Insights into the genome and proteome of *Sphingomonas paucimobilis* strain 20006FA involved in the regulation of polycyclic aromatic hydrocarbon degradation

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**Abstract**

In order to study the mechanisms regulating the phenanthrene degradation pathway and the intermediate-metabolite accumulation in strain *S. paucimobilis* 20006FA, we sequenced the genome and compared the genome-based predictions to experimental proteomic analyses. Physiological studies indicated that the degradation involved the salicylate and protocatechuate pathways, reaching 56.3% after 15 days. Furthermore, the strain degraded other polycyclic aromatic hydrocarbons (PAH) such as anthracene (13.1%), dibenzothiophene (76.3%), and fluoranthene. The intermediate metabolite 1-hydroxy-2-naphthoic acid (HNA) accumulated during phenanthrene catabolism and inhibited both bacterial growth and phenanthrene degradation, but exogenous-HNA addition did not affect further degradation. Genomic analysis predicted 126 putative genes encoding enzymes for all the steps of phenanthrene degradation, which loci could also participate in the metabolism of other PAH. Proteomic analysis identified enzymes involved in 19 of the 23 steps needed for the transformation of phenanthrene to trichloroacetic-acid intermediates that were upregulated in phenanthrene cultures relative to the levels in glucose cultures. Moreover, the protein-induction pattern was temporal, varying between 24 and 96 h during phenanthrene degradation, with most catabolic proteins being overexpressed at 96 h—e. g., the biphenyl dioxygenase and a multispecies (2Fe–2S)-binding protein. These results provided the first clues about regulation of expression of phenanthrene degradative enzymes in strain 20006FA and enabled an elucidation of the metabolic pathway utilized by the bacterium. To our knowledge the present work represents the first investigation of genomic, proteomic, and physiological studies of a PAH-degrading *Sphingomonas* strain.

**Keywords** Genomics · HNA accumulation · Phenanthrene pathway · Proteomics · Strain 20006FA

**Introduction**

Polycyclic aromatic hydrocarbons (PAH) constitute ubiquitous and serious worldwide pollutants since they are extremely harmful to human health (Vandermeersch et al. 2015) and may drastically affect the biodiversity of natural ecosystems (Zhang et al. 2015). Although several natural and anthropic sources contribute to the release of PAH into the environment, petrochemical activities account for the majority of those compounds contaminating soils and water bodies (Zafra et al. 2017). Microbial degradation is the main process for the ecologic restoration of PAH-contaminated sites (Peng et al. 2008); prokaryotes temporarily change their pattern of gene expression in response to environmental signals, such as sudden spikes in PAH (Fernandez-Luqueno et al. 2011).

The genus *Sphingomonas* includes many strains that are attractive as a result of their diverse environmental
adaptations and their capabilities to degrade xenobiotics and recalcitrant pollutants, including PAH, (Zhao et al. 2017; Khara et al. 2014; Dong et al. 2014; Stolz 2009; Vandermeer and Daugulis 2007). Sphingomonads also make use of original strategies to enhance PAH bioavailability (Fialho 2008; Johnsen and Karlson 2005). Despite the increase in the number or aromatic compounds that are known to be biodegraded by Sphingomonas, many critical aspects of the metabolism of those bacteria are still unknown, including the nature of the metabolites in degradation pathways, the evolution and dispersion of degradation-related gene clusters, and the regulatory mechanisms (Zhao et al. 2017).

S. paucimobilis 20006FA was originally isolated from a soil microcosm contaminated with phenanthrene and was selected for its ability to grow on several PAH (e.g., anthracene, phenanthrene, fluoranthene, and dibenzothiophene)—indeed, investigations of its ecological properties have indicated that the strain apparently enhances phenanthrene bioavailability by producing biosurfactants and by adhesion to the phenanthrene crystals (Coppotelli et al. 2010). Studies on pure cultures and contaminated microcosms inoculated with the strain have demonstrated its ability to degrade phenanthrene, though the intermediate metabolite in the phenanthrene-degradation pathway 1-hydroxy-2-naphthoic acid (HNA) was observed to accumulate both in soil and in pure cultures (Coppotelli et al. 2010). Until now, the regulation of the PAH biodegradation mechanism, the cause of that metabolite accumulation, even the proteomics of the resulting expression, however, have not been studied on S. paucimobilis 20006FA. The proteome of an organism provides perspicacious insight into bioremediation-related genes and their regulation (Fulekar and Sharma 2008). More complete annotation of genomes with the help of proteomic evidence is accordingly paving the way for integrated multomic approaches in microbiology (Armengaud 2013).

Our aim in the present work was to study the involvement of enzymes or metabolites in the stimulation or inhibition of PAH degradation by strain 20006FA. This objective was pursued through proteomic- and genomic-based approaches along with investigations on the influence of the key metabolite HNA on phenanthrene degradation. The present work represents the first investigation of genomic, proteomic, and physiological studies of a PAH-degrading Sphingomonas strain.

**Materials and methods**

**Chemicals**

Phenanthrene, anthracene, dibenzothiophene, fluoranthene, HNA, and phthalic acid were purchased from Carlo Erba (Milan, Italy, > 99.5% purity).

**Bacterial strain**

_S. paucimobilis strain_ 20006FA was isolated from a microcosm whose soil had been artificially contaminated with phenanthrene. That soil had been obtained from an area near La Plata city, Argentina (34°51′24.6″S, 58°06′54.2″W) (Coppotelli et al. 2008).

**Bacterial growth conditions**

_S. paucimobilis_ 20006FA was grown in either R3 or mineral medium (MM) with glucose, phenanthrene or HNA as described by Coppotelli et al. (2010).

**Carbon-source utilization**

Growth was tested in sterile liquid minimal medium (LMM) supplemented with phenanthrene or with HNA. Incubation and growth monitoring were performed according to Coppotelli et al. (2010).

**PAH degradation**

Phenanthrene degradation and HNA formation were analyzed in triplicate at a phenanthrene concentration of 0.84 g L. Cultures were seeded with 2 × 10⁷ colony-forming units (CFUs) mL⁻¹ of a 24-h-old prior culture and incubated at 26 °C for 160 h in a rotary shaker at 150 rpm. One no inoculated bioreactor was used as an abiotic control. The cultures were extracted with ethyl acetate and analyzed by reversed-phase high-performance liquid chromatography (HPLC; Coppotelli et al. 2010).

