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Microbiome enrichment from contaminated marine sediments unveils novel bacterial strains for petroleum hydrocarbon and heavy metal bioremediation

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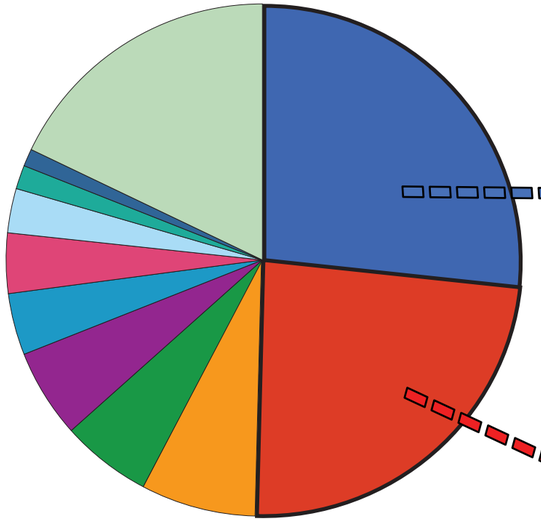
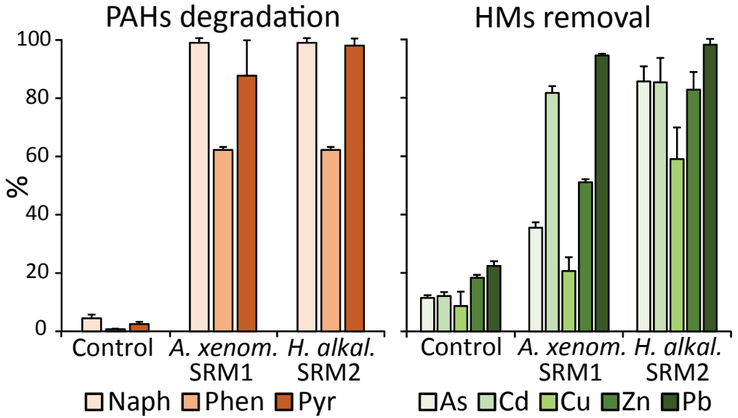
Graphical Abstract

Microbiome enrichment

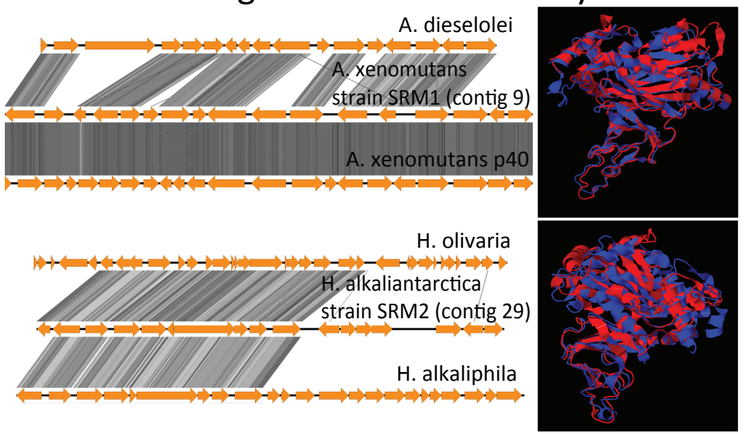


Bacterial isolation

Bioremediation experiments



Genomic basis of bioremediation and screening for novel useful enzymes



Abstract

Petroleum hydrocarbons and heavy metals are some of the most widespread contaminants affecting marine ecosystems, urgently needing effective and sustainable remediation solutions. Microbial-based bioremediation is gaining increasing interest as an effective, economically and environmentally sustainable strategy. Here, we hypothesized that the heavily polluted coastal area facing the Sarno River mouth, which discharges >3 tons of polycyclic aromatic hydrocarbons (PAHs) and ~15 tons of heavy metals (HMs) into the sea annually, hosts unique microbiomes including marine bacteria useful for PAHs and HMs bioremediation. We thus enriched the microbiome of marine sediments, contextually selecting for HM-resistant bacteria. The enriched mixed bacterial culture was subjected to whole-DNA sequencing, metagenome-assembled-genomes (MAGs) annotation, and further sub-culturing to obtain the major bacterial species as pure strains. We obtained two novel isolates corresponding to the two most abundant MAGs (*Alcanivorax xenomutans* strain-SRM1 and *Halomonas alkaliantarctica* strain-SRM2), and tested their ability to degrade PAHs and remove HMs. Both strains exhibited high PAHs degradation (60-100%) and HMs removal (21-100%) yield, and we described in detail >60 genes in their MAGs to unveil the possible genetic basis for such abilities. Most promising yields (~100%) were obtained towards naphthalene, pyrene and lead. We propose these novel bacterial strains and related genetic repertoire to be further exploited for effective bioremediation of marine environments contaminated with both PAHs and HMs.

Keywords: polycyclic aromatic hydrocarbons; heavy metals; bioremediation; next-generation sequencing; marine biotechnology.

1. Introduction

Pollution of coastal environments due to organic (e.g., polycyclic aromatic hydrocarbons, PAHs) and/or inorganic (e.g., heavy metals, HMs) contaminants can determine major detrimental effects on the marine food web and human health (Tashla et al., 2018; Fuentes-Gandara et al., 2018; Loflen et al., 2018; Buah-Kwofie et al., 2018). PAHs, HMs and metalloids are known to strongly affect biological systems such as cell membranes, organelles and enzymes, causing cell cycle alteration, carcinogenesis or apoptosis (Tchounwou et al., 2001; Sutton et al., 2002; Yedjou and Tchounwou, 2007a,b; Beyersmann and Hartwig, 2008; Patlolla et al., 2009; Kim et al., 2015; Costa et al., 2022). Such contaminants can persist in the environment for a long time and can be subjected to bioaccumulation and/or biomagnification processes, which increase their potential to cause harm (Oyetibo et al., 2017). Conventional methods for the removal of PAHs and HMs include chemical treatments (e.g., precipitation, oxidation and reduction), electrochemical techniques or physical adsorption (Fenyvesi et al., 2019). Unfortunately, such methods typically produce special wastes (e.g., toxic sludge and by-products) and are expensive, ineffective at low pollutant concentrations and highly energy-demanding (Joshi, 2017; Priyadarshanee and Das, 2021). The use of microorganisms (especially, bacteria, fungi and microalgae) may be a promising alternative or complementary strategy to such conventional tools, due to several microbial characteristics including high biodegradation/detoxification efficiency towards several contaminants, high surface area-to-volume ratio and the ability to grow at high concentrations of toxic pollutants (Zouboulis et al., 2004; Kordialik-Bogacka and Diowksz, 2014). Microbial-based remediation strategies are also considered to be one of the most sustainable approaches due to low carbon footprint of the overall decontamination process (Kuppusamy et al., 2017; Dell'Anno et al., 2020; Jain et al., 2022).

Microbes effectively degrade PAHs by several mechanisms including assimilation, intracellular detoxification, and/or co-metabolism mediated by the activity of specific enzymes such as oxygenase, dehydrogenase and ligninolytic enzymes (Johnsen et al., 2005; Ladino-Orjuela et al., 2016; Saravanan et al., 2021). Microbial-mediated HMs' sorption, leaching or transformation are well-known as

inexpensive and highly efficient HM bioremediation processes (Leung et al., 2000; Aryal et al., 2010; Sahmoune, 2018; Quiton et al., 2018; Cai et al., 2019). Co-contamination by PAHs and HMs is typically encountered in most marine polluted sites (El-Mufleh et al., 2014; Ali et al., 2021) and several microbes can exert a combined action towards such contaminants by bioleaching, biosorption and biodegradation, which increase their overall removal and/or detoxification capacity (Liu et al., 2017; Ali et al., 2021). Examples of this synergistic approach include microbial secretion - under mixed PAHs and HMs contamination - of enzymes with useful bioremediation activities (Chen et al., 2020) and of extracellular polysaccharides (EPSs). EPSs are particularly relevant targets for bioremediation studies, as they consist of a wide array of polymers and functional groups, that can simultaneously bind metals (thus enhancing HM extraction/removal from contaminated matrices) (Amoozegar et al., 2012; Ates, 2015; Little et al., 2014; Mohite et al., 2017; Gupta and Diwan, 2017; Cao et al., 2022; Cheng et al., 2022), and increase PAH solubilization and enzymatic degradation efficiency (Gutierrez et al., 2013; Alaba et al., 2018).

Environmental matrices that are naturally or experimentally enriched with organic and/or inorganic contaminants are an underexploited source of novel microbes resistant to contaminants and involved in their biodegradation/detoxification (Beolchini et al., 2009; Adams et al., 2015; Fodelianakis et al., 2015; Dell'Anno et al., 2021; Wang et al., 2021). Currently, high-throughput sequencing technologies help to uncover such microbial bioremediation potential, providing novel insights on the diversity of useful environmental microbes and their repertoire of genes involved in PAH and HM bioremediation (Czaplicki and Gunsch, 2016; Dell'Anno et al., 2021; Meng et al., 2022; Hassan et al., 2022; Sharma et al., 2022).

In this study, we selected the highly anthropically-impacted and severely contaminated coastal area at the mouth of the Sarno River (Gulf of Naples, Mediterranean Sea), one the most polluted rivers in Europe discharging large amounts of PAHs and HMs into the sea (Montuori and Triassi, 2012; Montuori et al., 2013). Based on such high inputs of contaminants at this site, we postulated that here, marine sediments could host microbiomes enriched in bacterial taxa that typically characterize marine

areas affected by oil-spills and/or industrial activities, and possibly useful for PAHs and HMs bioremediation. We collected marine sediments from one of the most polluted sites in this area, and we adopted a laboratory approach based on the enrichment of the sediment microbiome, subsequent selection and culturing of bacteria resistant to HMs, and next-generation sequencing coupled with laboratory experiments to unveil their potential for PAHs and HMs bioremediation.

