CFU/mL) was removed, the microplates were washed (3 ×) with 0.9% sterile saline, and the biofilm fixed (55 ºC/1h). Then, violet crystal (0.4%) was added at room temperature for 15 min, followed by three washes to remove excess dye. Finally, absolute ethanol was added for 30 min. From the optical density (OD) readings on a microplate reader (FLx800 ™ Multi-Detection - BioTec Instruments, Inc.) at wavelength 570 nm, there determined the mean of the absorbance values of each sample (OD_S) in comparison with the absorbance of the sterility control (OD_C). The samples were classified as strong ($4 \times OD_C < OD_S$), moderate ($2 \times OD_C < OD_S \leq 4 \times$ ODc), and weak ($OD_C < OD_S \leq 2 \times OD_C$) biofilm producer. For this assay, three independent and triplicate experiments were performed.

Fluorescence microscopy

An isolate (8945) was selected, capable of forming biofilm in the three media tested (BHI, LB, TSB) to confirm the biofilm formation. The tests, in triplicate, were repeated three times independently on polystyrene plates (six wells). 4 mL of each medium was added in separate wells, 0.5 mL of Milli-Q water and 0.5 mL of bacterial inoculum. For sterility control, the bacterial inoculum was replaced with distilled water sterilized. After incubation (37 ºC/24h), the plates were washed three times with 0.9% saline to remove planktonic cells. Then, SYBR Green (20 µL for each 1 mL of Milli-Q water) and Calcofluor White (1:1 with 10% KOH) were added in different wells to analyze the biofilm cells and the exopolysaccharide structure, respectively. The images were obtained under a fluorescence microscope (Leica DMLB Stand Tilting Trinocular Head) in filter 2 (BP 515-560) for SYBR Green and filter 1 (BP 480/40) for Calcofluor White.

Microbial adhesion test to hydrocarbons (MATH)

The Cell Surface Hydrophobicity (CSH) profile was determined based on the hydrocarbon bonding method described by Czerwonka et al. (2016), with some modifications. The bacterial isolates grown in BHI broth (37 °C/18h) were transferred to microtubes, centrifuged for 10 min (7,000 rpm), and the supernatant discarded. The resulting pellet was suspended in PUM buffer (phosphate potassium trihydrate and monobasic, urea, and sulfate magnesium heptahydrate) and adjusted to 0.5 (1.5×10^6 CFU/mL) obtaining the initial OD reading (OD_i) at 600 nm. Subsequently, there added p-xylene hydrocarbon (1: 0.2), and the mixture was vortexed for 2 min. After the phase separation, at room temperature for 30 min, the final OD (OD_f) was performed at 600 nm of the lower phase of each microtube. The following formula was used to determine CSH (%): (OD_i - OD_f) / OD_i x 100. Bacteria with CSH (%) below 30% were considered hydrophilic and with CSH (%) higher than 70% as hydrophobic. Samples with CSH between 30% and 70% were classified as moderately hydrophobic (Tendolkar et al. 2004).

Genomic DNA extraction

DNA extraction was performed according to the protocol described by Sambrook & Russell (2001), with some modifications. The quality of the extracted DNA was evaluated using the 1% agarose gel electrophoresis technique. After the run, the gel was observed in a UV transilluminator and photo-documented. The DNA concentration was determined by optical density in a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) at wavelengths 260 and 280 nm, obtaining an estimate of the amount of DNA present in the sample and the degree of purity.

Genes identification by PCR

The amplification of the fimbriae encoding genes was performed by Polymerase Chain Reaction (PCR). For the *fimH* gene, the following sequence was used: F 5' TCCACAGTCGCCAACGCTTC3' and R 5'GCTCAGAATCAACATCGGTAAC3'. The sample amplifications were prepared for a final volume of 25 µL. It contains genomic DNA (20 ng), primer pairs (10 pmol), $MgCl_2$ (25 mM), dNTP (2 mM), Milli-Q water, Taq DNA polymerase (1 U), and sample buffer (25 mM). The reactions were carried out in a thermocycler (C1000™ BioRad), programmed with the respective stages of denaturation, annealing, and extension (Stahlhut et al. 2009, Sahly et al. 2008). After amplification, the PCR was evaluated by electrophoresis on 1.2% agarose gel, using 100bp Ladder DNA marker (Invitrogen, Carlsbad, CA, USA). Then, the gel was observed in a UV transilluminator and photo-documented. A strain of *Klebsiella pneumoniae* (K5-A2),

obtained from the Bacterial Culture Collection of the Laboratory of Bacteriology and Molecular Biology - Department of Tropical Medicine at UFPE, was used as a reference control.

