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Bioremediation 3.0: Engineering pollutant-removing bacteria in the times of systemic biology

by

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Abstract

Elimination or mitigation of the toxic effects of chemical waste released to the environment by industrial and urban activities relies largely on the catalytic activities of microorganisms—specifically bacteria. Given their capacity to evolve rapidly, they have the biochemical power to tackle a large number of molecules mobilized from their geological repositories through human action (*e.g.*, hydrocarbons, heavy metals) or generated through chemical synthesis (*e.g.*, xenobiotic compounds). Whereas naturally occurring microbes already have considerable ability to remove many environmental pollutants with no external intervention, the onset of genetic engineering in the 1980s allowed the possibility of rational design of bacteria to catabolize specific compounds, which could eventually be released into the environment as bioremediation agents. The complexity of this endeavour and the lack of fundamental knowledge nonetheless led to the virtual abandonment of such a recombinant DNA-based bioremediation only a decade later. In a twist of events, the last few years have witnessed the emergence of new *systemic* fields (including systems and synthetic biology, and metabolic engineering) that allow revisiting the same environmental pollution challenges through fresh and far more powerful approaches. The focus on contaminated sites and chemicals has been broadened by the phenomenal problems of anthropogenic emissions of greenhouse gases and the accumulation of plastic waste on a global scale. In this article, we analyze how contemporary systemic biology is helping to take the design of bioremediation agents back to the core of environmental biotechnology. We inspect a number of recent strategies for catabolic pathway construction and optimization and we bring them together by proposing an engineering workflow.

1. Introduction

Increasing pollution of air, soils, ground and surface waters constitutes a major threat to public health both in developing countries as well as in industrialized countries including EU states, the USA, India and China. The majority of contaminants that affect soils and waters are heavy metals and organic compounds such as mineral oil hydrocarbons, polyaromatic hydrocarbons, benzene derivatives, and halogenated hydrocarbons. Many of organic polluting compounds for agricultural (the pesticides dichlorodiphenyltrichloroethane, atrazine, and pentachlorophenol), industrial (solvents such as dichloroethane or dielectric fluids such as polychlorinated biphenyls) or military use (explosives such as 2,4,6-trinitrotoluene) are xenobiotics of anthropogenic origin. There is also a spectrum of so-called *emerging contaminants* (**Table 1**), *i.e.*, substances long present in the environment whose presence and negative effects have only recently been recognized (Petrie et al., 2015). The list can be further broadened with petroleum-derived plastics and some chemicals originally considered to be *green*, including certain types of bioplastics or ionic liquids (Amde et al., 2015). Despite the recalcitrant nature of some of these polluting compounds, many are more or less susceptible to biodegradation (Alexander, 1999). In addition to these traditional causes of environmental deterioration, the recent decades have witnessed the onset of ramped-up levels of anthropogenic emissions of CO_2 and other greenhouse gases and their ensuing impact on climatic change. Whereas the chemicals themselves are simple (CO_2, CH_4, N_2O), the challenge here is less their biodegradation than their recapture in a non-gaseous form.

The major entity that causes large-scale transformations in the biosphere are microorganisms and their metabolic pathways. Microbes degrade toxic chemicals *via* complete mineralization or co-metabolism, in aerobic or anaerobic conditions. Advantageous properties such as small genome size, relative simplicity of the cell, short replication times, rapid evolution and adaptation to the new environmental conditions made microbes, and particularly bacteria, favourable candidates for bioremediation technologies, that is *in situ* or *ex situ* removal of polluting chemicals from the environment using biological agents. The removal of environmental pollution caused by the extensive activities of industrial society is a serious topic that draws the attention of biotechnologists. This is because beyond the medical and environmental consequences, the situation signs considerable potential for growth of eco-industry focused on clean-up technologies and removal of environmental contaminants. In fact, valorization of waste chemicals accumulating in industry is one of the pillars of the circular economy and the 4th Industrial Revolution (Schmidt, 2012; Wigginton et al., 2012).

Table 1 Emerging contaminants.

Groups of products	Classes of chemicals	Examples
Human and veterinary pharmaceuticals	Antibiotics, anti-parasitic agents, ionophores	Amoxicillin, erythromycin, metronidazol, tetracycline, lincomycin, sulfathiazole
	Stimulants and drugs including anti-inflammatory, anti-diabetic, anti-epileptic, anti-hypertensive, or anti-cancer drugs, anticoagulants, hallucinogens, analgesics, β-blockers, anti-depressants, lipid regulators, or erectile dysfunction drugs	Amphetamine, cocaine, caffeine, nicotine, propranolol, ibuprofen, codeine, carbamazepine, bezafibrate, metformin, fluoxetine, warfarine, valsartan, tramadol, morphine, methandone, diazepam, ephedrine, tamoxifen
	Hormones including natural and synthetic estrogens, androgens	Estrone, estriol, testosterone, progesterone, mestranol (ovulation inhibitor), cholesterol
Industrial and household wastewater products	Insecticides, plasticizers, detergents, flame retardants, polycyclic aromatic hydrocarbons, antioxidants, solvents, disinfectants, fumigants, fragrances, preservatives	Carbaryl, chlorpyrifos, diethylphtalate, *p*-nonylphenol, tri(2-chloroethyl)phosphate, naphtalene, anthracene, 2,6-di-*tert*-butylphenol, 1,2,3-trichloropropane, phenol, 1,4-dichlorobenzene, acetophenone
Personal care products	Insect repellents, polycyclic musks, sunscreen agents, fragrances, antiseptics	Bisphenol A, 1-benzophenone, methylparaben, *N*,*N*-diethyltoluamide, triclosan
Nanomaterials	Miscelaneous	Nanosilver, alumina nanoparticles, titanium dioxide, fullerenes, carbon black

Sources: http://toxics.usgs.gov, http://www.eugris.info (Petrie et al., 2015)

The earliest attempts at directed bioremediation, although not formalized as such at the time, dated back to the late 19th century with the origins of the first wastewater treatment plants (Litchfield, 2005). Bioremediation began in earnest some 45 years ago with the isolation of culturable bacteria from contaminated sites and studying their degradation pathways. The first report on enhanced *in situ* bioremediation of soil contaminated with peroleum-derived hydrocarbons was published in 1975 by Raymond and co-workers (Raymond et al., 1975). Natural microbial degraders were later applied with success in world-wide and local biotechnological processes including large-scale wastewater denitrification, uranium removal, and degradation of 1,2-dichloroethane from groundwater or the organophosphorus pesticide coumaphos from cattle-dip waste (Francis and Mankin, 1977; Lovley et al., 1991; Mulbry et al., 1998; Stucki and Thueer, 1995). The advent of technologies for pollutant removal using naturally emerging microorganisms could be called the era of *Bioremediation 1.0*. Even so, a number of specific chemicals, especially of anthropogenic origin, including persistent organic pollutants such as dichlorodiphenyltrichloroethane (DDT), trichloroethylene, 1,2,3-trichloropropane, some polychlorinated biphenyls (PCB) or dioxins continued to be resistant

to natural biodegradation due to lack of efficient microbial catabolic traits whose evolution was not sufficiently rapid or ended in a deadlock (Janssen et al., 2005).

Initial discoveries in molecular biology and progress in biological engineering disciplines seemed to provide a partial solution for such challenges through rational interventions in the metabolic networks of selected microbial hosts. The rise of recombinant DNA technology allowed the transformation of bioremediation from empirical practice into an excercise in genetic engineering, giving rise to what we might term *Bioremediation 2.0*. The goal of the new field was to engineer whole microbes, their biodegradation pathways, and the corresponding enzymes toward *in situ* mineralization of target pollutants. Such *superbugs* were expected to provide an economically feasible, environmentally friendly alternative to the costly conventional technologies for pollutant removal available at the time (Ramos et al., 2011). The late 1980s and early 1990s represented the golden era of biodegradation research, with numerous engineering attempts following the pioneering work by Chakrabarty and co-workers (Kellogg et al., 1981). They described the preparation of recombinant *Pseudomonas putida* strains able to break down crude oil by the *plasmid-assisted molecular breeding*, that is, propagation of novel catabolic capabilities through directed bacterial conjugation and plasmid transfer. The persistence of many xenobiotics was attributed mainly to the absence of complete degradative pathways in a single organism (Brenner et al., 1994; Reineke and Knackmuss, 1979). Recruitment of complementary enzyme sequences by conjugative gene transfer and so called *patchwork assembly* of several existing natural pathways in a suitable host was believed to generate functional synthetic routes that would allow for the complete mineralization of persistent target compounds such as PCB (Lehrbach et al., 1984; Ramos et al., 1987; Rojo et al., 1987).

Despite some success with the *patchwork* strategy and engineering of *superbugs* with extended substrate scope in laboratory conditions, this initial and rather naïve approach led to many disappointments as well (Cases and de Lorenzo, 2005; de Lorenzo, 2009). A prominent example was the case of engineered *Pseudomonas* strains that did not grow on 2-chlorotoluene as the only carbon source, even though they possessed all the genetic components presumed necessary for substrate mineralization (Haro and de Lorenzo, 2001). From a contemporary perspective, such failures can be explained by lack of insight into important factors such as: (i) thermodynamic feasibility of assembled catabolic networks, (ii) kinetic characteristics of enzymes and physicochemical properties of metabolites, (iii) expression levels of pathway modules, (iv) cross-talk between exogenous and endogenous metabolic routes, and (v) stress responses and changes in overall host cell physiology after introduction of new metabolic modules and exposure to toxic substrates and metabolites (de Lorenzo, 2009; Ramos et al., 2011).

Fortunately, the last decade has witnessed the onset of what can be called *systemic biology*, which merges different approaches of systems biology, metabolic engineering, and synthetic biology, for the sake of understanding and reprograming biological systems. Systemic biology has the potential to remove the unknowns and bottlenecks encountered in past trials and paves the way towards the era of *Bioremediation 3.0*. The joint power of the systemic biology disciplines can ensure that biodegradation and bioremedation using genetically modified microorganisms will remain a vital concept deserving of the full attention of new generations of bioengineers.

In this article we review the applications of novel engineering strategies to the design and evolution of microbial biodegradation pathways and whole-cell degraders from the last

decade, and propose an optimal workflow for pathway design, construction and optimization. In particular, we discuss the potential of state-of-the-art *systemic* technologies not yet fully employed for this purpose including new ways to genetically engineer superior CO_2 scavengers. Lastly, the perspectives of microbial cell factories tailored for biodegradation and bioremediation are critically evaluated.

2. Approaches and tools of systems biology and metabolic engineering for tailoring biodegradation pathways

One key objective of systems biology is to gain comprehensive, quantitative understanding of living cells by combining high-throughput technologies and computational methods to characterize and predict cell behaviour (Dai and Nielsen, 2015). Metabolic engineering, first defined as a new scientific discipline by James E. Bailey in 1991 (Bailey, 1991), is now understood as the practice of optimizing genetic and regulatory processes within the cells to (i) improve the yield and productivity of native products synthesized by organisms, (ii) establish the synthesis of products new to the host cell, and, two points especially relevant for biodegradation, to (iii) extend the range of substrates or improve substrate uptake, and (iv) improve overall cell robustness (Nielsen et al., 2014). These goals can be achieved by engineering natural metabolic pathways in the host cell or synthetic routes assembled from enzymes originating from different organisms. The aims of systems biology coincide fully with the objectives of metabolic engineering. These two disciplines are now inseparable, complement each other, and have even merged into a field of systems metabolic engineering (Lee et al., 2012). Metabolic engineers use systems biology computational tools and 'omic' techniques to gain deeper insight into the genetic and physiological background of target organisms, to model enzymatic reactions and to determine the constraints for efficient biocatalysis. To overcome these constraints, computational tools are applied together with established experimental protocols, now frequently strengthened with synthetic biology standards and fine-tuning adaptive laboratory evolution. The *Design-Build-Test-Analyze* (DBTA) cycle is repeated until performance of the engineered cell factory is optimized and a cost-effective process can be established (Paddon and Keasling, 2014). It is tempting and logical to use the same intellectual workflow to engineer biodegradation pathways (**Fig. 1**). The portfolio of systems metabolic engineering tools applicable for such purposes will be discussed in more detail in the Section 2 of the review.

Figure 1 here

2.1 Step 1: Get to know the contaminant and find a suitable catabolic pathway
The compound to be degraded or removed from the contaminated environment or industrial waste site is usually the very first component known in a project focused on biodegradation or bioremediation. The polluting chemicals show diverse physicochemical properties, can occupy heterogenous physical niches in the environment, and exist in concentrations ranging from ng/L to mg/L (Meckenstock et al., 2015). Industrial waste chemicals, prohibited pesticides, or warfare agents such as 1,2,3-trichloropropane, γ-hexachlorocyclohexane, or yperite, respectively, can be available for degradation as stock piles or mixed with environmental matrix. When bioremediation is a method of choice for removal of the target compound, another challenge arises: how to find the most suitable pathway for degradation and, ideally, complete mineralization of the chemical in the huge amount of genetic, biochemical and microbiological data available. As pointed out by Nobel laureate Sydney Brenner, we are currently "...drowning in a sea of data, thirsting for knowledge...". New computational tools and algorithms, combined with common sense, are the only way to cope with the complexity of life, find the needle in a haystack, and obtain valuable output – the

natural or synthetic pathway or set of pathways most suitable for a specific biodegradation task in a selected microbial host. Computational tools can provide the user with relevant information on physicochemical properties of the target compound and the basic building blocks of the catabolic pathway – enzymes and metabolites. A wide spectrum of databases and prediction systems that provide useful information on a number of chemicals and biodegradative or biosynthetic routes has been developed over the years. Comprehensive reviews that focus on evaluation of computational tools mainly for biosynthetic pathway design have been published in the last few years (Long et al., 2015; Medema et al., 2012). Chemical and biodegradation databases as well as pathway and toxicity prediction systems that might be useful for evaluating existing biodegradation pathways or engineering new ones were reviewed by Arora and co-workers (Arora and Bae, 2014). To avoid duplication, here we aim to discuss mainly the updates and applications of representative systems for biodegradation pathway design.

