



Genetic expression of *pobA* and *fabHB* in *Bacillus licheniformis* M2-7 in the presence of benzo[a]pyrene

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ABSTRACT. *Bacillus licheniformis* M2-7 is a heat-resistant bacterium able to biotransform polycyclic aromatic hydrocarbons. It can transform a wide range of these compounds as naphthalene, phenanthrene, pyrene and benzo[a]pyrene. Benzo[a]pyrene is a polycyclic aromatic hydrocarbon of high molecular weight considered as potentially toxic and carcinogenic for humans. Aiming to discover the genes involved in the biotransformation of benzo[a]pyrene, we made a *B. licheniformis* M2-7 genomic library in *E. coli*. We isolated two *E. coli* strains that were able to grow in minimal salt medium supplemented with benzo[a]pyrene. From the analysis of the DNA fragments in the clones H23 and H38, we identified open reading frames coding for 5 possible genes, among them *pobA* and *fabHB*, which products are the enzymes 4-hydroxybenzoate 3-monooxygenase and the ketoacyl-ACP synthase

III, respectively. To evaluate the role of these genes in the metabolism of benzo[a]pyrene in *B. licheniformis* M2-7, we estimated their relative expression through reverse transcription quantitative PCR. Finally, we observed that the genes *pobA* and *fabHB* were overexpressed after 3 h under induction with benzo[a]pyrene, suggesting that this strain could use these genes during the metabolism of this PAH, plus it does it in a faster time than that reported for other bacterial genera

Key words: 4-hydroxybenzoate 3-monooxygenase (*pobA*); Ketoacyl-ACP synthase III (*fabHB*); *Bacillus licheniformis* M2-7; benzo[a]pyrene; Reverse transcription quantitative (PCR)

INTRODUCTION

Benzo[a]pyrene (BaP) is a polycyclic aromatic hydrocarbon (PAH) of high molecular weight which solubility in water is very low. This produces a limited availability in the environment (Kanaly and Harayama, 2010; Banach-Szott et al., 2015). BaP is considered to be potentially carcinogenic for humans (IARC, 1983; Vázquez-Gómez et al., 2016) because during its biotransformation it is oxidized by the enzyme epoxide hydrolase to benzo[a]pyrene 7,8-diol-9,10-epoxide, a highly toxic compound (Harvey, 1996). Despite its toxicity there are studies indicating the activity of different microorganisms in the removal of BaP in low concentrations, where bacteria are the main organisms (Kanaly and Harayama, 2000; Moody et al., 2004). In the bacterium *Mycobacterium vanbaalenii* PYR-1, this organism initially oxidized benzo[a]pyrene with dioxygenases and monooxygenases at C-4,5, C-9,10, and C-11,12. The major intermediates of benzo[a]pyrene metabolism that had accumulated in the culture media after 96 h of incubation were benzo[a]pyrene cis-4,5-dihydrodiol, benzo[a]pyrene cis-11,12-dihydrodiol, benzo[a]pyrene trans-11,12-dihydrodiol, 10-oxabeno-[def]chrysen-9-one, and hydroxymethoxy and dimethoxy derivatives of benzo[a]pyrene. From this data, a hypothetical transformation pathway was generated where a wide number of enzymes were included (Moody et al., 2004). An interesting example is the bacterium *Novosphingobium pentaromativorans* US6-1 that metabolizes BaP by using ring hydroxylating dioxygenases to complete the degradation through different pathways until entering the tricarboxylic acid cycle. For *N. pentaromativorans* US6-1 it was reported that the HBMO gene coding for the enzyme 4-hydroxybenzoate 3-monooxygenase gets overexpressed after the incubation with BaP during 12 h (Lyu et al., 2014). Other studies have been shown that *B. licheniformis* grows in the presence of BaP, inducing the expression of genes coding for monooxygenases (Sowada et al., 2014). There are several reports about BaP metabolism, however most of them focus on the physical and chemical aspects, reporting the intermediary metabolites that are generated. But little is known about the genetic processes involved in the BaP degradative pathways (Kanaly and Harayama, 2000; Moody et al., 2004; Lyu et al., 2014; Sowada et al., 2014).

