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The pressing need for novel bioproduction approaches faces a limitation in the number and type of molecules accessed through synthetic biology. Halogenation is widely used for tuning physicochemical properties of molecules and polymers, but traditional halogenation chemistry often lacks specificity and generates harmful by-products. Here, we pose that deploying synthetic metabolism tailored for biohalogenation represents an unique opportunity towards economically attractive and environmentally friendly organohalide production. On this background, we discuss growth-coupled selection of functional metabolic modules that harness the rich repertoire of biosynthetic and biodegradation capabilities of environmental bacteria for in vivo biohalogenation. By rationally combining these approaches, the chemical landscape of living cells can accommodate bioproduction of added-value organohalides which, as of today, are obtained by traditional chemistry.

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Synthetic metabolism for biohalogenation
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The urgent need for bio-based processes as an alternative to traditional chemistry reaches beyond the customary bulk products that have been the object of intensive research thus far (e.g. organic acids, amino acids and alcohols—to name but a few). In particular, fine chemicals that contain ‘non-biological’ atoms in their structures are attracting attention as they find multiple applications in industry [8]. Cases in point are the non-radioactive halogen elements [i.e. fluorine (F), chlorine (Cl), bromine (Br), and iodine (I), all of them within group 17 of the periodic table]. Organic molecules containing halogens (i.e. organohalides) are widely used as ingredients of clasters, adhesives and sealants, pesticides, refrigerants, fire-resistant oils, solvents, electrically insulating coatings, plasticizers, and plastics. As an example, the global fluoropolymer market was valued at ca. 5100 M€ in 2018 and is expected to reach >7900 M€ by 2023 [9]. Specialty applications of this broad family of compounds can be likewise found in the pharmaceutical industry, in the form of drugs, and even in the food industry (e.g. sucralose, a sweetener, is a chlorinated molecule). Thus, the ever-increasing demand for organohalides is confronted with a narrow set of alternatives to the traditional chemical approaches currently employed for their synthesis. This scenario prompts the question of whether biotechnology can bridge this gap and provide novel solutions to organohalide production. In this review, we argue that synthetic metabolism tailored for the incorporation of halogen atoms into the biochemistry of microbial cells is a powerful approach towards fulfilling such an overarching goal. The article starts with an overview of the physicochemical properties imparted by the presence of halogens, with a focus on drug discovery. Next, we describe enzymatic mechanisms identified for
halogenation and explain how these can be harnessed in bottom-up and top-down synthetic biology strategies to establish synthetic biohalogenation in cell factories. We conclude by discussing future avenues for optimizing these pathways to establish biotechnological organohalide production.

**General physicochemical features brought about by halogenation on organic molecules**

Many living organisms produce organohalides as part of their secondary metabolism, owing to the special physicochemical properties of these molecules that define their specific biochemical role. Oftentimes, halogenation underlies bioactivity—as epitomized by vancomycin, a chlorinated glycopeptide antibiotic produced by the actinobacterium *Amycolatopsis orientalis*. Removal of one or both Cl atoms from the molecule decreases its binding affinity towards the target peptidoglycan, significantly reducing the antibiotic activity [10]. Predicting such physicochemical effects remains a complex and challenging task even with modern chemical and analytical tools [11,12], making it difficult to pinpoint advantageous positions for halogen substitution. Nevertheless, thanks to the information gathered over the last 60 years on the development of synthetic halogenated drugs (especially bioactive molecules containing F or Cl atoms), we can now predict some general effects that follow the incorporation of halogens into organic structures.

Halogens display a high electronegativity that decreases as the covalent radius increases (Figure 1a), with F as the most electronegative element in the periodic table. This trait can influence multiple physicochemical properties of organic molecules, and fluorinated pharmaceuticals represent a prime example in terms of enhanced bioavailability and binding affinity to targets. The strength of acid-base groups [measured as pKₐ, the negative, base-10 logarithm of the acid dissociation constant (K_a)] is a key aspect defining interactions between a drug and its target site. pH-properties influence binding affinities and membrane penetrability, thus impacting every aspect of pharmacokinetics [12]. These two physicochemical traits are often affected in opposite ways, e.g., the presence of a strong base group often allows for high drug affinity with the cognate target, but limits bioavailability by impairing lipophilicity. The electronegativity of a halogen atom significantly alters the electron density of neighboring functional groups, shifting their pKₐ value by up to several logarithms. Therefore, inserting a halogen near such a pH-active group can reduce basicity, enabling balanced target affinity and membrane permeability. These two parameters should be calibrated carefully: even if improved membrane penetration can be beneficial for drugs to reach the bloodstream or the central nervous system, excess lipophilicity will lead to poor solubility (resulting in incomplete absorption and lower bioavailability) or accumulation in adipose tissues. Besides, the carbon–halogen (C–X) bond can form intermolecular, non-covalent interactions with lone pairs of heteroatoms—similar to H bonding but with half the strength [13]. Such bonding improves target-binding affinity but only for the heavier halides, as F lacks the σ hole found on the three other non-radioactive halogens. The dipole in a C–X bond also influences a molecule’s 3D conformation, fostering new interactions with previously inaccessible, deep pockets on the target-binding site [14,15]. Lastly, halogenation modulates drug stability by hampering enzyme-catalyzed reactions, e.g., oxidations carried out by archetypal cytochrome P450 monooxygenases. Thus, halogens (and especially F [16]) are critical targets for the hit-to-lead phase of drug discovery, illustrated by the fact that ca. 20% of commercial pharmaceuticals contain at least one F atom [17]—with three out of the five top-selling drugs being fluorinated molecules (Figure 1b). F is also an essential component of agrochemicals, and the entire market of organohalides is largely dominated by chlorinated and fluorinated molecules (Figure 1b). While synthetic chemistry has harnessed halogenation to tune physicochemical properties of organic molecules (e.g. in bioactive compounds), organohalides are widespread in Nature—and the underlying enzymatic mechanisms for halogen incorporation serve as a toolset to build synthetic metabolism.

