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ISOLATED BACTERIA FROM HOT SPRINGS ABLE TO USE HYDROCARBONS AS CARBON SOURCE

Bacterias aisladas de aguas termales capaces de usar hidrocarburos como fuente de carbono

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Key words: *Bacillus licheniformis*, degradation, diesel, gasoline

ABSTRACT

Petroleum derivates used in energy production are gravely pollutants for the ecosystem, especially for aquatic environments and human health. This study aimed to isolate hydrocarbons-degrading bacteria from hot springs. Three strains of hydrocarbon-degrading bacteria strains, belonging to the *Bacillus* and one of the genus *Lysinibacillus* were isolated. These strains tolerate temperatures from 65 to 100 ºC and were able to degrade and grow on BH medium supplemented with gasoline and diesel. Strain M2-7 shared 100 % 16S rRNA identity with *Bacillus licheniformis* and was the only able to degrade pyrene and benzopyrene among these isolated strains. The results indicate that *B. licheniformis* M2-7 could degrade a wider range of hydrocarbons and some recalcitrant hydrocarbon components, which could be particularly helpful for the treatment and bioremediation of hydrocarbon-polluted systems.

Palabras clave: *Bacillus licheniformis*, degradación, diésel, gasolina

RESUMEN

Los hidrocarburos derivados del petróleo son los principales contaminantes del ambiente y tienen graves efectos perjudiciales para la salud humana y el ambiente acuático. El objetivo de este estudio fue aislar bacterias degradadoras de hidrocarburos a partir de aguas termales. Se lograron aislar tres cepas de bacterias degradadoras de hidrocarburos pertenecientes al género *Bacillus* y una del género *Lysinibacillus*.

Las cepas bacterianas aisladas toleran temperaturas de 65 a 100 °C y un análisis cinético indicó que son capaces de degradar la gasolina y el diésel. La cepa M2-7 comparte 100 % de identidad de ARNr 16S con *Bacillus licheniformis* y a diferencia de las otras tres cepas identificadas, M2-7 presentó capacidad para degradar pireno y benzoapireno. Estos resultados indican que *B. licheniformis* M2-7 puede degradar una gran variedad de hidrocarburos, así como algunos componentes de hidrocarburos recalcitrantes, y puede aplicarse para la biorremediación y el tratamiento de ambientes contaminados con petróleo.

INTRODUCTION

Extremophilic microorganisms survive in diverse extreme conditions of high or low temperatures, high salinity, acidic and alkaline pH environments, and even high radiation conditions (Aanniz et al. 2015). Microorganisms that inhabit and survive at high temperatures are classified according to the optimal growth temperature as thermophiles (Pikuta et al. 2007). Thermophiles live in temperatures from 45 to 70 °C, e.g., *Bacillus acidocaldarius* (Darland and Brock 1971) and *Geobacillus stearothermophilus* (Nazina et al. 2001). In contrast, extreme thermophiles thrive between 70 to 80 °C, e.g., *Thermus aquaticus* (Brock and Freeze 1969) and *Thermoanaerobacter ethanolicus* (Wiegel and Ljungdahl 1981). Finally, hyperthermophile microorganisms have an optimal growth temperature above 80 °C, e.g., *Thermotoga maritima* (Huber et al. 1986) and *Pyrococcus furiosus* (Fiala and Stetter 1986). Thermophile microorganisms have potential use for different applications such as petroleum industry, microbial recovery of oil (depletion, fluidization, consolidation of sands), preventing bio-corrosion, as well as environmental protection through bioremediation techniques and compost (Atomi et al. 2011, Aanniz et al. 2015, Zeldes et al. 2015, Gou et al. 2019).

The petrochemical industry has worldwide importance. However, it affects the environment and human settlements through oil spills during transportation and storage that impact the surface and underground water, soils, and atmosphere (Zeldes et al. 2015).

The petroleum hydrocarbons are mixtures of thousands of hydrophobic hydrocarbons, including n-alkane, cycloalkane, and aromatic hydrocarbons, most of which are bioaccumulative and toxic (Kaldor et al. 1984, Mehlman 1992, Nadal et al. 2004).