RESULTS

All investigated *P. stuartii* isolates showed the ability to form biofilm in polystyrene microplates, with a high formation in BHI and TSB media, except for isolate 04446, which proved to be weak in the three media tested. The formation intensity can be evidenced through the optical density represented in Figure 1. Of the total isolates analyzed, 22 were classified as strong forming both in BHI and in TSB. However, in the LB medium, 23 showed moderate biofilm formation capacity (Figure 1, Table I).

There used fluorescence microscopy to confirm the formation. According to the results obtained by the violet crystal method, isolate 8945 was selected, evaluated as a strong former in the three investigated media. It was possible to observe biofilm in the samples stained with SYBR Green for the three media (Figure 2a, b, c), as well as the formation of exopolysaccharide matrix (EPS) from staining with Calcofluor White (Figure 2d, e, f). In Figures 2g, b, i, there possible to verify the overlap of the previous images revealing the fundamental structures that characterize the bacterial biofilm.

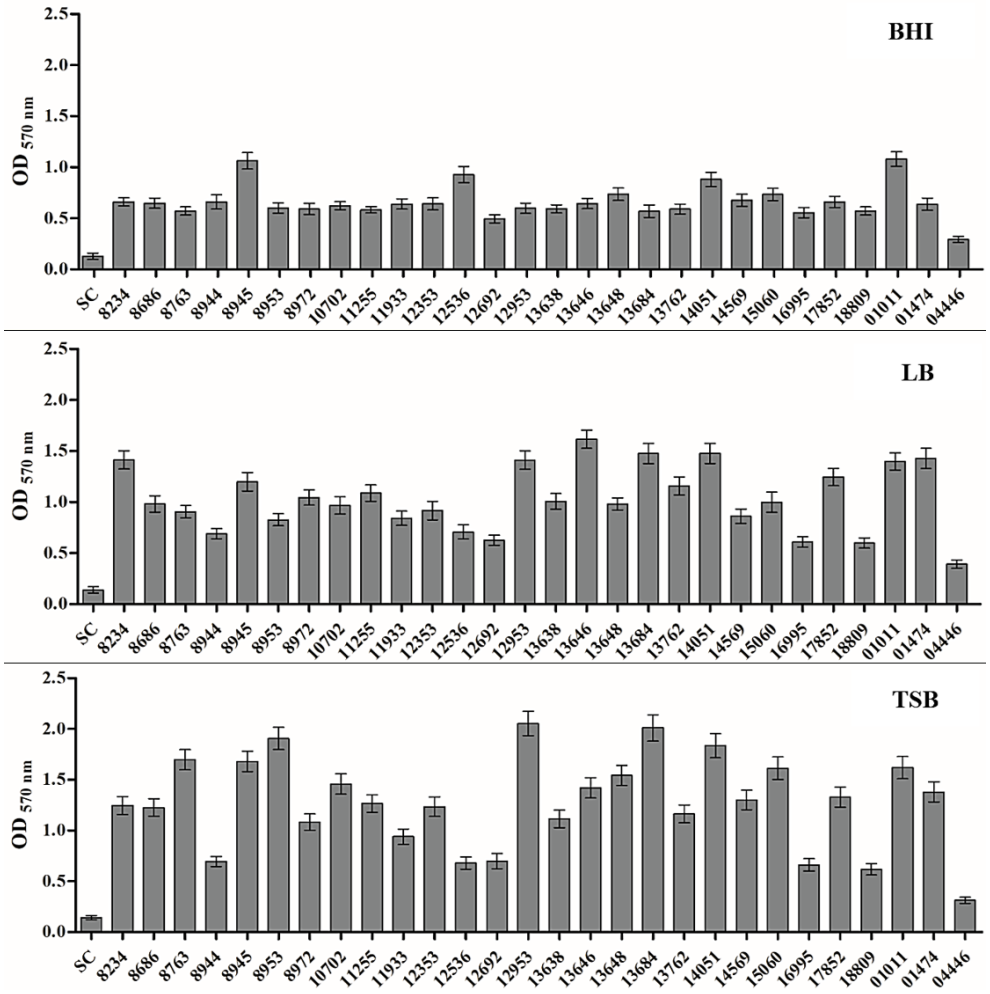

Figure 1. Biofilm formation revealed with violet crystal. Biofilms prepared in polystyrene microplates were expressed by the mean optical density (OD) 570 nm and compared with the sterility control (SC). Brain Heart Infusions (BHI), Luria Bertani (LB), Tryptic Soy Broth (TSB).