**Diversity of synthetic and natural organohalides**

Halogen elements are rather abundant on Earth in their halide forms (X⁻): while Cl and Br salts are found in the oceans, F-containing minerals form a significant part of the Earth’s crust components, where F is the 13th most abundant element. The Earth’s crust contains around 90 quadrillion tons of F, 45 quadrillion tons of Cl and 190 trillion tons of Br, with volcanoes emitting 3 million tons/year of Cl and 11 million tons/year of F. Organohalides were once thought to be primarily produced abiotically, for example, through biomass burning and volcanic activity, where extreme temperature and pressure conditions could allow for the formation of C–X bonds. Man-made organic chemistry was considered as the only other supply of organohalides (Figure 1c), e.g., through the production of compounds such as the chlorinated pesticide dichlorodiphenyltrichloroethane (DDT). The structural landscape of synthetic organohalides expanded significantly during the 19th and 20th centuries to include multiple halogenated bioactives (e.g. the brominated benzodiazepine bromazepam and the chlorinated anti-platelet drug clopidogrel; Figure 1c). Discoveries in bioactives were matched with the emergence of a large market for halogenated polymers, e.g., polyvinyl chloride (PVC) and polytetrafluoroethylene (PTFE). Although we mention these (few) selected examples for the sake of historical accuracy and to illustrate the industrial importance of organohalides, we refer the reader to the rich literature on synthetic organohalide chemistry and focus
Overview of natural and synthetic organic products containing halogens and their industrial relevance. (a) Key characteristics of the non-radioactive halogen atoms (X). The atomic radii (drawn to scale) and electronegativity (which dictates the strength of the C–X bond in an organohalide) are key to the unique physicochemical properties of halogenated molecules. (b) General figures for the presence of organohalides in the market according to recent estimations. The predominant role of fluorine (F) is highlighted in the agrochemical and pharmaceutical drug categories. (c) Structural diversity of synthetic organohalides, with examples of herbicides (e.g. ioxynil and DDT), pharmaceuticals (bromazepam, clopidogrel, and fluoxetine), industrial polymers (PCV and PTFE), and flame-retardants (hexabromocyclododecane). (d) Structural diversity of organohalides found in Nature. Most of these natural products are synthesized by marine organisms, and usually contain chlorine (Cl) or bromine (Br) atoms. A handful of organofluorines are produced by terrestrial organisms (e.g. fluoroacetic acid in Streptomyces sp. and some plants). Finally, iodine (I) is present in some hormones and related bioactive compounds.

on natural halogenated molecules henceforth. The rare documented instances of naturally occurring organohalides, as well as their perceived toxicity, led scientists to view them as Nature’s oddities for a long time. Yet, there was an inkling that experimental limitations in unraveling natural products could have veiled the landscape of halogenated molecules therein.

**Halometabolites at the seabed**

The development of research submersibles facilitated the exploration of the world’s seafloor environment, leading to the discovery of the largest array of unknown organisms thus far. With a plethora of new specimens at hand and improved sampling and identification techniques, >5000 natural halogenated products were identified, showcasing that organohalides are far from being biochemical irregularities [18]. Since marine seaweed, animals, bacteria, and fungi evolved in a Cl-rich and Br-rich environment, it does not come as a surprise that these organisms developed strategies to incorporate halides into their metabolite repertoire [19]—making them the biggest supply of halometabolites on Earth. These molecules display a wide structural diversity (Figure 1d), ranging from simple haloalkanes found in phytoplankton (e.g. chloromethane,
bromomethane, and iodomethane), to the complex halogenated terpenoids, polyketides, peptides, and alkaloids isolated from Actinobacteria [20]. Because of the solicessity of sponges, corals, and marine plants (and the consequent inability to evade predators), halometabolites were originally considered to be exclusively involved in chemical warfare or survival mechanisms. For instance, chloramphenicol, produced by *Streptomyces venezuelae*, played a remarkable role in the fight against bacterial infections together with the aforementioned vancomycin (isolated from the soil bacterium *A. orientalis*). Iodine-containing halometabolites proved to be equally valuable in the pharmaceutical industry (although they are drastically less prominent in Nature). An important example of this sort is that of calicheamicins (Figure 1d), enediyne anti-tumor antibiotics produced by the Actinobacterium *Micromonospora echinospora*, used in the treatment of acute myeloid leukemia. Although not as structurally diverse, organohalides are also present in terrestrial environments, as illustrated in the examples below.

