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# Identification of proteins induced by polycyclic aromatic hydrocarbon and proposal of the phenanthrene catabolic pathway in *Amycolatopsis tucumanensis* DSM 45259

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## ARTICLE INFO

## ABSTRACT

In the present study the polycyclic aromatic hydrocarbon removal and metabolic adaptation of *Amycolatopsis tucumanensis* DSM 45259 were investigated. Analysis of one-dimensional gel electrophoresis of crude cell extracts revealed differential synthesis of proteins which were identified by MALDI-TOF. To elucidate the phenanthrene metabolic pathway in *A. tucumanensis* DSM45259, two-dimensional electrophoresis and detection of phenanthrene degradation intermediates by GS-MS were performed. The presence of aromatic substrates resulted in changes in the abundance of proteins involved in the metabolism of aromatic compounds, oxidative stress response, energy production and protein synthesis. The obtained results allowed us to clarify the phenanthrene catabolic pathway, by confirming the roles of several proteins involved in the degradation process and comprehensive adaptation. This may clear the way for more efficient engineering of bacteria in the direction of more effective bioremediation applications.

## 1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds with two or more fused benzene rings, originating from natural "as well as" anthropogenic sources. They are widely distributed environmental contaminants and have detrimental biological effects, including toxicity, mutagenicity and carcinogenicity (ATSDR, 2005; Peng et al., 2008). PAHs are not easily degraded under natural conditions, their persistence increments with the increase in molecular weight (Cerniglia, 1992). Although they are the main air pollutants and soil acts as final depository of these compounds (Usman et al., 2016). PAHs released to the environment are removed via volatilization, photo-oxidation, chemical oxidation, adsorption to soil particles and leaching; however, the principal removal processes are thought to be microbial transformation and degradation (Baboshin and Golovleva, 2012).

Many of these compounds are extremely ubiquitous and recalcitrant pollutants, therefore a large number of bacterial species have evolved through metabolic versatility, adapting their catabolic activities in order to metabolize them (Vandera et al., 2015). Numerous bacteria isolated from soils and sediments are known to have the ability to use PAHs as their sole carbon and energy source (Bamforth and Singleton, 2005; Cerniglia, 1992; Haritash and Kaushik, 2009; Sutherland, 1992).

Additionally, proteomics and metabolomics have been recently employed in environmental and microbiological studies demonstrating a high impact in the field of biodegradation and bioremediation, leading to the identification of new catabolic pathways (Nesatyy and Suter, 2007). Analysis at protein level is important in order to understand bacterial physiology state and regulatory mechanisms during biodegradation processes, because proteins are the functional biomolecules in cellular activities (Graves and Haystead, 2002), and they

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should be characterized as part of a risk assessment of this process as well.

Most research work about enzymes involved in PAH metabolism and genetic regulation is focused on *Pseudomonas* and *Sphingomonas* species (Isaac et al., 2013; Lu et al., 2013; Singleton et al., 2011). However *Mycobacterium*, *Rhodococcus*, *Nocardioides*, and *Novosphingobium* are actinobacterium species and were found to be able to mineralize PAHs (Saito et al., 2000; Song et al., 2011; Zhong et al., 2011).

Actinobacteria of the genus *Amycolatopsis* are soil microorganisms and they are mainly recognized for their ability to produce secondary metabolites with pharmacological applications (Gallo et al., 2010). *Amycolatopsis tucumanensis* DSM 45259 was previously isolated from a copper-polluted area in the province of Tucumán, Argentina (Albarracín et al., 2010) and it was widely studied for its remarkable copper-resistance, chromium and organochlorine pesticides removal (Dávila Costa et al., 2012, 2011; Polti et al., 2014). More recently, *A. tucumanensis* DSM 45259 abilities for degradation of naphthalene, phenanthrene, alkane and monoaromatic hydrocarbons as sole carbon source were detected and hydrocarbons catabolic capacities were analyzed by microarray system (Bourguignon et al., 2016, 2014).

In the present study, a proteomic analysis of *A. tucumanensis* DSM 45259 exposed to naphthalene, phenanthrene and pyrene was conducted to identify novel proteins involved in the degradation of these PAHs.

Finally, a phenanthrene metabolic pathway by identification of catabolic intermediates and proteins using GC-MS and two-dimensional gel electrophoresis, respectively, is proposed.

The results presented here comprise the foundation of a protein index for *A. tucumanensis* DSM 45259, and provide fundamental information on PAH degradation "as well as" other metabolic characteristics in environmental strains of *Amycolatopsis* sp.

## 2. Materials and methods

### 2.1. Bacterial strain, chemicals and culture conditions

*Amycolatopsis tucumanensis* DSM 45259 was isolated from wastewater sediment from a copper plant filter in the Tucumán province, Argentina (Albarracín et al., 2005).

Naphthalene, phenanthrene and pyrene (> 99% purity) were purchased from Sigma Aldrich Co. (St. Louis, MO, US). All chemicals used in this study were analytical grade and acquired from standard manufacturers. PAHs stock solutions were 25 mM in acetone.

All assays were carried out in minimal media broth (MMb in g/L: $(NH_4)_2SO_4$, 2; $K_2HPO_4$, 0.5; $MgSO_4·7H_2O$, 0.2; $FeSO_4·7H_2O$, 0.01 and glucose, 1.25; pH 7) and Tryptone Soy Broth (TSB) was used for maintenance of the strain and pre-cultures.

For proteomic assays, flasks containing 30 mL of MMb supplemented with: naphthalene, phenanthrene or pyrene (0.1 mM) were inoculated with $2 g L^{-1}$ of wet biomass of *A. tucumanensis* DSM 45259 obtained from pre-cultures. Flasks 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 considering the PAH loss occurring in the control.

### 2.2. Protein extraction

*A. tucumanensis* DSM 45259 was cultured as previously described for biodegradation assays (point 2.1). The pellets were harvested at exponential phase by centrifugation (7728xg, 10 min, 4 °C), cells were washed twice with PBS (20 mM, pH 7) and re-suspended in 5 mL of extraction buffer (Tris-HCl 50 mM pH 8) with PMSF (1 mM) protease inhibitor. The cell disruption was performed through French press at 20.000 psi (1 psi = 6.895 kPa). The samples were centrifuged (12075xg; 10 min; 4 °C) for elimination of cell debris and whole cells. Total soluble protein content was determined by the Bradford method with BioRad reagents (Bradford, 1976) using BSA as reference protein.

