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Influence of both media carbon source and strains variety on the Biofilms formation ability by *Pseudomonas aeruginosa*

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ABSTRACT: The ability of biofilms formation was studied by utilizing fifty *Pseudomonas aeruginosa* strains isolated from both environmental and hospital samples. The support utilized for strains fixation was in polystyrene and contained two liquid media: Luria-Bertani and Mineral Medium supplemented with Hexadecane as the sole source of carbon and energy. The results obtained showed that the Biofilms-forming ability depend on both media carbon source and the *P. aeruginosa* strain variety. When the carbon source is hard to degrade or toxic, like hexadecane or derived substrates, the formed biofilms presented a high density; however, when the carbon is easy to degrade by the strains; like that one of L.B medium, the formed biofilms have a slight density. Furthermore, this density is also influenced by the strains although they belonged to the same species; at this purpose, the ERIC - PCR analysis, showed that *P. aeruginosa* strains studied are various because their profil ERIC present a percentage of similarity which not exceeds 60%.

KEYWORDS: Biofilm, carbon source, growth, *Pseudomonas aeruginosa*, ERIC-PCR.

1 INTRODUCTION

Among the strategies to clean up pollutants, it is widely recognized that biological treatments, so called bioremediation technologies, have the advantage over chemical and physical treatments in terms of their compatibility with global cycles and thus less impact on the environment [1].

One of the options for effective clean-up methods of contaminated sites is the use of carrier materials so as to maintain sufficient activity of inoculants during prolonged periods. There are several reports demonstrating that immobilized or encapsulated cells effectively degrade pollutants at the contaminated sites [2],[3]. However, the cellular and molecular mechanisms underlying these technologies are largely unknown [4].

In natural environments, bacteria often exist in closed association with surfaces and interfaces, where they form biofilms. Biofilms are structured microbial communities formed on the surface of solid materials or interfaces [5]. It has recently been pointed out that environmental microorganisms exist predominantly as biofilms and gain high tolerance to physical, chemical, and biological stresses [6].

Biofilm-associated cells exhibit specific gene expression, controlled many times by quorum sensing systems, or dormancy, to allow their increase in resistance [7], [8], [9], [10], [11]. Thus, forming biofilms is considered a natural strategy of microorganisms to construct and maintain a favorable niche in stressful environments [12], [13].

The aim of this study is to demonstrate the performance of some *Pseudomonas aeruginosa* (*P. aeruginosa*) strains isolated from both environmental and hospital samples to form biofilms on surface containing a carbon source toxic or hard to degrade. The genetic relationship between the *P. aeruginosa* strains forming biofilms was appreciated by the analysis of their Enterobacterial repetitive intergenic consensus (ERIC) sequences.

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2 MATERIALS AND METHODS

2.1 INOCULATION, ISOLATION AND IDENTIFICATION OF BACTERIAL STRAINS

All bacterial strains used in this study belonged to *P. aeruginosa* species. They are isolated in city and area of Meknes (Morocco) from clinical and environment samples. The clinical samples were coming from hospital services, they included pus and respiratory secretions and were taken by using sterile swabs. The environment samples include soil and water. All sampling were caring out during the year 2012.

Water samples were inoculated directly in Petri dishes containing King A, King B medium or citrimide agar. For soil samples, we performed a suspension of 10% (w/v) in sterile distilled water, and then the supernatant is inoculated as before. Swabs containing the pathological specimens are suspended in 1ml of sterile physiologic water; this later is inoculated as the water samples. All inoculated media were incubated at 30°C during 24h to 48h.

The bacterial colonies obtained were examined to identify those belonging to the species *P. aeruginosa*. The identification tests are primarily morphology, Gram, oxidase enzyme, production of pigments pyoverdin and pyocyanin and growth at 42° C. The confirmation was done by the gallery system "API 20NE" (BioMérieux, Marcy l'Etoile, France).

2.2 BIOFILMS ASSAYS

P. aeruginosa strains isolated during this work were tested for "Biofilm assay". The culture media utilized are different: the first is Luria-Bertani (LB) which enabled a good strains growth; the second is the mineral medium (M.M) supplemented with 0,5% of hexadecane as the sole source of carbon and energy. This medium was chosen because his carbon source is slightly toxic toward *P. aerugionsa* strains.

The M.M is containing in g/L: KH_2PO_4: 0.68; $MgSO_4.7H_2O$: 0.35; Na_2HPO_4: 1.7; $CaCl_2$: 0.02; NH_4NO_3: 1; $FeSO_4$: 0.004. The media was supplemented with a solution (0, 01% final concentration) of trace elements containing in g/L: $CuSO_4$: 0.05; H_3BO_4: 0.1; $MnSO_4$: 0.1; $ZnSO_4$: 0.1; Na_2MoO_4: 0.1; $CoCl_2$: 0.1. The pH was adjusted to 7.