**Halometabolites on the earth’s surface**

A handful of chlorometabolites and fluorometabolites are synthesized by Earth-bound organisms [21]. Plants and lichen proved to follow the same strategy as their marine cousins in that such compounds are typically produced as defense mechanisms. Some plants, e.g., the African shrub * Dichapetalum cymosum*, synthesize and accumulate fluoroacetic acid (and a few other fluorometabolites) in their seeds—possibly to defend themselves against foraging animals (and very effectively so: it has been estimated that ca. 150 g of plant dry weight would suffice to kill a 500-kg cow) [22]. Fluoroacetic acid, as other organohalides, has been used as a pesticide against rodents under the commercial trade name 1080™. As of today, fluoroacetic acid has been banned in several countries because of its impact on the environment, but is still used in Australia and New Zealand to fight non-native land mammals that threaten endemic ecosystems. As we will discuss later, low-molecular-weight halometabolites (e.g. haloacids) could be harnessed for the biosynthesis of more complex, value-added organohalides.

Although halometabolites are often involved in defense strategies, they are also required for peaceful (or passive) mechanisms, e.g., plant development. Such is the case of the auxin 4-chloroindole-3-acetic acid, a phytohormone found in peas and beans that triggers nutrient storage in seeds. The secondary metabolism of insects and higher animals also accommodates halometabolites. Social insects, for instance, are known for their peculiar pheromones-based communication—and termites have adopted chloroform (CHCl₃) as one of such signal molecules (although the biochemical synthesis mechanism is yet to be fully resolved). Termites are responsible for depleting the chlorine contained in the soil near their mounds by producing >100,000 tons of CHCl₃ per year, which accounts for ca. 15% of the global CHCl₃ emissions. Even humans rely on halogenated compounds as signal molecules. The iodine-containing thyroid hormone thyroxine (3,5,3',5'-tetraiodothyronine: Figure 1d) regulates the metabolic rate of the entire body, and 2-octyl-γ-bromoacetoacetate is involved in the induction of the rapid-eye-movement sleep. Taken together, these observations illustrate how Nature evolved biochemical mechanisms for incorporating halogen atoms into organic structures in organisms that inhabit strikingly different ecosystems. Over the years, the discovery and characterization of halogenases paved the way to halogen biocatalysis. Accordingly, the main mechanisms for enzymatic halogenation are disclosed in the next section.

**Halogenases mediate different biochemical mechanisms for halogen incorporation into organic structures**

Chemical processes for site-specific halogenation of organic structures remain challenging, involving harsh and environmentally hazardous reaction conditions, and relying on toxic reagents and solvents. Furthermore, these procedures are characterized by poor stereoselectivity, low space-time yields, and formation of unwanted side-products (as a consequence of the high reactivity of halogenating reagents) that necessitate tedious, low-yield separation methods [23,24]. In contrast, enzymatic halogenation (i.e. biohalogenation) offers an effective and environmentally friendly alternative to circumvent most of the problems typically associated with traditional chemistry [25,26]. Similarly to other enzyme-catalyzed processes, biohalogenation brings advantages in terms of regio-selectivity and stereo-selectivity while avoiding the need for protecting or activating chemical groups in target molecules or performing extensive by-product separation. In this section, we summarize key discoveries related to enzymes mediating halogenation chemistry within the three main known mechanisms of action—i.e. electrophilic, nucleophilic, and radical catalysis (Figure 2).

**Haloperoxidases**

Haloperoxidases are the first group of halogenases to be discovered when a chloroperoxidase was identified in the fungus *Caldariomyces fimago* in 1959 as the key enzyme involved in the biosynthesis of the Cl-containing antibiotic caldariomycin [27]. These enzymes are named after the most electronegative halogen that they can activate, i.e., a bromoperoxidase will be able to oxidize Br and I but not Cl. No fluorination reaction has been detected for this type of halogenase—probably due to the physicochemical properties of F, which render the formation of electro-negative F species impossible under biological conditions. Haloperoxidases are further classified depending on the cofactor required for their activity, either heme-dependent or vanadium-dependent. Heme-dependent haloperoxidases, e.g., the chloroperoxidase from *C. fimago*, oxidize X⁻ ions in presence of H₂O₂ to form the
corresponding hypohalous acids (HXO, an oxyacid containing a halogen atom), which diffuse outside of the active site and then react with electron-rich compounds, usually at the most reactive site of the substrate [28]. Vanadium-dependent haloperoxidases rely on the same reaction mechanism of production and release of a free hypohalous acid using vanadium as cofactor in the form of a vanadate prosthetic group. Regardless of how the hypohalous acid is produced, this highly reactive chemical species acts as the halogenating agent and generates an unspecific mixture of products displaying different halogenation degrees. This feature is illustrated by phenol (poly)chlorination catalyzed by CPO chloroperoxidase (Figure 2a). Haloperoxidases offer limited industrial applications since they lack a narrow substrate preference, and they display limited regio-specificity and stereo-specificity.