### 2.3. One-dimensional gel electrophoresis

For one-dimensional gel electrophoresis (1-DE), 5% SDS, 10 mM EDTA and 5% beta-mercaptoethanol were added to the protein homogenates, which were then incubated at 100 °C for 5 min. A solution containing sucrose (5%) and bromophenol blue (0.0125%) was added before rerunning the samples on 10–12.5% polyacrylamide gels. 15–25 µg of total protein was run at 15 and 20 mA. SDS-PAGE was carried out using the tris-glycine-SDS buffer system (Tris-HCl 25 mM pH 8.8, Glycine 192 mM, SDS 0.1%). Gels were stained with Coomassie Brilliant Blue R-250. Images were scanned with an Image Scanner III and analyzed with Quantity One (BioRad). Only the bands that showed any change (an increase or decrease) in their intensity of at least 1.5 times with respect to the control were selected to be identified.

### 2.4. Two-dimensional gel electrophoresis

Comparative study between the profile of proteins synthesized by *A. tucumanensis* DSM 45259 in presence of glucose ($1.25 g L^{-1}$) or glucose ($1.25 g L^{-1}$) plus phenanthrene (0.1 mM) was performed by two-dimensional gel electrophoresis (2-DE). In the same way, cell homogenates were obtained as described above. To minimize SDS concentration, total proteins were precipitated with 9 vol. of acetone for 45 min. Precipitated proteins were washed three times with acetone by centrifugation at 3018xg for 5 min and resuspended in 100 µL of 8 M urea, 4% (w $v^{-1}$) CHAPS, 50 mM dithiothreitol (DTT), 2% (v $v^{-1}$) ampholytes (pH 3–7; GE Healthcare Life Science), and bromophenol blue. The suspension was incubated for 3 h with gentle shaking and centrifuged at 8724xg for 5 min to remove insoluble proteins. Proteins were quantified by the BCA method (Sigma-Aldrich, Saint Louis, USA).

Isoelectric focusing was performed in 18 cm immobilized pH gradient strips (3–7NL, GE Healthcare Life Science), according to previous assays with this actinobacteria (Dávila Costa et al., 2012). The focused strips were stored at −20 °C until second-dimension electrophoresis was performed. Thawed strips were equilibrated for 15 min in buffer Tris–HCl 370 mM (pH 8.8) containing 6 M urea, 2% (w $v^{-1}$) SDS, 20% (v $v^{-1}$) glycerol, and 130 mM DTT, and then equilibrated for 15 min in the same buffer containing iodoacetamide135 mM instead of DTT. Second-dimension SDS-PAGE was performed on 12% polyacrylamide gels at 22 mA for 20 h. Gels were stained using modified colloidal Coomassie Brilliant Blue R-250 technique (Wang et al., 2007). Images were scanned with an Image Scanner III and analyzed with Image Master 2D Platinum v 7.0 (GE Healthcare Life Sciences).

### 2.5. Proteome analysis

Three replicates of each gel (1D or 2D) were obtained for each sample. To match the detected spots a synthetic master gel was created by matching representative gels with each other. Molecular mass (*Mr*) of protein spots was determined using Sigma Protein Marker (S8445) run at the acidic end of the IPG strip. Bands and individual spots were excised from the gels and subjected to mass spectrometry analyses, carried out by CEQUIBIEM (Centro de Estudios Químicos y Biológicos de Espectrometría de Masa), Facultad de Ciencias Exactas y Naturales, UBA, Argentina. To focus on the protein changes that could be associated with PAHs biodegradation, proteins whose synthesis appeared to be decreased or repressed upon PAH treatment were not considered in this analysis. Proteins were identified by peptide mass fingerprinting with MASCOT program (Matrix Science Inc., Boston, MA; http://www.matrixsciende.com/search-form-select.html). Fragmentation was

carried out with more intense MS peaks (MS/MS). When possible, MS and MS/MS information was combined for one or more peptide searches. De novo sequencing was inferred from BLAST results when peak fragmentation was allowed. The percentage of protein coverage was determined for each band or spot using the MASCOT search (Perkins et al., 1999).

*2.6. Analytical methods*

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 mm Bondapak HPLC column (4.6_250 mm, 50 A° pore size, 5 μm particle size). A methanol/water (9:1 v v$^{-1}$) solution at a flow rate of 1 mL min$^{-1}$ (Manohar et al., 2001) was established as mobile phase during 25 min. PAH concentrations were calculated by applying the external standard method.

Phenanthrene metabolites were extracted with hexane, then with ethyl acetate and the aqueous fraction after extraction was acidified with HCl 0.5 N to pH 1.5 and extracted again with ethyl acetate. The residual extracts were dried; the samples were separated and the components were identified in a Gas Chromatograph (Agilent Technologies 6890) equipped with a Network Mass Selective Detector (Agilent 5973), HP-5MS capillary column (30 m × 0.25 mm × 0.25 mm) and a split/splitless injector (Agilent 6850).

Running conditions consisted of: injector temperature: 280 °C, injection volume: 1 μL, carrier: helium gas, run mode: constant flow 1 mL min$^{-1}$, temperature detector: 280 °C and sweeping masses from 40 to 500 amu. The Mass Detector was used in Single Ion Monitoring. This assay was performed at the Laboratory of Analysis of Pesticide, Chemistry Section, Experimental Station Obispo Colombres, EEAOC, Tucumán.

## 3. Results and discussion

*3.1. Comparison of the overall proteome profile in presence of naphthalene, phenanthrene and pyrene*

In a previous work, fifteen actinobacterium strains belonging to *Streptomyces*, *Rhodococcus* and *Amycolatopsis* genus were described for their capacity to remove and grow in presence of PAHs. After 7 days *A. tucumanensis* DSM 45259 could remove 76.6%, 45.0% and 5.0% of naphthalene, phenanthrene and pyrene, respectively. To the best of our knowledge, this was the first report on PAHs degradation ability by a member of the *Amycolatopsis* genus (Bourguignon et al., 2014). Furthermore, the additional ability of DSM 45259 to produce bioemulsifiers (Colin et al., 2013) represents a great advantage for bioremediation process.

