Expression of pathogenicity genes in environmental strains of *Pseudomonas aeruginosa* in a model system contaminated with petroleum

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Abstract

Pseudomonas aeruginosa is a bacterium frequently isolated from a wide range of environments, including those contaminated with oil hydrocarbons. Because of this, it is widely used in hydrocarbon bioremediation processes. *P. aeruginosa* is also an opportunistic pathogen associated with many infections to humans, animals and plants. This pathogen has a multiplicity of molecular virulence determinants and global regulators to control their expression. Although there is a plethora of studies that evaluate the expression of pathogenicity genes in different environmental conditions, the behavior of *P. aeruginosa* under conditions of petroleum contamination has not been studied. In this work, we first standardized an RNA extraction protocol from liquid samples contaminated with hydrocarbons and subsequently evaluated the expression of nineteen genes related to pathogenicity under these conditions. Surprisingly, fifteen out of the nineteen genes evaluated were expressed in the contaminated system. This result suggests that the majority of pathogenicity genes of *P. aeruginosa* are expressed in the presence of oil hydrocarbons and prompts for this pathogen to be used with caution in bioremediation programs.

Pseudomonas aeruginosa is an important bacterium because of its environmental, industrial and medical implications. It has properties for degradation of many compounds as chloromethane [26], benzene [37], and oil [13, 29, 54], for this reason, it has been bioaugmented for bioremediation purposes. Programs of petroleum bioremediation are carried out by the Centro de Investigaciones Microbiológicas of Universidad de los Andes, Colombia; here *P. aeruginosa* is always isolated from these environments and it has been bioaugmented (unpublished data). It can also produce compounds for industrial purposes as antioxidant hydroxytyrosol [3], and polyhydroxyalkanoic acids and rhamnolipids [16], it is useful as a biological control agent for plant pathogens [8, 17], and also for growth promotion in plants [4, 63, 79]. At the same time *P. aeruginosa* is widely recognized as an opportunistic pathogen associated with nosocomial ear and eye infections [49, 50], as well as with colonization in patients with cystic fibrosis [10, 47]. Even when the majority of infections occur in immunocompromised patients or patients suffering from certain previous conditions as pneumonia [5, 27], acquired immunodeficiency syndrome [34], and also bacterium was isolated from abscess produced [76], there are also reports in healthy subjects [5, 11, 14, 25, 30, 42, 47]. Equally, *P. aeruginosa* has been reported as a pathogenic organism for other species, such as nematodes [63], plants

[63], birds [6, 28] and mammals like dogs [1, 74], cows [7, 85], pigs [12], guinea pigs [43], horses [33], sheeps [45], rats [73] and mouse models [83].

Many determinants of pathogenicity in *P. aeruginosa* have been identified. These include swarming, swimming and twitching motility, several proteases, rhamnolipids or hemolysins, elastase, lipase, the general type II secretion system, piocianine, S exoenzyme and exotoxin A [41, 57, 72]. Most of the genes coding for these determinants are under the control of a hierarchical system for regulation of expression known as quorum sensing [32, 55, 66]. In P. aeruginosa quorum sensing is a highly complex phenomenon. It is defined by two regulatory interrelated circuits (the so-called las and rhl systems) which depend on N-Acyl Homoserine Lactone (AHL) molecules. These, in turn, are modulated by a signal molecule of the quinolone type and by other transcriptional and post transcriptional regulators [19, 66, 84]. The las system is composed of the transcriptional activator LasR and the AHL synthase LasI, which controls the synthesis of N-3-oxo-dodecanoyl-homoserine lactone (3-oxo-C 12-HSL). The rhl system consists of the transcriptional regulator RhlR and of the AHL synthase RhI, which controls the synthesis of N-butanoyl-homoserine lactone (C 4-HSL). The two systems are interconnected, given that the LasR - 3- oxo-C 12-HSL complex positively regulates the transcription of RhlR and RhlI; additionally, the rhlR gene depends not only on LasR for its expression, but also on Vfr and RhlR itself [32]. Recently, the response regulator PprB and the transcriptional activator VqsM were described as additional modulators for a large number of genes, including *lasI, rhlI, rhlR* [19, 20]. This proves that, although quorum sensing is a widely investigated phenomenon, in this bacterium there are still many aspects to unveil.