2. Materials and Methods

2.1. Sediment sampling, microbiome enrichment and selection of bacteria useful for PAH and HM bioremediation

Sampling was performed at the mouth of the Sarno River (Fig. 1). Surface sediments were collected by a Van Veen grab, placed into sterile Whirl-Pak bags (Nasco), and then stored at 4°C in the dark until processing. One gram of sediment was added to a 1000 ml flask containing 200 ml of Marine Broth (Difco, Marine Broth 2216). Inoculated flasks were mixed and incubated at 28°C in the dark. After 2 weeks of incubation, the enriched microbiome was plated by streaking onto marine agar (Difco, Marine Agar) added with a mix of Pb^{2+} (500 ppm), As^{3+} (500 ppm), and Cd^{2+} (10 ppm) and incubated at 28°C for 48 hours. Above HMs were selected as the most relevant in the study area, and the applied concentrations were significantly higher than those determined in the sediments (Montuori et al., 2013), to isolate HM-resistant bacteria with possible PAH and HM bioremediation ability. PAHs were not added at this stage, as we hypothesized that bacterial PAH degraders were already abundant in the original contaminated sediments, and our rationale was to select those able to also tolerate high HM concentrations. As the diversity of potential PAH-degrading bacteria is potentially high in contaminated sediments, we acknowledge that alternative enrichment strategies (e.g., contextual addition of PAHs at this stage) may have led to different/additional bacterial isolates than those obtained in our study.

Following incubation, high microbial growth was observed, which was confirmed to be a multi-species bacterial culture by preliminary Sanger sequencing of the 16S rRNA genes, hence analyzed by whole DNA shotgun sequencing, as described below.

2.2. Whole DNA shotgun sequencing and analysis of bacterial metagenome-assembled genomes (MAGs)

The total genomic DNA (gDNA) obtained from the enriched mixed culture was extracted with the DNeasy Blood & Tissue kit, according to the manufacturer's instructions. The DNA concentration was determined using the Qubit™ dsDNA HS assay kit with a Qubit fluorometer (Thermo Fisher, Waltan, US). Sequence library preparation of gDNA was performed using the Nextera DNA Flex kit (Illumina, Hayward, USA) with 1 ng input DNA according to the manufacturer's instructions. The resultant libraries were sequenced on an Illumina MiSeq instrument using a MiSeq Reagent kit V2 (500 cycles) with a 10% phiX v3 spike, generating 2×250 bp reads. Preliminary metagenome processing and taxonomic and functional annotation were performed in MG-RAST under default settings (Meyer et al., 2008). Read assembly was performed using CLC Genomics Workbench version 11. Briefly, the raw reads were trimmed and demultiplexed, and contigs ≤500 bp were removed from the final assembly. Binning of metagenomic contigs was performed using MyCC (Lin and Liao, 2016) while completeness and contamination of MAGs and genome quality were determined using CheckM with the lineage-specific workflow and default parameters (Parks et al., 2015).

2.3. Isolation and identification of single bacterial strains

To obtain single colonies of pure bacterial strains, the enriched mixed culture was re-plated on marine agar (Difco, Marine Agar) added with HMs as described above, through serial dilution. After several re-streaking cycles of 48-hours incubation at 28°C, two main colony morphologies were observed, and each re-streaked until confirmed to be pure by PCR analysis and Sanger sequencing targeting

16S rRNA gene (by universal bacterial primers E9F-5′-GAGTTTGATCCTGGCTCAG-3′ and U1510R-5′-GGTTACCTTGTTACGACTT-3′; Rodriguez-Caballero et al., 2012). All polymerase chain reactions (PCR) were carried out in a Perkin Elmer Thermocycler (Gene Amp PCR system 6700) in a 50 µl reaction volume containing 1× PCR buffer, 200 µM of each dNTP, 0.5 µM of each primer, 0.2 U of Taq Gold polymerase (Applied Biosystems, Waltham, MA, US) and 1 ng of template DNA. Thermal cycling conditions were 5 min denaturation at 94 °C; 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72°C for 90 s; final elongation step at 72°C for 5 min. The PCR products were analyzed by agarose gel (1.2% w/v) electrophoresis in TAE buffer solution (40 mM Tris-acetate, 1 mM ethylenediaminetetraacetic acid, EDTA) containing 0.5 µg ml^{-1} (w/v) ethidium bromide. The amplicons were purified and sequenced using an ABI PRISM 377 automated sequencer (Applied Biosystems). The sequencing data were processed using Chromas Pro v. 1.5a software (Technelysium, South Brisbane, QLD, Australia) for alignment and manual editing of sequences. The consensus sequences of the isolates were compared with those deposited in GenBank using BLAST. The two bacterial strains were matched with the two corresponding MAGs obtained by whole-DNA shotgun sequencing by comparing their 16S rRNA gene sequences following nucleotide alignment performed through a local blast in the annotation system. The pure bacterial strains were then tested in experiments to assess their bioremediation ability to degrade PAHs and remove HMs, as described below.

2.4. Laboratory tests to assess the PAH degradation and HM removal ability of the bacterial isolates

Each bacterial isolate was incubated in flasks (T175, TPP tissue culture flasks, final volume 250 ml Marine Broth; starting inoculum of 8 x 10^7 cells ml^{-1}), and subjected to two treatments. These included: i) addition of naphthalene, pyrene and phenanthrene (ratio of 1:1:1 with a total concentration of 242 ppm); ii) addition of arsenic (As^{3+}; 14 ppm), lead (Pb^{2+}; 331 ppm), cadmium (Cd^{2+}; 1 ppm), copper (Cu^{2+}; 74 ppm), and zinc (Zn^{2+}; 899 ppm). Controls were included for each of

the two treatments, following the same procedure but without bacterial inoculum. All experimental microcosms were set up in triplicate. The concentration of the toxic compounds used for bioremediation experiments was selected based on the average values found in the surrounding marine area (Montuori and Triassi, 2012; Montuori et al., 2013). The flasks were incubated for 27 days at 28°C, and bacterial growth was monitored by OD_{600} at days 0, 9 and 27. The quantification of PAHs and HMs was conducted, respectively, by gas chromatography-mass spectrometry (GC-MS; EPA8270) (Casillo et al., 2018), and by inductively coupled plasma atomic emission spectroscopy (ICP-OES; EPA6010) (EPA, 2014) on aliquots from each experimental treatment. The PAHs degradation yield was calculated for each experimental treatment by comparing the concentrations of each contaminant at the beginning and at the end of the incubations. For HMs, aliquots of each experimental treatment at the end of incubations were first centrifuged (1000 x g, 5 min), and the HM concentration in the pellet was used to calculate the % of HMs removal, as the % of HM mass in the pellet compared to the HM mass added at the beginning of incubations.

2.5. MAGs functional annotation and comparative genomics

The genome taxonomy database (GTDB) (https://gtdb.ecogenomic.org/) implemented through K-Base (www.kbase.us) was used to perform the whole-genome based classification of the two MAGs obtained following whole-DNA shotgun sequencing and corresponding to the two bacterial strains used in the PAHs degradation and HM removal experiments in this study. Species relatedness was evaluated through the average nucleotide identity (ANI) analysis (Rodriguez and Konstantinidis, 2016; Han et al., 2016) by comparing the de novo genomes with the genomes selected following the GTDB output. The obtained genomes were annotated by RAST (Overbeek et al., 2014) providing an automated functional annotation or hypothetical protein annotation for each open reading frame (ORF) identified on the genome. KEGG was used for metabolic prediction (Kanehisa et al., 2017). Following automated identification of genes involved in hydrocarbon degradation or metal resistance/detoxification/removal, manual verification of the annotated ORFs was conducted against

the SwissProt database. In addition, the sequences flanking the genes of interest were manually annotated to better understand their genomic context, accurately delineate the regions involved in these functions and for synteny comparison, and visualized using Easyfig (Sullivan et al., 2011). The superimposition analysis of dioxygenases belonging to the two MAGs have been performed using the web portal for protein modelling, prediction and analysis Phyre2 (Kelley et al., 2015), and enzyme similarity was assessed according to Zhang and Skolnick, 2004.

2.6. Statistical analyses

To test for differences in the experimental results, Student T and Fisher-Snedecor tests were carried out using PAST3 software (Hammer et al., 2001).

3. Results and Discussion

3.1 Characterization of the enriched microbiome and of the bacterial isolates tested for PAHs and HMs bioremediation

The shotgun sequencing of the total DNA, extracted from the selectively enriched mixed bacterial culture obtained in this study from the contaminated marine sediments of the Sarno River mouth, resulted in >3.1 million high-quality reads (average sequence length 201±59 bp; Supplementary Table S1). Taxonomic annotation of this enriched metagenome showed a dominance of two Oceanospirillales (Gammaproteobacteria) genera: *Alcanivorax* (~24% of total reads) and *Halomonas* (~27% of total reads) (Fig. 2A). Several other bacterial taxa were detected in the metagenome, though at a much lower relative abundance. These included other Gammaproteobacteria (*Chromohalobacter*, *Marinobacter*, *Pseudomonas*, *Pseudoalteromonas* and *Marinomonas*), Clostridia (*Alkaliphilus*), Alphaproteobacteria (*Ruegeria*), and Betaproteobacteria (*Burkholderia*) (Fig. 2A). All of these bacterial taxa have previously been reported to typically increase rapidly in abundance during oil spills, to degrade hydrocarbons and to tolerate/detoxify heavy metals (Gutierrez et al., 2013; Dubinsky et al., 2013; Kumar et al., 2019; Dell'Anno et al., 2021; Huo et al., 2014; Liu et al., 2019;

Ramasamy et al., 2020; Ghosh et al., 2022). This suggests that our microbiome enrichment strategy was successful in selectively boosting autochthonous bacterial taxa with promising potential for petroleum hydrocarbon and HM remediation. This was also supported by the preliminary functional annotation of the metagenome (Fig. 2B), which highlighted that the functions putatively related to hydrocarbon degradation and to resistance/interaction with heavy metals represented an important portion of the overall reads count (>6%). The major role of such functional features was further suggested by the fact that their representation was quantitatively similar compared to fundamental cell processes such as cell respiration, DNA metabolism or membrane transport (Fig. 2B). It should be noted that our enrichment approach, resulting in the virtual absence of Archaea in the enriched metagenome, might have overlooked possible syntrophic relationships among bacteria and archaea in the original sediments, whose relevance in petroleum hydrocarbons degradation has been highlighted by recent independent studies (Liu et al., 2018, 2021; Harindintwali et al., 2022).

