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Haloalkalitolerant Actinobacteria with capacity for anthracene degradation isolated from soils close to areas with oil activity in the State of Veracruz, Mexico

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Summary. The use of native strains of microorganisms from soils is an excellent option for bioremediation. To our knowledge, until now there has been no other group working on the isolation of Actinobacteria from contaminated soils in Mexico. In this study, samples of soils close to areas with oil activity in the State of Veracruz, Mexico, were inoculated for the isolation of Actinobacteria. The strains isolated were characterized morphologically, and the concentrations of NaCl and pH were determined for optimal growth. Strain selection was performed by the detection of a phylogenetic marker for Actinobacteria located at the 23S rRNA gene, followed by species identification by sequencing the 16S rRNA gene. Several haloalkalitolerant Actinobacteria were isolated and identified as: *Kocuria rosea, K. palustris, Microbacterium testaceum, Nocardia farcinica* and *Cellulomonas denverensis*. Except for *C. denverensis*, the biomass of all strains increased in the presence of anthracene. The strains capacity to metabolize anthracene (at 48 h), determined by fluorescence emission, was in the range of 46–54%. During this time, dihydroxy aromatic compounds formed, characterized by attenuated total reflectance Fourier transform infrared spectroscopy bands of 1205 cm^{-1} and 1217 cm^{-1}. Those Actinobacteria are potentially useful for the bioremediation of saline and alkaline environments contaminated with polycyclic aromatic hydrocarbon compounds. [**Int Microbiol** 2016; 19(1):15-26]

Keywords: *Kocuria* · *Microbacterium* · haloalkalitolerant Actinobacteria · anthracene degradation · State of Veracruz, Mexico

Introduction

Contamination by crude oil spills is among the most concerning environmental problems worldwide. Oil is a complex mixture of hydrocarbons and related compounds classified as aliphatics, asphaltenes and polycyclic aromatic hydrocarbons (PAH) [39]. PAHs represent more than 10% of organic compounds in oil [52], and have been found to be widely distributed in the atmosphere because of their moderate vapor pressure, low solubility and low reactivity [22]. These hydrophobic organic compounds have two or more linear, angular or branched benzene rings [30]. Those with three or more rings tend to be strongly adsorbed to the soil [32].

Anthracene is a 3-ring PAH, which is among the priority environmental pollutants by the US Environmental Protection

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Agency (EPA). According to EPA, there is insufficient information to classify the anthracene as a substance that causes cancer [http://www.epa.gov/osw/hazard/wastemin/priority.htm] but the fact that its structure resembles to carcinogenic PAHs such as benzo[*a*]pyrene and benzo[*a*]anthracene arises concern [37]. Therefore, anthracene is usually used as a model PAH in studies of degradation [5,19,31]. During the last decades, bacteria capable of degrading several hydrocarbons, including anthracene, naphthalene, phenanthrene and/or pyrene, have been isolated from PAH-contaminated soils. Most of these bacteria belong to the genera *Burkholderia* [44], *Dietzia* [2] and *Sphingomonas* [8].

Depending on the physiological conditions regarding salinity and pH required for the growth of microorganisms, they are classified as halophile, halotolerant, alkaliphile or alkalitolerant. With respect to salinity, halophile microorganisms are those that require NaCl for living and growth [34]. Halotolerant microorganisms can grow in media with NaCl from concentrations as low as zero up to as high as 25% [20]. With respect to pH tolerance, a microorganism is classified as alkaliphile or alkalitolerant when it grows at pH values higher than 9 [17]. The classification of haloalkaliphile is given to microorganisms that require both NaCl (in concentrations up to 30%) and an alkaline pH (pH 9) for growth [17]. In this work, we call haloalkalitolerant to those microorganisms that can live and grow either in the absence or the presence of salt; they can even tolerate high NaCl concentrations (up to 25%) and grow optimally in a wide range of pH (8–9, or higher). Due to the salinity and pH ranges tolerated by haloalkalitolerant microorganisms, they are a good prospect for the bioremediation of soils contaminated with PAH.

Among bacteria, Actinobacteria can produce several extracellular enzymes that metabolize various complex organic compounds, and also produce biosurfactants [13,35]. These two characteristics make them good candidates for the bioremediation of sites contaminated with organic pollutants [13]. Several Actinobacteria, such as *Kocuria rosea, K. flava, Microbacterium marinilacus, Nocardia pneumoniae* and *Cellulomonas bogoriaensis,* have been isolated from soils and water contaminated with oil in Pakistan [1] and Kuwait [2], and their capacity to degrade naphthalene, phenanthrene and/or fluoranthene has been demonstrated.

The bioremediation of PAH-contaminated environments has been reported with native halotolerant or alkalitolerant Actinobacteria, as shown by studies performed with *Arthrobacter crystallopoietes* and *Arthrobacter arilaitensis* [36] and *Micrococcus* sp., *Dietzia* sp. and *Rhodococcus* sp. [14]. However, there are no reports about the use of Actinobacteria classified as haloalkalitolerant able to grow in polluted environments such as those affected by PAHs. Because PAHs have a relatively high quantum yield, fluorescence spectroscopy has been successfully used to monitor the processes of biodegradation of PAHs dissolved in aqueous solutions [54].

Mexico has several areas with oil activity. Therefore, it is expected that (i) the soils close to these areas are contaminated with oil and its derivatives, and (ii) some of the physicochemical conditions of these soils are usually found in contaminated environments. One option for bioremediation could be the use of local microbial strains adapted to live under these conditions, such as haloalkalitolerant Actinobacteria. To our knowledge, however, there have been no previous reports related to the isolation and characterization of such microorganisms from soils close to areas with oil activity, and we have published a preliminary study on the comparison based on sensitivity, linearity, and detection limits of the excitation, emission, and synchronous fluorescence methods, during the quantification of the residual anthracene concentration from the haloalkalitolerant Actinobacteria cultures [21]. The aim of this work was to isolate and genetically identify haloalkalitolerant Actinobacteria strains from soils close to areas with oil activity, and to assess their capacity to degrade PAHs by using the anthracene model and fluorescence emission.