Studies have shown that biodegradation of hydrocarbons-contaminated soils is possible by bacteria such as *Pseudomonas* sp. (Weissenfels et al. 1990), *Sphingomonas* sp. (Desai et al. 2008), *Cycloclasticus* sp. (Geiselbrecht et al. 1998), *Burkholderia* sp. (Kim et al. 2003), *Rhodococcus* sp. (Di Gennaro et al. 2001), *Polaromonas* sp. (Pumphrey and Madsen 2007), *Neptunomonas* sp. (Hedlund et al. 1999), and *Janibacter* sp. (Zhang et al. 2009). The use of bacteria for bioremediation is an attractive environmental-friendly alternative compared with traditional chemical approaches. Mesophilic bacteria have been previously reported for bioremediation, but their application for this process has been limited because of the slow mass transfer (Lu et al. 2011). Only a few studies have reported thermophilic bacteria able to use and metabolize hydrocarbons as carbon source (Wang et al. 2019). Thermophile microorganisms have advantages over mesophilic organisms, especially in biotechnological processes. At higher temperatures there is a better efficiency of hydrocarbon degradation, therefore, potential applications of thermophilic microorganisms should be further exploited (Viamajala et al. 2007).

The state of Guerrero, Mexico, has two important hot springs, and the microbial diversity in these places is still unknown; therefore, the aims of this study were *i)* to isolate thermophilic bacteria from these hot springs, *ii)* to determine the thermostability of these isolates, *iii)* recognize their capabilities to grow on hydrocarbons and media, and *iv)* to identify the phylogenetic affiliation of the isolated thermophilic Bacteria.

MATERIALS AND METHODS

Sampling sites

Samples were taken from two hot spring locations located at Coacoyul de San Marcos and Atotonilco de San Luis Acatlán, both in the Guerrero state in southern México (17° 38′ 30″ N and 101° 29′ 3″ W). Two samples were randomly collected

from the water surface and the bottom of the hot springs using aseptic screw-capped bottles. The pH and temperature were measured in situ using a pH meter (Orion Research, Beverly, MA) and thermometer, respectively.

Isolation of strains, media and growth conditions and screening of thermotolerance

A volume of 1 mL of the collected samples was plated on nutritive, trypticase soy and dextrose potato agar plates at pH 9.7. To recognize its thermotolerance, plates were incubated at 45 and 65 ºC for 48 hours. Afterwards, each strain was inoculated into the nutritive broth and then placed at different temperatures: 37, 45, 50, 55, 65, 70, 80, 90, and 110 °C for 1 hours. After incubation, 100 µL of the broth were poured into plates with nutritive medium and incubated at 50 °C for 24 hours.

The microbial growth for the essays was realized in Brain Heart Infusion broth (BHI) medium (in g/L): brain heart infusion 6, meat peptone 6, NaCl 5, dextrose 3, agar peptone 14.5, and KNa_2PO_4 2.5 (Guevara-Luna et al. 2018). To produce the viable count, trypticase agar was used, containing casein pancreatic digest 15 g, digested papain from soybean meal 5 g, NaCl 5 g, and agar 15 g dissolved in 1 L of distilled water. The strains were incubated at 50 °C for 24 hours.

The Bushnell-Hass (BH) culture medium containing (g/L) $MgSO_4$ 0.2, $CaCl_2$ 0.02, KH_2PO_4 1, K_2HPO_4 1, NH_4NO_3 1, and $FeCl_3$ 0.050 at pH 7, was used to make the selection of the thermophilic strain with better capacity to use hydrocarbons as the only source of carbon (Serrano-Ángel et al. 2021).

The modified minimum saline medium (MSM) (Zeinali et al. 2008) composition was (g/L) NH_4Cl 1, Na_2HPO_4 0.380, $KH_2PO_4.H_2O$ 0.380, $MgSO_4 \cdot {}_6H_2O$ 0.080, $CaCl_2$ 0.070, KCl 0.040, and $FeSO_4 \cdot 7H_2O$ 0.001 supplemented with a trace elements solution (2.5 mL/L of medium containing $MgCl_2 \cdot 4H_2O$ 0.027, H_3BO_3 0.031, $ZnCl_2$ 0.050, $CuCl_2 \cdot 2H_2O$ 0.01, $NiCl_2 \cdot 6H_2O$ 0.02, $CoCl_2 \cdot 6H_2O$ 0.036, and $Na_2MoO_4 \cdot 2H_2O$ 0.03). Naphthalene, phenanthrene, and pyrene or benzo[a]pyrene (BaP) at a final concentration of 10 µg/ml were used as carbon sources.