The reads assembly and MAGs reconstruction and annotation resulted in two dominant MAGs with genome completeness between 99-100%, which were classified as *Alcanivorax xenomutans* and *Halomonas alkaliantarctica* based on GTDB-Tk whole-genome based classification, and supported by 16S rRNA gene sequence identities (Supplementary Tables S2-S4). The ANI analysis indicated *Alcanivorax xenomutans strain* KS-293 (Barbato et al., 2015; ANI score 99.10%) and *Halomonas alkaliantarctica* strain CRSS (Poli et al., 2007; ANI score 97.13%) as their respective closest relatives. Two additional partial MAGs (4-32% completeness) were recovered from the metagenome and classified in the genera *Pseudoalteromonas* and *Alkalphilus*, but these were not analysed further. The completeness and coverage of the four reconstructed MAGs reflected the relative contribution of their reads to the overall sequence count (Fig. 2A), further suggesting that the obtained enriched mixed bacterial culture was dominated by *A. xenomutans* and *H. alkaliantarctica,* with minor contribution by other bacterial taxa.

The sequential and selective sub-culturing from the enriched mixed bacterial culture allowed us to obtain the two strains that matched the two full-reconstructed MAGs based on 16S rRNA gene

sequence identity (100%). We hence refer to the two novel strains and related genomes obtained in this study as *A. xenomutans* strain SRM1 and *H. alkaliantarctica* strain SRM2 (Suplementary Fig. S1, S2).

The laboratory tests conducted to assess their ability for PAHs and HMs remediation showed high PAHs degradation (ranging for both strains from 60% for phenanthrene to 100% for both naphthalene and pyrene; Fig. 3A), as well as high HMs removal yields (34-91% for As, 79-94% for Cd, 21-70% for Cu, 50-89% for Zn, and 94-100% for Pb), with highest values observed with *H. alkaliantarctica* strain SRM2 for As, Cu, and Zn; Fig. 3B). Notably, we observed that the culturing of *A. xenomutans* strain SRM1 and *H. alkaliantarctica* strain SRM2 with addition of PAHs resulted in growth rates almost double to those of control conditions (Supplementary Fig. S3), which agrees with previous independent evidence that these bacterial genera include taxa able to exploit hydrocarbons to produce cell biomass (Mnif et al., 2009; Rahul et al., 2014). Both strains exhibited resistance to the high concentrations of mixed HMs tested in our study (>1300 ppm, considering the sum of As, Cd, Cu, Zn, and Pb) (Fig. 3B, Supplementary Fig. S3), indicating that these strains can be particularly useful for PAH biodegradation of marine matrices that are simultaneously highly polluted with different HMs. Indeed, the use of bacterial strains able to both degrade organic contaminants and tolerate toxic inorganic compounds present in the target matrix can help to reduce failure risk in bioremediation of environments that display high loads of mixed toxic contaminants (Thompson et al., 2005; Nwuche and Ugoji, 2008; Alisi et al., 2009; Tyagi et al., 2011; Dueholm et al., 2015).

Overall, these results confirm several previous reports showing that *Halomonas* spp. and *Alcanivorax* spp. include members that display high resistance towards toxic organic and inorganic contaminants (Rahul et al., 2014; Fu et al., 2018, Catania et al., 2018; Dell'Anno et al., 2020) and can degrade PAHs (Budiyanto et al., 2018; Kadri et al., 2018).

The observed higher degradation rates of naphthalene and pyrene than phenanthrene (Fig. 3A) may be counterintuitive, as it is generally assumed that bacterial biodegradation of low-molecular-weight PAHs (such as naphthalene and phenanthrene, with ≤ 3 aromatic rings) occurs faster than for high-

molecular-weight PAHs (with ≥ 4 aromatic rings, like pyrene). Despite previous investigations support the expected pattern of a higher PAH recalcitrance with increasing molecular weight (Yu et al., 2005; Haritash and Kaushik, 2009; Thavamani et al., 2012; Nzila et al., 2021), other studies have reported the opposite trend, similar to that observed in the present study (Sohn et al., 2004; Wongwongsee et al., 2013; Vaidya et al., 2017, 2018). This can be explained by different enzymatic and metabolic pathways among microbes, by differences in the specific chemical-physical conditions applied in the laboratory (which can differentially influence the degradability of different PAHs; Leahy and Colwell, 1990; Bagby et al., 2017), as well as by possible interactions among different PAHs, such as inhibition or enhancement of the biodegradation of specific high-molecular-weight PAHs in the presence of specific low-molecular-weight PAHs (Guha et al., 1999; Vaidya et al., 2018). As PAHs-contaminated sites typically display mixtures of high- and low-molecular-weight PAHs (Bezza and Chirwa, 2017), we can conclude that our two bacterial strains, able to enhance biodegradation rates of high-molecular-weight PAHs in the presence of low-molecular-weight PAHs, can represent a significant advantage for environmental bioremediation applications.

Regarding the ability of the two tested strains to tolerate and remove HMs from contaminated matrices, our results confirm previous findings obtained using other *Halomonas* and *Alcanivorax* species. *Halomonas elongate*, *Halomonas halophila* and others displayed 50-94% removal yields towards Pb, Cd and/or Cr (Amoozegar et al., 2012; Murugavelh and Mohanty, 2012; Asksonthong et al., 2018; Abdel-Razik et al., 2020), while Pb removal ability has already been documented for some *Alcanivorax* sp. (da Costa Waite et al., 2016; Dell'Anno et al., 2020; Ramasamy et al., 2020). Notwithstanding, the current knowledge on the promising application of *Halomonas* sp. and *Alcanivorax* sp. for the bioremediation of HMs is still in its infancy and should be investigated further, also including tests for larger sets of HMs (Pennafirme et al., 2015; Verma and Kuila, 2019; Cecchi et al., 2021; Dell'Anno et al., 2020).

3.2. Genetic basis for PAHs biodegradation

Understanding the genetic basis and metabolic processes involved in microbial removal of petroleum hydrocarbons is fundamental to optimize bioremediation strategies, enabling tailored amendments to favor specific bacterial metabolism, genetic bioengineering, or discovery of useful enzymes/compounds (Schneiker et al., 2006; Dell'Anno et al., 2021; Sharma et al., 2022). Our results from high throughput sequencing allowed us to identify the genes and metabolic pathways potentially involved in the bioremediation ability of the tested *A. xenomutans* strain SRM1 and *H. alkaliantarctica* strain SRM2. In particular, several genes involved in the metabolism of aromatic compounds were identified through the automatic functional annotation of their MAGs (Supplementary Fig. S4). Even though the two strains possessed a different number of enzymes involved in these metabolic pathways (36 in *A. xenomutans* strain SRM1, 61 in *H. alkaliantarctica* strain SRM2*;* Supplementary Fig. S4), they performed similarly in the laboratory tests for PAHs degradation (Fig. 3A,B). We cannot exclude that additional genes for enzymes known to be involved in petroleum hydrocarbon degradation and apparently missing in the two MAGs were not identified through automatic annotation, due to divergence of DNA sequences from those available in current public databases. To gain a more complete picture of the main pathways for hydrocarbon degradation in the two novel strains, we manually checked the organization of the genomic regions that contain the genes of major interest, including those for hydrocarbon degradation peripheral pathways (*cis*-hydroxylation and *trans*-hydroxylation pathways) and central degradation routes (catechuate, protocatechuate, homoprotocatechuate, homogentisate and phenylacetic pathways), as detailed below.