2.1.1 Databases

The University of Minnesota Biocatalysis/Biodegradation Database and Pathway Prediction System (UM-BBD/PPS) is a remarkable tool that has garnered microbial pathways for xenobiotics for over 20 years and can be considered an advisable first-choice search engine (Gao et al., 2010). In 2014, UM-BBD/PPS rights were assigned to Eawag, the Swiss Federal Institute of Aquatic Science and Technology and the database got a new name EAWAG-BBD/PPS. EAWAG-BBD (http://eawag-bbd.ethz.ch) can not only search through 543 microorganism entries, 219 metabolic routes, 1503 reactions, 1396 chemicals, and 993 enzymes, but also allows prediction of novel biotransformations leading from target xenobiotic substrate to the molecule that can be metabolized by central catabolic pathways of a host cell. The enviPath tool (The Environmental Contaminant Biotransformation Pathway Resource; https://envipath.org/) was recently introduced as a rebuilt version of EAWAG-BBD/PPS (Wicker et al., 2016). Registered user can employ enviPath for design of particular biochemical routes and personal databases with biotransformation data as well as for prediction of new catabolic pathways. In 2017, the same team introduced a new database implanted in enviPath platform, Eawag-Soil (Latino et al., 2017). This public database is a unique repository of data collected during laboratory simulations on aerobic degradation of pesticides in soil, including biotransformation half-lifes.

Although they provide access to much more data in addition to biodegradation pathways and related content, MetaCyc and BioCyc databases by SRI International should be mentioned as well (Caspi et al., 2016). With its 2526 experimentally elucidated pathways from 2844 different organisms from all domains of life, MetaCyc (https://metacyc.org/) is one of the largest repositories of metabolism data and serves primarily as an on-line encyclopedia in which metabolic routes can be predicted from available sequenced genomes, browsed through, or mutually compared. The majority of listed compounds and reactions now also include the Gibbs free energy values (Caspi et al., 2016). BioCyc (https://biocyc.org/) is a collection of 9389 organism-specific Pathway/Genome Databases (PGDB; the number tripled in the last three years!). Each PGDB encompasses the sequenced genome and predicted metabolic network of single organism. Moreover, information on individual components such as predicted operons, metabolites, enzymes and their reactions, or transport systems is provided (Caspi et al., 2016). BioCyc also provides several useful tools for navigating, visualizing, and analyzing the PGDB, and for analyzing omics data. Both MetaCyc and BioCyc are linked to numerous other databases. UniProt (www.uniprot.org/), a collaboration between the European Bioinformatics Institute, the SIB Swiss Institute of Bioinformatics and the Protein Information Resource, supplies missing information on function, sequence, or

taxonomy of proteins from metabolic or signalling pathways and is further interconnected with more specific protein databases such as RCSB Protein Data Bank or BRENDA, thus completing the picture with structural or kinetic data (Berman et al., 2000; Chang et al., 2015; UniProt Consortium, 2015).

2.1.2 Pathway prediction systems and toxicity prediction algorithms

The development of reliable pathway prediction systems is of major importance for the field of biodegradation as for the numerous anthropogenic chemicals released into the environment the catabolic pathways are either completely unknown or poorly understood. These tools consider many factors including substrate specificities, binding sites or reaction mechanisms of enzymes, structural changes in substrate-product pairs and pathway distance from substrate to product (Medema et al., 2012). The pathway prediction system of EAWAG-BBD/PPS or enviPath, predicts biodegradation routes based on available biotransformation rules derived from reactions found in the EAWAG-BBD database or in the literature (Gao et al., 2010; Wicker et al., 2016). It can highlight initiating reaction types likely to occur in aerobic environments, which in some cases lead to the complete mineralization of the contaminant (**Fig. 2**), although, it does not define the thermodynamic feasibility of the proposed pathways or the specific enzymes that catalyse proposed reactions. This latter information can be supplemented by the complementary prediction algorithm of a new public database RAPID (http://rapid.umn.edu/rapid/), currently being developed by the group of Lawrence P. Wackett at University of Minnesota. The idea of future utility of combined EAWAG-BBD and RAPID algorithms was provided recently by Aukema and co-workers who used these tools to predict initial metabolism of a set of emerging contaminants that include recalcitrant pharmaceuticals, alkyl phthalates, or fragrance compounds previously shown to be degraded by *Paraburkholderia xenovorans* LB400 (Aukema et al., 2016).

In case of anthropogenic contaminants with unknown or uncomplete natural catabolic pathways, the Biochemical Network Integrated Computational Explorer (BNICE.ch) can be recommended as well (Hatzimanikatis et al., 2005). The authors from the Vassily Hatzimanikatis' group at the Swiss Federal Institute of Technology have been developing BNICE.ch, one of the first tools for uncovering and describing new enzymatic reactions based on known biochemistry, for more than 15 years. In the latest version, the method was coupled with a manually-curated KEGG database, generally recognized as one of the most complete repositories of metabolic data (Kanehisa et al., 2014). The catalogued biochemistry of the KEGG database was converted into 361 bidirectional generalized rules which were applied to explore the possible space of all metabolic reactions that link the compounds reported in the KEGG and are potentially found in nature (Hadadi et al., 2016). As a result, 6,528 KEGG reactions and 137,416 known or completely new enzymatic reactions between two or more KEGG compounds were organized in a reaction web-based database named ATLAS of Biochemistry (http://lcsb-databases.epfl.ch/atlas/). Importantly, the thermodynamic feasibility of *de novo* generated reactions was evaluated and the parameter of Gibbs free energy is a part of the majority of records in the ATLAS database. To the benefit of the reaction prediction system, according to authors, up to 80% of the newly added KEGG reactions in 2015 already existed in ATLAS as novel reactions.

In biodegradation pathway design, BNICE.ch was first used by Finley and co-workers to propose all possible catabolic routes for 4-chlorobiphenyl, phenanthrene, γ-hexachlorocyclohexane, and 1,2,4-trichlorobenzene (1,2,4-TCB), compounds that represent various classes of xenobiotics (Finley et al., 2009). BNICE.ch not only reproduced the

experimentally verified pathways, but also found completely novel reactions, taking into account the starting compound, the requested length of the pathway and the broader reaction rules of the Enzyme Commission classification system. The 15 novel pathways for 1,2,4-TCB were subsequently probed using thermodynamic analysis and found energetically feasible. In the follow-up study, the authors evaluated the new routes in the context of *Pseudomonas putida* KT2440 cellular metabolism (Finley et al., 2010). They expanded the available *P. putida* metabolic model by including the pathways obtained from BNICE.ch and metabolic flux analysis was used to predict the maximum biomass generated using 1,2,4-TCB as the sole carbon source. In this way, interesting alternative pathways were proposed that couple 1,2,4-TCB utilisation with biomass formation. Although this work was purely theoretical, it suggests the way for those who wish to use the increasing power of computational modelling to design biodegradation routes or explore the fate of xenobiotics in the environment.

Figure 2 here

Prediction tools for assisting the design of robust microbial biosensors are highly desirable as well. An interesting approach to engineering new effector specificities in *e.g.*, transcription factors was presented recently by Delépine et al. (2016). Their SensiPath algorithm (http://sensipath.micalis.fr/) stems from the concept of retrosynthesis introduced previously by the same laboratory (Carbonell et al., 2014). SensiPath applies more than 9,000 unique reaction rules collected from BRENDA, Metacyc, and Rhea (Morgat et al., 2015) databases to find enzymes (currently limited to max. two-step reactions) able match given organic chemicals to (known) small-molecule sensing system. In the theoretical part of the follow-up study published by Libis *et al.*, the authors applied SensiPath to expand the range of thereby detectable compounds among chemical structures collected from several databases (Libis et al., 2016). The method almost tripled (from 169 to 477) the number of theoretically detectable compounds in the TOX21 database (Krewski et al., 2009). The predictive power of the method was validated in the experimental part of the study. Metabolic modules of up to two enzymes proposed by SensiPath was introduced into *E. coli* bearing a sensing module for the product of enzymatic conversion at stake. Functional whole-cell biosensors for cocaine, nitroglycerin, or parathion were constructed in such way. Despite this remarkable contribution, the number of undetectable organic chemicals remains at a high > 94 % of all compounds in TOX21 database. Strengthening the computational part of tools such as SensiPath will hopefully improve the situation in the near future.

The predicted reaction networks can also be pruned using toxicity estimation algorithms, to prevent the formation of compounds highy toxic to the host (Benfenati, 2007). Besides quantitative structure activity relationship (QSAR) models, which calculate toxicity based on the physical characteristics of the chemical structures (molecular descriptors) (Eriksson et al., 2003), other models have recently been developed to predict toxicity based, for example, on chemical-chemical interactions (Chen et al., 2013). QSAR models nonetheless remain the paradigm in the field. An interesting example of a QSAR model-based computational tool (https://absynth.issb.genopole.fr/Bioinformatics/) is the toxicity prediction web server EcoliTox (Planson et al., 2012). The dataset obtained from screening a diversified chemical library of 166 compounds for toxicity in *Escherichia coli* was used to develop a predictor of toxicity of metabolites throughout the metabolome of this popular bacterial host. Tools similar to the EcoliTox server could be integrated into a computational framework for improved design of native or heterologous biodegradation pathways and used to fine-tune their expression in selected microbial hosts. A powerful virtual screening approach, currently applied predominantly in drug design studies (Buryska et al., 2016), could be used similarly

for synthetic pathway predictions to avoid selection of enzymes whose catalytic machinery might be inhibited by molecules present in a host cell.

2.1.3   Detection and quantification of pathway building blocks

Once the suitable pathway is selected, another issue arises, that of how to detect and quantify the pathway building blocks - metabolites and enzymes. Detection and accurate quantification of metabolites in complex reaction mixtures and of functional enzymes produced within the cell provide valuable information on pathway performance and its bottlenecks, as well as a background for further optimization of the route. Here, the experimental tools and approaches come into play for the first time in our workflow scheme (**Fig. 1**). The best sensitivity and separation power is currently provided by chromatographic separation of metabolites followed by mass spectrometry analysis (Büscher et al., 2009). Information on physicochemical properties of compounds (boiling point, polarity, molecular weight) generated in the pathway is nonetheless crucial for correct choice of a detection method, be it gas chromatography, liquid chromatography, or capillary electrophoresis (Büscher et al., 2009). Of the numerous chemical databases, PubChem (https://pubchem.ncbi.nlm.nih.gov/), created in 2004 by the US National Institutes of Health, is the world's largest free chemistry repository, into which anyone can deposit data on structures and their properties (Kim et al., 2016). The database has grown to more than 92 million structures. Bioactivity data of chemical substances, manually withdrawn from the scientific literature, are saved in PubChem Substance and Compound databases. Integration with other databases allows including pharmacology, toxicology, drug target information, safety or handling information to the annotation of chemical records. PubChem also hosts data from important regulatory agencies, including the US Environmental Protection Agency. In 2007, another free repository, ChemSpider (http://www.chemspider.com/), was created and later purchased by the UK Royal Society of Chemistry; it provides access to over 59 million entries from 487 diverse data sources and can be recommended as another rich source of knowledge on experimental or predicted physicochemical properties of compounds.

For pathway enzyme detection and absolute quantification, SDS-PAGE or better, Western blot analysis, followed by determination of enzyme activity have been standard protocols in metabolic engineering for many years (Kurumbang et al., 2014). When applied to multiple-enzyme pathways, however, these methods are tedious and time-consuming. Selected Reaction Monitoring Mass Spectrometry protocols developed in the past few years seem to provide a promising alternative for absolute protein quantification in the future, as they allow rapid simultaneous quantification of many proteins in the cell, with good selectivity and sensitivity regardless of organism of origin (Batth et al., 2012).

2.2   Step 2: Select and get to know a suitable microbial host

In addition to catabolic pathway choice or design, the selection of a suitable host is a crucial initial step in any engineering project focused on the development of a whole-cell degrader. Over the years, several microbial hosts have been considered for application in biodegradation processes, but no single, naturally isolated bacterial strain possesses all the desired characteristics of the optimal degrader. Soil bacteria could satisfy many of these requirements because of the conditions they face naturally in the niches in which they thrive, including exposure to environmental contaminants and to competing and predatory species. These microorganisms have versatile metabolic lifestyles that allow them to adapt to changing conditions, sometimes adverse (oxidative stress, temperature challenges, osmotic perturbations). *P. putida* is a ubiquitous rhizosphere colonizer that belongs to the wide (if somewhat fuzzy) group of fluorescent *Pseudomonas*. *P. putida* KT2440 is the best-

characterized saprophytic laboratory pseudomonad that has retained its ability to survive and thrive in the environment. Its entire chromosome sequence is available since 2002 (Belda et al., 2016; Nelson et al., 2002; 2014). This strain derives from *P. putida* mt-2, a naturally occurring species able to degrade several aromatic compounds through the activities encoded in the catabolic TOL plasmid. The physiological and metabolic properties of pseudomonads argue for their selection as the starting point for engineering of biodegradation processes. In addition to rapid growth and low nutrient demand, their extremely versatile metabolism and high capacity to supply redox power, providing endurance to oxidative stress, are several advantages that render *P. putida* an interesting host for degradation applications (Nikel et al., 2016a). Most of these properties arise from a very robust central metabolism (**Fig. 3**), in which the combined activity of enzymes from different pathways (*i.e.*, the EDEMP cycle; Nikel et al., 2015)) endows the cells with a large NADPH supply (Chavarría et al., 2013). The operativity of the EDEMP cycle enables the formation of ATP and NADPH at different rates depending on the amount of trioses phosphate recycled. Assuming that *P. putida* has (i) a NAD^+ dependent glyceraldehyde-3-*P* dehydrogenase and a $NADP^+$ dependent glucose-6-*P* dehydrogenase, (ii) pyruvate as the end product of glycolytic pathways, and (iii) a negligible flux through 2-ketogluconate, the yields of pyruvate, ATP, and NADPH are as indicated in the inset to **Fig. 3**. The ability to sustain high NADPH formation rates is a requisite needed in a bacterial host for the implantation of biodegradation pathways (Lorenzo and Loza-Tavera, 2011). As will be shown in part in following sections, these beneficial properties are reflected in the increasing number of metabolic engineering studies in which *P. putida* appears as one major player.

