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Review Article

A prescription for engineering PFAS biodegradation

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Per- and polyfluorinated chemicals (PFAS) are of rising concern due to environmental persistence and emerging evidence of health risks to humans. Environmental persistence is largely attributed to a failure of microbes to degrade PFAS. PFAS recalcitrance has been proposed to result from chemistry, specifically C-F bond strength, or biology, largely negative selection from fluoride toxicity. Given natural evolution has many hurdles, this review advocates for a strategy of laboratory engineering and evolution. Enzymes identified to participate in defluorination reactions have been discovered in all Enzyme Commission classes, providing a palette for metabolic engineering. *In vivo* PFAS biodegradation will require multiple types of reactions and powerful fluoride mitigation mechanisms to act in concert. The necessary steps are to: (1) engineer bacteria that survive very high, unnatural levels of fluoride, (2) design, evolve, and screen for enzymes that cleave C–F bonds in a broader array of substrates, and (3) create overall physiological conditions that make for positive selective pressure with PFAS substrates.

Defining the problems and why natural bacteria have largely not evolved to biodegrade PFAS

More than twenty-two million fluorinated chemicals are listed by the PubChem database [1] almost all of which have been devised by humans and within the last one hundred years. Thousands of organofluorine compounds have entered commercial use. The last decade has seen a steeply rising concern per- and polyfluorinated alkyl substances (PFAS) for their persistence, bioaccumulation, and health effects including infertility and cancer [2,3]. According to the 2021 OECD definition, PFAS are considered, with some exceptions, to be substances with at least one perfluorinated methyl group ($-CF_3$) or a perfluorinated methylene group ($-CF_2-$). Thus, in the broadest PFAS definition, even commonly used antidepressants (e.g. prozac) and other pharmaceuticals meet the definition of PFAS, although their chemical and biological properties vary widely. Moreover, >50% of recently introduced pesticides are fluorinated [4]. Human exposure to fluorinated chemicals is heightened by their extreme persistence in the environment. Persistence is largely due to resistance to microbial degradation.

Microbes biodegrade most anthropogenic chemicals when the chemicals are composed of functional groups found in natural products [5], but PFAS are different. No natural products currently known contain more than a single fluorine substituent [6]. In this context, PFAS are inherently foreign to nature. This has led some to suggest that PFAS are largely resistant to the catalytic capacity of enzymes [7–10]. But other reasons for PFAS persistence may be more compelling. One alternative explanation posits that evolutionary forces have constrained PFAS biodegradation.

In general, microorganisms have evolved to biodegrade structurally diverse anthropogenic chemicals, largely driven by selective pressure to acquire metabolic energy and elements for cell replication such as C,N,P, and S. There are many examples of enzymes involved in intermediary metabolism being recruited to liberate essential elements from anthropogenic chemicals [11–13]. The propensity for new enzyme evolution in nature is partly driven by the sheer abundance of microorganisms. Greater than 10^{30} prokaryotes exist on earth and their rapid replication and gene transfer abilities ensure that useful metabolic innovations spread globally [14]. This was recently observed for the

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biodegradation of the pharmaceutical metformin, with almost identical genes identified and characterized in wastewater treatment plants globally [15–17]. Metformin can provide either carbon or nitrogen, or both, to support growth [18].

PFAS are different [19]. Fluorine is a toxin, not a nutrient. Perfluorinated carbon chains are highly oxidized such that their oxidation to carbon dioxide affords little energy to drive cell metabolism. To handle perfluorinated carbon chains, multiple evolutionary innovations are required to remodel metabolic pathways and cell physiology. In total, these evolutionary hurdles contribute to making PFAS environmentally persistent.

Targets for fluoride robustness bioengineering

It is well-established that fluoride anion is highly toxic to prokaryotes and eukaryotes [20,21]. Although fluoride is more abundant than carbon, nitrogen, and all the other halides in the Earth's crust [22], it is mostly eschewed by living things. In almost all cell types, chloride is a major anion in ionic and osmotic balance [23]. Organic compounds containing chlorine, bromine and iodine number in the thousands and are particularly common in marine organisms [24]. In contrast, known organofluorine compounds number on the order of tens, and some, such as 5-fluorouracil, are now believed to have been environmental contaminants from pharmaceuticals rather than biosynthetic products [25].