3.2.1 Peripheral pathways for hydrocarbon degradation

The *cis*-hydroxylation pathway usually starts following the activity of ring hydroxylating dioxygenase enzymes (RHDs) (Peng et al., 2019), which have large (α) and small (β) subunits (Kauppi et al., 1998). The alpha subunit (RHDα) contains two conserved regions (the [Fe2-S2] Rieske center and the mononuclear iron-containing catalytic domain), which promote the incorporation of molecular oxygen into the aromatic ring forming a *cis*-dihydrodiol. We found RHDs in both *A.*

xenomutans strain SRM1 and *H. alkaliantarctica* strain SRM2 MAGs (Fig. 4A-D). The sequence of *A. xenomutans* strain SRM1 showed ~100% identity with that of *A. xenomutans* P40 (Fu et al. 2018) (Fig. 4A). Comparison with *A. dieselolei* B5 (Lai et al., 2012) highlighted a lower correlation but still high overall similarity (homology of ≥ 70%) with 11 genes (including the RHD gene) (Fig. 4A). Other proteins involved in hydrocarbon detoxification and degradation processes were found in *A. xenomutans* strain SRM1 when analyzing the flanking region of RHD, including glutathione s-transferase, linear amide C-N hydrolase, aldo-keto reductase and nitrite reductase (Lloyd-Jones and Lau, 1997; Al-Turki, 2009; Cao et al., 2015; Imperato et al., 2019; Salam and Ishaq, 2019). The comparison of the sequences flanking the RHD in *H. alkaliantarctica* strain SRM2 MAG, with those of close relatives (Fig. 4B) highlighted that in all genomes the genes encoding the RHD and ferredoxin (both directly involved in the degradation of hydrocarbons), as well as serin hydroxymethyl transferase, sarcosine oxidase α,β,γ-subunits and formyltetrahydrofolate deformylase, were all located in the same genomic region. Such genomic organization supports previous evidence that genes associated with glycine and serine metabolism are involved in hydrocarbon degradation (Yan and Wu, 2017). The 3D superimposition analysis revealed a high similarity for both RHDs with naphthalene 1,2 dioxygenases (Fig. 4C,D), which are enzymes involved in the first ring hydroxylation of multiple PAHs, including naphthalene, phenanthrene, anthracene, dibenzothiophene and fluorene (Park and Crowley, 2006). Although it will be necessary to confirm the specific function of the RHDs identified, the results of 3D modeling suggest a similar ability in degrading PAHs (based on TM scores of 0.89 and 0.73 respectively for *A. xenomutans* strain SRM1 and *H. alkaliantarctica* strain SRM2). Surprisingly, such high similarity between the two enzymes' structures corresponded to only 30% identity in their secondary sequences. This further suggests that the lack of matches of our MAGs DNA sequences for some of the other major genes involved in hydrocarbon degradation may actually be due to evolutionary genetic divergence from currently known bacterial genomes deposited in public databases.

An alternative to the *cis*-hydroxylation pathway is represented by the *trans*-hydroxylation pathway, in which the cytochrome P450 system (CYP450) catalyzes a *trans*-dihydrodiols formation by the epoxidation of the aromatic ring by epoxide hydrolase (Moody et al., 2005). For the *A. xenomutans* strain SRM1 MAG, we found highly conserved homologs of the CYP450 within the 3 closest relatives retrieved from NCBI (100% identity with *Alcanivorax xenomutans* sp 40, and >70% identity with *Alcanivorax* sp N3-2A and *Alcanivorax dieselolei* B5) (Fig. 5A). Within the same genome region containing CYP450, we also found two genes involved in the detoxification and biodegradation of xenobiotics: glutathione-disulfide reductase (Moron et al., 1979) and a Rieske domain non-heme oxygenase (Barry and Challis, 2013). For *H. alkaliantarctica* strain SRM2, the BLASTp analysis of the CYP450 sequence found within its MAG showed no homologs within the 3 most similar reference sequences retrieved from NCBI (of *H. axialiensis* Althf1, *H. olivaria* TYRC17 and *H. aestuari* Hb3), despite the flanking region included other conserved genes (e.g., cytochrome C, nitrogen metabolism and membrane transporters genes) (Fig. 5B). As the abovementioned three closest relatives were not isolated from matrices heavily polluted by petroleum hydrocarbons and do not show genetic bases for PAHs degradation (Tsurumaki et al., 2019; Nagata et al., 2019; Kim et al., 2018), we can expect that this CYP450 system acquired by this novel *H. alkaliantarctica* strain SRM2 may have conferred specific PAH-degradation abilities to this strain. As CYP450s are broadly distributed across the tree of life and are considered the most versatile biocatalysts in nature because of the wide variety of substrate structures they can react with (Nelson, 2018; Yeom et al., 2021; Haas et al., 2022), further studies are needed to test the possible degradation/detoxification activity of the enzymes we identified towards other contaminants besides petroleum hydrocarbons.

3.2.2. Central pathways for hydrocarbon degradation

The activity of RHD generates salicylate that enters the catechol pathway, a classic bacterial central hydrocarbon degradation route mainly found in proteobacteria and actinobacteria (Nešvera et al., 2015). In both MAGs, we identified the salicylate hydroxylase, which catalyzes the formation of catechol acid (a substrate of the catechol 1-2 dioxygenase enzyme; Nešvera et al., 2015), as well as

the terminal enzymes (β-ketoadipate succinyl-CoA transferase or β-ketoadipyl thiolase), which convert β-ketoadipic acid in acetyl-CoA and succinyl-CoA (Peng et al., 2008). Additionally, we found a hydroxiquinol 1,2 dioxygenase (BLASTp e-value 0, score 1461, id. 95.5%) suggested to promote the formation of β-ketoadipic acid (Ferraroni et al., 2005). These findings suggest that the catechol pathway could contribute to the observed PAH degradation ability of both strains.

Following CYP450-mediated trans-hydroxylation, the degradation of hydrocarbons proceeds *via* the protocatechuate metabolic pathway, which starts with an hydroxybenzoate hydroxylase that transforms 4-hydoxybenzoate into 3,4 hydroxybenzoate (Fuchs et al., 2011). This enzyme has been identified only in *H. alkaliantarctica* strain SRM2 MAG (Suplementary Fig. S5), which also possessed a protocatechuate 3,4-dioxygenase, 3-carboxy-*cis,cis*-muconolactone cycloisomerase and 4-carboxymuconolactone decarboxylase. Again, we cannot exclude that genes with similar function but low sequence-similarity may be present also in *A. xenomutans* strain SRM1 MAG. Conversely, both MAGs possessed the terminal enzymes of the protocatechuate pathway (including β-ketoadipate enol-lactonase, 3-oxoadipate CoA transferase, β-ketoadypil-CoA thiolase) capable of catalyzing the production of succinyl-CoA. The comparison of the *H. alkaliantarctica* strain SRM2 gene sequences involved in this pathway with their homologs, generally showed high conservation (>70%) (Supplementary Fig. S5). To the best of our knowledge, this is the first evidence for genes associated with the protocatechuate degradation pathway organized as an operon in a *Halomonas* sp. (Corti Monzón et al., 2018). Further analyses are needed to assess if this feature is peculiar to the strains considered here, or common across *Halomonas* spp. and/or other bacterial taxa**.**

Several other genes of the homoprotocatechuate pathway (Méndez et al., 2011) were also identified in both MAGs (Fig. 6A,B). Notably, the genome region containing the genes for the homoprotocatechuate pathway in *A. xenomutans* strain SRM1 was highly conserved with that of close *Alcanivorax* sp. relatives (Fig. 6A), while that of *H. alkaliantarctica* SRM2 showed no apparent homologs in currently known *Halomonas* sp. genomes (Fig. 6B). We thus suggest that deeper investigation of the homoprotocatechuate pathway of *H. alkaliantarctica* strain SRM2 through

mutation, heterologous expression or proteomics may unveil novel mechanisms for PAH biodegradation.

Finally, *H. alkaliantarctica* strain SRM2 also displayed a complete set of genes for the phenylacetic pathway within a specific genomic region, highly conserved with closest *Halomonas* sp. genomes available for comparison (Supplementary Fig. S6), indicating that this strain possesses a particularly wide array of genes for PAHs degradation. Additional enzymes involved in the homogentisate pathway (Arias-Barrau et al., 2004; Guazzaroni et al., 2013), were detected in both MAGs, including maleylacetoacetate isomerase, fumarylacetoacetate hydrolase, and 4-hydroxyphenilpyruvate dioxygenase (data not shown).

3.3. Genetic basis for HMs removal/detoxification

The blastp search against the SwissProt database identified in both MAGs the genetic basis for exopolysaccharide (EPS) biosynthesis, which may not only facilitate PAH degradation by enhancing the efficiency of all abovementioned PAH-degrading enzymes (Gutierrez et al., 2013; Alaba et al., 2018), but also confer metal-binding properties contributing to explain the observed ability of these strains to resist to and to sequester HMs (Amoozegar et al., 2012; Gupta and Diwan, 2017; Cao et al., 2022; Cheng et al., 2022). Specifically, a UDP-glucose 4-epimerase (e-value 0, score 1709, 97% identity), a UDP-glucose pyrophosphorylase (e-value 7.5 e^{-180}, score 1300, 90% identity), and a poly-beta-1,6-N-acetyl-D-glucosamine synthase (e-value 0, score 2011, 81% identity) were identified in *A. xenomutans* strain SRM1, with the latter two also present in *H. alkaliantarctica* strain SRM2 (respectively, e-value $2.6e^{-165,}$ score 1206, identity 77.3%, and e-value 0, score 1977, identity 86.1%). A deeper insight on the production of EPS by the tested strains would likely allow optimizing of their use for simultaneous bioremediation of PAHs and HMs in different environmental conditions.

The annotation of the two MAGs by the automated RAST pipeline, followed by blastp check against the SwissProt database, shed light on further mechanisms possibly involved in the ability of both strains to resist to and to remove/detoxify HMs (Supplementary Fig. S7). In particular, several genes

coded for efflux pumps that selectively and non-selectively regulate the transport of multiple metals, including *czcA*, *czcB*, *czcC*, *czcD* able to transport cobalt, zinc and cadmium, as well as *corC*, specific for the transport of cobalt and magnesium. In addition, several genes were identified encoding for proteins involved in the detoxification of copper, including a copper chaperone, copper homeostasis protein CutE and CutF, copper resistance protein B, C, D, copper ATP ase, Cu-sensing two-component system response regulator and Cu-responsive transcriptional regulator. Notably, the automated RAST pipeline also identified multicopper and blue multicopper oxidases (Supplementary Fig. S7), which may be synergistically involved both in Cu-detoxification/sequestration and act as laccase-like multicopper oxidases for the degradation of PAHs and other organic contaminants (Cooksey, 1994; Arregui et al., 2019; Ramasamy et al., 2020; Zhang et al., 2020).

