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Highlights

Metabolic and regulatory networks are finely tuned for biodegradation of aromatics

New pathways and widespread bacterial biodegradation capabilities revealed by omics

Full characterization of hybrid pathways expands the scope of aromatic biodegradation

The metabolism of aromatics plays a pivotal role in cell to cell communication

Computational and synthetic biology approaches design novel biodegradation pathways

Aerobic degradation of aromatic compounds

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Abstract

Our view on the bacterial responses to the aerobic degradation of aromatic compounds has been enriched considerably by the current omic methodologies and systems biology approaches, revealing the participation of intricate metabolic and regulatory networks. New enzymes, transporters, and specific/global regulatory systems have been recently characterized, and reveal that the widespread biodegradation capabilities extend to unexpected substrates such as lignin. A completely different biochemical strategy based on the formation of aryl-CoA epoxide intermediates has been unraveled for aerobic hybrid pathways, such as those involved in benzoate and phenylacetate degradation. Aromatic degradation pathways are also an important source of metabolic exchange factors and, therefore, they play a previously unrecognized biological role in cell-to-cell communication. Beyond the native bacterial biodegradation capabilities, pathway evolution as well as computational and synthetic biology approaches are emerging as powerful tools to design novel strain-specific pathways for degradation of xenobiotic compounds.

Introduction

Microbial degradation of aromatic compounds, which represent about 20% of the earth biomass, has been extensively studied due to its importance in the biogeochemical carbon cycle. Since many aromatic compounds are major environmental pollutants, their detection and removal from contaminated sites are of great biotechnological interest. Moreover, the use of aromatic compounds, e.g., lignin-derived compounds, as feedstock for the bioproduction of a number of substances in the pharmaceutical, industrial, agricultural, food and health sectors stresses the study of aromatic bioconversion processes [1-3].

Two major biochemical strategies are used by bacteria to activate and cleave the aromatic ring depending primarily on the availability of oxygen. Whereas in the absence of oxygen reductive reactions take place, in the aerobic catabolism oxygen is not only the final electron acceptor but also a co-substrate for some key catabolic processes [1]. In this review we will focus in some recent advances related to the aerobic aromatic degradation pathways.

The genes that encode the enzymes involved in a particular aromatic catabolic pathway, i.e., the catabolic genes, are usually physically associated in operons and/or clusters. Aromatic catabolic genes most often lay adjacent to transport genes, responsible for the up-take of the aromatic substrate, and regulatory genes that encode specific transcriptional regulators which co-evolve along with the enzymes that form the catabolic machinery [1, 4] (Figure 1). The first part of this review will deal with the catabolic, transport and regulatory genes of the aromatic catabolic clusters.

The modern ‘omic’ tools have enabled to investigate the metabolism of aromatic compounds from a systems biology perspective [2, 3]. Thus, the catabolic operons are tightly connected with the global metabolism of the particular recipient cell and they are subject to varied, host-dependent influences. Since many aromatic compounds are not only nutrients but also important chemical stressors for the bacteria, they constitute a nice model system to study different aspects about the evolution/adaptation mechanisms in life systems [4]. On the other hand, aromatic degradation pathways are an important source of metabolic exchange factors and, therefore, they

play a previously unrecognized biological role in cell-to-cell communication (Figure 1). Recent findings regarding all these issues will be presented in the second part of this review.

The catabolic and transport genes

The mechanisms developed by microbial cells to assimilate aromatic compounds were fixed and optimized by natural selection, giving rise to the current enzymes, their organization into functionally separable modules, and to the general trend of a catabolic funnel-like topology. Thus, a wide diversity of aromatics are channeled (activated) via different peripheral pathways to a few key central intermediates that suffer dearomatization and further conversion to intermediary metabolites, such as acetyl-CoA, succinyl-CoA or pyruvate, via some central pathways that are conserved in evolution and function [1]. Following the first metabolic reconstruction of aromatic acids metabolism in *Pseudomonas putida* KT2440 [5], other aerobic aromatic-degrading bacteria have been evaluated at genome-scale, e.g., *Cupriavidus necator* JMP134 and *Burkholderia* spp. [6*], and *Corynebacterium glutamicum* [7].