Evaluation of the growth of strains in hydrocarbons media

The strains were inoculated in 25 mL of LB broth for 12 hours at 50 °C and 120 rpm. Centrifugation of the LB broth was performed at 3,400 rpm to obtain a pellet, which was washed twice with BH medium. Optical density, OD_{600} nm, was adjusted at 0.5. The strains were inoculated in BH medium supplemented with 0.1 % (v/v) gasoline (95 octane), diesel (51 cetane) or burned diesel oil and incubated at 50 °C with stirring at 150 rpm. At 5, 7, and 13 days of incubation, 1 mL aliquots were taken to measure the OD_{600} nm and protein; all these experiments were performed by triplicate. The proteins were quantified using the Bradford method (Bradford 1976)

Organic extraction and thin-layer chromatography

After 13 days, BH medium supplemented with diesel or gasoline was transferred to 50 mL Falcon tubes, which were centrifuged at 3500 rpm for 10 min. The supernatant was separated from the cell pellet into a new 50 mL Falcon tube. To monitor the BaP transformation process by the strain *Bacillus licheniformis* M2-7, at 24 h culture in MSM was collected in 50 mL Falcon tubes to obtain the supernatant with the procedure described by Guevara-Luna et al. (2018). After extraction, the organic phase was collected in a 1000 mL ball flask, and the solvent was evaporated with the aid of a rotary evaporator for 15 min. The extract was collected by resuspending with ethyl acetate and deposited in 5 mL vials. The extracts were analyzed in HPTLC Silica Gel F254 chromatography using a hexane: acetone mobile system (7:3) and revealed on a UV light lamp (365 nm).

DNA extraction, sequencing, phylogenetic analyses

Genomic DNA was extracted by using a GeneJeT™ Genomic DNA purification kit, according to the manufacturer´s instructions (Thermo Scientific). Bacterial 16S rDNA was amplified by PCR using the universal bacterial 16S rDNA primers fd1 5´-AGAGTTTGATCCTGGCTCAG-3´ and rd1 5´-AAGGAGGTGATCCAGCC-3´ (Rodicio and Mendoza 2004, Ndip et al. 2008). The PCR was performed with a 25 µL reaction mixture containing 1 µL (10 ng) of DNA as a template, each primer at a concentration of 5 µM, 2.5 mM $MgCl_2$ and dNTPs at a concentration of 2.5 µM, Taq polymerase and a buffer used as recommended by the manufacturer (Thermo Scientific). The PCR conditions consisted of an initial denaturing step at 95 ºC for 7 min, 30 cycles of 95 ºC for 40 sec, 55 ºC for 40 sec, 72 ºC for 2 min, and a final extension at 72 ºC for 15 min. The PCR products were electrophoresed and purified from 1 % agarose gels (0.56 TAE buffer; 2.42 g Tris–HCl, 0.57 mL glacial acetic acid, 1 mL 0.5 M EDTA (pH 8.0), and 1 L distilled and deionized water) using the GeneJET™ PCR Purification Kit (Thermo Scientific). DNA sequences were determined by the dideoxy chain termination method (Sanger et al.

1977) with a Perkin Elmer/Applied Biosystems DNA Sequencer. The 16S rDNA sequences were aligned and compared with other 16S rDNA genes in Ribosomal Database Project II (RDP) by using SEQMATCH (Wang et al. 2007) and GeneBank by using NCBI basic local alignment search tools BLAST software (Benson et al. 2000). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test, 1000 replicates (Felsenstein 1985). The evolutionary distances were computed using the Jukes-Cantor method (1969). The rate variation among sites was modeled with a gamma distribution. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013). GeneBank accession numbers for 16S rDNA sequences are: M2-5 (MK656091), SF11 (MK656092), M2-7 (MK656090), and APF3 (MK656093).

RESULTS

A total of 14 strains were collected from hot springs, of which 12 showed temperature tolerance at 65 ^{o}C. The strains MISA-1, MISA-2, MISA-3, MISA-4, M2-5, M2-6, M2-7, APF23, ANF21, and ASF11 tolerated heat up to 100 ^{o}C. Afterward, these 10 strains were screened to grow on hydrocarbons; only M2-5, ASF11, M2-7, and APF23 grew on gasoline and diesel. Growth was evaluated by protein quantification from a culture in BH medium with gasoline (**Table I**) or diesel (**Table II**) as the only carbon source, at 50 °C and 150 rpm, with an incubation period of 13 days.