Flavin-dependent halogenases
The first flavin-dependent halogenase (FDH) was isolated in *Streptomyces aureofaciens* in 1995, and FDH was recognized as being involved in the pathway for 7-chlorotetracycline biosynthesis. The most studied FDH members are tryptophan halogenases, for which mechanistic details have been elucidated in detail. PrnA from *Pseudomonas fluorescens*, for instance, mediates a specific chlorination at positions 5, 6, or 7 in the indole ring of tryptophan [29]. RebH, another well-characterized tryptophan 7-halogenase, can install a Cl atom at position 7 of the aromatic ring (Figure 2a), and this enzyme has been identified as the key catalytic element in the biosynthesis of the antitumor agent rebeccamycin by the Actinobacterium *Lechevalieria aerocolonigenes*. Other members of the relatively broad FDH family of halogenases are involved in biosynthetic pathways of antibiotics (e.g. vancomycin) and high-molecular-weight halometabolites—mostly chlorinated species. The mechanism catalyzed by FDHs relies on a reduced flavin adenine dinucleotide (FADH2) cofactor directly reacting with molecular O2, subsequently reacting with an X− ion and generating the hypohalous acid that acts as the halogenating species. Compared to the above-described haloperoxidases, HXO is specifically guided by FDH towards an organic substrate, thereby achieving highly specific halogenations. Recent efforts have exploited this circumstance for in vivo combinatorial biosynthesis of indolocarbazole alkaloids in engineered strains of *S. albus* [30].

**S-Adenosyl-L-methionine (SAM)-dependent halogenases**
Yet another mechanism of enzyme-mediated halogenation relies on SAM, the universal methyl donor, as the organic substrate. Methyl halide transferases are the most studied class among the group of enzymes collectively known as SAM-dependent halogenases, which target the methyl group of SAM to catalyze the halogenation reaction (thus yielding Me–X) [31]. The catalytic mechanism...
involves the nucleophilic attack of SAM by the halide anion (X⁻) to form Me–X while releasing 3'-adenosyl-1-L-homocysteine (SAH) in the process. Importantly, SAM-dependent halogenases seem to be the only group of enzymes able to catalyze the formation of a C–F bond. The SAM-dependent fluorinase from Streptomyces cellvata (FIA, S'-fluoro-5'-deoxyfluoroadenosine synthase or adenosyl-fluoride synthase; Figure 2b) has a strong substrate preference for the F⁻ ion over Cl⁻ (and no reported activity on Br⁻ or I⁻) [32,33]. Because of the strong solvation energy of halide ions in an aqueous environment (especially important for the compact F⁻ ion), SAM-dependent halogenases need to overcome a high-energy barrier to convert X⁻ ions into potent nucleophiles within the active site. Therefore, SAM-dependent halogenases are sluggish enzymes (kcat, SAM ≈ 0.1 min⁻¹). The substrate scope of these halogenases seems to be largely limited to SAM-related substrates, e.g. 2-deoxy-SAM analogs. Besides FIA, another prominent SAM-dependent halogenase is the Sall chromolase, identified in Salinispora tropica as to be involved in the biosynthesis of salinosporamide (a promising anti-tumoral agent) and displaying a very low preference for F⁻ as a nucleophile [34]. Biosynthesis routes where these halogenases are present (often, as the first enzyme in the sequence) usually involve halonucleotides and halosugars as intermediates and low-molecular-weight halocids as end-products—a circumstance that can be exploited to build synthetic metabolisms in surrogate hosts.

Non-heme Fe(II) 3'-ketoglutarate-dependent halogenases

This group of halogenases belongs to the broad category of α-ketoglutarate-dependent oxygenases that catalyze hydroxylation, epoxidation, epimerization, demethylation, ring formation, C-C cleavage and desaturation reactions [35]. These enzymes follow a radical mechanism that proceeds through the formation of a high-valence, short-lived Fe(IV)-O-ferryl intermediate, a powerful oxidant that abstracts an H from non-activated C–H bonds to create a substrate radical and another Fe(III)–OH/Cl⁻ intermediate. The Cl⁻ residue then binds to the substrate radical, thereby yielding the halogenated product. An archetypal example of this sort is the CmaB halogenase (Figure 2c), involved in the biosynthesis of coronic acid (a cyclopropyl amino acid component of coronatine, a phytotoxin produced by Pseudomonas syringae pv. glycinea) [36]. Although these biocatalysts display a significant degree of regio-specificity and stereo-specificity, side reactions were reported (e.g. aliphatic hydroxylation, nitration, and azidation of the substrate). Structural studies revealed a remarkable feature shared among non-heme Fe(II) α-ketoglutarate-dependent halogenases: they utilize substrates covalently tethered to acyl or peptidyl carrier proteins within a phosphopantetheine arm. Prominent applications of these halogenases are mostly restricted to in vitro reconstruction of biosynthetic pathways composed of purified enzymes, e.g. for the one-pot biosynthesis of the functionalized lipopeptide jamaicamide A [37].

Bottom-up and top-down approaches to biohalogenation

The wealth of halogenation reactions in Nature can be exploited by using these as functional modules to assemble synthetic metabolism. We divide these synthetic biology strategies into two major categories: (i) bottom-up approaches, which start by the creation of a C–X bond by means of a halogenation reaction, and then connect the halometabolite to the rest of the host biochemical network, and (ii) top-down approaches, where a fluorinated substrate is fed to microbial strains (either wild-type or engineered) in a biotransformation setup. Relevant examples of either category are discussed in the sections below (with a focus on biofluorination), starting by a general framework to crimp halogenation reactions in cell factories by exploiting growth-coupled designs.