Materials and methods

Sample collection. A random sampling was performed on soils close to 4 areas with oil activity in the State of Veracruz, Mexico (Fig. 1). Soil samples were taken from the surface, at a maximum depth of 15 cm, maintaining sterile conditions, placed in sterile polyethylene bags and transported to the lab under refrigeration conditions. Major oil activities close to the sampling area included oil drilling, extraction of liquids, refining and petrochemical production. Facilities where oil activities are carried out have risks of leaking oil, diesel and gasoline either by pipeline rupture, or oily water filtration. These activities potentially contaminate the soil mainly by discharges and spills of oily water or flooding in the rainy season.

Physicochemical characteristics of the soil samples. Soil samples were separately placed on aluminum plates and dried in an oven at 40 ºC (Scientific, H-71) for 7 days. The lumps present were grounded in a porcelain mortar and in a pestle and passed through a 2-mm-pore sieve to standardize the particle sizes. Physicochemical variables measured for each sample were NaCl concentration and pH. One gram of dried soil was placed in an assay tube, 9 ml of distilled water was added, and the sample was agitated in a vortex for 10 min and filtered overnight through Whatman 2 filter paper. Both NaCl concentration and pH were measured from the filtered solution using a refractometer (Hann, HI931100) and a pH-meter (Hanna, HI98128), respectively.

Culture media. Six cultures recommended for the isolation of halophilic bacteria and Actinobacteria were initially used: culture media for moderate halophilic microorganisms (MH) [38], SAUTON-UAM [42], Czapek agar,

Fig. 1. Two locations near the area with oil activity in the State of Veracruz, Mexico where the samples for the isolation of the strains included in this study were obtained.

soil extract agar [16], NZ amine A [28], GA agar [33], and yeast and malt extract agar (YME) [45]. MH medium was used as reported by Quesada et al. [38]: 10 g/l yeast extract, 5 g/l proteose peptone; 1 g/l glucose, 18 g/l agar; 10% NaCl, pH 7. This medium was also adjusted to pH 10 before use. Other two variations were to replace 10% NaCl for 3% NaCl and to adjust the pH to either 7 or 10. YME agar was used as reported by Shirling and Gottlieb [45]: 4 g/l yeast extract; 10 g/l malt extract; 4 g/l glucose; 20 g/l agar; pH 7. This medium was also used with 10% NaCl added and after adjusting the pH to 7. All media were sterilized in an autoclave at 121 ºC for 15 min, cooled to 45 ºC and poured in petri dishes.

Isolation, purification and morphological characterization of Actinobacteria. One gram of soil from each sample was weighed and placed individually in assay tubes, after which 9 ml of 10% NaCl solution was added. The mixture was agitated in a vortex for 30 s and subsequently diluted until obtaining a final concentration of 8-orders lower than the initial concentration. A 200-µl aliquot of this dilution was inoculated in a series of 6 petri dishes containing the culture media previously described. The mixture was homogenized until dried, and the culture media were incubated at 37 ºC for 7 days. The selection of strains was performed according to the morphological characteristics reported for Actinobacteria in the Bergey's Manual [4]. The strains selected were purified in the culture medium from which they were isolated. The colonies were described according to their size, color, shape, texture, aspect, height, the presence of vegetative or aerial mycelium and diffusible pigment in the culture medium. To confirm the purity of the strains, Gram staining was performed. The stained strains were observed under a microscope (Leica 5605) to identify the presence of filamentous, branched, coccoid, Gram-positive cells.

Genetic identification. DNA extraction was performed according to the protocol of the Promega Wizard Genomic DNA Purification kit (Promega, A1120). A phylogenetic marker, 250/350 base pairs (bp) in length, located in the 23S rRNA gene was amplified from the isolated strains using polymerase chain reaction PCR. The sequences of the primers used were as follows: 23 insF: 5′-(AC)AGCGTAG(AGCT)CGA(AT)GG-3′ and 23S insR: 5′-GTG(AT)CGGTTT(AGCT)(GCT)GGTA-3′ [40]. The reaction was performed using commercial Taq DNA polymerase (Dongsheng Biotech, P1082). PCR amplification of the 16S rRNA gene was performed on strains that showed amplification of the 350-bp phylogenetic marker located in the 23S rRNA gene. For this amplification, the sequences of the primers used were as follows: 8f: 5′-AGAGTTTGATCMTGGCTCAG5′- and 1492r: 5′-TACGGYTACCTTGTTACGACTT5′. The reaction was performed using commercial Taq DNA polymerase (Dongsheng Biotech, P1082). The amplified fragments of the 16S rRNA gene were filtered using PCR Amicon Ultra 0.5 ml purification equipment (Merck Millipore, UFC503096) following the methodology provided by the manufacturer. The amplified products were sent to a sequencing service (Macrogen, USA), and the sequences obtained were checked and corrected. Consensus sequences were built from the forward and reverse fragments using BioEdit software version 7.0.9 [15]. To determine the percentage of similarity of the consensus sequences, they were compared with sequences already deposited in the GenBank database (National Center for Biotechnology Information–NCBI) using Basic Local Alignment Search Tool (BLAST) [3].

Determination of the concentration of NaCl and pH value for the optimal growth of the isolated strains (physiological characterization). The range and optimal concentration of NaCl for the growth of purified strains were determined by inoculating each strain in the medium from which they were isolated. The media were adjusted to the following NaCl concentrations: 0, 0.5, 3, 5, 10, 15, 20, 25 and 30% at pH 7.0. The inoculum was streaked and incubated at 37 ºC. The plates were checked for growth every day for 10 days. The liquid medium, from which the strain was isolated, with the corresponding NaCl concentration previously determined for optimal growth, was used for pH determinations. The pH was adjusted to values of 5, 6, 7, 8, 9, 10, 11 and 12. The strain was inoculated from solid medium in the culture medium, and the flasks were incubated at 37 ºC. Microbial growth was determined by measuring the optical density at 600 nm [11] in a spectrophotometer (Perkin-Elmer UV-Vis, model 551S). Readings were taken at 0 h and subsequently every 24 h for 10 days. The group of pH values in which an optical density equal to or higher than 0.2 was obtained was established as the pH range for growth. The pH value at which the highest optical density was obtained was considered as the optimal pH for growth.