To the CSH profile, there classified 27 isolates as hydrophilic, with a hydrophobicity below 30%. Only the isolate 12353 showed a percentage between 30% and 70% and was therefore classified as moderately hydrophobic (Figure 3, Table I).

Once analyzed the capacity for biofilm formation and the hydrophobicity profile of the isolates, there also investigated genes related to the biofilm formation mechanism such as *mrkD* and *fimH* and their prevalence in the main species from the *Enterobacteriaceae* family. Interestingly, none of the isolates investigated in the present study were positive for these genes (Figure 4, Table I).

Table I. Biochemical and genetic characteristics of the investigated isolates.

Isolates	Biofilm			Hidrophobicity	Genes	
	BHI	LB	TSB		*fimH*	*mrkD*
8234	+++	++	+++	Hydrophilic	–	–
8686	+++	++	+++	Hydrophilic	–	–
8763	+++	++	+++	Hydrophilic	–	–
8944	++	++	++	Hydrophilic	–	–
8945	+++	+++	+++	Hydrophilic	–	–
8953	+++	++	+++	Hydrophilic	–	–
8972	+++	++	+++	Hydrophilic	–	–
10702	+++	++	+++	Hydrophilic	–	–
11255	+++	++	+++	Hydrophilic	–	–
11933	+++	++	+++	Hydrophilic	–	–
12353	+++	++	+++	MO Hydrophobic	–	–
12536	++	+++	++	Hydrophilic	–	–
12692	++	++	++	Hydrophilic	–	–
12953	+++	++	+++	Hydrophilic	–	–
13638	+++	++	+++	Hydrophilic	–	–
13646	+++	++	+++	Hydrophilic	–	–
13648	+++	++	+++	Hydrophilic	–	–
13684	+++	++	+++	Hydrophilic	–	–
13762	+++	++	+++	Hydrophilic	–	–
14051	+++	+++	+++	Hydrophilic	–	–
14569	+++	++	+++	Hydrophilic	–	–
15060	+++	++	+++	Hydrophilic	–	–
16995	++	++	++	Hydrophilic	–	–
17852	+++	++	+++	Hydrophilic	–	–
18809	++	++	++	Hydrophilic	–	–
01011	+++	+++	+++	Hydrophilic	–	–
01474	+++	++	+++	Hydrophilic	–	–
04446	+	+	+	Hydrophilic	–	–

Strong biofilm producer (+++), moderate biofilm producer (++), weak biofilm producer (+). Brain Heart Infusions (BHI), Luria Bertani (LB), Tryptic Soy Broth (TSB). Hydrophobic MO: moderately hydrophobic. Absent gene (–).