The degradation of anthracene, dibenzothiophene, or fluoranthene, as the sole carbon and energy source was tested in duplicate in sterile LMM. Cultures were incubated at 28 °C, 150 rpm for 7 days.

**HNA degradation**

The degradation of HNA was measured by HPLC, was performed in triplicate in 50-mL glass bioreactors containing 10 mL of LMM supplemented with 0.05 or 0.14 g L⁻¹ of the carbon source.

**Statistical analysis**

The statistical evaluation of the degradation and counting data were performed by parametric one-way analysis of variance (ANOVA), followed by Tukey’s honestly significant difference (HSD) post-hoc test, through the use of the...
XLStat-Pro statistical package v7.5.2 (Addinsoft SARL, France).

Whole-genome sequencing, assembly, and annotation

The genome of strain 20006FA was sequenced by the HiSeq 1500 Illumina (2 × 100-bp paired-end reads) sequencing technology. The raw Illumina-sequence data were quality trimmed and filtered by means of the Nextera® XT Illumina protocol. The de-novo assembly of the reads was performed with Illumina’s A5-miseq Assembly Pipeline (v. 2.0) platform (Tritt et al. 2012). To annotate the genes, Rapid Annotation with the Subsystem Technology (RAST) server version 2.0 was used (Aziz et al. 2008). A functional analysis of genes was subsequently performed with the KEGG database and a manual curation by means of the genome viewer Artemis (Rutherford et al. 2000) (http://www.sanger.ac.uk/Software/Artemis). To determine each probable coding sequence a BLASTX alignment NCBI database was performed (http://blast.ncbi.nlm.nih.gov). The draft genome sequence of *S. paucimobilis* strain 20006FA was deposited in GenBank Whole-Genome Shotgun Project (listed as WGS) under the Accession Number LYMJ00000000.

Preparation of the cell lysates for proteomic studies

Cells grown in glucose or phenanthrene were harvested for protein analysis in the stationary phase (96 h) or the log phase (24 h). Triplicate cultures were analyzed under each condition.

The preparation of cell lysates was performed according to Pérez Vidakovics et al. (2007). The procedure stated in brief: *S. paucimobilis* strain 20006FA was grown in LMM supplemented with 0.84 g L⁻¹ of the corresponding carbon source. Cells from 1000 mL of culture were collected, washed and resuspended in MilliQ water with 5 mM phenylmethylsulfonylFluoride (PMSF) as a protease inhibitor. The cell suspension was disrupted with a Precellys 24-bead beater (Bertin Technologies, location) and solubilized in a solubilization solution containing Amberlite. The protein concentration in the extracts was determined by the BioRad Protein Assay Dye Reagent Concentrate and 2-D Quant Kit (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden).

Proteomic analysis

For isoelectric foccusing (IEF), precast 7- and 18-cm pH 4–7 immobilized-pH-gradient (IPG) gels (Immobiline DryStrips, GE Healthcare) were used. Samples were prepared according to Pérez Vidakovics et al. (2007), mixed with rehydration buffer containing Pharmalyte™ pH 3–10, bromophenol blue sodium salt ultrapure, PMSF, and IPG Buffer with a reducing agent (dithiothreitol; GE Healthcare). The final protein concentration was 2 µg µL⁻¹. The IPGs pH 4–7 were rehydrated with the samples as described (Sánchez et al. 1999).

The IEF was performed with the Ettan IPGphor 3 IEF (GE Healthcare Bio-Sciences) according to Pérez Vidakovics et al. (2007). After the IEF, the IPG strips were treated with equilibration buffer with dithiothreitol (PlusOne) and iodoacetamide (GE Healthcare). Thereafter the IPG strips were mounted on a sodium-dodecyl-sulfate–polyacrylamide gel and overlaid with a 12.5% (w/v) resolving polyacrylamide gel. The separation was carried out at 40 V and 20 °C in a PROTEAN II xi 2-dimensional electrophoresis unit (Bio-Rad, Hercules, CA) connected to a Multitemp II cooling bath (GE Healthcare) for large gels or a Mini PROTEAM Tetra Cell (Bio-Rad, Hercules, CA) for small ones. The gels were stained with Coomassie brilliant blue G-250 the 2-dimensional–gel images captured with Universal Hood II; and spot detection, matching, quantification, and normalization performed by means of the ProteomeWeaver 4.0 program (both from Bio-Rad). The proteins with significant changes in representation were processed for mass-spectrometry (MS) analysis. All the studies were performed in triplicate.

The spots of interest were manually excised and sent to the Mass Spectrometry Facility (CEQUIBIEM) at the School of Exact and Natural Sciences, University of Buenos Aires. A tryptic digestion was performed and the resulting peptides analyzed by ultraviolet matrix-assisted laser desorption ionization-time of flight/time of flight (MALDI-TOF/TOF; UltraflexII BrukerDaltonics). The software used for spectra visualization and tandem-MS (MS/MS) protein identification were Flex Analysis (v. 3.3) and BioTools (Bruker Daltonics), linked to MASCOT (Matrix Science, Boston, MA©2016 http://www.matrixscience.com/) in order to search for the protein-sequence databases listed under the NCBI number. The protein score was calculated as \(-10 \times \log(P)\), where *P* is the probability at which the observed match was a random event. The proteins identified by MALDI-TOF/TOF were subjected to bioinformatic analysis including similarity searches with predicted 2006FA proteins from the genome in ARTEMIS.

Results

Bacterial growth and the degradation of phenanthrene and other PAH

In order to quantify the phenanthrene degradation and the resulting formation of HNA during strain-2006FA growth, cultures with phenanthrene as the sole carbon and energy source were prepared and chemical analysis performed by HPLC. Figure 1, panels A and B, depict,
respectively, the phenanthrene remaining and the HNA produced as a function of incubation time in these cultures. Phenanthrene degradation reached 49.1% after 161 h of incubation, but at around 120 h an interruption in that process was recorded.