B. licheniformis M2-7 is a heat-resistant bacterium, isolated from the surface of hot springs in Guerrero, Mexico. Recent investigations have shown that these bacteria have the ability to grow in crude oil and its derivatives as well as in the presence of different PAHs (naphthalene, phenanthrene, pyrene and BaP) (Guevara-Luna et al., 2018, under review). In addition, our group did an analysis by gas chromatography–mass spectrometry to the extracts of the *B. licheniformis* M2-7 strain after 3 h of incubation with BaP. We discovered that the bacterium transforms the BaP with the catechol 2, 3-dioxygenase (C23O), generating 3 intermediary metabolites, one of them being the phthalic acid. It was suggested that the BaP hypothetical transformation pathway of *B. licheniformis* M2-7 starts with the enzyme C23O, which incorporates molecular oxygen into the carbons 9 and 10 of the BaP, generating benzo[a]pyrene cis-9,10-dihydrodiol, subsequently metabolized by the *meta* pathway to phthalic acid (Guevara-Luna et al., 2017, under review). It is for this metabolic versatility that the strain M2-7 has been proposed as a potential candidate for bioremediation of contaminated areas with PAHs. The aim of this work was to identify the genes related to the BaP biotransformation in *B. licheniformis* M2-7. This was achieved through the generation of a *B. licheniformis* genomic library in *E. coli* cells. Finally, we analyzed the expression of the genes *pobA* and *fabHB* (encoding the enzymes 4-hydroxybenzoate 3-monooxygenase and the ketoacyl-ACP synthase III, respectively), in the strain M2-7 induced with BaP.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Cultures of *B. licheniformis* M2-7 and *E. coli* XL1-Blue were grown in LB medium (composition in g/L: casein peptone 10, sodium chloride 10, yeast extract 5 for liquid medium, supplemented with 15 g of agar for solid medium) at a temperature of 37°C for 24 h. The antibiotic used for *E. coli* was ampicillin 200 mg/mL. The BaP used was from Sigma-Aldrich HPLC purity ($\geq 96\%$), and the stock solution was prepared with acetone at 1 mg/mL concentration. To confirm the metabolic capacity of *E. coli* to grow in BaP or to measure the genetic expression of *B. licheniformis* M2-7, the strains were grown in minimal salt medium (MSM) developed by Zeinali et al., (2008) with some modifications (composition in g/L NH₄Cl, 1; Na₂HPO₄, 0.380; KH₂PO₄.H₂O, 0.380; MgSO₄.6H₂O, 0.080; CaCl₂, 0.070; KCl, 0.040; FeSO₄.7H₂O, 0.001; and 20% glucose or 10 µg/mL BaP as the only carbon source) supplemented with a solution of trace elements (2.5 mL per liter of medium, composition in g/L MgCl₂.4H₂O, 0.027; H₃BO₃, 0.031; ZnCl₂, 0.050, CuCl₂.2H₂O, 0.01; NiCl₂.6H₂O, 0.02; CoCl₂.6H₂O, 0.036 and Na₂MoO₄.2H₂O, 0.03) and adjusted to pH 7.

Growth analysis of *E. coli* strains H23 and H38 in the presence of benzoapyrene

The *E. coli* strains H23 and H38 were cultured in liquid LB medium at 37°C for 16-18 h. The cells were harvested, washed three times with H₂O and inoculated in 50 mL of MSM with 10 µg/mL of BaP, to an OD₆₀₀=0.3. The cultures were incubated at 37°C in the dark and mixed using a rotary shaker at 200 rpm. The controls used were as follows: 1) the MSM with the same concentration of BaP with no inoculate; 2) the MSM with the same concentration of BaP and inoculated with the *E. coli* strain harboring pUC18. The assays were done in duplicate and the growth was measured through the protein concentration by the Bradford method (Bradford, 1976) every 3 days for 16 days

Sequencing of the DNA fragments inserted in pUC18 that conferred the ability to grow in BaP to the *E. coli* strains H23 and H38

A plasmid DNA extraction of the strains H23 and H38 was performed and the inserted fragments from *B. licheniformis* M2-7 were released by digestion with the restriction enzyme *Hind*III (Thermo Scientific). For the capillary automated sequencing, we used the primer pair m13/pUC-40 Forward 5'-GTT TTC CCA GTC ACG TTG TA-3' y m13/pUC Reverse 5'-TTG TGA GCG GAT AAC AAT TTC-3'. A mix of 10 pmoles of the oligo plus 300 ng of the plasmid in a final volume of 16 µL was done. The nucleotide sequences recovered from the strains were analyzes through alignments with the genome of the *B. licheniformis* strain, DSM13=ATCC 14580.

Reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

We cultured *B. licheniformis* M2-7 in MSM with 10 µg/mL of BaP to a OD₆₀₀=0.5. The cultures were incubated at 50°C for 12 h in the dark and mixed using a rotary shaker at 200 rpm. As control, we used the MSM under the same conditions but without BaP. The samples were done by triplicate. We then extracted and purified the total RNA of *B. licheniformis* M2-7 according to the protocol developed by Damm et al., (2015) with some modifications. The residual DNA contaminant was degraded with DNase I (Thermo Scientific). The RNA was stored at -70°C. For the generation of complementary DNA (DNAc) we used the kit RevertAid (Thermo Scientific). The amplification reaction was done as follows: 1 µg of total RNA, 20 pmol/µL of primer reverse (Table 1), 2 µL of dNTP's (10 mM), 4 µL of reaction buffer 5X, 1 µL of Rnase inhibitor (40 U) and 0.5 µL of reverse transcriptase. The reaction was incubated at 42°C for 1 h and then inactivated at 70°C during 10 min. The cDNA was stored at -20°C.

The qPCR was done in a *LightCycler® 96 Instrument II* from Roche. The PCR reaction was done in a volume of 20 µL as follows: 1 µL of cDNA, 10 µL of Master Mix 2x (*LightCycler® 480 SYBR Green I*, from Roche) and 2.5 mM of primer (Table 1). Each reaction was done by triplicate. The amplification was performed done as follows: denaturation at 95°C during 10 min, followed for 40 cycles at 95°C for 10 s and 60°C for 60 s. The analysis of relative expression was done using the method $2^{-\Delta\Delta CT}$.

Table 1. Primer used in this study

Primer	Gene and target protein		Sequence 5'-3'	Amplicon size (bp)
HBMo Fw	<i>pobA</i> (4-hydroxybenzoate 3-monooxygenase)		AGA AGC GCC TGT CAT CAC TT	117
HBMo Rev	<i>pobA</i> (4-hydroxybenzoate 3-monooxygenase)		TCC TTC GGA ATG CTT TGT CT	117
KASi Fw	<i>fabHB</i> (ketoacyl-ACP synthase III)		ACT TCG GAT GGA ACG AAG TG	105
KASi Rev	<i>fabHB</i> (ketoacyl-ACP synthase III)		TAT GCA GTC CGG AAG TGA TG	105
CDox Fw	<i>catE</i> (catechol 2,3-dioxygenase)		AGC AGG GGA ACT ACG TGA TG	114
CDox Rev	<i>catE</i> (catechol 2,3-dioxygenase)		GGA CGT GTC CGA TTT TTG TT	114
rpoB Fw	<i>rpoB</i> (beta subunit of the RNA polymerase)		GGT TTT ACC GCG ACT GTC AT	115
rpoB Rev	<i>rpoB</i> (beta subunit of the RNA polymerase)		GAA CCG TAA CCG GCA ACT TA	115

RESULTS

Analysis of the growth of *E. coli* strains H23 and H38 in the presence of benzoapyrene

A total of 1668 *E. coli* transformant were obtained from *B. licheniformis* library. We carried out a first selection filter by growing them in solid MSM supplemented with BaP (data not shown). We selected two strains, which were incubated in liquid MSM supplemented with BaP for 16 days (Figure 1). Both strains showed an adaptation period to the culture medium conditions during the first 4 days of growth, after that, we observed an increase in the growth rate and it was different for each one. H23 increased its growth rate at day 7th (3.58 µg/mL of protein); reaching its maximum growth at day 16 (4.38 µg/mL of protein); in contrast to the other strains. The strain H38 showed a constant growth during the 16 days, having a concentration of 3.67 µg/mL of protein in the last measurement. The growth rate of both transformant strains was compared to the growth of the wild type control strain (Figure 1).