The Biofilms assay was performed in microtiter plates of polystyrene according to the crystal Violet method as described by Stepanovic *et al*. [14]. Briefly, 4 µL of overnight culture was inoculated into 200 µL of medium culture in a 96 polystyrene microstate plate wells. All the samples were prepared in triplicate for each sampling time with a negative control containing no strains; the plates were incubated at 37˚C during 24 hours. They were subsequently washed threefold with sterile distilled water, left to dry at room temperature, added with crystal violet (200µL/well) and incubated for 15 minutes at room temperature. Then, each well was washed with sterile distilled water threefold and left to dry at room temperature.

Biofilms formation was evaluated by reading the absorbance (optical density) of each well using a spectrophotometer (BIO-RAD Laboratories PR 2100), at a wavelength of 490 nm. Based on the optical density (ODi) of the samples and on the average of optical density of the negative control (ODc), the samples were classified as strong (4xODc < ODi), moderate (2xODc < ODi ≤ 4xODc), weak (ODc < ODi ≤ 2xODc) or non-producer of biofilm (ODi < ODc).

2.3 MOLECULAR BIOLOGY AND PCR AMPLIFICATION TECHNIQUES

2.3.1 PREPARATION OF DNA TEMPLATE FOR PCR

Preparation of DNA template for PCR: Total DNA was extracted by suspending 4-5 colonies of overnight culture of *P. aeruginosa* isolates growing on LB agar (Bio-Rad, Marnes-la-Coquette, France) in 500µL of DNase- and RNase-free water (Invitrogen, Paisley, UK). The suspension was boiled at 100°C for 10min in thermal block (Polystat 5, Bioblock Scientific, France), then centrifuged at 19000 x *g* for 5min. An aliquot of 1 µL of the supernatant was used as DNA template for PCR.

2.3.2 GENOTYPING OF *P. AERUGINOSA* STRAINS BY ERIC- PCR

Enterobacterial repetitive intergenic consensus (ERIC) analysis was performed using the primer ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') and ERIC 1R (5'- ATGTAAGCTCCTGGGGATTCAC-3') as previously described [15]. Each PCR reaction was carried out in a 25 µL volume using 1.5 U of *Taq* DNA polymerase (Promega, Madison, Wis, USA) in the reaction buffer provided by the manufacturer containing 2.5 mM of $MgCl_2$, 50 µM of each deoxynucleoside triphosphate, 10% of dimethyl sulfoxide, 1.7 mg/mL of bovine serum albumin, 2 μM of the selected primer and 5 μL of the DNA template. Aliquots (10 μL) of each PCR product were subjected to an electrophoresis on 1.5% agarose gel.

The similarity of the ERIC-PCR banding patterns was analyzed by the Dice coefficient, and the data obtained were analyzed by the unweighted pair group method with arithmetic average (UPGMA) clustering using the Pearson correlation coefficient (Biochemistry and Biotechnology Department, Rovira i Virgili University, Tarragona, Spain) (http://genomes.urv.cat/UPGMA/index.php).

2.4 STATISTICAL ANALYSIS

The results were analyzed with descriptive statistics wherever appropriate Khi square test and Fisher's exact test were used to evaluate the statistical significance of the differences in the result. A *p*-value of <0.05 was considered statistically significant. Statistical analysis was performed using SPSS v20.0 software.

3 RESULTS

3.1 ISOLATION AND IDENTIFICATION OF BACTERIAL STRAINS

Among bacterial strains isolated, a total of 50 strains are Gram-negative, oxidase positive, showing growth at 42 ° C and producing pyoverdine and pyocyanin on king A and king B as well as on citrimide agar mediums. They were capable of denitrification and were identified as *P. aeruginosa* strains using classical tests. Thirty five of them are from the environment and fifteen are from hospitals. Theses strains are indicated Pa in the text and preceded with the letter E or H in case of Environmental or Hospital origins.

3.2 ATTACHMENT OF *P. AERUGINOSA* STRAINS TO POLYSTYRENE SURFACE

When using LB medium, all *P. aeruginosa* strains showed a high growth, but their attachment to the surface polystyrene varied greatly according to incubation time. Thus, at first incubation time (2h to 4h) the fixation amount unregistered was high (about 50%) and then began to decrease when incubation time increased; it reached negligible percentages after 18h of incubation. The attachments experiences results for the 50 *P. aerugionsa* strains are illustrated in figure 1.