Phylogenetic analysis. Sequences from well-known collections, such as the Deutsche Sammlung von Mikroorganismen (DSM), the American Type Culture Collection (ATCC), the Biological Resource Center of the Na-

tional Institute of Technology and Evaluation (NBRC), and the Centers for Disease Control and Prevention (CDC), were obtained, corresponding to the species that showed the highest percentage of similarity. The consensus sequences and sequences from GenBank collections DSM, ATTC, NBRC and DCD were aligned using BioEdit Sequence Alignment Editor version 7.0.9 [15]. The phylogenetic analysis was performed with the maximum parsimony method using Molecular Evolutionary Genetics Analysis (MEGA) software version 5.2 [49]. The bootstrap was calculated with 1000 repetitions. (The nucleotide sequence data reported are available in GenBank database under the accession numbers from KP100512 to KP100519.)

Growth kinetics in Czapeck and minimum salts media with anthracene. To characterize the tolerance of the isolated strains to growth in the presence of anthracene, all strains were cultivated in two Czapeck media containing 0.01% of glucose as growth starter and 100 mg/L or 200 mg/L of anthracene respectively. Czapeck media were supplemented with 0.5%, 3% or 10% NaCl for allowing the optimal growth of the strains. Strains M2C, M3H, M3I and M5B were inoculated in this medium supplemented with 0.5% NaCl. Strains M1B, M4A and M10A were inoculated in the medium supplemented with 3% NaCl, and strain M11A was inoculated in the medium supplemented with 10% NaCl. In all cases, pH was adjusted at 8. Each solution was sterilized in an autoclave at 121 ºC for 15 min. The inoculated flasks were maintained in an incubator with agitation at 150 rpm in the dark. Microbial growth was determined by measuring optical density at 600 nm [11] using a spectrophotometer. The capacity of each strain to use anthracene was related to the increase in the medium's turbidity.

Once the exponential phase of the growth in Czapeck was determined, a volume corresponding to an OD_{600} of 0.25 at the 50% of this phase was taken and inoculated in two minimum salt medium, one without anthracene and another with 1 µg/ml of anthracene respectively, both supplemented with NaCl (as described for Czapeck medium). As in the previous cases, the inoculated flasks were maintained in an incubator with agitation at 150 rpm in the dark. Microbial growth was determined by measuring the OD_{600} [11] using a spectrophotometer. The capacity of each strain to use anthracene was related to the increase of the medium's turbidity.

The composition of the minimal salts medium (MSM) [43] was: anthracene, 0 or 1 µg/ml; $(NH_4)_2SO_4$, 1000 mg/l; Na_2HPO_4, 800 mg/l; K_2HPO_4, 200 mg/l; $MgSO_4 \cdot 7H_2O$, 200 mg/l; $CaCl_2 \cdot 2H_2O$, 200 mg/l; $FeCl_3 \cdot H_2O$, 5 mg/l; $(NH_4)_6Mo_7O_{24} \cdot H_2O$, 0.5 mg/l. This MSM was supplemented with NaCl in three different variants, adding 0.5%, 3% and 10% NaCl, respectively. In all cases the pH was adjusted to 8. Each solution was sterilized in an autoclave at 121 ºC for 15 min.

Analysis of the concentration of anthracene by fluorescence emission and identification of functional groups by ATR-FTIR. Primary inocula were prepared by transferring the strain from solid medium into the corresponding flasks containing 60 ml of MSM with 1 µg/ml of anthracene and the percentage of NaCl and pH required for their growth, as described previously. Screw-cap flasks were covered with aluminum foil and were maintained in an incubator with agitation at 150 rpm in the dark until reaching half the exponential growth phase. The exponential growth phase was already known from the kinetics growth curves.

At the time corresponding to half of the exponential growth phase from each primary inoculum, the volume corresponding to an OD_{600} of 0.25 was transferred to 60 ml of MSM with 270 ng/ml of anthracene and the required percentage of NaCl and pH as previously indicated. This optical density corresponds to 0.5 nephelometric turbidity units (NTU). The flasks were incubated in the dark at 150 rpm. Five-ml aliquots of each culture were taken at 0, 1, 24 and 48 h and were centrifuged at 10,000 rpm for 5 min to separate biomass. The concentration of anthracene was measured in the supernatant by fluorescence emission. In order to identify the functional groups of the of anthracene degradation, the infrared spectrum by attenuated total reflectance–Fourier transform infrared spectroscopy (ATR-FTIR) of the supernatant was recorded. The strains were also inoculated in the MSM medium supplemented with the required NaCl concentration for optimal growth without anthracene in order to verify that metabolites that could interfere with the anthracene signal had not been produced during the incubation time of cultures. These control cultures were analyzed in the same manner as the culture samples.

The fluorescence emission spectra of the supernatants were read in a spectrofluorometer (Horiba, Fluoromax-3). In all cases, the recording conditions of the spectra were as follows: excitation wavelength of 340 nm, 2-nm resolution, integration time of 0.5 s and 2-nm slits. An emission signal of 401 nm was used for strains M1B, M10A, M2C, M5B, M3H and M3I; for strain M11A, the emission signal was 419 nm. Infrared spectra were obtained in an ATR-FTIR spectrometer (Brucker, Alpha) equipped with a diamond ATR module for individual reflection (Platinum ATR single reflection diamond ATR module) in a range of 500–4000 cm^{-1}, with a resolution of 4 cm^{-1}. Each spectrum was obtained from 5 µl of supernatant and is the mean of 24 scans. All assays of fluorescence emission and ATR-FTIR were repeated 5 times.

Statistical analysis. The residual anthracene concentrations in the media inoculated with microorganisms were statistically compared by a bivariate analysis. The two studied variables were strains (7 levels) and time (3 levels). Each experiment was performed five times (five independent inoculations for each strain).

Results

Isolation and characterization of Actinobacteria. The eleven soil samples analyzed contained from 1 to 3% NaCl and their pH ranged from 7.8 to 8.6. No other analyses of the samples collected were conducted.