We observed that the strains M2-7 and APF23 had an apparent increase of ~0.1 mg of protein per milliliter of culture, thus, both strains were chosen to continue with the following experiments.

To validate the use of hydrocarbons as carbon source, a liquid/liquid organic extraction was performed with ethyl acetate. The extracts obtained from the cultures in Bushnell-Haas medium supplemented with gasoline or diesel from the M2-7 and APF23 strains were run on an HPTLC Silica Gel F254 plate. **Figure 1** and **2** show the different migration patterns of the extracts of M2-7 and APF23, respectively. The extracts of the gasoline medium (**Fig. 1A**) are shown through the chromatographic plate, observing the extract of strain M2-7 in BH medium supplemented with gasoline (Lane 2G) presents a migration pattern different from that of gasoline (Lane 3G). In addition, the migration pattern of the 2G lane extract is not similar to the untreated cell extract (Lane 1G) or BH

TABLE I. PROTEIN CONCENTRATION EVOLUTION OF CULTURES USING GASOLINE AS CARBON SOURCE AFTER 13 DAYS OF INCUBATION AT 50 °C AND 150 RPM.

Strain	Protein (mg/mL)	
	Initial	Final
Bacillus licheniformis M2-5	0.326	0.367±0.006
Lysinibacillus fusiformis ASF11	0.329	0.334±0.02
Bacillus licheniformis M2-7	0.256	0.318±0.009
Bacillus cereus APF23	0.239	0.331±0.007

TABLE II. PROTEIN CONCENTRATION EVOLUTION OF CULTURES USING DIESEL AS CARBON SOURCE AFTER 13 DAYS OF INCUBATION AT 50 °C AND 150 RPM.

Strain	Protein (mg/mL)	
	Initial	Final
Bacillus licheniformis M2-5	0.326	0.396±0.003
Lysinibacillus fusiformis ASF11	0.329	0.345±0.01
Bacillus licheniformis M2-7	0.256	0.338±0.008
Bacillus cereus APF23	0.239	0.355±0.009

medium extract without inoculum or hydrocarbon (Lane 4G), meaning that strain M2-7 may be able to transform any gasoline derivative.

Similarly, in panel B of **figure 1**, the migration pattern of the extracts of the strain M2-7 treated with diesel (2D lane) was different from the diesel lane (3D lane) and in the controls 1D and 4D. The above data suggest that the strain M2-7 can transform the components present in gasoline and diesel.

The same test was performed for the APF23 strain treated with gasoline and diesel. In **figure 2A**, the slides of the organic extracts obtained from the APF23