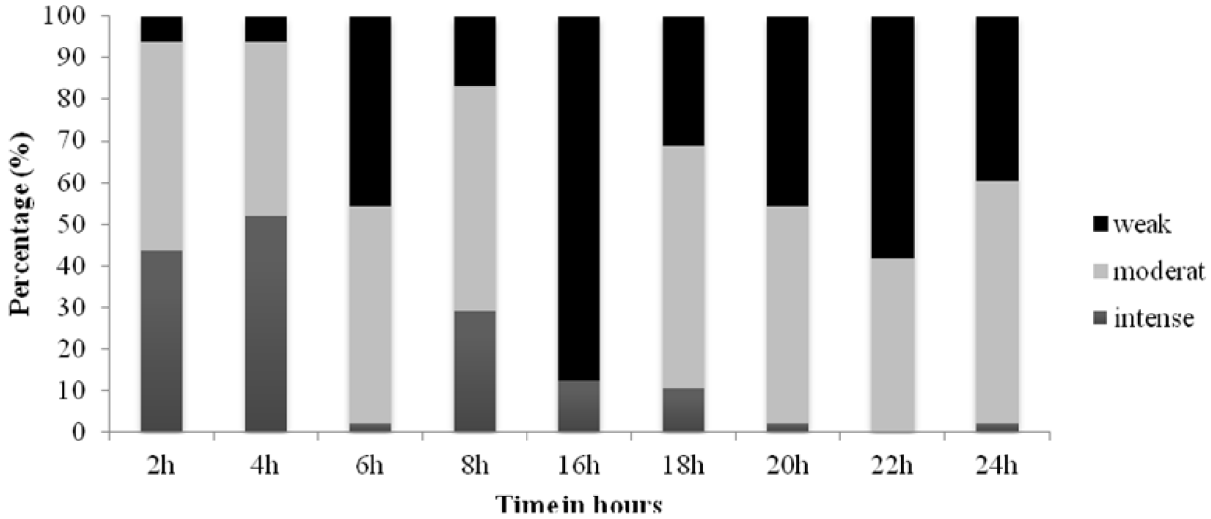


Fig. 1. Kinetic of biofilm-forming ability in L-B Medium for 50 P. aeruginosa strains studied

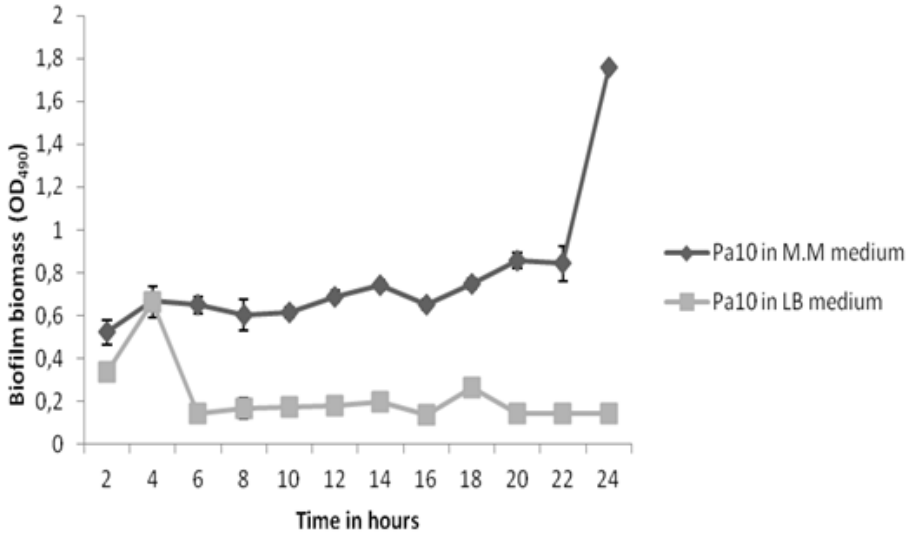
We have compared the ability of environmental and hospital *P. aeruginosa* strains to form biofilms in L.B medium at different incubation times; thus, we have noted various biofilm-forming abilities, although the strains have the same origins (Environmental or hospital). At this purpose, some strains formed biofilms strongly, and other moderately or weakly. The results obtained are registered in table I.