Studies from several groups have shown that bacteria from both clinical and environmental sources have highly preserved genomes [21, 48, 60, 80]. Previous works in our group intended to find differences in the virulence of environmental and clinical isolates; no difference was detected between the two groups of strains, as assayed in two models (*Drosophila melanogaster* and *Lactuca sativa*) [77]. This result is in agreement with other reports in which no significant differences were detected in the sequences of specific genes and specifically in pathogenicity determinants as resistance to antibiotics [2], type III secretion system [23], type IV pili [38, 69], flagellum [51], and *oprD* gene [61]. Considering the wide range of potential hosts, the introduction of high concentrations of *P. aeruginosa* to the environment would represent a risk for wild life and human population in places where the bioremediation processes take place.

Although there are many studies about expression of genes associated with pathogenicity in infection models, the expression of genes of *P. aeruginosa* during a bioremediation process is unknown. Gene expression depends on the growth conditions of the organism. This was shown for *P. aeruginosa* by means of

several studies, which showed that the expression profile is altered depending on the presence of inhibitor agents as peracetic acid [15], hydrogen peroxide [56, 64], arsenite [58]; growth phase [78], different culture conditions [24, 78], culture system [82], or the strain itself [56]. To the best of our knowledge, studies of gene expression when *P. aeruginosa* is degrading petroleum have not been carried out, partially due to the lack of a method to obtain good quality RNA from this kind of samples. In this work, RNA was extracted from *P. aeruginosa* cells grown in a liquid system with crude oil as the sole carbon and energy source, and the expression of genes associated with pathogenicity was assessed qualitatively. This information gives insight about the metabolism of *P. aeruginosa* in the environment and it is useful as a risk indicator when the bacterium is been used in bioremediation processes.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals

Four *P.aeruginosa* strains (M8A.1, M8A.2, M8A.3 and M8A.4) were used for analysis of gene expression. All strains were isolated from a water sample contaminated with petroleum. The strains were pooled and cocultured in static liquid culture systems in 250-ml Erlenmeyer flasks at 30ºC in 100 ml of salt minimum medium (0,5gr K_2HPO_4, 1gr NH_4Cl, 2gr Na_2SO_4, 0,001gr $CaCl_2$.6H2O, 1gr $MgSO_4$, 0,0004gr $FeSO_4$ for 1L) with 1% petroleum as carbon source; the control culture was supplemented with 1% succinate (Sigma) instead of petroleum. The cultures were grown at 30ºC to a cell density of 10 CFU/ml, required to obtain enough amounts of RNA.

RNA extraction

To standardize the RNA extraction protocol, samples of 10 ml, 15 ml, 25 ml, 40 ml and 50 ml from contaminated and control cultures were assayed. Finally, 50ml was the volume chosen. Bacterial cells were pelleted by centrifugation at 4ºC for 30 min at 8,000 x g, and the supernatant was removed. Bacterial cells were washed from contaminants with1% PBS and pelleted by centrifugation at 4ºC for 10 min at 12,000 x g. Total RNA was isolated with Trizol Reagent (Invitrogen) according to the manufacturer`s instructions. Chloroform was added to each sample and the mix was vortexed for 30 sec and kept at room temperature for 5 min. After centrifugation at 12,000 x g for 15 min at 4ºC, the clear supernatant was saved on ice. RNA precipitation was carried out by the addition of isopropanol to each sample, incubation at 4ºC for 15 min and centrifugation at 12,000 x g for 15 min at 4ºC. The RNA pellet was washed with 75% ethanol in DEPC-treated water, followed by centrifugation at 12,000 x g for 5 min. The pellet was air dried and re-suspended in 25 µL of DEPC-water. RNA was treated with DNase I (Biorad). Subsequently, RNA was purified with a Micro-to-Midi Total RNA Purification System kit (Invitrogen). DNA contamination was evaluated by PCR. To determine the efficiency of the extraction method, RNA quality and quantity

was assessed using three methods: 1) 1.5% agarose gel electrophoresis and ethidium bromide staining, 2) 260/280 ratio using a spectrophotometer ND-1000, Nanodrop Technologies and 3) RT-PCR amplification.

