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The bacterial diversity associated with biofilm-forming ability was studied. Eighteen bacterial strains were isolated from a microbial film collected from the roof of an old house located in Sfax, Tunisia. The purity of these microorganisms was confirmed by microscopic observation after repeated streaking on a Tryptic Soy agar medium. Biofilm formation was estimated using preliminary tests including a motility test, microbial adhesion to solvents (MATS), and the Congo Red Agar method (CRA). Since these tests showed no significant result, microplate tests, such as crystal violet and resazurin assays, were used. The results obtained showed that strain S61 was able to form a biofilm within 24 h (OD_{570} = 4.87). The viability of the S61 biofilm with resazurin assessed with fluorescence measurement was about 1.5×10^3. The S61 strain was identified as *Staphylococcus epidermidis*. In the biofilm studied here, it was the most biofilm-forming bacterium and will be used as a bacterial model for studying anti-biofilm activity.

Key Words: biofilm; crystal violet; resazurin; *Staphylococcus epidermidis*

Introduction

Most of the biomass of microorganisms on the planet live in biofilms, and more than 75% of all infections are caused by biofilms (Davies, 2003; Richards and Melander, 2009). A biofilm is a heterogeneous and structured community of aerobic or anaerobic microorganisms adhering to each other and to an inert or biological surface (Drenkard, 2003). A biofilm is surrounded by a protective and adhesive matrix of exopolysaccharides (Hall-Stoodley et al., 2004). The first biofilms ever described were in natural aquatic environments by Zobell (1943). In the natural environment and in the human host, bacteria fluctuate between two forms: mobile cells and cell-forming biofilms, the latter being the most common mode (Jefferson, 2004). Biofilms are found ubiquitously, since they contaminate a wide variety of surfaces, including food contact surfaces, distribution systems (Davey and O'Toole, 2000), dental plaques (Rickard et al., 2002), medical implants (Stewart and Costerton, 2001), aquatic systems (Rickard et al., 2000), and oil refineries and plumbing (Stewart and Costerton, 2001). Living bacteria within the biofilm are less susceptible to antibiotics and disinfectants than planktonic cultures of the same organisms (Simoes, 2011). In fact, the required concentrations of antibiotics needed to inhibit bacteria within biofilms may be up to 1,000 times greater than that used to inhibit the same bacteria in planktonic states (Simoes, 2011).

Biofilms can cause various problems in different areas, such as medicine and the agri-food sector (Fux et al., 2005). In addition to being more resistant to antibiotics, biofilms are more resistant to phagocytosis and other components of the body defense system, than are planktonic cells (Høiby et al., 2010). The comparison between the sessile and planktonic forms of some pathogenic bacteria for antibiotic resistance shows that biofilm production is the main cause of the appearance of infections and diseases (Cerca et al., 2005). These bacterial infections are very hard to eradicate, particularly in the case of multiresistant hospital bacteria. In this regard, it would be interesting to isolate biofilm-forming bacteria from non-hospital environments.

In Tunisia and in many countries in the world, mainly

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where water is scarce, a great majority of houses are equipped with a roof-harvested rainwater tank, to be used as drinking water (Meera and Ahammed, 2006). For that, the study of the microbial biofilms colonizing the house roof is of great importance, either to get an idea of the microbial diversity within the biofilms, and, hence, the species which could contaminate and form biofilms in tank rainwater (Kim and Han, 2014), or for using the biofilm-forming microbes for an evaluation of anti-biofilm-forming activities of some interesting agents and molecules. In this context, the aim of the present work is to select biofilm-forming bacteria from the natural environment that could be used as a model for studying anti-biofilm activities.

Material and Methods

Bacterial strains and chemicals. The *Escherichia coli* (*E. coli*) laboratory strain DH5α is a non-biofilm-forming bacterium and has been used as a negative control (Houdt et al., 2004), whereas three strains *Pseudomonas aeruginosa* PAO1 (*P. aeruginosa*, M1), *Staphylococcus aureus* ATCC33581 (*S. aureus*, M2) and *Pseudomonas aeruginosa* ATCC 27853 (*P. aeruginosa*, M3) have been used as positive controls. For growth and biofilm-formation assays, the strains were grown in a Tryptic Soy Broth (TSB) medium at 30°C in a rotary shaker (150 rpm) before inoculation in 96-well flat bottom polystyrene plates (Orange scientific). All strains were stored at –80°C in a 20% (v/v) glycerol medium until use. Crystal violet and resazurin were purchased from Loba-chemie, India. Congo Red was obtained from Sigma-Aldrich, Germany. Safranin was obtained from Protulab.

Isolation of biofilm-forming bacteria. Bacterial strains were isolated from the roof of an old house located in Sfax, Tunisia (34°44′ N, 10°43′ E). The roof surface was scratched with a sterilized spatula and a sample was collected in a clean and sterile bottle. The sample was first suspended in sterile distilled water for 24 hours. The overnight suspension was serially diluted with sterile distilled water up to 10^{-8} dilution and 100 μL of each dilution was spread on Tryptic Soy agar plates and incubated at 30°C for 24 h, until colony formation. Single colonies were picked and purified by repeated streaking on a Tryptic Soy agar medium followed by microscopic observations. All isolation experiments were performed under sterile conditions using a laminar flow cabinet (Nuaire, USA).