The graph shows the growth measured by protein quantification of the strains H23 and H38, and the control strain (pUC18). Bacteria were incubated at 37°C for 16 days. The experiments were done in duplicate. Standard deviation is shown.

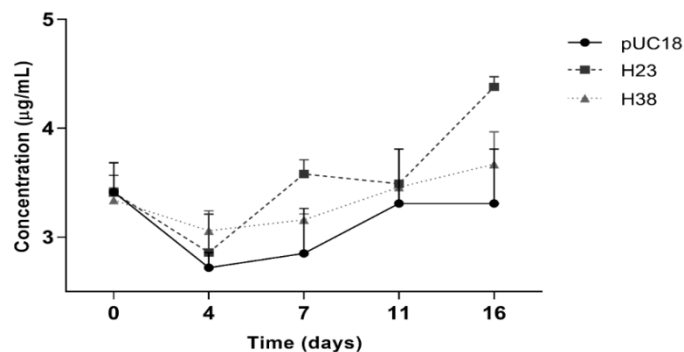


Figure 1. Growth kinetics of *E. coli* strains in the presence of benzo(a)pyrene.

Sequencing analysis of the DNA fragments inserted in pUC18, that conferred the transformants the ability to grow in medium with BaP

In order to determine the genetic elements that confer the ability to the strains H23 and H38 to grow in medium containing BaP, we did a restriction analysis of the purified plasmids to observe the pattern of the inserted fragments. The strain H23 showed three fragments bigger than 1 Kb and the strain H38 one fragment of 1.5 Kb (Figure S1). This result suggested the presence of at least one gene in the plasmids, which were then sent for sequencing. In this analysis we identified five possible open reading frames (ORF) that code for different proteins (Table 2). This suggests that the acquired ability to grow in BaP is due to the presence of at least one of these proteins. In the fragment from the strain H23, we identified the genes *prpB*, *TRNA_RS41120* and *pobA* that encode a membrane transporter type MFS, the enzyme methyl isocitrate lyase and the enzyme 4-hydroxybenzoate 3-monooxygenase, respectively. On the other hand, in the fragment from the strain H38, the genes identified were *TRNA_RS26940* and *fabHB* that encode for a hypothetical protein and the enzyme ketoacyl-ACP synthase III, respectively.

Table 2. Genes identified from the plasmid DNA sequencing in the *E. coli* strains.

Strain	Gene	Description	Protein	Coverage(%)	Identity (%)	Accession number
H23	<i>prpB</i>	Methyl isocitrate lyase	prpB	99	99	WP_011198423.1
		Transporter MFS	TRNA_RS41120	65	99	WP_009329802.1
	<i>pobA</i>	4-hydroxybenzoate 3-monooxygenase	pobA	26	99	WP_011198399.1
H38		Hypothetical protein	TRNA_RS26940	94	100	WP_009328937.1
	<i>fabHB</i>	Ketoacyl-ACP synthase III	fabHB	56	99	WP_011197721.1

^aThe similarity level was based on the alignments with BLASTX of non redundant proteins from the NCBI database.

The strain used as reference for the alignments was *B. licheniformis* DSM13=ATCC 14580. The analysis of the fragments DNA with the biodegradation rates (Figure 1), suggest that during the growth in MSM with BaP, these enzymes promote the development of the strains H23 and H38. According to different authors, the degradation metabolism of high molecular weight PAHs involves at least 23 different proteins, among them monooxygenases and dioxygenases, that participate in the initial pathway of degradation; and enzymes that participate in the fatty acids metabolism as part of the final pathway of degradation. Interestingly, these two

strains harbor the genes *pobA* (H23) and *fabHB* (H38) in their plasmids, encoding for the enzymes 4-hydroxybenzoate 3-monooxygenase and the ketoacyl-ACP synthase III, respectively (Table 2). In order to determine if the transcription of the genes *pobA* and *fabHB*, is induced by the presence of BaP in the *B. licheniformis* strain M2-7, we analyzed their expression. In addition, we measured the relative expression of the gene *catE* that codes for the enzyme C23O (TRNA_RS25790) as a positive control of a gene directly involved in the biotransformation of benzoapyrene, because the activity of this enzyme in *B. licheniformis* M2-7 was previously observed after 3 h of incubation with BaP (Guevara-Luna et al., 2017, under review).

