1- Project goal:

Bioremediation of polycyclic aromatic hydrocarbon (PAH)

2- Project description:

In this project, we are focused on engineering microorganisms with the capability of removing crude oils. Crude oils are predominantly composed of hydrocarbons (hydrogen (H) and carbon (C)) made of alkanes with carbon chain lengths ranging from C8 ~ C40 and aromatic hydrocarbons based on a 6-carbon ring account for approximately 80% of the total petroleum hydrocarbons. Aromatic carbons such as polycyclic aromatic hydrocarbons (PAHs) tend to be the molecular compounds in oil that are the most toxic as they can be quite persistent in the environment due to their intrinsic stability provided by the aromatic ring.

After review of the literature, we have identified several PAHs that are major components of crude oils: Naphthalene Phenanthrene Fluorene and Pyrene Anthracene Biphenyl

The degradation pathway of each of these PAHs includes a first step of oxygenation followed by degradation of the aromatic ring. Each of these pathways includes a complex set of 10-15 genes encoding different classes of enzymes, mainly oxygenase, hydrogenase, and carboxylase. Additional genes are also involved in the regulation of expression of the pathway (activator), genes involved in secreting surfactants to allow the bacteria to mix well with crude oil, and genes involved in the transport of PAH inside the microorganism.

The overall scheme of degradation of PAH, that contains 2 or more rings, includes an initial attack of the hydrocarbons by oxygenases that add oxygen (s) followed by degradation by peripheral pathways. The addition of oxygen by monooxygeneases or dioxygenases to the alkyl moiety or aromatic ring convert the PAH to a few **central intermediates** such as catechol, salicylic acid, and gentisate through convergent pathways.

To be able to degrade as many aromatic components as possible, our approach would be to converge the pathways and do what is called gene augmentation. This approach is possible because there are some intermediates that are common amongst the pathways. So we would clone the genes that are upstream of the intermediates and introduce them into a bacteria that would already contain one degradation pathway already containing the genes that are downstream of the intermediate. For example, the degradation maps of anthracene and fluorene can converge at 3 points: cathecol or salicylate or phthalate. So, we could introduce the genes upstream of cathecol in a strain that is degrading anthracene via the cathecol degradation pathway. So in sample words, the host strain already has a piece of the pathway, and by a process of engineering, we are augmenting its pool of genes and thus its capacity of degradation. The advantage of this approach is that the host strain is already adapted to grow in the environment (in the “wild”). Furthermore, the host microorganism already have some of the elements in place for the catabolic system to take place (transport, electron transfer functions, oxygenase functions, dehydrogenase functions, etc.).

The aim is to enhance the ability of the newly engineered strains to degrade a broader range of PAHs and to eventually increase the degradation effectiveness in comparison with wild or natural strain.

3- Background:

Since there are no single bacteria/fungi which have all the catabolic capacity to efficiently degrade all the crude oil components, in particular polycyclic aromatic hydrocarbons, there is the need to create one.

Bioaugmentation is defined as the enhancement of performance of microorganism populations through the addition of genetically engineered bacteria with specific catabolic activities, isolated bacterial strains or enrichment consortia to increase the rate of degradation. The criteria for choosing microbes are the physiology and metabolic ability of the microbes to degrade the crude oil. The efficiency of crude oil degradation of individual bacterial cultures and designed bacterial consortium was tested with five bacterial strains (Micrococcus sp. GS2-22, Corynebacterium sp. GS5-66, Flavobacterium sp. DS5-73, Bacillus sp. DS6-86 and Pseudomonas sp. DS10-129) and data have shown an increase of 78% of oil degradation efficiency compared to the individual strains. The data were also suggesting synergistic promotion of PAH degradation by a mixture of monoculture isolates.

Individual microorganisms can degrade only a limited range of substrates, so assemblages of population with overall broad enzymatic capacities are required to increase the rate and extent of oil biodegradation. Bioaugmentation is especially relevant in the case of more recalcitrant chemicals such as PAH.

4- Examplse of possible augmentation:

The common biochemical pathways for the bacterial degradation of PAHs such as naphthalene, phenanthrene, anthracene and acenaphthene have been well investigated. Biodegradation mechanisms require the presence of molecular oxygen to initiate the enzymatic attack of PAH rings. In the initial step, Dioxygenases (Aromatic) catalyzed oxidation reactions. Dioxygenase enzyme breaks the benzene ring and formed cis-dihydrodiols as early byproduct. It is a multi component enzyme system which involved many coenzyme and metal ions (as a co factors).

