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ORIGINAL PAPER

Metaproteomic and gene expression analysis of interspecies interactions in a PAH-degrading synthetic microbial consortium constructed with the key microbes of a natural consortium

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Abstract Polycyclic Aromatic Hydrocarbons (PAHs) impose adverse effects on the environment and human life. The use of synthetic microbial consortia is promising in bioremediation of contaminated sites with these pollutants. However, the design of consortia taking advantage of natural interactions has been poorly explored. In this study, a dual synthetic bacterial consortium (DSC_AB) was constructed with two key members (*Sphingobium* sp. AM and *Burkholderia* sp. Bk), of a natural PAH degrading consortium. DSC_AB showed significantly enhanced degradation of PAHs and toxic intermediary metabolites relative to the axenic cultures, indicating the existence of synergistic relationships. Metaproteomic and gene-expression analyses were applied to obtain a view of bacterial performance during phenanthrene removal. Overexpression of the Bk genes, *naph, biph, tol* and *sal* and the AM gene, *ahdB,* in DSC_AB relative to axenic cultures, demonstrated that both strains are actively participating in degradation, which gave evidence of cross-feeding. Several proteins related to stress response were under-expressed in DSC_AB relative to axenic cultures, indicating that the division of labour reduces cellular stress, increasing the efficiency of degradation. This is the one of the first works revealing bacterial relationships during PAH removal in a synthetic consortium applying an omics approach. Our findings could be used to develop criteria for evaluating the potential effectiveness of synthetic bacterial consortia in bioremediation.

Keywords Synthetic bacterial consortium · Phenanthrene (PHN) degradation pathway · Microbial degradation potential · Bioaugmentation · Bioremediation

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Introduction

Polycyclic Aromatic Hydrocarbons (PAH) are ubiquitous in the environment with well documented negative effects on living organisms, showing toxic, mutagenic, and carcinogenic properties, both in aquatic and in terrestrial ecosystems (Nzila 2018; Davin et al. 2021). Since biological degradation of

these pollutants is regarded as the principal mechanism of detoxification and removal, bioremediation is an efficient and safe technology for the restoration of polluted ecosystems (Martínez-Ávila et al. 2021). In particular, in recent years the consortium-based concept has become a research trend for applied microbiology and biotechnological processes related to environmental restoration (Ghosh et al. 2016) since they are promising as resilient and cost-effective biotechnologies. Those attributes are due to key species that can fulfill desired functions cooperatively based on the natural principles of microbial interaction (Che and Men 2019). The higher complexity, compared to monocultures, allows consortia to be more resistant to environmental perturbations, increasing the chances of successful environmental establishment when inoculated.

In microbial life, a cell phenotype is determined by the properties encoded in its genome and by its environment, which is strongly influenced by the presence or absence of other species in the community. Several studies have also reported that certain metabolic intermediates with relatively high solubility produced from the degradation of hydrocarbons by bacteria may have higher cytotoxicity than the parent molecules and therefore can hinder bacterial performance (Hou et al. 2018). Natural consortia may contain members that metabolize inhibitory and/or toxic by-products of primary substrates, which if not further consumed will waste energy and carbon and inhibit biomass production due to acidification and anion accumulation (Che and Men 2019).

Substrate degradation by consortia is controlled by key microbes, whose action is due to their metabolic abilities and to the level of interaction among populations. Any assembly of bacteria needs to consider the ecological mechanisms modulating these interactions, and to link them with the degradation process. Studies manifesting gene expression profiles, enzymatic activities, growth of key members and degradation rates, are fundamental to elucidate the ecological processes occurring in microbial consortia (Jiménez et al. 2017). The interactions between the microbial partners in these mixed cultures are expected to have a significant impact on the combined performance of the microorganisms and the bioprocess as a whole (Xu 2021). Delineation of interaction modes between microorganisms in natural niches could facilitate engineering and design of novel synthetic microbial consortia and their traits (Song et al. 2014).

Consortia with high species richness could negatively affect the processes due to enhanced competition (Puentes-Téllez and Falcao Salles 2018). One of the ways to identify the mechanisms that regulate the formation, diversity and stability of a community is to study "minimal" native consortia, where key microorganisms have been enriched from a complex ecosystem (Gilmore et al. 2019). Synthetic microbial consortia designed with two or three species/strains between which their interaction is known, constitute an incentive for their application in biotechnological processes, including biosynthesis and bioremediation (bioaugmentation) (Wanapaisan et al. 2018; Che and Men 2019).

Knowledge of microbial interactions is critical in consortia related to the degradation of pollutants. Application of multi-omic tools has become the most useful strategies to predict the functioning and interactions in different microbiomes (Malla et al. 2022). Genomics identifies the metabolic potential of a consortium, transcriptomics offers insight into the genes that are being expressed and metaproteomics offers the advantage of confirming functionalities by quantifying the presence of the gene expression products. This linkage to function suggests that proteomics approaches may be appropriate for answering ecological questions about the actual activities and interactions of detected species (Siggins et al. 2012). Metaproteomic studies provide high confidence when applied to cultures of species with annotated genomes and offer advantages when used on synthetic communities, like greater confidence in peptide-protein matches, greater metaproteome coverage, better validation of proteomics results and more manageable datasets (Chignell et al. 2017).

In our previous studies, the bacterial composition of a natural phenanthrene (PHN)-degrading enrichment culture (named CON) was studied (Festa et al. 2013, 2016) and five bacterial strains were isolated; from them, two strains are key members in PAH degradation (*Sphingobium* sp. AM and *Burkholderia* sp. Bk) (Macchi et al. 2021). The enrichment culture CON eliminates 65% of phenanthrene in 15 days. In order to improve phenanthrene degradation by consortia and taking into account the advantage of easily propagate synthetic microbial assemblages in relation to natural consortia under

experimental conditions (Dolinšek et al. 2016) we propose the strategy of generating a minimal functional bacterial consortia, whose members are specialized in certain biodegradative steps. The conservation and culture of single strains and so the construction and propagation of a synthetic consortium represent an important advantage in relation to natural consortia.

In this work, a co-culture constituted by *Sphingobium* sp. AM and *Burkholderia* sp. Bk, a Dual Synthetic Consortium AM-Bk (DSC_AB), was constructed to gain insight and unravel the potential interactions between them during PAH removal applying omics approaches.

Hence, the present study is the first which evaluated individual strain roles, interaction among strains and stages during PHN removal through the time-driven changes in gene expression and metaproteome patterns in a designed PAH-degrading synthetic consortium. The understanding of the relationships among degradation efficiency and individual strain role into the designed PAH-degrading dual synthetic bacterial consortium AM-Bk (DSC_AB) will allows us to build relevant criteria to be applied in the combination of the strains to be used in bioremediation processes.

Material and methods

Bacterial strains and dual synthetic bacterial consortium (DSC) cultures

Each strain (AM and Bk) was grown in R2A medium (0.5 g of yeast extract, 0.5 g of proteose peptone, 0.5 g of casamino acids, 0.5 g of glucose, 0.5 g of soluble starch, 0.3 g of K_2HPO_4, 0.05 g of $MgSO_4$ $7H_20$, 0.3 g of sodium pyruvate, and 15 g of agar per litre of distillate water) for 48 h at 30 °C and 150 rpm, centrifuged at 6000 rpm during 10 min, then washed three times with 0.85% NaCl and resuspended in the same solution. The amount of the inoculum was determined by OD_{580} inoculating $1*10^7$ CFU.ml^{-1} for AM and Bk cultures and $2*10^7$ CFU.ml^{-1} for DSC_AB culture in a proportion of 65:35 of AM:Bk (to mimic their proportion in natural consortium CON) (Festa et al. 2016).