In the classical aerobic catabolism, the hydroxylation and oxygenolytic cleavage of the aromatic ring is carried out by hydroxylating oxygenases and ring-cleavage dioxygenases, respectively. Most classical aerobic pathways converge to catecholic substrates which undergo either *ortho* or *meta* cleavage by intradiol or extradiol (type I and II) dioxygenases, respectively (Figure 2). However, a number of bacterial degradation pathways generate noncatecholic intermediates, e.g., gentisate, homogentisate, monohydroxylated aromatic acids, *O*-heteroaromatic flavonols, that are subject of ring cleavage by devoted type III extradiol dioxygenases [8]. Ring-cleavage of *N*-heteroaromatic compounds can be carried out by additional types of dioxygenases, e.g. the CO-forming 1-*H*-3-hydroxy-4-oxoquinaldine 2,4-dioxygenase (HOD) and 1-*H*-3-hydroxy-4-oxoquinoline 2,4-dioxygenase (QDO), are homologous metal- and cofactor-independent dioxygenases that possess a classical α/β -hydrolase fold core domain evolved to host and control dioxygen chemistry [9**].

Despite many classical central pathways have been known for long, the genes encoding some of these pathways are still uncharacterized. Some examples of recently uncovered genes clusters

encoding the central pathways for *meta* cleavage of gallate and 2,3-dihydroxybenzoate in *Pseudomonas* strains have been reported [10**, 11].

Aromatic peripheral pathways appeared later in the evolution, are usually more tightly regulated, and show broader substrate specificity than central pathways. The complex polymer lignin was considered an almost exclusive substrate of fungal laccases and peroxidases. Recent works have shown that lignin can be the substrate of bacterial dedicated peripheral pathways releasing low molecular weight phenolic products that finally lead to protocatechuate or some derivatives of the latter [12]. In the biphenyl-degrading *Rhodococcus jostii* RHA1 strain an extracellular Dyp-type peroxidase has been shown to be active for lignin degradation, and it has homologues in a wide range of actinomycetes and α - and γ -proteobacteria (12, 13*). The catabolism of aromatic compounds plays also an essential role in the degradation of some terpenoids, such as steroids and resin acids, that generate an aromatic intermediate during their degradation. Cholesterol degradation has been shown to be critical to the pathogenesis of *Mycobacterium tuberculosis*, and the close similarity between some of the enzymes involved in the catabolism of cholesterol and aromatic-degrading enzymes may facilitate the design of new drugs to deal with tuberculosis [14].

A second aerobic strategy for cleaving the aromatic ring relies on the use of oxygenases, but solely to form a non-aromatic epoxide. In these aerobic hybrid pathways, as in the anaerobic catabolism, all metabolites are activated to CoA thioesters through the action of an initial CoA ligase, the ring cleavage is carried out hydrolytically rather than oxygenolytically, and further metabolism of the non-aromatic CoA thioesters involves β -oxidation-like reactions yielding β -ketoadipyl-CoA, a common intermediate with the conventional β -ketoadipate pathway [1] (Figure 2). The aerobic hybrid pathways are widely distributed among bacteria for the degradation of benzoate (box pathway) (Figure 2) and phenylacetate (paa pathway) [1, 15**, 16]. In some bacteria the classical benzoate degradation pathway coexist with the box pathway, and it was suggested that the latter could be advantageous under less favorable energetic conditions, i.e., low oxygen and benzoate concentrations [1]. Key enzymes of aerobic hybrid pathways are class I di-iron proteins that catalyze the ring epoxidation (dearomatization) of the first intermediate of the catabolic pathway, i.e., benzoyl-CoA and phenylacetyl-CoA from benzoate