We have identified a couple of bacteria candidates that could be the host with already one or 2 pathways.

- Candidate 1: Janibacter YY-1 (biosafety level 1, so safe in the lab and in the environment) that degrades anthracene and phenanthrene efficiently. We would add the upper genes of the fluorene and/or pyrene degradation pathways.
- Candidate 2: *Novosphingobium aromaticivorans* strain F199 that degrades biphenyl and naphthalene. We would introduce genes for the phenanthrene and/or the fluorene degradation pathways.
- We are looking at additional candidates.
- Note: the microorganisms have to be commercially available (for instance through the American Tissue Culture Collection, ATCC)

The engineering steps would include:

- Cloning of the genes in an expression vector (this is done in E.coli). The genes would be amplified by PCR from bacteria or they would be designed synthetically using publically available genomic sequences from degrading bacteria known to be efficient at degrading phenanthrene.
- Characterize the host strain that already contain one pathway
    - study its natural level of resistance to antibiotic to make sure that we can introduce the expression vector

Commented [RP1]: I sort of agree, but then you have picked a group of very similar aromatics, so actually I would expect most isolates capable of growing on 1 to grow on all of them. Pyrene may be the exception here… if you look at its structure you can see that it is much more 'fused'. The four-ring chrysene goes reasonably quickly in the environment, but it also might be dufficiently different from phenanthrene that it might need a different enzyme

Commented [RP2]: If you do a Google Scolar search you will see that this organism, or at least something with a very similar name, may be hazardous, at least to old people. Kaplan MM. Novosphingobium aromaticivorans: a potential initiator of primary biliary cirrhosis. The American journal of gastroenterology. 2004 Nov 1;99(11):2147.

    - Determine its propagation time (how long it takes to grow and on which medium with and without PAH)
    - Verify the PAH that it can degrade
- Introduce the expression vector in a strain already containing a natural degradation pathway and then test the degradation

5- Project:

Project Plan: We propose to work on the following:

- Select/ rank the PAHs of interest
- Identify bacteria that are **efficiently** degrading the selected PAHs
- Identify the genes involved in the corresponding catabolic (degrading) pathways and understand if their sequences are publically available
- Select the pathways and matching bacteria/fungi that have common central intermediates between the selected pathways
    - Select several PAH that are relevant for a program of bioremediation
    - Identify their catabolic pathways with the chemical molecules and genes involved
    - Try to find common chemical molecules between pathways.
- Find the corresponding microorganism in the ATCC database
    - Search the collection ATCC (American Tissue Culture Collection) and see if it is commercially available. These microorganisms could be used (i) as a source of genes (genes to be cloned by PCR amplification) and/ or (ii) as a host for gene augmentation (recipient of cloned genes to broaden the number of catabolic pathways).
- Assays
    - Find source of PAH and search literature for protocols to detect PAH degradation
    - Write protocol to detect microorganism growth in media with or without PAH (use of PAH as a carbon source)
- Select pathways that share common intermediates (for instance, catechol, phthalate, benzoate) so we clone only the genes upstream of the common intermediates. We will then introduce the genes into 2 or more bacteria that have the genes downstream of the common intermediate.

Status of the project: We are conducting a comprehensive literature search to identify the pathways, bacterial genes annotated to have a potential function in the degradation of polycyclic aromatic hydrocarbons (PAH).