Harnessing the power of evolution to nest synthetic metabolism in cell factories

Engineering novel synthetic metabolic pathways in microbial hosts entails substantial metabolic modifications, and a combination of rational design and evolution is often required to attain meaningful titers and yields. In this section, we present a general framework for establishing synthetic biohalogenation—an approach that has been successfully implemented for pathway engineering in several bacterial hosts [38,39,40]. This framework is based on three core aspects: (i) pathway modularization, facilitating the fine-tuning of gene expression as well as enzymes levels and catalytic activities thereof; (ii) metabolic rewiring of microbial strains for growth-coupled selection, enabling high-throughput, growth-based experiments for testing and optimizing pathway components; and (iii) adaptive laboratory evolution (ALE), exploiting a selective pressure to mediate network-wide rearrangements towards efficient synthetic metabolism. This approach forms the core of bottom-up strategies to establish bihalogenation in vivo.

Because of the nature of the reactions involved, engineering biohalogenation modules in cell factories can be problematic. The number of challenges increases as the traditional ‘copy-and-paste’ metabolic designs become more complex, involving novel pathways and transformations—which can expand the number and structural diversity of new-to-Nature products [42]. Toxicity, caused by surrogate substrates, alien pathway intermediates, or the very organohalide products of interest, is a major hurdle towards establishing synthetic biohalogenation in cell factories. Moreover, as organohalides are foreign to central carbon metabolism, screening strategies based on direct selection are ineffective, requiring extensive, time-consuming analytical procedures [43,44].
However, dividing a synthetic pathway into simpler modules, each comprising few reaction steps, emerges as a smart strategy to overcome these multifactorial challenges. The mere overexpression of genes encoding pathway enzymes is not sufficient to establish a strong flux due, among other reasons, to suboptimal enzyme levels (and activities), depletion of intermediates in the form of unwanted by-products, limited precursor supply and the toxicity issues indicated above [45]. Modularization of a metabolic route, in contrast, allows for the study and optimization of different pathway segments by plugging-in each segment in a dedicated selection strain, where the encoded activities complement a metabolic deficiency (i.e. synthetic auxotrophy) [46]. Once each module proves functional, individual parts can be assembled to build a complete biohalogenation pathway—increasing the chances of successful screening and selection.

As indicated, growth-coupled selection adopts selection strains—where a synthetic auxotrophy is established by introducing multiple gene deletions so that microbial growth is made dependent on the tested module’s activity under specific cultivation conditions (e.g. a minimal culture medium amended with selected carbon sources). In fact, selection strains can be exploited as whole-cell biosensors for the very products of the synthetic module, since the activity of the pathway is assessed through growth as a simple read-out, i.e. by following changes in the optical density of the culture. Thus, organohalide biosynthesis must be intertwined with the host native metabolism to exploit growth-coupled selection. Non-halogenated by-products of the biosynthetic pathway can be exploited to relieve the engineered auxotrophies in the selection strain—rather than repurposing the metabolic network around the alien (and, often, toxic) halogenated products. A synthetic biohalogenation module easily selectable in growth-coupled schemes would comprise a halogenation reaction (e.g. catalyzed by the fluorinase enzyme), which yields a first organohalide (X–R). X–R is subsequently converted into one or more halogenated intermediates (X–R') in preparation for a cleavage reaction (e.g. an aldolase acting on a halosugar) that releases the halogenated product (X–R') and a non-halogenated side product (R''; Figure 3a). Metabolic rewiring of the host (e.g. by eliminating conditionally essential genes) leads to a synthetic auxotroph for metabolite R'', such that microbial growth will be impaired or altogether prevented in the absence of an alternative source of R''. Expressing the synthetic biohalogenation module in the selection strain yields R'' as a side-product—thereby relieving the auxotrophy and allowing biohalogenation-coupled growth. Under these selective conditions, microbial growth (both at the level of specific growth rates and biomass yields) is proportional to pathway activity and, consequently, organohalide biosynthesis. Furthermore, the same synthetic module can be tested in different rewired strains with increasing (i.e. tighter) selective pressure to foster higher fluxes through the module [46]. Accordingly, the side product R'' can be used either to synthesize a single amino acid or to feed a small portion of central carbon metabolism (i.e. low to medium selection strength), or to provide an abundant biomass precursor critical for the operation of the entire biochemical network (i.e. high selection strength). Such selection schemes can be adjusted around essentially any metabolite generated from a synthetic module—not only biomass precursors. Alternative strategies can be established to generate selective pressure, e.g. by providing reduced cofactors for energy production in energy-deficient selection strains. One way or the other, and since the readout of the engineering approach is bacterial growth, high-throughput testing of multiple module designs in a given selection strain (e.g. diversifying promoters and ribosome binding sites that drive the expression of target genes) can be easily implemented in microtiter plate reader experiments.

If expressing biohalogenation modules results in poor or absent growth, different optimization strategies can be applied—e.g. fine-tuning module expression, re-routing fluxes to improve precursor supply and eliminate ‘sinks’ for intermediates, or replacing enzymes with orthologous variants displaying superior kinetics or stability. In parallel to rational design efforts, ALE can be implemented in selection strains carrying the synthetic biohalogenation module. ALE builds on natural selection, allowing microbial populations to accumulate adaptive changes under specific growth conditions [47]. In a typical ALE experiment, bacterial cells require ca. 100 to 2000 generations to accrue changes leading to improved phenotypes. Depending on the organism and doubling times, this could take from weeks up to months [48]. The main applications of ALE are accelerating growth rates, increasing production yields, optimizing substrate consumption, and boosting tolerance towards stress agents [49]—all of which are crucial for biohalogenation. In this case, ALE could be useful to improve tolerance towards the halogenated precursor (e.g. F salts), toxic halometabolites formed during the process of biohalogenation or the halogenated end-products themselves, as well as to enhance the productivity of the growth-coupled biofluorination pathway.