and phenylacetate, respectively (Figure 2) [17, 18*]. The multicomponent phenylacetyl-CoA epoxidase (PaaABCDE) not only transforms phenylacetyl-CoA into 1,2-epoxyphenylacetyl-CoA but also mediates an unprecedented NADPH-dependent deoxygenation of the epoxide regenerating phenylacetyl-CoA when oxygen becomes limiting. Presumably, this bifunctionality plays an important biological role to avoid toxic intracellular epoxide levels if the subsequent catabolic steps are impeded [19**]. On the other hand, the two-component benzoyl-CoA epoxidase (BoxAB) converts benzoyl-CoA into 2,3-epoxybenzoyl-CoA, a reaction significantly enhanced by the interaction with the BoxC ring-cleavage dihydrolase (Figure 2) [18*]. As part of a general detoxification strategy, aerobic hybrid pathways have developed salvage mechanisms to avoid the accumulation of CoA-thioesters and depletion of the intracellular CoA-pool, e.g., the PaaI and PaaY thioesterases of the phenylacetate degradation pathway [19**].

The aromatic acid:H⁺ symporters (AAHS) of the major facilitator superfamily are the main family of transporters involved in the uptake of aromatic acids, and they become essential for growth on some aromatic compounds that are easily oxidized and difficult to uptake by passive diffusion, e.g., the GalT permease for gallic acid uptake and chemotaxis in *P. putida* [10]. Genes encoding multicomponent ABC transporters are also commonly found within or close to catabolic clusters, and in some cases they were shown to be involved in the uptake of aromatic acids [20, 21]. In the α -proteobacterium *Rhodopseudomonas palustris*, periplasmic solute-binding proteins belonging to four different ABC-type transporters of aromatic compounds have been biochemically characterized [22*]. Other family of periplasmic solute-binding proteins (the Bug family) has been identified in β -proteobacteria, where they can be involved in aromatic compound detection by tripartite transporters, e.g., phthalates transporters in *Comamonas* strains [23]. Transporters of aromatic compounds in gram-negative bacteria that are adapted to nutrient-poor conditions are often accompanied by nearby specific outer membrane substrate specific channels (porins) that accelerate transport [10**]. The recent structural and functional characterization of some porins of the OccK subfamily in *Pseudomonas aeruginosa*, such as the OccK1 porin that shows high structural similarity to the putative BenF benzoate channel from *Pseudomonas fluorescens* [24], reveal that they mediate an efficient uptake of a substantial number of monocyclic carboxylic compounds and certain medium-chain fatty acids (adipate, octanoate, etc.) [25**].

The regulatory genes

Specific transcriptional factors belonging to a wide range of distinct families of regulators have been recruited and evolved to control the expression of particular aromatic catabolic operons, thus ensuring the production of the enzymes and transporters at the right place and time (Figure 1). Although both transcriptional activators and repressors have been shown to regulate aromatic catabolic pathways, those pathways that use CoA-derived aromatic compounds are mainly controlled by transcriptional repressors that recognize CoA-derived effector molecules [26, 27, 28**]. This observation may reflect that repressors are generally preferred to control low demand genes whose unspecific transcription can decrease the overall fitness of the cell by spending valuable resources, such as CoA and ATP, on futile processes [29]. In some cases, the transcription factors control a set of different functionally related metabolic clusters, e.g., the PhhR regulon that assures the homeostasis of aromatic amino acids in *P. putida* [30].

Some specific regulators have more than one effector binding-pocket, and the cognate effector molecules may have peculiar synergistic effects on transcriptional activation [31]. On the contrary, efficient recognition of molecules (antagonists) that show structural similarity to the inducers (agonists) by certain transcriptional regulators, e.g., the TodS/TodT like two-component regulatory systems, leads to a lack of activation of the target promoter, which may compromise an efficient degradation response when bacteria are exposed to complex mixtures of aromatic pollutants some of which behave as agonists and other as antagonists [32]. To prevent the gratuitous induction by non-metabolizable analogues, some regulatory proteins are coupled to the aromatic degradation enzymes in order to induce gene expression when there is an efficient catabolic flux in the cell. For instance, in the tetralin biodegradation pathway in *Sphingopyxis macrogolita* the activity of the transcriptional activator ThnR is under the control of the *thnY* gene, which has been recruited by the regulatory module and encodes an electron transport component whose redox state may be modified by the ThnA3 ferredoxin of the tetralin dioxygenase [33**].