Several genes coding for proteins known to confer resistance to and/or detoxify arsenic were found in both MAGs (Supplementary Fig. S7), and our manual annotation of flanking gene sequences highlighted operon-like structures (Figure 7A-B) similar to "arsenic islands" previously described (Wu et al., 2018). In *A. xenomutans* strain SRM1, this MAG region (Figure 7A) included an arsenic resistance protein ArsH, arsenic transporter ArsB, arsenate reductase ArsC and ArsR regulator (Rosen and Liu, 2009; Chang et al., 2018). This genome region of *A. xenomutans* strain SRM1 showed on average ≥70% similarity with its closest *Alcanivorax* sp. relatives (Figure 7A), indicating high conservation of this As operon and flanking regions across different *Alcanivorax* species. Similarly, in *H. alkaliantarctica* strain SRM2, the region comprising the arsenic operon displayed high conservation with the homologous sequences of close *Halomonas* sp. relatives (Figure 7B). Notably, all the ORFs of the arsenic resistance operon in *H. alkaliantarctica* strain SRM2 MAG are contiguous, whereas additional ORFs (of un-identified function) are present in the three reference genomes, that separate ORF 7 from ORFs 8-9. As the three reference *Halomonas* genomes were obtained from matrices not contaminated by As (Nagata et al., 2019; Williamson et al., 2016) we can argue that this observed simplification of the arsenic operon may have enhanced the ability of *H. alkaliantarctica* strain SRM2 to detoxify/remove As. The functional implications of the observed differences in the

structure of the arsenic resistance operon across different *Halomonas* species remains to be further investigated, to understand which gene asset may perform better for different bioremediation purposes.

A complete mercury-resistance operon (Boyd and Barkay, 2012) was also identified in *H. alkaliantarctica* strain SRM2 (Fig. 7C), whose coding sequence was highly conserved within the closest relatives retrieved from NCBI (*H. axialiensis* Althf1 and *H.* sp. ZM 3, which were isolated from HM-rich hydrothermal vents in the Pacific Ocean and from a mineral waste repository, respectively; Dziewit et al., 2013; Tsurumaki et al., 2019). The structure of the identified mercury operon showed typical features identified in other *Halomonas* species (Boyd and Barkay, 2012), including two transcriptional regulators MerR, a mercuric transport protein MerT (able to transport Hg(II) to the cytoplasm), a periplasmic Hg-binding protein MerP, a mercuric reductase MerA, and an organomercurial lyase MerB. Based on the presence of this Mer operon, and especially of MerP, we can expect that, even if not directly assessed in our bioremediation tests, *H. alkaliantarctica* strain SRM2 may also perform Hg^{2+} biosorption (Huang et al., 2003).

Notably, several of the genes of the arsenic and mercury operons we identified have been documented to cross-react with other toxic metals. For instance, ArsH has been reported to also detoxify and enhance the precipitation of chromium by reducing Cr(VI) to Cr(III) (Xue et al., 2014), ArsB to also detoxify the hazardous metalloid antimony (Meng et al., 2004), and MerP to be involved in the biosorption of other HMs such as nickel, chromium, copper and zinc (Kao et al., 2008; Hsueh et al., 2017). This suggests that similar cross-reactivity with multiple HMs may contribute to explain the overall high removal efficiency towards the different HMs observed in our bioremediation experiments, possibly extending our findings to additional HMs not directly investigated here.

Nevertheless, we acknowledge that other processes such as extracellular electron transfer and electrocatalysis, not assessed in the present study, may be involved in HMs bioremediation (Liu et al., 2018) and as such deserve further investigations.

4. Conclusions

In summary, our multidisciplinary study based on an integrated approach that combines experimental microbiome enrichment, next-generation sequencing and selective culturing, allowed us to obtain two novel *Alcanivorax* and *Halomonas* strains with promising bioremediation potential. Both strains were shown to be resistant to, and to detoxify or remove multiple PAHs and HMs, and hence represent promising candidates for developing bioremediation applications (e.g., bioaugmentation or *ex situ* treatments) in environments contaminated by combinations of toxic pollutants. Finally, the contextual analysis of their genomic repertoire highlights the presence of genes and/or operons that are proposed as possible bioengineering targets, to further enhance the observed ability of these or other bacterial strains to serve for environmental bioremediation purposes.

CRediT authorship contribution statement

AD, ER, and FD conceived the study. FD, MP, ER, and CS conducted the field work. FD, ER, LJZ, and MT conducted the laboratory analyses. FD, ER, LJZ, MT, AC, EB, PNG and AD contributed to data elaboration. ER and FD wrote the draft of the manuscript. All authors critically revised the article and contributed to its finalization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at XXX

Data Availability Statement

The sequencing data of the two bacterial strains described in this work have been deposited in NCBI (accession number: PRJNA899357). The bacterial strains obtained in our study are maintained at the Stazione Zoologica Anton Dohrn, National Institute for Marine Biology, Ecology and Biotechnology, and are available upon request of collaboration.

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Figure Legends

Figure 1. Map of the study area with the position of the sampling site at Sarno River Mouth (Tyrrhenian Sea; 40°43'42.01"N, 14°28'0.45"E).

Figure 2A-B. Taxonomic and functional annotation of the enriched metagenome. A) taxonomic classification at the genus level based on M5NR database (standard cutoff: alignment length 15bp; e-value e^{-5}; id. 60%). B) functional classification based on Subsystems ontology (level 1; standard cutoff: alignment length 15bp; e-value e^{-5}; id. 60%), with functions putatively related to hydrocarbon degradation and to resistance/interaction with heavy metals contained in the subsystems "stress response" and "metabolism of aromatic compounds" subsystems.

Figure 3A-B. Removal of PAHs and HMs by the two novel bacterial strains isolated from the enriched metagenome. A) Degradation of PAHs (naphthalene, phenanthrene and pyren) under control (no bacteria) and treated conditions (i.e., addition of *A. xenomutans* strain SRM1 or *H. alkaliantarctica* strain SRM2). B) Removal of HMs (As, Cd, Cu, Zn, Pb) under control (no bacteria) and treated conditions (i.e., addition of *A. xenomutans* strain SRM1 or *H. alkaliantarctica* strain SRM2). Reported are average values and standard deviations.

Figure 4A-D. Comparative genomics of genetic regions for peripheral pathways for hydrocarbon degradation with ring hydroxylating dioxygenases enzymes (RHDs). A) Comparison of contig 9 from *A. xenomutans* strain SRM1 MAG (containing the ORF 4 encoding for RHD) with three closest sequences from the NCBI database. In the right, the complete list of genes encoded within contig 9 (ORFs 1-10) and those encoded by the 3 other reference genomes (ORFs 11-29). B) Comparison of contig 29 from *H. alkaliantarctica* strain SRM2 MAG (containing the ORF 2 encoding for RHD) with three closest sequences from the NCBI database. In the right, the complete list of genes encoded within contig 29 (ORFs 1-17) and those encoded by the 3 other reference genomes (ORFs 18-30). C) Superimposition of RHD of *H. alkaliantarctica* strain SRM2 (in red) with *n*aphthalene 1,2-dioxygenase crystal structure from *Pseudomonas* sp. strain C18 (in blue) [www.rcsb.org/structure/2hmm]. D) Superimposition of RHD of *A. xenomutans* strain SRM1 (in red) with naphthalene 1,2-dioxygenase crystal structure from *Rhodococcus* sp. NCIMB 12038 (in blue) [www.rcsb.org/structure/2b1x].

Figure 5A-B. Comparative genomics of genetic regions for peripheral pathways for hydrocarbon degradation with cytochrome P450 (CYP450).A) Comparison of contig 76 from *A. xenomutans* strain SRM1 MAG (containing the ORF 12 encoding for CYP450) with three closest sequences from the NCBI database. In the right, the complete list of genes encoded within contig 76 (ORFs 1-20) and those encoded by the 3 other reference genomes (ORFs 21-29). B) Comparison of contig 15 from *H. alkaliantarctica* strain SRM2 MAG (containing the ORF 6 encoding for CYP450) with three closest sequences from the NCBI database. In the right, the complete list of genes encoded within contig 15 (ORFs 1-14) and those encoded by the 3 other reference genomes (ORFs 15-27).

Figure 6A-B. Comparative genomics of genetic regions for central pathways for hydrocarbon degradation with genes for the homoprotocatechuate pathway. A) Comparison of contig 65 from *A. xenomutans* strain SRM1 MAG (containing the ORF 7-17 encoding for the genes of the homoprotocatechuate pathway) with three closest sequences from the NCBI database. In the right, the complete list of genes encoded within contig 65 (ORFs 1-20) and those encoded by the 3 other reference genomes (ORFs 21-38). B) Comparison of contig 29 from *H. alkaliantarctica* strain SRM2 MAG (containing the ORF 4-14 encoding for the genes of the homoprotocatechuate pathway) with three closest sequences from the NCBI database. In the right, the complete list of genes encoded within contig 29 (ORFs 1-17) and those encoded by the 3 other reference genomes (ORFs 18-47).

Figure 7A-C. Comparative genomics of genetic regions for heavy metal removal/detoxification. A) Comparison of contig 20 from *A. xenomutans* strain SRM1 MAG (containing the ORFs 11-18 encoding the arsenic resistance operon-like genomic region) with three closest sequences from the NCBI database. In the right, the complete list of genes encoded within contig 20 (ORFs 1-18) and those encoded by the 3 other reference genomes (ORFs 19-24). B) Comparison of contig 68 from *H. alkaliantarctica* strain SRM2 MAG (containing the ORFs 2-12 encoding the arsenic resistance operon-like genomic region) with three closest sequences from the NCBI database. In the right, the complete list of genes encoded within contig 68 (ORFs 1-15) and those encoded by the 3 other reference genomes (ORFs 16-29). C) Comparison of contig 30 from *H. alkaliantarctica* strain SRM2 MAG (containing the ORFs 6-11 encoding the mercury-resistance operon) with three closest sequences from the NCBI database. In the right, the complete list of genes encoded within contig 30 (ORFs 1-15) and those encoded by the 3 other reference genomes (ORFs 16-23).