To gain a better understanding of the metabolic capabilities of *A. tucumanensis* DSM 45259, the proteome profiles of *A. tucumanensis* DSM 45259 after incubation with naphthalene, phenanthrene and pyrene were compared to screen and detect proteins involved in PAHs biodegradation. The protein profiles in Fig. 1. show polypeptides with several sizes ranging from 6.4 to 200 kDa and it is seen that PAHs exposure altered protein patterns. The variability of the number of bands and differences in the intensity of the bands with respect to the control, were observed (Fig. 1.b.). Most of the differential bands in *A. tucumanensis* DSM 45259 were detected in presence of naphthalene and phenanthrene, the degree of difference in protein synthesis could be explained by the remarkable capability of this strain to degrade both PAHs compared to pyrene (Bourguignon et al., 2014).

At least, six specific bands were observed in the proteome profiles of *A. tucumanensis* DSM 45259 with higher intensities after exposure to the three PAHs compounds compared with the control (glucose-growth). They were identified using peptide mass fingerprinting or peptide
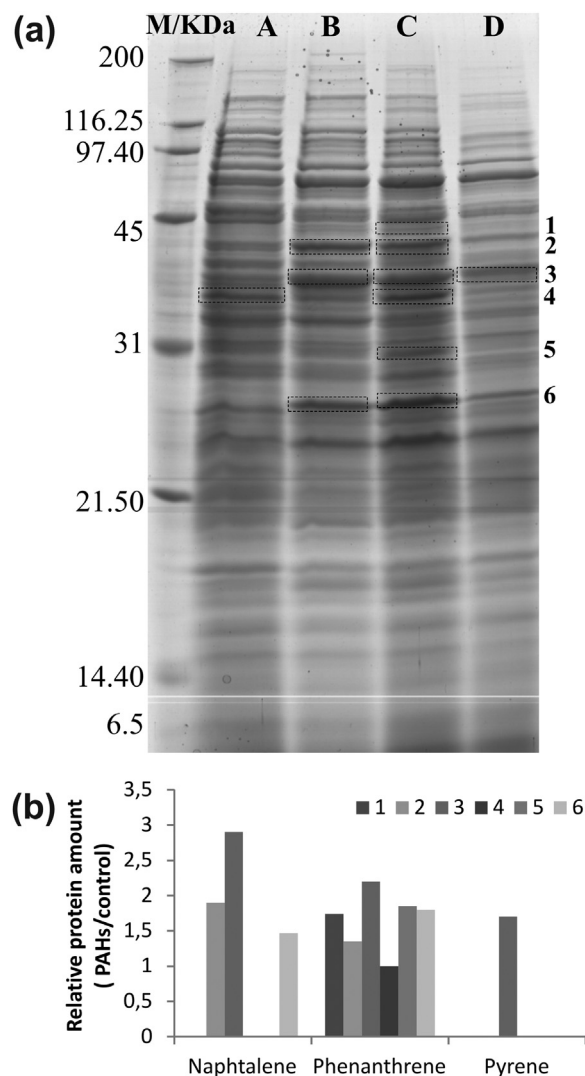


Fig. 1. The total proteins of Amycolatopsis tucumanensis DSM 45259 separated by SDS-polyacrylamide gel electrophoresis. (a) Proteome profiles where M, marker; A, control; and supplemented with B, naphthalene C, phenanthrene; D, pyrene. Differentially detected bands are indicated by dotted lines boxes. (b) Ratio of protein amount synthetized in presence of each PAHs and the control condition.

sequencing after MS/MS analysis (Table 1).

Bands 2, 3 and 6 were detected to be 1.8, 2.8 and 1.4-fold more intense in the culture supplemented with naphthalene compared to the control condition (Fig. 1.b). The molecular chaperone GroEL (band 2) is located in the bacterial cytosol bound at partially folded proteins and it is involved in restoration of native conformation proteins denatured by stress (Deuerling and Bukau, 2004). The expression of this enzyme would be considered as an adaptive response of the cells to PAHs exposure stress. Methane monooxygenase and a domain of methane / phenol / toluene family proteins (band 3) are proteins involved in aromatic compounds metabolism, indicating their possible role in the initial monooxygenation of PAHs. These enzymes are bacterial multicomponent oxygenases (BMOs) that catalyze oxidation of aromatic ring carbons (Borodina et al., 2007; Patel et al., 1982), resulting in mono- and dihydroxylated intermediates products (Sarma et al., 2009). Within ring hydroxylating monooxygenases (RHMO catabolic gene family), the gene Z36909 that encodes a phenol hydroxylase, that catalyzes the first step in the degradation of phenol into catechol, was previously found in the genomic microarray study of *A. tucumanensis* DSM 45259 (Bourguignon et al., 2016). Furthermore, the synthesis of

**Table 1**
Identification of differentially synthesized proteins in *Amycolatopsis tucumanensis* DSM 45259 incubated with naphthalene, phenanthrene or pyrene.

| Band | MASCOT score[a] | NCBI accession number | Protein | Organism | Identification technique[b] | MW (kDa) | Peptides matched[c] |
|---|---|---|---|---|---|---|---|
| 1 | 142 | gi\|521091944 | Enoyl-CoA hydratase | *Amycolatopsis* sp. ATCC 39116 | MS-MS/MS | 71.35 | 13 |
| 2 | 606 | gi\|521088446 | Molecular chaperone GroEL | *Amycolatopsis* sp. ATCC 39116 | MS-MS/MS | 57.840 | 14 |
| 3 | 202 | gi\|516606654 | Methane monooxygenase | *Amycolatopsis* multiespecies | MS-MS/MS | 64.07 | 25 |
|  | 142 | gi\|464806391 | Methane / phenol / toluene family proteins | *Gordonia* sp. NB4–1Y | MS-MS/MS | 63.20 | 21 |
| 4 | 280 | gi\|516609489 | Aldehyde dehydrogenase | *Amycolatopsismethanolica* | MS-MS/MS | 54.58 | 17 |
| 5 | 154 | gi\|739955750 | Methane monooxygenase | *Amycolatopsis* sp. ATCC 39116 | MS-MS/MS | 64.09 | 23 |
| 6 | 365 | gi\|516606658 | Amidohydrolase | *Amycolatopsis methanolica* | MS-MS/MS | 39.72 | 21 |

[a] MASCOT protein scores > 89 were significant ($p < 0.05$). MS/MS: individual ion scores > 22 indicate peptides with significant homology and individual ion scores > 30 indicate identity or extensive homology ($p < 0.05$).