Some facultative anaerobic bacteria can degrade aromatic compounds either aerobically or anaerobically depending on the presence or absence of oxygen, respectively. The expression of the *box* and *bzd* genes for the aerobic and anaerobic degradation of benzoate in *Azoarcus* sp. CIB

is controlled by the cognate BoxR and BzdR regulators, respectively, which function synergistically to assure a tight repression in the absence of the common intermediate and inducer molecule benzoyl-CoA (Figure 2). The cross-regulation between the aerobic and anaerobic degradation pathways could be an adaptive advantage for cells that drive in changing oxygen environments [28**]. The observed expression of the *box* genes under anaerobic conditions [28**] may constitute an alternative oxygen scavenging mechanism when the cells face low oxygen tensions that could inactivate the highly oxygen-sensitive anaerobic reductase, and also a strategy to rapidly shift to the aerobic degradation if oxygen levels become high.

There is a hierarchical use of aromatic compounds when bacteria grow in mixtures of these carbon sources in the environment, e.g., benzoate is usually a preferred carbon source over 4-hydroxybenzoate. Whereas the 4-hydroxybenzoate transporter (PcaK) has been proposed as the main target of the repression in *Acinetobacter baylyi* and *P. putida*, the 4-hydroxybenzoate hydroxylase (PobA) is the key controlled element in *C. necator*, being benzoate itself the molecule mediating the repression [34]. Interestingly, the aromatic preference profile can change even between closely related strains [35].

The host cell

Genomic, transcriptomic and proteomic studies in *Pseudomonas* spp. have provided new insights into the host-cell response towards the presence/metabolism of aromatic compounds, that involve i) a metabolic response that connect the specific aromatic catabolic pathway with the energetic/biosynthetic metabolism of the cell, and ii) a stress response for protection from the toxic effect of aromatics and adaptation to suboptimal growth conditions (Figure 1). These different types of response are intimately connected and influence each other [4, 36, 37]. Proteomic studies in *C. glutamicum*, *Geobacillus thermodenitrificans* and *Mycobacterium vanbaalenii* growing in the presence of aromatics have shown a common response to these compounds between Gram-negative and Gram-positive bacteria [38, 39*, 40]. Nevertheless, the adaptation to the central carbon metabolism and the stress responses may differ depending on the particular aromatic compound tested, and also on the bacterial cell under study [7, 38].

Some bacteria are able to grow in the presence of high concentrations of organic solvents that impede the growth of most microbes. This extremophile behavior can be attributed to the presence of efficient efflux pumps, that in the case of the solvent tolerant *P. putida* DOT-T1E strain are encoded in the pGRT1 plasmid which contains several other stress resistance genes [37, 41*, 42]. Chemotaxis-related proteins are also up-regulated when bacteria grow in the presence of aromatics [36], suggesting that chemotaxis plays an important role in the degradation of aromatic compounds (Figure 1). The McpT chemoreceptor, encoded also in the self-transmissible pGRT1 plasmid, has been found responsible of an extreme chemotaxis response (hyperchemotaxis) to high concentrations of toluene and several other hydrocarbons, including crude oil [37, 41*]. The hyperchemotactic phenotype was found gene-dose dependent, strongly supporting the notion that gene duplication is an effective mechanism to respond properly to the presence of aromatic compounds [41*]. A more complex chemotactic response that involves three different types of taxis has been described for 2-nitrotoluene (2NT) in *Acidovorax* sp. JS42 [43].

The different host-dependent response programs for the catabolism of aromatic compounds are controlled in a coordinated manner by complex regulatory networks that ensure that the trade-off between metabolic gain and stress endurance imposed by the aromatic compounds is not detrimental to the general cell physiology. Such global regulatory systems govern and adjust the specific regulation of the catabolic operons to the physiological and metabolic state of the cell (Figure 1). For instance, in natural environments some microorganisms display a sequential substrate utilization strategy (carbon catabolite repression, CCR) initiating the catabolism of a preferred substrate and inhibiting the uptake/metabolism of other non-preferred compounds. Usually, the catabolism of aromatic compounds is subject to CCR by other carbon sources such as glucose or low-molecular weight organic acids and amino acids [44]. Interestingly, whereas the cAMP-Crp complex controls CCR of aromatic degradation pathways in *E. coli*, in soil bacteria CCR of aromatic catabolic pathways appear to integrate different regulatory systems, such as those mediated by the Crc global translational repressor, the CyoB terminal oxidase, the phosphorylation-responding PtsN regulator, the (p)ppGpp synthesizing RelA enzyme, and the BphQ transcriptional regulator [44, 45*, 46**, 47, 48]. On the contrary, in *C. glutamicum* the simultaneous utilization of aromatic compounds and other carbon sources has been reported [7].