Microbial enumeration of the dual synthetic bacterial consortium AM-Bk (DSC_AB) and growth interaction

Bacterial counts were carried out at different incubation times (0, 1, 3, 7, 10 and 15 days). DSC_AB was incubated in 100 ml of Liquid Mineral Medium (LMM) (5 g of NaCl, 1 g of K_2HPO_4, 1 g of $(NH_4)H_2PO_4$, 1 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4$ $7H_20$ and 3 g of KNO_3 per litre of distillate water) (Vecchioli et al. 1990) supplemented with 200 mg.l^{-1} of PHN and incubated at 30 °C and 150 rpm. The determination of total heterotrophic bacteria was performed in triplicate on R2A medium plates.

Growth inhibition assay

For inhibition assay co-cultures are inoculated in solid medium using the 2 species at a time (binary co-cultures) The inocula are deposited in 5 µl of a microbial suspension (overnight culture in the same medium) in a punctual manner (spots) on a minimal culture medium such as R2A Reasoner and Geldreich (1985), 3 spots of species A are placed separated by 1 cm and 3 spots of species B separated in the same way. The distance between the spots of both species is 0.5 cm. This scheme allows zones of different degrees of interaction to be established and the ends to serve as controls. The plates are incubated for 7 days and the evolution is observed daily (increase or inhibition of growth, modification in colony characteristics, pigmentation, motility) (Stubbendieck and Straight (2015).

PHN-degradation and 1-hydroxy-2-naphthoic acid (HNA) production

The kinetics of PHN-degradation (200 mg.l^{-1}) and HNA production of AM and Bk axenic cultures (culture in which only a single species of organism is present) and of DSC_AB were performed in triplicate at 0, 1, 2, 3, 4, 7 and 15 days of incubation (30 °C and 150 rpm). Since PHN degradation could produce de accumulatios of toxic intermediaries as HNA, its production was also evaluated. Chemical extraction with ethyl acetate were performed and PHN and HNA were measured by HPLC (Waters® XBridge C18 3.5 µm, 4.6×65 mm) as detailed in our previous works (Coppotelli et al. 2010; Festa et al. 2013).

Degradation of other PAH

The degradation of fluorene (FLU), dibenzothiophene (DBT) and anthracene (ANT) was also determined in DSC_AB and compared to AM and Bk axenic cultures. In LMM, 100 $mg.l^{-1}$ of each compound was supplemented as a sole carbon and energy source. The degradation was carried out in triplicates during 15 days (30 °C and 150 rpm). The chemical extraction and the quantification were performed as it was mentioned in the previous section.

RNA isolation and quantitative real-time polymerase chain reaction (RT-qPCR) assays

Cell cultures of AM and Bk strains and DSC_AB were prepared to study the gene expression profile through time. The strains were starved in LMM without any carbon source during 7 days and later inoculated in LMM with 200 $mg.l^{-1}$ of PHN as sole energy and carbon source. The amount of the inoculum was determined by OD_{580} inoculating $1*10^9$ total cell for AM and Bk cultures and $2*10^9$ total cell for DSC_AB and sampled at 2, 8, and 72 h for RNA extraction. Cultures were performed in triplicate. PHN crystals were removed by filtration and cells were then washed, resuspended in RNAlater® (Sigma Aldrich) and stored at − 80 °C to prevent RNA degradation (Macchi et al. 2021).

Total RNA extraction was performed using RNeasy Protect Bacteria Mini Kit (Qiagen) following the manufacturer's instructions. The RNA concentration was determined in NanoDrop 2000 (Thermo-Scientific™) and the quality was assessed by measuring $A_{260}/_{A280}$ ratios as well as by electrophoresis in a formaldehyde denaturing agarose gel (1%). Genomic DNA was removed by incubating with DNase I (Promega RQ1) for 50 min at 37 °C. The DNA-free RNA was then used as template to synthesize cDNA with M-MLV (Invitrogen) Reverse Transcriptase and Random Hexamer Primers (Thermo Scientific) following the manufacturer's instructions. RNA extraction and cDNA synthesis were carried out in independent cultures of each condition run in triplicate.

Phenanthrene pathway can be divided in upper PHN-degradation pathway which describes the conversion from PHN to HNA and the lower PHN-degradation pathway which describes the conversion from HNA to Krebs cycle intermediaries (Khara et al. 2014). A set of genus-specific primers targeting the 16S rRNA gene of S*phingobium* sp. (AM strain) and *Burkholderia* sp. (Bk strain), used as internal control, (Table S2) and *Sphingobium* representative genes (proven to be involved in the upper and lower PHN-degradation pathway) (Table S1) (Khara et al. 2014) were used for RT-qPCR assays. The representative genes involved in PHN-degradation pathway for Bk were chosen according to Pérez-Pantoja et al. (2012) and to KEGG database. The catabolic genes primers used for Bk were design for this study using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and OligoAnalyzer tool (https://www.idtdna.com/pages/tools/oligoanalyzer). The primer pair specificity was verified in silico by BLAST® (Basic Local program BLAST alignment SearchTool) and by Artemis genome browser annotation tools against the AM and Bk genomes and by PCR with the same program detailed below, followed by gel electrophoresis. Additionally, the primers were tested against the other strain to rule out any non-specific amplification. The primer pair specificity was confirmed by sequencing PCR products in MACROGEN service (Korea).

Melting curve and the efficiency of the primers pairs were evaluated by RT-qPCR, using Rotor-Gene Q Qiagen. The reactions were performed following Macchi et al. (2021). The cycling parameters of reactions consisted of an initial step of 10 min at 95 °C, 45 cycles at 95 °C for 30 s, 60 °C for 15 s and 72 °C for 15 s, and a final stage at 72 °C during 10 min. Technical RT-qPCR triplicates of each biological replicate were performed and the relative expression levels were quantified according to the average values.

The results of relative gene expression were quantified with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001; Macchi et al. 2021). We used 16S rRNA gene of each strain as internal control and the lower incubation time during degradation (2 h) as reference sample.

Metaproteomic analysis

The metaproteomes of DSC_AB and axenic cultures of AM and Bk strains were analysed. A mix of the AM and Bk strains was prepared by cultivating the strains separately and mixing them in the same proportion as in the DSC_AB; this mix was used as a

control to discount the mixing effect in the abundance calculation.

Protein isolation

The cultures were grown in 500 ml LMM supplemented with 200 mg.l^{-1} of PHN during 72 h at 30 °C and 150 rpm in triplicate. Protein isolation, extraction and digestion and LC–MS/MS analysis of protein extracts were carried out following Macchi et al. (2021).

Protein digestion and mass spectrometry analysis were performed at the Proteomics Core Facility CEQUIBIEM (University of Buenos Aires-National Research Council). Triplicates of the raw data obtained with Q Exactive (Thermo Scientific) were searched against the draft genome sequence databases under the accession numbers NHOM01 (Bk) and LRUK01 (AM) using Proteome Discoverer and analyzed according to Macchi et al. (2021). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (PXD024411) partner repository with the dataset identifier.

Proteins areas were processed with the Perseus program (Max Planck Institute of Biochemistry, 1.5.5.3 version, available for free) for a deeper statistical analysis.

Functional categorization of significantly expressed proteins was carried out using BlastKOALA (Kanehisa et al. 2016) and complemented with manual curation.

Statistical analysis

The statistical data analysis was performed with parametric one-way ANOVA, using the SigmaPlot/SigmaStat software program (SPSS Inc., Chicago, Illinois, USA). Results represent means ± standard deviations.