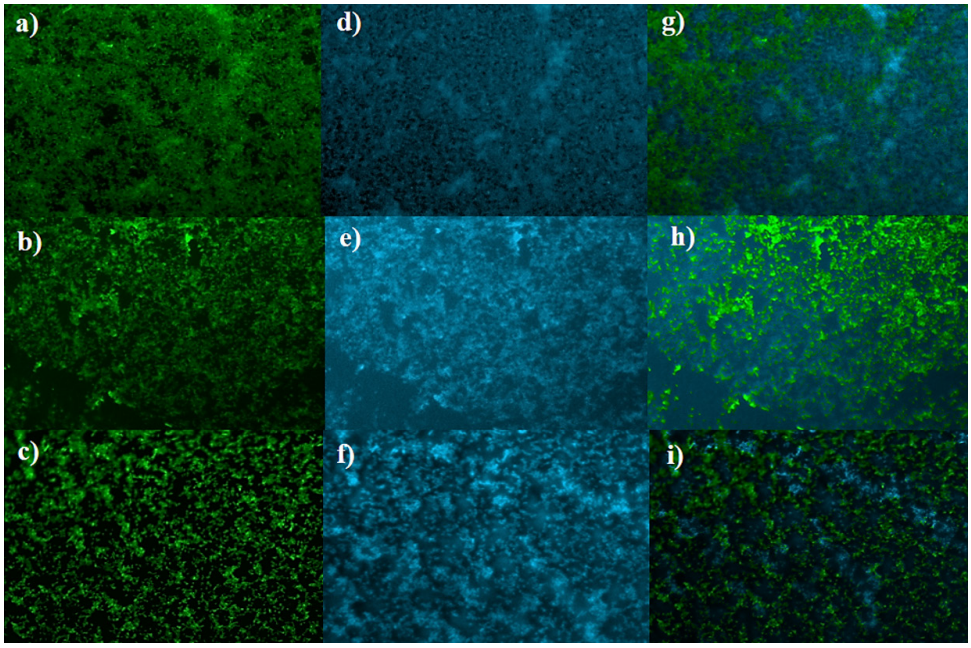

Figure 2. Biofilm of isolate 8945 under fluorescence microscopy. a), b) and c): biofilm formed in BHI, LB and TSB and Stained with SYBR Green. d), e) and f): exopolysaccharides stained with Calcofluor White, respectively. g), h) and i): biofilm cells stained with SYBR Green and Calcofluor White, respectively.

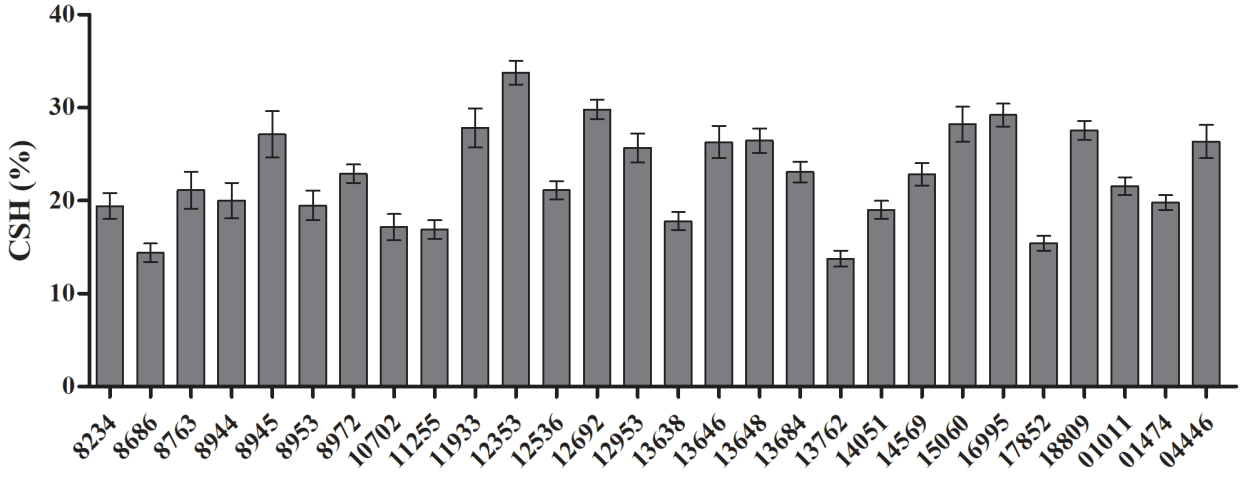

Figure 3. Percentage of the cell surface hydrophobicity (CSH) profile of *P. stuartii* isolates, represented by the mean of three bacterial suspensions in p-xylene (1: 0.2).

DISCUSSION

The structural stability of the biofilm gives the involved bacteria a suitable microenvironment for their survival. It acts as a barrier and, consequently, protecting the entire resident community from antimicrobials and the host immune system. In this way, there characterized biofilm as a great virulence mechanism (Flemming et al. 2016).

In the present study, all isolates were able to biofilm form but with different intensities, especially in BHI and TSB media. However, under the conditions tested, there characterized the isolate 04446 as a weak producer. Our data corroborate Silva et al. (2020) study that analyzed the biofilm formation capacity of *Proteus mirabilis* isolates, a species of *Enterobacteriaceae*, as well as *P. stuartii*. In which there observed a higher formation in these two culture media. Similarly,

another study evaluated the biofilm formation in *Acinetobacter baumannii* and *Pseudomonas aeruginosa* in LB and TSB media in standard concentrations, supplemented with glucose and diluted (25% of the standard) (Lima et al. 2020). Some isolates of these species responded to stress treatments only in TSB. For the authors, the medium dilution that resulted in the nutrient reduction may have induced a higher biofilm formation. Based on these results, isolate 04446 from *P. stuartii* may be capable of form more biofilm if subjected to some stress condition.