Expression studies

cDNA was generated using random hexamers as primers for reverse transcription with Iscript cDNA Synthesis Kit (Biorad). Nineteen pathogenicity-related genes were amplified from this cDNA; reaction mix was prepared with 2.5µl of the supplied buffer, 0.75µl $MgSO_4$, 2.5 µM 5dNTPs, 10 µM forward primer, 10 µM reverse primer, 1.25U Platinum Taq DNA polymerase (Invitrogen), and 18.5 µl water. The amplification reactions were carried out in an iCycler Thermocycler (Biorad). The cycles consisted of 4 min at 95ºC, then 30 cycles (90sec at 95ºC, 90 sec at the appropriate annealing temperature for each pair of primers, and 90 sec at 72ºC), followed by a final 10-min extension at 72ºC. Controls without reverse transcriptase were performed for each set of PCRs. The 19 pathogenicity-related genes evaluated were : *toxA, mexA, oprM, mexC, oprJ, mexX, mexY, lasA, lasB, lasI, lasR, rhlI, rhlR, exoS, exoT, rhlA, rhlB, pilB* and, *fliA* ; we also tested for the expression of two alkane degradation genes (*alkB1* and *alkB2*), and nadB as a housekeeping gene. Primers were designed with Primer3 program [71]; the sequences of the primers used are listed in table 1 and their secondary structure was verified with the mfold web server (http://mfold.bioinfo.rpi.edu/). Amplification products were visualized in 1% - 2.5% agarose gel stained with ethidium bromide, in order to check the quality of the amplicons and corroborate the expected product size. PCR
products were purified with a Wizard SV Gel and PCR Clean-Up System kit and sequenced using ABI PRISM® 310 Genetic Analyzer and its compatible chemistry.

Table 1. List of Genes evaluated by RT-PCR and primers used in this work

Gene	Primers	Encoded Protein	Amealing Temperature (℃)	Product Size (pb)	Reference
toxA	F5'AAGCCTTCGACCTCTGGAAC3' R5'ACCCGGTAGATCTTGCCTTC3'	Exotoxin A	54	673	This study
toxA qPCR	F5'GGAGCGAACTATCCCACT3' R5'TGGTAGCCGACGAACACATA3'	Exotoxin A	60	90	Reference 31
mexA	F5'CCATGCGTGTACTGGTTCC3' R5'GTCGGCGTACTGCTGCTT3'	Structural gene for MexAB-OprM efflux pump	56	392	This study
oprM	F5'AGGCCTACGGGCAGAACAC3' R5'CTGTTCGGTCGCCAGGTACT3'	Membrane porine for MexAB-OprM	54	380	This study

		efflux pump			
mexC	F5'CAGGCCCTCGGTGATCTG3' R5'CCGCTGGTGAAGATCCAG3'	Structural gene of MexCD-OprJ efflux pump	53	663	This study
oprJ	F5'CGCCTCGATGTACCACCA3' R5'CAGAAACAGCAGGCATTCAA3'	Membrane porine for MexCD-OprJ efflux pump	53	712	This study
mexX	F5'CGTGCTGTTCCAGATCGAC3' R5'GTGTACTCGCGTTCGCTGAT3'	Structural gene for MexXY-OprM efflux pump	53	159	This study
mexY	F5'CCAAGGACCATTACCAGGAA3' R5'AACAGCGGTACCAGGAACAC3'	Structural gene for MexXY-OprM efflux pump	53	226	This study
lasA	F5'CGGCGCTCTGGTTGTAGA3' R5'GGAGCGGCTACTACAGCATC3'	Las A Protease	53	921	This study
lasB	F5'TCATCACCGTCGACATGAAC3' R5'TGTACAGCTTGTGGGTCAGC3'	Las B Elastase	53	204	This study
lasR	F5'AAGGACAGCCAGGACTACGA3' R5'CCGATATCTCCCAACTGGTC3'	Transcriptional activator of the las quorum sensing system	53	472	This study
lasI	F5'GTATCGAGAATTCGCCAGCA3' R5'GGGCGAGATGCACAAGTT3'	AHL synthase of the las quorum sensing system	53	180	This study
rhlR	F5'AGGAATGACGGAGGCTTTTT3' R5'GTCAGCTTCTGGGTCAGCA3'	Transcriptional activator of the rhl quorum sensing system	54	497	This study
rhlI	F5'GCTCTCTGAATCGCTGGAAG3' R5'GTCTCGCCCTTGACCTTCT3'	AHL synthase of the rhl quorum sensing system	54	489	This study
exoS	F5'CTCAGCAGAACCCGTCTTTC3' R5'ACCTGGCCCTCTTTCACC3'	Exoenzyme S (effector of TIIISS)	53	997	This study
exoT	F5'CTCAGCAGAACCCGTCTTTC3' R5'CTTGCGCTGAGTACATGCTG3'	Exoenzyme T (effector of TIIISS)	53	589	This study
rhlA	F5'GCGCGAAAGTCTGTTGGTA3' R5'AACAGCACCACGTTGAAATG3'	Protein involved in Hemolysins production	53	171	This study