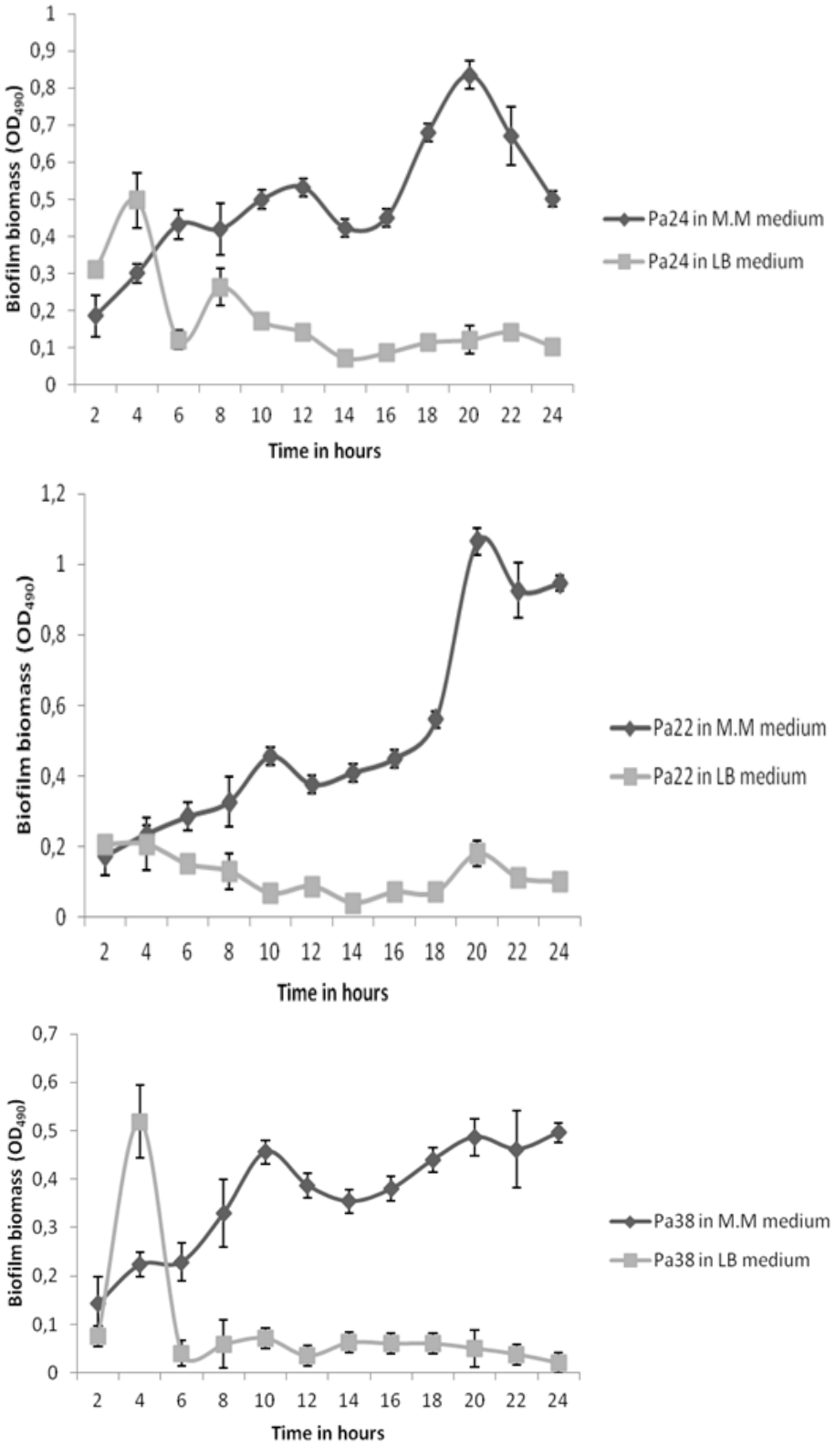
Table 1. Comparison of biofilm-forming ability in LB medium for P. aeruginosa strains isolated from hospital and environmental samples

Time in hours (h)	Biofilm-forming ability (%)								
	Strong			Moderate			Weak		
	Environmental strains	Hospital strains	*p*	Environmental strains	Hospital strains	*p*	Environmental strains	Hospital strains	*p*
2h	51.4	23.1	0.07	48.6	53.8	0.74	0	23.1	0.01
4h	54.3	54.3	0.61	42.9	38.5	0.78	2.9	15.4	0.17
6h	2.9	0	1	54.3	46.2	0.61	42.9	53.8	0.49
8h	25.7	38.5	0.7	62.9	30.8	0.04	11.4	30.8	0.18
16h	0	0	-	11.4	15.4	0.65	88.6	84.6	0.71
18h	11.4	7.7	1	60	53.8	0.7	28.6	38.5	0.50
20h	2.9	0	1	54.3	46.2	0.6	42.9	53.8	0.49
22h	0	0	-	40	46.2	0.7	60	53.8	0.70
24h	2.9	0	1	60	53.8	0.7	37.1	46.2	0.57

