

Research Paper

Enhanced polyaromatic hydrocarbon degradation by adapted cultures of actinomycete strains

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Fifteen actinomycete strains were evaluated for their potential use in removal of polycyclic aromatic hydrocarbons (PAH). Their capability to degrade of naphthalene, phenanthrene, and pyrene was tested in minimal medium (MM) and MM with glucose as another substrate. Degradation of naphthalene in MM was observed in all isolates at different rates, reaching maximum values near to 76% in some strains of *Streptomyces*, *Rhodococcus* sp. 016 and *Amycolatopsis tucumanensis* DSM 45259. Maximum values of degradation of phenanthrene in MM occurred in cultures of *A. tucumanensis* DSM 45259 (36.2%) and *Streptomyces* sp. A12 (20%), while the degradation of pyrene in MM was poor and only significant with *Streptomyces* sp. A12 (4.3%). Because of the poor performance when growing on phenanthrene and pyrene alone, *Rhodococcus* sp. 20, *Rhodococcus* sp. 016, *A. tucumanensis* DSM 45259, *Streptomyces* sp. A2, and *Streptomyces* sp. A12 were challenged to an adaptation schedule of successive cultures on a fresh solid medium supplemented with PAHs, decreasing concentration of glucose in each step. As a result, an enhanced degradation of PAHs by adapted strains was observed in the presence of glucose as co-substrate, without degradation of phenanthrene and pyrene in MM while an increase to up to 50% of degradation was seen with these strains in glucose amended media. An internal fragment of the *catA* gene, which codes for catechol 1,2-dioxygenase, was amplified from both *Rhodococcus* strains, showing the potential for degradation of aromatic compounds via salicylate. These results allow us to propose the usefulness of these actinomycete strains for PAH bioremediation in the environment.

Keywords: Actinomycete / Polycyclic aromatic hydrocarbons / Enhanced PAH removal / *catA* gene

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are environmentally ubiquitous and recalcitrant organic contaminants produced naturally or as a result of incomplete combustion of organic materials. The persistence of PAHs is of environmental concern because they have

been reported to produce carcinogenic, teratogenic, and mutagenic effects [1].

PAHs present different chemical properties and bioavailability according to the number of benzene rings in the molecule. Usually, low-molecular-weight PAHs such as naphthalene and phenanthrene are easily degradable. Pyrene, a tetracyclic PAH, has been widely used as indicator and model compound to study biodegradation of high-molecular-weight PAHs [2]. Several researchers demonstrated the difficulty to degrade pyrene [3], since high-molecular-weight PAHs are regarded recalcitrant and thermodynamically stable [4]. Furthermore, PAHs are usually present in the

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environment as a mixture of several aromatic compounds, where each PAH has the capacity to influence others affecting their bioavailability and increasing the difficulty for degradation by microorganisms [5, 6]. Information on the degradation of these PAHs in bacteria mostly is available for Gram-positives, suggesting that these organisms play an important role in the environmental degradation of these aromatic compounds [7].

In general, actinomycetes are promising candidates for bioremediation procedures because they have a great potential for biodegradation of organic and inorganic toxic compounds in soils [8]. In addition, several culture conditions and composition of the culture media, which have a direct influence on degradation, could be manipulated in order to accelerate the process of biodegradation of PAH [9].

Catechol 1,2-dioxygenase is known to accomplish the first step of catalysis of the aromatic ring by ortho-cleavage. The enzyme is encoded by the *catA* gene, present in the genomes of a large number of PAH-degrading bacteria. However, information on *catA* prevalence in Gram-positives is limited [10].

The aim of this work was to select bacteria with the ability to degrade naphthalene, phenanthrene, and pyrene, and to improve the degradation by using glucose as co-substrate. Furthermore the *catA* gene was identified to show potential for degradation of aromatic compounds in the actinomycete strains.