Regarding the hydrophobicity profile, there observed no relevant differences between CSH and biofilm formation. The only isolate classified as moderately hydrophobic (12353) presented a percentage closer to the hydrophilic profile and formed biofilm similarly to other isolates. Unlike most studies, our data agree with previous studies that observed higher formation when the bacteria had hydrophilic cell surfaces (Czerwonka et al. 2016, Araújo et al. 2019, Silva et al. 2020). Moreover, a study has shown that *P. stuartii* is capable of adhering and invading epithelial cells effectively, according to its growth stage (Kurmasheva et al. 2018). Therefore, the data presented here is relevant, mainly because CSH is one of the main physicochemical factors that influence the process of microbial adhesion on substrates. It is noteworthy that most bacterial cells and some surfaces are negatively charged, thus enabling electrostatic repulsion. This fact has contributed significantly to the synthesis of anti-infectious clinical devices, thus preventing microbial adhesion and, consequently, pathogens dissemination in the hospital environment (Trentin et al. 2015, Ren et al. 2018).

For the formation and structure of biofilms, there required a variety of physical-chemical and genetic factors. A study that investigated the biofilm of *P. stuartii* found that in the planktonic state, this species forms communities of floating cells that precede and later coexist with biofilms attached to the surface, showing the specificity of this species related to this virulence factor (El-Khatib et al. 2018). Another study investigated the flexibility of the main porins of this bacterium (OmpPst1 and OmpPst2) and suggested that the movements, especially of OmPst1, may contribute to the adaptation of this species in the environment by interacting with other membrane components such as LPS, important exopolysaccharides in the processes of biofilm formation (Tran et al. 2017). Possibly, the diversity of amino acid residues present in

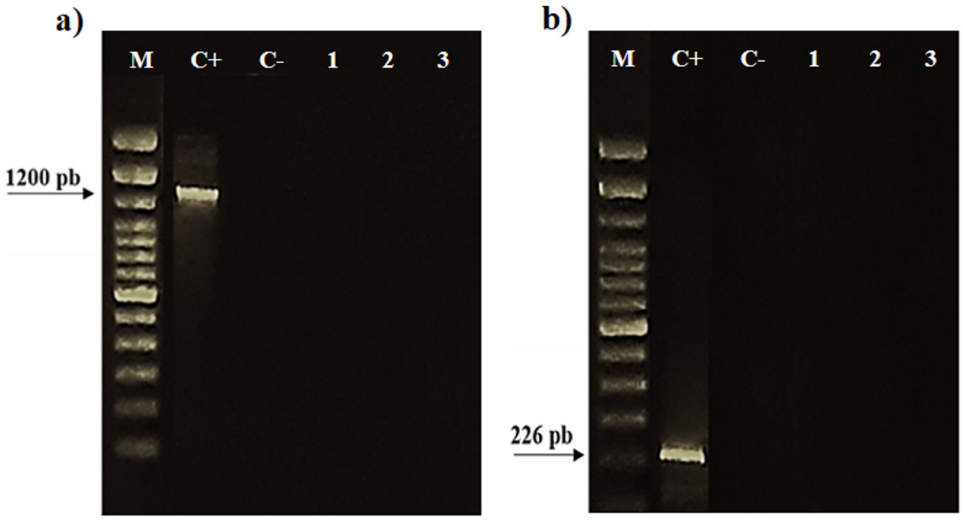

Figure 4. Gel representative of the amplification products of the a) *fimH* and b) *mrkD* genes. M: Molecular marker; C+: Positive control (*K. pneumoniae* / K5-A2), C–: Negative control (milliQ water). 1: 8234, 2: 8945, 3: 01011.

the external domain of this porin helps charge movements and electrostatic interactions with other components of the cell membrane (Arunmanee et al. 2016).

Among the genetic factors related to biofilm formation, a diversity of fimbrial-adhesins stand out, especially in gram-negative bacteria. It can promote cell adhesion and contribute to the development of biofilm. These adhesins interact with specific receptors present on inert and biotic surfaces and can be encoded by chromosomal or plasmid genes (Zamani & Salehzadeh 2018). In the present study, there investigated genes encoding fimbriae of type 1 (*fimH*) and type 3 (*mrkD*), which are identified in different species of the *Enterobacteriaceae* family (Table II), as well as in bacteria from other families (Mohajeri et al. 2016, Tavakol et al. 2018).