[b] MS–MS/MS indicates a combination of MS and MS/MS.

[c] Number of peptides observed in mass spectra contributing to identification.

amidohydrolase enzyme (band 6) was detected and it could implicate in the bacterial degradation of PAHS. The amidohydrolase superfamily is comprised of functionally diverse enzymes that catalyze the cleavage of the C–N, C–C, C–O, C–Cl, C–S, or O–P bond of structurally distinct organic compounds (Chowdhury et al., 2016), however Chowdhury et al. (2016) described the oxygen-insensitive 2H1NA nonoxidative decarboxylase that is the first bacterial enzyme belonging to the amidohydrolase superfamily that catalyzes an irreversible decarboxylation of a hydroxynaphthoic acid, an aromatic acid. Other authors also reported 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase (ACMSD) detected in bacteria belonging to the amidohydrolase 2 family and involving in aromatics compounds degradation pathway (Kirimura et al., 2010; Li et al., 2006; Xu et al., 2013).

Knowing that *A. tucumanensis* DSM 45259 has a great ability to remove naphthalene, it is inferred that the over-synthetized identified enzymes with high values could play an important role in this catabolic process.

Six differential protein bands intensified compared to the control condition were observed in presence of phenanthrene. The over-synthesis of Enoyl-CoA hydratase (band 1) is related with fatty acids metabolism, which is a common phenomenon during bacterial PAHs metabolism (Lee et al., 2007), additionally this enzyme was reported to have a key role in phenylacetic acid catabolism in Gram positive bacteria: *Rhodococcus* sp. RHA1 and *Arthrobacter phenanthrenivorans* Sphe3 (Navarro-Llorens et al., 2005; Vandera et al., 2015). Moreover, the over-synthesis of aldehyde dehydrogenase (band 4), which function is dehydrogenation of dihydroxylated intermediate (Sho et al., 2004), was previously reported during PAHs catabolism and generally its gene was described along with the *nidA* gene for the large subunit of dioxygenases, suggesting it is a key enzyme in the PAHs pathway (Khan et al., 2001; Lee et al., 2007; Navarro-Llorens et al., 2005). Both enzymes, Enoyl-CoA hydratase and aldehyde dehydrogenase, were detected to be more abundant uniquely upon exposure to phenanthrene. The molecular chaperone GroEL (band 2), methane monooxygenase and a domain of methane / phenol / toluene family proteins (band 3) with other methane monooxygenase (band 5) and amidohydrolase enzyme (band 6) were also detected in a range of 0.8–2.1 folds of over-synthesized in presence of phenanthrene. Thereby *A. tucumanensis* DSM 45259 seems to require different enzymes for the catabolism of this PAH whose marked removal was verified.

Only band 3 was detected with high intensity in presence of pyrene in the proteome profile (Fig. 1). The lowest removal percentage and less differential protein synthesis were observed in presence of this four-ring PAH.

### 3.2. Proteins from Amycolatopsis tucumanensis DSM 45259 differentially synthesized in presence of phenanthrene

Since the greater number of over-synthesized bands was observed under phenanthrene supplementation, and all the selected bands were related to the exposure to this PAH, we decided to deepen into the study of the degradation of phenanthrene by *A. tucumanensis* DSM 45259.

Crude cell-free protein extracts of cultures in presence of phenanthrene *vs* control condition (glucose) were separated on 2-DE gels. The analysis of the gel images revealed at least 22 proteins synthesized only under phenanthrene exposure in comparison with the control condition in *A. tucumanensis* DSM 45259. Spots numbered from 1 to 22 shown in the representative gel (Fig. 2) were successfully identified and it could be considered that these proteins are likely to represent key proteins related to PAH biodegradation.

The identity of these proteins witch repeatedly showed differences in protein amount in the presence of phenanthrene are described in Table 2. In 2-DE studies, key aromatic metabolism proteins and others indirectly related with those could be identified, but some of other proteins identified may not be involved in PAH degradation (Kim et al., 2004). Some of the identified proteins had similar sequence and functions to those described in the 1-DE electrophoresis study described above. All identified proteins from 1-DE and 2-DE studies showed high homology with those belonging to *Amycolatopsis* strains.

It is important to highlight the presence of another BMO named toluene hydroxylase (spot 1), which is related with the metabolism of aromatic compounds. Aldehyde dehydrogenase (spot 9) and alcohol dehydrogenase (spot 14) are PAH-induced proteins presumably involved in the PAH catabolic pathways and generally the genes that
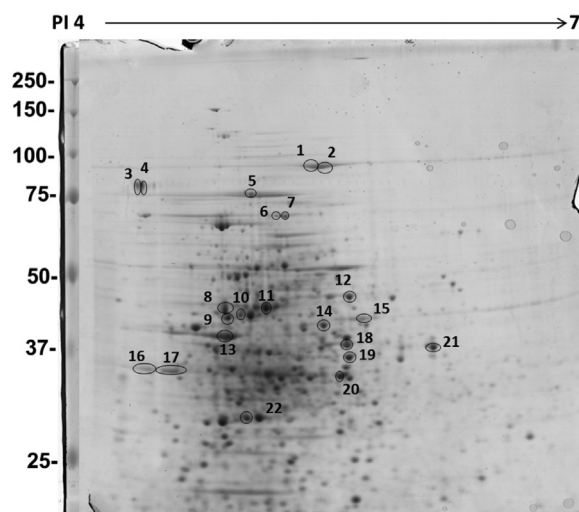


**Fig. 2.** *Amycolatopsis tucumanensis* DSM 45259 proteome in presence of phenanthrene. The PAH over-synthesized spots were numbered and indicated with circles. The pH gradient (linear) and molecular weight range marker (25–250 kDa) are indicated at the top and left of the image, respectively.