Materials and methods

Strains, culture media, and chemicals

Streptomyces strains with the capacity to growth, remove and use different organochlorine pesticide were previously isolated from an illegal storage of organochlorine pesticide in Argentina. The strains, named A1, A2, A3, A5, A6, A8, A11, A12, A13, A14, and M7, showed typical actinomycete characteristics such as vegetative mycelium and in some cases aerial mycelium and spore formation [11, 12]. *Streptomyces coelicolor* A3(2) [13] was included as reference strain. *Amycolatopsis tucumanensis* DSM 45259 isolated from copper polluted sediments [14]. *Rhodococcus* sp. 20 and *Rhodococcus* sp. 016 recovered from chronically hydrocarbon-contaminated soil in Patagonia, Argentina. Macroscopic and microscopic observations, chemotaxonomic analysis, and 16S rDNA sequencing confirmed the placement of these strains with the genus *Rhodococcus*, closest related to *Rhodococcus erythropolis* CCM2595 (CP003761) and *Rhodococcus jostii* RHA1 (CP000431), both with 99% of identity (data not shown).

Luria Bertani Broth (LB) and Tryptone Soy Broth (TSB) were used for precultures. Liquid MM was used for the biodegradation assays (g L^{-1} : $(\text{NH}_4)_2\text{SO}_4$, 2.0; K_2HPO_4 , 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 and glucose, 1.25, pH 7.2 ± 0.2). All media were sterilized by autoclaving at 121°C for 20 min. Naphthalene, phenanthrene, and pyrene were purchased from Sigma–Aldrich (St. Louis, MO, USA). All chemicals used in this study were analytical grade and acquired from standard manufacturers.

Screening for PAH degrading actinomycetes

For degradation assays, bottles containing 30 ml of MM supplemented with: naphthalene, phenanthrene, or pyrene (0.2 mM : stock solution in acetone, 25 mmol L^{-1}) were inoculated with $5\% \text{ v v}^{-1}$ of cell suspension or $10\% \text{ v v}^{-1}$ of spore suspension containing 10^7 spores ml^{-1} . Bottles were incubated in the dark, at 30°C on a rotary shaker (150 rpm) for 7 days. Before bacterial inoculation, PAH solution was added and acetone was permitted to evaporate at room temperature. All experiments were conducted in triplicate, and a control sterile flask was prepared for each set-up in order to evaluate abiotic loss. Biodegradation was calculated taking into account the PAH loss occurring in the control.

After incubation, residual PAHs were extracted by adding 30 ml of acetone and filtered using a $0.22 \mu\text{m}$ –nylon membrane (Microclar, Argentina). The samples were stored at -20°C until analysis. PAH analysis was carried out by reverse-phase high performance liquid chromatography (RP-HPLC) using a Waters e2695 HPLC equipment with a PDA detector (Waters 2998, Waters Corporation, MA, USA) operating at 276 nm of fixed wavelength. Filtered solutions were injected into C18 μm Bondapak HPLC column ($4.6 \times 250 \text{ mm}$, 50 \AA pore size, $5 \mu\text{m}$ particle size). A methanol/water ($9:1 \text{ v v}^{-1}$) solution at a flow rate of 1 ml min^{-1} [15] was established as mobile phase during 25 min. PAH concentrations were calculated applying the external standard method. Biomass was estimated by washing the pellets with 25 mM Tris–EDTA buffer (pH 8.0) and drying to constant weight at 105°C .

Morphological observations

Streptomyces sp. A12, *A. tucumanensis* DSM 45259, *Rhodococcus* sp. 20, and *Rhodococcus* sp. 016 were grown for 48 h in rich medium (LB or TSB) supplemented with phenanthrene and the biomass was observed in the cryo-scanning electron microscopy (cryo-SEM). This technique allowed us to observe morphological cell characteristics when bacteria culture was exposed to the toxic compound.

DNA extraction and PCR amplification of *catA* gene

Total DNA from *Streptomyces* sp. A2, *Streptomyces* sp. A12, *A. tucumanensis* DSM 45259, *Rhodococcus* sp. 20, and *Rhodococcus* sp. 016 was extracted using GenElute Bacterial Genomic DNA Kits (Sigma–Aldrich). Cultures were incubated in LB at 30 °C for 48 h and the DNA extraction was carried out according to protocols supplied by the manufacturer. The DNA was used as template for PCR amplification of catechol 1,2-dioxygenase gene (*catA*). Using primers C12OF and C12OR [10]. The reference strains *Gordonia amarae* CECT 5704 and *Gordonia amicalis* DSMZ 44461 were used as negative and positive control, respectively. All amplification products were checked by electrophoresis on 1% agarose gels.