It appears that more than 50% (51 to 54%) of environmental strains possess a strong ability to form biofilms during the first hours (2 to 4h) of incubation, but this percentage decreased and reached 2,9 % after a period of 18h. For strains having the same origin, the ability of biofilms-forming is also variable. So, we note the values of 54,3% and 2,9% for the same incubation time (4h). However, it is important to note than more of 80% of *P. aeruginosa* strains showed a weak biofilm-forming ability just at 16h of incubation and then began to decrease with time for reaching the value around 37%.

Then, we have tested the biofilm-forming ability in M.M supplemented with 0,5% hexadecane but only for five *P. aeruginosa* strains. These strains grew in presence of hexadecane as the sole source of carbon and energy but at variable intensity. Three of them are from environmental origin: *EPa10, EPa22* and *EPa45*; the two other are from Hospital origin: *HPa 24* and *HPa38*. The results obtained varied according to strains. So, after 24h of incubation, the maximum O.D reached the value of 1.8 for the strain *EPa10* and the minimum was 0.9 for the strain *HPa24* (figure 2). Surprisingly, the strain that reached a high D.O value was that presented a high growth. Furthermore, the difference of biofilm-forming between M.M. and L.B. medium was very clear and outstanding. Thus, the five *P. aeruginosa* strains studied formed a developed biofilm under hexadecane growth.




Biofilm biomass (OD_{490})
Time in hours
Pa24 in M.M medium
Pa24 in LB medium
Pa22 in M.M medium
Pa22 in LB medium
Pa38 in M.M medium
Pa38 in LB medium

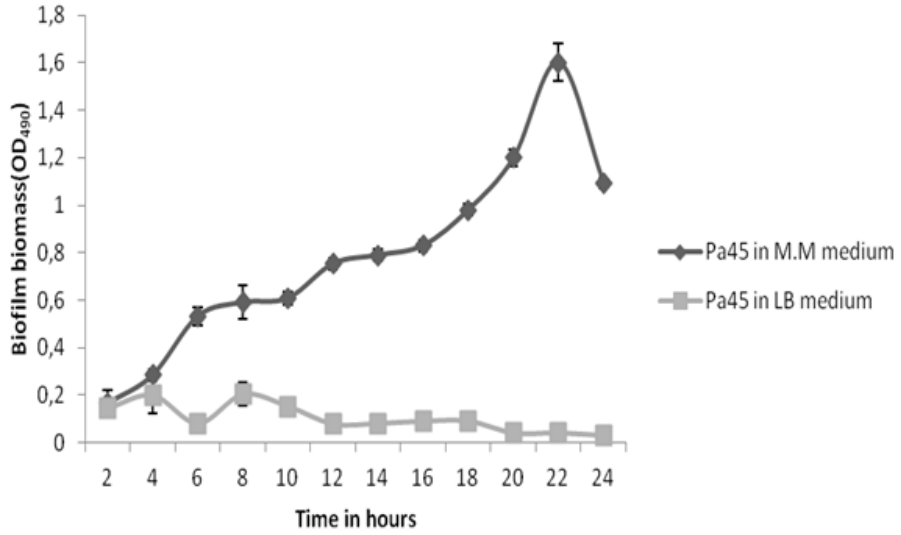


Fig. 2. Quantification of the biofilm biomass by crystal violet staining (OD_{490}) in the presence of L.B medium or M.M supplemented with hexadecane in microtitre plates during 24h of incubation.

The formation of biofilms has been visualized with the crystal violet staining, particularly designed as a ring more usually dense around the top of the wells in the surface liquid (S-L) interface. However, a small amount of biofilms has been observed in the bottom of wells. This was observed for the two mediums studied. But the visual difference was manifested by the fact that L.B. medium biofilms formation was discontinuous over the time, while for the M.M. media, the biofilms formation was low at the beginning and then continues to increase until the end of the experiment, especially for *EPa10* strain (Figure 2).

Therefore, we noted that L.B. medium, which allow good growth for all bacterial strains, enabled only a weak biofilm density. In contrast, the M.M medium, which contains carbon source not easily utilized by the bacterial strains, enabled high biofilms density. So, it emerges from these results that the nature of carbon source influenced the ability of biofilm formation and these ability is influenced by the variety of *P. aeruginosa* strain. In another way, we can say that the heterogeneity in the Biofilm-forming ability demonstrates the genetic diversity into the species of *P. aeruginosa* studied.