Figure 3 here

2.2.1. **Omics** *techniques in studies of bacterial degraders*

Systems biology, and particularly 'omic' techniques, contributed markedly to rational selection of suitable hosts for metabolic engineering. The enormous amount of genetic information read with first- or next-generation sequencing techniques together with powerful new algorithms and computational tools to aid with data mining, allowed considerable progress in our understanding of dynamic interactions inside and in between cells and the environments they inhabit (Bouhajja et al., 2016; Vilchez-Vargas et al., 2010). Available genomic data also enabled new insights into the evolution of microbial metabolism toward biodegradation of well-recognized xenobiotics such as 1,3,5-triazine (Shapir et al., 2007). 16S rRNA-phylogenetic microarrays and the functional gene microarrays have been used to monitor microbial community dynamics and to track activities of microbes in the polluted environments, respectively (Chakraborty et al., 2012). The reconstructed genome-scale metabolic models, together with transcriptomic, proteomic, metabolomic, and flux analyses in various growth and stress conditions, completely changed our view of popular biodegradative bacterial hosts including the paradigmatic *P. putida* (Sohn et al., 2010).

For instance, fluxomic analyses applied to *P. putida* KT2440 helped to reveal its distinct strategies for dealing with carbon sources, *e.g.*, by favouring the routes that generate NADPH, which promotes greater resistance to oxidative stress (Chavarría et al., 2013; Nikel et al., 2013). Examination of transcriptomic data uncovered the significant portion of the *P. putida* genome ($\geq 20\%$) that is differentially expressed when the cells are grown on diverse substrates and emphasized the role of a suite of global regulators (J. Kim et al., 2013). Recent complete structural re-annotation of the KT2440 strain genome allowed identification of 1485 CDSs associated to 1898 chemical reactions, prediction of catabolic pathways for 92 compounds (carbon, nitrogen, and phosphorus sources) and upgrading of the available

genome-scale metabolic model (Belda et al., 2016; Puchałka et al., 2008). 'Omic' analyses greatly increased insight into the genetic and physiological background of some other pseudomonads besides KT2440, including *P. pseudoalcaligenes* CECT5344, which might be used for biodegradation of industrial cyanide-containing wastes, or the 2,4,6-trinitrotoluene biotransforming *P. putida* JLR11 (Pascual et al., 2015; Wibberg et al., 2016). At the time of writing this article, there are 3133 drafted and 215 completed *Pseudomonas* genomes currently available in the *Pseudomonas* Genome Database (http://www.pseudomonas.com/), perhaps the most comprehensive genomic database dedicated to a single bacterial genus (Winsor et al., 2016).

Complete genomic sequences are currently available for many other potentially useful degraders such as *Cupriavidus necator* JMP134, a bacterium with nearly 300 genes involved in the catabolism of aromatics, including chloroaromatics (Lykidis et al., 2010). Other examples include, but are not limited to, the PCBs-degrading *Acidovorax* sp. strain KKS102 (Ohtsubo et al., 2012), the cyclic hydrocarbons-degrading *Alicycliphilus denitrificans* strains BC and K601 (Oosterkamp et al., 2013), or the oil-degrading bacterium *Oleispira antarctica,* whose genome has provided useful information as to how bacteria mitigate oil spills in cold environments (Kube et al., 2013). Genomic sequences of these and many other microorganisms with biodegradation capabilities can be searched through in comprehensive databases such as NCBI genome database (https://www.ncbi.nlm.nih.gov/genome/) or MicroScope (www.genoscope.cns.fr/agc/microscope/home/), a microbial genome annotation and analysis platform (Vallenet et al., 2016), developed in the Laboratory of Bioinformatics Analyses for Genomics and Metabolism at the National Sequencing Centre (Evry, France); it provides a complete pipeline for annotations and comparative analyses of up to 6000 microbial genomes, of which hundreds were manually curated for accuracy.

Despite interest in study of individual bacterial species, the fascinating *in situ* systems biology exercise that followed one of the most alarming ecological disasters in modern human history, the 2010 BP Deepwater Horizon spill in the Gulf of Mexico, emphasized the irreplaceable role of bacterial communities in natural bioremediation. Bacteria that can convert oil-derived alkanes and aromatics into the biomass were identified based on (i) genome reconstructions via metagenome sequencing of the DNA from the stable-isotope-probing experiments (Dombrowski et al., 2016), and (ii) cultivation trials using oil samples from sea surface and deep sea collected during the outflow (Gutierrez et al., 2013). Only the concerted action of many microbial species including *Cycloclasticus*, *Alcanivorax*, members of *Rhodospirillales*, *Alteromonas* and others, augmented by the specific environment of the oil spill, allowed the degradation of the complex mixture that consisted of as many as 1,000 compounds. Metagenomic strategies help to shed light also on the composition and dynamics of microbial consortia that participate in natural biodegradation of chorinated ethenes in contaminated groundwater (Adetutu et al., 2015) or polycyclic aromatic hydrocarbons in soils (Guazzaroni et al., 2013).

2.3 **Step 3: Build the pathway and optimize its performance in the context of host metabolism**

Optimization of the proposed biochemical pathway in the context of host cell metabolism is often the most demanding part of the engineering project. This is especially true for *de novo* synthetic pathways composed of enzymes from different sources, implanted into a new bacterial host. Codon composition of introduced genes might not be optimal for expression in a heterologous host, enzyme activities might not be well balanced or might cross-talk with the

native metabolic network, pathway intermediates can accumulate and inhibit the growth of cells, or cofactors, ATP or redox carriers might be lacking. The portfolio of systemic tools that can help overcome these issues is expanding constantly. Here, we will center on the major concepts that have proven useful in tailoring biodegradation pathways and microbial degraders. We will discuss the application of several of these modern tools including the example of engineering the synthetic catabolic route for 1,2,3-trichloropropane (TCP, **Fig. 2**), an industrial byproduct of anthropogenic origin now being recognized as a groundwater contaminant (Samin and Janssen, 2012). TCP is representative of difficult-to-degrade halogenated aliphatic compounds, a tough case for remediation technologists due to its physicochemical properties, toxicity in living organisms, and absence of efficient natural catabolic pathway (Janssen et al., 2005; Samin and Janssen, 2012). The enzymes later used to compose the first synthetic pathway that allowed mineralization of TCP by an engineered bacterium had already been described in the late 1980s and early 90s (Bosma et al., 1999). With a history spanning more than 25 years, the engineering of this biodegradation pathway thus represents one of the most systematic efforts of its kind.

2.3.1 Computational tools for pathway and strain optimization

The tremendous increase in the number of sequenced genomes (the number of complete genomes in the NCBI database has more than doubled in the last two years) of unicelluar and multicellular organisms, including the microbial degraders mentioned above, led to the reconstruction of genome-scale metabolic models representing all (anotated) biochemical reactions that take place in the living cell (King et al., 2015). Mathematical models of metabolic networks have a central role in metabolic engineering, and in pathway and strain optimization. Modelling can be used to analyze the selected pathway (*e.g.,* to determine the distribution of metabolic fluxes) and identify reactions that must be modified to improve its performance. Genome-scale metabolic models are built by compiling data on genes related to biochemical reactions from databases such as KEGG and BioCyc. The genes are compared to already finalized reconstructions of related organisms to find homologous reactions; the draft model is then refined and verified by simulations. Flux Balance Analysis (FBA) and Metabolic Flux Analysis (MFA) are two major approaches that use metabolic models to predict intracellular fluxes (Stephanopoulos, 1999). FBA is a theoretical concept that uses the stoichiometric coefficients for each reaction in the system as the set of constraints for optimization. MFA determines intracellular fluxes from measurable rates of metabolite uptake and secretion in growth medium. In a typical experiment, substrates labelled with the non-radioactive isotope ^{13}C are used in chemostat-grown cultures to trace fluxes through a cellular network. Computational tools such as Cobra 2.0 for Matlab, OptKnock, or k-OptForce can implement constraint-based flux calculations and suggest strategies based on knock-out, upregulation or downregulation of target genes to optimize metabolite production without compromising cell growth (Burgard et al., 2003; Chowdhury et al., 2014; Schellenberger et al., 2011). In a study by Izallalen and colleagues, OptKnock was used together with the constraint-based *in silico* model of *Geobacter sulfurreducens* to determine gene deletions which could lead to increased respiration rates (Izallalen et al., 2008). *Geobacter* species are well recognized for their ability to degrade radioactive and toxic metals and have been explored for their utility in microbial fuel cells (Shi et al., 2016). These processes are nonetheless limited by electron transfer rates in cells. OptKnock calculations followed by genetic constructions by the authors resulted in recombinant *Geobacter* cells with decreased ATP levels, slower growth rates, and predicted higher respiration rates (Izallalen et al., 2008). Subsequent genome-wide analysis of gene transcript levels also verified the *in silico* predictions.

Although FBA- and MFA-based approaches can handle genome-scale metabolic models and require no information on enzyme kinetics, their output only gives a steady-state approximation of the dynamic reality in the living cell. Kinetic modelling is an alternative to static flux calculations when reliable kinetic parameters of pathway enzymes and some other input data are available, *e.g.,* specific surface area of the cell, permeability coefficients for substrates, metabolite concentrations, or approximate enzyme amount in the cell (Chowdhury et al., 2015). Kinetic constants can be obtained from enzyme databases such as BRENDA or can be determined experimentally. One should keep in mind that most kinetic constants deposited in databases are not standardized and also measured in *in vitro*, which might be a limiting factor when such data are applied to kinetic modelling in living systems (Costa et al., 2011). Computational tools such as COPASI or E-Cell are used to assemble the pathway model in the form of kinetic and differential equations, and to simulate reaction time courses for various conditions (Mendes et al., 2009; Takahashi et al., 2003). Kinetic models of whole metabolic networks are the holy grail of metabolic engineering but their application in strain design is limited by factors such as unreliability of parameters, non-universality of rate laws, need to implement regulatory events, or extremely high demand on computational power (Chowdhury et al., 2015). In contrast, the use of kinetic modelling to engineer individual metabolic pathways or simpler *in vitro*-assembled networks is currently more feasible (Muschiol et al., 2015).

Recent studies by Dvorak, Kurumbang and colleagues are notable example of kinetic modelling applied in engineering of synthetic biodegradation pathways (Dvorak et al., 2014b; Kurumbang et al., 2014). As a model, these studies centered on detoxification of the industrial waste compound and water pollutant TCP to glycerol - reaction catalysed by the haloalkane dehalogenase DhaA from *Rhodococcus rhodochrous* NCIMB 13064 and the haloalcohol dehalogenase HheC and the epoxide hydrolase EchA from *Agrobacterium radiobacter* AD1 (Bosma et al., 1999). *In vitro* steady-state kinetic data obtained for HheC, EchA, and three variants of DhaA were utilized to develop a kinetic model of the reaction that proved useful for revealing major bottlenecks in the pathway, that is (i) low activity of wild-type DhaA on TCP and (ii) unbalanced enantioselectivity and enantiospecificity of the first and the second enzyme in the route, DhaA and HheC, respectively (Dvorak et al., 2014). Modelling was further applied to predict optimal enzyme stoichiometry and fine-tuning overall pathway efficiency, both in *in vitro* conditions and in the heterologous microbial host *E. coli*. In *E. coli*, the pathway was established as a modular, tunable system (Kurumbang et al., 2014). Absolute amounts of pathway enzymes in the cell, copy numbers of used plasmid vectors, and toxicity levels of pathway substrate and intermediates were included in the mathematical model as additional parameters. Variants of the pathway, predicted to promote better host survival in a medium with toxic TCP, were verified experimentally. The observed precise matching between calculated and experimental data confirmed the performance of the pathway in *E. coli* and stressed the power of kinetic modelling when reliable parameters are available. Finally, the kinetic parameters of an optimal DhaA variant that would ensure sufficient TCP conversion to glycerol as well as host cell growth on the toxic substrate were both predicted by mathematical modelling.

This study shows that, for engineering and implanting heterologous pathways, complete knowledge of the host cell metabolic background is not a must, and a reliable strain design can be achieved with a relatively simple mathematical model (Dvorak et al., 2014b). Even so, similar studies that combine theoretical and experimental approaches in the design of pathways and strains for biodegradation are still rare. Most recent work in the biodegradation

field is restricted to application of purely experimental techniques from a repertoire of metabolic engineering, discussed in the following section.

2.3.2. Experimental tools for pathway and strain optimization

Once the bottleneck reaction steps are identified and a solution is proposed, experimental techniques are applied to target the appropriate genes or regulatory mechanisms. Engineering is traditionally conducted at the level of gene expression, which affects the quantity of protein. Genes in native or synthetic pathways are usually overexpressed by introducing plasmid vectors with extra copies of desired coding sequences. One popular approach, also used for modular assembly of the TCP biodegradation pathway described above, is to combine Novagen Duet plasmid vectors (Merck Millipore, Germany) or derivatives such as ePathBrick vectors, which carry compatible replication origins and antibiotic markers (Kurumbang et al., 2014; Tolia and Joshua-Tor, 2006; Xu et al., 2012). This system allows effective propagation and maintenance of up to four plasmids in a single *E. coli* BL21 cell with λDE3 lysogen and modular assembly of multi-gene pathways. SEVA (Standard European Vector Architecture) plasmids with standardized architecture and nomenclature are an alternative modular vector system tailored to allow cloning or expression of heterologous genes in *P. putida, E. coli* and other Gram-negative bacteria, with no specific need for strain pretreatment (Martínez-García et al., 2015). Up to four plasmids, each with one of the six available antibiotic markers (ampicillin, kanamycin, cloramphenicol, streptomycin, tetracycline, gentamicin), four independent replication origins (RK2, pBBR1, pRO1600/ColE1, RSF1010), and diverse cargoes, can be combined in a single host cell to fulfill the user's needs. A rich collection of constructs is already freely available (http://seva.cnb.csic.es/).