The typical response of organisms to fluoride anion is to manage the toxicity when it gets into cells and expel the ion as quickly as possible. In prokaryotes, as little 0.0007 M intracellular fluoride has been demonstrated to be toxic [26], whereas intracellular chloride levels of 0.1 M are common and 4.0 M is tolerated by some microbes [27]. The toxicity of fluoride is largely attributed to coordination of the anion to essential enzymes which utilize calcium and magnesium. Those cations are common in enzymes involved in phosphoryl group transfer reactions such as enolase, pyrophosphatase and ATPases [21,28]. Fluoride binding to enzymes is typically reversible and that underlies the widespread cellular strategy of using membrane fluoride exporters to help shift the binding equilibrium and expel the toxic anion [29]. The majority of microbes, including fungi, which have been subjected to genomic sequencing and analyzed show genes clearly identified as fluoride exporters [30,31]. Microbes express two independently evolved fluoride exporters. The more widespread, that constitute 60%, are known as Fluc, or CrcB, type exporters. They function as channels that are driven by ionic equilibrium. The other type are known as CLC^F membrane proteins and function as fluoride/proton antiporters. For all classes, fluoride exporters have been shown to serve as major protective measures. Genetic knockouts render the cell much more susceptible to fluoride toxicity [31–33]. Moreover, the expression of fluoride exporters has been shown to be significantly up-regulated following exposure to fluoride [20,33].

Enzymes cleave C–F bonds via different mechanisms

In the last few years, there has been an explosion of interest in enzymatic and non-enzymatic defluorination [34–38]. Studies on non-enzymatic defluorination can help reveal plausible mechanisms for new natural and engineered defluorinating enzymes that are being uncovered. There have also been numerous reports of PFAS defluorination by microorganisms in which the responsible enzymes have not been identified [39,40]. These studies, and reports of PFAS biodegradation by microbial consortia, have been recently reviewed [41] and will not be covered here.

The focus of this review will be on the breadth of enzyme types known to participate in defluorination, organized by the Enzyme Commission classification and covering all seven major classes: (1) Oxidoreductases, (2) Transferases, (3) Hydrolases, (4) Lyases, (5) Isomerases, (6) Ligases, and (7) Translocases (Table 1). The most well-represented are oxidoreductases and lyases. The most well studied individual enzymes are the hydrolases. The translocases do not catalyze a C–F bond cleavage reaction but serve to remove fluoride from cells after enzymatic defluorination. Table 1 illustrates representative reactions, and it is not meant to be comprehensive. For additional examples, we have compiled more than one hundred enzymes that have fluoride as a product for this paper as part of a PFAS biodegradation enzyme resource (z.umn.edu/pfas-biodeg). The resource primarily sourced from various literature sources as well as the BRENDA Enzyme Database [65]. We emphasize that these natural proteins primarily conduct biochemical reactions with monofluorinated compounds rather than PFAS. Nonetheless, this resource provides a consolidated set of enzymes generally capable of defluorination as starting points for engineering or evolution.

Oxidoreductases are the first major class depicted in Table 1 and they highlight widely divergent mechanisms of defluorination. Pyruvate dehydrogenase uses thiamine pyrophosphate to mediate decarboxylation with elimination of fluorine from the β-carbon of 3-fluoropyruvate [42]. This theme of β-elimination is also observed