Different methods can be used in ALE experiments towards improving biohalogenation pathways. Once a biohalogenation module has been implanted into a rewired bacterial strain, ALE can be performed by stepwise subculturing in shaken-flask (batch) cultures or by running chemostats as continuous cultivations (Figure 3b). In contrast to chemostats, batch cultivation in flasks (moderate volumes) or microtiter plates (small volumes) is characterized by technical simplicity and a low operation cost. In this case, an aliquot of the culture is
transferred at regular intervals into a flask containing fresh medium. However, this technique makes it difficult to maintain constant growth rates and population densities due to fluctuations in the environment (e.g. nutrients, pH and dissolved O₂), which, instead, can be kept constant in a chemostat setup. Nutrient availability plays a major role in ALE. For example, cells from batch cultures in a ‘relaxing’ medium (i.e. nutrient-sufficient but pathway-dependent) are transferred into fresh medium before reaching stationary phase, adapting the strain to exponential growth under conditions where the activity of the pathway is essential to support growth. In chemostats, the growth rate is kept constant (or stepwise increased) by limiting a growth nutrient (i.e. ‘stressing’ medium)—leading to the appearance of potential growth trade-offs in relaxing conditions. Thus, ALE experiments for
biohalogenation are commonly based on the use of stressing media to pre-adapt cells and increase their fitness and tolerance to (organohalides [50]. A recent example of this sort is the implementation of ALE to adapt Escherichia coli strains auxotrophic for tryptophan to both utilize fluorinated amino acids and incorporate them into the proteome [51]. During such experiments, genetic diversity is achieved through the acquisition and fixation of mutations. In order to select bacterial cells endowed with improved fitness, evolved strains are constantly characterized by implementing multi-omic analyses and comparing the results to those obtained with the ancestral, “naïve” strain. Beneficial adaptive mutations are typically identified by next-generation sequencing (NGS) complemented with technologies such as high-resolution transcriptional, metabolic, or proteome profiling. The last step in these optimization efforts usually entails the reverse-engineering of the beneficial mutations in the naïve strain in an attempt to validate the genotype-phenotype relationship.

To date, only a few examples are available on bottom-up halogenation, including E. coli and Pseudomonas putida strains engineered to express fluorinase genes for biosynthesis of fluoronucleotides and fluorosugars [52,53]. In the case of engineered P. putida, the engineered strain bears a genetic circuit controlled by a fluoride-responsive riboswitch, triggering the production of the fluorinase (and accessory enzymes) through the addition of NaF to the culture medium—i.e. the very substrate of the biofluorination reaction.

**Exploiting the natural diversity of biochemical reactions towards synthetic metabolism for biohalogenation**

**Biodegradation pathways as a treasure trove of activities to build synthetic metabolism**

As indicated in previous sections, F is an integral structural component of numerous chemicals with industrial, agrochemical, or pharmaceutical applications [54,55]. As a result of the use and disposal of these compounds, microorganisms inevitably came into contact with organofluorines—oftentimes, in the form of xenobiotics. Environmental bacteria (e.g. species of the genus Pseudomonas) exhibit a wide catabolic funnel as part of a bowtie metabolic structure, in which a broad spectrum of structurally diverse substrates can be utilized to provide essential biomass building blocks [56–58]. Because of the steric size similarity between F, H and OH, catabolic enzymes often display biochemical activity on organofluorines. Biotransformations can yield difficult-to-synthesize intermediates and products, e.g. derivatives of bioactive secondary metabolites. Thus, microbial organofluorine metabolism can be harnessed for the incorporation of F via top-down synthetic biohalogenation. Several pathways involved in microbial degradation of fluorinated compounds have been identified [59]. The rich biochemical repertoire of aerobic bacteria (and, in particular, that of Pseudomonas species [60–62]) provides access to a wide range of fluorometabolites with multiple functional groups that are difficult to produce through traditional chemistry (Figure 4).