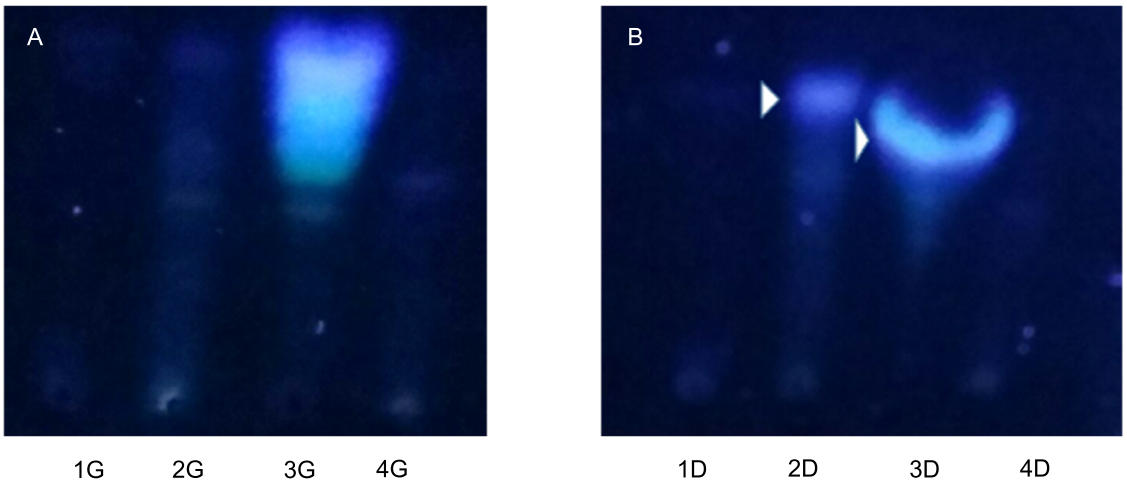

Fig. 1. Thin-layer chromatography of extracts obtained with ethyl acetate from strain *B. licheniformis* M2-7. Panel A, lane 1G: cells in culture with nutrient medium; Lane 2G: extract of medium BH supplemented with gasoline; Lane 3G: gasoline; Lane 4G: medium BH without inoculum or hydrocarbon. Panel B, lane 1D: cell sin culture with nutrient medium; Lane 2D: extract of BH medium supplemented with diesel; Lane 3G: diesel; Lane 4D: medium BH without inoculum or hydrocarbon. The white triangles point out the change in the migration pattern in the lanes.

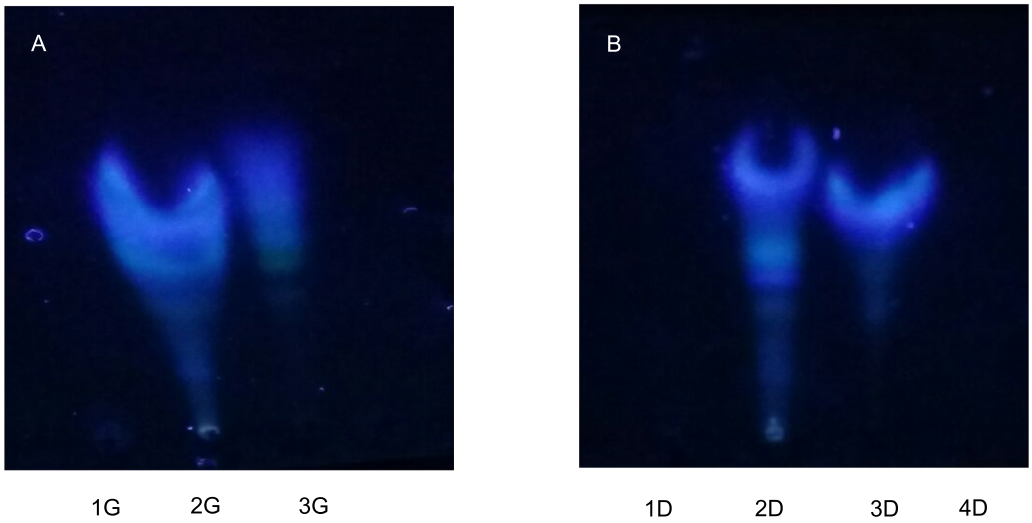

Fig. 2. Thin layer chromatography of extracts obtained with ethyl acetate from the *B. cereus* APF23 Panel A, lane 1G: cells in culture with nutrient medium; Lane 2G: extract of medium BH supplemented with gasoline; Lane 3G: gasoline; Lane 4G: medium BH without inoculum or hydrocarbon. Panel B, lane 1D: cells in culture with nutrient medium; Lane 2D: extract of BH medium supplemented with diesel; lane 3D: diesel; Lane 4D: medium BH without inoculum or hydrocarbon.

strain are shown in BH medium supplemented with gasoline. There are differences in the banding pattern of the organic extracts (Lane 2G) compared to the controls (Lanes 1G and 4G); however, the change was not so evident in the degradation of the components of the gasoline (Lane 3G). In **figure 2B**, the extracts from the cultures of BH medium with diesel (**Fig. 2B**, 2D lane) and diesel reference (3D lane) show a different banding pattern, which reflects the fractionation of the diesel components (2D lane). However, there is no appreciable decrease compared to the control (3D lane), suggesting that the APF23 strain did not perform a noticeable hydrocarbon transformation after 13 days of incubation.

Once the capacity of the *B. licheniformis* strain M2-7 to transform hydrocarbon mixtures (gasoline and diesel) was estimated, the ability of the strain to biotransform four PAH (naphthalene, phenanthrene, pyrene, and BaP) with environmental importance was evaluated. As shown in **figure 3**, strain M2-7 transformed the starting compound of pyrene and BaP into different intermediates during the growing period. **Figure 3A** shows the migration pattern of the extracts obtained from the MSM supplemented with naphthalene and is different from the migration pattern shown by the naphthalene reference observed in lane 2A. A loss of naphthalene from the system was observed by volatilization due to the high temperature used in the study model, indicating that no biotransformation of PAH occurred. In **figure 3B**, the chromatographic plate of phenanthrene shows a similar phenomenon to the one observed with naphthalene (**Fig. 3A**). In **figure 3C**,