From the six culture media employed, only MH and YME allowed the isolation of Actinobacteria and eubacteria, with no growth of fungi or yeasts, indicating that the nutrients present in these media, the NaCl concentration and the pH were appropriate. From the isolated strains, eight were Actinobacteria. These strains were identified as M1B, M10A, M2C, M3H, M3I, M5B, M4A and M11A. From MH medium at pH 7 supplemented with 3% NaCl, strains M1B, M10A, M4A, M2C and M5B were isolated. From MH medium at pH 7 and supplemented with 10% NaCl, M11A was isolated, and from YME medium at pH 7 with 0% NaCl, M3H and M3I were isolated. The eight isolated strains formed small or medium border-raised, soft colonies, which were cream or yellow and in some cases orange; circular or irregular; opaque or brilliant; with vegetative mycelium; and without pigment diffused into the medium. Under the microscope, they could be seen as unique cocci and in tetrads, small bacilli, or branched filamentous cells. They were Gram-positive.

Genetic and physiological identification. Eight strains showed amplification of a 350-bp fragment, indicating

Table 1. Identification and physiological characterization of the eight strains of Actinobacteria isolated in this work. All of them are haloalkalitolerant and have an optimal growth at pH of 8. Major similitude with known strains (DSM or ATCC) is shown as percentage (%)

Strain	bp of the amplified fragment	Species	Strains with higher similitude	%	g/100 ml NaCl range	g/100 ml NaCl for optimal growth	pH range
M1B	1360	*Kocuria rosea*	DSM 20447	99.0	0–10	3	5–11
M11A	1415	*Kocuria palustris*	DSM 11925	98.0	0–20	10	5–12
M10A	1372	*Microbacterium testaceum*	DSM 20166	98.0	0–10	3	5–11
M2C	1350	*Nocardia farcinica*	ATCC 3318/DSM 43665	99.0	0–5	0.5	5–10
M3H	1356	*Nocardia farcinica*	ATCC 3318/DSM 43665	99.0	0–5	0.5	5–10
M3I	1325	*Nocardia farcinica*	ATCC 3318/DSM 43665	99.0	0–5	0.5	5–10
M5B	1363	*Nocardia farcinica*	ATCC 3318/DSM 43665	98.0	0–5	0.5	5–10
M4A	1355	*Cellulomonas denverensis*	DSM 15764	99.0	0–3	3	5–10

that they were Gram-positive bacteria with high G+C content. The comparative analysis of 16S rRNA sequencing resulted in the identification of 2 strains belonging to the *Kocuria* genus, of which, strain M1B was similar to the *Kocuria rosea* species and strain M11A to *Kocuria palustris*. Strain M10A was identified as *Microbacterium testaceum*. Strains M2C, M3H, M3I and M5B were identified as *Nocardia farcinica*. Strain M4A was identified as *Cellulomonas denverensis*. The similarity percentages obtained from BLAST analysis are shown in Table 1. The table also shows the % NaCl range, the % NaCl for optimal growth, and the pH range.

All strains were capable of growing within a range of NaCl concentrations which was as narrow as 0–3% up to as wide as 0–20%. In addition, they could also grow within a wide range of pH. Therefore all these eight Actinobacteria species were classified as haloalkalitolerant. Within the haloalkalitolerant category, they were classified, according to Russell [41], as low haloalkalitolerant (M2C, M3H, M3I, M5B and M4A), moderate haloalkalitolerant (M1B and M10A) and extreme haloalkalitolerant (M11A).

Phylogenetic analysis. The phylogenetic tree shows the formation of solid groups for genera and species (Fig. 2). The locations of the strains identified inside of the groups confirmed their identification and phylogenetic relationships, meaning that changes over time in their nucleotide sequences have maintained them in their corresponding genera.

Growth kinetics in MSM with anthracene: Figure 3 shows the growth over time of *K. rosea, K. palustris, M. testaceum, N. farcinica* and *C. denverensis* in Czapeck medium containing 100 and 200 mg/ml (Fig. 3A,B), as well as in MSM without and with anthracene (1 µg/ml) (Fig. 3C,D). Except for *C. denverensis*, all strains could grow in these culture media.

The growth of the strains in the Czapeck medium with anthracene was accelerated, the stationary phase was reached between 1 and 1.5 h with 100 and 200 mg/l of anthracene, the stationary phase was stable up to at least 48 h. The bacterial population (log DO/DO_0) increased slightly with anthracene (200 mg/l), showing that anthracene is tolerated and utilized by the bacteria. To confirm quantitatively this result, the fluorescence experiment was designed (see results below). Czapeck medium had a high background signal in fluorescence. For this reason, fluorescence experiments were carried out in MSM medium and the kinetic growth curves in MSM were determined.

To study the growth kinetics in MSM medium, strains were transferred from Czapeck to MSM medium without anthracene to cultivate a primary inoculum, which was subsequently transferred to MSM medium with 1 µg/ml anthracene. From Czapeck to MSM without anthracene, and from this to MSM with 1 µg/ml anthracene, the volume transferred used as primary inoculum corresponded to an OD of 0.25 of culture strains taken at 50% of the logarithmic phase. In MSM medium without anthracene (Fig. 3C), the growth of the strains was due to the carbon source that bacteria accumulated as a reserve at the time that were cultured in medium Czapeck (Fig. 3A,B).