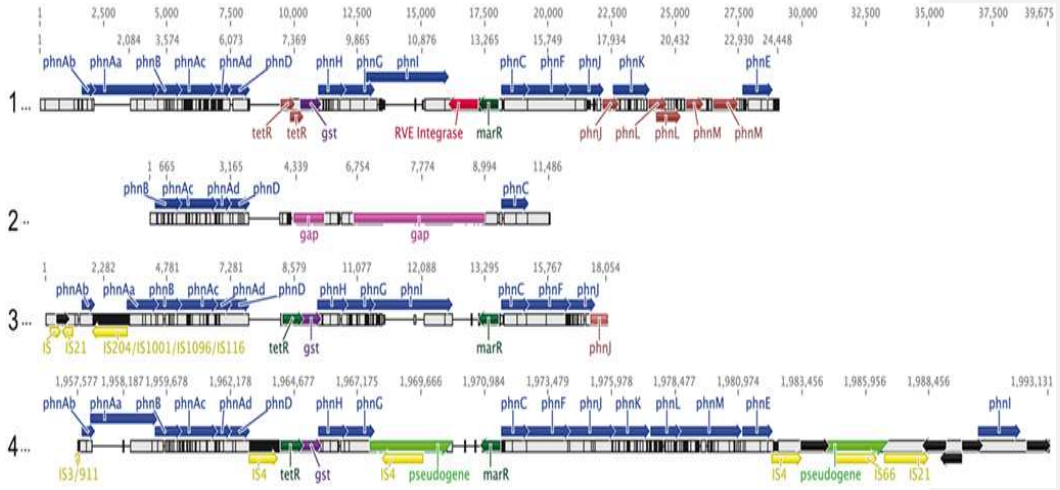
- We are starting to rank PAH by order of relevance.
- We are starting to look at protocols to set up the assays to perform the testing (bacterial growth, chemistry, etc.). We are also looking at sources of crude oils and PAHs.

6- Example: The Phenanthrene Pathway:

a. **Background:**

Representative of the genus Sphingomonas seem to have a wide range of substrates than other organisms. For instance, *Novosphingobium aromaticivorans* strain F199 can degrade biphenyl, naphthalene, fluorine, salicylate, benzoate, toluene, and xylene. The complete sequence of a 184-kb catabolic plasmid, pNL1, from *Novosphingobium aromaticivorans* strain F199 has been determined. The plasmid carries 79 genes distributed in at least 6 operons that are associated with the degradation and transport of PAHs.

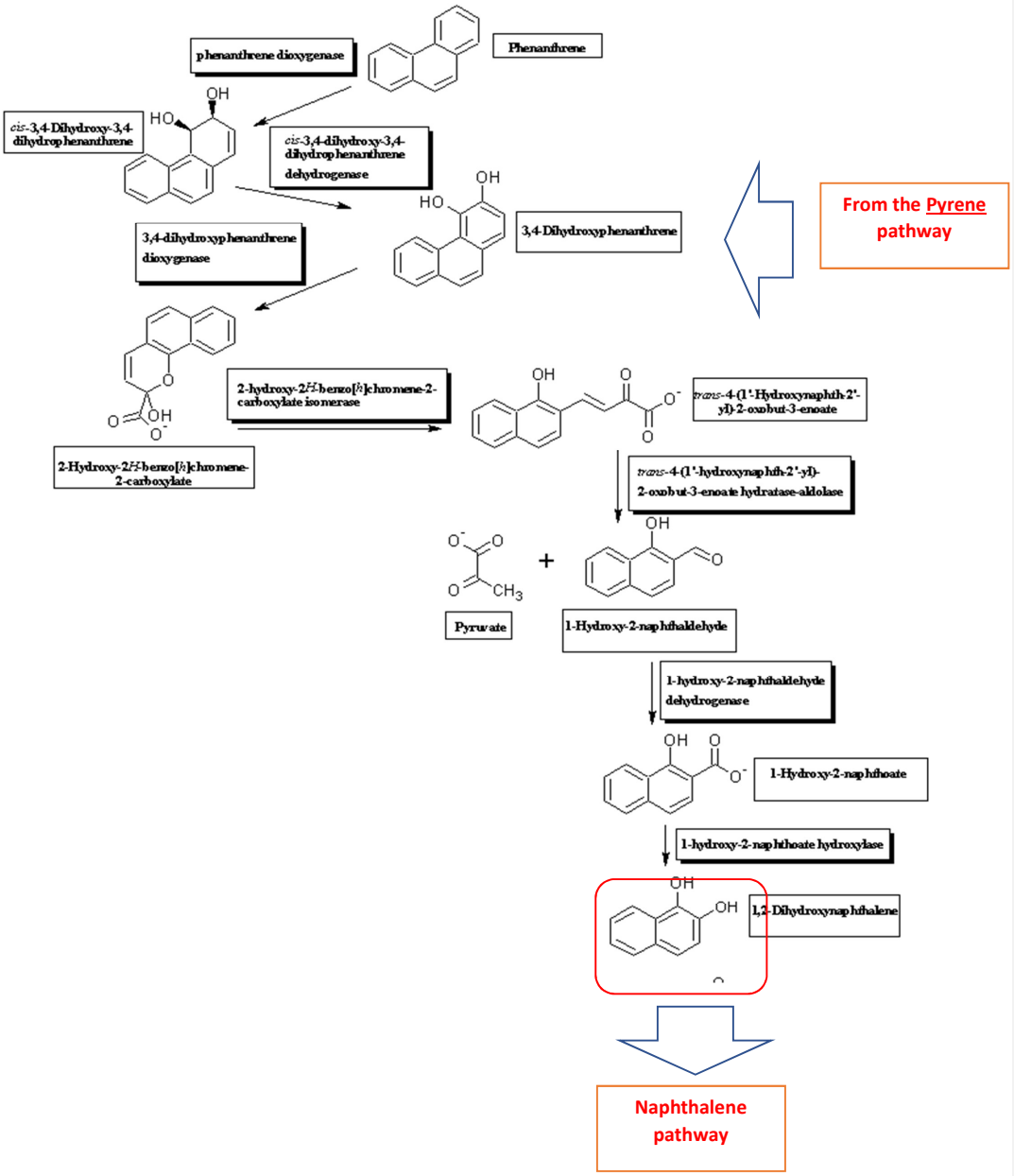
b. **Comparison of phenanthrene clusters between 4 strains**. The cluster encoding phenanthrene catabolism to one of the final product o-phthalate encompasses 17 genes (phnAa to phnI) interspersed with regulatory elements and other hypothetical proteins, so the genes are not all clustered together. Also, for a given PAH, each microorganism may exhibit a slightly different catabolic pathway with different set of genes.