Biochemical Journal (2024) **481** 1757–1770
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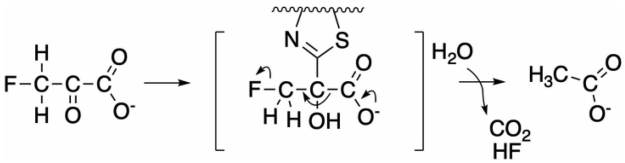
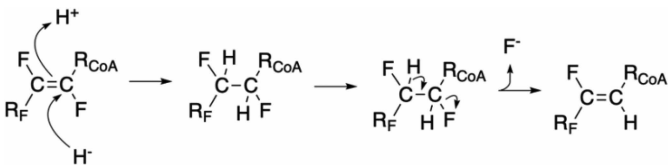
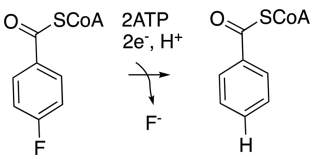
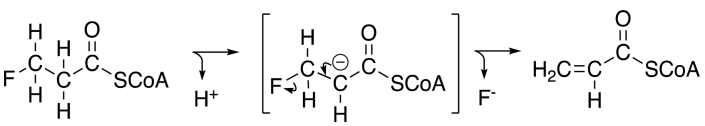
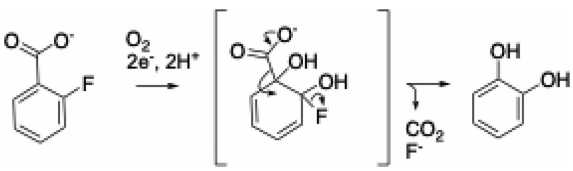
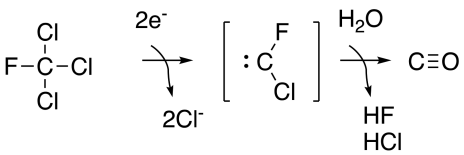
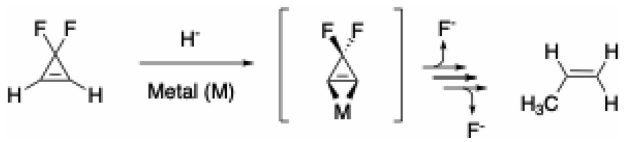
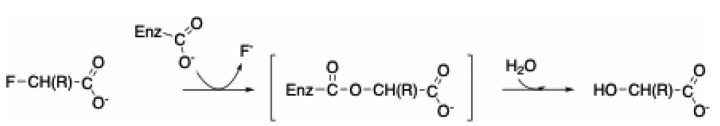
Table 1. Representative enzymes catalyzing C–F bond cleavage. Part 1 of 2

Enzyme	E.C. #	Reaction catalyzed	References
Oxidoreductase	1		
Pyruvate dehydrogenase	1.2..4.1		[42]
Caffeoyl-CoA reductase	1.3.1.108		[43]
Succinate dehydrogenase	1.3.5.1	$^{-}O_2CH_2CF_2CO_2^{-} \longrightarrow {}^{-}O_2CH{=}CFCO_2^{-} + HF$	[44]
Benzoyl-CoA reductase	1.3.7.8		[45]
Short-chain acyl-CoA reductase	1.3.8.1		[46]
2-Fluoro-benzoate-1,2-dioxygenase	1.14.12.13		[47]
Cytochrome $P450_{CAM}$	1.14.15.1		[48]
Nitrogenase	1.18.6.1		[49]
Glycine reductase-type defluorinase	1.21._	$F\text{-}CH_2CO_2^{-} + 2e^{-} + 2H^{+} \longrightarrow H\text{-}CH_2CO_2^{-} + HF$	[50]
Transferases	2		
Glutathione transferase	2.5.1._	$GSH + FCH_2CCO_2^{-} \rightarrow GSCH_2CCO_2^{-} + HF$	[51]
GST-Dichloro-methane dehalogenase	2.5.1._ or 4.5.1.3	$GSH + CH_2ClF \rightarrow [GS\text{-}CH_2F + Cl^{-}] \rightarrow GSH + HCHO + F^{-}$	[52,53]
Hydrolases	3		
(S)-2-Haloacid dehalogenase	3.8.1.2		[54]