Results

PHN biodegradation and HNA production

PHN and HNA concentrations in AM and Bk cultures and in DSC_AB were evaluated during 15 days of incubation. As shown in Fig. 1, the strain AM degraded 44% of the PHN supplemented after 1 day of incubation, 88% after 7 days and more than 97% after 15 days. During this process, the intermediary HNA was generated, and the highest production (73.21 ± 5.86 mg.l^{-1}) was observed around

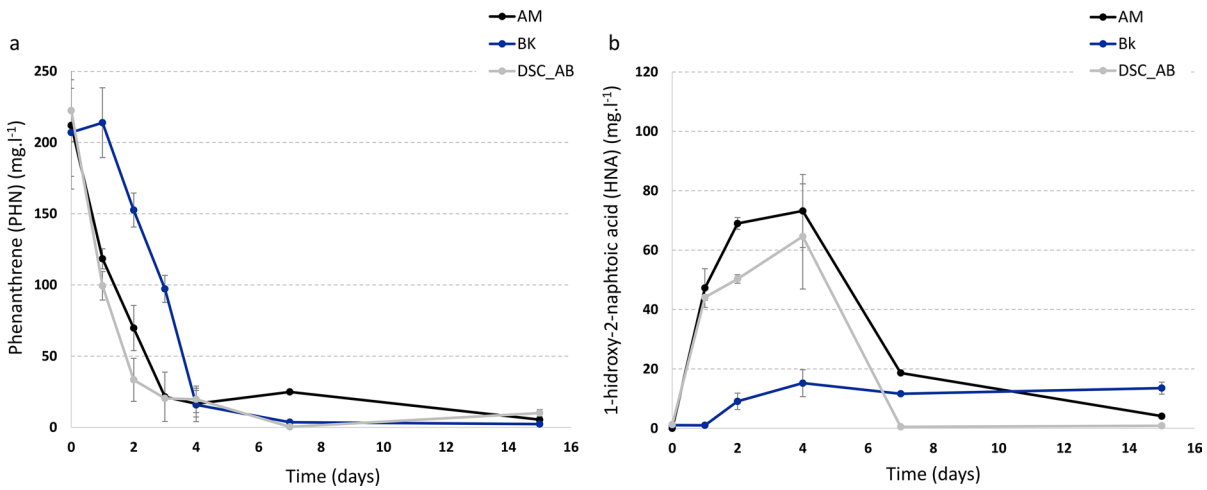


Fig. 1 Phenanthrene (PHN) (**a**) and 1-hydroxy-2-naphthoic acid (HNA) (**b**) concentrations in cultures of *Sphingobium* sp. (AM), *Burkholderia* sp. (Bk) and Dual Synthetic Consortium AM-Bk (DSC_AB) grown in LMM with PHN as sole carbon and energy source (200 mg.l^{-1}) during 15 days of incubation. Results are means of independent experiments performed in triplicate. Error bars represent standard deviations

day 4 and it was almost completely metabolized at the end of the incubation (4.10 ± 0.10 mg.l^{-1}). The strain Bk did not degrade PHN during the first day of incubation but reached 97% of degradation after 7 days and more than 99% after 15 days. HNA was also generated, but in a significantly minor quantity, its highest production was also observed around day 4 (15.23 ± 4.51 mg.l^{-1}). However, in this case that value was maintained until the end of the experiment.

DSC_AB degraded around 55% of PHN supplemented after 1 day of incubation, more than 99% after 7 days and after 15 days PHN was not detected (Fig. 1a). Of the analysed time points, the highest HNA production value (64.62 ± 17.72 mg.l^{-1}) was observed around day 4, and its concentration decreased until it was not detected after day 7 of incubation (Fig. 1b). Comparison of axenic cultures and DSC_AB showed significant differences ($P < 0.05$) in PHN-degradation after 7 days of incubation, with DSC_AB having the highest efficiency (99% degradation), followed by Bk (97% degradation) and AM (88% degradation).

Regarding HNA production, DSC_AB and AM axenic culture had the same amount of accumulated intermediary at day 4 of incubation, but Bk axenic culture showed a significantly ($P < 0.05$) lower HNA production. This scenario changed after day 7, when HNA production showed significant differences ($P < 0.05$) among cultures; the highest value was observed in AM culture (18.66 mg.l^{-1}), followed by Bk culture (11.65 mg.l^{-1}) and DSC_AB culture, where the HNA was not detected. At the end of the experiment, the HNA concentration in Bk culture remained constant, while in AM culture decreased to a final concentration of 4.10 mg.l$^{-1} \pm 0.10$ (Fig. 1b).

Bacterial population dynamics in DSC_AB during PHN-degradation

Given that some interactions depend on bacterial density, in order to study the changes in the population abundance in co-culture during PHN-degradation, DSC_AB was constructed with an unequal proportion of the strains (AM:Bk 65:35) to mimic the abundances found in the natural consortium CON (Festa et al. 2013) where *Sphingomonadales* order was the majority. To discard antagonistic response between AM and Bk strains, the growth interference was evaluated in R2A plates (Ebadi et al. 2021). No growth inhibition was observed between the strains (Fig. S1).

Population dynamics in DSC_AB were analysed based on the morphological analysis of colonies on R2A plates (Bk forms white round colonies and AM form round yellow colonies) (Fig. S1) during 15 days of incubation.

In DSC_AB culture, AM population remained in the same order in which was inoculated and in a higher proportion than Bk population during the first 4 days of incubation. However, the Bk population increased significantly during the same period. The proportion of both strains corresponds with a density around $1.3*10^8$ CFU/ml after day 7 and until the end of the incubation time (Fig. 2).

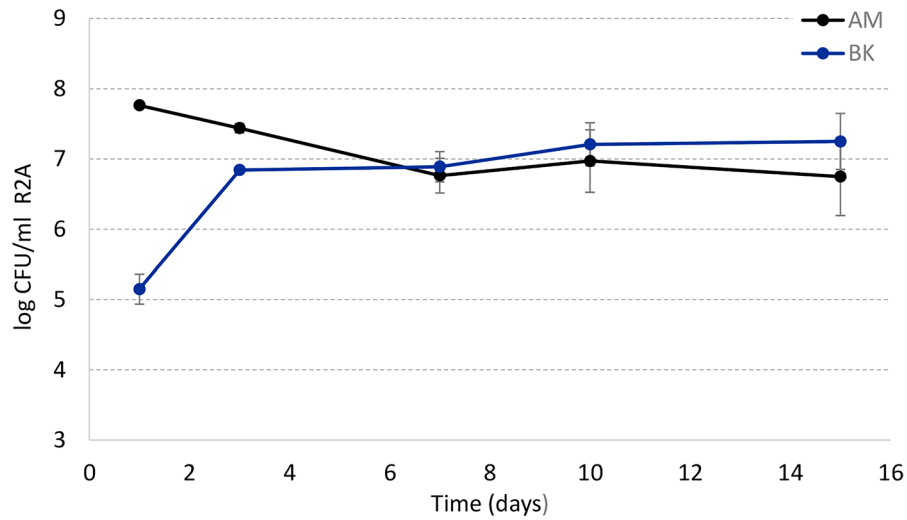


Fig. 2 Differential count of *Sphingobium* sp. (AM) and *Burkholderia* sp. (Bk) populations in DSC_AB cultures during PHN-degradation. Results are means of independent experiments performed in triplicate. Error bars represent standard deviations

Anthracene, dibenzothiophene and fluorene degradation

Other PAH degradation potential of DSC_AB was studied and compared with AM and Bk axenic cultures. Anthracene (ANT), dibenzothiophene (DBT) and fluorene (FLU) degradation supplemented as sole carbon and energy sources was studied over 15 days (Fig. 3).

AM strain degraded 75% and 85% of DBT and FLU respectively, while it did not show any significantly degradation of ANT in 15 days. Bk strain degraded 35%, 89% and 65% of the supplemented ANT, DBT and FLU respectively after 15 days of incubation. The consortium DSC_AB degraded 45%, 93% and 95% of the supplemented ANT, DBT and FLU respectively in 15 days. These results indicate a synergistic degradative capacity in DSC_AB. Colonies with morphologies typical of the two strains were found in all DSC-AB cultures after 15 days of incubation (data not shown).

Relative expression of PAH catabolic genes in DSC_AB by RT-qPCR

With the aim of revealing the individual roles played by each population in DSC_AB during PHN-degradation, the kinetics of gene expression and the relative expression levels of the AM and Bk strains specific genes (upper and lower PHN-degradation pathway) were compared to axenic AM and Bk axenic cultures during PHN-degradation (from 2 to 72 h).