*Pseudomonas* species hydrolyze the highly stable, recalcitrant C–F bond in a variety of ways. Some low-molecular-weight organofluorines (e.g. fluoroacetate and 2-fluoropropionate and 3-fluoropropionate) are subjected to enzymatic dehalogenation, whereas fluoro-substituted aromatic compounds undergo a series of conversions before mineralization. In some instances, F⁻ is released from the organic structure during biotransformation. Structurally diverse arenes (e.g. toluene, cinnamate, nitrobenzene, biphenyl, phenol or aromatic amino acids) are funnelled through peripheral pathways that converge in a handful of central intermediates [63]. Aerobic bacteria cleave the aromatic ring via incorporation of two hydroxyl (O–H) groups, performed by dioxygenases. Fluorinated derivatives of protocatechuate, a common intermediate of arene degradation, are the convergence point for the degradation of 2-fluoro-4-hydroxybenzoate and 3-fluoro-4-hydroxybenzoate (P. fluorescens) or 2-fluoro-5-hydroxybenzoate (Comamonas testosteroni, previously classified as P. testosteroni). 2-Fluoroprotocatechuate can be converted into 2-fluorocarboxylic acid and 2-fluoro-3-oxoacid to eventually yield fluoroacetate and succinate. Another bacterium of this clade is known to metabolize 4-fluorophenylacetate to 3-fluorohex-3-enedioate and, subsequently, 2-fluoresuccinate. The nicotinate-degrader P. fluorescens strain N-9 can also oxidize 5-fluoronicotinate. Quantification on the total O₂ uptake of the cells under bioconversion conditions suggested further oxidation of 5-fluoro-6-hydroxynicotinic into the fluorinated analogs of pyruvate and acetate. Thereby, the inability of the strain to utilize 5-fluoronicotinate as carbon substrate further attested to the conservation of the F atom within the end products. Other studies on microbial organofluorine degradation by single bacterial species elucidated the mechanism of fluoro benzene, fluorobenzoates and fluorophenols degradation. In this context, the position of F on the aromatic ring determines substrate biodegradability and compatibility with enzymes involved in their catabolism. For example, 2-fluorobenzoate, 3-fluorobenzoate, and 4-fluorobenzoate are dihydroxylated with concomitant decarboxylation into 3-fluorocatechol or 4-fluorocatechol. Further conversion of the catechols via additional dioxygenation results in intradiol ring fission and the formation of 2-fluorocumate or 3-fluorocumate. An alternative set of fluorinated substrates with functional groups can be accessed from 3-fluorocatechol and 4-fluorocatechol, as described in *Rhodococcus* strains. In contrast to direct ring cleavage, *Rhodococcus opacus* 1cp metabolizes catechols into trihydroxyfluorobenzenes which are further converted into fluorinated end products (e.g. 2-pyrene-4-fluoro-6-
A metabolic blueprint of biochemical reactions involving fluorometabolites. The reactions shown in this diagram are categorized as part of either biodegradation routes (top panel) or biosynthetic pathways (bottom panel), and they are involved in the breakdown, detoxification, and bioconversion of fluorinated compounds. Note that some of these enzymatic transformations have been predicted based on phenotypic evidence (e.g., growth of a microbial isolate on the corresponding fluorometabolite), with several enzymatic steps yet to be fully elucidated and characterized. Some key references are given in the diagram close to the corresponding biotransformation described therein; the exhaustive list of examples and references is provided in Table S1 in the Supplementary material.
carboxylate is the endproduct of 1,2,3-trihydroxy-5-fluorobenzene breakdown). Other *Rhodococcus* isolates act on various monofluorophenols, difluorophenols, and trifluorophenols — yielding the corresponding monofluorophenols, difluorocatechols and trifluorocatechols and, following the *ortho*-cleavage route, monofluorocatechols, difluorocatechols and trifluorinated derivatives of muconate. Very few reports describe biotransformations of benzoates and catechols substituted with a trifluoromethyl (CF₃) group — the most commonly used (and valuable) fluorinated group in pharmaceuticals and agrochemicals. Establishing a synthetic metabolism for their metabolism by engineering the canonical *ortho*-cleavage and *meta*-cleavage pathways of *Pseudomonas* seems to be feasible. The wealth of biodegradation pathways that can process fluorinated compounds is detailed in Table S1 in the Supplementary material.

**Biosynthesis of fluorinated molecules by precursor-directed biosynthesis**

Besides exploiting microbial biodegradation pathways that start with organofluorines, precursor-directed biosynthesis can be used to produce complex fluorinated structures [64]. Within this approach, the culture medium of a producing microbial strain (either natural or engineered) is supplemented with a fluorinated analog of a precursor molecule, resulting in the formation of corresponding derivatives of the natural product. Often, these derivatives are not accessible through synthetic methods and display enhanced properties, e.g. pharmacokinetics. Successful incorporation of fluorinated moieties in precursor-directed biosynthesis and mutasynthesis (i.e. using mutant strains) studies have been described by Murphy *et al.* [64,65]. Fluorinated polyketides (e.g. 16-fluoroerythromycin A, *ciao* 4-fluorobutyrate feeding) and peptides ([3-fluoro-r-Ala]-cyclosporin A, *ciao* fluoroalanine feeding) are two remarkable examples of precursor-directed biosynthesis using fungal species. *Acinetobacter calcoaceticus* incorporates fluorinated fatty acids into the exopolysaccharide bioemulsifier emulsan, leading to polymers with improved emulsification properties. Similarly, *P. putida* could utilize fluorinated hydroxyphenoxoyalkanoates to produce fluorinated polyhydroxyalkanoates (PHAs), yielding crystalline polymers with higher melting points than the non-fluorinated counterpart [66]. A similar approach has been exploited in engineered *E. coli* strains tailored for the production of fluorinated polyketides [67] and fluoropolymers [68**] by feeding fluoromalonate.