Figure 1

Figure 2

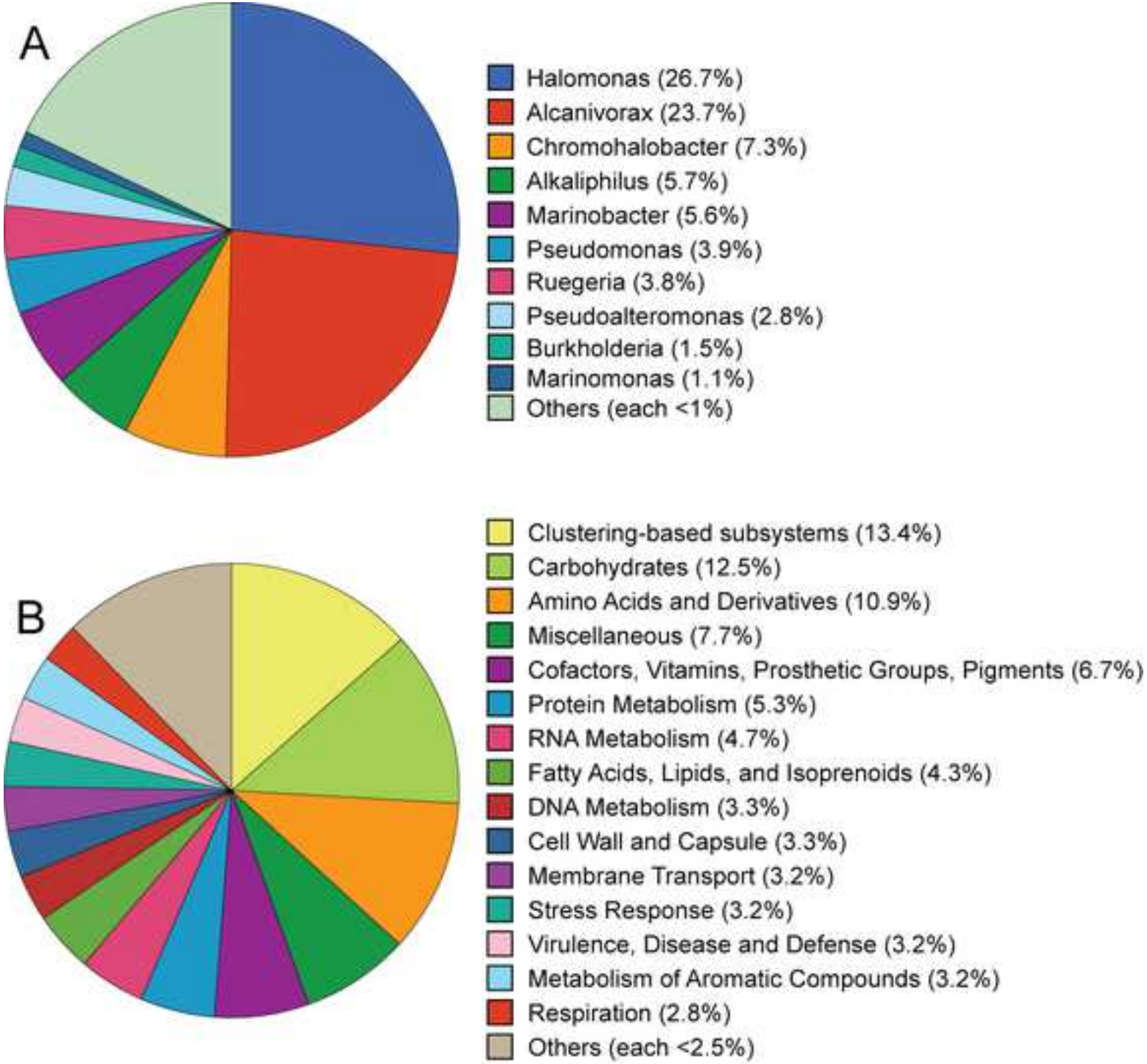

Figure 3

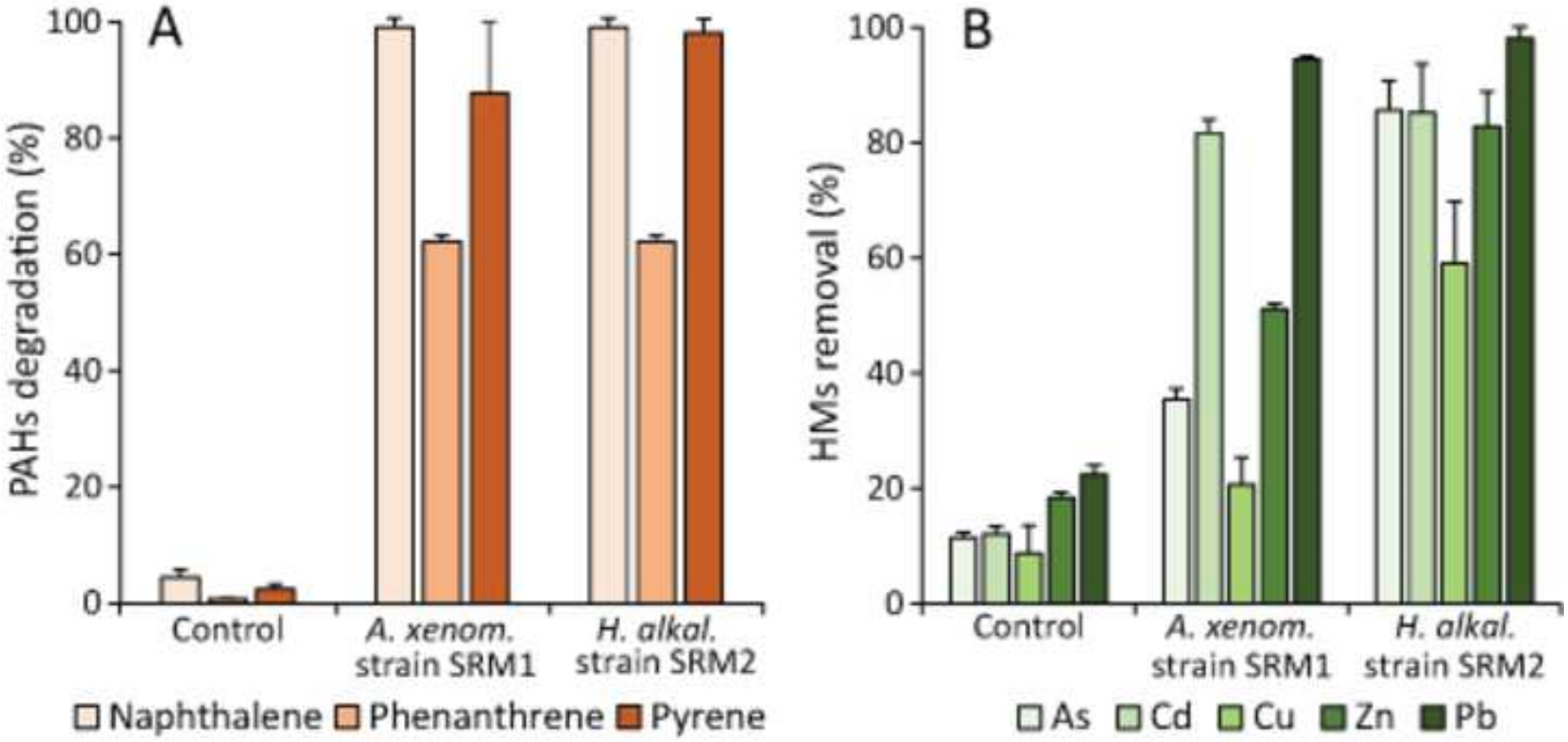

Figure 4

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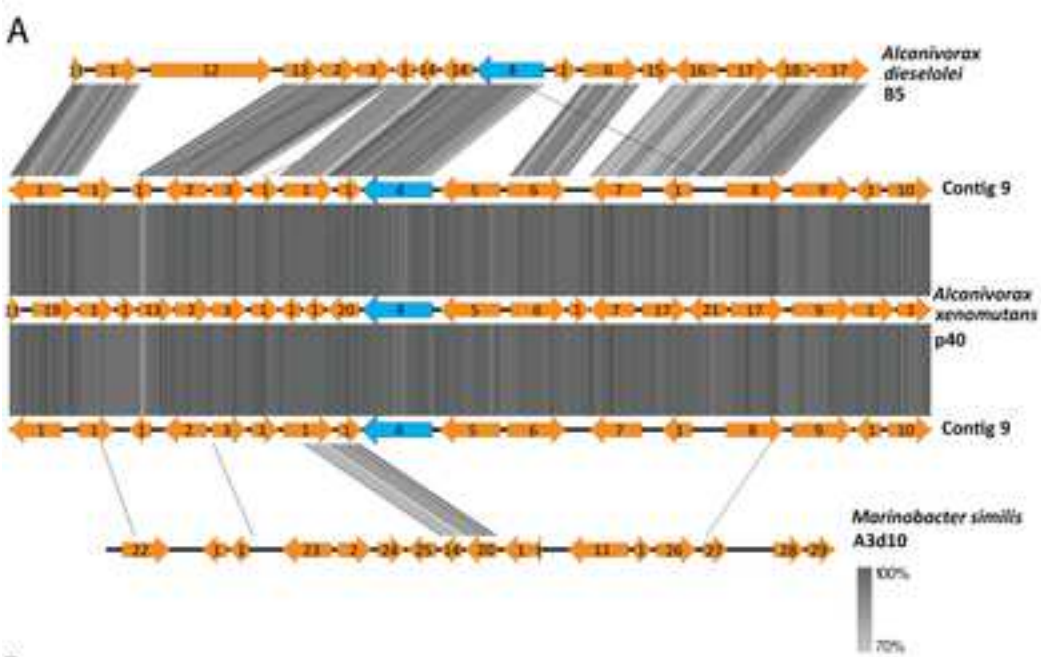
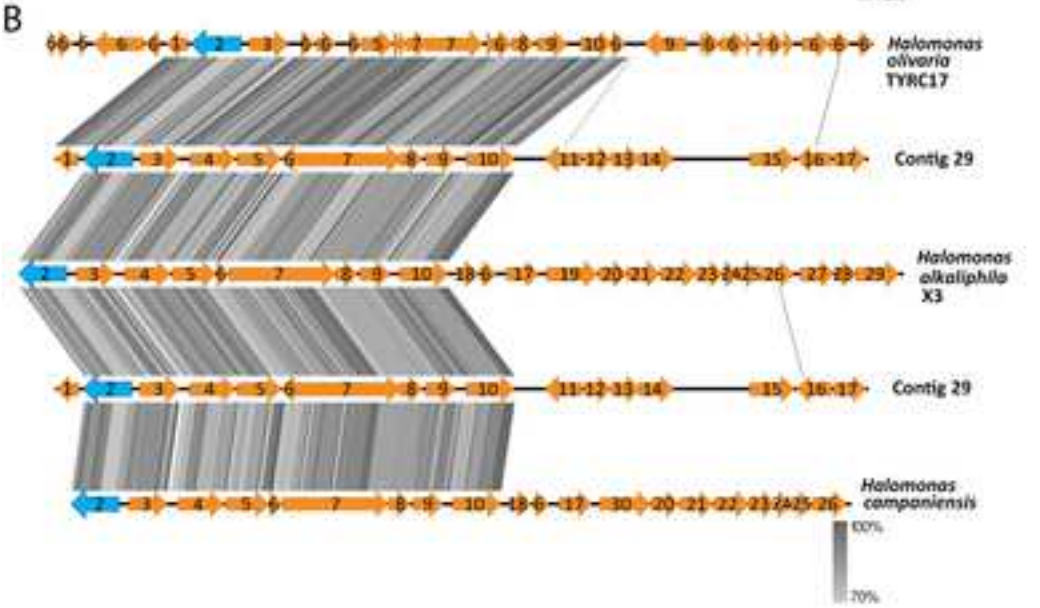


1. hypotetical protein
2. LysE family translocator
3. glutathione S-transferase
4. aromatic-ring-hydroxilating dioxygenase
5. linear-amide C-N hydrolase
6. aldo-keto reductase
7. sulfite exporter TAU- Saf E family protein
8. glxA family trascriptional regulator
9. nitrite reductase
10. SCO family protein
11. α/β hydrolase
12. Ton-B siderophor receptor
13/14. epimerase/proteinase
15. phenilacetic acid degradation protein
16/17. membrane protein/Ara C
18/19. hydratase/nucleotydil transferase
20/21. glutamine amido tranferase/ThiJ
22. enoyl-CoA hydratase
23. Lys-R trascriptional activator
24. EF-P translational elongation factor
25. glycoside hydrolases
26. short-chain dehydrogenase
27. organic hydroperoxide resistance protein
28. 2'-5' RNA ligase
29. nitrilase/cyanide hydratase and apolipoprotein N-acyltransferase