Identification of bacterial strains. Molecular identification of the different strains was carried out by 16S rRNA amplification and sequencing. Genomic DNA was extracted with the WiZard Genomic DNA purification Kit (Promega). To determine the 16S rRNA gene sequence, PCR was conducted using the universal primers 16S-27F (5′AGRGTTTGATCMTGGCTCAG3′) and 16S-1492R (5′TACGGYTACCTTGTTAYGACTT3′) in a thermal cycler (ABI, Applied Biosystems). A polymerase chain reaction was performed in 10-μL volumes comprising 0.4 μL of each primer (10 μM), 5 μL of KAPA2G ready Mix (2X) (Clinisciences) (containing $MgCl_2$, Buffer and dNTP), 3.2 μL ultrapure water and 1 μL DNA. Reaction without DNA was used as a negative control. PCR amplification was carried out as follows: initial denaturation for 5 min at 94°C followed by 35 cycles of 15 s at 94°C, 15 s at 50°C, 20 s at 72°C and a final elongation for 10 min at 72°C. The amplified product (10 μL) was resolved on 1% (w/v) agarose gel at 100 V. In order to remove unincorporated dNTPs, excess primers and salts, the PCR products were purified with the AMPure PCR purification kit according to the manufacturer's instructions (Agencourt Bioscience Corporation). The cleaned PCR products were then used as templates for the sequencing reaction using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and the reaction was run for 40 cycles in a thermocycler (AB2700) as follows: 10 s at 95°C, 5 s at 50°C and 2 min 30 s at 55°C. The total volume of the sequencing reaction mix was 10 μL containing 0.5 μL of the ready mix (Big Dye Terminator v3.1 cycle sequencing) from the kit, 1 μL of the purified PCR product, 1.75 μL BDT sequencing buffer (5X), 5.75 μL ultrapure water and 1 μL of the primer (907r) (3.2 μM). The results were analyzed by the codon code Aligner software and the 16S rRNA gene sequences were compared with the NCBI GenBank database by Blast analysis in order to find identities between sequences. Phylogenetic analysis was conducted using MEGA, version 4.

Detection of biofilm formation.

Preliminary tests:

Bacterial motility test. The motility study consisted of three swimming, swarming and twitching tests. These tests were performed in Petri dishes using the solid medium Tryptic Soy with 0.3% (w/v), 0.5% (w/v) and 1.5% (w/v) agar, respectively (Rashid and Kornberg, 2000). Swim and swarm plates were inoculated with bacteria from an overnight culture in TSB medium. For this purpose, 5 μL of bacterial culture was deposited with the tip of the cone on the surface of sterile agar. In the twitching plate, 5 μL of bacterial culture was injected into the center of the agar with the cone tip. After incubating the Petri dishes for 24–48 h at 30°C, the bacterial mobility area was determined by measuring the migration of strain diameters.

Microbial adhesion to solvents (MATS). Microbial adhesion to solvents was measured according to the method described by Bellon-Fontaine et al. (1996) with slight modifications made by Grasland et al. (2003). Three organic solvents were used: chloroform, ethyl acetate and hexane. In brief, a bacterial culture was grown overnight in TSB medium at 30°C in a rotatory shaker (150 rpm). The culture was centrifuged at 6,000 rpm for 10 min to obtain the pellet which was subjected to two successive washes with water and subsequently resuspended in 1.5 mM NaCl to obtain an OD of approximately 0.8 at 400 nm (A0). The bacterial suspension (2.4 mL) was vortexed separately with 0.4 mL of each solvent for 1 minute. After 15 minutes of decantation at room temperature, the optical density of the aqueous phase (A1) was measured. In order to complete this test, the percentage of microbial adherence to each solvent was calculated as follows: $(1 - (A1/A0)) * 100$. Each measurement was carried out in triplicate.

Congo Red Agar Method (CRA). The test was carried out as previously described by Freeman et al. (1989), re-

quiring the use of CRA, a specially prepared solid medium. The CRA medium was composed of Brain-Heart Infusion broth (37 g L^{-1}, sucrose 50 g L^{-1}), agar 10 g L^{-1} and Congo Red stain 0.8 g L^{-1}. The Congo Red solution was prepared and autoclaved separately from the other constituents and then added when the agar was cooled to 55°C. Plates were inoculated with different tested microorganisms and incubated for 24 hours at 37°C. A positive result was indicated by the production of black streaks or colonies with a dry crystalline consistency, whereas the presence of pink or red colonies was regarded to be a negative result (Freeman et al., 1989).

Microplate tests:

Crystal violet assay. For the biofilm formation assay, strains from fresh agar plates were inoculated in 5 mL of Tryptic Soy Broth and incubated for 24 hours at 30°C. Biofilm formation was conducted in 96-well flat bottom plates (with lid) (Mathur et al., 2006). In order to increase biofilm formation (Moreira et al., 2013), culture strains were diluted with a fresh medium (which commonly contains 0.25% (w/v) glucose) supplemented with 2.25% glucose (Hola et al., 2004) until a final OD_{600nm} of 0.1 in each well was reached. The microplate wells were filled with 200 μL of diluted culture, whereas only broth with glucose served as a control to check the sterility of the medium and non-specific attachment. *E. coli* DH5α was used as a negative control, and *P. aeruginosa* M1, *S. aureus* M2 and *P. aeruginosa* M3 were used as positive controls. Each inoculated plate was covered with a lid and incubated for 24–48 h at 30°C under static conditions. After incubation, the wells were emptied by tapping the bottom plates into a disposal vessel. Each well was washed twice with 250 μL of sterile phosphate buffered saline (PBS, pH 7.2) using a suitable micropipette to remove all planktonic cells and the medium, while preserving the integrity of the biofilm (Beenken et al., 2003). After washing, the attached bacteria were left to dry at 60°C for 60 min to promote biofilm fixation. In order to visualize the biofilm formation, the remaining bacteria attached to the bottom of wells were stained for 15 min at room temperature with 150 μL of 0.2% crystal violet solution prepared in 20% ethanol (v/v) (Vasudevan et al., 2003). Subsequently, crystal violet was eliminated and excess stain was rinsed three times with sterile water. The microplates were then inverted to remove any dye that was not staining the attached bacteria. Finally, 200 μL of glacial acetic acid were added to each well. The plates were incubated for 1 h at room temperature to promote the biofilm bursting and solubilization of crystal violet that had already penetrated into the cells. All tests were performed in triplicate. The optical density (OD) of each well was measured at 570 nm using a microplate reader. For the classification of isolates according to their ability to form biofilms, a cut-off value was calculated for the negative control strain DH5α, by the following formula: OD_c = Average OD of negative control + 3 × SD (Standard Deviation) of negative control. Based on the cut-off OD calculated, strains were classified into the following categories: non-biofilm producers (0): ($OD < OD_c$), weak biofilm producers (+): ($OD_c < OD < 2 \times OD_c$), moderate biofilm producers (++): ($2 \times OD_c < OD < 4 \times OD_c$) and strong biofilm producers (+++): ($OD > 4 \times OD_c$) (Stepanovic et al., 2007).

Resazurin assay. Viability was determined using the blue-colored dye resazurin, which was reduced by viable bacteria to the pink, fluorescent compound resorufin (Toté et al., 2010). After removing the TSB medium and non-adherent cells and washing with PBS, resazurin, prepared in sterile water at a concentration of 10 μg mL^{-1}, was added directly to the wells. The plate was incubated for 30 min in darkness at room temperature followed by measurement of the resorufin fluorescence using an excitation at 560 nm and an emission at 590 nm with a microplate reader (Varioscan Flash, Thermo Scientific).

Confocal microscopy: Biofilm visualization was performed using confocal scanning microscopy. Biofilms were grown overnight on glass coverslips. After the coverslips were washed with distilled water to remove the non-adherent bacteria, they were stained using a Live/Dead Light bacterial viability kit (Molecular Probes, Invitrogen). The kit contained a mixture of two different nucleic acid fluorescents represented as two components: component A composed of 1.67 mM Syto9 plus 1.76 mM propidium iodide (PI) and component B composed of 1.67 mM Syto9 plus 18.3 mM PI. Syto9 stained all viable bacteria in green, while PI stained in red dead bacteria whose membranes were damaged. The mixture used for staining the biofilm was prepared by the dilution of 4 μL of component A and 6 μL of component B in 1 mL of distilled water. Biofilm staining required the deposition of 200 μL of the mixture stain on the coverslips. After 30 min of incubation in the dark at room temperature, the coverslip was gently washed with distilled water. Biofilms were observed under a Zeiss LSM confocal laser scanning microscope 510 using a 488 nm laser line of argon, with emission wavelengths from 500 to 550 nm and from 598 to 660 nm for Syto 9 and PI, respectively. A 63X water immersion objective lens was used. Images were assessed using Zeiss Software, version 3.20.070, recorded with a picture size of 640–424 pixels and arranged using a Photo Filter (Bauer et al., 2013).

Statistical analysis: Statistical analysis was performed using the IBM SPSS Statistics Version 22. The comparison between 24 h and 48 h biofilm formation in the crystal violet assay data was analyzed by a paired sample T-test. A level of significance set at 5% was used to perform the analysis.

Results and Discussion

Isolation and characterization of biofilm-forming bacteria

Eighteen biofilm-forming bacteria were isolated on Tryptic Soy agar medium after purification based on successive streaking using microscopic observations to ensure bacterial strain purity. These strains were isolated from the roof of an old house located in the region of Sfax, Tunisia. Phylogenetic analysis of the 16S rRNA gene sequences allowed identification of eighteen isolates of aerobic strains. Twelve bacterial strains (S2, S5, S11, S12, S17, S19, S21, S31, S91, S111, S112 and S181) belonged to *Serratia proteamaculans*. Two strains (S4 and S41) were affiliated to *Klebsiella variicola*. Strains S14 and S22 were phylogenetically related to *Pseudomonas*