rhlB	F5'GCATCTCGCAGGTGATCTTG3' R5'CGACGACGTGATGAACCTC3'	Protein involved in hemolysin production	53	545	This study
pilB	F5'TACGAGGAGGACCAGAAGGA3' R5'GGATTGACGTTGACCTGGTT3'	Hexameric ATPase required for type IV pili biogenesis	55	206	This study
fliA	F5'GTTACGCACCACTGGTCAAG3' R5'CCTGGAAACGTTCGTCGAG3'	Sigma Factor involved in the expression of flagellum	55	480	This study
alkB1	F5'GCCGTTCAACTACTGGATGG3' R5'GGATACTGGTAGACCGACTGG3'	C16 - C24 alkane hydroxylase	55	500	This study
alkB2	F5'TGCTCGACGTAGTTGACGAT3' R5'GACTGGGTCGGCCAACTG3'	C12 - C-20 alkane hydroxylase	55	479	This study
nadB	F5'CTTCACCGTGAGCATAGC3' R5'GCCTTCCTCGTGGTTGTG3'	Nicotinate nucleotide phosphorylase (housekeeping gene)	60	94	Reference 53

Real-time quantitative polymerase chain reaction

To analyze the relative amounts of *toxA* expression in the contaminated system compared to the control, real-time quantitative polymerase chain reaction (RT- qPCR) was performed. This process was carried out using 2U SuperScript IIIPlatinum SYBR Green One-Step qRT-PCR Kit of Invitrogen. The reaction mixture consisted of 12.5 µl SYBR Green, 10 µM forward primer, 10 µM reverse primer, 76µl nuclease-free water and 1µg RNA.

Reaction conditions for toxA and *nadB* were an initial stage of heating at 50℃ for 3 minutes, then 95℃ for 5 min, fo llowed by 40 cycles of 95℃ for 15 seconds, 60° for 30 seconds , one cycle of 40℃ for 1 minute . A melting curve using 1℃ increments was performed following the 40 cycles. All reactions were carried out in iQ5 Real-Time PCR iCycler (Biorad). Standard curves were made with five points for each gene. Controls included PCRs without total RNA and without SuperScript III. Data of relative expression of toxA was calculated using
REST-mcs program [59].

RESULTS and DISCUSSION

RNA extraction

There are no reports in the literature for RNA extraction protocols from samples contaminated with petroleum. In this study, we standardized an RNA extraction protocol from liquid cultures that included a pre-wash of the cells with

PBS, a cellular lysis and RNA isolation using Trizol (Invitrogen), and an additional column-based RNA purification step. With this protocol, we got RNA of high quality (Figure 1, a and b) amplifiable by PCR, and in concentrations ranging between 70 and 705.6 ng/µl.

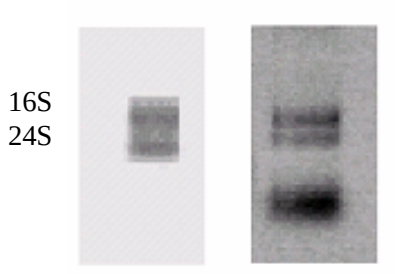

Figure 1. RNA extracted from liquid cultures. (a) Contaminated with petroleum and (b) not contaminated, with succinate as carbon source. RNA isolated from a culture with 10^7 CFU/ml.