In the MSM medium, the growth of bacteria was lower than in the Czapeck medium, which was an expected result because MSM has a lower nutrient content. However, growth behavior was similar in both media. In three cases (Fig. 3A–C), a rapid growth was observed during the first h, and the stationary phase began at some tim between 1.5 and 2 h. In MSM media with 1

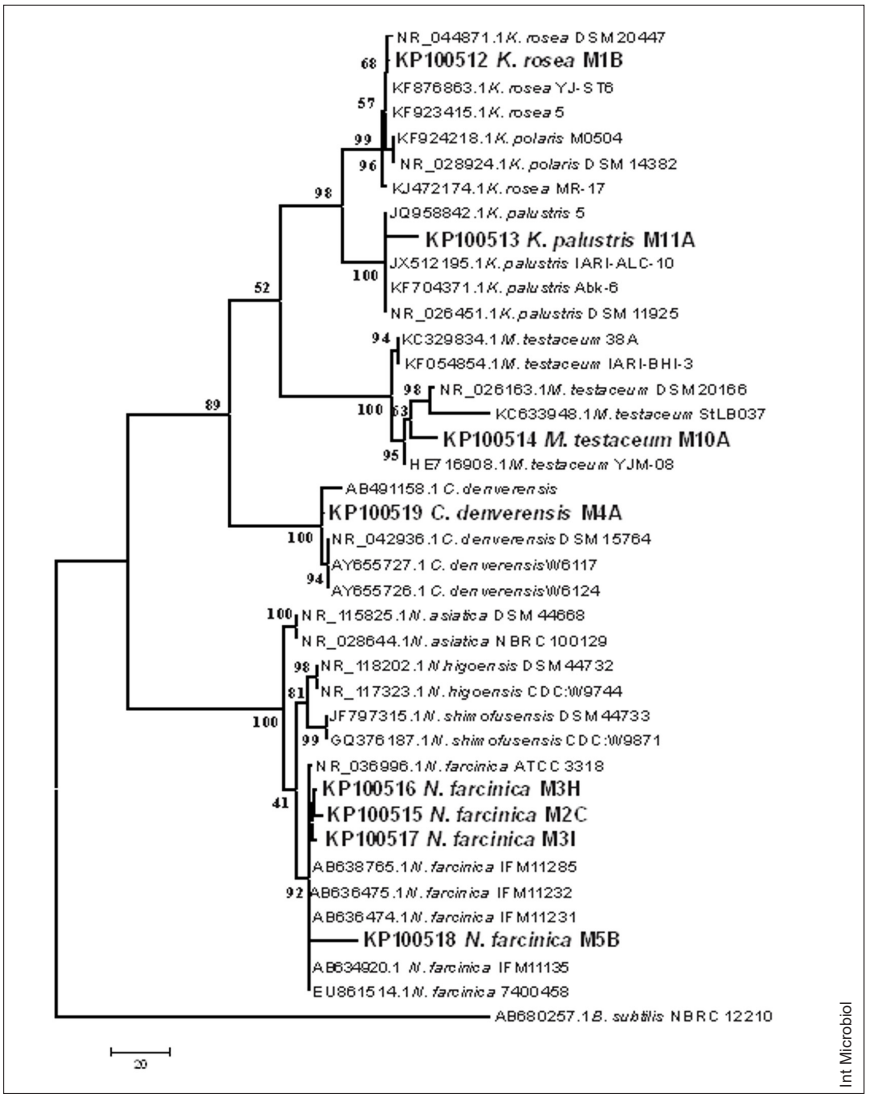

Fig. 2. Phylogenetic tree of haloalkalitolerant Actinobacteria.

μg/ml anthracene (Fig. 3D), the growth curve for *K. rosea*, *M. testaceum* and *N. farcinica* strains showed an abrupt growth within the first hour, followed by a slower growth until 7.5 h, when the stationary phase began, and, from then, it was constant for at least 24 h (Fig. 3D), (measurements are not continued after this time). In the case of *K. palustris*, the exponential growth phase finished at 10 h. Therefore, the primary inocula used for the spectrofluorimetry and ATR-FTIR assays were obtained at 4 and 5 h, respectively.

Analysis of the concentration of anthracene by fluorescence emission and identification of functional groups by ATR-FTIR. The concentrations of anthracene at 0, 1, 24 and 48 h present in the culture medium of *K. rosea, K. palustris, M. testaceum* and *N. farcinica* strains were determined by fluorescence emission. The results obtained (Fig. 4) show steep decreases in anthracene concentration (40–55%) during the first hour for all strains. This reduction was not further increased during the 1 to 24 h and 24 to 48 h intervals, respectively. This result agrees with the abrupt bacteria growth (Fig. 3) within the first hour that was later stabilized for at least 48 h. A bivariate analysis of the anthracene residual concentration shows statistically differences ($P < 0.05$) among the strains and the studies time interval.

The ATR-FTIR spectra of all strains were very similar and are shown in Fig. 5. The MSM with anthracene at 0 h and 48 h, as well as the cultures with anthracene after 48 h of incubation presented the band at 1237 cm^{-1} which decreased in intensity over time and did not appear in the medium inoculated for 48 h without anthracene. However, this band did not correspond to any vibration of the anthracene molecule; instead,