c. **Proposal.**

Considering that strain *Novosphingobium aromaticivorans* strain F199 contains the pathway for biphenyl , naphthalene, fluorine, salicylate, benzoate, toluene, and xylene, the upper genes encoding for phenanthrene catabolism could be introduce into it.

d. The phenanthrene pathway with all the different pathways convergence.



7- Sources:

Source: Phenanthrene degradation pathway http://eawag-bbd.ethz.ch/pha/pha_map_1.gif)

Source: Naphthalene degradation pathway http://www.genome.jp/kegg-bin/show_pathway?map00626

Source: Biphenyl degradation pathway http://www.genome.jp/kegg-bin/show_pathway?map00363

Source: Polycyclic aromatic degradation pathway http://www.genome.jp/kegg-bin/show_pathway?map00624

Source: All pathways http://eawag-bbd.ethz.ch/servlets/pageservlet?ptype=allpathways

Source: Bacteria from ATCC

Designation: NAG2N113 (ATCC® 700638™)
Deposited Name: *Neptunomonas naphthovorans*, Hedlund et al., Janibacter sp. : Medium
ATCC® Medium 2: Marine agar 2216 or marine broth 2216
Growth Conditions Temperature: 20.0°C Atmosphere: Aerobic
Propagation Procedure
1. Open vial according to enclosed instructions.
2. Using a single tube of #2 broth (5 to 6 ml), withdraw approximately 0.5 to 1.0 ml with a Pasteur or 1.0 ml pipette. Rehydrate pellet. 3. Aseptically transfer this aliquot back into the broth tube. Mix well.
4. Incubate the tube at 20oC for 48 to 72 hours.
Colonies are white, shiny, moist, and entire. The longer the culture is incubated on agar the easier it is to detect the white pigmentation.

Novosphingobium aromaticivorans (ATCC® 700278™) Designation: SMCC F199; DSM 12444 Deposited Name: *Sphingomonas aromaticivorans* Balkwill et al.
Medium ATCC® Medium 18: Trypticase Soy Agar/Broth Growth Conditions Temperature: 30°C
Atmosphere: Aerobic
Propagation Procedure
1. Open vial according to enclosed instructions.
2. Using a single tube of #18 broth (5 to 6 mL), withdraw approximately 0.5 to 1.0 mL with a Pasteur or 1.0 mL pipette. Rehydrate the entire pellet.
3. Aseptically transfer this aliquot back into the broth tube. Mix well.
4. Use several drops of the suspension to inoculate a second tube of broth, a slant, and/or plate.
5. Growth occurs after 24-48 hours at 30°C.
Colonies on #18 agar are size variable, entire, glistening, circular, smooth, low convex, and yellow in color.