Expression of genes required for the degradation of alkanes

We first evaluated the expression of genes involved in degradation of hydrocarbons in *P. aeruginosa*. For this, the expression of alkB1 and alkB2, which encode alkane hydroxylases, was tested by RT-PCR. The AlkB1 alkane hydroxylase oxidizes n-alkanes in the range of C 16 to C 24, while AlkB2 is active on C 12 to C 20 n-alkanes [44, 68]. Detectable levels of expression were obtained for these two genes in the culture contaminated with petroleum. On the other hand, no expression was detected in the culture with succinate as the sole carbon source (Figure 2). This indicates that the technique used can detect metabolic differences in *P. aeruginosa* when it is in different culture conditions.

Expression of genes associated with pathogenicity

We evaluated the expression of 19 genes associated with the pathogencity of *P. aeruginosa*. Fifteen out of the nineteen genes were expressed in both culture conditions (with and without oil hydrocarbons), two were expressed only in the oil-contaminated culture (*mexX* and *mexY*), and two (*mexC, oprJ*) were not detected in any treatment (Figure 2). Four of the twenty genes are quorum sensing regulators (*lasI, rhlI, lasR, rhlR*), five are regulated under this system (*toxA, lasA, lasB, rhlA, rhlB*), and nine genes are regulated by other mechanisms (*exoS, exoT, pilB, fliA, mexA, oprM, mexX, mexY, mexC, oprJ*).

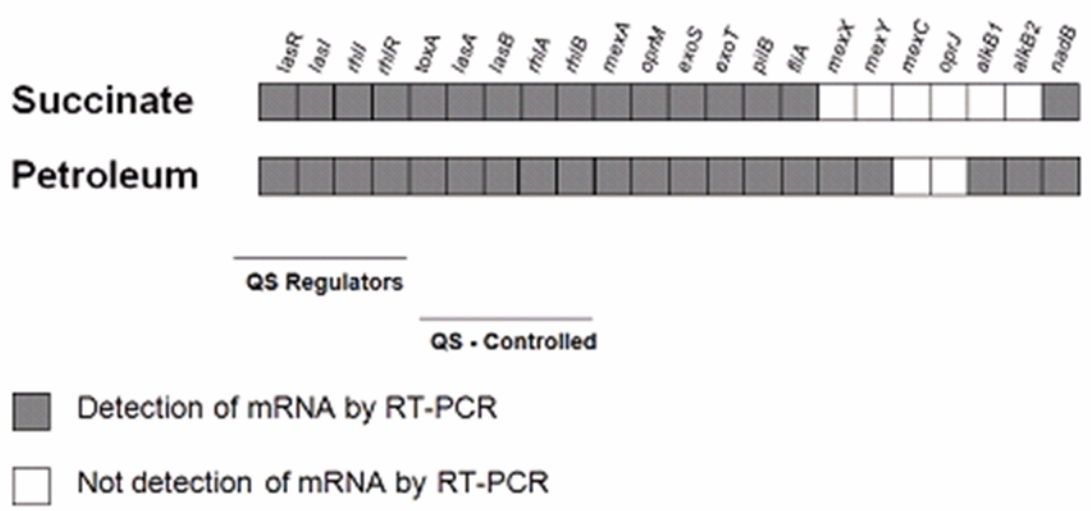

Figure 2. Expression of genes evaluated in contaminated (petroleum) and control (succinate) culture

The five genes regulated by quorum sensing (*toxA, lasA, lasB, rhlA and rhlB*) and four quorum sensing regulators genes were expressed in both culture systems. Figure 3 shows amplification gene of toxA in both system. The results were not surprising because of the cell density with which the RNA extractions were started (10^7 - 10^7 UFC/ml), and quorum sensing system begin to function when bacterial density increase, 3OC12-HSL (LasI generated signal) builds to a critical concentration, at which point it interacts with LasR and the system is turned on [66, 84]. Nevertheless, this is a biologically relevant result because bioremediation conditions require that bacteria are present at these cell densities. Additionally, this can indicates that the presence of petroleum does not "quench" quorum sensing signaling in *P. aeruginosa.*