In order to appreciate the phylogenetic relationship between five *P. aeruginosa* strains chosen for this study, we have analyzed the bacterial genome by ERIC technique. The ERIC-PCR profile obtained is illustrated in figure 3.

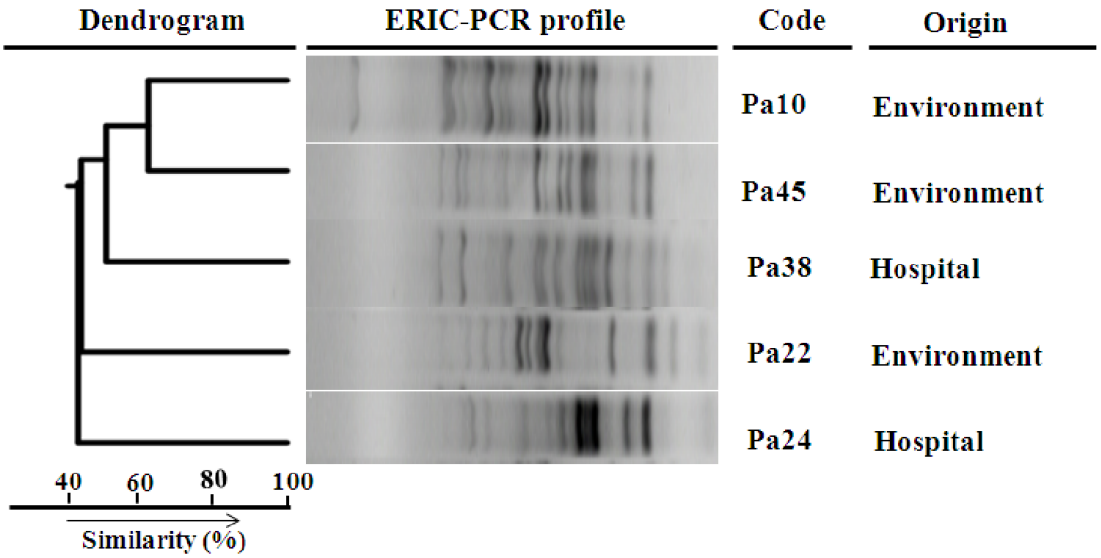


Fig. 3. Dendrogram based on ERIC-PCR profile of five hexadecane degrading P. aeruginosa

The interpretation of ERIC-PCR patterns was based on the criteria of Tenover et *al.* [14]. Thus, the strains showing more than three DNA fragment variations and a similarity of <80% at dendrogram analysis were considered to represent different ERIC-PCR types, while one- to three-fragment differences and a similarity of >80% upon dendrogram analysis were considered to represent ERIC-PCR pattern subtypes.

Based on these criterions, we can suggest that the similarity of the studied strains is about 40% to 60%. So, these strains represent various subtypes belonging to *P. aeruginosa* species.

4 DISCUSSION

The formation of biofilms by *P. aeruginosa* strains has been reported by many workers. Thus, Gross *et al.* [16], Davey et *al.* [17], Ramsey and Whiteley [18], Toutain *et al.* [19] reported that many bacterial species enabling O.D like or higher than 0,8 belonged to *Pseudomonas* genus. These reports are consistent with our results (Figure 2).

Meliani and Bensoltane [20] reported that all bacterial strains are able to produce biofilms; however, these latter varied from an almost invisible film to a thick structure depending on the presence of carbon source. Furthermore, according to Stepanović *et al.* classification [14] the *Pseudomonas* isolates having an OD_{590} ranging from 0.29 to 0.35 were categorized as having moderate biofilms. These results are also consistent with those obtained in our study but in the presence of LB medium. However, when the culture medium become relatively toxic (such growth in hexadecane presence), *P. aeruginosa* strains reply to stress by forming strong biofilms. This latter phenomenon can be explained by the fact that medium containing hexadecane become toxic after 10h of incubation, because the accumulation of short chain alkanes derived from initial substrate (hexadecane).

Previous studies indicated that Pseudomona*s* biofilms formed in a 96-well microtiter dish declines after about 10h of incubation, probably because of presence and increase of cells but in planktonic forms [21]. However, in this study, and in presence of hexadecane medium, *P. aeruginosa* strains decreased in planktonic forms and simultaneously increased in fixated form after a period of 8 to 10h. During this period, the liberation of short chains alkanes (alkanes with carbon C6 to C10) in medium stimulates probably the biofilms formation.