In general, two key steps take place in the aerobic biodegradation of PAH. In the first step, the rings are activated by incorporation of one or two oxygen atoms upon the action of monooxygenases or dioxygenases, respectively. The second step is achieved by an extradiol- or intradiol-dioxygenase type enzyme, which produces a limited number of metabolites that will be later transformed into intermediaries of the tricarboxylic acid cycle (TCA) (Bertrand et al. 2015). As described in our previously work (Festa et al. 2016; Macchi et al. 2021) many ORFs (Open Reading Frames) analysed in AM and Bk genomes encoded proteins potentially involved in PAH degradation.

To obtain information of the whole process, relative expression of AM and Bk genes encoding enzymes for the upper (from PHN to HNA) and the lower (from HNA to TCA intermediaries) PHN-degradation pathway (Tables S1 and S2) were analysed. Figure 4h shows the PHN-degradation pathway (23 steps) and the relative expression levels of 7 specific catabolic genes of AM (Fig. 4a–g) and 5 specific catabolic genes of Bk (Fig. 4i–m) both in axenic cultures and in DSC_AB during PHN-degradation (2, 8 and 72 h).

The analysed specific catabolic genes of AM encode: (i) enzymes for the first steps of the upper PHN-degradation pathway, large subunit naph/bph dioxygenase *ahdA1f* (steps 1 and 3) and

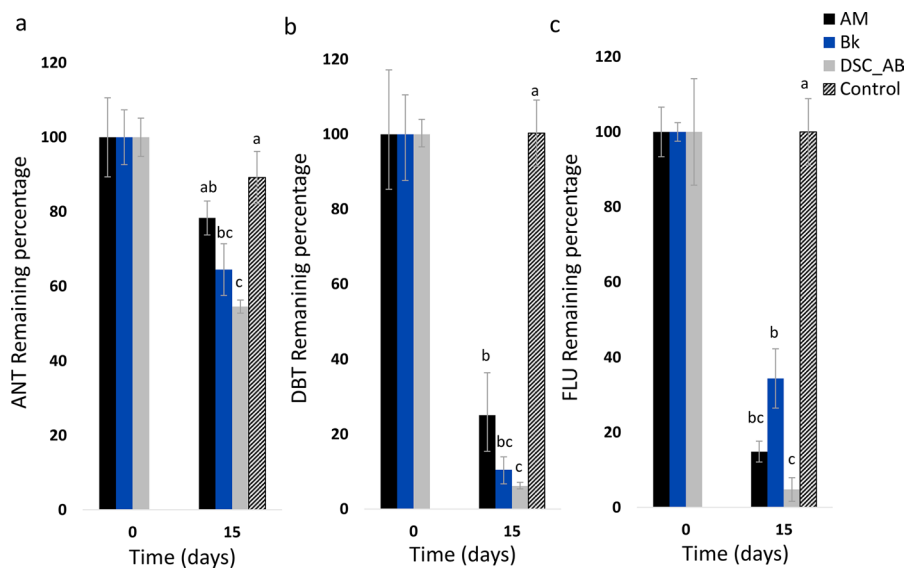
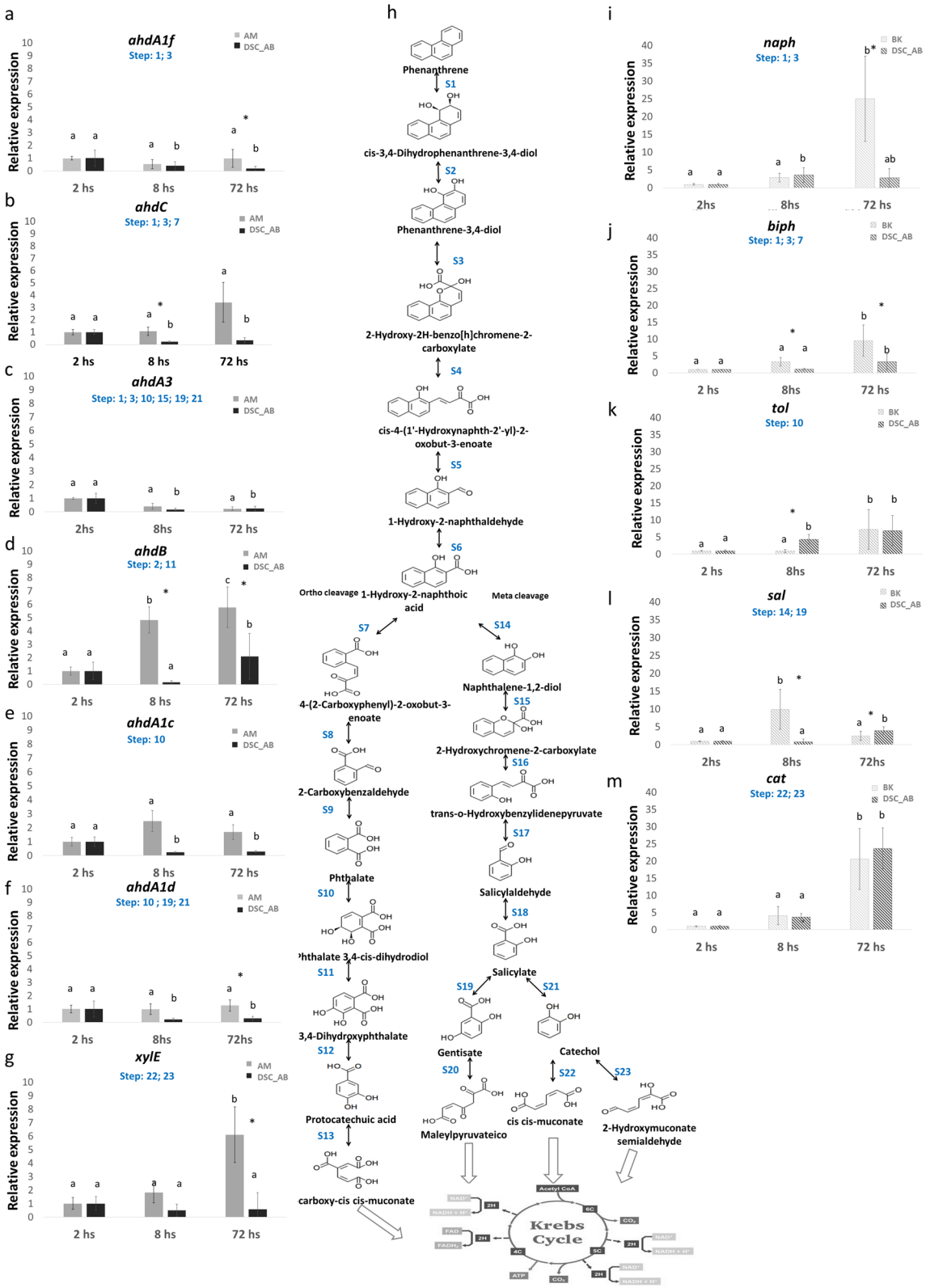