A set of compatible SEVA plasmids was used, for instance, in a study of Nikel and de Lorenzo (2013), who described extrication of *P. putida* KT2440 from its strictly aerobic nature, which prevents its use in industrial anaerobic fermentors and certain bioremediation applications (Nikel and de Lorenzo, 2013). Two scenarios were considered to explain the inability of *P. putida* to grow in anoxic conditions, (i) unbalanced energy charge due to limited activity of the respiratory chain, and (ii) lack of appropriate pathways for anoxic NADH re-oxidation. This two-fold problem was tackled by recruiting the pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adhB*) genes from the anaerobe *Zymomonas mobilis* and the acetate kinase (*ackA*) gene from the facultative aerobe *E. coli*, to manipulate energy generation and redox balance in anoxic conditions. To evaluate the potential of the recombinant host for anoxic biotransformations, the authors used it as a host for the haloalkane dehalogenase genes from *P. pavonaceae* strain 170. The resulting recombinant, which bore two synthetic operons on compatible plasmids pSEVA234 and pSEVA428, not only survived in anoxic conditions, but also degraded the environmental pollutant 1,3-dichloropropene. These results highlight the possibility of harnessing the full potential of *P. putida* KT2440 as a robust biocatalyst by precise control of its energy and redox metabolism.

Despite the widespread use of recombinant plasmids in proof-of-concept studies, metabolic engineers must bear in mind that the introduction and massive expression of heterologous genes can affect host fitness, due either to additional metabolic load or to depletion of essential cofactors in redox reactions (Dvorak et al., 2015; Glick, 1995; Wu et al., 2016). This is even more important in the design of robust bacterial degraders that must cope with a spectrum of toxic substrates and/or pathway intermediates, and might need an additional energy charge and reducing cofators to cope with oxidative stress (Dvorak et al., 2015; Nikel

et al., 2013). By-passing such difficulties is possible by tuning inducer concentration, applying lower-copy-number plasmids with weaker promoters, or by enzyme-mediated co-factor recycling through overexpression of NAD^+ kinase, transhydrogenases or dehydrogenases (Dvorak et al., 2015; Nikel et al., 2016b).

As an alternative, expression of heterologous genes directly from the host chromosome can be beneficial for greater stability of the desired genotype/phenotype and improved host viability (Martínez-García et al., 2014; Santos and Yoshikuni, 2014; St-Pierre et al., 2013; Zobel et al., 2015). Homologous recombination and site-specific or random transposition-based techniques have been developed to enable chromosomal insertion of single genes, gene clusters or whole synthetic operons, or knockouts of genes that encode competing metabolic pathways (Loeschcke et al., 2013; Martínez-García et al., 2014a; Santos and Yoshikuni, 2014; St-Pierre et al., 2013; Zobel et al., 2015).

In a study by Samin and co-workers, a mutant variant of haloalkane dehalogenase DhaA was introduced into the chromosome of 2,3-dichloro-1-propanol-degrading *P. putida* MC4 under the control of a strong constitutive promoter using the Tn5 transposon-based delivery vector (Samin et al., 2014). Although the complete mineralization pathway in strain MC4 is not yet fully understood, the recombinant *Pseudomonas* was the first microorganism shown to grow aerobically on TCP, both in shaken flasks and in packed-bed reactor in continuous-flow conditions. Mini-transposon systems have also been used successfully for chromosomal integration of the whole catabolic cluster, as in the study by Wittich and Wolf who transplanted 11 genes from three distinct bacteria into *Cupriavidus necator* H850, giving rise to the first designer bacterium to show aerobic growth on a wide range of PCB, including the two commercial pesticides Aroclor 1221 and Aroclor 1232 (Wittich and Wolff, 2007).

Gene expression balancing is undoubtedly a powerful metabolic engineering concept. In addition to the approaches mentioned above for rather rough manipulation of gene expression, numerous techniques for fine-tuning expression have been developed in recent years. Surgical cuts, including computationally designed ribosome binding sites, refined architecture of the whole expression cassette, mutagenesis of promoter regions, tailored stability of mRNA molecules, or altered gene order in the operon were proven useful for optimizing bisynthetic pathways (Boyle and Silver, 2012). In several cases, expression adjustment was also considered in biodegradation pathway design. de la Peña Mattozzi and co-workers achieved more rapid paraoxon hydrolysis in a recombinant *P. putida* strain by altering the order of three genes in the operon for enzymes that convert one of the initial pathway intermediates (de la Peña Mattozzi et al., 2006). Expression of the *dszB* gene, which encodes 2-hydroxybiphenyl-2-sulfinate sulfinolyase, the last rate-limiting enzyme in the dibenzothiophene biodesulfurization pathway, was improved by rearranging gene order and removing the overlapping structure in the *dsz* operon (Li et al., 2007). Nonetheless, especially in cases of synthetic metabolic pathways composed of biocatalysts from diverse organisms, the activity, selectivity, stability or inhibition bottlenecks of individual enzymes can be too far-reaching to be solved by tuning expression of the corresponding genes. Moreover, overexpression of endogenous or exogenous genes often results in a metabolic burden and lower host viability due to overconsumption of metabolic precursors (amino acids, rRNA, ATP, reducing cofactors) to fuel the synthesis of non-essential proteins (Glick, 1995; Wu et al., 2016). Protein engineering of bottleneck enzymes then becomes a more reasonable approach.

2.3.3 *Protein engineering to eliminate bottlenecks of biodegradation pathways*

Over the last two decades, three distinct strategies were developed to allow the construction and identification of mutant enzymes with desirable properties (Bornscheuer et al., 2012). The earliest approach, *rational design*, exploits various computational techniques such as*,* molecular docking, homology modelling and molecular dynamics simulations, together with site-directed mutagenesis protocols to generate targeted mutations that result in single or several modified variants of an enzyme (Damborsky and Brezovsky, 2014). In contrast, *directed evolution* uses random mutagenesis methods and is beneficial especially in cases when neither the structure nor the catalytic mechanism of the enzyme is available (Brakmann, 2001). Random methods of directed evolution are combined with elements of rational enzyme modification to bypass certain limitations of both approaches. This focused directed evolution approach targets several specific residues or certain protein regions selected on the basis of prior structural and functional knowledge (Bornscheuer et al., 2012). Mutation hot spots are chosen experimentally and, on a much larger scale, computationally, using powerful new algorithms and statistical tools such as HotSpot Wizzard or 3DM tool (Bendl et al., 2016; Damborsky and Brezovsky, 2014; Kuipers et al., 2010). A clear trend in recent years is the introduction of protein engineering into the metabolic engineering workflow.

The activities of many enzymes that act on anthropogenic compounds are derived from promiscuous activities that are generally very inefficient (Khersonsky and Tawfik, 2010). In such cases, protein engineering is the only possible solution. Numerous reports from the first decade of the 2000s describe application of common protein engineering methods such as error-prone PCR, DNA shuffling, site-directed or saturation mutagenesis for engineering activity, or selectivity of individual catabolic enzymes to halogenated hydrocarbons (Parales and Ditty, 2005; Wittich et al., 2010). Recently, the new challenge of accumulating plastic waste has attracted the attention of protein engineers, who try to enhance the activities of certain bacterial or fungal enzymes such as esterases, lipases or polyster hydrolases to synthetic polymers, including polyethylene terephthalate (PET) or polyurethanes (Wierckx et al., 2015). For instance, Wei and colleagues applied a comparison of crystal structures and molecular docking combined with side-directed mutagenesis to improve the activity of cutinase TfCut2 from *Thermobifida fusca* KW3 towards PET at elevated temperatures that promote degradation of this oil-derived plastic (Wei et al., 2016). Subsequent kinetic and *in silico* energetic analyses confirmed that the improvement in PET hydrolysis was the result of a relief of product inhibition caused by single point mutation in the enzyme's surface-exposed active site. New unique PETase and MHETase, shown to help bacterium *Indonella sakaiensis* to depolymerize and utilize PET, are promising candidates for protein and metabolic engineering exercises that might lead to biotechnological recycling of the polymer (Bornscheuer, 2016; Yoshida et al., 2016a). Alas, such a promise is not devoid of controversy about the very enzymes that could do the job (Yan et al., 2016; Yoshida et al., 2016b)

The studies reporting the implantation of constructed mutants in the context of the whole biodegradative pathway are nonetheless rather rare. Iwakiri and co-workers successfully applied *Alcaligenes sp.* KF711 harbouring engineered monooxygenase $P450_{CAM}$ for the dehalogenation of pentachloroethane to trichloroethane in anoxic conditions (Iwakiri et al., 2004). In another study, promiscuous toluene *ortho*-monooxygenase and epoxide hydrolase were engineered using DNA-shuffling and saturation mutagenesis; the use of mutant genes allowed more rapid aerobic degradation of chlorinated ethenes, with less accumulation of stress-inducing intermediates in recombinant *E. coli* bearing the synthetic pathway (Lee et al., 2006; Rui et al., 2004).

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Mutants of DhaA, the enzyme that initiates dehalogenation of TCP, were used in three of the previously mentioned studies that focused on TCP pathway engineering (Dvorak et al., 2014b; Kurumbang et al., 2014; Samin et al., 2014). The DhaA31 variant, with 29-fold improved catalytic efficiency towards the chlorinated substrate, was prepared by combining molecular dynamics simulations of product release from the buried active site with site-directed and saturation mutagenesis in selected hot spots (Pavlova et al., 2009). Another promising mutant with the potential to remove the second serious bottleneck in the synthetic TCP pathway—enantioselectivity of DhaA and high enantiospecificity of HheC—originated from the work of van Leeuwen and colleagues (van Leeuwen et al., 2012). The authors targeted DhaA31 to obtain variants that convert TCP predominantly into (*R*)-DCP, which can be further converted by HheC at a much higher rate than the (*S*)-enantiomer. Five rounds of focused directed evolution using saturation mutagenesis with restricted codon sets provided mutant DhaA r5-90R with 13 new amino acid substitutions, and substantially improved (*R*)-selectivity. Mutagenesis nevertheless affected the activity with TCP, which dropped to wild-type DhaA levels; the mutant was later shown in *in vitro* and *in vivo* studies to be of no value for further improvement of TCP pathway efficiency (Dvorak et al., 2014b; Kurumbang et al., 2014). Additional engineering input is thus needed to obtain DhaA variants with improved activity and modified enantioselectivity that would promote smooth flux through the synthetic pathway (**Fig. 4**) and allow growth of bacterial recombinants in minimal medium with the toxic substrate (Kurumbang et al., 2014).

Figure 4 here

It will be necessary to accelerate the processes of laboratory evolution and screening of new enzymes with enhanced properties in mutant and metagenomic libraries to make protein engineering of greater value in optimizing natural and synthetic catabolic pathways (Bouhajja et al., 2016). Selection couples an improved enzyme property with host survival, which allows even more than 10^9 clones/enzyme variants to be tested in a reasonable time. New selection assays based on toxic substrate conversions into a harmless utilizable metabolite, coupled with fluorescence-activated sorting of surviving cells could, for example, be applied for the enrichment of new dehalogenase variants from libraries prepared by directed evolution or a semi-rational approach (Fernández-Álvaro et al., 2011; Fibinger et al., 2015). Several recent studies presented workflows for high-throughput screening and characterization of improved enzyme variants or novel enzyme activities. These test schemes are based on: (i) bioinformatic pre-screening combined with high-throughput experimental characterization of candidate proteins (Bastard et al., 2014), (ii) completely automated robotic platforms manipulating clones growing in microtitre plates (Dörr et al., 2016), (iii) microcapillary arrays coupled with fluorescent assays and laser extraction for recovery of live clones (Chen et al., 2016), or (iv) single-cell sorting based on a fluorescent signal from clones with an implemented synthetic genetic circuit responding to a specific metabolite (Choi et al., 2014). *In silico* screening of molecules that fit the active site cavity of an available enzyme structure, or reverse screening of enzymes that can accommodate target substrate could supplement experimental methods and reduce the time needed for discovery of new biocatalysts for recalcitrant chemicals. Indeed, the potential of *in silico* screening methods was demonstrated when investigating cytochrome P450-mediated metabolism of xenobiotics (Raunio et al., 2015). In a recent study by Aukema and colleagues, docking and molecular dynamics simulations allowed selection of biphenyl dioxygenase from *Paraburkholderia xenovorans* LB400 as the best candidate enzyme for metabolizing carbamazepine, one of the most commonly identified recalcitrant pharmaceuticals in rivers (Aukema et al., 2016). The

experimentally verified rate of carbamazepine degradation by *P. xenovorans* cells was 40-times greater than the best reported rates so far.

As shown in the preceding sections, the available toolbox of systems biology, metabolic engineering and protein engineering applicable for re-factoring microbes and their catabolic pathways towards more efficient biodegradation of waste chemicals and recalcitrant pollutants is becoming inordinately large. Even so, the defined workflows, standards and universally applicable principles for strain optimization were long missing in the field of metabolic engineering, despite which, the aim to engineer living systems on a rational basis remained prevalent in the community. This goal led metabolic engineers to adopt standards and strategies from another closely related field of synthetic biology, that was established primarily to program living systems with a high predictability.

3. Synthetic biology approaches and tools for biodegradation pathway engineering

The principal underlying thought in synthetic biology is that any living system can be considered a set of separate usable components that can be combined by the means of biological engineering in new arrangements to alter existing features or generate new ones (de Lorenzo and Danchin, 2008). Such biological engineering can be simplified by applying principles adopted from electronic engineering and computer science to produce predictable, robust systems (genetic control systems, metabolic pathways, chromosomes and whole cells) with non-natural functions (Paddon and Keasling, 2014). This would be achieved through the fabrication of thoroughly characterized, standardized, recyclable parts. From its very beginnings, synthetic biology has been confronted with the need to define its practice precisely and to develop clear, generally applicable strategies, as this was the only way to guarantee and implement engineering principles in a field as stochastic as biology. With its tools, standards and the DBTA cycle strategy, synthetic biology has contributed as has no other scientific discipline to rationalize metabolic engineering. Its bottom-up approaches and synthetic devices are being implemented to make more robust, better controllable microbial cell factories. Synthetic biology can, and to some extent already contributes, the same service with its new concepts to the fields of biodegradation and bioremediation. In the the Section 3, we will discuss examples of beneficial inclusion of synthetic biology into strategies for engineering pathways and whole cells for biodegradation of waste and polluting chemicals, and perspectives for these initiatives.