**Table 2**
Identification of proteins in *Amycolatopsis tucumanensis* DSM 45259 differentially synthesized in presence of phenanthrene.

| Spot | MASCOT score[a] | NCBI accession number | Protein | Organism | Identification technique[b] | MW (kDa) | pI | Peptides matched[c] |
|---|---|---|---|---|---|---|---|---|
| 1 | 164 | gi\|516606658 | Amidohydrolase | *Amycolatopsis methanolica* | MS-MS/MS | 39.72 | 5.17 | 8 |
|  | 202 | gi\|521088442 | Toluene hydroxylase | *Amycolatopsis* sp. ATCC 39116 | MS-MS/MS | 41.25 | 5.32 | 8 |
| 2 | 115 | gi\|654459929 | Aconitate hydratase | *Amycolatopsis thermoflava* | MS-MS/MS | 102.22 | 4.86 | 10 |
| 3 | 143 | gi\|654464362 | Pyruvate dehydrogenase | *Amycolatopsis thermoflava* | MS-MS/MS | 62.14 | 4.63 | 3 |
| 4 | 143 | gi\|516609056 | Dihydrolipoamideacetyltransferase | *Amycolatopsis methanolica* | MS-MS/MS | 60.58 | 4.60 | 2 |
| 5 | 482 | gi\|521087461 | Molecular chaperone DnaK | *Amycolatopsis* sp. ATCC 39116 | MS-MS/MS | 66.24 | 4.82 | 8 |
| 6–7 | 191–228 | gi\|521086885 | F₀F₁ ATP synthase subunit alpha | *Amycolatopsis* sp. ATCC 39116 | MS-MS/MS | 59.27 | 4.98 | 10 |
| 8 | 101 | gi\|654470746 | DNA-directed RNA polymerase alpha subunit | *Amycolatopsis taiwanensis* | MS-MS/MS | 38.24 | 4.61 | 8 |
| 9 | 519 | gi\|521089349 | Aldehyde dehydrogenase | *Amycolatopsis* | MS-MS/MS | 54.64 | 5.07 | 15 |
| 10 | 295 | gi\|521085329 | DNA polymerase III beta subunit | *Amycolatopsis* sp. ATCC 39116 | MS-MS/MS | 39.52 | 4.75 | 5 |
|  | 146 | gi\|521091411 | GTP binding protein YchF | *Amycolatopsis* sp. ATCC 39116 | MS-MS/MS | 38.94 | 4.73 | 5 |
| 11 | 356 | gi\|516612430 | Aldehyde dismutase | *Amycolatopsis methanolica* | MS-MS/MS | 43.11 | 5.36 | 5 |
| 12 | 285 | gi\|516611761 | Elongation factor Tu | *Amycolatopsis* | MS-MS/MS | 43.73 | 5.17 | 12 |
| 13 | 318 | gi\|516610552 | Hypothetical protein | *Amycolatopsis* | MS-MS/MS | 37.44 | 4.76 | 16 |
| 14 | 465 | gi\|521088445 | Alcohol dehydrogenase | *Amycolatopsis* sp. ATCC 39116 | MS-MS/MS | 36.32 | 5.18 | 12 |
| 15 | 284 | gi\|516612465 | F0F1 ATP synthase alpha subunit | *Amycolatopsis* | MS-MS/MS | 59.30 | 4.92 | 9 |
| 16–17 | 350–349 | gi\|516607007 | Binding substrate protein ABC sugar transporter | *Amycolatopsis methanolica* | MS-MS/MS | 38.17 | 5.68 | 4 |
| 18 | 157 | gi\|654465929 | Gryceraldehyde 3-phosphate dehydrogenase | *Amycolatopsis* sp. ATCC 39116 | MS-MS/MS | 35.42 | 5.21 | 8 |
| 19 | 631 | gi\|516612750 | Elongation factor Ts | *Amycolatopsis* | MS-MS/MS | 29.59 | 5.23 | 10 |
| 20 | 228 | gi\|521089238 | Universal stress protein | *Amycolatopsis* sp. ATCC 39116 | MS-MS/MS | 31.99 | 5.39 | 19 |
| 21 | 114 | gi\|516611878 | Succinyl-CoA synthase subunit alpha | *Amycolatopsis* | MS | 30.41 | 5.44 | 8 |
| 22 | 352 | gi\|654466186 | Enoyl-CoA hydratase | *Amycolatopsis* sp. ATCC 39116 | MS-MS/MS | 26.89 | 4.82 | 5 |

MASCOTprotein scores > 89 were significant (p < 0.05). MS/MS: individual ion scores > 22 indicate peptides with significant homology and individual ion scores > 30 indicate identity or extensive homology (p < 0.05).

[b]MS–MS/MS indicates a combination of MS and MS/MS.