Sequencing and phylogenetic analysis of the *catA* amplicons

The PCR amplicons were sequenced using IBMCP sequencing service (UPV, Spain) and sequences were analyzed in Chromas software (Technelysium, Tewantin, Australia). The identity and similarity to the nearest neighbor of nucleotide sequences were obtained by using the BLAST tool, from NCBI [16]. The translated CatA amino acid sequences were aligned and compared to CatA sequences of other actinobacteria available in the GenBank database. A phylogenetic tree was constructed with the aid of MEGA 5.2 software using neighbor-joining and the Jukes-Cantor distance correction methods [17]. Only unambiguously aligned positions from all sequences were used and gaps were not included in the match/mismatch count.

Adaptation and improvement of PAH degradation

Five successive streaks on solid medium were performed with *Streptomyces* sp. A2, *Streptomyces* sp. A12, *A. tucumanensis* DSM 45259, *Rhodococcus* sp. 20, and *Rhodococcus* sp. 016 in MM with decreasing concentrations of glucose (from 10 to 1.25 g L⁻¹) and supplemented with phenanthrene or pyrene (0.05 mM). Then, adapted strains were pre-cultured in rich media (TSB) or MM with glucose (10 g L⁻¹) for a subsequent inoculation (2 g L⁻¹ or 5% v v⁻¹) in flasks containing 30 ml of MM supplemented with phenanthrene or pyrene (0.1 mM) and glucose (1.25 g L⁻¹). The flasks were incubated at 30 °C, 150 rpm for 7 days. PAH residual fraction was determined by HPLC and the bacterial growth by dry weight.

Results

Screening of actinomycete strains for the ability to degrade PAH in MM

PAH degradation potential of the fifteen actinomycete strains was estimated by quantifying residual hydro-

carbons after 7 days of incubation in liquid MM. Using RP-HPLC, the analyzed PAHs (naphthalene, phenanthrene, and pyrene) were well separated at retention times of 7.20, 8.30, and 13.05 min, respectively. Naphthalene removal was observed in all strains at different rates (between 13.1 and 76.5%), but *A. tucumanensis* DSM 45259, *Rhodococcus* sp. 016, *Streptomyces* sp. A1, and *Streptomyces* sp. A2 showed the best performance of naphthalene removal (73.0–76.6%).

Maximum degradation values of phenanthrene in MM occurred in cultures of *A. tucumanensis* DSM 45259 (36.2%) and *Streptomyces* sp. A12 (20%), while the degradation of pyrene in MM was poor in most strains, reaching significance only in *Streptomyces* sp. A12 (4.3%: Fig. 1).

Biomass production was variable between the strains growing in the presence of each PAH. In some cases, even though no evident removal of PAH was observed, it would seem that the aromatic compound was capable of supporting growth (Fig. 2). According to average values and error bars, the highest biomass was produced by *Streptomyces* strains and *Rhodococcus* sp. 016. Particularly *Streptomyces* sp. A3, reached maximal biomass values

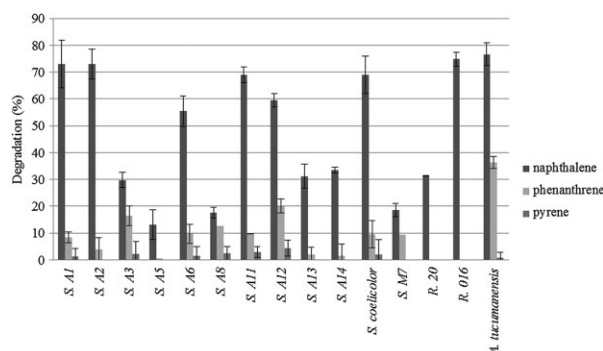


Figure 1. Removal of naphthalene, phenanthrene, and pyrene by actinomycete strains in MM.

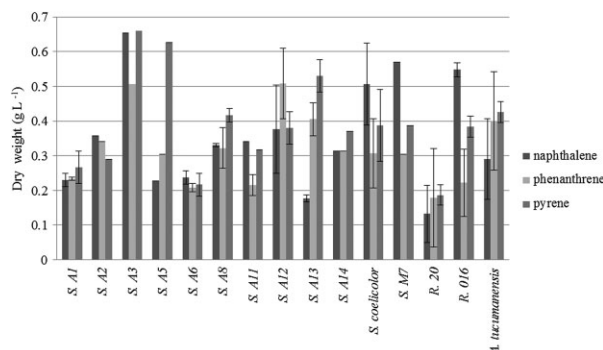


Figure 2. Growth of actinomycete strains with naphthalene, phenanthrene, and pyrene in MM.

when growing with naphthalene and pyrene as the sole carbon and energy sources (0.66 g L^{-1}), while these aromatic hydrocarbons did not reach high degradation rates (Fig. 2).