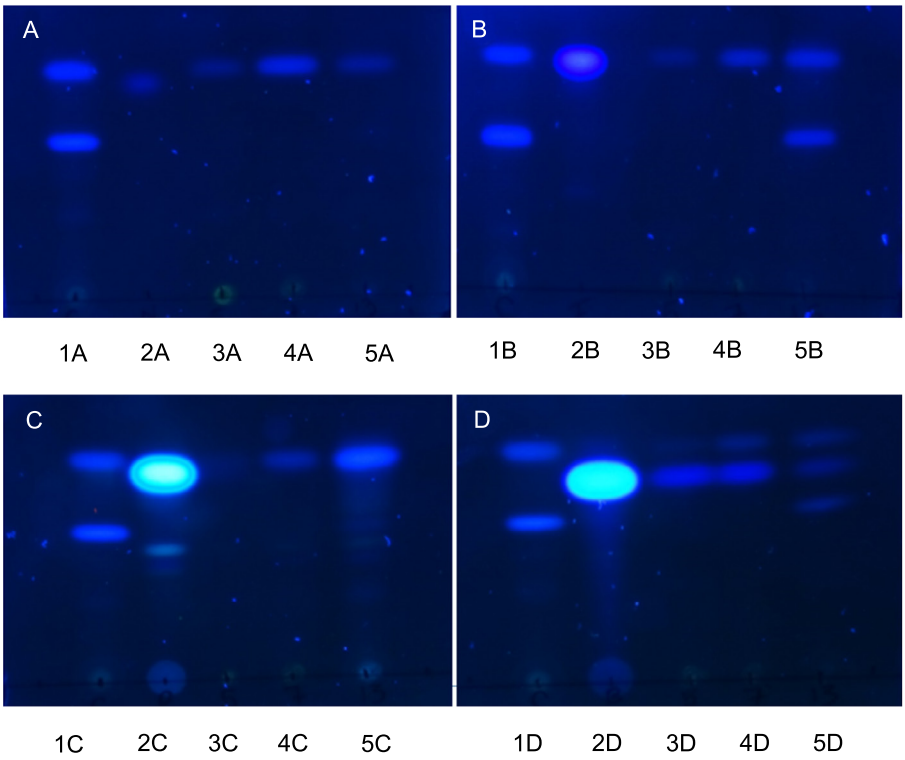

Fig. 3. Thin-layer chromatography of extracts obtained with ethyl acetate from the culture of strain *B. licheniformis* M2-7 in different hydrocarbons. Panel A, naphthalene: 1A culture medium without inoculum and naphthalene, 2A, naphthalene reference, 3A, 4A, and 5A represent extracts of 5, 7, and 13 days of culture, respectively. Panel B, phenanthrene: 1B culture medium without inoculum or phenanthrene; 2B, reference of phenanthrene; 3B, 4B, and 5B represent extracts of 5, 7, and 13 days of culture, respectively. Panel C, pyrene: 1C, culture medium without inoculum or pyrene; 2C, pyrene reference; 3C, 4C, and 5C represent extracts of 5, 7, and 13 days of culture, respectively. Panel D, benzo [a] pyrene: 1D, culture medium without inoculum or BaP; 2D, reference of benzo [a] pyrene, 3D, 4D, and 5D represent extracts of 5, 7, and 13 days of culture, respectively.

after 13 days of culture, pyrene showed degradation (lane 5C); **figure 3D** shows the biotransformation of the BaP from the fifth day (lane 3D) of culture, and a second biotransformation from the 13th day (lane 5D).

The results from the analysis using the Ribosomal Database Project II (RPD II, data not shown), revealed that the 16S rDNA sequences of the four isolates able to grow on gasoline and diesel, three belonged to the *Bacillus* genus, and one to the *Lysinibacillus* genus. The species similarities were: strain ASF11 to *Lysinibacillus fusiformis*, strains M2-5 and M2-7 to *B. licheniformis*, and strain APF23 to *B. cereus*.