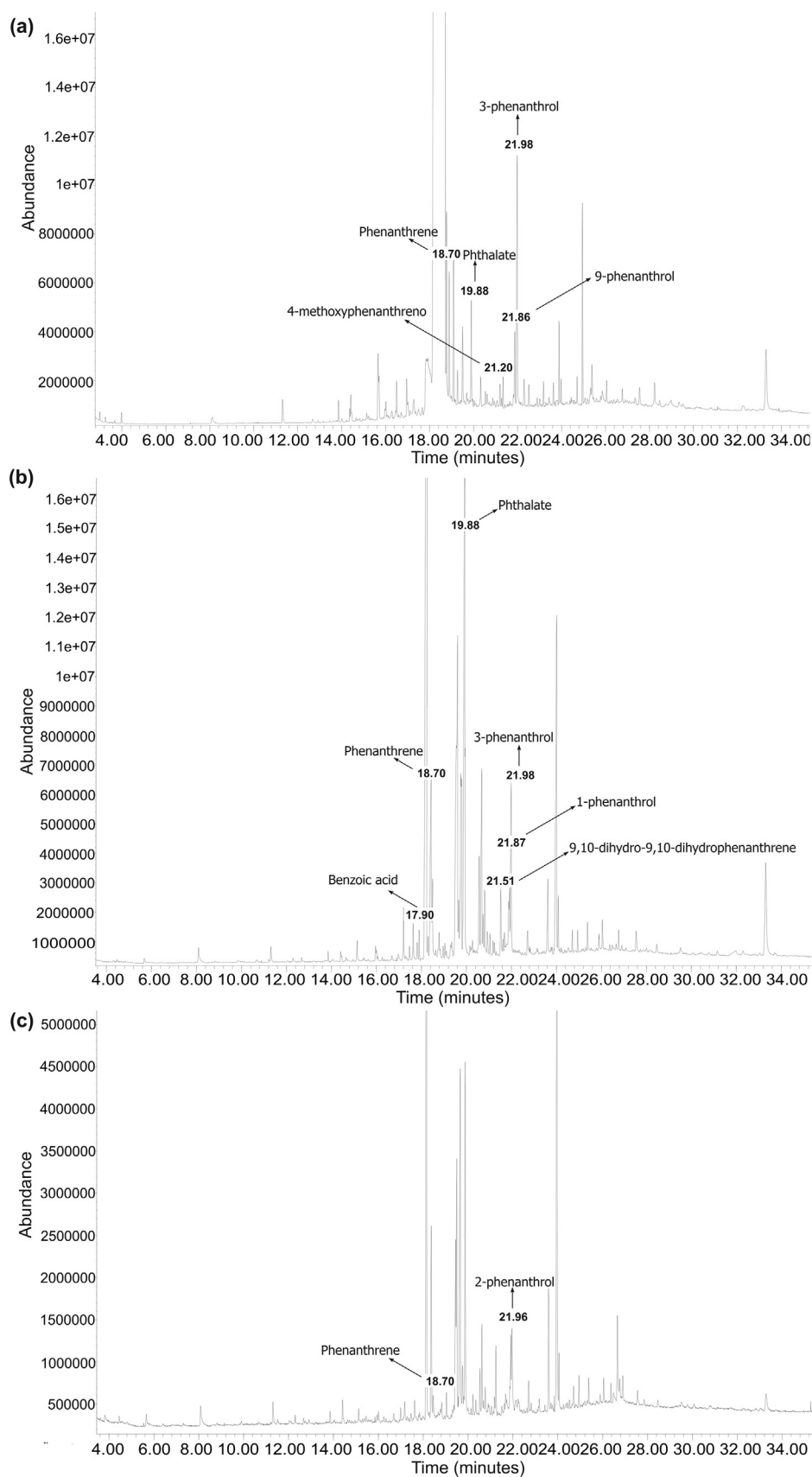[c]Number of peptides observed in mass spectra contributing to identification.

**Fig. 3.** GC-MS chromatograms of (a) hexane, (b) ethyl acetate and (c) ethyl acetate in acidic condition at 7 days of incubation, with phenanthrene as the sole carbon source.

encode them are located close to those related to key enzymes of the catabolism of these compounds as dioxygenases (Khan et al., 2001; Lee et al., 2007; Navarro-Llorens et al., 2005).

Binding substrate protein ABC sugar transporter (spots 16 and 17) was synthetized in phenanthrene-culture condition in *A. tucumanensis* DSM 45259 proteome and it is inferred that this enzyme could be involved in the uptake of the hydrocarbon. Although it is known that the phenanthrene and other PAHs are partitioned into the cell membranes by passive diffusion (Bugg et al., 2000), PAHs active uptake mechanisms in *Pseudomonas*, *Arthrobacter* and *Mycobacterium* strains were demonstrated, in which ABC transport systems would be involved (de Menezes et al., 2012; Vandera et al., 2015).

Moreover, universal stress protein (spot 20) and molecular chaperone DnaK (spot 5) were more abundant in presence of phenanthrene too. DnaK belongs to the Hsp70 heat-shock system, aiding the co-translational folding of proteins by binding to hydrophobic segments (Vandera et al., 2015), and cooperates in the folding of newly synthesized proteins and interacts with both nascent polypeptides and polypeptides released from the ribosome (Deuerling and Bukau, 2004). Also, DnaK was reported specifically associated at cellular stress by aromatics compounds in *Pseudomonas* spp. (Park et al., 2001). DnaK and GroEL (band 2 of 1-DE analysis) were more abundant during phenanthrene degradation by *Arthrobacter phenanthrenivorans* Sphe3 (Vandera et al., 2015). Some authors hypothesized that the synthesis of these proteins is included in a bacterial response in order to ensure survival in natural environments under different stress conditions (Lee et al., 2007).

Additionally, Enoyl- CoA hydratase (spot 22) and amidohydrolase (spot 1) were over-synthetized when cells were grown on phenanthrene as in 1-DE study (band 1 and 6, respectively). Pyruvate dehydrogenase multienzyme complex (spot 3), which included dihydro-lipoamide-acetyl-transferase (spot 4), catalyzes pyruvate decarboxylation to acetyl-CoA and it is the enzymatic step that connects glycolysis and the tricarboxylic acid (TCA) cycle. Aconitate hydratase (spot 2), glyceraldehyde 3-phosphate dehydrogenase (spot 18) and succinyl-CoA synthase (alpha subunit) (spot 21) belonging to TCA cycle, were found to be over-synthetized. These enzymes are involved in carbohydrates metabolism being the TCA cycle a main source of energy (ATP) and reducing power needed under stress conditions. Similar results have been reported for the proteomes of *Mycobacterium vanbaalenii PYR-1* grown on PAHs (Kim et al., 2004), *Rhodococcus* sp. RHA1 grown on phenyl acetate (Navarro-Llorens et al., 2005) and *Halomonas* sp. AAD12 grown on phenol-(Ceylan et al., 2011).

In addition, the synthesis of GTP binding protein YchF (spot 10) and $F_0F_1$ ATP synthase subunit alpha (spot 15) were enhanced with phenanthrene, both involved in energy production. The presence of DNA polymerase III beta subunit (spot 10) implicated in replication of DNA, suggested an active metabolic state with high replication rate upon phenanthrene degradation, in comparison with the control (Seo et al., 2009).