Relative expression of genes *catE*, *fabHB*, and *pobA* in *B. licheniformis* M2-7

The degradation pathway of BaP is complex and in order to understand its metabolism, many microorganisms have been used in trials to analyze if they can use it as a carbon source. Moreover, the genes that participate in the degradation of the BaP in *B. licheniformis* M2-7 are not known. For this reason, we determined the relative expression levels of three genes in the genome of *B. licheniformis* M2-7 under the induction of BaP (Figure 2).

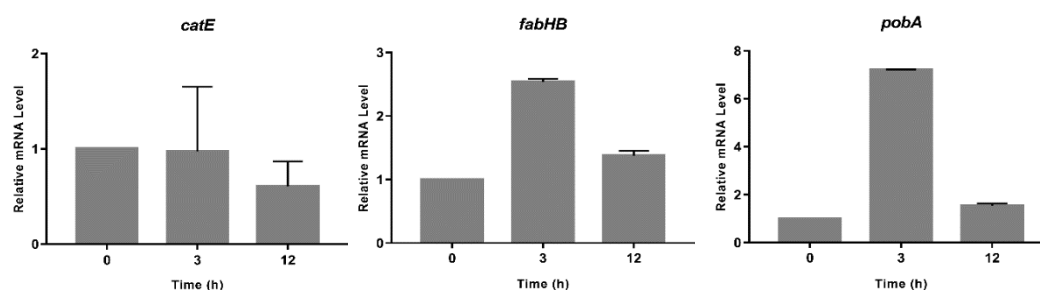


Figure 2. Quantitative PCR.

Relative expression quantification of the genes *catE* (catechol 2,3-dioxygenase), *fabHB* (ketoacyl-ACP synthase III), and *pobA* (4-hydroxybenzoate 3-monooxygenase) in *B. licheniformis* M2-7 after induction with BaP.

The expression of the gene *catE* was basal during the three first hours of exposure (0.97-fold), after 12 h decreased by 0.6-fold after induction in BaP. (Figure 2A). In the Figure 2B, there is an overexpression of the gene *fabHB* after 3 h (2.5-fold higher) and 12 h (1.3-fold higher) of exposure to BaP. In the Figure 2C, we can observe an overexpression of the gene *pobA* in the first 3 h with BaP (7.2-fold higher), then decreased by 1.5-fold after 12 h. These results all together suggest that the overexpression of the genes *pobA* and *fabHB* after the first 3 h of exposure to benzo[a]pyrene is driven by their participation in the metabolism of PAH.

DISCUSSION

A total of 1668 *E. coli* transformant were exposed to BaP. From those, strains H23 and H38 showed better growth compared to the negative control. Based on this analysis and the sequencing of DNA we were able to relate the ORFs harbored in the H23 and H38 plasmids, encoding for different proteins, with the BaP metabolism.

The strain H23 harbors three enzymes. The first of them is a methyl isocitrate lyase, a protein involved in the metabolism of glycolipids and probably in the production of biosurfactants (Rooney, 1979; Kato and Watanabe, 2009). There are studies reporting the bacterial degradation of alkanes by *Alcanivorax borkumensis* MM1 and *Pseudomonas aeruginosa* PG201 using glycolipids to increase the alkane's biodisponibility (Abraham et al., 1998; Holden et al., 2002). The second ORF codes for a membrane transport protein type major facilitator superfamily (MFS), which are proteins that facilitate movement of small solutes across cell membranes in response to chemiosmotic gradients in prokaryote cells. They participate in the detoxification of endogenous and exogenous metabolites (Dean et al., 2001; Biemans-Oldehinkel et al., 2006; Oldham et al., 2008; Kim et al., 2017). This last antecedent suggests that the BaP can get into the cell causing toxic effects. As an example, there is *Euplotes crassus*, where it was observed that an ABC transporter allowed the access of BaP into the cell

which then inhibited the activity of the transporter, causing the cellular toxicity of the protozoan (Kim et al., 2017). However, in the strain H23 we observed that the presence of BaP did not have any toxic effect and this result is attributed to the last ORF that encodes for a monooxygenase protein. This effect could be due to the 4-hydroxybenzoate 3-monooxygenase present in the plasmid DNA from strain H23, incorporates molecular oxygen to the BaP benzene rings and in that way allowing the strain's survival. Finally, we suggest that the strain H23 uses the enzyme methyl isocitrate lyase to make the BaP more bio-available, then the membrane transporter facilitates the intake to the cell and the enzyme 4-hydroxybenzoate 3-monooxygenase metabolizes the PAH.