Phenanthrene biodegradation (Fig. 1, Panel A) was modelled by using a three-parameter logistic function described by the equation: $PHN = P + ae^{-bt}$ (where $t$ is the treatment time and $PHN$ is phenanthrene concentration at time $t$ and $a$ and $b$ are constants that define the curve in accordance with the input raw data). In that equation, the parameter ($P$) modulates the phenanthrene-elimination rate over time. That parameter could be represented by an influence that depends on PHN concentration and could thus involve at least one of the metabolites accumulated (Fig. 1, Panel B). A decrease in the elimination rate is also indicated in the Supplemental Material (Table S1). A determination of the phenanthrene concentration after 15 days of incubation indicated a final degradation of 59.6%, thus confirming that a progressive decrease in the degradation rate occurred over time. A previous study had demonstrated that this strain could efficiently degrade an initial concentration of 2 g L$^{-1}$ of phenanthrene to 52.9% in 20 days (Coppotelli et al. 2010). Although, in another experiment performed in the present work with cultures initially containing 0.2 g L$^{-1}$ of phenanthrene, the strain was able to attain a degradation of 99% in 15 days. This result may suggest that parameter $P$ depends on the initial phenanthrene concentration.

HNA became detectable from 26 h on and continued to accumulate during the incubation, reaching a concentration of 0.166 g L$^{-1}$ in 161 h. Subsequently, on Day 15, the HNA concentration was ca. 0.087 g L$^{-1}$, thus indicating the occurrence of degradation. For HNA production (Fig. 1, Panel B), the adjustment curve was obtained by using a two-parameter logistic function $Y = a \cdot (1 - e^{-bt})$—with an exponential rise to a maximum, where $t$ is the treatment time and $a$ and $b$ are constants that define the curve in accordance with the input raw data.

From the phenanthrene-degradation and HNA-accumulation curves, we clearly observed that the elimination rate of phenanthrene decreased significantly over time (Fig. 1, Panel A), conjointly when the accumulation of intermediate metabolites occurred (i.e., after 24 h; Fig. 1, Panel B). Thereafter, even though the HNA was further degraded, the degradation of phenanthrene did not continue.

Figure 1, Panel C is a plot of the growth of strain 20006FA in those same cultures; growth was determined by CFU counts as a measure of the viable bacteria present at each time point. Under those conditions, the growth rate was 0.146 h$^{-1}$ ($r^2 = 0.995$) and the doubling time 4.72 h. That, at 20 h, the culture entered in a stationary phase while the degradation rate was still high (Fig. 1, Panel A) is indeed noteworthy.

![Fig. 1 Concentration of phenanthrene (Panel A) and 1-hydroxy-2-naphthoic acid (HNA; Panel B) and counts of heterotrophic viable bacteria (Panel C) in the strain-20006FA culture growing in LMM with phenanthrene as the sole carbon and energy source (0.84 g L$^{-1}$) during 161 h of incubation. The results are the means of three biological replicates. The bars represent standard deviations. The mathematical fitting of the curves to growth equations was performed by the SigmaPlot V10, Systat software, Inc., San Jose, CA. In panels A and B, the respective concentrations of phenanthrene (black squares) and HNA (black circles) in g L$^{-1}$ are plotted on the ordinate as a function of the incubation times in h on the abscissas for the bacterial cultures or, in Panel A, abiotic controls (gray triangles). The solid curves in panels A and B (Estimated concentrations) are the loci of points representing the functions best fitting the data. In Panel C, the growth of viable bacteria (black circles), expressed as CFUs per mL, is plotted on the ordinate as a function of incubation time in h on the abscissa.](image-url)
In addition, we determined that the strain was able to degrade anthracene (13.1%) and dibenzothiophene (76.3%), plus a qualitative analysis furthermore demonstrated the production of colored metabolites after 15 days of growth on 0.2 g L\(^{-1}\) of fluoranthene. In this regard, a previous publication from our laboratory had documented the metabolization of salicylic acid (Coppotelli et al. 2010); and we now report here that phthalic acid was also metabolized, as judged by an observed and recorded support of bacterial growth (data not shown). Thus indicating that strain 20006FA could be utilizing either the *meta* or the *ortho* pathway for the degradation of phenanthrene (Fig. 3).

**HNA—bacterial growth and degradation**

In order to study the strain’s capability of using HNA as the sole carbon and energy source, cultures in LMM supplemented with HNA at three different concentrations (0.05, 0.14, and 0.3 g L\(^{-1}\)) were prepared and monitored to determine microbial growth and remnant-HNA levels. The concentrations were chosen near the values obtained during phenanthrene degradation (Table 1). In the cultures supplemented with 0.05 g L\(^{-1}\) of HNA, strain 20006FA grew slowly after a lag phase of 6 h, exhibiting a specific growth rate of 0.148 h\(^{-1}\) and a doubling time of 4.72 h. When grown on 0.14 g L\(^{-1}\) of HNA, however, the lag phase increased significantly up to 30 h, but the specific growth rate was 0.153 h\(^{-1}\) and the doubling time 4.52 h (Fig. 2). At both concentrations of HNA, the medium took on a yellow cast, indicating HNA-ring cleavage. When strain 20006FA was inoculated in LMM supplemented with 0.3 g L\(^{-1}\) HNA, no growth was detected (not shown). That failure could be indicative of HNA to be toxic to the strain at that concentration.

The degradation of HNA was determined after 24 h in cultures growing in LMM with HNA as the sole carbon and energy source, at concentrations of 0.05 and 0.14 g L\(^{-1}\). A significantly higher degradation value of 94 ± 9% was obtained in the culture at 0.05 g L\(^{-1}\); compared to that attained at 0.14 g L\(^{-1}\), where 44 ± 8% became degraded. These results, in addition to the considerably longer lag phase observed at 0.14 g L\(^{-1}\) of HNA, could indicate an inhibition of cellular metabolism at increasing concentrations of HNA.

**Influence of HNA on phenanthrene-degradation kinetics**

In order to investigate if the delay in phenanthrene degradation was caused by an accumulation of HNA, which could inhibit the growth of strain 20006FA; we studied the effect of HNA on phenanthrene degradation. Cultures of the strain in LMM were supplemented with phenanthrene at 0.84 g L\(^{-1}\) along with HNA added at a concentration of either 0.05 or 0.14 g L\(^{-1}\). After 24 and 96 h of incubation, the concentrations of HNA and phenanthrene remaining were determined by HPLC. During growth, an orange-to-yellow cast appeared in both media.