Fig. 3 Anthracene (ANT) (**a**), dibenzothiophene (DBT) (**b**), and fluorene (FLU) (**c**) remaining percentage in LMM with each PAH as sole carbon and energy source (100 mg.l^{-1}) in control (culture media without cells) and cultures of *Sphingobium* sp. (AM), *Burkholderia* sp. (Bk) and Dual Synthetic Consortium AM-Bk (DSC_AB), during 15 days of incubation. Results are means of independent experiments performed in triplicate. Error bars represent standard deviations


a
ahdA1f
Step: 1; 3
b
ahdC
Step: 1; 3; 7
c
ahdA3
Step: 1; 3; 10; 15; 19; 21
d
ahdB
Step: 2; 11
e
ahdA1c
Step: 10
f
ahdA1d
Step: 10 ; 19; 21
g
xylE
Step: 22; 23
Relative expression
AM
DSC_AB
2 hs
8 hs
72 hs
h
Phenanthrene
S1
cis-3,4-Dihydrophenanthrene-3,4-diol
S2
Phenanthrene-3,4-diol
S3
2-Hydroxy-2H-benzo[h]chromene-2-carboxylate
S4
cis-4-(1'-Hydroxynaphth-2'-yl)-2-oxobut-3-enoate
S5
1-Hydroxy-2-naphthaldehyde
S6
1-Hydroxy-2-naphthoic acid
Ortho cleavage
Meta cleavage
S7
4-(2-Carboxyphenyl)-2-oxobut-3-enoate
S8
2-Carboxybenzaldehyde
S9
Phthalate
S10
Phthalate 3,4-cis-dihydrodiol
S11
3,4-Dihydroxyphthalate
S12
Protocatechuic acid
S13
carboxy-cis cis-muconate
S14
Naphthalene-1,2-diol
S15
2-Hydroxychromene-2-carboxylate
S16
trans-o-Hydroxybenzylidenepyruvate
S17
Salicylaldehyde
S18
Salicylate
S19
S21
Gentisate
Catechol
S20
S22
S23
Maleylpyruvateico
cis cis-muconate
2-Hydroxymuconate semialdehyde
Acetyl CoA
Krebs Cycle
i
naph
Step: 1; 3
j
biph
Step: 1; 3; 7
k
tol
Step: 10
l
sal
Step: 14; 19
m
cat
Step: 22; 23
BK
DSC_AB
2hs
8hs
72hs

◂**Fig. 4** Relative expression values of catabolic genes involved in PHN-degradation pathway in axenic cultures of AM (**a** *ahdA1f*, **b** *ahdC*, **c** *ahdA3*, **d** *ahdB,* **e** *ahdA1c*, **f** *ahdA1d,* **g** *xylE*) and Bk (**i** *naph*¸ **j** *biph,* **k** *tol,* **l** *sal,* **m** *cat*) and in DSC_AB, according to PHN-degradation pathway (h). The values are expressed as 'fold change' ± STD and represent the averages of three independent replicates and indicate the change in mRNA levels of the studied genes compared with the control conditions (value of 1). Significant difference in gene expression between conditions (different times) of the same culture (AM, Bk or DSC_AB) are shown with different letters (a, b and c) and significant differences between cultures (AM and DSC_AB, or Bk and DSC_AB) for each time are shown with an asterisk (*). The Student's test was used, setting significance at ± 0.05 (two-way ANOVA, Tukey test). Error bars correspond to standard deviations

2,3-dihydroxybiphenyl 1,2-dioxygenase *ahdC* (steps 1, 3 and 7); (ii) enzymes for the lower PHN-degradation pathway, large toluate/benzoate dioxygenase subunit *ahdA1c* (steps 10 and 21), *ahdA1d* (steps 10,19 and 21) and catechol 2,3-dioxygenase *xylE* (steps 22 and 23); (iii) enzymes that could participate in both pathways, dihydrodiol dehydrogenase *ahdB* (steps 2 and 11) and ferredoxine and ring-hydroxylating dioxygenases *ahdA3* (steps 1, 3, 10, 15, 19 and 21) (Table S1).

Figure 4a–g shows the mRNA fold change of the 7 AM specific genes for the higher and the lower PHN-degradation pathway in samples from axenic AM culture compared with DSC_AB at different times in relation to the internal control (16 s rRNA gene specific for *Sphingobium* genus) using the Delta-Delta Ct method. The gene expression of AM (grey bars) and DSC_AB (black bars) is shown in relation to the expression at 2 h of incubation (value of one).

The 7 genes studied in AM were expressed early during the PHN-degradation, after 2 h of incubation (control condition). In the axenic AM culture, the expression of *ahdB* (Fig. 4d) and *xylE* (Fig. 4g) genes (lower pathway) was significantly up-regulated after 8 h and after 72 h, respectively. The expression of *ahdA1f*, *ahdC, ahdA3, fahdA1c* and *ahdA1d* genes did not show significant ($P < 0.05$) changes at the times analysed (Fig. 4a, b, c, e, f).

In DSC_AB**,** *adhA1f* (Fig. 4a) and *adhC* (Fig. 4b) genes for upper pathway, and *ahdA1c* (Fig. 4e) and *ahdA1d* (Fig. 4f) genes for lower pathway were down-regulated both at 8 h and 72 h of incubation compared to 2 h. Compared to AM culture, *adhA1f* (Fig. 4a) and *ahdA1d* (Fig. 4f) genes at 72 and *adhC* (Fig. 4b) gene at 8 h were also significantly ($P < 0.05$) down-regulated. While *xylE* (Fig. 4g) gene in DSC_AB culture did not present significant differences in the analysed times, it was significantly down-regulated at 72 h as compared with AM culture. Regarding genes for both the upper and lower pathways, *ahdA3* (Fig. 4c) was significantly down-regulated at 8 h and 72 h in DSC_AB, but did not show significant differences in relation to the axenic AM culture*; ahdB* (Fig. 4d) gene was significantly ($P < 0.05$) up-regulated at 72 h and a marked down-regulation was observed compared to AM at both 8 h and 72 h. Overall, in DSC_AB an under-expression of most of the analysed genes was observed relative to the axenic AM culture.

For the analysis of specific catabolic genes of Bk, five sets of primers were designed encoding enzymes for gene sequences related to PHN-degradation found in Bk genome (Table S2). The analysed catabolic genes of Bk encode: (i) enzymes for the first steps of the upper PHN-degradation pathway, naphthalene/biphenyl dioxygenase *naph* (steps 1 and 3) and biphenyl 2,3 dioxygenase *biph* (steps 1, 3 and 7); (ii) enzymes for the lower pathway, toluate/benzoate dioxygenase *tol* (step 10), salicylate 1,2 hydroxylase *sal* (steps 14 and 19) and catechol 2,3-dioxygenase *cat* (steps 22 and 23) (Table S2).

Figure 4i–m shows the mRNA fold change of the 5 Bk specific genes in axenic cultures compared with the cultures of DSC_AB at different times in relation to the internal control (16S rRNA gene specific for *Burkholderia* genus). The 5 genes studied in Bk were detected early (2 h) during PHN-degradation. In the axenic Bk culture, *naph, biph* and *tol* (Fig. 4 i–m) genes were significantly ($P < 0.05$) up-regulated after 72 h in relation to 2 h, and *sal* and *cat* (Fig. 4l, m) genes were significantly ($P < 0.05$) up-regulated at 8 h and 72 h, respectively.

In DSC_AB, Bk *naph* (Fig. 4i) *and biph* (Fig. 4j) genes for the upper pathway were up-regulated at 8 h and 72 h, respectively, in relation to 2 h, and the expression of both genes was significantly ($P < 0.05$) down-regulated at 72 h in relation to the axenic Bk culture. Additionally, in DSC_AB, *tol, sal* and *cat* (Fig. 4k–m) genes for the lower pathway, were mainly up-regulated at 72 h in relation to 2 h; *tol* (Fig. 4k) and *sal* (Fig. 4l) genes showed significant ($P < 0.05$) up-regulation at 8 h and 72 h, respectively, in relation to Bk.

Overall, in DSC_AB, the Bk genes analysed for the upper and lower pathway were up-regulated throughout the incubation time. In addition, only the genes for the lower PHN-degradation pathway showed up-regulation in relation to Bk, while the upper PHN-degradation pathway were down-regulated.

Metaproteomic analysis

The abundance of proteins in axenic and DSC_AB cultures was compared to assess proteomic modulation of AM and Bk strains when grown in DSC_AB. Around 641, 962 and 820 proteins were identified in AM, Bk and DSC_AB cultures, respectively**.** Significantly expressed proteins were identified and categorized according to different metabolic pathways (Table S3) using the BlastKOALA software (Kanehisa et al. 2016); the analysis was complemented by manual curation of the annotated genomes data in the NCBI database. In DSC_AB, proteins belonging to both strains were expressed at 72 h during PHN-degradation. Particularly, enzymes for many steps of PHN-degradation pathway belonging to both AM and Bk were detected (Figs. 5, 6; Table S3).