Degradation of PAH by adapted cultures of actinomycete strains with glucose

To increase the degradation rates for phenanthrene and pyrene, we performed an adaptation schedule with *Streptomyces* sp. A2, *Streptomyces* sp. A12, *A. tucumanensis* DSM 45259, *Rhodococcus* sp. 20, and *Rhodococcus* sp. 016. After adaptation, the strains were assayed for PAH removal in MM with glucose (1.25 g L^{-1}) supplemented with phenanthrene or pyrene.

Rhodococcus strains were capable to remove phenanthrene and pyrene only if another carbon and energy source was available. Although this hydrocarbon was not used as sole carbon source for growth, the ability to remove it appeared to be intact. No removal of phenanthrene or pyrene was observed in MM. However, when glucose was used as growth co-substrate, *Rhodococcus* sp. 20 was able to degrade 6.26% of phenanthrene and 8.96% of pyrene while *Rhodococcus* sp. 016 was able to degrade 25.78% of phenanthrene and 19.30% of pyrene. In our study, pyrene therefore could be degraded by *Rhodococcus* strains only if a co-metabolic substrate was present.

Streptomyces sp. A12 improved removal efficiency for both PAHs by approximately 50% after adaptation on glucose increasing phenanthrene degradation from 20.04 to 30.4%, and pyrene from 4.30 to 6.65%, and *Streptomyces* sp. A2 improved its phenanthrene degradation from 3.91 to 7.36%, however, no pyrene removal was increased, even with glucose addition.

The addition of glucose increased the efficiency of degradation of phenanthrene in *A. tucumanensis* DSM 45259 from 13.8 to 50% of the initial phenanthrene concentration after 7 days of incubation. Under these culture conditions, *A. tucumanensis* DSM 45259 showed the highest degradation of phenanthrene among all the actinomycete strains evaluated in this study. Addition of glucose did not further improve pyrene removal by this strain (Fig. 3).

Most strains showed a biomass increment in the presence of glucose and PAH, compared to MM supplemented only with PAH, except for *Streptomyces* sp. A12 and *A. tucumanensis* DSM 45259 (Fig. 4).

Cell morphology in presence of PAH

Cryo-SEM images showed cell morphology of the strains when grown in presence of phenanthrene. In general, no cell damage was observed, while an extracellular substance was observed around cells of *Streptomyces* sp

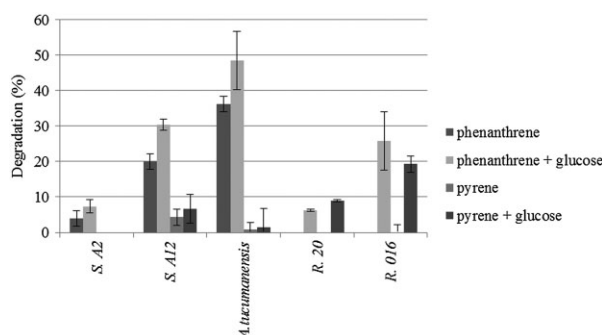


Figure 3. Removal of phenanthrene and pyrene by actinomycete strains in MM with and without glucose as a co-substrate.

A12. Also, cells of *A. tucumanensis* DSM 45259 appear to be aggregated, forming clusters or bunches of cells. Hemolytic activity (clear zone around the colonies when grown on blood agar plates; [18] confirmed the production of a biosurfactant in *Streptomyces* sp A12 and *A. tucumanensis* DSM 45259.

This substance could be involved in PAH degradation or be produced as a result of the stress conditions in the presence of PAHs.

Presence of *catA*

Partial nucleotide sequences of *catA* (382 bp) were obtained from *Rhodococcus* sp. 20 and *Rhodococcus* sp. 016. The set of primers had been designed to amplify the *catA* gene from *Gordonia* strains isolated from oil-enriched samples [10]. However, the gene was not detectable in *Streptomyces* strains and *A. tucumanensis* DSM 45259. Thus the conceptually translated amino acid sequence was used to identify isofunctional proteins from other well-known Gram-positive bacteria. High homology at amino acid level (98.3 and 87.4%) was detected with catechol 1,2-dioxygenase of *R. erythropolis* CCM2595 (FM995530) and *Rhodococcus opacus* 1CP (X99622), respectively (Figs. 5 and 6).