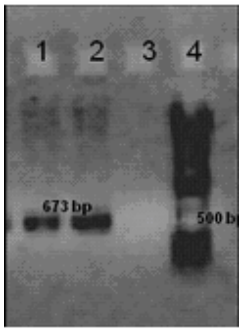

Figure 3. Amplification of toxA of *P. aeruginosa* in contaminated with petroleum culture and control culture

In addition, we evaluated nine genes not regulated by quorum sensing, six of which were expressed in both culture systems. The six genes were *exoS, exoT, pilB, fliA, mexA* and *oprM*.

The exoenzymes ExoS and ExoT are effectors translocated into the host cell by the type III secretion system from *P. aeruginosa*. ExoS y ExoT inhibits phagocitosys by disrupting actin cytoskeletal rearrangement, focal adhesines and signal transduction cascades important for phagocytic function [9]. Transcripts of *exoS* and *exoT* were found in both culture systems. This was a surprising result, since the aim of this secretion system is to inject toxic proteins directly into the cytoplasm of eukaryotic cells. For this, the system is activated by bacterial contact with host cell surfaces [67]; although fungal organisms were detected in the oil-contaminated culture (the petroleum used was not esterilized), no eukaryotic cells were observed in the control treatment. One possible explanation for the expression of these genes in the control culture is the composition of the salt minimum medium used, calcium concentration in medium was 0.009 mM and TIIISS can be activated at calcium concentrations under 5 mM [81].

The two genes involving motility evaluated in the present study (*pilB* and *fliA*) showed no differential expression; this suggest that the presence of a contaminant such as petroleum does not inhibit the expression of these determinants of pathogenicity, directly related to biogenesis of type IV pili (pilB) and the formation of the flagellum (*fliA*). It is important to mention that succinate is associated with increased expression of flagella genes [65]. The expression of these proteins in *P. aeruginosa* allows the bacteria to stick and move on several surfaces. Although these genes are not regulated by quorum sensing, there are reports concluding that flagellum and the type IV pili make *P. aeruginosa* develop the swarming movement [36]. This type of movement is related to quorum sensing by the rhamnolipids production, because swarming movement is facilitated by rhamnolipids production. They diminish the superficial tension of surfaces inducing internalization, and this production is controlled by quorum sensing [36]. This would be consistent with results of genes related to quorum sensing found in this study. Alternatively, we cannot rule out the possibility that the presence of hydrocarbon directly induces these genes independently from QS activation.

mexA and *oprM* genes, from the efflux pump MexAB-OprM, also were expressed in both culture conditions. This pump is expressed in wild strains of *P. aeruginosa*, and it contributes to the intrinsic resistance to a large number of antimicrobials [35], detergents [70], organic solvents as aromatic hydrocarbons [39, 40] and molecules of the homoserin lactones type [22, 70]. This result is important because of expression of this efflux pumps can be up expressed in presence of toxic substances inducing stress [40, 53]. It could implicate that antibiotics resistance is raised.

mexX and *mexY* genes were detected only in contaminated culture (Figures 2 and 4). These genes belong to the efflux pump MexXY-OprM of *P. aeruginosa*. This pump is classified within the RND -resistance nodulation-division-family [75]. It is important because it provides the capacity for the extrusion of antibiotics, biocides and organic solvents [46]. Expression of *mexXY* is inducible by aminoglycosides, tetracycline, erythromicine and tigecycline [61] even whenMexAB-OprM pump (which is the most used pump in P. aeruginosa) is functional [18, 46].This result suggests that MexXY-OprM is induced by the petroleum. If this were the case, this would be the first report of a gene differentially expressed in the presence of hydrocarbons in *P. aeruginosa*. Alternatively, the differential expression might be due to the presence of antibiotics in the environment (petroleum is not sterile, although there are no reports of antibiotics produced by fungus and/or bacteria present in the contaminant). This is because this pump has the capacity for the extrusion of antibiotics [46].