A linear model for the statistical evaluation of the results obtained from mass spectra of the total proteins of the three cultures (AM, Bk and DSC_AB) was created to reveal the distribution of significantly differentially expressed proteins involved in different cellular systems (Fig. 5a, b). We plotted log p value (-Log Student T-test p-value AM_DSC_AB or Bk_DSC_AB) on the y axis versus Student T-test Difference AM_DSC_AB or Bk_DSC_AB in x axis. Proteins that appear in the volcano plot with a fold change greater than 2 (less than -1 or greater than 1 on the x axis of the graph) and a p value below 0.05 (above 1.3 on the y axis of the graph) were considered as differentially expressed in DSC_AB relative to AM and Bk axenic cultures of (Figs. 5a, 6a).

Only 5 of the total proteins quantified for AM were significantly over-expressed and 39 proteins were significantly under-expressed in DSC_AB in relation to the axenic culture (Table S3, Fig. 5b, black squares). These proteins were categorized mainly in the metabolic pathways of xenobiotic degradation, cellular stress, and carbohydrate metabolism (Fig. 5b). Similarly, 20 proteins of the total proteins quantified for Bk were significantly over-expressed and 110 proteins, were significantly under-expressed in DSC_AB compared to the axenic culture (Table S3; Fig. 6a, black squares). These proteins were categorized mainly in the amino acid metabolism, xenobiotic degradation, transporters and cellular stress pathways (Fig. 6b).

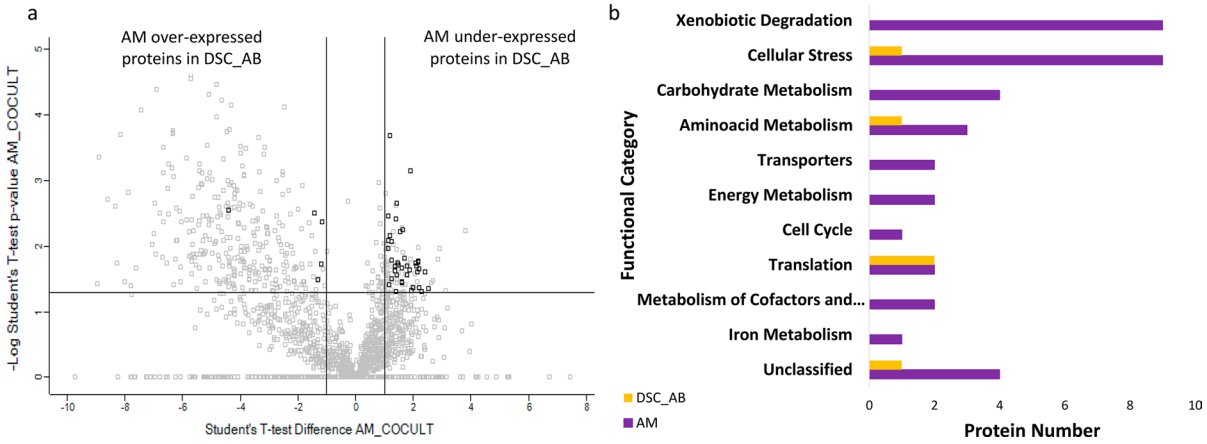


Fig. 5 **a** Volcano plot of protein abundance differences as a function of statistical significance with fold-changes greater than 2 (less than − 1 in DSC_AB or greater than 1 in AM on the x axis of the graph) and with t-test p-values lower than 0.05 (of values greater than 1.3 on the y axis of the graph). The black squares indicate significantly expressed AM proteins in DSC_AB. Proteins with no statistically significant difference in abundances between the two lineages are shown in grey. **b** Functional categorization of over-expressed (yellow) and under-expressed (violet) AM proteins in DSC_AB according to their abundance

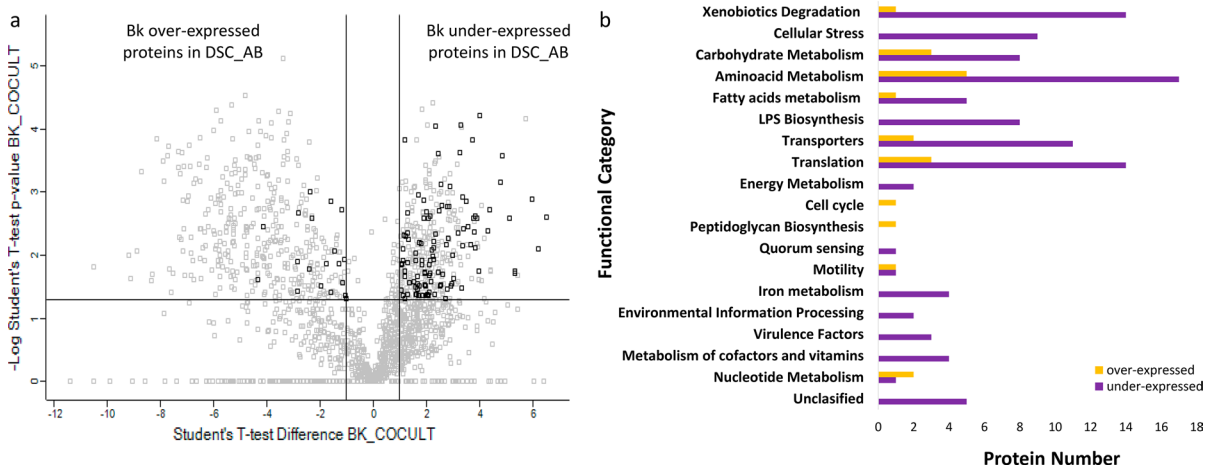


Fig. 6 **a** Volcano plot of protein abundance differences as a function of statistical significance with fold-changes greater than 2 (less than − 1 in DSC_AB or greater than 1 in Bk on the x axis of the graph) and with t-test p-values lower than 0.05 (of values greater than 1.3 on the y axis of the graph). The black squares indicate significantly expressed Bk proteins in DSC_AB. Proteins with no statistically significant difference in abundances between the two lineages are shown in grey. **b** Functional categorization of over-expressed (yellow) and under-expressed (violet) Bk proteins in DSC_AB according to their abundance

Xenobiotic degradation

In relation to *xenobiotic degradation* pathways, 9 proteins linked to the degradation of aromatic compounds of AM strain were under-expressed in DSC_AB relative to axenic cultures and belonged to the lower aromatic compound degradation pathway (Table S3, Fig. 5). Among them, 4-hydroxy-2-oxovalerate aldolase (step 5, 8 and 17), 4-oxalocrotonate decarboxylase (step 8), 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (step 9), benzoate/toluate 1,2 dioxigenase (step 10), and 1,2-dioxygenase anthranilate (step 10) could participate in the degradation of benzoate and xylene (intermediate metabolites of PHN-degradation pathway) and belong to the ortho-cleavage pathway. The aldolase (step 17) also participates in the catabolism of naphthalene and is the only enzyme found that belongs to the metha-cleavage pathway. One of the proteins found, 4-cresol dehydrogenase, could participate in the degradation of toluene and related compounds (Table S3). No over-expression of any enzyme belonging to AM linked to aromatic compound degradation was observed in DSC_AB (Fig. 5).

On the other hand, only one Bk protein linked to degradation of aromatic compounds, the aldehyde dehydrogenase family protein, was over-expressed in DSC_AB, it could act in the step 9 of PHN-degradation pathway (Fig. 6; Table S3). In addition, 14 Bk enzymes linked to the degradation of aromatic compounds were under-expressed in DSC_AB (Table S3). Within this group, biphenyl-2,3-diol 1,2-dioxygenase (steps 1, 3 and 7) and aromatic-ring-hydroxylating dioxygenase subunit beta (step 1 and 10) are involved in aromatic ring breaking and could act in the upper degradation pathway.