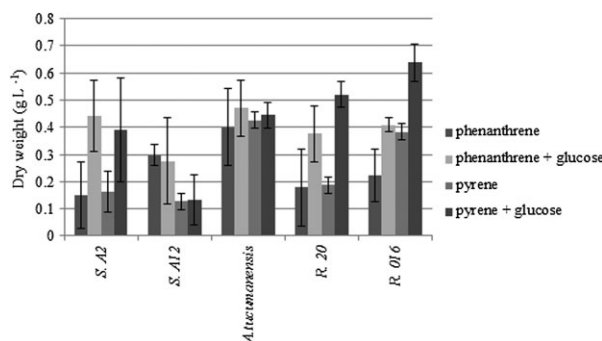


Figure 4. Growth of actinomycete strains in the presence of phenanthrene and pyrene in MM with and without glucose as a co-substrate.

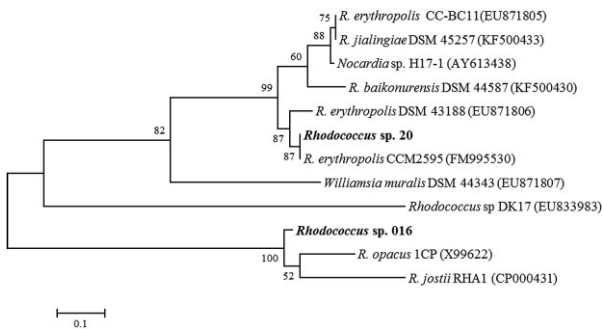


Figure 5. Phylogenetic tree based of CatA sequences.

Most CatA type sequences form two distinct and highly supported groups, one of them includes the sequences from *R. erythropolis*, *Nocardia*, and other actinobacteria; the other clade includes sequences belonging to *R. jostii* and *R. opacus* (named previously *R. erythropolis*).

Discussion

PAH degradation by actinomycetes has been well documented in several genera, but little is known about the ability of bacteria of the genus *Streptomyces* in the

removal of these contaminants. Chaudhary et al. [19] has described one *Streptomyces* strain able to degrade several PAHs, while in our study, 12 *Streptomyces* strains were able to remove naphthalene, 11 strains removed phenanthrene, and 9 strains also degraded pyrene, the latter with low percentages of removal. Our results showed that degradation rates decreased with increasing structural complexity of PAHs, which was previously noted [19]. The adaptation schedule allowed us to increase PAH tolerance and decrease co-substrate dependency. Adaptive laboratory evolution strategies are known to be highly effective in the activation of latent metabolic pathways [20]. *Rhodococcus* strains were capable to remove phenanthrene and pyrene only if another carbon and energy source was available. Pizzul et al. [21] observed that *Rhodococcus* and *Gordonia* strains degraded phenanthrene to different extents only when a co-substrate was present, although never reaching reduction values higher than 20%. Co-metabolic degradation may occur in strains that cannot use PAH as their source of carbon and energy, but can effectively degrade them [22]. Furthermore, Teng et al. [23] observed that the addition of glucose stimulated and increased the number and activity of PAH-degrading microorganisms, and it enhanced the degradation of phenanthrene and benzo(a)pyrene in soil. In this