Table 1 documents that phenanthrene degradation in both cultures was around 12–14% after 24 h and 41–45% after 96 h. Under these conditions, phenanthrene degradation was not significantly affected by externally added HNA (Table 1), in comparison to the results obtained in the

**Table 1** Percent degradation of phenanthrene and concentration of HNA (g L\(^{-1}\)) accumulated in cultures of the strain 20006FA growing in LMM with phenanthrene (PHN, 0.84 g L\(^{-1}\)) and 1-hydroxy-2-naphthoic acid (HNA) externally added, after 24 and 96 h of incubation

|                  | PHN           | PHN + HNA (0.05 g L\(^{-1}\)) | PHN + HNA (0.14 g L\(^{-1}\)) |
|------------------|---------------|-------------------------------|--------------------------------|
| PHN degradation (%) | 24 h 15 ± 2  | 24 h 12 ± 4                  | 24 h 14 ± 4                   |
|                  | 96 h 44 ± 5   | 96 h 45 ± 9                  | 96 h 41 ± 4                   |
| HNA accumulation (g L\(^{-1}\)) | 24 h 0.07 ± 0.01 | 24 h 0.09 ± 0.01 | 24 h 0.13 ± 0.01 |
|                  | 96 h 0.138 ± 0.007 | 96 h 0.15 ± 0.04  | 96 h 0.19 ± 0.02  |

Results are means ± standard deviations of three biological replicates.
control cultures (PHN), where the degradation was about
15 and 44% after those respective times. After 96 h, HNA
had accumulated up to a concentration of 0.15 g L\(^{-1}\) in the
culture with HNA at 0.05 g L\(^{-1}\) and up to a concentration
0.19 g L\(^{-1}\) in the culture with HNA at 0.14 g L\(^{-1}\). Since
HNA accumulation did not reach values significantly higher
than those observed during phenanthrene degradation in cul-
tures with no added HNA (Table 1, column PHN)—i.e., the
HNA concentrations measured were less than the sum of the
added HNA and the HNA produced in the cultures without
HNA addition—this difference could be showing that the
cells could be maintaining the concentration under values
that would be toxic. Concentrations of HNA within the range
0.15–0.19 g L\(^{-1}\) might be suspected to be toxic to the cells
that would be toxic. Concentrations of HNA within the range
of strain 20006FA. Despite the decline in phenanthrene-deg-
radation rate observed (Fig. 1, Panel A and supplementary
Table S1), the HNA concentration remained within a con-
stant range (around 0.15 g L\(^{-1}\)) that allowed a continuation
of phenanthrene degradation over time.

Genomic analysis

In-silico analysis of the genome of *S. paucimobilis* 20006FA
revealed a high number of open-reading frames (ORFs) pre-
dicted to encode enzymes for aromatic-hydrocarbon de-
gradation (Tables 2, 3). Table 2 lists the general features of
the genome. A genome sequencing yielded 144 scaffolds
along with 4862 predicted coding genes. The strain pos-
sesses multiple ORF homolog for enzymes with similar
functions that are implicated in PAH degradation; they are
distributed throughout the bacterial chromosome, and are
not organized in gene clusters. On the basis of this analysis,
we have predicted 126 putative genes encoding enzymes for
all the steps in the degradation of phenanthrene to acetyl-
CoA and succinyl-CoA. The gene products of selected ORFs
that were likely to be involved in both the ortho and the meta
phenanthrene-degradation pathways, are summarized
in Table 3. The steps in the scheme of Fig. 3 in which these
enzymes could be implicated are also indicated in Table 3.
The ORFs that were confirmed by the proteomic analysis
described below to be coding for the enzymes involved in
the degradation steps are shadowed in gray.

We found six ORFs that shared a significant identity with
components of ring-hydroxylating dioxygenases that cata-
lize the initial step of phenanthrene degradation and the con-
version of specific intermediate metabolites. Several genes
are a flanked by, transposon or transposon-like sequences.
A particular region, corresponding to scaffold number 19,
has a high frequency of ORFs for PAH-degrading enzymes.
This region exhibits a 92% similarity to and a 76% coverage
of the sequence of plasmid pNL1 from *NovoSphingobium*
(originally *Sphingomonas*) *aromaticivorans* F199 (Romine
et al. 1999).

### Proteomic analysis

In order to study the effect of phenanthrene on the cellular
response, the proteome of *S. paucimobilis* 20006FA grown
in batch cultures under two different conditions—utilizing
either glucose or phenanthrene as the sole carbon source—
were analyzed by two-dimensional gel electrophoresis.

#### Proteomic background of biodegradation

The growth curves of strain 20006FA on glucose or phenan-
threne as the sole carbon source indicated that the cultures
reached the stationary phase of growth after 96 h (Supple-
mental Material Fig. S1). At that time, quantitative analysis
of biodegradation (Fig. 1) revealed that more than 40% of
the initial phenanthrene had been removed and the prin-
cipal derivative HNA had reached a concentration of ca.
0.13 g L\(^{-1}\). On the basis of the growth phase and degree
of phenanthrene degradation illustrated in Fig. 1, we estab-
lished the optimal culture conditions for comparative pro-
teome analysis of strain 2006FA after 96 h of growth. Upon
consideration of the kinetics of phenanthrene-derivative
formation, we determined the optimal culture conditions
for comparative proteome analysis of strain 2006FA with
respect to phenanthrene degradation to be at ca. 24 and 96 h.

#### Proteomic response to phenanthrene

The proteins obtained from the bacterial lysates of cultures
containing glucose or phenanthrene as the sole carbon
source were analyzed by two-dimensional-electrophoresis
in order to identify the enzymes expressed during phenan-
threne degradation. Accordingly, under those conditions, the
electrophoreses resolved ca. 250 protein spots (Fig. 4). The
differentially expressed protein spots were then excised and
analyzed by MALDI-TOF/TOF (MS/MS). The MASCOT

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**Table 2** General genome feature of *S. paucimobilis* strain 20006FA

| Genome feature                          | Value       |
|-----------------------------------------|-------------|
| Size (bp)                               | 5,409,713   |
| GC content (%)                          | 64.3        |
| Gene number                             | 5077        |
| Protein-coding sequences (CDSs)         | 4882        |
| RNA gene number                         | 56          |
| rRNA gene number                        | 4           |
| tRNAs gene number                       | 49          |
| ncRNAs gene number                      | 3           |
| Pseudogene number                       | 115         |

*CDS* coding sequence, *INSDC* International Nucleotide Sequence Database Collaboration, *ncRNAs* noncoding RNAs
program and the database of the translated proteins of the strain-20006FA genome were used for protein identification. The protein profiles revealed an overexpression of multiple structural genes belonging to the phenanthrene catabolic pathway in the phenanthrene-grown cultures compared to the corresponding profile of the control cultures (the glucose-grown cells), with the more pronounced differences being demarcated by the black rectangle in Fig. 4. Table 4 details the identification of those proteins.