3.1 Development of robust microbial chassis for biodegradation of toxic chemicals

Genetic instability and negatively affected fitness are frequently encountered drawbacks of the native microbial hosts tailored by metabolic engineering for specific biodegradation purposes. Synthetic biology offers possible solutions for these problems by implementing DNA synthesis and genome editing strategies. Both curiosity and prospective practical applications drive synthetic biologists to generate microbial cells with deleted parts of their genomes that encode redundant, cryptic, or even deleterious functions. Such cells, endowed with a reduced genome as foundation to house and support heterologous genetic parts, are collectively known as *chassis* (Adams, 2016). An extreme case of a *chassis* is the so-called *minimal cell* (Glass et al., 2006). Chassis can be prepared by *de novo* synthesis of a reduced target genome and its implantation into a suitable cell envelope, or through systematic deletions of non-essential genes in the genomes of an existing natural host. Microarray-based oligonucleotide synthesis is currently used to provide a substrate (usually 5-50 oligos) for construction of larger (usually 200-3,000 bp) synthetic fragments (Kosuri and Church, 2014). Scarless methods including the popular Gibson assembly, uracil assembly, Golden Gate technology, ligase cycling reaction, or yeast recombination are used to combine sequence-

verified gene-length fragments in even larger complexes (Casini et al., 2015). DNA synthesis also allows preparation of standardized genetic parts (promoters, ribosome binding sites, genes, terminators and so on) with verified codon-optimized sequences. Despite breakthroughs in gene synthesis and DNA assembly methods, this last approach for *chassis* construction seems far more feasible at the moment, considering the lesser demand of chromosome editing experiments, the constantly expanding portfolio of genetic tools, and the growing list of microorganisms with intentionally reduced genomes (Martínez-García and de Lorenzo, 2016; Si et al., 2015).

Discarding non-essential cell functions shows considerable potential not only for biosynthetic purposes (Hutchison et al., 2016), but also for designer biodegradation and bioremediation. A recent report on the systematic deletion of 11 non-adjacent genomic regions in *P. putida* KT2440 (**Fig. 5**) is a unique example of genome streamlining in a popular bacterial host with well-defined biodegradation capabilities (Martínez-García et al., 2014b). In all, 300 genes were eliminated, that is, 4.3% of the entire KT2440 strain genome, using the scar-less deletion procedure based on homologous recombination after *in vivo* DNA cleavage by the homing *Saccharomyces cerevisiae* nuclease I-*SceI* (Martínez-García and de Lorenzo, 2012). A suite of functions was targeted, including the complete flagellar machinery, whose assembly and function drains ATP from the cells and consumes NAD(P)H. Four prophages, two transposons, and three components of DNA restriction-modification systems were also eliminated to minimize genetic instability. The resulting strains designated *P. putida* EM42 and EM383 (the latter lacks the *recA* gene that encodes recombinase A) showed clearly superior growth properties and improved overall physiological vigour compared to wild-type KT2440. Moreover, due to the higher NADPH/NADP$^+$ ratio, the reduced-genome strains also better tolerated endogenous oxidative stress, a property that provides a crucial advantage for catalysing harsh biodegradation reactions such as aerobic dehalogenation of chlorinated pollutants (Nikel et al., 2013).

Despite their reliability and robustness, the procedures based on these genome editing tools are time- and labour-intensive, and restricted to a limited number of model microorganisms. This problem is now being challenged by new protocols that profit from the type II bacterial Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein (Cas), Multiple Automated Genome Editing (MAGE), and combinations thereof (Barrangou and van Pijkeren, 2016; Wang et al., 2009). Nonetheless, these technologies also suffer from some weaknesses such as generation of numerous off-target mutations. The off-target problem of the CRISPR-Cas system was recently mitigated by engineering high-fidelity Cas9 nucleases (Kleinstiver et al., 2016). In a similar manner, the number of off-target mutations during MAGE was reduced substantially when temperature-sensitive control of the endogenous methyl-directed mismatch repair system was introduced into the *E. coli* host together with a dominant negative mutator allele of the *E. coli* mismatch repair protein MutL (Nyerges et al., 2016). By placing this highly conserved allele together with λ Red recombinase genes and a temperature-sensitive λ repressor on a single broad-host range plasmid, Nyerges and co-workers provided a simplified MAGE variant (pORTMAGE) that allowed genome editing and mutant library generation in several biotechnologically and clinically relevant bacterial species, with no need for prior modifications of parental strains. The recent discovery of the Ssr protein from *P. putida* DOT-T1E, a functional homologue of the β protein of the λ Red recombination system, paved the way for oligonucleotide-mediated multiplex genome engineering in pseudomonads, including *P. putida* KT2440 and its derived platform strains EM42 and EM383 (Aparicio et al., 2016). Continuing facilitated reduction of their genomes (deletions of multiple repetitive sequences or certain proteases) and genomes of

other relevant bacterial hosts will provide more reliable, robust *chassis* for biodegradation pathway engineering.

Figure 5 here

3.2 Development of synthetic microbial consortia for enhanced biodegradation and bioremediation of pollutants

An alternative strategy that allows for the emergence of more robust microbe-based bioprocesses is just the opposite of the systematic development of bacterial *chassis* based on individual wild-type strains. Natural microbial consortia perform complicated biocatalytic tasks such as lignocellulose degradation, wastewater purification, or intestinal food digestion. By dividing the labour and metabolic burden, the consortium members become more flexible when facing complex environmental conditions. Distribution of catabolic capacity among cells of a single organism, among different bacterial strains, or even among species from different kingdoms is pivotal; this is especially true for biodegradation of complex toxic chemicals, which encompasses many steps and harmful intermediates with diverse physicochemical properties. The idea of exploiting consortia for directed biodegradation of polluting compounds and bioremediation of contaminated sites thus lies ready to hand.

Application of natural microbial consortia have already shown promise for bioremediation of sites polluted with anthropogenic pesticides such as diclofop methyl or atrazine (Baghapour et al., 2013; Wolfaardt et al., 1994). Together with the new field of synthetic biology, attempts are being made to modify the structure of original natural consortia or develop completely new artificial organizations of co-operating microorganisms. For example, co-culture of engineered *E. coli* SD2 and *P. putida* KT2440 was used successfully to mineralize the insecticide parathion in shaken flasks and in biofilm culture (Gilbert et al., 2003). In another study, complete mineralization of 2,4,6-tribromophenol, a flame-retardant intermediate and pesticide, was achieved using an artificial anaerobe consortium of reductive debrominator *Dehalobacter sp.* FTH1, a hydrogen supplier *Clostridium sp.* Ma13, and the 4-chorophenol-degrading strain *Desulfatiglans parachlorophenolica* DS (Li et al., 2015). Recently, Martínez and co-workers introduced an attractive alternative strategy for engineering the 4S pathway (**Fig. 6A**), a paradigmatic bioprocess for removal of the recalcitrant sulphur from aromatic heterocycles in fuels (Martínez et al., 2016). The pathway encoded by *dszABCD* genes converts the model compound dibenzothiophene (DBT) into sulphur-free 2-hydroxybiphenyl (2HBP). The authors initially synthesized and re-arranged the original genes and optimized transcriptional and translational signals for *P. putida* KT2440. By dividing the pathway into three separate modules expressed individually, they were able to define previously unreported bottlenecks in the pathway; that is, the inhibitory effect of intermediate 2HBP-sulfinate (HBPS) on DszA and DszC monooxygenases and HBPS leakage into the culture medium, from which it could not be transported back into the cells for further processing. To prevent HBPS accumulation, two *P. putida* strains bearing *dszC1-D1* or *dszB1A1-D1* modules were mixed as a suspension of resting cells. When the strains were combined at a 1:4 ratio and added with cell-free extract containing extra DszB, almost 100% of DBT was transformed into 2HBP in assay conditions.

Considerable attention is currently being dedicated to developing strategies for biodegradation of oil and oil-derived chemicals. Patoway and co-workers designed a potent microbial consortium for prospective decontamination of sites exposed to polycyclic aromatic hydrocarbons from crude oil (Patowary et al., 2016). The authors first inspected the biodegradative capacity for total petroleum hydrocarbons of 23 bacterial isolates from

petroleum-contaminated soils, and subsequently designed 14 artificial consortia based on the five most efficient isolates. The best designer consortium, comprised of two biosurfactant-producing, hydrocarbon-degrading strains of *Bacillus pumilus* and *B. cereus*, showed up to 84% degradation of total petroleum hydrocarbons after five weeks, as verified by gravimetric, FTIR and GC/MS analyses. Application of natural or re-designed bacteria and their consortia also holds considerable promise for decomposition of oil-derived plastic waste (Skariyachan et al., 2016; Yoshida et al., 2016).

New tricks for more efficient biodegradation strategies could be adopted from inspiring current research on synthetic consortia designed to decompose and valorize lignocellulosic biomass (Minty et al., 2013). Tozakidis and co-workers reported application of an engineered consortium of three *P. putida* strains displaying thermophilic endoglucanase, exoglucanase, or β-glucosidase for concerted hydrolysis of cellulose at elevated temperatures (Tozakidis et al., 2016). Some studies describe surface display of whole designer cellulosomes – synthetic enzymatic nanomachines whose natural counterparts are produced by certain cellulolytic bacteria that exploit clustered surface-attached cellulases for efficient depolymerization of cellulose and hemicellulose (S. Kim et al., 2013; Moraïs et al., 2014). The vast majority of the work that exploits surface display systems for whole-cell removal of environmental contaminants has been restricted to individual proteins such as laccases, methyl parathion hydrolase, or triphenylmethane reductase (Gao et al., 2014; Liu et al., 2016; Yang et al., 2012). It is tempting to expand available surface display technologies with cellulosome parts for the design of synthetic consortia that would catalyse more complex biodegradation reactions, including polymeric substrates or toxic metabolites, outside the cells.

Despite their true biotechnological potential, microbial consortia remain too complex for directed long-term application, either for biosynthesis or for biodegradation. It is still difficult to engineer homeostasis and evolutionary stability in an artificial multi-cellular system, whose behaviour becomes unpredictable with time (Escalante et al., 2015). Attempts to adopt principles of intercellular communication for synthetic consortia are just at their beginnings (Hennig et al., 2015; Scott and Hasty, 2016). Intensive co-operation of genetic engineers and evolutionary biologists with microbial ecologists and chemists will obviously be needed to derive a deeper understanding of the processes that rule the formation and survival of natural microbial consortia. Lessons learnt from nature can be implemented and combined with completely anthropogenic orthogonal genetic devices for more reliable design of stable multicellular systems.

3.3 Development of orthogonal systems in bacteria to enhance pollutant biodegradation and bioremediation

Orthogonal, or in other words parallel, independent systems[a] is a key concept in synthetic biology as long as such quality makes live systems more amenable to *bona fide* engineering. Orthogonalization involves *e.g.* use of unnatural genetic codes (for example, quadruplet rather than triplet), alternative transcription-translation machineries, toggle switches, and genetic circuits for assembly of novel metabolic and signalling pathways from proteins containing non-natural amino acids (An and Chin, 2009; Wang et al., 2012). Such metabolic pathways could have yet new functions and would synthesize or catabolize a fresh spectrum of

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[a] The mathematical and geometrical concept of *orthogonality* has been adopted first by computing science and then by synthetic biology to signify operative independence. System A is orthogonal to system B if A does not influence B—and *vice versa.*

compounds. Ideally, orthogonal pathways/modules interact minimally with their natural counterparts in the host cell. This improves predictability of the component behaviour and prevents inhibitory cross-talk following introduction of the pathway or circuit into an existing metabolic network (Kim and Copley, 2012; Kurumbang et al., 2014). In an orthogonal module the input and output molecules of genetic circuits—or enzymes and metabolites of synthetic biochemical routes—are not degraded or inactivated by side-reactions or physicochemical conditions of the host cell. While complete orthogonality is virtually impossible in biological systems, a practicable level of context-independence is indeed feasible. Some examples of claimed orthogonal systems include simple synthetic genetic circuits, switches, or designed metabolic pathways. These have the potential to improve function of individual engineered bacteria or synthetic consortia as whole-cell biosensors and degraders of recalcitrant chemicals.

Sensitivity, robustness and applicability of microbial biosensors for detecting heavy metals or halogenated hydrocarbons can be greatly improved by implementing orthogonal genetic devices (Bereza-Malcolm et al., 2015). Thus far, simple microbial biosensor designs based on one input and two-part regulatory systems with promoter and reporter providing a coloured, fluorescent, bioluminescent, or electric signal prevail in the literature (Ravikumar et al., 2017). Reports on more complex multi-input systems based on Boolean logic gates are still rare, but give us a clue to future developments in the field. As first outlined by the work of Wang and colleagues (2016), orthogonally acting multi-input/multi-output logic gates will be especially valuable for detecting mixtures of polluting chemicals (organic, inorganic, or both) by providing a specific signal for each of the constituents (He et al., 2016; Wang et al., 2011). Developing such biosensors is of practical importance, as polluted environments are usually burdened with numerous contaminants that have diverse physicochemical properties. The authors engineered a set of two-input *E. coli*-based biosensors with synthetic AND gates using signalling sensory modules from native two-component signal transduction pathways, as well as *hrpR* and *hrpS* genes along with their HrpL promoter element from *P. syringae*. Using these sensors, they detected arsenic, mercury, copper, and zinc ions, as well as quorum sensing molecules in aqueous environment (Wang et al., 2011). The most advanced design resulted in a triple-input AND logic-gated biosensor that formed a synthetic bacterial consortium in which each of the two members acted as a double input sensor (**Fig. 6B**). In the presence of arsenic and mercury, the first strain formed a quorum sensing molecule ($3OC_6HSL$) that diffused freely into the medium and was sensed together with Cu^{2+} by the second strain, which provided a red fluorescent signal (Wang et al., 2013). The fluorescent response was seen only when all three metal ions were present in the culture.

Figure 6 here

Reliable orthogonal devices will be crucial for engineering artificial cell-cell communication, for instance, taking advantage of bacterial quorum sensing systems (Hennig et al., 2015; Scott and Hasty, 2016). Programming the dynamics of subpopulations of synthetic consortia that perform complex tasks will be only possible with parallel genetic circuits and signalling molecules unknown to the host cells (Chen et al., 2015). As stressed by Silva-Rocha and de Lorenzo (2014), catabolic pathways for recalcitrant and xenobiotic compounds could be a reliable source of wiring devices and molecules These pathways are frequently found in specific types of organisms, and metabolic crosstalk can thus be avoided by implementing components of such networks in suitable hosts. For instance, aromatic molecules such as benzoate or phenol can be used as signal inducers that are recognized by regulatory proteins that trigger expression of target genes. The recent determination of the crystal structure of

the sensory domain in the phenol-responsive transcription activator PoxR ilustrates how aromatics are sensed in bacteria (Patil et al., 2016). This study paves the way for wider application of rationally engineered transcription regulators responsive to aromatics in the design of synthetic genetic circuits.