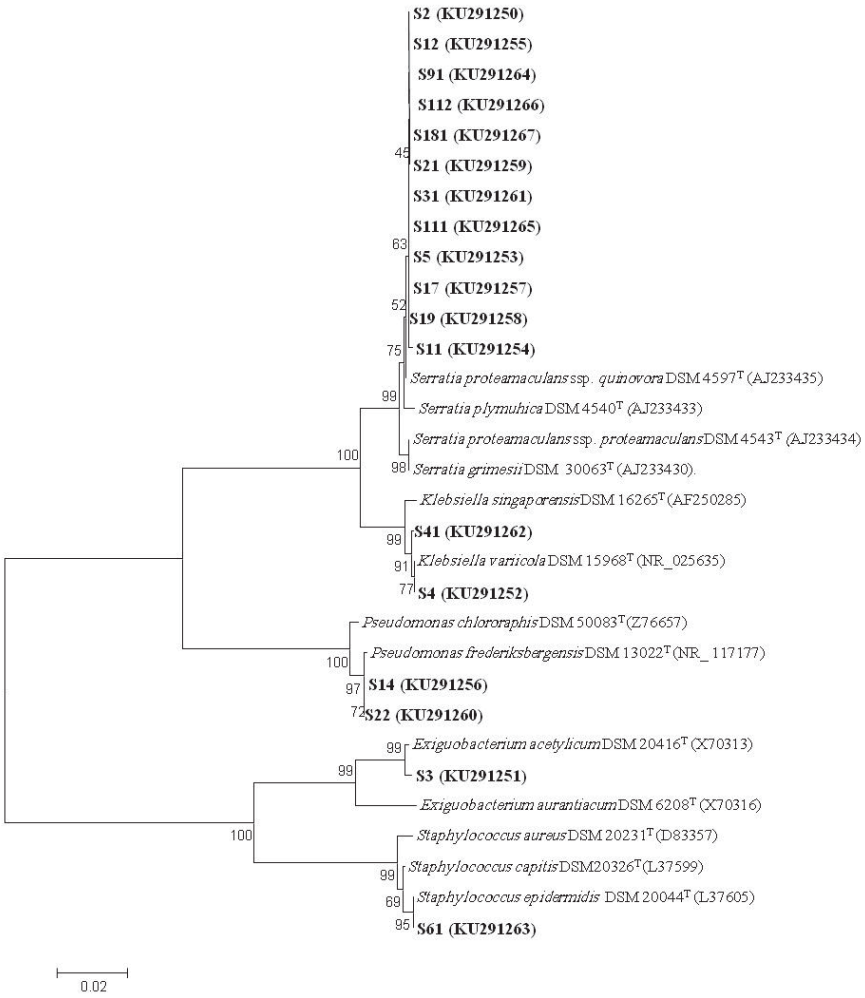

Fig. 1. Phylogenetic positions of the aerobic microorganisms isolated from the roof of an old house in relation to other species.

Sequence accession-numbers are given in parentheses. The bar represents 2 nucleotide substitutions per 100 nucleotides.

frederiksbergensis. The last two strains (S3 and S61) were affiliated to the species *Exiguobacterium acetylicum* and *Staphylococcus epidermidis*, respectively. The phylogenetic tree of the aerobic isolated strains and their closely related species is shown in Fig. 1. The 16S rRNA gene sequences of 18 isolates have been deposited with the Genbank database under accession numbers from KU291250 to KU291267.

Evaluation of biofilm-forming bacteria

Preliminary tests.

Motility test: It is crucial to assess the motility of bacteria, and several authors have highlighted its importance by the presence of bacterial flagella in the early stages of biofilm formation (Moreira et al., 2003). Bacterial motility was illustrated by measuring the migration distances in Petri dishes. The results of swimming, swarming and twitching assays are shown in Fig. 2. All strains migrated more by swimming than swarming and twitching. In the swimming test, the majority of the tested strains showed very significant migration distances and covered the entire Petri dish. A comparison of the migration distance for the three tests indicated that the majority of the isolated strains migrated more than the negative control DH5α, known as a low-motile strain (Wood et al., 2006). In fact, some authors reported that six to ten flagella were required for swimming motility, whereas swarming motility required an increased number of flagella (Rashid and Kornberg, 2000). Other studies demonstrated that the motility, by either swimming or swarming, required the presence of flagella but with a difference between these two modes. In fact, motility by swimming is favored by the movement of individual cells in three dimensions, while swarming is a multi-cellular movement in two dimensions (Darnton et al., 2010) favored by a collision with neighboring bacteria (Kearns, 2010).

Twitching motility is the result of the extension and the retraction of the pili filaments (Merz et al., 2000). The type IV pili are responsible for movement by twitching (Mattick, 2002), and consequently for biofilm formation on abiotic surfaces (Touhami et al., 2006). The migration diameters obtained with the twitching motility were obviously less than those obtained with the two other motility tests (Fig. 2). Even so, strains M2, M3, S4, S14, S91 and S112 showed a migration diameter in twitching motility much higher than that of the negative control DH5α (Fig.