Continued

Biochemical Journal (2024) **481** 1757–1770
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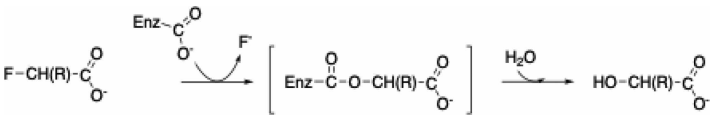
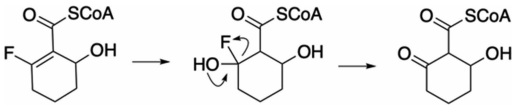
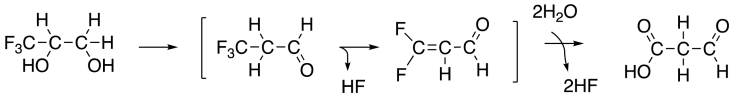
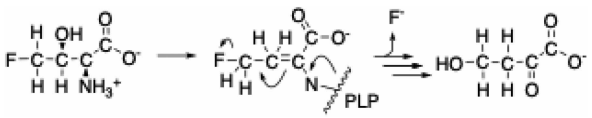
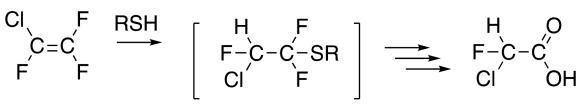
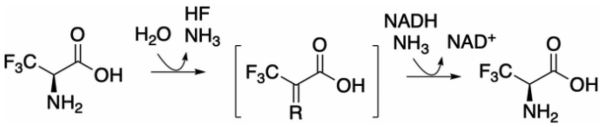
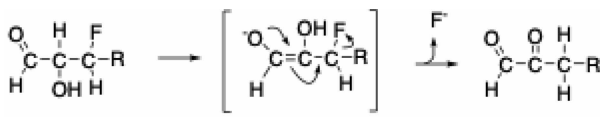
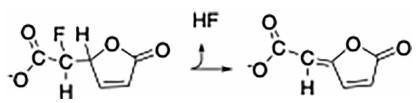
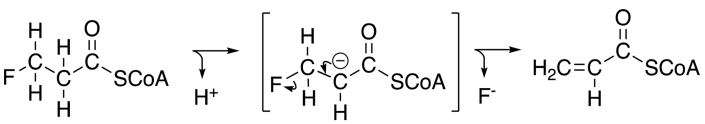
Table 1. Representative enzymes catalyzing C–F bond cleavage. Part 2 of 2

Enzyme	E.C. #	Reaction catalyzed	References
Fluoroacetate dehalogenase	3.8.1.13		[55,56]
Lyases	4		
Enoyl-CoA hydratase	4.2.1.17		[57]
Propane diol dehydratase	4.2.1.28		[58]
Threonine ammonia lyase	4.3.1.19		[59]
Cysteine conjugate β-lyase	4.4.1.13		[60]
Isomerases	5		
Alanine racemase	5.1.1.1		[61]
Uronate isomerase	5.3.1.12		[62]
Fluoromucono-lactone cycloisomerase	5.3.3.4		[63]
Ligases	6		
Propionyl-CoA carboxylase	6.4.1.3		[64]
Translocases	7		
CrcB fluoride exporter	7.2._	$F^-_{side1} \longrightarrow F^-_{side2}$	[32]
CLC^F F^-/H^+ antiporter	7.2._	$F^-_{side1} \rightleftharpoons H^+_{side2}$	[29]

Enzymes are organized according to the numbering of the Enzyme Commission (E.C.). While the entire reaction pathway could not be represented, a key mechanistic feature or the overall reaction was represented. Protein sequences and other metadata are available for download at z.umn.edu/pfas-biodeg.

with succinate dehydrogenase that is proposed to effect deprotonation as a prelude to a physiological dehydrogenation reaction [44]. A variation of this mechanism occurs with caffeoyl-CoA reductase that catalyzes a flavin-dependent double bond reduction as part of an electron-bifurcating reaction designed to conserve metabolic energy for the host bacteria, *Acetobacterium* spp [43]. It has been proposed that certain perfluorinated olefinic carboxylic acids can bind and undergo double bond reduction followed by deprotonation and β-elimination of fluoride.

Laccase mediator systems (LMS) are chemo-enzymatic processes by which laccases oxidize a chemical mediator such as a nitroxyl radical, which subsequently oxidizes a substrate, while molecular oxygen is reduced to water [66]. LMS were reported to be effective in the removal of PFAS [67]. Recent evidence suggests that this removal may be linked to experimental artifacts arising from PFAS sorption onto the laccases rather than actual degradation [68]. Regardless, the mechanisms of LMS in PFAS binding or catalysis warrant further investigation to assess laccases as a strategy for remediation.

Oxygenases catalyze a different type of defluorination with aryl fluorides which can undergo mono- or dioxygenation at the carbon atoms bearing fluorine and/or those adjacent. There are many examples of such reactions as recently reviewed [69], one example is shown in Table 1. 2-Fluorobenzoate-1,2-dioxygenase catalyzes an aromatic *cis*-hydroxylation of a fluorinated benzene that is set up for facile decarboxylation and defluorination at the adjacent carbon to generate catechol [47]. Cytochrome P450Cam functions physiologically as a monooxygenase, but under anaerobic conditions can catalyze reductive dehalogenation as shown in Table 1. The enzyme catalyzes dechlorination followed by defluorination through a carbene intermediate that may be partially stabilized by the enzyme heme iron [48].