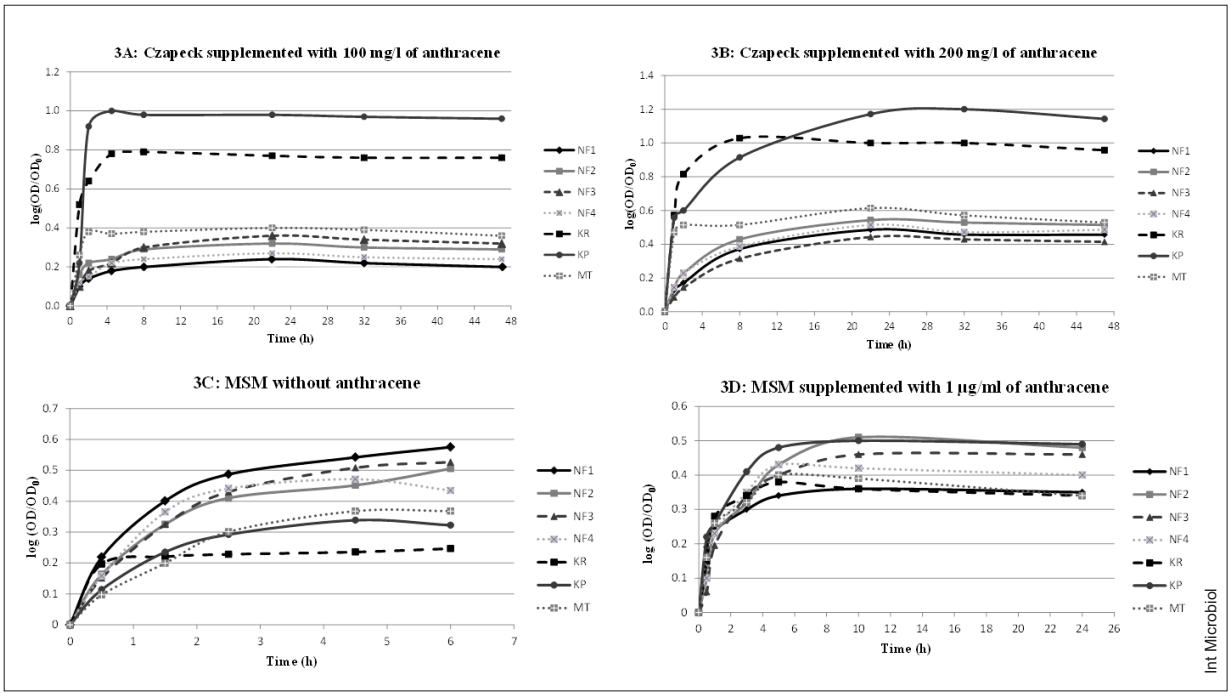

Fig. 3. Kinetic growth curves in Czapeck and MSM media with anthracene of the haloalkalitolerant identified Actinobacteria.

it was related to the decrease in its concentration, as shown in Fig. 5. The reduction of the 1237 cm^{-1} signal at 48 h is in agreement with the reduction in the residual anthracene concentration (Fig. 4). The anthracene was dissolved in MSM at pH 8. This solution had both high salt content and high ionic force.

Therefore, this band at 1237 cm^{-1}, which corresponds to a C–C–O–phenol asymmetric *stretching* vibration [46], is expected to be due to some interaction or direct effect of the solvent (medium) on the anthracene molecule, either solvating or hydroxylation. The decrease of this band over time indicates a molecular transformation in the anthracene. Another transformation of the anthracene is the emergence of bands at 1205 cm^{-1} and 1217 cm^{-1}, which are not initially present in either the MSM with anthracene at 0 h or the microorganism solution incubated with anthracene for 1 and 24 h, nor they

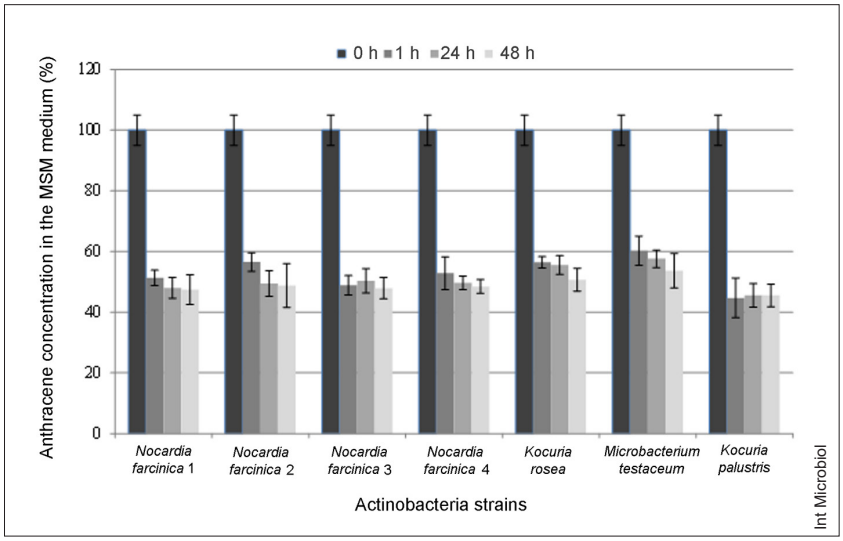

Fig. 4. Residual anthracene concentration in the Actinobacteria cultures by emission fluorescence (n = 5).