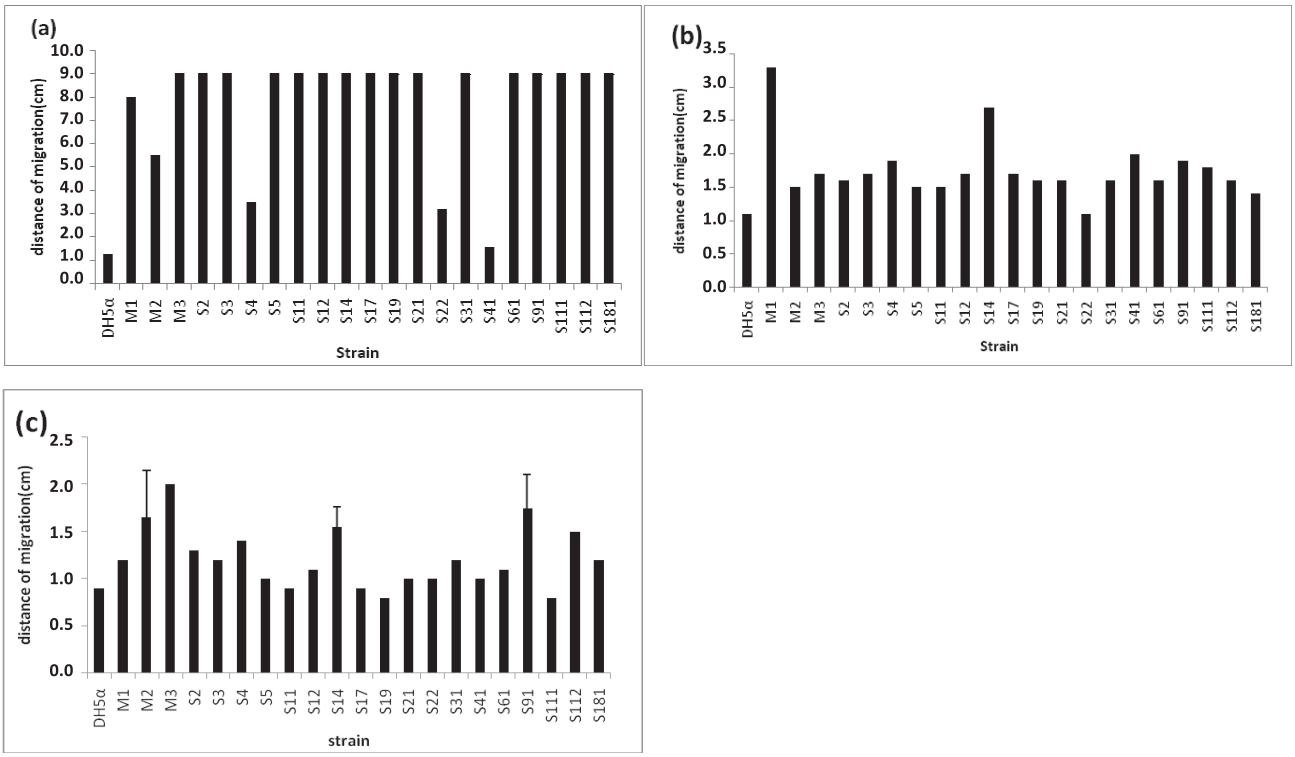

Fig. 2. Evaluation of motility assays swimming (a), swarming (b) and twitching (c).

2).

Microbial adhesion to solvents (MATS): It is important to study the physicochemical properties of the cell surface, because the adherence of microbial cells to a solid support is the result of physico-chemical interactions between the cell surface and that of the inert support. Adhesion to solvents is based on the comparison between microbial cell surface affinity to different solvents, which enables the assessment of the Lewis acid/base properties and the hydrophilic/hydrophobic nature of bacterial surfaces (Bellon-Fontaine et al., 1996). This test involves the use of three different solvents: chloroform (acidic), ethyl acetate (basic) and hexane (apolar). The affinities of the strains to different solvents are presented in Fig. 3. Hexane was used as a reference solvent for evaluating the hydrophobicity of the bacterial strains. In fact, the adhesion to hexane enabled the determination of the hydrophilic/hydrophobic character of the bacterial strain. The percentage of adhesion to hexane varied widely between the strains within the range 2.4% to 89.6%, and bacteria are considered to be hydrophobic when the percentage of adhesion to hexane exceeds 50%, and they are considered to be hydrophilic if the percentage is below 20% (Krepsky et al., 2003). If the percentage is within these two values, the bacteria are moderately hydrophobic (Krepsky et al., 2003). According to the degree of hydrophobicity, the strains were divided into three categories: The hydrophobic strains *Staphylococcus epidermidis* S61 and *Serratia proteamaculans* S21 which represent 11.1% of all strains; the moderately hydrophobic strains representing 38.9% of all the strains, were *Serratia proteamaculans* S2, S11, S91, S111, S112, S181 and *Pseudomonas frederiksbergensis* S14, with the two positive control strains *P. aeruginosa* M1 and *S. aureus* M2 being classified in this category; and, finally, the class of hydrophilic strains, which constituted half the tested strains, were *Exiguobacterium acetylicum* S3, *Klebsiella variicola* S4 and S41, *Pseudomonas frederiksbergensis* S22 and *Serratia prtoteamaculans* S5, S12, S17, S19, S31, with the negative control strain DH5α and the positive control strain *P. aeuruginosas* M3 being classified in this group. The first stage of biofilm formation is facilitated by the hydrophobicity of the bacterial surface (Pagedar et al., 2010). In fact, the more hydrophobic bacteria are, the more prone they are to aggregate onto a surface (Donlan, 2002). A high percentage of adhesion to chloroform (acid and electron acceptor solvent) was observed for most tested strains, which is superior to that for hexane (apolar solvent) and that for ethyl acetate (basic solvent and electron donor). These comparisons demonstrated the basic character and, hence, the electron donor property for most strains (Preedy et al., 2014).

CRA method: The slime production of the isolates was evaluated by the Congo Red Agar method (CRA), according to the protocol of Freeman et al. (1989). This method enables the differential detection of slime-forming strains (black colonies on the red agar) and non-slime-forming strains (red-colored colonies). CRA plates showed that about 61.1% of the isolated strains formed rough and black colonies and were considered as slime-producing strains and 39.9% of the isolates were classified as non-producers (red colonies). Most *Serratia proteamaculans* strains were slime-producing, except for the strain *Serratia proteamaculans* S181. The strains *Klebsiella variiloca* S41 and S4, *Pseudomonas frederiksbergensis* S14 and S22, and *Exigobacetrium acetylicum* S3 and *Staphylococcus epidermidis* S61, did not produce any slime. The reference strain, *S. aureus* M2, was positive, whereas the two