A comparison of the gels yielded 17 potentially relevant protein spots—proteins differentially expressed under the influence of the presence of PAH as the sole carbon source—from which spots 15 different proteins could be identified by mass spectrometry (marked with gray circles in Fig. 4). Those identifications revealed that all the indicated proteins had a similarity to those of other strains of *Sphingomonas*. The latter proteins included one NahA1f (naphthalene dioxygenase alpha subunit; Spot 29), one catechol 2,3-dioxygenase (Spot 8), one 2,3-dihydroxybiphenyl 1,2-dioxygenase (Spot B), two glutathione S-transferases (spots U and 24), and enzymes for the lower metabolic pathway—e.g., 2-hydroxymuconic semialdehyde hydrolase (spots 12, 17 and 26), 4-oxalocrotonate decarboxylase (Spot 11), along with others (Table 4).

**Table 3** Predicted enzymes belonging to PAH degradation pathways in *S. paucimobilis* strain 20006FA strain, obtained with the RAST server and KEGG database and curated by the National Center for Biotechnology Information (NCBI) database

| Steps of degradation pathway | Enzymes predicted from the Upper and Lower pathway of PAH degradation in the strain *Sphingomonas paucimobilis*20006FA | ORFs* | Identified Proteins (2D-PAGE**) |
|------------------------------|-------------------------------------------------------------------------------------------------|------|-------------------------------|
| 1, 10                        | aromatic-ring-hydroxylatingdioxygenase alpha and beta subunit (EC 1.14.12.--)                 | 4    | J L                           |
| 1, 3, 7                      | 2,3-dihydroxybiphenyl 1,2-dioxygenase (EC 1.13.11.39)                                        | 1    | B 29                         |
| 1, 3, 10                     | biphenyl 2,3-dioxygenase (EC 1.14.12.18)                                                      | 1    | B 29 J                       |
| 2, 11, 18                    | 1,2-dihydroxycyclohexa-3,5-diene-1-carboxylate dehydrogenase (EC 1.3.1.25)                  | 1    | 31                           |
| 2, 11, 18                    | 1,6-dihydroxycyclohexa-2,4-diene-1-carboxylate dehydrogenase (EC 1.3.1.25)                  | 1    | 31                           |
| 4, 13                        | maleylacetatoacetateseromerase (EC 5.2.1.2)                                                 | 1    |                               |
| 5, 12, 17                    | 4-carboxymuconolactone decarboxylase (EC 4.1.1.44)                                          | 1    | 11                           |
| 5                            | carboxymuconolactonedecarboxylase                                                             | 4    | 11                           |
| 5, 8, 17                     | 4-hydroxy-2-oxovalerate aldolase (EC 4.1.3.39)                                              | 3    | 6, 7                         |
| 6, 18                        | acetalddehydedehydrogenase (EC 1.2.1.10)                                                    | 4    | 31 27 C                     |
| 8                            | carboxymuconolactonedecarboxylase                                                             | 5    | 11                           |
| 8                            | 4-oxalocrotonate decarboxylase                                                               | 4    | 11                           |
| 9                            | aldehydedehydrogenase (EC 1.2.1.3)                                                          | 12   | C 27                         |
| 9                            | 2-hydroxymuconic semialdehydedehydrogenase (EC 1.2.1.60)                                     | 2    | C 27                         |
| 10                           | benzoate 1,2-dioxygenase alpha and beta subunit (EC 1.14.12.10)                              | 1    | L                            |
| 10                           | anthranilate 1,2-dioxygenase small subunit (EC 1.14.12.1)                                    | 2    | L                            |
| 13, 15, 20                   | homogenitiate 1,2-dioxygenase (EC 1.13.11.5)                                                | 1    | 8 B 29                      |
| 14, 19                       | ciclohexanonemonooxygenase (EC 1.14.13.22)                                                   | 1    |                               |
| 16                           | muconolactone delta-isomerase (EC 5.3.3.4)                                                   | 1    |                               |
| 16, 13                       | muconatecycloisomerase (EC 5.5.1.1)                                                          | 1    |                               |
| 17                           | 2-keto-4-pentenoate hydratase (EC 4.2.1.80)                                                  | 2    | 10                           |
| 18                           | 5-carboxymethyl-2-hydroxymuconate semialdehydedehydrogenase (EC 1.2.1.60)                   | 2    | 27                           |
| 21                           | salicylatehydratase (EC 1.14.13.1)                                                          | 1    |                               |
| 22, 23                       | catechol 2,3-dioxygenase (EC 1.13.11.2)                                                       | 2    | 8                            |
| 22                           | catechol 1,2-dioxygenase (EC 1.13.11.1)                                                       | 2    | 8                            |
| ***                          | (2Fe-2S)-bindingprotein                                                                     | 15   | M                            |

*ORF: of open-reading frames for that protein in the genome. **2D-PAGE, two-dimensional polyacrylamide-gel electrophoresis.

***: Component of the dioxygenase complexes that act in different steps of the pathway.

The ORFs that were confirmed by the proteomic analysis are shadowed in gray.

Bacterial growth on phenanthrene thus produced an upregulation of proteins involved in that compound’s degradation, including enzymes from both the upper (spots 6, 7, 11, 27, 29, 31, B) and the lower (spots 8, 10, 12, 17, 26) catabolic pathways along with other enzymes involved in cellular detoxification (spots U and 24).
Fig. 3 The reconstructed phenanthrene-degradation pathway based on the genome and proteome analyses on *S. paucimobilis* 20006FA. The Ss over the conversion arrows indicate step numbers.
The spots marked were analyzed by MALDI-TOF/TOF. The black circles indicate the proteins identified. The gray-shadowed rows indicate proteins that belong to the phenanthrene degradation pathway (24 and 96 h). The unshadowed proteins belong to central metabolism.