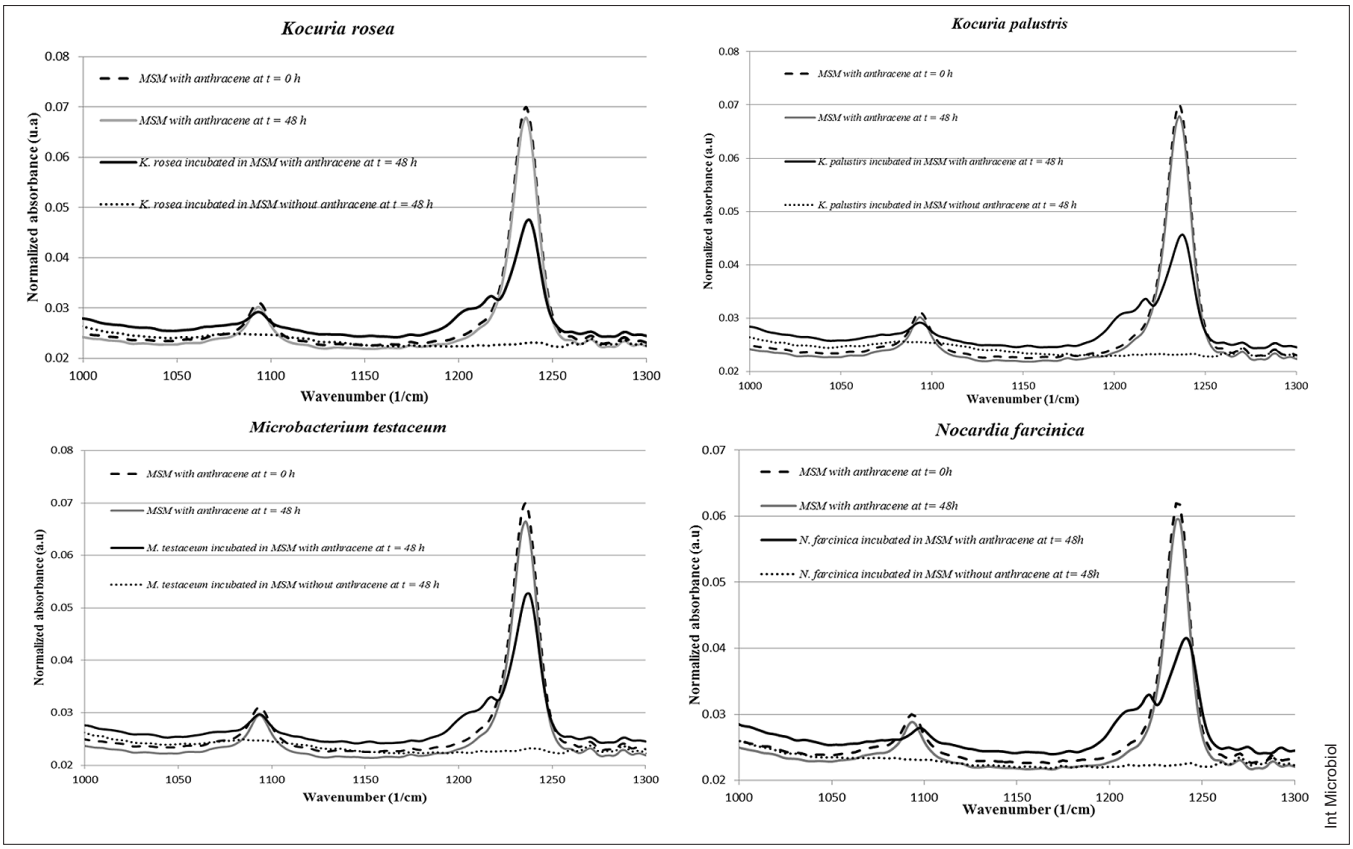

Fig. 5. ATR-FTIR spectra of the Actinobacteria species incubated with and without anthracene at t = 48 h, and the MSM medium with anthracene at t = 0 h and t = 48h.

are present in the microorganism solution incubated for 48 h without anthracene. These bands only appear in the samples inoculated with anthracene for 48 h. These last two bands correspond to the C–C–O–phenol asymmetric stretching vibration [46]. This band indicates that the anthracene is being hydroxylated, and the emergence of 2 bands indicates that the anthracene in fact is being dihydroxylated.

Discussion

The main objective of this study was to identify genetically haloalkalitolerant Actinobacteria isolated from soils close to oil activity and to evaluate their potential PAH degradation capacities. Bioremediation of PAH-contaminated environments is possible when using native halotolerant and alkalitolerant microorganisms [14,36]. Therefore, microorganisms with these two physiological properties should be able to grow in polluted environments presenting a wide range of NaCl concentrations and pH. Soils contain the highest numbers of existing phylogenetic groups; there are more than 10^9 bacterial cells per gram of soil [12]. However, it have been estimated that only 1% of the microbial soil populations can be cultured using traditional methods [50], which makes isolation a very difficult task. Therefore, in this study, we initially prepared MH culture medium [38], SAUTON-UAM [42], Czapek agar, soil extract agar [16], NZ amine A [28], GA agar [33] and YME [45], which are the most used media for the isolation of halophilic microorganisms and Actinobacteria. By modifying pH and NaCl concentration in these media, we obtained 22 different combinations (data not shown). Under these conditions, 45 strains were isolated, of which only 8 were haloalkalitolerant Actinobacteria according to the morphological and physiological characterization, as well as the genetic identification.

The M1B strain, identified as *K. rosea*, had been previously classified inside the *Micrococcus* genus and recently has been re-classified in the *Kocuria* genus [47]. It has been mainly isolated from water and soil samples. Mahjoubi et al. [23] reported its isolation in Bushnell Hass mineral salts (BHMS) medium supplemented with 1% petroleum from sediments and seawater collected near a refinery in Tunisia.

The authors also evaluated its capacity for the production of biosurfactants and concluded that it could be a potential candidate for bioremediation. Ahmed et al. [1] isolated *Kocuria rosea* from artificial seawater (ASW) medium from water and soil samples contaminated with hydrocarbons in Pakistan. They found and reported *K. rosea* capacity to grow on naphthalene, phenanthrene and fluoranthene and to degrade the first. In this study, *K. rosea* was isolated from a soil sample containing 1% NaCl at pH 8.3.

Strain M11A was identified as *K. palustris*. This species was described by Kovács et al. [18] after being originally isolated from *Typha angustifolia*. In 2007, Mariano et al. [25] isolated *Kocuria palustris* from the soil of a gas station in PCA medium (plate count agar), and one year later, Mariano et al. [24] reported the degradation of commercial diesel using this strain. To our knowledge, there have no other reports on its isolation from other environments. In this study, *K. palustris* was isolated from a soil containing 3% NaCl at pH 8.4.

Strain M10A was identified as *M. testaceum*. This species was first isolated from Chinese rice paddies and classified in the *Aureobacterium* genus but subsequently re-classified in the *Microbacterium* genus [48]. De Vasconcellos et al. [9] isolated the genus *Microbacterium* sp. from coastal oil wells in nutritive broth and reported its capacity to degrade dihydrophenanthrene. To our knowledge, there have no other reports on the isolation of *Microbacterium testaceum* from contaminated environments. In this study, *Microbacterium testaceum* was isolated from a soil containing 3% NaCl at pH 8.6.

Strains M2C, M3H, M3I and M5B correspond to *Nocardia farcinica*. This species was originally isolated from a lesion on a horse by Trevisan [51]. Al-Awadhi et al. [2] isolated it along with *N. pneumoniae* in mineral medium from samples of soils contaminated with hydrocarbons in Kuwait. *N. farcinica* was isolated particularly from a soil with 1.6% of salinity and pH 6.4. Additionally, Zeinali et al. [53] reported the isolation of *N. otitidiscaviarum* from soils of different oil-industrial sites. This species is capable of degrading naphthalene. In this study, *N. farcinica* was isolated from four soil samples containing 2%, 3%, 3% and 2% NaCl at pH values of 7.8, 7.8, 7.8 and 8.1, respectively. The salinity and pH values from our soil samples are slightly higher than the salinity and pH of the soil from which Al-Awadhi et al. isolated *N. farcinica* [2]. These results agree with the physiological characterization carried out in this work (Table 1), which showed that *N. farcinica* tolerated % NaCl in the range 0–3 and pH in the range 5–10.