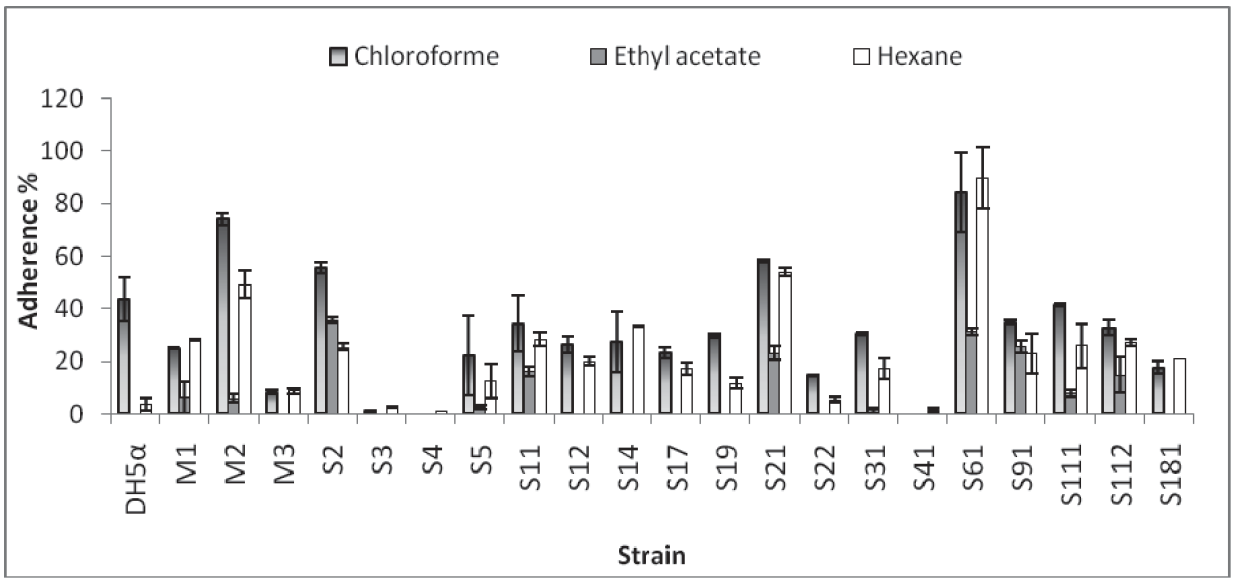

Fig. 3. Microbial adhesion to solvents (MATS) evaluated by the percentage of adhesion to three solvents: Chloroform, Ethyl acetate and Hexane.

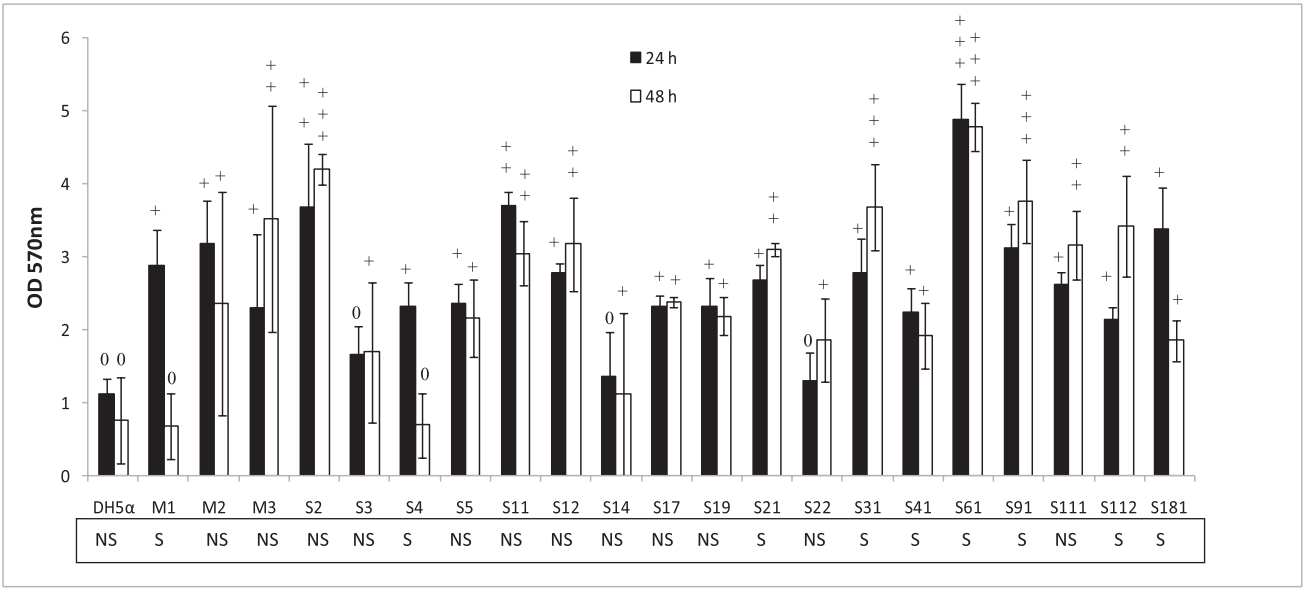

Fig. 4. Differences in biofilm production in 24 and 48 hours.

The classification of biofilm-forming bacteria based on OD cut-off in 24 h and 48 h is shown above histograms: 0: Non-biofilm producer; +: Weak biofilm producer; ++: Moderate biofilm producer and +++: Strong biofilm producer. The comparison between 24 h and 48 h biofilm formation based on the OD values is given below the names of the strains: S, significant ($P < 0.05$); NS, not significant ($P > 0.05$). *P* value was calculated by paired sample T-test SPSS.

positive control strains, *P. aeruginosa* M1 and M3, showed negative results compared with the negative control DH5α which showed a positive result in this test. Several studies have shown that this method has a low accuracy, due to the evaluation criteria being based on the visual analysis of the color of the colonies (Hassan et al., 2011). According to Bozkurt et al. (2009), the CRA method shows erroneous results in the case of the biofilm formation *in vitro* of *Staphylococcus epidermidis*. In contrast, other researchers consider the method as a reliable and specific test for the detection of biofilm formation (Arciola et al., 2005) of *Staphylococcus aureus* and *Staphylococcus epidermidis* (Jain and Agarwal, 2009). The CRA method allows the determination of exopolysaccharide production by the variation in the color of colonies in the medium (Freeman et al., 1989). The formation of colored colonies is due to the reaction of Congo Red with some polysaccharides. In fact, the appearance of black colonies is the result of metabolic changes in the dye, which promote the formation of a secondary product (Arciola et al., 2001). According to the literature, the researchers believe that the presence of a glucose source in the culture medium may play a role in the synthesis of Polysaccharide Intercellular Adhesin (PIA) and may subsequently promote the expression of positive phenotypes in biofilm formation (Dobinsky et al., 2003).