Table 4: Summary of Mascot search results

| Spot label | Protein description | Theoretical migration | Score | Coverage | CDS |
|------------|---------------------|-----------------------|-------|----------|-----|
| 1          | chaperonin GroEL    | g|94497512| 52427| 5.15| 138| 36| 2981349| 2983001|
| 2          | ribosomal protein S1| g|94497990| 61616| 5.01| 89| 32| 4728086| 4729816|
| 3          | Chaperone DnaK       | g|94496879| 67507| 4.77| 173| 7| 2535305| 2537254|
| 4          | DNA-directed RNA polymerase, alpha subunit | g|94496225| 37939| 4.94| 115| 28| 4586713| 4587807|
| 5          | 3-hydroxybutyrate dehydrogenase | g|148556334| 27479| 6.51| 150| 26| 1586603| 1587706|
| 6          | dihydrodiolapamide succinyl transferase | g|94496913| 43776| 5.33| 68| 5| 4137048| 4138325|
| 7          | Electron-transfer flavoprotein alpha subunit, (ETF5L) | g|94498756| 31569| 4.98| 237| 10| 4215886| 4216854|
| 8          | succinyl-CoA synthetase subunit alpha | g|490321670| 29976| 5.51| 62| 5| 4141180| 4142103|
| A          | 2,3-dihydroxybiphenyl 1,2-dioxynagen [Sphingobium changbukense] | g|4007893| 33421| 5.54| 120| 10| 3496244| 3497146|
| B          | ATP synthase subunit alpha [Sphingomonadaceae] | g|294012668| 54727| 5.60| 188| 40| 4995782| 4997329|
| C          | ring-hydroxylating dioxynagen large subunit [uncultured bacterium] | g|406718203| 35464| 5.64| 164| 12| 3546301| 3547812|
| D          | 2,3-dioxygenase subunit [Sphingomonas sp. LH128] | g|158346884| 47324| 5.57| 173| 19| 3491834| 3491885|
| E          | MULTISPECIES: (2Fe-25)-binding protein [Sphingomonadaceae] | g|496103618| 47477| 5.31| 92| 35| 3498818| 3499292|
| F          | Ubiquinone-biosynthesis protein Ubid [Novosphingobium] | g|500246287| 52942| 5.17| 103| 10| 3510553| 3512076|
| G          | 4-hydroxy-2-oxovalerate aldolase [Sphingomonas changbukensis] | g|4007416| 37010| 5.10| 181| 42| 3483998| 3485104|
| H          | Putative 2-hydroxy-benzylpyruvate aldolase [Sphingomonas sp. P2] | g|28971850| 35759| 5.12| 187| 15| 3466433| 3467443|
| I          | catechol 2,3-dioxygenase [Sphingomonas agrestis] | g|151128| 34543| 5.11| 337| 82| 3488745| 3489866|
| J          | 2-hydroxyxenyl-2,4-dienoate hydratase [Sphingomonas changbukensis] | g|2316027| 28142| 5.09| 354| 32| 3485987| 3486793|
| K          | 4-oxaloacetoate decarboxylase [Sphingomonas changbukensis] | g|4091975| 27280| 5.02| 147| 57| 3483226| 3484017|
| L          | 2-hydroxymuconic semialdehyde dehydrogenase [Sphingobium changbukense] | g|1923245| 31340| 5.40| 178| 63| 3489687| 3490640|
| M          | XylF (2-hydroxymuconic semialdehyde dehydrogenase) [Sphingomonas sp. LH128] | g|158346887| 30953| 5.50| 311| 26| 3489687| 3490640|
| N          | translation elongation factor [Sphingomonas sp. SKA58] | g|94498474| 42980| 5.21| 85| 31| 2755441| 2756637|
| O          | glutathione S-transferase [Sphingobium changbukense] | g|158346886| 21470| 5.34| 156| 51| 3490706| 3491353|
| P          | 2-hydroxymuconic semialdehyde dehydrogenase [Sphingobium changbukense] | g|1923245| 27695| 5.28| 178| 87| 3480363| 3481265|
| Q          | acetaldehyde dehydrogenase (acylating) [Sphingobium changbukense] | g|4007415| 33138| 5.64| 241| 80| 3485026| 3486093|
| R          | 2-hydroxydiolapamide dehydrogenase [Sphingobium japonicum U7265] | g|294012415| 48666| 5.68| 148| 32| 3415260| 3416663|
| S          | 3-hydroxybutyrate dehydrogenase [Sphingobium changbukense] | g|158346890| 52139| 5.47| 162| 39| 3546301| 3547509|
| T          | benxaldehyde dehydrogenase [Sphingobium changbukense] | g|6136053| 54650| 5.98| 270| 51| 3481195| 3482736|
| U          | acetaldehyde dehydrogenase [Sphingobium sp. C100] | g|566034322| 34452| 5.28| 86| 34| 3485026| 3485955|
| V          | glutamate-cysteine ligase, partial [Kanthonomas euvesicatoria] | g|733444971| 27612| 5.29| 119| 16| 3485026| 3485955|
| W          | BphS [glutathione S-transferase] [Sphingomonas sp. LH128] | g|2316034| 21518| 5.52| 148| 63| 3490706| 3491353|

*The list indicates the proteins identified (high score and/or high sequence coverage) found in two-dimensional–electrophoresis gels of cultures in GLU, PHN, 24 h and PHN 96. The gray-shadowed: proteins belong to the phenanthrene degradation pathway (24 and 96 h). The unshadowed proteins belong to central metabolism. y indicates proteins present under each condition. NCBI, National Center for Biotechnology Information; CDS, coding sequence.

The gray-shadowed rows indicate proteins that belong to the phenanthrene degradation pathway.
recorded the following differences (Fig. 5). Certain overexpressed proteins were found in the cultures at 24 h relative to the levels present at 96 h; but, of the former, only two could be identified, a glutamate–cysteine ligase (Spot R) and a glutathione S-transferase (GST; Spot U; Fig. 5; Table 4).