Furthermore, several proteins involved in the synthesis of new proteins were found to be positively synthesized in cultures with phenanthrene: DNA-directed RNA polymerase alpha subunit (spot 8), elongation factor Tu (spot 12) and elongation factor Ts (spot 19), which participate in DNA-transcription and RNA-translation. Thus, protein synthesis is necessary to counteract oxidative stress "as well as" to carry out hydrocarbon metabolism (Kim et al., 2004; Seo et al., 2009).

### 3.3. Identification of intermediate metabolites in degradation of phenanthrene

Fig. 3 shows the chromatograms obtained from hexane, ethylacetate and ethylacetate in acidic condition extracts of *A. tucumanensis* DSM 45259 phenanthrene-cultures. Eight intermediates of phenanthrene degradation by *A. tucumanensis* DSM 45259 were identified by gas chromatography coupled to mass spectroscopy (GC-MS). Phenanthrene

(RT: 18.70 min) was detected at all conditions because of its incomplete degradation at 7 days of incubation.

Dioxygenases are involved in the first step of phenanthrene bacterial degradation, these enzymes could incorporate oxygen atoms to carbon atoms at positions 1,2; 2,3 or 9,10 (Peng et al., 2008). However, dioxygenation at 9,10 positions were reported as the most common in Gram positive bacteria resulting in 9,10-dihydro-9,10-dihydrophenanthrene (RT: 21.51 min); which is degraded to 9-phenanthrol (RT: 21.86 min) (Moody et al., 2001), both compounds were detected as intermediates in phenanthrene degradation by *A. tucumanensis* DSM 45259 (Fig. 3a and b).

1-phenanthrol (RT: 21.87 min), 2-phenanthrol (RT: 21.96 min) and 3-phenanthrol (RT: 21.98 min) were also detected (Fig. 3), they are monohydroxylate compounds and these results could be explained by dioxygenation at 1, 2 ó 3, 4 phenanthrene position. Similar results were observed in phenanthrene degradation by *Rhodococcus* sp. P14 (Song et al., 2011). Moreover, *L. adecarboxylata* PS4040 initiates its attack on pyrene by mono-oxygenation of C-1 position with1-hydroxypyrene as an initial ring oxidation product (Sarma et al., 2009). The detection of the high variety of monohydroxylated intermediates, the over-synthesis of monooxygenase (methane monooxygenase and toluene hydroxylase) detected in proteomics studies above and the presence of ring hydroxylating monooxygenase gene in microarray study (Bourguignon et al., 2016), propose that *A. tucumanensis* DSM 45259 has diverse monooxygenase, dioxygenase and/or monooxygenase-dioxygenase systems combined, with broad specificity to degrade phenanthrene suggests that phenanthrene initial attack would be at all the positions by *A. tucumanensis* DSM 45259. Similar results were obtained with mixed bacterial cultures in hydrocarbon degradation (Zhong et al., 2011).

4-methoxyphenanthrene (RT: 21.20 min) was detected at 7 days of incubation in the hexane extract (Fig. 3.c.) as novel metabolite for phenanthrene biodegradation studies; however other authors probed the formation of o-methylated intermediates in phenanthrene, anthracene, fluoranthene, pyrene and benzo[*a*]pyrene degradation (Moody et al., 2001). Kinetic studies indicate that these methoxylated derivatives are dead-end metabolites, which are formed to prevent the redox cycles between catechol and quinone compounds which produce reactive oxygen species, a phenomenon that occurs in parallel to degradation (Kim et al., 2005; Moody et al., 2001). *Mycobacterium vanbaalenii* PYR-I oxidizes pyrene in 1 and 2 positions by initial dioxygenation to form o-methyled 1,2-pyrene diol, as a detoxic process (Kim et al., 2007; Peng et al., 2008).

In the bibliography, two lower pathways alternative to phenanthrene microbial catabolism were reported; in the first one, an aromatic ring is degraded to synthesize 1,2-hydroxynaphthalene, which is then transformed into salicylic acid as in the degradation of naphthalene (Deverychetty and Phale, 2010). In the other degradation pathway, ring opening results in the formation of phthalic acid and protocatechuic acid, the protocateuic acid is degraded to pyruvic acid which is optionally incorporated into the TCA cycle (Tomás-Gallardo et al., 2006). The detection of phthalic acid in the culture extracts (RT: 19.88 min) suggests that further phenanthrene degradation of *A. tucumanensis* DSM 45259 proceeds via o-phtalic acid pathway and while pyruvate was not detected, its presence can be inferred by over-synthesis of the pyruvate dehydrogenase enzyme observed in proteomic studies described above, which serves to transform pyruvate into TCA intermediates (Fig. 2). Moreover, Bourguignon et al. (2016) reported the degradation of phthalate by *A. tucumanensis* DSM 45259 by identifying the increasing abundance level of protocatechuic acid and 3-oxoadipic acid (phthalic-degradation products) and in combination with the identification of a gene encoding a phthalate 4,5-dioxygenase in the microarray demonstrate that the catabolism of phthalate takes place via the proto-catechuic ortho cleavage pathway in which a protocatechuate 3,4-dioxygenase may be implicated, as is proposed in the novel pathway (Fig. 2). The phthalic acid degradation to catechol and muconic acid and then to TCA intermediates also was documented (Balachandran

et al., 2012). Alternatively, phthalic acid could be transformed through β-ketoadipatepathway and finally resulting in the production of TCA cycle intermediates (Singh *et al.* 2013).

At retention time of 17.90 min the benzoic acid was detected (Fig. 3.b) which is the product of oxidative catabolism of several aromatic hydrocarbons (Prabhu and Phale, 2003), it was reported as a metabolite in naphthalene degradation by *Streptomyces* sp. ERI-CPDA-1 (Balachandran et al., 2012) and in phenanthrene degradation by *Nocardia otitidiscaviarum* TSH1 (Zeinali et al., 2008). Several authors propose the benzoic acid as product of the decarboxylation of phthalic acid (Zeinali et al., 2008) and as was described above this metabolite was also found in the catabolism of phenanthrene by *A. tucumanensis* DSM 45259 (Fig. 3a and b). In addition, the use of benzoate as the sole carbon source by *A. tucumanensis* DSM 45259 was previously demonstrated and the gene encoding benzoate 1,2-dioxygenase related to ring hydroxylating dioxygenases (RHDO catabolic gene family) was detected with high intensity of hybridization in microarray analysis of strain DSM 45259 (Bourguignon et al., 2016).