The dynamic behaviour of the whole biodegradation pathway can also be better studied when the route is transferred into a distant host chassis where it acts orthogonally. The orthogonal nature of the synthetic pathway for TCP biodegradation in the surrogate host *E. coli* BL21 (DE3), described in Section 2.3.1., allowed a very good match between *in silico* predicted and *in vivo* metabolite concentrations and precise description of the bottlenecks (Kurumbang et al., 2014), as well as deciphering the contribution of metabolic burden and substrate/metabolite toxicity to the fitness cost of TCP biotransformation by whole-cell catalysts (Dvorak et al., 2015). Despite their benefits for such mechanistic and proof-of-concept studies, laboratory *E. coli* strains might not be optimally suited for the harsh conditions that accompany biodegradation and bioremediation processes (Adams, 2016; Nicolaou et al., 2010). Development of reliable *chassis* amenable to implantation of orthogonal genetic devices is therefore desirable. These new cell platforms should be based on robust environmental strains of *Pseudomonas*, *Rhodococcus*, *Deinococcus*, and other microorganisms with broad metabolic versatility and natural resistance to organic solvents or heavy metals (Adams, 2016).

One can also take advantage of other specific properties of environmental bacteria including formation of a robust biofilm. As shown recently by Benedetti and colleagues, the actual physical forms of whole-cell biocatalysts can be mastered using orthogonal genetic parts (**Fig. 6C**) through the so-called *synthetic morphology* approach (Benedetti et al., 2016). *Pseudomonads* generate biofilms with biophysical properties that depend on the species. Cyclic di-GMP (c-di-GMP) is the key signal molecule that rules the complex regulatory network mediating the transition between planktonic cells and biofilm formation. This feature was exploited to design an orthogonal genetic device for manipulating the native c-di-GMP biochemistry in *P. putida*, thereby controlling biofilm formation (Benedetti et al., 2016). The *E. coli yedQ* gene (encoding diguanylate cyclase, the enzyme that synthesizes c-di-GMP from GTP) was placed under control of a tightly regulated cyclohexanone-responsive expression system. A synthetic operon, encoding the enzymes needed for 1-chlorobutane biodegradation, was also introduced in the engineered, biofilm-forming *P. putida* strain. Upon activation of the corresponding genetic modules with appropriate inducers, the resulting *P. putida* biocatalyst displayed high dehalogenase activity in robust biofilms.

The modern approaches of biological engineering accelerate the development of whole-cell biocatalysts for *in situ* bioremediation or biosensing of emerging environmental pollutants. Field applications of genetically modified microorganisms are nonetheless restricted by current legislation and hindered by the unreliable behaviour of recombinant microbes in complex, fluctuating environments. Synthetic biology can help to tackle the major problems through the means described at the beginning of this chapter, *i.e.*, non-canonical genetic codes and xenobiochemistry, which can prevent diffusion of undesired sequences into the environmental gene pool, differentiate recombinants from their natural counterparts, and improve robustness and reliability of prepared genetic devices, whole-cell degraders and biosensors. The examples of such endeavours were recently reviewed extensively (Schmidt, 2010; Schmidt and de Lorenzo, 2016). These systems could be combined with bioluminiscence, fluorescence, or DNA watermarking technologies to track the environmental

fate of designer degraders, or with inducible suicide systems for discarding degraders once their mission is completed (Liss et al., 2012; Liu et al., 2010; Paul et al., 2005). Yet the path to applied xenobiology is still crooked, and many ethical questions remain. Another concept from the portfolio of synthetic biology that will be discussed in the following section has the potential to fill the time gap until we obtain reliable whole-cell catalysts and alternative solutions also acceptable for GMO critics will be provided.

3.4 Engineering microbial biodegradation pathways in vitro

The major principle of cell-free synthetic biology is that purified biomolecules or components in crude cell extracts replace intact cells for constructing complex biomolecular systems (Hodgman and Jewett, 2012). To date, metabolic networks that encompass more than 10 enzymes have been reconstructed *in vitro* (Rollin et al., 2015; Schwander et al., 2016). Cell-free metabolic pathways allow easy verification of biocatalyst function, determination of kinetic parameters, as well as evaluation of the network by kinetic modelling (Santacoloma et al., 2011). Note, however, that assemby of degradative pathways in cell-free systems (whether with purified enzymes or cell extracts) is not only to prototype and parametrize new routes, but also for direct use in the field (Karig, 2017). Despite the drawbacks that cell-free systems cannot self-propagate, that certain biomolecules can be sensitive to oxidizing environments, and that their encapsulation and large-scale production might be costly, these systems offer an appealing means to circumvent problems associated to GMO release to the environment. Apart from increasing safety, they offer other clear benefits. For instance, *in vitro* systems can operate in the presence of toxins that would inhibit or kill live cells, and metabolites, regulators and enzymes can be produced in optimized concentrations without interfering with cell components. Most important for release and predictability, the evolutionary opportunities of such non-live agents are zero, making the emergence of unexpected properties virtually impossible.

In vitro metabolic networks can suffer from suboptimal efficiency due to the lower enzyme concentration compared to the extremely dense cell cytoplasm (Hodgman and Jewett, 2012). This issue can be mitigated by enzyme immobilization and improved spatial organization *via* synthetic protein or DNA scaffolds that reduce diffusion of pathway intermediates and promote substrate channeling (Siu et al., 2015). Alternatively, biocatalysts can be precipitated and covalently interconnected in cross-linked enzyme aggregate particles (Sheldon, 2011); this method was applied successfully to synthesize toxic nucleotide analogues by an immobilized five-enzyme synthetic pathway (Scism and Bachmann, 2010). The biochemical route described was completed with functional ATP regeneration, which demonstrate that *in vitro* technologies can cope with another possible drawback – limited co-factor recycling.

Thus far, mainly single immobilized enzymes or whole-cell biocatalysts have been exploited in biotechnological processes intended to remove polluting chemicals such as polyethylene terephthalate, 2,4-dinitrophenol, atrazine, or inorganic nitrates (Barth et al., 2016; Dehghanifard et al., 2013; Mutlu et al., 2015; Trögl et al., 2012). *In vitro* assays with cell-free extracts or purified enzymes, respectively, helped to identify an unknown anaerobic pathway for complete phthalate degradation to CO_2 in *Thauera chlorobenzoica* 3CB-1 or to decipher sequestration of the highly toxic intermediate tetrachlorobenzoquinone in pentachlorophenol degradation pathway in *Sphingobium chlorophenolicum* (Ebenau-Jehle et al., 2017; Yadid et al., 2013). Geueke *et al.* showed the benefits of an *in vitro* engineering approach in the model of γ-HCH biodegradation pathway (Geueke et al., 2013). They used a system of two purified enzymes, the dehydrochlorinase LinA and the haloalkane dehalogenase LinB, which initiate biotransformation of γ-HCH, a prohibited insecticide. They separately incubated five isomers

that form technical HCH (which used to be applied frequently instead of pure γ-HCH) with various LinA:LinB ratios and determined metabolic profiles of sequential biotransformations. Analyses of these profiles helped determine the environmental fate of HCH isomers, and showed that the original HCH degradation pathway is optimized by evolution for γ-HCH, but not for other isomers that co-pollute contaminated sites.

The first report of *in vitro* assembly of a fully functional synthetic biodegradation pathway was published by Dvorak and co-workers (Dvorak et al., 2014a). The authors adopted the cell-free strategy using immobilized engineered haloalkane dehalogenase DhaA31, halohydrin dehalogenase HheC and epoxide hydrolase EchA for TCP biotransformation to harmless, valuable glycerol in contaminated water (**Fig. 6D**). The practical utility of bacterial recombinants that mineralize TCP (desccribed in Sections 2.3.1 and 2.3.2) is limited by substrate toxicity and by legislative barriers on the application of GMO (Kurumbang et al., 2014; Samin et al., 2014). Probing the function of the pathway in *in vitro* conditions therefore lied ready to hand. The authors successfully immobilized the pathway in the form of purified enzymes or cell-free extracts in cross-linked enzyme aggregates and lens-shaped polyvinyl alcohol particles. The immobilized pathway showed almost the same efficiency of TCP-to-glycerol conversion as mixture of free enzymes. Physicochemical properties of polyvinyl alcohol lentils allowed recovery from the reaction mixture and recycling of the pathway. In addition, the immobilized enzymes retained more than 50% of their initial activities for over 2 months of continuous operation in a bench-top packed bed reactor. The study indicated that the immobilized route removes TCP from heavily contaminated water with a pollutant concentration (~ 1 g/L) that would be detrimental to the living degraders. One can anticipate that such conversions of toxic TCP to useful glycerol could even pave the way for prospective valorization of this waste chemical.

Although this biotechnology requires further validation and tuning, the work showed that *in vitro* assembly of natural or synthetic enzymatic pathways with engineered enzymes is a promising concept for the biodegradation of polluting compounds and toxic industrial waste products. Transport of such pathways *via* non-live carriers is related conceptually to efforts to produce and release complex therapeutic agents for human use. In both cases, their application demands the development of a sort of *environmental Galenic science* that enables supply of the engineered biological remedy when and where needed.

4. Towards planet-wide bioremediation interventions: CO$_2$ capture as a large-scale challenge

Environmental deterioration due to emissions of recalcitrant chemicals seems to pale when compared to the problem of global warming caused by the release of greenhouse gases that originate in human activities. The bulk of these gases comprise four natural molecules, carbon dioxide (CO$_2$), methane (CH$_4$), nitrous oxide (N$_2$O) and ozone (O$_3$), as well as one class of xenobiotics, the chlorofluorocarbons or CFC. Given the diluted, aereal and global nature of this problem, the strategies contemplated for tackling these chemical species differ considerably from those that centre on site-specific pollution issues. Most proposals thus far focus much more on reducing emissions than on capture and returning to innocuous chemical or mineral forms. All these compounds have their own natural biogeochemical cycle and, in theory, simply reducing their input into the biosphere should restore the original equilibrium (Rockström et al., 2017). Recent ecological thought nonetheless postulates that the impact of climate change in many ecosystems is already irreversible by natural means, and call for

large-scale interventions that could help to resolve this impasse (de Lorenzo et al., 2016). In this case, what types of options could be considered from a systemic biology perspective?

Some incipient attempts have appeared recently, mostly in the field of engineering bacteria with a greater capacity for non-photosynthetic fixing of CO_2 (Erb and Zarzycki, 2016). One option is to use the six alternative pathways of autotrophic carbon fixation known in nature (Fuchs, 2011; Hicks et al., 2017) as a starting point to optimize such natural processes. Another possibilty is the invention of new routes by rewiring or modifying existing enzymes in a new configuration. A remarkable example of this solution is the work of Antonovsky and co-workers (Antonovsky et al., 2016), who showed that combining metabolic rewiring, recombinant protein expression, and laboratory evolution enables the production of sugars and other biomass constituents by a fully functional Calvin-Benson-Bassham (CBB) cycle in *E. coli*. In the bacteria designed in this way, carbon is fixed via a refactored CBB cycle, whereas reducing power and energy are derived from pyruvate oxidation (**Fig. 7**). It is interesting to note how evolutionary approaches adjust fluxes and generate connections between the designed CBB cycle and the host biochemical network, that cannot be predicted or designed.

A separate approach was recently developed by Schwander and colleagues (Schwander et al., 2016), who developed a synthetic route for continuous CO_2 fixation *in vitro* by composing a cycle formed of 17 enzymes that convert CO_2 into organic molecules. The cycle was draughted by metabolic retrosynthesis with enzymes from nine organisms from three domains of life, further optimized through rounds of protein engineering and metabolic proofreading. Although this completely designed pathway has not yet been implemented *in vivo* (*e.g.*, in a bacterial carrier) it is a question of time until it is done and propagated throughout an entire microbial community (de Lorenzo et al., 2016).

Finally, today's systemic approaches enable the invention of enzymes from scratch through *de novo* protein design followed by directed evolution, which opens perspectives for improving the natural state of affairs regarding fixation of CO_2 (and other greenhouse gases) as well as valorization of this waste as biotechnological feedstocks (Huang et al., 2016; Renata et al., 2015). A separate matter is the large-scale deployment of the microbial agents for CO_2 retrieval. Existing technology allows their implementation in biofilters and bioreactors that capture emissions at source. But how can we spread such desirable catalytic properties at a very large, even at planetary level? Such technologies are not yet available—let alone that they raise a large number of safety questions. But they must necessarily be developed if we are not just to control greenhouse gas discharges, but also aim at their eventual reduction to pre-industrial levels (de Lorenzo et al., 2016).

Figure 7

Unfortunately, greenhouse gases are not the only globally widespread molecules to be concerned about. Much before global warming associated to CO2 emissions became so conspicuous, widespread pollution by endocrine disruptors (e.g. alkylphenols, bisphenols, DDT, PCBs, polybrominated diphenyl ethers, phthalates and perfluorooctanoic acid) was well documented. Moreover, other *silent pollutants* originate in the pharmaceutical industry e.g. antibiotics and hormones. They end up in a large variety of ecosystems in a diluted but still active form. Finally, plastics (e.g. polycarbonate, polystyrene, polyethylene terephthalate, low/high-density polyethylene, polypropylene and polyvinyl chloride) are currently receiving a considerable focus as a major environmental problem. A number of microbial strains able to

totally or partially act upon these compounds have been isolated (see above) but in general the biodegradation process is very slow. The global nature of these types of pollution thus requires the development of new, efficient microbial pathways for their elimination, quite a challenge for contemporary synthetic biologists and metabolic engineers (de Lorenzo, 2017). But pathways and hosts able to deploy them are not enough. Effective strategies for global dispersion of biodegradative activities of interest are badly needed—perhaps inspired in gene drives or some type of self-propagating, massive horizontal gene transfer (de Lorenzo et al., 2016).