The strain H38 harbors two enzymes, the first one is a hypothetical protein while the second one is the ketoacyl-ACP synthase III. This protein has been described in Gram-positive bacteria and participates in the fatty acid and polyunsaturated fatty acid biosynthesis (Zaccari et al., 2008; Johnson et al., 2011). As mentioned before it is known that the metabolism of high molecular weight PAHs, specifically that of the BaP, involves the cleavage of 2 or more benzene rings by monooxygenases like enzymes (Kweon et al., 2011; Sowada et al., 2014). In this study we identified two strains harboring in the plasmids to the genes *pobA* (H23) and *fabHB* (H38), encoding for the enzymes 4-hydroxybenzoate 3-monooxygenase and the ketoacyl-ACP synthase III, respectively.

In the real time PCR analysis, we observed the overexpression of the genes *pobA* and *fabHB* after 3 h of exposure to the BaP although it was not the case for the gene *catE*. The gene *pobA* that codes for the 4-hydroxybenzoate 3-monooxygenase was 7.4-fold higher than the control, which indicates that the activity of this gene continues to occur until 3 h later to the exposure. In the strain *N. pentaromaticivorans* US6-1, the overexpression of the gene *HBMO* that codes for the enzyme 4-hydroxybenzoate 3-monooxygenase was 6-fold higher than the control after 12 h of exposure to BaP (Lyu et al., 2014). The comparison of these results suggests that *B. licheniformis* M2-7, under the culture conditions that we used, overexpresses the protein 4-hydroxybenzoate 3-monooxygenase in the earliest stages of the cellular growth in the presence of BaP. The overexpression of the gene *fabHB* that codes for the enzyme ketoacyl-ACP synthase III after 3 h of incubation with BaP, suggests that this gene also participates in the metabolism of PAHs. Although it has not yet been described in the genus *Bacillus*, it is proposed that under aerobic conditions and in the β -keto adipate pathway, is where the gene *fabHB* participates. Finally, we quantified the expression of the gene *catE*, which we consider to be a control gene involved in the biodegradation of benzo(a)pyrene, that codes for the enzyme catechol 2,3 dioxygenase, finding a basal expression during the exposure to BaP, however in previous analysis the activity of the enzyme from *B. licheniformis* M2-7 was determined, finding 0.33 U/mg of protein after 3 h of incubation with BaP (Guevara-Luna et al. 2017, under review). This suggests that the expression of the gene *catE* occurs in earlier growth stages (within the first 3 h) in the strain M2-7. Many authors have proposed a several bacteria as being able to use BaP, for example *Mycobacterium* sp. RGJII-135 degrades 40% of BaP after 32 days of incubation and supplemented with different carbon sources; *M. vanbaalenii* PYR-1, transforms BaP after 96 h of incubation; and *Burkholderia cepacia* degrades 20-30% of BaP after 63 days of incubation (Juhász et al., 1986; Schneider et al., 1996; Juhász et al., 1997; Moody et al., 2004). Other reports have used consortia as the one formed by *Ochrobactrum* sp., *Stenotrophomonas maltophilia* and *Pseudomonas fluorescens* that was able to degrade 44.07% of 10 ppm of BaP after 14 days of incubation (Luo et al., 2009). These results indicate that the degradation metabolism of BaP is complicated, additionally it is very slow, which makes it difficult to use these microorganisms in bioremediation. However, our system strongly suggests that it can metabolize the BaP within a period of 3 h under the tested conditions. With the last in mind we propose *B. licheniformis* M2-7 as an excellent alternative for application in bioremediation.