Regarding the lower degradation pathway, both ortho-cleavage and meta-cleavage enzymes, hydratase (steps 8, 17) and Alpha/beta hydrolase (step 9) were under-expressed in DSC_AB. Among ortho-cleavage enzymes were 4-oxalocrotonate decarboxylase (step 8), aldehyde dehydrogenase (step 9), (2Fe-2S) -binding protein of anthranilate 1,2 dioxygenase large subunit (step 10), and hypothetical protein BWU74_31040 (anthranilate 1,2-dioxygenase small subunit) (step 10). Among meta-cleavage enzymes were aldolase (OWJ56339.1) (step 17), 2-keto-4-pentenoate hydratase (step 17), acetaldehyde dehydrogenase (step 6 and 18) and catechol 2,3-dioxygenase (step 22 and 23). The enzyme 3-oxoadipyl-CoA thiolase, could act in the last steps of the pathway, to give place to compounds that can enter in TCA.

Three enzymes, (2Fe-2S)-binding protein (OWJ56343.1) (steps 10),

1,6-dihydroxycyclohexa-2,4-diene-1-carboxylate dehydrogenase (step 2 and 11), were expressed only in Bk axenic culture (Table S4).

Stress response

To further identify possible molecular mechanisms of the consortium in response to the presence of PAH, expression proteins related to stress response were analysed in detail.

Only an AM protein, ATP-dependent chaperone ClpB was over-expressed in DSC_AB, and other 9 proteins were under-expressed in DSC_AB. Among them, PhyR regulator, which intervenes in the activation of a chain of phospho-relays that trigger the activation of other cellular stress resistance proteins. In addition, different enzymes linked to glutathione metabolism, the phage shock protein PspA and an exodeoxyribonuclease III, linked to the double-stranded DNA repair, in particular, due to H_2O_2 damage (Table S3) were also under-expressed DSC_AB.

A similar pattern for Bk stress proteins was observed: 9 proteins linked to cell stress were under-expressed in DSC_AB. Some of them were transposase, peroxirredoxin, DNA starvation / stationary phase protection protein and the organic hydroperoxide resistance protein (Fig. 6).

Other proteins

Proteins interveners in other biological processes are described briefly and detailed in Table S3.

One AM protein linked to cell cycle regulation, histidine phosphotransferase ChpT was under-expressed in DSC_AB and proteins related to ribosome maturation and post-transcriptional modification were over-expressed in DSC_AB (Table S3). In contrast, Bk proteins linked to cytokinesis the production of peptidoglycan and the nucleotide metabolism were over-expressed in DSC_AB. Other Bk proteins related to transcription and translation, were also over-expressed in DSC_AB. However, other 11 Bk proteins linked to ribosomes and translation were under-expressed in DSC_AB (Table S3).

In relation to *carbon metabolism*, AM proteins linked to glycolysis and the citric acid cycle, were under-expressed in DSC_AB. Additionally, proteins linked to the electron transport chain were also under-expressed in DSC_AB (Table S3). Bk proteins related to glycolysis were also under-expressed in Bk, but it showed over-expression proteins linked to the oxidation of pyruvate (e.g. pyruvate oxidase) (Table S3).

Regarding *transport proteins*, 2 AM proteins, efflux transporter periplasmic associated with antibiotic resistance in gram-negative bacteria, were under-expressed in DSC_AB. On the other hand, 2 transporters Bk proteins, such as amino acid ABC transporter substrate-binding protein and sugar ABC transporter substrate-binding protein were over-expressed in DSC_AB. Other 12 Bk transporters, were under-expressed in DSC_AB.

AM strain showed under-expression of three enzymes linked to amino acid biosynthesis and over-expression of a protein linked to amino acid degradation in DSC_AB. In the case of Bk strain, 19 proteins linked to the metabolism of aminoacids were under-expressed in the co-culture (Fig. 6, Table S3).

Discussion

The latest research has shown that the clean-up of soil pollutants through microbial consortium is a very promising method. In this work, a two-species synthetic microbial consortium (DSC_AB) was constructed harnessing naturally occurring microbial interdependence, in order to easily investigate the interaction and regulation mechanisms between these two bacterial populations through metaproteomic and gene expression analysis.

DSC_AB showed its potent contaminant and toxic intermediary metabolites conversion ability that accelerated PHN-degradation, reaching a total elimination, significantly higher than the obtained in the axenic cultures of AM and Bk after 7 days of incubation (Fig. 1). Efficient metabolic cooperation is reached by bacterial populations that have developed molecular mechanisms for interactions and communication. This higher elimination rate of PHN in DSC_AB, and its extended PAH degrading capability (Fig. 3), demonstrates the existence of synergistic relationships among AM and Bk. Concordantly in proteomic pattern, enzymes for the upper and lower PHN-degradation pathway of both strains were significantly expressed (Figs. 5, 6), indicating that the increased efficiency in PHN and HNA degradation (Fig. 1) could be caused by the cooperation between Bk and AM. Some hints of this have been previously

observed in gel-dependent proteomic analysis for the original enrichment culture CON where proteins from both Sphigomonadales and Burkholderiales orders were expressed, indicating their active metabolism during PHN-degradation (Festa et al. 2017). Given that both strains have genes for the complete PHN-pathway (Festa et al. 2016; Macchi et al. 2021) and the ability to deplete the resource when grown isolated was determined (Fig. 1), according Tilman's resource-ratio theory, it is expected that the bacteria depleting the resource to the lowest concentration will exclude the other in co-culture. However, bacteria release metabolic by-products that can be used by other populations, which allows coexistence of outcompeted species able to exploit this new niche (Pascual-García et al. 2020). The greater degradation efficiency and the lower accumulation of intermediate products observed in DSC_AB would indicate that the assembly of these two strains optimized the functions associated with the PHN metabolism.

Regarding HNA accumulation in DSC_AB during the first days of incubation, since AM strain is predominant (Fig. 2), it could be using higher carbon source quantities and producing higher quantities of HNA than Bk. This idea is supported by the fact that the HNA production pattern of DSC_AB is similar to that of AM, and also that Bk did not show degradation at day 1, while AM and DSC_AB achieved 45% and 55% of PHN-degradation, respectively (Fig. 1). A total degradation of HNA occurred later in DSC_AB culture, which could be a consequence of the complementation action of the enzymes of both strains on HNA degradation. This was a positive effect, because HNA could cause cell stress due to chemical toxicity (Carney et al. 2008; Roell et al. 2019). Although we have evidence that AM degraded HNA and Bk cannot use exogenous HNA as the sole source of carbon and energy, Bk can metabolize salicylic acid and 2,3-dihydroxybiphenyl (Macchi et al. 2021); AM strain could be protagonist in HNA degradation in DSC_AB, which is in agreement with AM proteins for the lower degradation pathway found expressed in this work (Table S3).