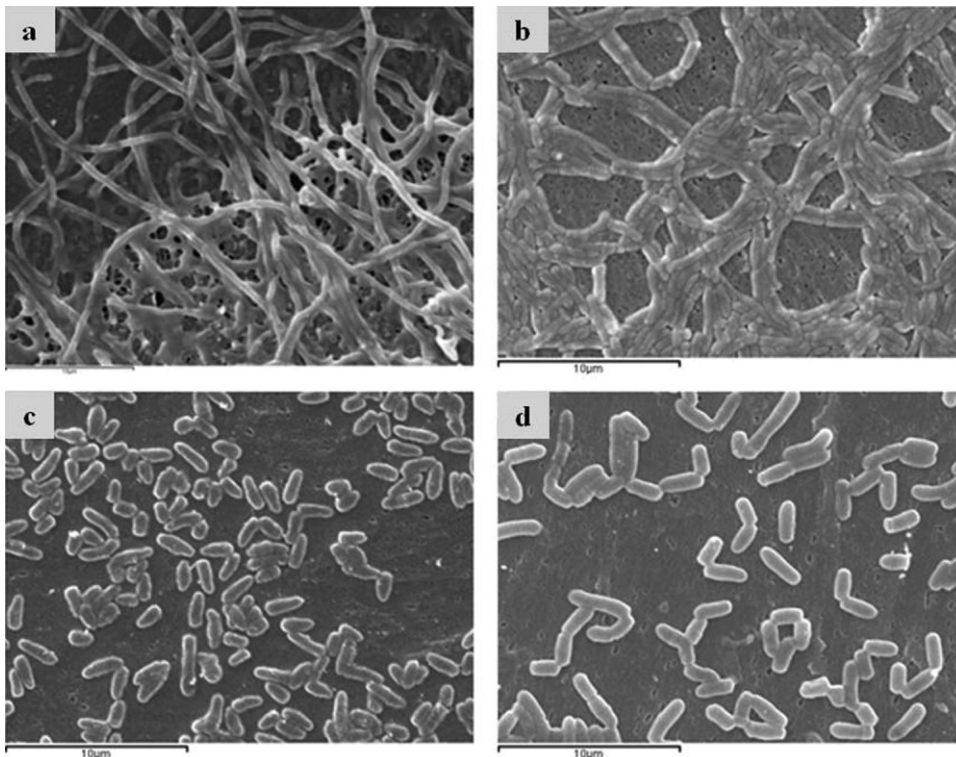


Figure 6. Observation (cryo-SEM) of actinomycete grow on phenanthrene: (a) *Streptomyces* sp. A12, (b) *A. tucumanensis* DSM 45259, (c) *Rhodococcus* sp. 20, and (d) *Rhodococcus* sp. 016.

way, glucose as a co-substrate seems to play a dynamic role in enhancement of high molecular weight PAH degradation [9].

Most of aromatic compounds, including polycyclics, are known to be metabolized to a common intermediate, catechol, which is further oxidized through the two ring-cleavage pathways, in which an exceptionally large number of peripheral pathways converge. Catechol can be further oxidized through the two ring *ortho* and *meta* cleavage pathways, catalyzed by catechol 1,2-dioxygenase and catechol 2,3-catechol-dioxygenase, respectively [24]. In this work, an internal fragment of the gene coding for catechol 1,2-dioxygenase could be amplified only from DNA of the two *Rhodococcus* strains. In *Rhodococcus* sp. 20 and *Rhodococcus* sp. 016, it seems that naphthalene or phenanthrene, are metabolized via salicylate [25]. The fact that this gene has not been detected in the other strains can be attributed to a completely unrelated and uncharacterized enzyme for aromatic ring cleavage. This result confirms the high diversity of catabolic genes in actinobacteria.

Another notable feature was observed with surfactin production. To overcome the low aqueous solubility of phenanthrene, the strains might produce extracellular polymeric substances that promote the availability and uptake of the hydrophobic compounds. In addition to the strains identified in this study, this feature had also been observed in other PAH-degrading bacteria like *Pseudomonas putida* ATCC 17514 while growing in presence of solid phenanthrene and fluorene [26].

In conclusion, we report the use of indigenous actinomycete strains isolated from different contaminated environments, able to remove naphthalene, phenanthrene, and pyrene when grown in minimal culture media. The selected microorganisms showed enhanced degradation of phenanthrene and pyrene after adaptation in the presence of glucose, which acted as co-substrate of growth. *Rhodococcus* strains were unable to remove phenanthrene and pyrene in MM without glucose, whereas removal rates of 25 and 19% of these PAHs were achieved in presence of this alternative carbon source. *A. tucumanensis* DSM 45259 and *Rhodococcus* sp. 016 showed the highest rates for phenanthrene (50%) and pyrene (19%) degradation. In addition, our study describes for the first time the ability of *Amycolatopsis* to remove PAHs.

The primer set C12OF and C12OR used by Shen et al. [10] to detect the *catA* gene in *Gordonia* species could be used to detect the presence of *catA* sequences in *Rhodococcus* strains. These indigenous strains isolated from contaminated soil could be a reservoir for the hydrocarbon-degrading genes, and the *catA* gene may

serve in the future as a marker to monitor biodegradation of PAH in soil.

Acknowledgments

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Conflict of interest

The authors declare that there is no conflict of interest.

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