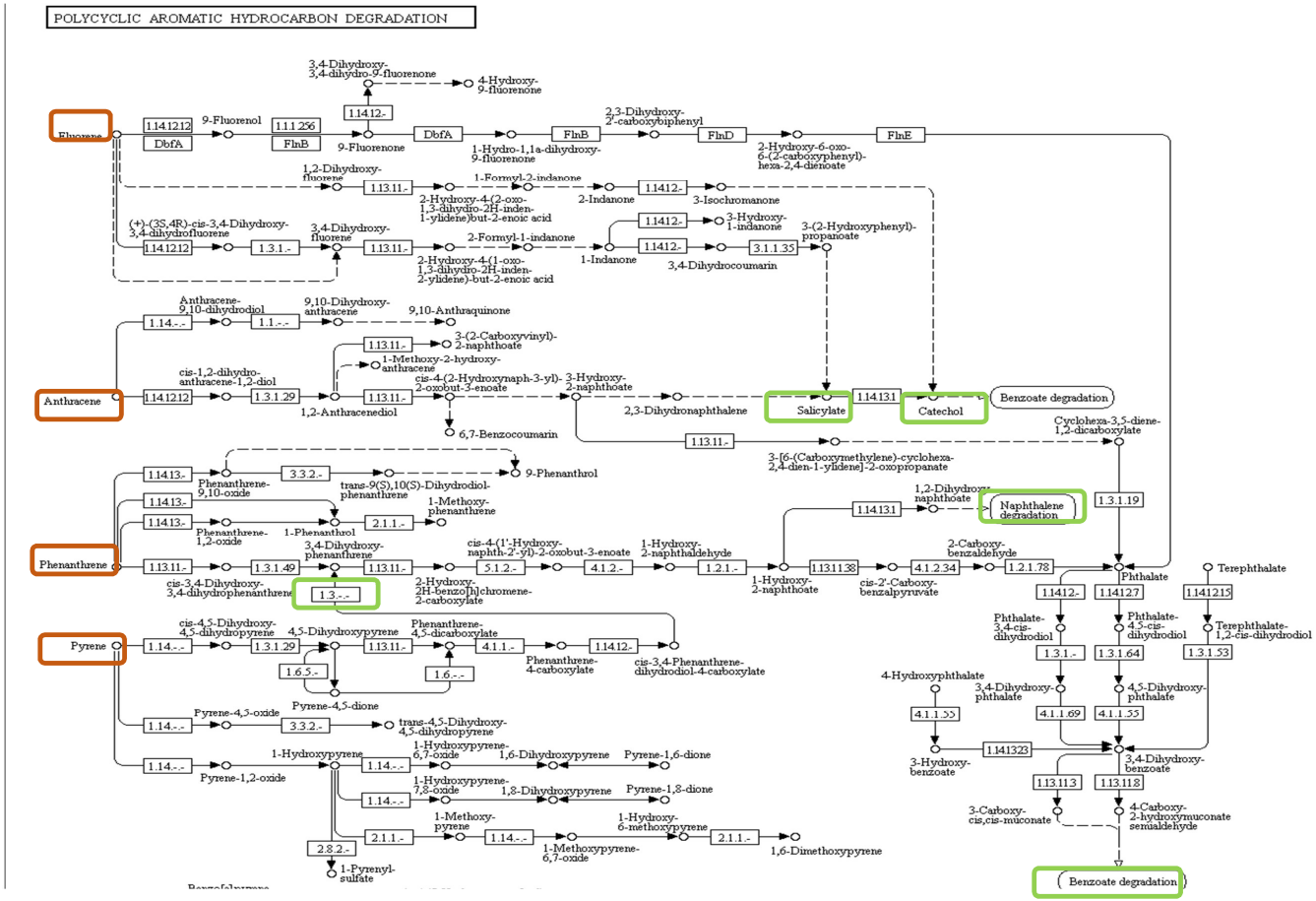


Figure: Polycyclic aromatic hydrocarbon (Chemical intermediates that are common to pyrene, phenanthrene, anthracene, and naphthalene are highlighted in green).