The co-occurrence of benzoic acid and phthalic acid has also been reported during metabolism of naphthalene by thermophilic *Bacillus thermoleovorans* (Annweiler et al., 2000) and the metabolism of fluoranthene by *Mycobacterium* sp. PYR-1 and *Pasteurella* sp. IFA (Kelley et al., 1993; Sepic et al., 1998).

Fig. 4 shows the catabolic pathway proposed for phenanthrene degradation by *A. tucumanensis* DSM 45259 based in differentially synthetized proteins and intermediates detected. The catabolic pathway is also supported by experimental evidence as presence of catabolic genes, enzymatic activity, use of phenanthrene and intermediates as the only carbon source, previously reported and referred throughout this work (Bourguignon et al., 2016, 2014).

Thus, it proposes that phenanthrene can enter in the cell by passive or active transport (ABC transporter sugar), after the initial attack by mono or dioxygenase enzyme, which gives rise to mono- and dihydroxylated phenanthrene compounds. It can also form 4- methoxyphenanthrene as o-methylated intermediate. Then it found the lower degradation pathways comprising phthalate pathway and catechol pathway. Finally, tricarboxylic acid cycle intermediates were originated to conclude the central metabolism.

## 4. Conclusions

This study is the first report on the proteome of *A. tucumanensis* DSM 45259 involved in PAHs degradation, and furthermore, provided new insights into the catabolic abilities of strain DSM 45259. Several proteins were differentially synthesized during growth on naphthalene, phenanthrene, pyrene and glucose and multiple cellular functions in response to the exposition at the different PAHs.

The combination of proteomic analysis data and phenanthrene metabolites detected, have also allowed us to clarify the phenanthrene catabolic pathway. Our results have shown that DSM 45259 degraded PAH through the monooxidation of aromatic rings. In addition, the presence of PAH triggered the synthesis of proteins involved in the defense against oxidative stress and other related to protein biosynthesis, transport and energy gain.

This is the first work that has comprehensively studied the protein composition to reveal the metabolism of phenanthrene in DSM 45259. Further investigations are required to elucidate the regulatory mechanisms into the complex metabolic processes involved in PAH degradation by *A. tucumanensis* DSM 45259.
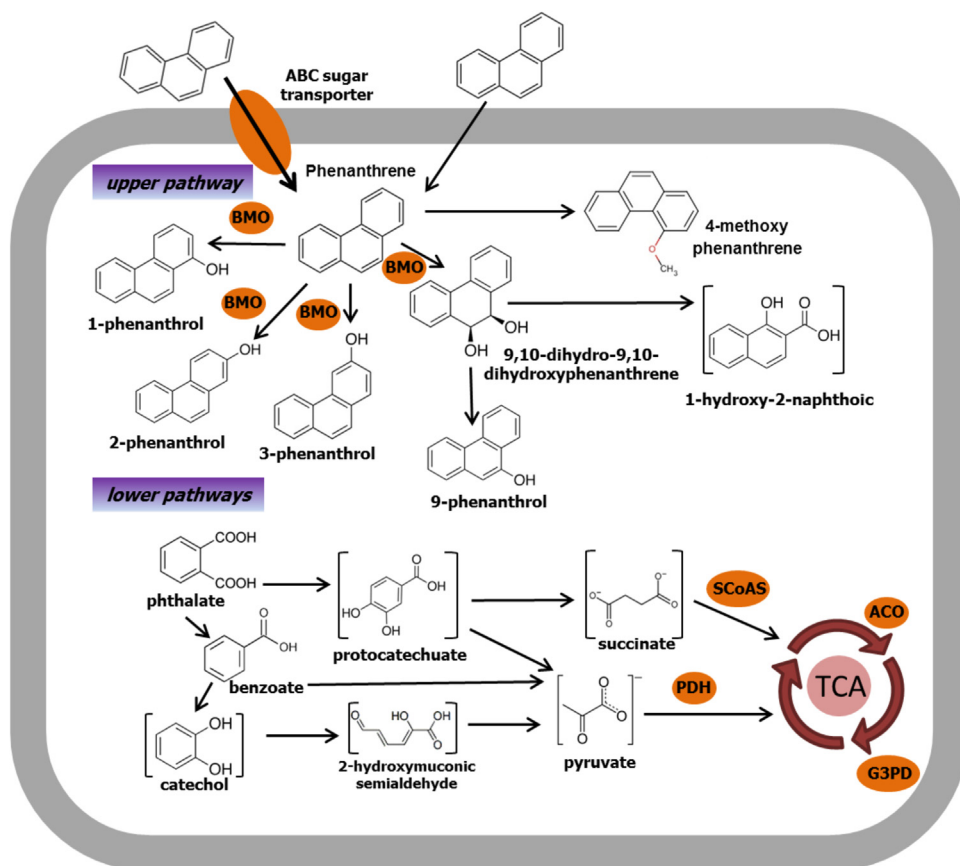
## Acknowledgments

**Fig. 4.** Proposed phenanthrene degradation pathway in *Amycolatopsis tucumanensis* DSM 45259 based on proteomic analyses and intermediates detected. In circles enzymes oversynthesized in the presence of phenanthrene: ABC sugar transporter, bacterial monooxygenase oxygenase (BMO): methane monooxygenase/ toluene hidroxylase, pyruvate dehydrogenase (PDH), succinyl-CoA synthase (SCoAS), aconitate hydratase (ACO) and griceraldehyde 3-phosphate dehydrogenase (G3PD). Metabolites in brackets were proposed, but not detected.

contribution to the knowledge of *A. tucumanensis* DSM 45259 and others actinobacteria.

## Conflict of interest

None declared.

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