In some bacteria, such as in *P. putida* strain CSV86, aromatic compounds (and organic acids) are a preferred carbon source over glucose and they act by repressing the expression of glucose transport proteins [49]. Some environmental signals, such as temperature, may modulate the CCR response and help to face cold stress. Thus, in *P. putida* a new regulatory link between sRNAs, temperature and Crc-dependent CCR in the metabolism of benzoate has been shown [50*].

Although the construction and analysis of genome-scale metabolic models (GEMs) suppose a step forward to comprehensive understanding of metabolic processes, few examples of these strategies to analyze the aromatic degradation potential of a cell are available thus far [51], and they have focused in *P. putida* [52, 53]. Since the metabolism of aromatic compounds is a multifactorial feature involving metabolic and regulatory networks (Figure 1), its systems understanding requires a multilayer approach. In an attempt to merge regulatory and metabolic networks in the same biological system, a *boolean* formalism, which adopts binary logic gates for describing both regulatory and metabolic actions, has been successfully applied to capture with high accuracy the behavior of the *m*-xylene catabolic pathway driven by the TOL plasmid in *P. putida* [54, 55*].

Cell-to-cell communication

Degradation of aromatic compounds in the ecosystem is usually accomplished by microbial consortia where syntrophic interactions between species involve interchange of byproducts. Function-driven metagenomic approaches have also highlighted the role of microbial consortia in biodegradation [56]. The existence of syntrophic interactions between genetically identical but phenotypically different cell subpopulations from the same bacterial culture has been shown in the metabolically versatile *R. palustris* species growing in benzoate and *p*-coumarate, and it resembles the behavior of the different tissues in a multicellular organism [57].

An emerging and previously unrecognized biological function for the aromatic catabolic pathways is the production of secondary metabolites or metabolic exchange factors, some of which are critical for establishing complex microbial interactions and as initiators of

multicellular behavior in monospecies bacterial communities, triggering a cell-to-cell communication response (Figure 1). Quorum sensing (QS) involves the production of chemical signals, e.g., acylhomoserine lactones (AHL) and alkyl-quinolones in gram-negative bacteria, which coordinate global gene expression in a population density-dependent manner [58]. The catabolism of aromatic compounds plays a relevant role in the synthesis and degradation of such QS signals, and itself is also subject to QS regulation (Figure 3). In *P. aeruginosa*, the kynurenine pathway for tryptophan degradation generates anthranilate, which is a pivotal branch-point metabolite that can be directed for energy metabolism via the ant-cat pathway or into biosynthetic routes such as the *Pseudomonas* quinolone signal (PQS) synthesis pathway. The flux of anthranilate into the diverging pathways is tightly controlled by transcriptional and posttranscriptional regulatory mechanisms as well as by AHL-based signaling systems that depend on LuxR-type signal receptors. A new LuxR-independent AHL gene regulation of the *ant* and *cat* operons has been reported via the control of the specific *antR* regulatory gene [59*]. Certain metabolites or enzymes from aromatic degradation pathways may achieve attenuation of QS (quorum quenching). Thus, non-toxic doses of protoanemonin, an antibiotic generated by misrouting during the aerobic degradation of some chloroaromatic compounds via the classical β -ketoadipate pathway, are capable of inhibiting QS, opening an interesting new function for this molecule present in active concentrations in certain pseudomonads-dominated consortia [60*]. On the other hand, some aromatic ring-cleavage dioxygenases behave as quorum quenching enzymes, e.g., Hod has the ability to cleave PQS, an unprecedented function for these key aromatic degradation enzymes [61] (Figure 3).