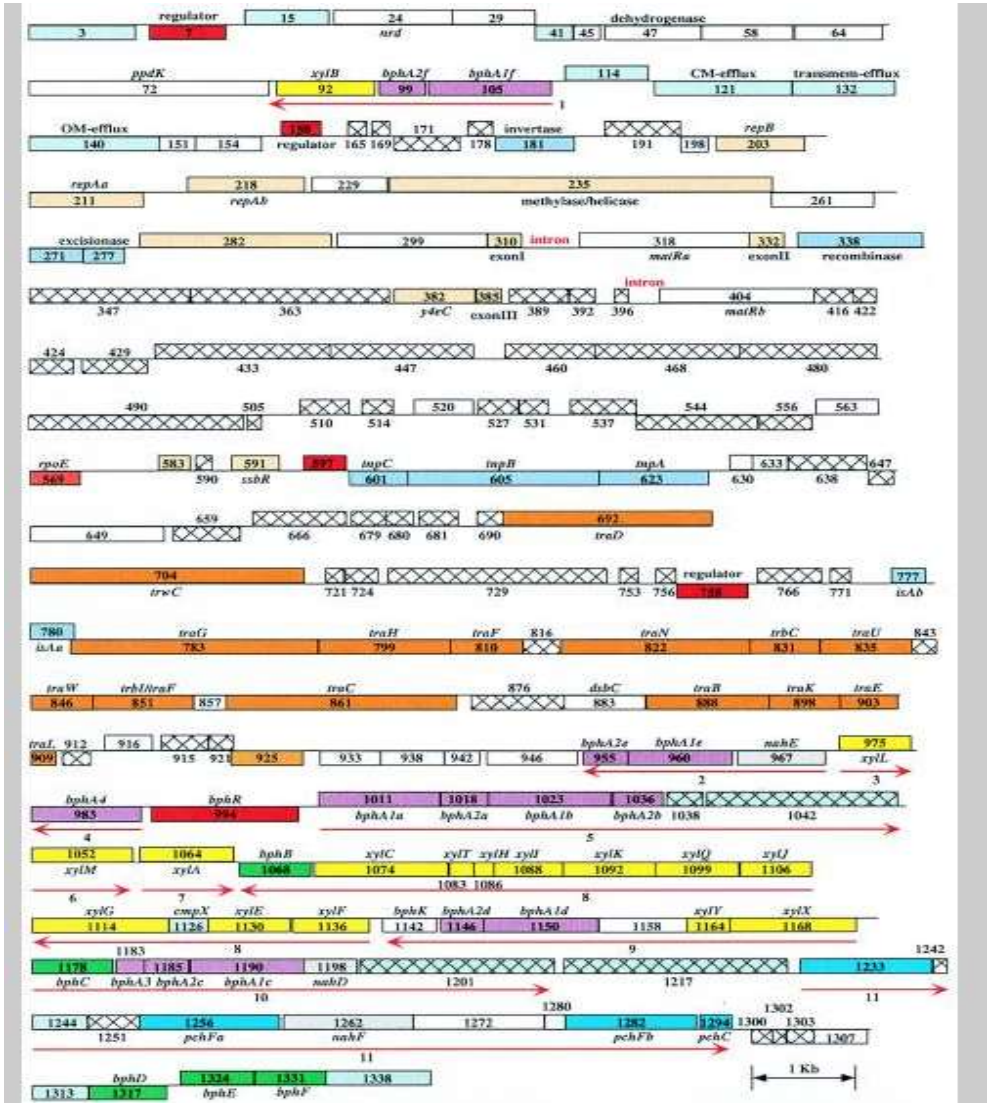


Figure: Graphical depiction of Genes (=open reading frames=ORF) of the 184-kb catabolic plasmid, pNL1, from *Novosphingobium aromaticivorans* strain F199. ORFs are depicted as boxes placed either above (frames 1 to 3) or below (frames 4 to 6) the axis. Red arrows indicate genes (1 to 11) which encode enzymes involved in biphenyl, *m*-xylene, and naphthalene degradation. Colors are used to highlight putative regulators (red); aromatic oxygenase subunits (purple); enzymes in biphenyl (green), naphthalene (gray), *m*-xylene (yellow), and *p*-cresol (turquoise) pathways; possible aromatic transport proteins (light blue); transposases and recombinases (medium blue); plasmid partitioning and replication (light orange); and conjugative genes (dark orange). Hatched boxes depict ORFs with no detected homologs outside pNL1.

Note: This illustrates that the genes of a pathway are not necessarily clustered together.