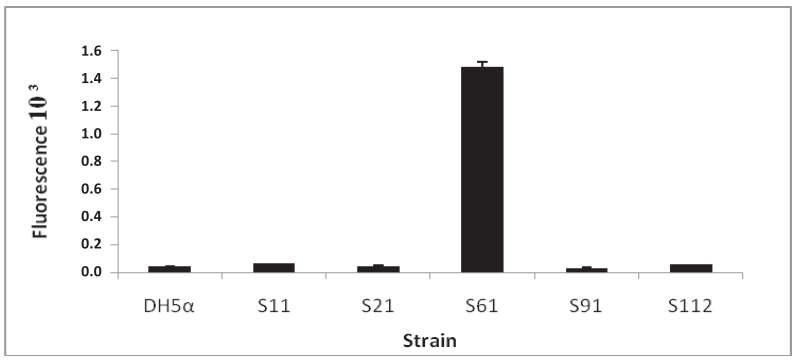

Fig. 5. Evaluation of the cellular viability within the biofilms by the resazurin assay.

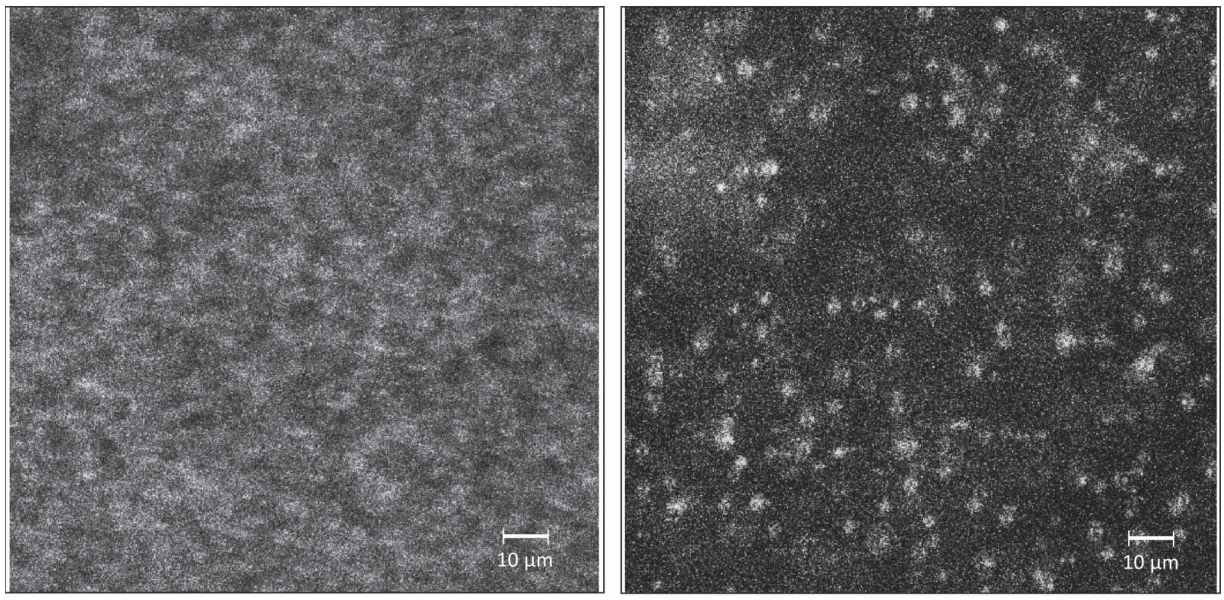

Fig. 6. Confocal microscopy images of biofilm from S61 and biofilm from *S. aureus*.

Biofilms were stained with 481 SYTO9 (green; viable cells) and propidium iodide (red; dead cells). Bar equals 10 μm. (For the interpretation of black and white version: light grey → viable cells; dark grey → dead cells.)

Preliminary tests of motility and microbial adhesion to solvents enabled the characterization of the physicochemical properties of the bacterial surface and motility, in order to obtain an idea about the ability of a given strain to form biofilms. The CRA method is a qualitative test that can lead to false results, due to difficulties in differentiating between moderate biofilm-producing strains and those which do not yield biofilms. Eventually, uncertain results made it necessary to adopt a more reliable quantitative approach to detect and measure the abilities of strains to form biofilms.

In vitro test.