It would be interesting to determine if MexXY-OprM is required for the adaptation of *P. aeruginosa* in the presence of limited concentrations of toxic hydrocarbons, before a substantial amount of degradation takes place. This was not assayed in this work.

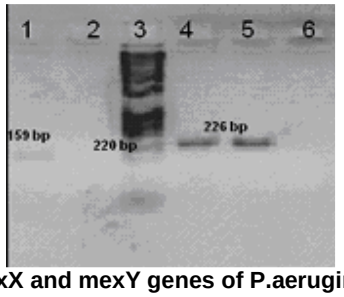

Figure 4. Amplification of mexX and mexY genes of P.aeruginosa in culture contaminated with petroleum and control culture

Expression of MexCD-OprJ can reduce susceptibility to a variety of antibiotics, for this reason *mexC* and *oprJ* genes were evaluated and they were the only two that did not show expression in any of the systems. These belong to the efflux pump MexCD-OprJ, which is reported as inducible by antibiotics such as flouroquinolone and non-antibiotic substrates as tetraphenylphosphonium chloride, ethidium bromide, rhodamine, acriflavine and the biocides benzalkonium chloride and chlorhexidine [75]. This pump is inducible in wild strains of *P. aeruginosa* by disinfectants as tetraphenylphosphonium chloride, ethidium bromide and rhodamine and it is not induced by antibiotics, although they are substrates for it [52]. Substances that can induce this pump are not present in any culture system used in this study.

Real-time quantitative polymerase chain reaction of toxA

Being a very useful technique and relatively easy to implement, RT-PCR might not show small differences in gene expression. To assess this, we evaluated the expression of one gene, toxA, using real-time quantitative RT-PCR. We chose toxA because is one of the most important pathogenicity determinants in *P. aeruginosa*. *nadB* gene was used as housekeeping, and it displayed similar levels of expression in both systems, giving C t values of 15.5 in control system, and 14.7 in contaminated system. C t for toxA was 20.0 in control and 23.8 in the contaminated system. Amplification efficiency for toxA was 2.3 (130%) and for *nadB* was 2.4 (140%). A randomization assay was carried out with REST-cms program [59], and was calculated P-value by obtaining the proportion of random allocations of the mean observed data to the control and treated sample groups. This value was of 0.65 indicating that the data are random.

This was a preliminary assay, as efficiencies were suspiciously over 110% allowed by majority of researches, and data are random, it is still not possibly to detect the relative expression, for this it is necessary to carry out other assays to standardize the qPCR reaction.

In summary, out of the 19 genes related to pathogenicity evaluated in the present study, 15 were expressed in the culture contaminated with hydrocarbon. Out of those fifteen, nine are related to the quorum sensing system, which indicates that, if there are high concentrations of this bacterium, the genes under its regulation will probably be expressed regardless the presence of oil hydrocarbons. This is consistent with the studies that show that there is no difference in the virulence or the genome content between environmental and clinical isolations of *P. aeruginosa* [77, 80]. This indicates that *P. aeruginosa*, besides being capable of degrading hydrocarbons can also be a potential risky organism for the health of people or other life forms that can enter in contact with it. It is necessary to take biosafety measures for the living organisms that find themselves in the environments where bioremediation processes take place, especially those involving bioaugmentation given that, under this conditions, the population of the bacteria will increase. With the bioaugmentation, the probability of activation of quorum sensing system will increase and, in the same way, the majority of genes related to pathogenicity in *P. aeruginosa*. Up to now there are no clinical reports about infections caused by *P. aeruginosa* while degrading hydrocarbons, for this reason it can not be removed from the degradation pool, but evidently, the biosafety measures should be increased.

Conclusions

In this study, we report a protocol to isolate RNA from liquid samples contaminated with petroleum. To our knowledge, this is the first time a protocol of this kind is reported.

In our expression analyses, we also showed that the expression of pathogencity genes dependent and independent of QS is not suppressed by petroleum. Because of this, it is necessary to take biosafety measures for the living organisms of the environments where bioremediation processes take place. It is important that organisms that can be targets for *P. aeruginosa*, such as humans, animals, and plants are not in contact with bacteria in bioremediation settings.

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