Proposal of the degradation pathway of the BaP in *B. licheniformis* M2-7

According to the results reported recently by our group (Guevara-Luna et al., 2017, under review), and together with the identification and the expression analysis of the genes *pobA* and *fabHB*, we suggest a transformation pathway for the BaP in *B. licheniformis* strain M2-7. The reactions would start with the incorporation of

molecular oxygen on the carbons 9 and 10 from BaP through the enzyme catechol 2, 3-dioxygenase, generating the benzo[a]pyrene *cis*-9,10-dihydrodiol (Guevara-Luna et al., 2017, under review), this last metabolite undergoes another dioxygenation through the catechol 2,3-dioxygenase producing *cis*-4-(hydroxy-pyrene-8-yl)-2-oxobut-3-enoic acid via *meta* (Cerniglia, 1992; Schneider et al., 1996). This is followed by the formation of 7,8-pyrene-dihydro-7-carboxylic acid (Schneider et al., 1996), through the high molecular weight PAHs degradation path described by Mahaffey et al., (1988), which involves a several enzymes and intermediaries metabolites.

Based on the results of the expression analysis of the genes *pobA* and *fabHB*, coding for the enzymes 4-hydroxybenzoate 3-monooxygenase and the ketoacyl-ACP synthase III, respectively. It is suggested that the second stage of degradation is similar to the one proposed in *M. vanbaalenii* PYR-1 for the degradation of pyrene (Heitkamp et al., 1988; Khan et al., 2001; Kim et al., 2005; Stingley et al., 2004a; Stingley et al., 2004b) and starts with the metabolism of the 7,8-pyrene-dihydro-7-carboxylic acid, through the oxygenation of the carbons 4 and 5 (K region) (Heitkamp et al., 1988), that we suggests it is carried out by the 4-hydroxybenzoate 3-monooxygenase (gene that was 7.4-fold higher than the control, Figure 2C). The subsequent formation of secondary metabolites that involve dioxygenation enzymatic reactions, results in the production of phthalic acid esters (Heitkamp et al., 1988; Kim et al., 2005; Stingley et al., 2004b; Guevara-Luna et al., 2017, under review). Although it has not been yet described in the genus *Bacillus*, it is believed that the gene *fabHB*, coding for the ketoacyl-ACP synthase III, participates under the β -keto adipate pathway and in aerobic conditions (the gene showed an overexpression of 2.5 times more than the control, Figure 2B). Finally, under the β -keto adipate pathway there is protocatechuate being generated from the phthalate (phthalic acid), until intermediaries that can enter the tricarboxylic acid cycle (Ornston and Stanier, 1966; Iwagami et al., 2000; Sim et al., 2013).

CONCLUSION

In conclusion, this investigation showed that during the growth of *Bacillus licheniformis* M2-7 under induction with BaP, the bacterium overexpresses the genes *pobA* and *fabHB*, encoding the enzymes 4-hydroxybenzoate 3-monooxygenase and the ketoacyl-ACP synthase III, respectively. Moreover, previous analysis discovered the intermediary metabolite phthalic acid and the activity of the enzyme C23O was measured. In the same way, it was also shown that in the *B. licheniformis* strain M2-7, this effect is achieved in a very short period after induction with BaP, making this bacterium an excellent alternative to apply in bioremediation. This investigation provides a basis for understanding the BaP metabolism in this bacterium, however, characterization of catabolic genes and identification of key enzymes will enable the full characterization in *B. licheniformis* M2-7.

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Gene's expression in *B. licheniformis* M2-7 in BaP

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Appendix

Supplementary material

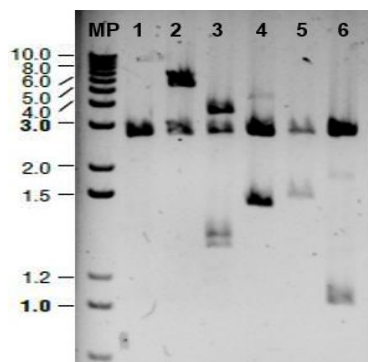


Figure S1. Analysis of the inserts cloned in pUC18.

Gene's expression in *B. licheniformis* M2-7 in BaP

Lane MP: molecular ladder 1Kb; Lane 1: linear pUC18 digested with *HindIII*; lane 2: plasmid from strain B24 digested with *BamHI*; lane 3: plasmid from the strain H23 digested with *HindIII*; lane 4: plasmid from the strain H38 digested with *HindIII*; lane 5: plasmid from the plasmid in the strain H66 digested with *HindIII*; and lane 6: plasmid with the strain H218 digested with *HindIII*. 1% agarose gel at 120 volts.