5. Conclusions and perspectives

The practical examples described in the preceding sections show that technologies of systemic biology offer promise not only for the renaissance of bioremediation using anthropogenically enhanced microbial degraders, but also for entry to the new era of Bioremediation 3.0. The bottlenecks described at the beginning of this text are one by one being released or removed with the help of new computational and experimental tools. Databases and pathway prediction systems help the user to select suitable chemically and energetically feasible traits for desired tasks. Multi-omic analyses provide valuable information that facilitates choice of a suitable host. Genome-scale and kinetic models applied hand-in-hand with genetic engineering techniques enable tailoring of gene expression, reduction of metabolic burden, and pathway optimization in the context of host metabolism. The visionary concepts of synthetic biology and global scale CO_2 bioremediation open up new dimensions for engineering microbial degraders. General work schemes and standards for design, building, and fine-tuning of selected biochemical routes, networks and whole-cell biocatalysts are simultaneously becoming better defined and accepted by the community.

Despite indisputable progress over the last decade of biochemical and biological engineering, the vast complexity of the living cell remains the major hurdle for any attempt for fully rational pathway design. In the case of biosensing and biodegradation pathway design and prospective applications of the genetically-modified microbes, this problem is exacerbated by the complexity of intercellular and interspecific interactions and by the still poorly understood interplay between the biotic and abiotic factors that govern contaminant biodegradation in polluted ecosystems (de Lorenzo, 2008; Meckenstock et al., 2015). We assume that, in principle, there are two ways to address this challenging complexity, now and in future.

The first anticipates that humans are unlikely to resolve the complexity of life and will partially give up the struggle for fully rational designs by providing space for natural evolution at the end of the DBTA cycle, thus expanding it to DBTAE (Design-Build-Test-Analyze-Evolve). Adaptive laboratory evolution (ALE) is a *high calibre* tool of this approach. ALE is now frequently used by academic and industrial laboratories to evolve desired properties in environmental bacteria and for fine-tuning recombinant microorganisms, but its principles have been well known since the time of the very first attempts to modify microbial phenotypes (Kellogg et al., 1981). In biodegradation research, exposing microorganisms with desirable catabolic traits to the target chemical(s) for prolonged intervals in chemostat or shaken-flask cultures enabled isolation of bacteria able to use persistent compounds such as 2,4,5-trichlorophenoxyacetic acid (the defoliating Agent Orange), or helped improve atrazine degradation rates by *Pseudomonas* sp. ADP or 2,4-dichlorophenoxyacetic by *Ralstonia* sp. TFD41 (Devers et al., 2008; Kellogg et al., 1981; Nakatsu et al., 1998). Nonetheless, the potential of ALE in engineering microbial degraders has yet not been fully exploited. ALE will be an ideal tool for empowering rationally pre-designed recombinants with resistance to diverse environmental stresses encountered in directed biodegradation processes or in

polluted ecosystems (Nicolaou et al., 2010; Oide et al., 2015; Wang et al., 2016). When combined with whole-genome sequencing and reverse engineering, ALE as an evolutionary "blind" approach can paradoxically contribute to further rationalizing the field by exposing otherwise unpredictable complex changes in multitrait phenotypes.

The second approach bets on technology without compromise, and will take advantage of continuing technological revolution in biology and related sciences. We recently witnessed the attempt of synthetic biologists to implement automation as we know it from electronic engineering in the design of orthogonal genetic circuits that would coordinate the actions of selected microbial chassis (Nielsen et al., 2016). The genetic "guts" and pathway enzymes of the chassis can be designed and synthesized *de novo* or be tailored rapidly, with surgical precision, using new genome editing and protein engineering tools (Huang et al., 2016; Hutchison et al., 2016; Nyerges et al., 2016; Ran et al., 2013; Renata et al., 2015). Although the circuit design tools still work predominantly with simple logic gates, the robustness and utility of the available minimal cells is limited, and *de novo*-designed enzymes have low activity, we can assume that the efficiency, reliability and time demand of such exercises will be further improved by progress in computer science and artificial intelligence development that could crack the complexity of life. A purely technological approach would omit any unknowns from the DBTA cycle with help of reliable computational simulations, thus expanding it to DSBTA (Design-*Simulate*-Build-Test-Analyze) and providing space for construction of whole-cell catalysts perfectly tailored for specific biodegradation tasks.

The challenges of both approaches applied to directed *in situ* bioremediation encompass the development of test schemes that will expose engineered organisms to mimicked environmental conditions, including limiting carbon sources, lack of oxygen or redox recycling, mass transfer perturbations, or interspecific predation (Meckenstock et al., 2015). A critical issue will be the choice and establishment of new chassis organisms better suited to field biotransformations than the currently available laboratory strains in terms of resistance to harsh conditions including extreme pH, temperature, osmotic pressure or fluctuating concentrations of toxic chemicals (Adams, 2016). These new strains must simultaneously be reliable, amenable to genetic manipulation, have mapped genomes and, ideally, metabolic models at hand. The *in situ* bioremediation quests will be complicated by the varying nature of polluting compounds, their mixtures and transformation products, all of which can be present at a single site (Sarigiannis and Hansen, 2012). Future microbial degraders must therefore be designed to cope with several problem compounds in parallel, and not only in terms of degradation or mineralization. The succesful conversion of dangerous contaminants into usable molecules that can be further valorized by whole-cell or cell-free biocatalysts is a desirable starting point for the development of new technologies that extend standard concepts of biodegradation and bioremediation (Dvorak et al., 2014a; Wigginton et al., 2012). Microbe-driven trash-to-treasure conversions are known from established biomass valorization processes, but this concept can also be implemented in wastewater treatment, metal and plastic recycling, or in direct valorization of industrial by-product streams or captured greenhouse gases (Bornscheuer, 2016; Koutinas et al., 2014; Nancharaiah et al., 2016; Wierckx et al., 2015).

Even so, the major challenge to the entire field of engineered degraders is not related to continued progress in the development of improved technologies, which is inevitable, but rather to the dissemination and popularization of achievements already attained. Public environmental concerns and regulatory constraints have not just restricted field tests of genetically modified microbes, but also affect the quality of fundamental research and, by the

same token, overall progress in the field. The exercises described in this review will only have a chance to become real enterprises if the general population is inspired by biotechnology and accepts it as an inseparable part of our daily routine, as it did for information technology. The new systemic disciplines that make life less puzzling as well as emerging do-it-yourself movement in biotechnology can undoubtedly contribute to a general change of attitude (de Lorenzo and Schmidt, 2017). Such change is vital, because our ability to minimize production of waste and polluting chemicals, remove existing waste or use it in bioprocesses to form value-added compounds will shape the fate of our society on a global scale.

Acknowledgements

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CAPTIONS of FIGURES

Fig. 1. Proposed workflow for engineering biodegradation pathways using systemic biology approaches and tools. The Figure sketches the roadmap to contructing superior bacterial catalysts for environmental bioremediation that capitalize on systems and synthetic biology, as explained in this review.

Fig. 2. Initial aerobic biotransformations of the emerging contaminant 1,2,3-trichloropropane (TCP) generated by the EAWAG-BBD Pathway Prediction System. Reactions are tagged with one of the 249 biotransformation rules used for the predictions. Reactions proven experimentally to be catalysed by haloalkane dehalogenase DhaA, epoxide hydrolase EchA and halohydrin dehalogenase HheC in aerobic conditions are highlighted with thick arrows (Bosma et al., 1999). The scheme was adopted and modified from http://eawag-bbd.ethz.ch/predict/. Aerobic likelihood specifies whether the reaction will occur in aerobic conditions, exposed to air, in soil (moderate moisture) or water, at neutral pH, 25°C, with no competing compounds. Abbreviations: DCP, 2,3-dichloropropane-1-ol; ECH, epichlorohydrin; CPD, 3-chloropropane-1,2-diol; GDL, glycidol.

Fig. 3. The action of the EDEMP cycle is illustrated with a theoretical example of flux distribution in glucose-grown *P. putida* KT2440. Total carbon uptake is considered to be 100 (arbitrary units), and glucose can be split into the phosphorylative (Glk) or oxidative (*x*) branches. The net formation rates (*v*) of pyruvate (Pyr), ATP, and NADPH are indicated in the inset table, with *r* representing the amount of triose phosphates recycled to hexose phosphates through the action of the EDEMP cycle. Note that part of the glucose can also be oxidized through the action of the so-called *peripheral reactions*, represented by the overall flux *x* in the scheme, and that two trioses are formed per glucose consumed, *i.e.*, a glucose uptake flux of 100 (arbitrary units) will result in a pyruvate flux of 200. Abbreviations: ED pathway, Entner–Doudoroff pathway; EMP pathway: Embden–Meyerhof–Parnas pathway; PP pathway, pentose phosphate pathway; G6P, glucose-6-P; F6P, fructose-6-P; FBP, fructose-1,6-P$_2$; DHAP, dihydroxyacetone-*P*; GA3P, glyceraldehyde-3-*P*; 6PG, 6-phosphogluconate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; 2KG, 2-ketogluconate; and 2K6PG, 2-keto-6-phosphogluconate. Figure was adapted and modified from (Nikel et al., 2016a).

Fig. 4. Hypersurface plot describing the effect of catalytic efficiency (k_{cat}/K_m) and enantioselectivity (*E*-value) of haloalkane dehalogenase DhaA on the production of glycerol in the TCP pathway. The hypersurface was calculated using the mathematical model of the TCP pathway (Dvorak et al., 2014b) using the constraints of Kurumbang and co-workers (Kurumbang et al., 2014). Positions of four DhaA variants, DhaAwt (wild type, $k_{cat}/K_m = 70$ M^{-1}s^{-1}, *E*-value = 1), DhaA r5-90R ($k_{cat}/K_m = 20$ M^{-1}s^{-1}, *E*-value = 10), DhaA31 ($k_{cat}/K_m = 600$ M^{-1}s^{-1}, *E*-value = 1), and hypothetical DhaAXX ($k_{cat}/K_m = 700$ M^{-1}s^{-1}, *E*-value = 10), are indicated by red dots. Note that the majority of mutations (orange spheres) introduced into DhaA r5-90R and DhaA31 are adjacent to the active site (red spheres) and to the access tunnels (in green). Hypothetical enzyme DhaAXX with 10-fold improved catalytic efficiency and 10-fold improved enantioselectivity compared to DhaAwt, would enable production of 1 mM glycerol (red line) from 2 mM TCP in the culture of recombinant *E. coli* BL21(DE3) within 24 h time interval. Such glycerol concentration will be sufficient to support the cell growth. Figure was adopted and modified from (Kurumbang et al., 2014).

Fig. 5. Engineered *Pseudomonas putida* KT2440 as a new robust chassis for biodegradation and bioremediation. Martínez-García and colleagues used in-house genome editing tools to

streamline the chromosome of *Pseudomonas putida* KT2440 (Martínez-García et al., 2014b). Deletion of 4.3% of the original genome gave rise to the new platform strains EM42 and EM383, whose advantageous properties make them useful chassis for engineering biodegradation pathways and other applications.

Fig. 6. Examples of synthetic biology approaches applied to engineering biodegradation pathways and whole-cell degraders. (a) Martínez and co-workers divided the 4S pathway for sulphur removal from aromatic heterocycles into two modules (*dszC1-D1* and *dszB1A1-D1*) that were expressed individually in two *P. putida* KT2440 strains to form a synthetic consortium (Martínez et al., 2016). Combining the strains at a 1:4 ratio and mixing them with cell-free extract from the third strain producing DszB resulted in almost complete conversion of dibenzothiophene into sulphur-free 2-hydroxybiphenyl in assay conditions. (b) Wang et al. constructed a synthetic bacterial consortium in which each of the two *E. coli* strains functioned as a double input sensor with synthetic AND gates for detecting arsenic, mercury, and copper ions and *quorum sensing* molecules (3OC$_6$HSL; Wang et al., 2013). Strain 2 generated red fluorescence only in the presence of all three metal ions in culture. (c) Benedetti and colleagues achieved directed transition between planktonic and biofilm lifestyles of *P. putida* KT2440 by controlling cyclic di-GMP levels with an orthogonal genetic device composed of the *yedQ* diguanylate cyclase gene from *E. coli* under the control of the cyclohexanone-responsive ChnR/P_{chnB} regulatory node from *Acinetobacter johnsonii* (Benedetti et al., 2016). Designed cells with a synthetic operon for 1-chlorobutane biodegradation formed inducible biofilms with higher dehalogenase activity than free, planktonic bacteria. (d) Dvorak and co-workers assembled the first *in vitro* synthetic biodegradation pathway for 1,2,3-trichloropropane (TCP) by combining engineered haloalkane dehalogenase DhaA31, halohydrin dehalogenase HheC, and epoxide hydrolase EchA, immobilized in cross-linked enzyme aggregates (CLEAS) and polyvinyl alcohol particles (LentiKats) (Dvorak et al., 2014a). The immobilized pathway converted high concentrations of toxic TCP into glycerol in contaminated water for more than two months of continuous operation in a bench-top packed bed reactor.