Strain M4A corresponds to *C. denverensis*. This new species was first isolated from a blood sample of a patient with endocarditis [7]. Brito et al. [6] suggested that *Cellulomonas* sp., isolated from sediments of mangroves in Brazil, could degrade pyrene in ASW medium. In this case, the species was isolated from a soil sample containing 3% NaCl at pH 7.8. Note that this is the first time that the isolation of *C. denverensis* has been reported from soil.

Considering the interest in determining the residual concentration of anthracene present in the culture medium over time using fluorescence emission, initially Czapeck medium was employed but later MSM was used for this purpose. MH and YME media, used for the strains' isolation and purification, are not colourless and could affect the analytical determination. Czapeck is colourless but showed high fluorescence background.

Except for *C. denverensis*, which did not grow in Czapeck and MSM media, the remaining seven strains increased the OD_{600} when anthracene was present (Fig. 3). This indicates that the strains increased their bacterial biomass so that anthracene could be used as the sole carbon source. The results of our study do not discard the possibility that *C. denverensis* could grow in the presence of anthracene given that Brito et al. [6] reported the capacity of *Cellulomonas* to degrade pyrene. This strain simply did not grow under our study conditions.

The fluorescence emission study demonstrated that *K. rosea*, *K. palustris*, *M. testaceum* and *N. farcinica*, isolated from soils near areas with oil activity, were capable of using and transforming anthracene (Figs. 4,5). The residual anthracene values measured in this study were in the range of those reported for other PAHs (naphthalene, phenanthrene, dihydrophenanthrene) [1,2,9]. For example, Ahmed et al. [1] reported residual naphthalene and phenanthrene concentrations after 10 days of incubation in the presence of *K. rosea* and *Kocuria flava* of 64% and 91% and 47% and 91%, respectively. In our study, residual anthracene concentrations after 48 h of incubation with *K. rosea* and *K. palustris* were 49% and 54%, respectively. De Vasconcellos et al. [9] reported residual dihydrophenanthrene concentrations from 40-day cultures of the genera *Microbacterium* sp., *Bacillus* sp., and *Halomonas* sp. of 56%, 59%, and 79%, respectively. In our study, the residual anthracene concentration after 48 h of incubation with *M. testaceum* was 36%. Al-Awadhi et al. [2] reported, after 15 days of incubation of *Nocardia pneumoniae* and *Streptomyces cellulosae* with phenanthrene, residual concentrations of 85% and 30%, respectively. In our case, residual anthracene concentrations after 48 h of incubation with 4 different *N. farcinica* samples were 48%, 52%, 51% and 53%.

The decrease in the fluorescence signal of the residual an-

thracene during the first hour indicated a rapid transformation, which coincided with the accelerated bacteria growth. From this point until 48 h, the observed fluorescence signal stabilized. The emergence of signals at 1205 cm^{-1} and 1217 cm^{-1} in the ATR-FTIR spectra at 48 h were associated with the formation of dihydroxy-aromatic intermediate compounds. In fact, these compounds have been reported as the initial phase of the anthracene degradation mechanism for Actinobacteria [10, 29]. They were probably formed from the first hour but the FTIR detection limit in our conditions (no chemical separation of anthracene was involved) did not allow their detection. The initial anthracene concentration in the cultures was 270 ng/ml. In 1 h it was reduced to about 135 ng/ml. The ATR-FTIR analysis was carried out with 5 µl of culture containing about 0.675 ng (135 ng/ml × 0.005 ml), which was a very small mass to be detected by ATR-FTIR without chemical separation. The determination of PAH (including anthracene) by the SW-846 Test Method 8410 EPA by GC-FTIR has a detection limit of tenths ng [27], and it involves the separation of PAH by capillary gas chromatography and subsequent detection by FTIR. The kinetic growth curves showed that the accelerated growth occurred during the first hour, but the stationary phase was present at least up to 48 h. Therefore metabolic activity continued in the culture and made it possible the detection of the dihydroxy-aromatic compounds at 48 h.

The reported anthracene degradation by Actinobacteria continues with the formation of COOH and COH groups [10,29]. In our ATR-FTIR spectra, however, there was no evidence of this step, indicating that this process might occur after 48 h. The total PAH oxidation by microorganisms is a slow process that requires several days or even weeks, as demonstrated by Martin et al. [26]. In our study, the concentration of residual anthracene at 48 h, was in the order of other reported studies of PAH biodegradation by Actinobacteria at 10, 15 and 40 days [1,2,9].

The capacity to transform anthracene by *N. farcinica, K. rosea, M. testaceum* and *K. palustris* strains was demonstrated at their optimal growth conditions (0.5%, 3% and 10% NaCl, respectively, and pH 8). However, they could growi at a wide range of pH and NaCl concentrations. Thus, they are potential candidates for the treatment of saline and alkaline environments contaminated with polycyclic aromatic hydrocarbons compounds. Note that *N. farcinica* should be used for biodegradation purposes only in places where it is native; it should not be seeded in foreigner locations due to its pathogenic nature.

To sum up, in this study eight strains of haloalkalitolerant Actinobacteria were isolated from soils close to areas with oil activity in Mexico. Analysis of the 16S rRNA gene revealed the presence of species *K. rosea, K. palustris, M. testaceum, N. farcinica* and *C. denverensis*. The first four species had already been isolated from soils in other parts of the world, but, to our knowledge, this report describes the first isolation of *C. denverensis* from this kind of environment. These strains were classified as haloalkalitolerant (based on the NaCl % and pH range), although *K. palustris* was classified as extreme haloalkalitolerant. Their biomass could increase in the presence of anthracene, this increase correlates with the decrease in the residual anthracene concentration in the culture. ATR-FTIR spectroscopy indicated that *K. rosea, K. palustris, M. testaceum* and *N. farcinica* transformed anthracene in dihydroxy-aromatic compounds during the first 48 h. These seven strains had the capacity to transform anthracene; thus, they have the potential to be employed for biodegradation processes using native haloalkalitolerant Actinobacteria in saline and alkaline environments contaminated with PAH.

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Competing interests. None declared.

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