Crystal violet test: This test was used to detect microbial attachment to an abiotic surface. The biofilm formation was quantified at two different time points, 24 h and 48 h using the crystal violet test. In fact, crystal violet is a basic dye that stains the bacteria and exopolysaccharides in the biofilm matrix (Pantanella et al., 2013). This test is a commonly used method for biofilm quantification based on optical density measurement (Pitts et al., 2003). A comparison between the OD values after 24 h and 48 h of incubation showed that there was a significant difference between 44.4% (8/18) of the isolated strains and the positive control *S. aureus*, M1 ($P < 0.05$), whereas 55.5% (10/18) of the isolated strains, the negative control DH5α and the two positive control strains *S. aureus*, M2, and *P. aeruginosa*, M3, did not show a significant difference between 24 h and 48 h ($P > 0.05$) (Fig. 4). The OD obtained for the DH5α strain served for OD_c determination at 24 h and 48 h. The results are presented in Fig. 4. Regarding biofilm formation at these two times, based on the OD_c value, no differences were found for 38.9% (7/18) of the isolated strains and for the positive control strain *S. aureus*, M2. While, for 55.5% (10/18) of the isolated strains and the positive control *P. aeruginosa*, M3, a slight improvement in biofilm production was observed after 48 h of incubation. For the remaining strains, 5.5% (1/18) of the isolated strains, a decrease in the biofilm formation was obtained at 48 h compared with that obtained at 24 h. These results show that the time necessary for biofilm formation varies between the strains. Some strains form a biofilm rapidly after 24 h, whereas others require 48 h to generate a mature biofilm. After 24 h of incubation, most isolates formed a biofilm, except in the case of three strains:

Exigobacterium acetylicum S3, *Pseudomonas frederiksbergensis* S14 and *Serratia proteamaculans* S22. On the other hand, after 48 h of incubation, only *Klebsiella variicola* S4 and the positive control *S. aureus*, M1, can be considered as non-biofilm producers. However, *Staphylococcus epidermidis* was the only strain which qualified as a strong biofilm producer after 24 h of incubation while, after 48 h of incubation, 4 strains were strong biofilm producers (S2, S31, S61 and S91). *Staphylococcus epidermidis* strain S61 was most efficient than the three positive control strains to generate a biofilm.

According to the literature, *Staphylococcus epidermidis* can form a multilayer biofilm by adhering to surfaces (Fey and Olson, 2010). However, the total biomass measured by the crystal violet test is reported to include viable and dead bacteria (Christensen et al., 1985). Therefore, it is important to determine the viability of bacteria within the biofilm. To do this, we used the resazurin test. Based on the comparison between the results of the different assays used, 5 strains, *Serratia proteamaculans* S11, S21, S112, S91 and *Staphylococcus epidermidis* S61, showed slight correlations between preliminary, and crystal violet, microplate tests. Therefore, these strains were selected as the most productive biofilm-producing strains to test biofilm cell viability by the resazurin assay.

Resazurin test: The intensity of the fluorescent pink color is directly proportional to the quantity of living cells, and reflects the viability of the biofilm (Pettit et al., 2005). This test showed that the strain *Staphylococcus epidermidis* S61, which presented the most important biofilm formation by the crystal violet assay, presented the most important viability within the biofilm. Indeed, for this strain, the fluorescence value was in the order of 1.5×10^3, much higher than was the case with the other biofilm-forming strains and the negative control strain DH5α (Fig. 5). The pink coloration explained the high cell viability within the biofilm matrix after 24 h and indicated a low mortality rate. The results obtained with the crystal violet test were not correlated with those obtained with the resazurin assay. In fact, the crystal violet quantifies the biofilm matrix, including viable and dead bacteria (Christensen et al., 1982), whereas the resazurin assay quantifies only the viable bacteria within the formed biofilm (Toté et al., 2010).

Confocal microscopy. Biofilms formed on glass cover slips were observed after staining with live/dead fluorophores. Figure 6 shows two typical dimensional pictures obtained of the biofilm of strain S61 and the biofilm of the reference strain *S. aureus* M2. The viability of S61 within the biofilm matrix was comparable with that obtained with M2 (Fig. 6). *Staphylococcus epidermidis* has been frequently isolated in a hospital environment. It is usually classified as one of the major causes of catheter-associated infections (Cerca et al., 2005; Krepsky et al., 2003). One of the causes of the antibiotic resistance of this strain is its ability to form a biofilm. The antibiotic resistance found in hospital environments could also be explained by mutations or the acquisition of genetic mobile elements, such as plasmids, integrons, transposons, etc. (Arciola et al., 2001). However, it is not surprising to find *Staphylococcus epidermidis* in a biofilm on the roof of an old house. According to the literature, *Staphylococcus epidermidis* has also been isolated from a variety of natural environments, such as the marine environment (Gunn and Colwell, 1983), drinking water distribution systems (Faria et al., 2009), a saltwater lake (Ghasemi et al., 2011), soil from a wastewater discharge (Yu et al., 2014), and textile wastewater (Ayed et al., 2010). The strain isolated here is considered to be a promising model for the study of anti-biofilm molecules. It is expected that this strain will be less resistant than those found in hospital environments, and therefore less dangerous to work with.

Conclusion

A cultural approach was used in this study to screen, identify and select biofilm-forming bacteria from a roof of an old house. The most efficient strain for forming a biofilm was identified as *Staphylococcus epidermidis*. The isolation of *Staphylococcus epidermidis* from a non-hospital environment presents an advantage. In fact, this strain can be used as a biofilm-forming model to study anti-biofilm activities while avoiding the manipulation risk of multi-resistance clinical strains. A study of the physico-chemical characteristics of the strains was used to gain preliminary ideas on the ability of the strains to form biofilms, but it was found that most tests did not reflect the actual biofilm-forming potential of the strains. For example, *Staphylococcus epidermidis* S61 showed no correlation between motility, MATS, the CRA method and crystal violet assay. In this study, the crystal violet assay and viability with resazurin were considered to be the most reliable methods for the detection and quantification of the biofilm-forming potential.

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