Whereas proteins belonging to the phenanthrene degradation pathway were already expressed at 24 h, many others were overexpressed at 96 h (spots circled in white within the black rectangle in Fig. 5). The latter included a 2,3-dihydroxybiphenyl 1,2-dioxygenase (Spot B), the large subunit of a biphenyl dioxygenase (BphA1; Spot L), the large subunit of a ring-hydroxylating dioxygenase (Spot J), and multispecies—i.e., the (2Fe–2S)-binding protein (Spot M; Fig. 5; Table 4). We found enzymes for the first steps of the phenanthrene pathway at both time points. While only one initial dioxygenase (Spot B) was present at 24 h, three overexpressed dioxygenases became detectable at 96 h (spots J, L, and 29) in addition to the (2Fe–2S)-binding protein (Spot M), the alpha subunit of a succinyl-CoA synthetase (Spot A) and a ubiquinone-biosynthesis protein UbiD (Spot P; Tables 3, 4). Other enzymes catalyzing the first steps of the pathway were likewise overexpressed at 96 h (spots 12, 27, and 31). On the contrary, many of the enzymes catalyzing the lower steps of the pathway were expressed at both those time points during phenanthrene degradation (e.g., spots 6, 7, 8, 10, 11 and C; Fig. 5; Table 4).

Such observations probably result from a complex system of regulation controlling the expression of the catabolic genes involved in PAH degradation in strain 20006FA that involves an increasing rate of gene transcription with the time of exposure to the contaminant.

As illustrated in Table 4 and Figs. 4 and 5, by this approach we were able to identify the enzymes required for 19 of the 23 steps of the complete phenanthrene-degradation pathway (Fig. 3). These proteins included 10 enzymes involved in the degradation of phenanthrene to HNA; 10 in the degradation of HNA to salicylate (i.e., the meta pathway); 11 in the degradation of HNA to protocatechuate (i.e., the ortho pathway); and 3 that could be responsible for the transformation of salicylate to acetyl coenzyme A (acetyl-CoA) and succinyl-CoA. These results agree well with the ORFs assigned in the genomics analysis listed in Table 3, thus suggesting that those loci could be undergoing an induced transcription to give rise to proteins.

**Discussion**

Our previous studies on *S. paucimobilis* strain 20006FA, demonstrated that bacterium to be a suitable candidate for bioaugmentation in soil because the strain could efficiently degrade phenanthrene to HNA and salicylic acid as major metabolic products (Coppotelli et al. 2010). Since the accumulation of metabolites occurs during degradation, we attempted to find any regulation that could clarify that point.

In this work, the draft genome sequence enabled the identification of the genes related to phenanthrene metabolism in strain 20006FA, although the information on the genome is far from complete. We undertook an approach involving...
physiological and proteomic studies in order to improve our understanding of the biological process. The proteomics determined substantiated the predictive information obtained by genomics analysis and enabled the elucidation of the metabolic pathway utilized by the bacterium.

Physiological studies showed that strain 20006FA possessed enzymes that degraded PAH such as anthracene, dibenzo thiophene, and fluoranthene. When the strain was grown in liquid MM with phenanthrene as the sole carbon source, the bacterium could efficiently degrade 52.9% of the phenanthrene present at an initial concentration of 2 g L⁻¹ in 20 days (Coppotelli et al. 2010), 59.6% of phenanthrene at 0.84 g L⁻¹ in 15 days, and more than 99% at 0.2 g L⁻¹ in 15 days. These results agree with Waigi et al. (2015), who have also observed that the concentration of a contaminant like phenanthrene is known to play a critical role in bacterial growth and as consequence, could produce lower rates of degradation at higher concentrations of pollutant.

The rate of degradation observed could be related to the accumulation of the intermediate metabolites. During the degradation occurring at 0.84 g L⁻¹, an accumulation of the intermediate acid HNA was observed (Fig. 1) along with a concomitant decrease in the phenanthrene-degradation rate. We postulated that the presence of HNA could have been inhibiting phenanthrene degradation by a possible negative-feedback mechanism or, alternatively, may have acted as a toxic compound for the strain. It was resolved in cultures with HNA present as the sole carbon source where an increase in the lag phase occurred along with an inhibition of cell growth at increasing concentrations of HNA (Fig. 2). Other authors had also observed that an accumulation of phenanthrene metabolites might lead to a toxicity that affected cell growth (Yuan et al. 2000; Zhao et al. 2008).

The enzyme salicylate hydroxylase catalyzes both the salicylate hydroxylation and the conversion of 1-hydroxy-2-naphthoate to 1,2-dihydroxynaphthalene (Cho et al. 2005). The latter reaction is a key step in the biodegradation of phenanthrene by Gram-negative bacteria. Jouanneau et al. (2007) purified the enzyme and found that 1-hydroxy-2-naphthoate was not a substrate, but rather induced NADH oxidation at a high rate in an uncoupled reaction that, in turn, might inhibit PAH degradation. They observed an inhibition of salicylate hydroxylation by the presence of 1-hydroxy-2-naphthoate and postulated that inhibition could be deleterious to bacterial cells growing on PAH. In our studies, on cultures with phenanthrene where HNA was added externally, no significant additional accumulation of HNA was observed at either concentration investigated (Table 1). This result indicated that the presence of exogenous HNA did not inhibit the degradative ability of the cells. Transport mechanisms have to be taken into consideration when designing superior biocatalysts for bioremediation purposes (Pieper and Reineke 2000). Since xenobiotic compounds are usually transported by specialized systems (Pao et al. 1998), a saturation of such a transporter could be the cause of an inhibition of the uptake of HNA into the cytoplasm.

The draft genome analysis of S. paucimobilis 20006FA predicted the existence of a complete catabolic pathway for phenanthrene (Table 3; Fig. 3). S. paucimobilis 20006FA possesses multiple ORFs for enzymes implicated in PAH degradation that are highly dispersed throughout the genome, as had been observed for other sphingomonads (Zhao et al. 2017; Demaneche et al. 2004). The arrangement of degradative genes in sphingomonads is complex with genes scattered across several gene clusters in contrast to the coordinately regulated organized operonic structure of genes in Burkholderia, Pseudomonas and Rhodococcus (Khara et al. 2014).

An analysis of the genome sequence indicated that at least one ORF could be assigned to each of the enzymatic steps required for the complete phenanthrene-degradation pathway (Table 3; Fig. 3). The genes could also be involved in the degradation of other PAH, as revealed by physiological studies and simple sequence comparisons. The phenanthrene degradation assay (Fig. 1a) and the analysis of the assembled draft genome the strain revealed that this strain could be considered a single generalist that can fully metabolize the carbon source (Festa et al. 2017).