There is a large number of additional metabolic exchange factors other than quorum-sensing signals, e.g., siderophores, surfactants, indole (Figure 3), and sublethal concentrations of antibiotics, that act as intraspecies and interspecies signals [58]. In the aerobic catabolism of phenylacetate different bioactive compounds and communication signals can be generated (Figure 3). Thus, an oxepin-CoA derivative can be used for the synthesis of ω -cycloheptyl fatty acids and is also the precursor of tropodithietic acid (TDA) and related tropone derived compounds with broad antibacterial activity [62, 63**]. The phenylalanine/phenylacetate degradation pathway's versatility has been also exploited by some algal symbionts, e. g, the roseobacter *Phaeobacter gallaeciensis*, to shift from health-promoting to an opportunistic

pathogen of its algal host [64**] (Figure 3). Some tropone-related molecules, such as TDA, have been shown to act also as QS signals in roseobacters [65]. In *P. gallaeciensis*, redundancy of different pathways that lead to the formation of phenylacetyl-CoA highlight the physiological relevance of this compound as a precursor for tropone-related molecules under different nutritional and physiological conditions [62]. Certain pathogens, like *Burkholderia cenocepacia*, use also the phenylacetate degradation pathway as a source of compounds that are part of the pathogen's arsenal against host cells, which may explain the increasing evidence for the phenylacetyl-CoA multicomponent oxygenase as a virulence factor [66]. Engineering the early phenylacetate catabolic pathway enzymes to efficiently produce TDA and related bioactive compounds is a straightforward biotechnological application that can be derived from the basic studies of the pathway [63**].

Pathway evolution and computational design of novel pathways

Bacteria that dwell in polluted environments are often capable to evolve, from pre-existing pathways that cope with natural compounds, novel enzymes and regulators for the degradation of anthropogenic (xenobiotic) analogues that have been in the biosphere for only a few years but whose toxic and mutagenic character impose a strong selective pressure [67**]. In *Acidovorax* sp. JS42, the key initial dioxygenase and its LysR-type transcriptional regulator (NtdR) involved in the degradation pathways of synthetic nitroaromatics, e.g., 2-nitrotoluene (2NT) and 2,4-dinitrotoluene (2,4DNT), appear to evolve from a previously existing naphthalene degradation pathway. Using long-term laboratory evolution experiments, mutants in the initial dioxygenase were obtained that gained the ability to grow on 4-nitrotoluene (4NT) but did not lose the ability to grow with nitrobenzene or 2NT [68**]. In *Burkholderia* sp. DNT, the regulation of the 2,4DNT degradation pathway is in an earlier stage of evolution since the NtdR regulator still recognizes salicylate, an effector of its NagR-like ancestor, but does not respond to 2,4DNT. That a useless but still active transcriptional factor occurs along enzymes that have already evolved a new substrate specificity points to the fact that the emergence of novel catalytic activities precedes the setting of a specific regulatory device for their expression, not vice versa, shading some light to the chicken-and-the-egg dilemma between regulators and enzymes that recognize the same compounds [67**]. The evolution of transcriptional regulators has been also

assessed by *in vitro* experimental evolution/selection setups. For instance, the XylR regulator from *P. putida* was evolved first to an effector-promiscuous variant and then to a more specific regulator where the natural response to *m*-xylene was decreased and the non-native acquired response to the synthetic 2,4-DNT was increased. The new XylR28 version may be used to develop more efficient 2,4-DNT responsive reporter systems to engineer whole cell biosensors for explosives [69].

The promiscuity or specificity of inducer recognition might be also tuned in a regulatory network just by changing the promoter architecture and without requiring the evolution of new transcription factors with altered inducer specificity, e.g., the 3-methylbenzoate dependent induction of the *ben* operon for benzoate degradation [70], or the participation of some global regulators in the activation of certain promoters, e.g., the ppGpp/DksA independent co-stimulation of the *dmpR* regulatory gene that controls phenol degradation [71] in *P. putida*.