Fig. 7. Hemiautotrophic growth of engineered *E. coli* allowed by decoupling energy production and carbon fixation. The hemiautotrophic growth was observed after performing three distinct steps: (i) Calvin-Benson-Bassham (CBB) cycle for the biosynthesis of sugars from CO_2 was completed by introducing only two exogenous enzymatic activities, phosphoribulokinase (*prkA*) and RuBisCO, (ii) phosphoglycerate mutase genes *gpmA* and *gpmM* were deleted to separate central metabolism into two sub-systems, *i.e.*, a CO_2-fixing part that included PrkA, RuBisCO, upper glycolysis, and the pentose phosphate pathway, and an energy-supplying module that employed lower glycolysis and the TCA cycle, (iii) the engineered strain bearing further mutations (Δ*pfkA*, Δ*pfkB*, Δ*zwf*) was exposed to the strong selective pressure towards greater carbon fixation in xylose-limited chemostat with a surplus pyruvate and CO_2. Emerged mutations made designed CBB cycle functional and the hemiautotrophic strain that replaced utilisation of xylose with CO_2 fixation dominated the population. Figure adapted from Antonovsky et al. (2016).
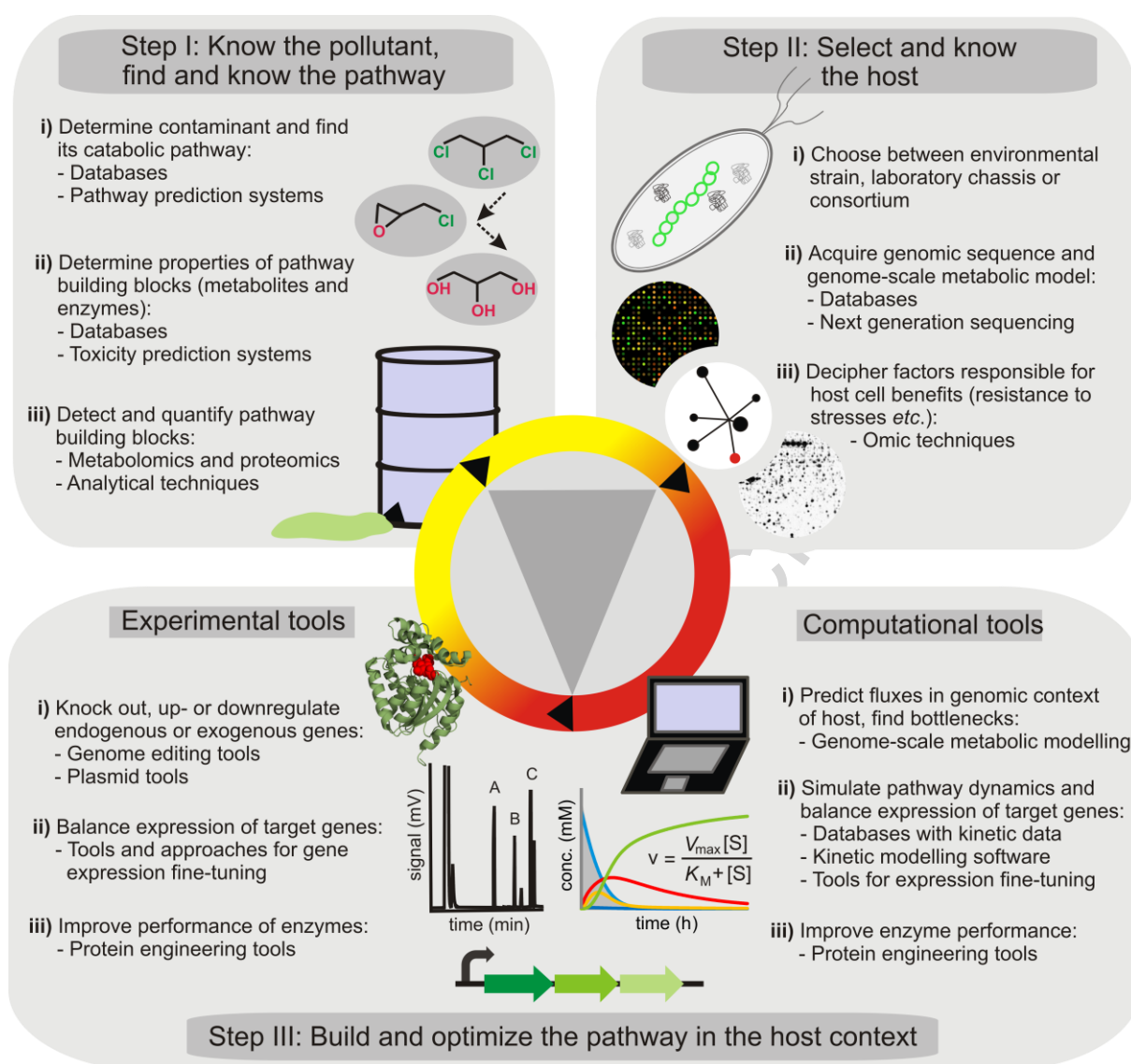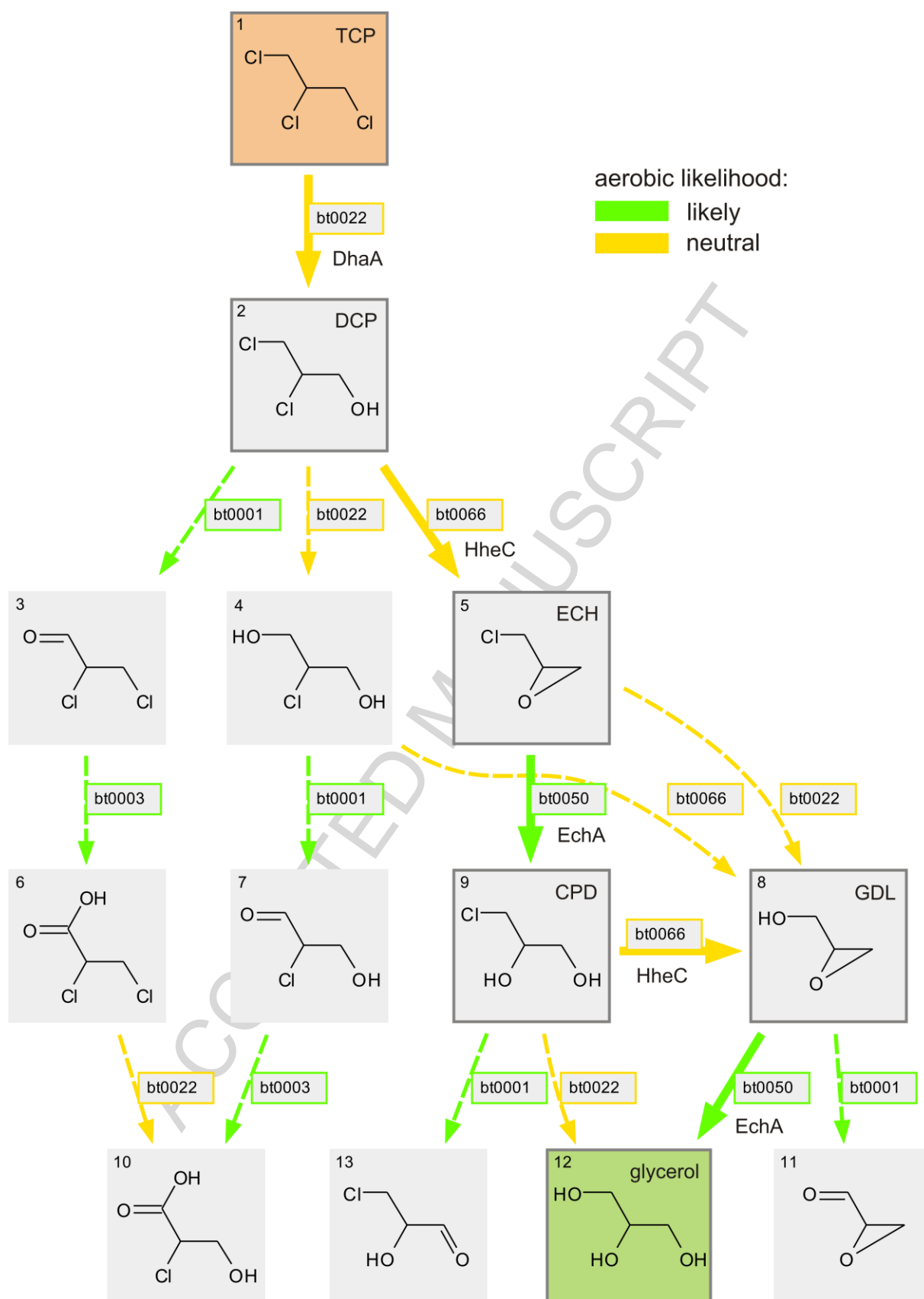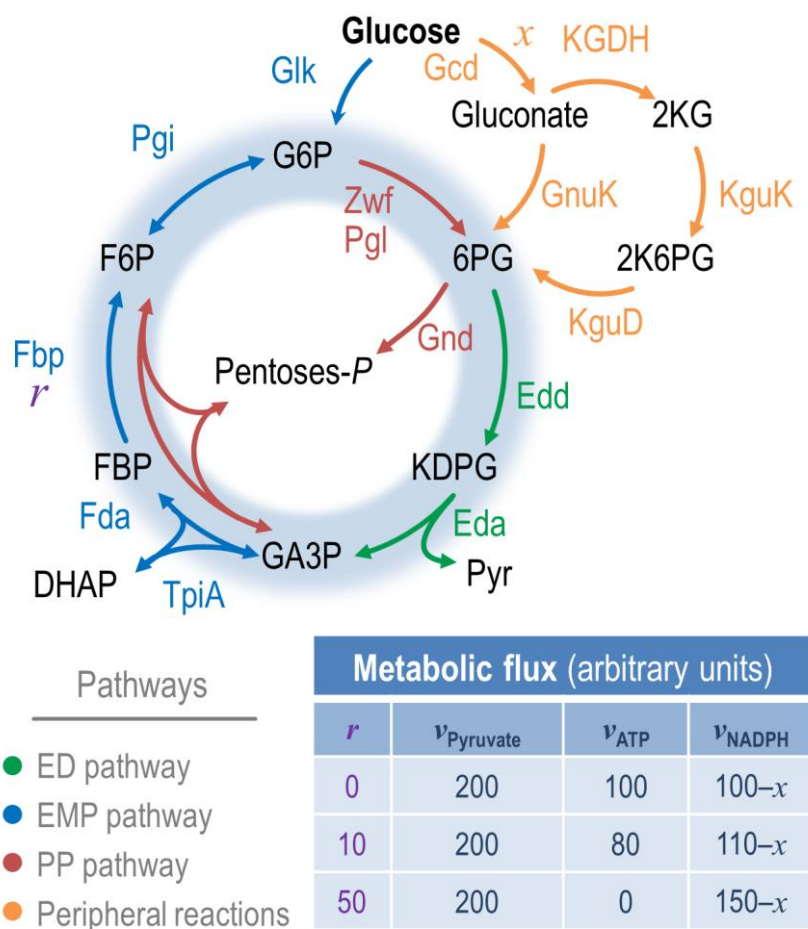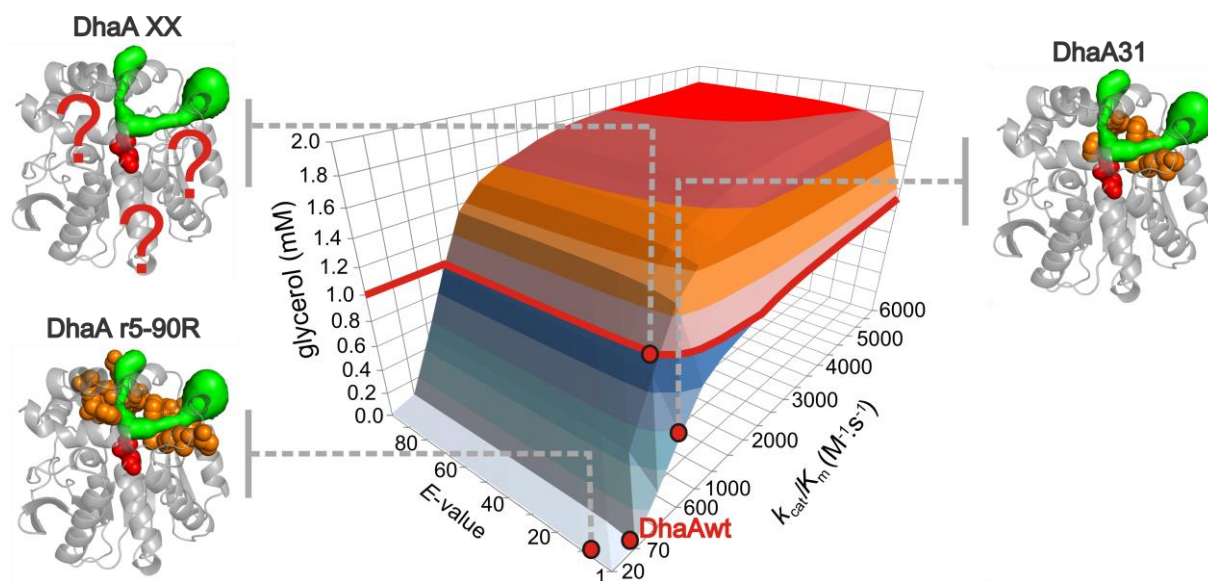
Figure 1

Figure 2

Figure 3

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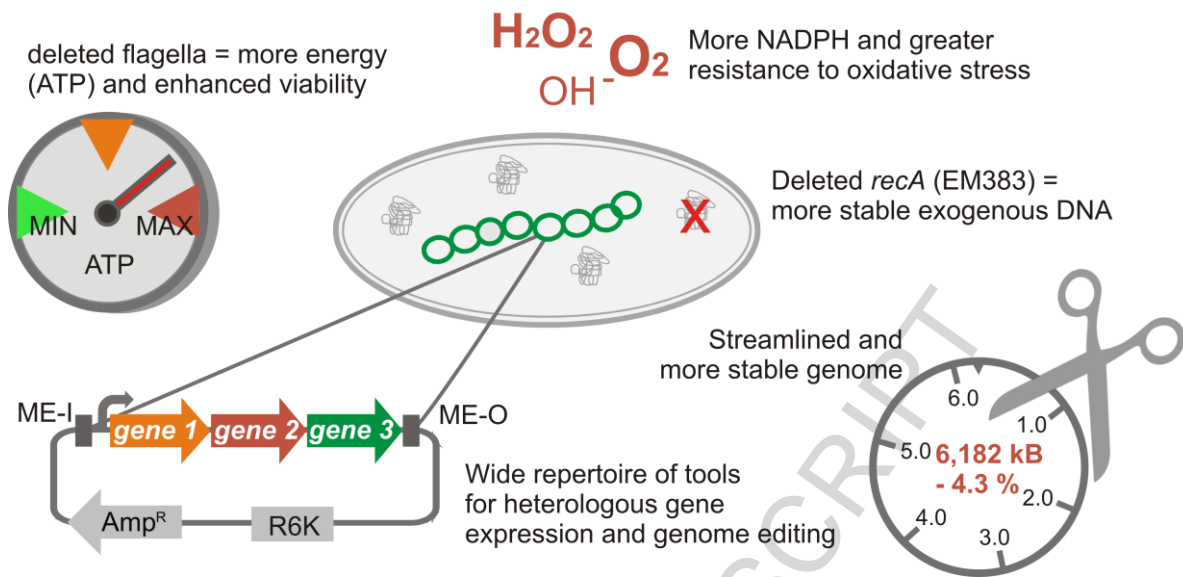
Figure 5

Figure 6

Figure 7