The catabolic versatility of the enzymes used in PAH degradation by sphingomonad strains has been well established: these bacteria exhibit a flexible organization of genes (different combinations with some conserved gene clusters), which plasticity aids in prompt and efficient adjustments to the presence of novel compounds in contaminated terrestrial sites (Basta et al. 2005).

In S. paucimobilis 20006FA as in other sphingomonads (Zhao et al. 2015, 2017) the gene clusters are flanked by plasmid-derived insertion sequences that promote the transfer of genes and provide peripheral metabolic functions. The transfer of large plasmids responsible for the degradation of PAHs has been observed in Sphingomonas and Sphingobium strains (Basta et al. 2004). Other studies on PAH-degrading strains indicated that certain catabolic genes were located on plasmids (Basta et al. 2005). The sequence similarities observed between the strain 20006FA phn genes (phenanthrene-catabolic genes) and the corresponding bph genes (biphenyl-catabolic genes) found on plasmid pNL1, seem to imply that the gene arrangement was the same. Moreover, several PAH-induced proteins found in strain-20006FA cell extracts displayed strong sequence similarity to the gene products found in the pNL1 plasmid—including xylQ (acetaldehyde dehydrogenase), nahE (a putative 2-hydroxy benzylpyruvate aldolase), and bphK (glutathione S-transferase). The gene clusters bph and xyl, also present in the plasmid pNL1 had been found in the genomes of other PAH-degrading strains belonging to the genus Sphingomonas and
Sphingobium such as Sphingobium sp. C100 (Dong et al. 2014) and Sphingobium yanoikuyae B1 (Zhao et al. 2015, 2017).

The predicted enzymes—such as the ring-hydroxylating dioxygenase alpha and beta subunits, 2,3-dihydroxy-biphenyl-1,2-dioxygenase, and anthranilate 1,2-dioxygenase (Table 3)—could attack phthalate; while enzymes like benzoate 1,2-dioxygenase alpha and beta subunit and biphenyl 2,3-dioxygenase (Table 3) could act on protocatechuate and even be involved in the ortho pathway for phenanthrene degradation. This supposition was confirmed with the physiological observations of strain 20006FA growing on o-phthalate as the sole carbon source, as mentioned above.

Most of the genes in the PAH-degrading sphingomonads have been annotated through different approaches—such as through cloned DNA fragments and target-protein expression or by functional evidence (Demanche et al. 2004)—but a proteomic elucidation of the pathway has never been realized. Until the present work, only one PAH-degrading S. paucimobilis (EPA 505; Desai et al. 2008; Story et al. 2004) had been fully sequenced (WGS: JFY00000000), but no proteomic studies had been undertaken on that strain.

The proteomic data obtained in this work enabled the identification of enzymes catalyzing 19 steps required for the degradation of phenanthrene to intermediates entering the TCA cycle in strain 20006FA. The results of phenanthrene-degradation experiments and the determination of protein expression (Figs. 1, 3; Table 4) indicated that specific catabolic enzymes were differentially regulated during growth on phenanthrene in comparison to those in the bacterium grown on glucose. Those enzymes allowed the strain to follow the meta-as well as the ortho-cleavage pathway for phenanthrene degradation, as had been reported for other sphingomonads (Cerniglia and Yang 1984; Gibson 1984).

The pattern of protein induction varied between 24 and 96 h of exposure to PAH. A higher number of enzymes for the upper metabolic pathway were overexpressed at 96 h (Fig. 5; Table 4). Although most of the phenanthrene-pathway enzymes were overexpressed at 96 h, the HNA nevertheless accumulated (Fig. 1). Enzymes that could act in the production of HNA (Step 6 of the pathway)—such as the ones corresponding to acetaldehyde dehydrogenase (Spot C), expressed at either 24 or 96 h, or the acetaldehyde and benzaldehyde dehydrogenases (spots 27 and 31), overexpressed at 96 h (spots B, J, L, M, 8, 29) compared to those at 24 h (spots B, 8) suggests that the regulation of the phenanthrene degradation rate occurs at the transcriptional level. In the degradation of PAH, the genes involved in the same pathway for a given aromatic compound are specifically regulated at the transcriptional level by regulatory proteins that are activated by aromatic compounds (Fernández et al. 1994). Based on the prediction of regulation, it has already been suggested that multiple inducers are required for the expression of aromatic catabolic enzymes in sphingomonads. The regulation of genes for various aromatic degradation in sphingomonads is quite complex (Khara et al. 2014).

The fact that at 96 h most of the phenanthrene-pathway enzymes were overexpressed could also be an indicator of the regulation of enzymatic activity, because a regulation by negative-feedback can also occur since despite the increased enzymatic expression (Fig. 5; Table 4), phenanthrene degradation rate did not increase (Fig. 1).

Despite not finding the salicylate hydroxylase in the proteome results, we included that enzyme in the degradation pathway of Fig. 3 based on the genetic location and functional confirmation of the enzymatic activity, since salicylate was catalysed by this strain (Coppotelli et al. 2010). The enzyme GST was found overexpressed in phenanthrene-containing cultures in comparison to cultures with glucose. The upregulation of GST had also been reported for other PAH-stressed Sphingomonas strains (Cavalca et al. 2007; Xia et al. 2005) and is in agreement with the production of oxidative damage by oxygenases involved in the degradation of xenobiotics (Favaloro et al. 2000).

**Conclusions**

The present work represents the first investigation of genomic, proteomic, and physiological studies of the strain S. paucimobilis 20006FA. A large number of genes coding for dioxygenase enzymes were present in the genome suggesting a considerable aromatic-biodegradation potential. Integration of studies indicated that the degradation occurs via the salicylate and protocatechuate pathways. In the proteomic studies, we identified enzymes of the phenanthrene-degradation pathway of the strain that enabled the construction of a complete pathway of phenanthrene degradation. Many of those proteins were expressed differentially during phenanthrene degradation or when compared with those from cultures with glucose as the sole carbon and energy source, thus suggesting that a regulation occurs at those loci. The first hints on regulation of the expression and activity of PAH degradative enzymes were found. The results will facilitate a more complete understanding of the biodegradation mechanism of the strain, which knowledge is essential for achieving a successful removal of the pollutant.

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