Computational approaches are being also used to design novel aromatic catabolic pathways. The ongoing characterization of new catabolic pathways requires of well-curated and continuously updated databases such as the University of Minnesota Biocatalysis/Biodegradation Database (UM-BBD) (<http://umbbd.ethz.ch/>) [72]. The Pathway Prediction System (PPS), Chemical similarity (PathPred), and rules derived from the Enzyme Commission (EC) classification system (BNICE) are examples of computational efforts to predict new biodegradation pathways [73, 74]. However, limitations to this approach include the need to evaluate the feasibility of the new pathways obtained as well as their subsequent implementation and *in vivo* testing. In an interesting example, the feasibility of novel biodegradation pathways for 1,2,4-trichlorobenzene predicted by BNICE was evaluated by using the genome-scale model of *P. putida*, and the subset of new pathways more suitable to be engineered into this host organism was identified [75**]. These computational approaches, together with the recent advances in the synthetic DNA technology and genome-scale metabolic engineering, will facilitate the design *à la carte* of novel strain-specific biodegradation pathways in the years to come (Figure 4).

Conclusions and future prospects

The advent of omic age has allowed a broader view of true bacterial potential towards the aerobic degradation of aromatic compounds, unraveling new and unsuspected catabolic pathways, and showing that this ability is more widespread than previously thought. However, there are a number of exciting issues that still require further studies, e.g., the role of genes of unknown function present in aromatic gene clusters, the exploration of the degradative capabilities of non-cultivable bacteria (metagenomics), and the role of auxiliary proteins integrating aromatic catabolic pathways with other cellular processes, among others.

While metabolism is relatively well conserved in different organisms, regulation shows a wider diversity and, therefore, the whole understanding of the regulatory network of a given organism is a challenging task. The physiological relevance of environments and/or metabolic perturbations, that may hinder the efficient expression of catabolic genes when bacteria are exposed to complex mixtures of aromatic pollutants, the potential cross-regulation between classical and hybrid pathways, the ecophysiological meaning of the diversity found in the regulation of the hierarchical utilization of aromatic compounds among closely related strains sharing ecological niches, and a more complete view of the molecular mechanisms underlying CCR in bacteria, are some regulatory aspects that should be explored further.

Aromatic degradation pathways are also an important source of metabolic exchange factors and, therefore, they play a previously unrecognized biological role in cell-to-cell communication. The role of quorum sensing in controlling aromatic catabolic pathways and the latter as a source of metabolic signals and/or quorum quenching mechanisms that modulate bacterial communication in microbial communities, are interesting topics that should be addressed and that point to the aromatic degradation pathways as possible targets for drug development.

From a biotechnology point of view, systems and synthetic biology approaches, which allow the integration of metabolic and regulatory networks as well as the prediction and design of novel strain-specific biodegradation pathways, will be a further step towards a more rational design of biocatalysts. The *in vitro* evolution of new enzymes and regulators is also an interesting way to track the evolutionary roadmap of these proteins and to engineer new synthetic

pathways/regulatory circuits. Advances in programming multicellular-like traits in microbial populations by using custom-made genetic systems, and engineering synthetic microbial consortia are also new approaches that should be accomplished.

In summary, a deeper understanding of the aromatic metabolism will pave the way for the forward engineering of bacteria as efficient biocatalysts for bioremediation of chemical waste and/or biotransformation to biofuels and renewable chemicals, for detection of toxic molecules (biosensors), and for biomedical applications.

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Figure legends

Figure 1. Scheme of the major genetic/biochemical elements and cell responses associated to the bacterial catabolism of aromatic compounds. The catabolic (C_A - C_D), transport (T) and specific regulatory (R) genes that constitute the aromatic catabolic cluster (grey box) are indicated in blue, grey and red arrows, respectively. Auxiliary genes (A) are those host-encoded outside the catabolic cluster and that are also necessary for an efficient biodegradation process. The degradation of aromatic compounds generates three main types of responses in the host cell, i.e., a metabolic response (blue), a stress response (violet) and a cell-to-cell communication response (green). The regulatory and metabolic elements involved in the biodegradation process are organized in complex networks (orange and green ovals) which, in turn, are interconnected and influence each other.