Applications in which hosts have been optimized through strain engineering for organofluorines biosynthesis are relatively scarce. A recent study, however, demonstrated the feasibility of creating versatile bioproduction platforms for specific applications. *E. coli* was engineered to overexpress a biosynthetic gene cluster from *Streptomyces* sp., and the resulting strain was employed in mutasynthesis to condense 3-hydroxyanthranilate and various fluorinated benzoate derivatives (i.e. 3-fluorosalicylate and 5-fluorosalicylate, 2-fluorobenzoate, and 3,5-difluoropyridine-2-carboxylate) into their respective benzoazole analogs [69]. As observed in microbial biotransformation studies, catabolic activities on fluorinated analogs often require the co-feeding of pathway-inducing, non-fluorinated substrates. Along this line, by following a rational, combinatorial approach, we recently engineered *P. putida* KT2440 to convert 3-fluorobenzoate into 2-fluoromuconate at the maximum theoretical yield relying on tight orthologous regulation of the involved genes [70**]. The bioconversion process constituted a first-case example of harnessing the natural ability of *Pseudomonas* to process organofluorines and optimizing the cognate pathways for biosynthesis of a novel fluorinated building block.

**Conclusions and future prospects**

As the strategies brought about by contemporary synthetic biology evolve in complexity, so does our ability to engineer living systems involving (bio)products that have been out of reach thus far. Satisfying market demands by bioproduction, however, still requires the adoption of efficient engineering strategies that could not only lead to the product(s) of interest (i.e. organohalides) but also circumvent the problems intrinsically associated with molecules alien to the host metabolism. Establishing F chemistry in cell factories is perhaps the best example of this problem, as it involves the most electronegative atom in the periodic table. Growth-coupled strategies emerge as an attractive option, since they build on the driving force of evolution — rather than simply adding a set of biochemical reactions to the extant metabolic network, which seldom results in efficient bioproduction upon direct selection [57]. The emerging and consolidating field of metabolite damage and repair is expected to support these developments [71,72]. The introduction of halometabolites in microbial cell factories could bring about unexpected side reactions that should be kept at bay to ensure sufficient production of the compounds of interest. The inclusion of synthetic modules that recycle or preempt highly reactive, toxic chemical species will become a routine component of metabolic designs to construct robust cell factories for biohalogenation.

While bottom-up biofluorination relies essentially on a single reaction (i.e. catalyzed by the fluorinase enzyme), top-down approaches, rooted on biodegradation pathways of environmental bacteria, can support the synthesis of useful fluorinated building-blocks. The ever-expanding use of F in pharmaceuticals (together with the increasing presence of fluorochemicals in diverse environmental niches) has broadened studies on microbial organofluorine transformation and degradation. Furthermore, some dehalogenases can be adopted for tailoring added-value building blocks while circumventing the accumulation of toxic by-products [73]. Members of the haloacid
dehalogenase superfamily, for instance, can be utilized both for biocatalysis and metabolic repair [74]. Next to the selection of biocatalysts, the development of analytical technologies that enable detection of minute amounts of halogenated compounds will be critical for developing novel approaches for their biosynthesis. 19F-NMR, for instance, is ideal for screening biotransformations of fluorinated compounds as it allows for the detection of fluorometabolite concentrations in the μM range without prior purification of analytes from culture supernatants. With analytic and gene editing technologies becoming more accessible, a vast enrichment of the available organofluorine spectrum, enabled through efficient bioproduction processes, can be expected in the near future.

Conflict of interest statement
Nothing declared.

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Antonin Cros: Conceptualization, Visualization, Writing - original draft. Gabriela Alfaro-Espinoza: Conceptualization, Visualization, Writing - original draft. Alberto De Maria: Conceptualization, Visualization, Writing - original draft. Nicolas T Wirth: Conceptualization, Visualization, Writing - original draft. Pablo I Nikel: Conceptualization, Funding acquisition, Project administration, Writing - review & editing.

Appendix A. Supplementary data
Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.copbio.2021.11.009.

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References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

• of special interest
**of outstanding interest


A thought-provoking account on how slight changes in the chemical build-up of living cells reveal general principles of life’s physics—opening avenues for rational manipulation of the chemical repertoire of cell factories.


The authors discuss the use of artificial intelligence to expand the biochemical capabilities of cell factories, which has important consequences for the biosynthesis of complex molecules that require novel chemistries.


Timely and detailed account of the key advances in utilizing halogenases to generate both in vivo and in vitro biosynthetic pathways.


General methods for growth-coupled optimization of cell factories are discussed at the light of accelerating the design-build-test-learn cycle of synthetic biology.


Long-term propagation of Escherichia coli in a ‘fluorinated habitat’ yielded strains naturally adapted to live on fluorinated amino acids.


Biofluorination engineered in Pseudomonas cell factories by adopting genetic circuits that respond to inorganic fluoride, the substrate for fluorination.


59. Moreira IS, Amorim CL, Murphy CD, Castro PML: Strategies for biodegradation of fluorinated compounds. In Approaches in Bioremediation: The New Era of Environmental Microbiology and...


Elegant example of target incorporation of fluorine into complex molecules (a fluorinated derivative of polyhydroxybutyrate) by feeding fluoromalonate to engineered *E. coli*.


70. Wirth NT, Nikel PI: *Combinatorial pathway balancing provides biosynthetic access to 2-fluoro-cis,cis-muconate in engineered Pseudomonas putida*. *Chem Catal* 2021, 1:1234-1259

Recessive rewiring of native biodegradation pathways in *P. putida* led to bioconversion of fluorinated aromatic substrates into 2-fluoro-cis,cis-muconate at theoretical yields.


A first-case example of repurposing a designer dehalogenase for photo-biocatalytic cross-coupling of aryl halides.