1. TetR family protein trascriptional regulator
2. aromatic-ring-hydroxilating dioxygenase
3/4. ferrodoxin/ Serin hydroxymethyl transferase
5. sarcosine oxydase sub unit β
6. hypothetical protein
7/8. sarcosine oxydase subunit α and γ
9. formyl tetrahydrofolate deformylase
10. serine ammonia-lyase
11/12. zinc transporter /ABC transporter
13. zinc uptake system membrane protein
14. GTP hydrolase
15. aminotransferase class I/II
16. ureohydrolase
17. LysR trascriptional regulator
18. sulfurtransferase
19. 2-hydroxymuconic semialdehyde dehydrogenase
20. 2-keto-4-pentenoate hydratase
21. acetaldehyde dehydrogenase
22. 4-hydroxy-2-oxovalerate aldolase
23. 4-oxalocrotonate decarboxylase
24. RidA family protein
25. 3-hydroxyanthranilate 3,4-dioxygenase
26. aminocarboxymuconate-semialdehyde decarboxylase
27. C4-dicarboxylate ABC transporter substrate
28. C4-dicarboxylate ABC transporter permease-binding protein
29. TRAP transporter large permease
30. betaine-aldehyde dehydrogenase

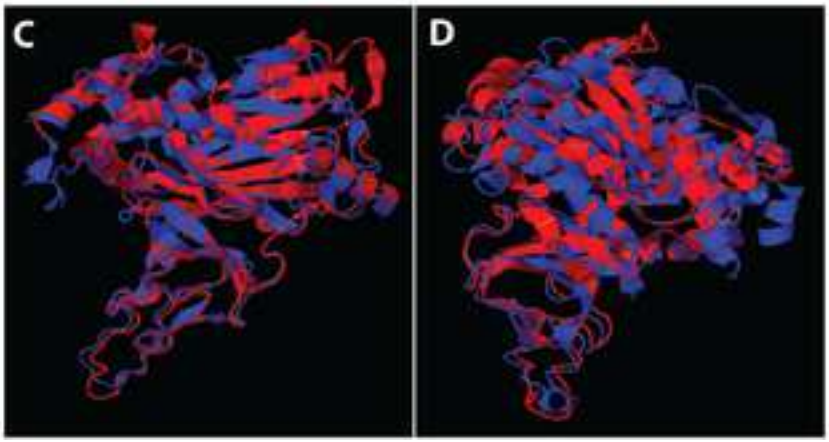


Figure 5

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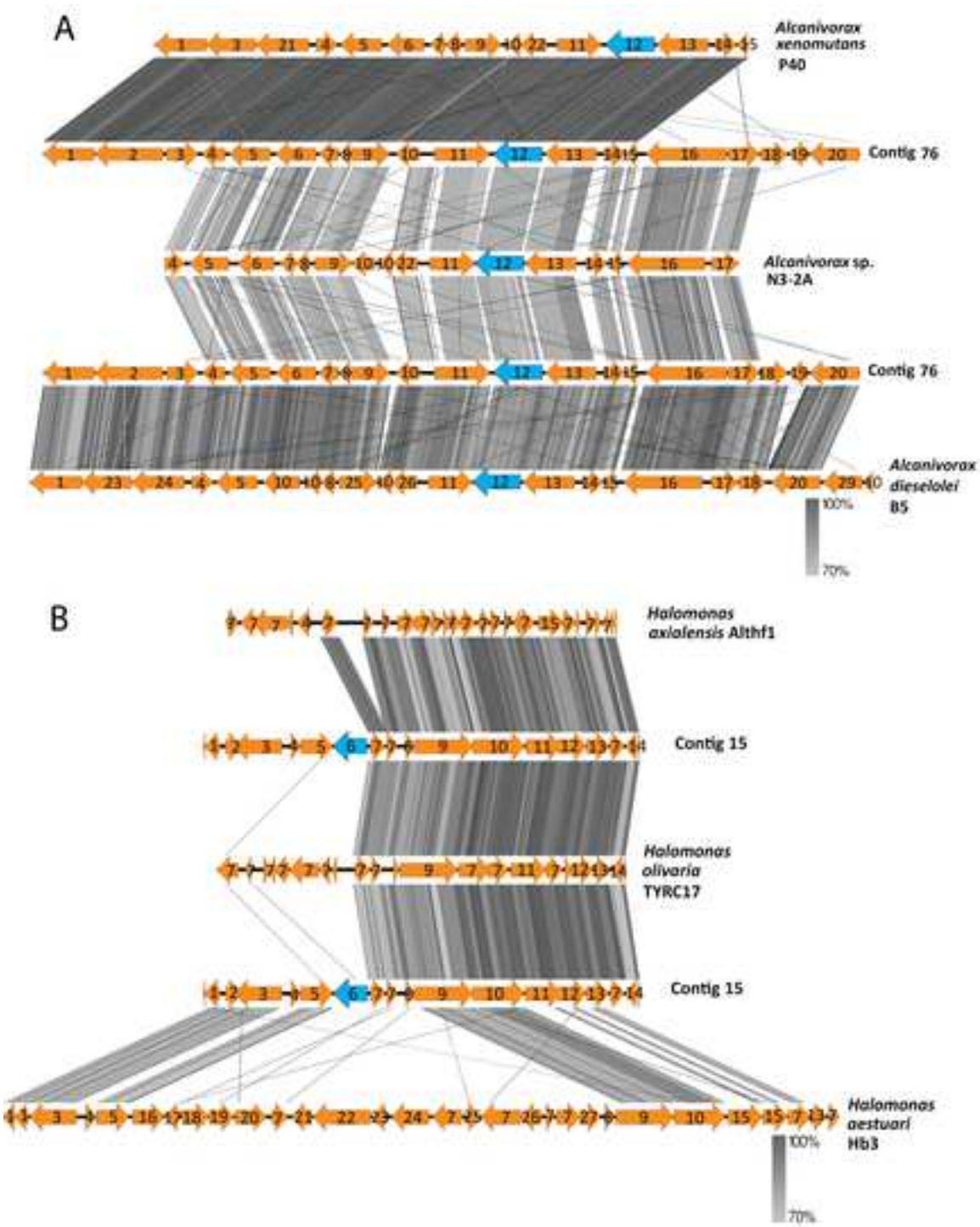