Recently, many other works in which the degradation of PAH by cocultures was studied showed efficient degradation but the molecular mechanisms of the interaction were not studied (Mawad et al. 2021; Zhang et al. 2022). To deepen the study of the role that each bacterium plays, a detailed analysis of PHN-related transcripts during PHN-degradation was performed. In AM axenic cultures, gene *ahdB* (Fig. 4d) was significantly up-regulated after 8 h of incubation, when still was a significant PHN-concentration in the culture (Fig. 1a) and *xylE* (Fig. 4g) gene (lower pathway) was also up-regulated after 72 h, when intermediates of the lower pathway increased (HNA concentration was over 50 mg l^{-1}) (Fig. 1). Meanwhile in Bk axenic cultures gene *sal* (Fig. 4l) was up-regulated after 8 h of incubation, and *naph, biph, tol* and *cat* (Fig. 4i, j, k, m) genes were up regulated after 72 h, when most of the PHN was consumed and HNA concentration increased above 15 mg l^{-1} (Fig. 1). In DSC_AB, overexpression along incubation time of *naph, biph, tol* and *sal* (Fig. 4i–l) Bk genes could indicate an active participation of Bk in the upper and lower pathways. Similarly, over expression along incubation time of *ahdB* (Fig. 4d) AM gene, that could participate in upper and lower pathways, steps 2 and 11 (for which none gene of Bk was analysed), in DSC_AB indicates that the encoded enzyme could be actively participating in PHN-degradation. Nonetheless, the relative expression of Bk genes (*naph* and *biph*) and AM genes (*adhA1f, ahdC, ahdB, ahdA1d* and *xylE*) for the upper pathway in DSC_AB showed a decrease compared to the respective axenic cultures (Fig. 4), however the co-culture exhibited the higher efficiency in the degradation in relation to the axenic cultures (Fig. 1). These findings reinforce the evidence that both strains collaborate in degradation. A co-culture of *Stenotrophomonas* sp. N5 and *Advenella* sp. B9 has shown an improvement in the biodegradation of phenol due to the synergistic effect of its constituents in relation to monocultures, where induction of extradiol ring-cleavage dioxygenase and aromatic ring-opening dioxygenase of a microorganism and extradiol ring-cleavage dioxygenase from the other, were detected. At the same time, phenol hydroxylase in N5 axenic culture was not expressed in the co-culture (Li et al. 2020). Only one work was published on molecular mechanisms of syntrophic associations in a defined PHA-degrader bacterial consortium (Laothamteep et al. 2021). The authors identified through transcriptome analysis the synergistic interactions among the bacterial members (PO1, PO2 and PY1) of the consortium OPK promoted the simultaneous degradation of two high molecular weight (HMW) PAHs. They showed that, in addition to key enzyme for PAH degradation,

functional genes encoding ribosomal proteins, an iron transporter, ABC transporters and stress response proteins were induced in some members of the consortium.

In agreement, our metaproteomic results showed that many metabolic pathways underwent significant modifications in the level of protein expression between axenic and DSC_AB cultures (Figs. 5, 6).

Despite the fact that most of the enzymes of the aromatic compound's degradation pathway were expressed at a lower level in the co-culture at the time studied, it is especially notable that proteins of the PHN-degradation pathway were expressed by both strains, which indicates that both could be actively participating in the degradation (Figs. 5, 6 Table S3). Regarding their role, all under-expressed proteins of AM corresponded to the lower degradation pathway (Fig. 5, Table S3), while for Bk there was an under-expression of enzymes for both the upper (biphenyl-2,3-diol 1,2-dioxygenase) and the lower pathways (e.g.: 4-oxalocrotonate decarboxylase, anthranilate 1,2 dioxygenase large subunit, etc.) (Fig. 6, Table S3). The under-expression of biphenyl-2,3-diol 1,2-dioxygenase agrees with the result obtained by RT-qPCR assays (Fig. 4j) where, at 72 h, an under-expression of *biph* gene was observed in DSC_AB with respect to the axenic Bk culture.

These results are consistent with the gene expression pattern, where some enzymes from both strains for the upper and lower degradation pathways were significantly under-expressed. Due to the fact that both strains under-expressed catabolic enzymes in DSC_AB, the increase in the efficiency of PHN and HNA degradation (Fig. 1) reinforces the concept of cooperation between Bk and AM. It is noteworthy that 1,6-dihydroxycyclohexa-2,4-diene-1-carboxylate dehydrogenase from Bk was expressed only in the axenic Bk culture (Table S4). This enzyme can act in the steps 2 and 11 of the PHN-degradation pathway. Its absence of expression in the DSC_AB coupled with the overexpression *of ahdB* in AM (Fig. 4d) in the DSC_AB is another evidence that both strains are cross-feeding.

To have a better understanding of the response of each strain to co-culturing, we analysed the behaviour of some proteins not directly related to xenobiotic degradation. For AM in DSC_AB, the overexpression of the chaperone ClpB, whose function is to prevent protein aggregation, would indicate that the strain is in stationary phase (Schramm et al. 2019). Additionally, the under-expression of the histidine phosphotransferase ChpT enzyme, which participates in the regulation of the cell cycle (Fioravanti et al. 2012) was observed (Table S3). These results are in agreement with the data shown in Fig. 2, where no significant changes were observed in the number of AM cells throughout the incubation time. For Bk in DSC_AB, the overexpression of 3 proteins closely related to cell division, involved in peptidoglycan production, transcription, and cytokinesis was observed (Table S3). This result matches with the significant increasing number of Bk cells throughout the incubation time shown in Fig. 2.

In DSC_AB, several proteins of both AM and Bk strains related to cellular stress were under-expressed in relation to the axenic cultures (Figs. 5, 6, Table S3). Among AM proteins, only the chaperone ClpB related to cell longevity, was over-expressed in DSC_AB. There was under-expression of several stress-related AM proteins in the DSC_AB, particularly, the Phyllosphere-induced regulator PhyR, a modulator of the general response to stress through the activation of histidine kinases in alphaproteobacteria. This regulator modulates the enzyme expression such as glutathione S-transferase and alkene reductase (Gottschlich et al. 2019) linked to protection against oxidative damage, which were also under-expressed. The enzymes expression linked to the response to reactive oxygen species (ROS) has been documented in relation to bacterial PAH-degradation, since they are expressed as a consequence of the products of the activity of aromatic compounds degradation enzymes (Santos et al. 2004; Macchi et al. 2018).

The under-expression of stress-related Bk proteins in the DSC_AB, such as DNA starvation/stationary phase protection protein, peroxidase and organic hydroperoxide resistance protein, is also directly associated with oxidative stress (Almiron et al. 1992), while transposase (Vandecraen et al. 2017; Vigil-Stenman et al. 2017) and the ProP effector are related to general stress conditions and osmotic stress, respectively. Overall, DSC_AB culture did not show significant stress response or over-expression of membrane transport systems related to PAH, mediated by proteins of the strains during PHN-degradation (Fig. 5, Table S3). This could indicate that, in co-culture, the division of labour could decrease the production of ROS, linked to the oxidation of

aromatic compounds. The under-expression of proteins linked to oxidative stress during the pollutants degradation was previously observed in a co-culture constituted by *Pseudomonas reinekei* and *Achromobacter xylosoxidans* in comparison with the axenic culture of the former, where no significant accumulation of toxic metabolites was observed and consequently there was a lesser stress response, resulting in improved fitness (Fazzini et al. 2010).

The fact that in DSC_AB the proteins related to cellular stress of both strains were under-expressed can explain the higher performance reached by the co-culture. Since metabolic stress is reduced in each strain, cell fitness is improved correspondingly (Zhang and Wang 2016; Bhatt et al. 2021).

Conclusion

Understanding the interrelationships into PAH-degrading consortia is important for their design and the management of a successful bioremediation. Most of the studies related to the design of PAH-degrading consortia focus on the pollutants degradation kinetics and the specific catabolic genes behaviour of degradative metabolic pathways. The combination of the study of degradation kinetics and application of omic strategies allowed us to understand the mechanism underlying the improvement in the degradation process of DSC_AB compared to the axenic cultures. In addition, we confirmed the roles of the consortium members during PHN exposure and concluded that both strains are actively participating in PHN-degradation in DSC_AB, obtaining evidence of cross-feeding. The division of labour among the strains reduces the cellular stress, which seems to decrease the metabolic burden increasing the degradation efficiency. Results showed that DSC_AB could be a good candidate to be used in bioaugmentation processes in soil.

Author contributions EEN: Investigation, Conceptualization, Software Methodology, Formal analysis, Data Curation Writing - original draft Writing - Review & Editing -MM: Investigation, Software, Methodology, Formal analysis, Validation, Data Curation, Writing - Original Draft, Review & EditingSF: Investigation, Formal analysis, Validation, ReviewMPV: Investigation, Software, Formal analysis, Validation, Review & EditingISM: Supervision, Resources, Funding acquisition, Review & Editing.BMC: Investigation, Conceptualization, Project administration, Methodology, Resources, Funding acquisition, Writing - original draft, Writing - Review & Editing, Visualization.

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Declarations

Competing interests The authors declare no competing interests.

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