DISCUSSION

The identification and characterization of bacteria capable to degrade and grow in hydrocarbons is a part of our bioremediation strategy (Pérez Vargas et al. 2017). Most of the bacteria isolated are mesophilic bacteria, such as *Microbacterium pretrolearium*, isolated from hydrocarbon-polluted soils from the Burgos basin with a recognizable hydrocarbon-degrading potential (Cruz-Hernández et al. 2020). However, thermophile microorganisms have advantages over mesophilic organisms in some biotechnological processes (Zhang et al. 2012). Some studies support our decision to work with bacterial strains with thermophilic lifestyle and the capacity to degrade hydrocarbons and high molecular weight PAH (Viamajala et al. 2007, Zeinali et al. 2007, Zheng et al. 2011). In the present study, hot springs from our locality were sampled at 70 °C and pH 8.7. These conditions define this habitat as an extremophilic ecosystem. From the 14 bacterial strains isolated from the sampling site (Antranikian et al. 2005), we identified their ability to grow in the presence of hydrocarbons and polycyclic aromatic hydrocarbons. The results showed that only 4 strains could grow in gasoline (**Table I**) and diesel (**Table II**). The evidence suggests that only the M2-7 strain was able to transform the gasoline, diesel, pyrene (**Fig. 3C**), and BaP (**Fig. 3D**). Through 16S rRNA nucleotide sequence analysis it was identified that ASF11 was closely associated with *Lysinibacillus fusiformis*, M2-5 and M2-7 strains presented a high identity (99 %) to *Bacillus licheniformis*, whereas APF23 showed a similar identity with *Bacillus cereus*.

Some species of the genus *Bacillus* are well known to degrade hydrocarbons, pyrene, and other PAH (Sowada et al. 2014). *B. thuringiensis* strain was able to degrade anthracene (Tarafdar et al. 2017), whereas *B. thuringiensis* B3, and *B. cereus* B6, were able to live and metabolize crude oil-contaminated sites in Ecuador, metabolizing PAHs from diesel. In 2017 it was reported that some strains of *Bacillus* spp. were able to degrade total petroleum hydrocarbons from contaminated soil (Raju et al. 2017). In our study, only one *B. licheniformis*, isolated from hot springs in southern Mexico, presented the ability to utilize PAH as the sole carbon source.

The alternative metabolism to degrade benzo [a] pyrene was previously proved by Sowada et al. (2014), who isolated *B. licheniformis* from human skin, and showed their metabolic capabilities, degrading BaP under mesophilic conditions. Another work supporting this metabolic diversity was that of Bahuguna et al. (2012), which analyzed growth kinetics of *B. licheniformis* BMIT5ii MTCC 9446 able to grow on naphthalene (0.5 mg/mL).

Recently, it has been proposed the BaP transformation pathway and reaction mechanism in *B. licheniformis* M2-7 (Guevara-Luna et al. 2018) along with the *catE*, *pobA* and *fabHB* gene expression in hydrocarbon degradation identification (Rojas-Aparicio et al. 2018). The reactions would start with the incorporation of molecular oxygen at carbons 9 and 10 of BaP through the enzyme catechol 2,3-dioxygenase, generating BaP cis-9,10-dihydrodiol (Guevara-Luna et al. 2018), subsequently, the metabolite undergoes another dioxygenation via catechol 2,3-dioxygenase, producing cis-4-(hydroxypyrene-8-yl)-2-oxobut-3-enoic meta-acid via (Schneider et al. 1996). This is followed by the formation of 7,8-pyrene-dihydro-7-carboxylic acid (Schneider et al. 1996) via the degradation pathway of high molecular weight PAH described by Mahaffey et al. (1988), involving several enzymes and intermediary equivalent metabolites. The second stage of this process is to produce 4-hydroxybenzoate 3-monooxygenase and ketoacyl-ACP synthase III enzymes, by the *pobA* and *fabHB* genes. These enzymes (Rojas-Aparicio et al. 2018) would participate in the formation of protocatechuate under the β-ketoadipate pathway, and generate phthalic acid, additionally generating intermediates that need to enter the tricarboxylic acid cycle to terminate the degradation (Sim et al. 2013). This suggests that *B. licheniformis* M2-7 may also have a degradation pathway for naphthalene, phenanthrene, and pyrene, which needs to be further investigated for its characterization and optimization of the metabolism.

CONCLUSIONS

Four thermophilic strains able to grow on gasoline and diesel as a carbon source were isolated from hot springs in southern Mexico at high 65°C.

Of them, *B. licheniformis* M2-7 was the only able to metabolize naphthalene, phenanthrene, and pyrene.

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