1. carbhoydrate kinase
2. glycerol 3-phopshate dehydorgenase/oxydase
3. FAD-binding oxidoreductase
4. cell envelope biogenesis protein
5. MSF 1 transporter
6. ferritin-like domain
7/8. paal family tioesterase
9. poly hydroxyalcanoate syntase
10. hypotetical protein
11. PAR A family protein
12. cytochrome P450
13. glutathione-disulfide reductase
14. glutamate synthase family protein
15. lytic transglycolase domain
16. rieske-domain non-heme oxygenase
17. phosphoenol pyruvate synthase
18. kinase phosphorilase
19 . thioesterase family protein
20. 2-methyl citrate
21. FAD-linked oxidase
22. cobyric acid synthase
23. FAD-dependent oxidoreductase domain protein
24. FAD-linked oxidase
25. hydrolase α/β domain protein
26. ParA family ATPase
27. pyridine nucleotide-disulfide oxidoreductase domain protein
28. conserved region in glutamate synthase superfamily
29. 2-methylaconitate cis-trans isomerase

1. Lrp/AsnC family trascriptional regulator
2. dissimilatory nitrite reductase
3. nitrite reductase
4. cytochrome C
5. protein Nirf
6. cytochrom P450
7. hypothetical protein
8. cytocrhome C5 family protein
9. regulatory protein Nosr
10. nitrous oxide reductase
11. nitrous oxide reductase NosD
12. ABC transporter
13. nitrous reductase accessory protein Nos L
14. invasion gene expression Sir B
15. copper ABC transporter ATP binding protein
16. nitric oxide dioxygenase
17/18/19. globin/protease/peptidase
20. AraC family transcriptional regulator
21. ribonucleoside-diphosphate reductase
22. ribonucleoside-diphosphate reductase, adenosylcobalamin-dependent
23. sterol-binding protein
24. oxygen-independent coproporphyrinogen III oxidase
25. Rrf2 family transcriptional regulator
26. DNA-binding response regulator
27. molybdopterin-binding protein

Figure 6

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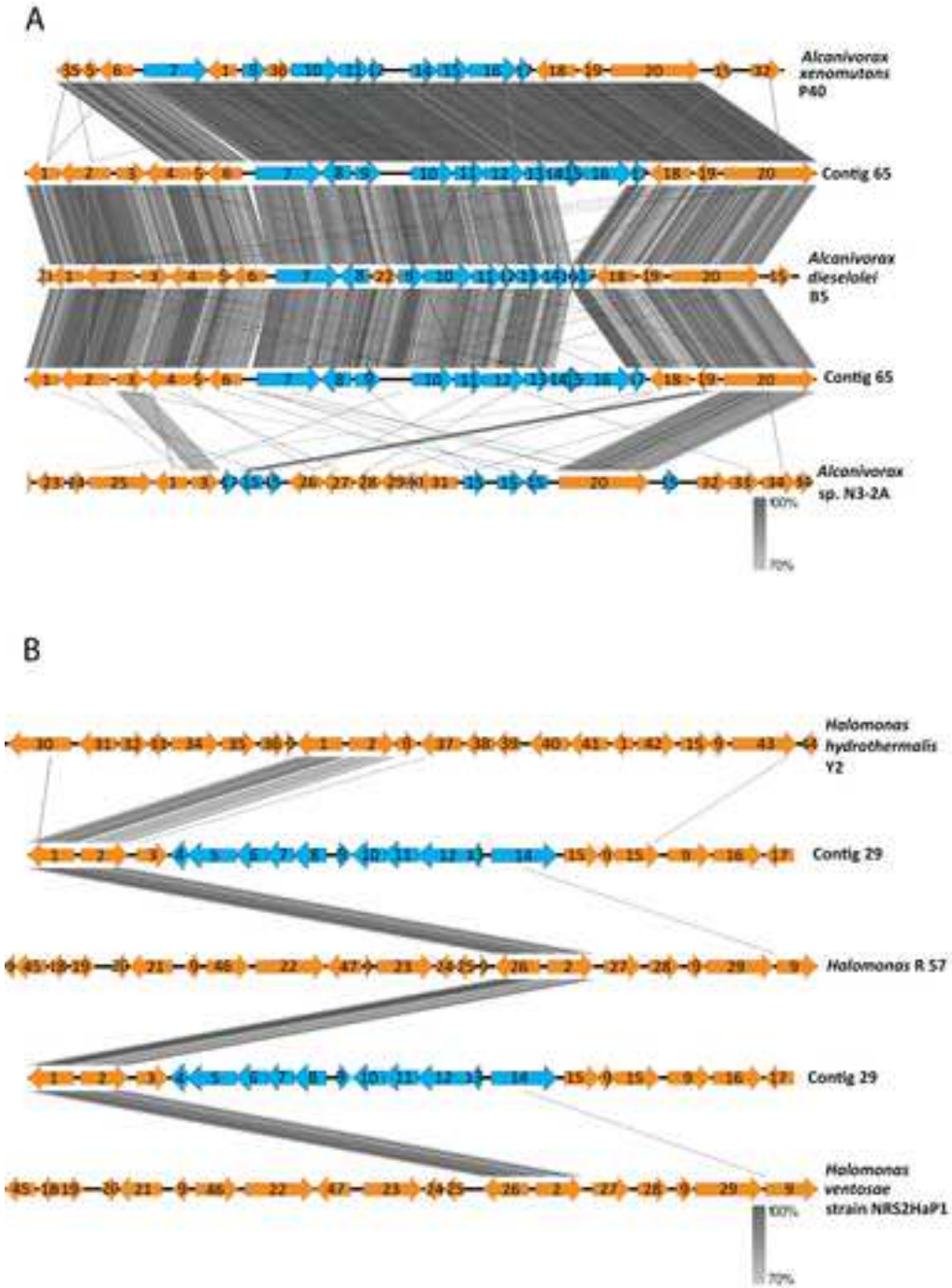

1. ARA C family trascriptional regulator
2/3. Na/Pi efflux pump / Sterol desaturase
4/5. TRAP transporter large/small permease
6. TRAP transporter substrate binding protein
7. 3-phenol 2-monooxygenase
8. hydroxyphenylacetate catabolism regulator protein
9. 4-hydroxyphenylacetate isomerase
10. 5-carboxymethyl-2-hydroxymuconate semialdehyde dehydrogenase
11. 3,4-dihydroxyphenylacetate 2,3-dioxygenase
12. 5-carboxy methyl 2 hydroxy muconate isomerase
13. 2-oxo hepta-3-ene 1-7-dioic acid hydratase
14/15. 4-hydroxy-2-oxoheptanedioate aldolase / hypot. protein
16. NAD-dependent succinate-semialdehyde dehydrogenase
17. homoprotocatechuate degradation operon regulator Hpa R
18/19.ABC transporter / TerC family protein
20/21. aconitate hydratase AcnA / Transporter CPA 2 family
22. fumarylacetoacetate hydrolase family protein
23/24. gluconolactonase / damage-inducible protein DinB
25/26. potassium proton antiporter / 4-hydroxy-2-oxovalerate aldolase
27. acetaldehyde dehydrogenase (acetylating)
28. 4-oxalocrotonate decarboxylase
29. 2-keto-4-pentenoate hydratase
30. 4-oxalocrotonate tautomerase
31/32. MFS transporter / glutathione ABC transporter permease GsiC
33. ABC transporter permease
34. peptide ABC transporter ATP-binding protein
35. C4-dicarboxylate ABC transporter permease
36. 2-hydroxyhepta-2,4-diene-1,7-dioate isomerase

1. GNTR family trascriptional regulator
2/3. cytochrome c oxydase / Pas domain
4. homoprotocatechuate degradation operon regulator
5. semyaldheide deidrogenase
6. 2,4-dihydroxyheptadiene 1,7-dioic acid aldolase
7. 3,4-dihydrophenyl acetate 2,3-dyoxigenase
8. 4-hydroxyphenyl acetate catabolism regulator protein
9/10. hypoth. protein / 2-hydroxyheptadiene 1,7-dioate isomerase
11. 3-hydroxyphenyl acetate 2-3 dioxygenase
12. 5-carboxymethyl 2-hydroxymuconate semialdeyde deydrogenase
13. 5-carboxymethyl 2-hydroxymuconate isomerase
14. 3-hydroxy benzoate 4-monoxygenase
15. C4 decarboxylate transporter
16. NAD-dependent phenilacetaldeyde dehydrogenase
17. trascriptional regulator Factor
18/19. Cupin 2 / Transcriptional regulator, IclR family protein
20. nucleoside diphosphate kinase regulator
21. NADP-specific glutamate dehydrogenase
22. meiotically up-regulated protein
23/24. Phosphodiesterase / FMN-binding split barrel
25. lysine exporter protein LysE/YggA
26. PLP-dependent aminotransferase family protein
27. HTH-type transcriptional regulator CdhR
28. peptidase M19, renal dipeptidase
29. putative N-methylproline demethylase
30. type VI secretion system tip protein VgrG
31/32. monooxygenase / NAD-dependent deacetylase
33. 3-methyladenine DNA glycosylase
34/35/36. YdiU family protein /DNA polymerase / α/β Hydrolase
37. 4-aminobutyrate--2-oxoglutarate transaminase
38. sensor domain-containing diguanylate cyclase
39. diguanylate phosphodiesterase
40/41. CoA transferase / acyl-CoA dehydrogenase
42/43/44. L-2-hydroxyglutarate oxidase/Permease/penicillin-binding protein
45. tripartite tricarboxylate transporter substrate binding protein
46/47. MFS transporter / ADP-ribosylglycohydrolase family protein

Figure 7

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