Some biosurfactants (such rhamnolipids molecules) stimulate the attachment and/or detachment of bacteria from the surface of xenobiotics by influencing the hydrophobicity of the bacterial cell surface or the surface of the xenobiotic [22], [23], [24]. We hypothesized; therefore, that the surfactants could influence *P. aeruginosa* cell-to-cell and/or cell-to-surface interactions [25]. However, it's important to signal that biosurfactants are generally synthesized by bacterial strains for faciliting hydrocarbon biodegradation and growth. So, their levels increase with high levels of biodegradation. Nevertheless, these molecules seem also taking place in cases of cell-cell interaction or cell-surface interaction and subsequently in formation of biofilms; this phenomenon occurred at the end of bacterial growth when the carbon source become in failure [17].

The biofilms formation implies the fixation of bacterial strains to a given support. During our study, the *P. aeruginosa* strains tested did not have the same fixation degree, because the O.D recorded was variable according to strains; some strains posses a high potential fixation. This result can be explained, among others things, by the fact that probably these strains expressed highly the genes involved in biofilms formation, such Pilis and Flagels synthesis genes. It is interesting to note that, if such strains, colonized hospital environmental, they can generate a serious problem for health public because of nosocomial infections (infections acquired at a hospital). At this purpose, about 60–70% of nosocomial infections are associated with biofilms formation [28]. Furthermore, Biofilm infections are difficult to eradicate with antimicrobial treatment, and in vitro susceptibility tests show considerable resistance of biofilm cells to killing [29].

The strains studied are various and this is confirmed by their variable ERIC-PCR profile. So, the patterns amplification obtained showed a genetic diversity within strains of *P. aeruginosa* studied. The similarity percentage varied between strains without exceeding the value of 65% (case of strains EPa10 and EPa45). Taken as whole, the *P. aeruginosa* strains isolated are not similar and belong at variables subtypes.

The ERIC-PCR technique appears to be a more reliable typing strategy for *P. aeruginosa* than are other novel PCR-based typing methodologies [26]. Also Wolska and Szweda [27] confirm that among all currently used methods, the ERIC-PCR seems to be a powerful tool for the study of *P. aeruginosa* isolates diversity.

5 CONCLUSION

The *P. aeruginosa* strains isolated in Morocco from environmental and hospital samples possess the ability to form biofilms in presence of both L-B and MMH mediums. However, this ability varied according to medium and strain type. When the medium carbon source is relatively toxic (like hexadecane) toward *P. aeruginosa* strains, theses later grew and formed a developed biofilm as the response to this stress. The kinetic of biofilm formation increased according to time and toxicity intensity of carbon source. The type of strains influenced also the biofilm formation ability, indeed the strain *EPa10*, although it belonged to *P. aeruginosa* species, it presented a biofilm type greatly developed in comparison with the others of the same species.

REFERENCES

[1] Alexander M., Biodegradation and Bioremediation, *Academic Press*, 2nd Ed (1999) London.

[2] Moslemy P., Neufeld R.J., Giot S.R., Biodegradation of gasoline by gellan gum-encapsulated bacterial cells. *Biosci. Bioeng*, 80 (2002) 175–184.

[3] Mrozik A., Piotrowska-Seget Z., Bioaugmentation as a stratergy for cleaning up soils contaminated with aromatic compounds. *Microbiol. Res*., 182 (2010) 2675–2679.

[4] Shimada K., Itoh Y., Washio K., Morikawa M., Efficacy of forming biofilms by naphthalene degrading Pseudomonas stutzeri T102 toward bioremediation technology and its molecular mechanisms. *Chemosphere* 87 (2012) 226–233.

[5] Watnick P., Kolter R., Biofilms, city of microbes, *J. Bacteriol*. 13 (2000) 20–26.

[6] Gorbushina A.A., Broughton W.J., Microbiology of the atmosphere-rock interface: how biological interactions and physical stresses modulate a sophisticated microbial ecosystem. *Annu. Rev. Microbiol.* 63 (2009) 431–450.

[7] Gilbert P., Collier P.J., Brown M.R., Influence of growth rate on susceptibility to antimicrobial agents: biofilms, cell cycle, dormancy, and stringent response. *Antimicrob. Agents Chemother*. 34 (1990) 1865–1868.

[8] Donlan R.M., Costerton, J.W., Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev*. 15 (2002) 167–193.

[9] Rani S.A., Pitts B., Beyenal H., Veluchamy R.A., Lewandowski Z., Davison W.M., Buckingham-Meyer K., Stewart P.S., Spatial patterns of DNA replication, protein synthesis, and oxygen concentration within bacterial biofilms reveal diverse physiological states, *J. Bacteriol*. 189 (2007) 4223–4233.