There frequently identified the type 1 fimbriae in uropathogenic *E. coli* (UPEC), and it is one of the relevant factors in the adhesion of these pathogens (Zamani & Salehzadeh 2018). But they are also identified in other enterobacteria such as *Klebsiella* spp. and *Samonella* spp. among others (Araújo et al. 2019, Uchiya et al. 2019). Likewise, type 3 fimbriae in some other bacteria in this family (Stahlhut et al. 2013, Azevedo et al. 2018), as seen in Table II.

Given this information and considering the specificities already related to the virulence mechanism described for *P. stuartii*, the present study investigated the *fimH* and *mrkD* genes related to bacterial invasion and colonization processes in the urinary tract. However, in the present study, there found no presence of neither of these two genes. To the *fimH,* there are no reports of investigation in this species, even

Table II. Detection of *fimH* and *mrkD* genes in species from *Enterobacteriaceae* family.

Gene	*Enterobacteriaceae*	References
fimH	*Edwardsiella piscicida*	Zhang et al. (2019)
	Enterobacter aerogenes	Azevedo et al. (2018)
	Escherichia coli	Feenstra et al. (2017), Chen et al. (2018), Rabbani et al. (2018), Zhang et al. (2020)
	Klebsiella pneumoniae	Ferreira et al. (2019)
	Klebsiella oxytoca	Ghasemian et al. (2018)
	Salmonella enterica Enterica	Rakov et al. (2019)
	Salmonella enterica serovar Enteritidis	Kuźmińska-Bajor et al. (2012, 2015)
	Salmonella enterica serovar Choleraesuis	Grzymajlo et al. (2013), Lee & Yeh (2016)
	Salmonella enterica serovar Typhimurium	Zeiner et al. (2012), Uchiya et al. (2019)
mrkD	*Citrobacter freundii*	Ong et al. (2010)
	Citrobacter koseri	Ong et al. (2010)
	Enterobacter aerogenes	Azevedo et al. (2018)
	Enterobacter cloacae	Amaretti et al. (2020)
	Escherichia coli	Ong et al. (2010), Stahlhut et al. (2013)
	Klebsiella oxytoca	Ong et al. (2010), Ghasemian et al. (2018)
	Klebsiella pneumoniae	Ong et al. (2010), Stahlhut et al. (2013), Ferreira et al. (2019), Imai et al. (2019), Amaretti et al. (2020)
	Klebsiella quasipneumoniae	Imai et al. (2019)
	Klebsiella variicola	Imai et al. (2019)

though it encodes an essential structure for the invasion and colonization by bacteria, especially the urinary tract. While for *mrkD,* the possible association of this gene with the persistence of *P. stuartii* in cases of bacteriuria was reported in the 1980s (Mobley et al. 1988). However, there found no more recent experimental studies using molecular tools, that could confirm the presence of this sequence in this pathogen genome.

The absence of the two investigated genetic sequences in the present study indicates that, possibly, these genes do not correspond to determining factors for the formation of biofilms in *P. stuartii*, differently from what was found for other representatives of the *Enterobacteriaceae* (Table II). This information is interesting and reveals that this species has specific genetic and biochemical features not yet described or little known in species of that family.

These data demonstrate that understanding and clarifying possible mechanisms involved in bacterial biofilm formation is essential for understanding the composition of the extracellular matrix, subtypes, and cell behaviors. Also, the adhesion mechanisms involved in the survival and permanence of *P. stuartii* in the colonization process to, eventually, prevent or control chronic infections caused by this pathogen. The data presented here reinforce the need for further studies due to the specificities found in this species. These particularities have revealed an increase in the resistance profile (Silva et al. 2020) and survival of this bacterium in the hospital environment, making it difficult, therefore, to fight the possible infections.

CONCLUSIONS

In conclusion, our results confirm and demonstrate some specificities of *P. stuartii* regarding its potential for biofilm formation. The hydrophilic cell surface of this bacterium indicates that interaction with the abiotic surface investigated in this study may occur and, consequently, may facilitate cell-cell interaction. Additionally, other types of fimbrial adhesins not yet reported in the literature may be related to your biofilm formation. Thus, this study reinforces the need for further research to eventually prevent chronic bacterial infections and assist clinical treatments.

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