Figure 2. Scheme of the main biochemical strategies to degrade benzoate in bacteria. Benzoate can be aerobically catabolized following two major strategies, i.e., a classical aerobic biodegradation pathway (A) and an aerobic hybrid pathway (B). In both strategies, an activation step (blue), dearomatization/ring-cleavage step (red) and further degradation to central metabolites, i.e., lower pathway step (green), can be identified. The *ortho* cleavage of catechol (β -ketoadipate central pathway) and the benzoyl-CoA hybrid pathway converge into the common β -ketoadipyl-CoA intermediate. On the other hand, the anaerobic degradation of benzoate shares a similar initial reaction with the aerobic hybrid pathway catalyzed by a benzoate-CoA ligase, but then a strict anaerobic ring-reduction step catalyzed by a devoted CoA reductase and further β -oxidation like reactions lead to central metabolites (orange arrows). Abbreviations are: OX, ring-hydroxylating oxygenase; DH, dihydrodiol dehydrogenase; DOX, ring-cleavage dioxygenase; EX, aryl-CoA epoxidase.

Figure 3. The metabolism of aromatics influences cell to cell communication. A) Some bacterial strains produce quorum sensing signals, such as 2-heptyl-3-hydroxy-4-quinolone (PQS), or other metabolic exchange factors, such as indole, by the action of the Pqs enzymes during the catabolism of tryptophan (Trp) via anthranilate (AA), or through the enzyme tryptophanase (TnpA), respectively. As a result, cellular communication in the community is promoted (green cells and arrows). However, communication can be quenched (red cells and arrows) by

inactivation of the signals (red hammers). Indole can be removed through degradation pathways or by oxidation to indigoid compounds via some unspecific ring hydroxylating oxygenases (e.g., toluene-*o*-monooxygenase). PQS could be inactivated by the action of some ring-cleavage dioxygenases (e.g., *Hod*). Some antibiotics, e.g., protoanemonin, have been shown to behave also as quorum quenching factors. B) *Phaeobacter gallaeciensis* (yellow) waxes and wanes with the algae *Emiliana huxleyi* (green) displaying a mutualistic behavior (green arrows). When *E. huxleyi* is healthy, it provides dimethylsulfoniopropionic acid (DMSP) and a biofilm surface to the bacteria, which in turn secrete the auxin phenylacetic acid (PA) that promotes algal growth. Some intermediates produced in the catabolism of PA, such as oxepin-CoA (OCoA), are used to produce the broad range antibiotic tropodithietic acid (TDA) that prevents external bacterial aggressions. However, when *E. huxleyi* senesces it releases degradation products, like *p*-coumaric acid (pCA), that induce *P. gallaeciensis* to synthesize the algacide roseobacticide (RB) by using phenylacetyl-CoA (PACoA) and OCoA as precursors, and thus the bacteria turn into pathogens (red arrows and cells).

Figure 4. Overall workflow for *the novo* engineering of aromatic degradation pathways. Strictly computational or experimental steps are shown in a single color blue or orange, respectively while steps requiring both approaches are indicated in blue and orange. Many pathway prediction methods based on chemical reaction rules and/or information from biochemical databases can be applied to generate a set of novel degradation pathways for a particular compound, e.g., a non-biodegradable xenobiotic. To select the most suitable pathways, several criteria can be applied, e.g., thermodynamic feasibility, number of chemical transformations required to connect the target aromatic with the central metabolism of the host cell (pathway distance), and performance within the context of cellular environment by using genome-scale metabolic models (GEMs), among others. The selected pathway(s) are further implemented by identification of the gene families encoding the required biochemical transformations and up-take in the cell, and by optimizing their substrate specificity and production. An additional level of optimization including the design of a synthetic regulatory network would be desirable for a well-balanced expression in the host strain. The selected design(s) is then synthesized and expressed in the host strain. Finally, the new synthetic network is evaluated and optimized *in vivo* through different

approaches e.g., high-throughput data contextualization by using GEMs and Adaptive Laboratory Evolution (ALE) experiments.

Figure 1

Fig. 1

Figure 2

Fig. 2

Figure 3

A

B

Fig. 3

Figure 4

Fig. 4.