[10] Iijima S., Washio K., Okahara R., Morikawa M., Biofilm formation and proteolytic activities of Pseudoalteromonas bacteria that were isolated from fish farm sediments, *Microbial. Biotechnol.* 2 (2009) 361–369.

[11] Dickschat J.S., Quorum sensing and bacterial biofilms. *Nat. Prod. Rep*. 27 (2010) 343–369.

[12] Thompson I.P., van-der-Gast C.J., Ciric L., Singer A.C., Bioaugmentation for bioremediation: the challenge of strain selection. *Environ. Microbiol*. 7 (2005) 909– 915.

[13] Shemesh M., Kolter R., Losick R., The biocide chlorine dioxide stimulates biofilm formation in *Bacillus subtilis* by activation of the histidine kinase KinC. *J. Bacteriol*. 192 (2010) 6352–6356.

[14] Stepanovic S., Vukovic D., Hola V., Di Bonaventura G., Djukic S., Irkovic I. C. And Ruzicka F, Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *Journal Compilation.* 115 (2007) 891–9.

[15] Versalovic J., Koeuth T., and Lupski J. R., Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes, *Nucleic Acids Research*, 24 (1991) 6823 -6831.

[16] Gross R., Hauer B., Otto K., Schmid A., Microbial Biofilms: New Catalysts for Maximizing Productivity of Long-Term Biotransformations, *Biotechnol. Bioeng*. 98 (2007) 1123–1134.

[17] Davey M.E., Caiazza N.C., O'Toole G.A., Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1, *J. Bacteriol*. 185 (2003) 1027–1036.

[18] Ramsey M.M., Whiteley M., *Pseudomonas aeruginosa* attachment and biofilm development in dynamic environments, *Mol. Microbiol*. 53 (2004)1075–1087.

[19] Toutain C.M., Caiazza N.C., O'Toole G.A., Molecular basis of biofilm development by Pseudomonads, (2001), In: Ghannoum M., O'Toole G.A., editors, Microbial biofilms, Washington, *DC: ASM Press*, p 43–64.

[20] Meliani A. and Bensoltane A., Enhancement of Hydrocarbons Degradation by Use of Pseudomonas Biosurfactants and Biofilms, *J. Pet. Environ. Biotechnol.* 5 (2014) 2157-7463.

[21] O'Toole G. A., and Kolter R., The initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signaling pathways: a genetic analysis, *Mol. Microbiol*. 28 (1998) 449–461.

[22] Rosenberg E., Exploiting microbial growth on hydrocarbons- new markets, *Tibtech.* 11(1993) 419–424.

[23] Al-Tahhan R.A., Sandrin T.R., Bodour A.A., and Maier R.M., Rhamnolipid-induced removal of lipopolysaccaride from *Pseudomonas aeruginosa*: effect on cell surface properties and interaction with hydrophobic substrates, *Appl. Environ. Microbiol.* 66 (2000) 3262–3268.

[24] Garcia-Junco M., De Olmedo E., and Ortego-Calvo J.J. Bioavailability of solid and non-aqueous phase liquid (NAPL)-dissolved phenanthrene to the biosurfactantproducing bacterium *Pseudomonas aeruginosa* 19SJ. *Environ. Microbiol.* 3 (2001) 561–569.

[25] Neu T. R., Significance of bacterial surface-active compounds in interaction of bacteria with interfaces, *Microbiol. Rev*. 60 (1996) 151–166.

[26] Kidd T. J., Grimwood K., Ramsay K. A., Rainey P. B., and Bell S. C., Comparison of Three Molecular Techniques for Typing *Pseudomonas aeruginosa* Isolates in Sputum Samples from Patients with Cystic Fibrosis, *Journal Of Clinical Microbiology*. 49 (2011) 263–268.

[27] Wolska K. and Szweda P., A comparative evaluation of PCR ribotyping and ERIC PCR for dertemining the diversity of clinical *Pseudomonas aeruginosa* isolates, *Polish Journal of Microbiology*, 57 (2008) 157- 163.

[28] Bryers, J.D., Medical Biofilms, *Biotechnol Bioeng.*, (2008) 100(1): 1–18.

[29] Maira-Litran T, Allison D G, Gilbert P. An evaluation of the potential of the multiple antibiotic resistance operon (*mar*) and the multidrug efflux pump acrAB to moderate resistance towards ciprofloxacin in *Escherichia coli* biofilms. *J Antimicrob Chemother*. (2000) 45: 789–795.