A rare direct reductive cleavage is indicated in a recent report [50]. An anaerobic bacterium was shown to use the natural product fluoroacetate as an electron sink via reduction to acetate. Based on genomic analysis, the responsible enzyme is proposed to be a glycine reductase-type defluorinase [50]. Another reductive reaction is represented by benzoyl-CoA reductase [45]. That enzyme formally replaces an aryl fluorine with a hydrogen atom and that is proposed to be mediated by aryl double bond reduction and subsequent HF elimination (Table 1). A novel reductive defluorination of 3,3-difluorocyclopropene to propene is carried out by the molybdenum-iron nitrogenase from *Azotobacter vinelandii* OP [49]. This didefluorination reaction is proposed to proceed via initial reduction and cleavage of the carbon-carbon double bond with further electron inputs leading to fluoride displacement (Table 1). 2-Fluoropropene was observed as a minor product. This novel reductive defluorination mechanism highlights an untapped potential of biological catalysis.

Transferases known to catalyze defluorination are more limited (Table 1). In one example, fluoroacetate detoxification is furnished by glutathione transferases that catalyze a thiolytic displacement of fluoride [51]. In that case, the tripeptide glutathione that provides the thiol is sacrificial and eliminated from the host animal to protect against the toxin. Another example of transferase catalyzed defluorination uses the glutathione catalytically. With dihaloalkanes, one halide is displaced, generating a *gem*-halo thioether that decomposes to an aldehyde and re-releasing glutathione [52,53]. The transferases were reactive with chlorofluoromethane and catalyze defluorination but were not reactive with difluoromethane.

With the natural product fluoroacetate as a major enzyme evolutionary driver, the largest collection of characterized defluorinating enzymes are hydrolases which are reactive with fluoroacetate (Table 1). Some of those enzymes are non-selective with respect to their substrate. These hydrolases emanate from two divergent enzyme superfamilies. One group, fluoroacetate dehalogenases [55,56], maps to the α/β-hydrolases, and the other group is in the haloacetate dehalogenase superfamily [54]. Of the latter, there are members that are (*S*)-specific or (*R*)-specific and some enzymes work on either enantiomer. A subclass of the (*S*)-specific haloacid dehalogenases are the ones shown to date to be reactive with fluorinated substrates [59].

While the fluoroacetate dehalogenases and defluorinating haloacid dehalogenases derive from different ancestral enzymes, they are mechanistically similar, a good example of convergent evolution (Table 1). Both enzymes utilize an aspartic acid for nucleophilic attack on the carbon that is alpha to the substrate carboxylate to generate a covalent enzyme-substrate ester intermediate with displacement of fluoride. The ester is subsequently hydrolyzed as part of the catalytic cycle. Both enzymes facilitate C–F bond cleavage by providing amino acid side chains with some partial or full positive charge character. In both cases, the fluoride must be displaced from the active site fluoride pocket into the bulk water phase where hydration largely prevents rebinding and inhibiting enzyme activity. Some fluoroacetate dehalogenases have been shown to be reactive with difluoroacetate [70].

Lyases are an important enzyme class to look to because they typically involve elimination chemistry which is among the more facile mechanisms for displacing fluorine atoms from carbon chains, including perfluorinated chains [71]. Enoyl-CoA hydratase hydrates a fluorinated double bond leading to elimination of fluoride [57]. Propanediol hydratase is notable for catalyzing a triple defluorination of a trifluoromethyl group [58]. This cobalamin enzyme catalyzes a 1,2-shift of a hydroxyl that is thought to activate an adjacent fluoride for elimination. The subsequent difluoro-ene readily accepts water to generate an acyl fluoride that rapidly undergoes hydrolysis. In total, the enzyme transforms 3,3,3-trifluoro-1,2-propanediol to malonic semialdehyde.

Biochemical Journal (2024) 481 1757–1770
https://doi.org/10.1042/BCJ20240283

Threonine ammonia lyase catalyzes an elimination of fluoride via a double shift facilitated by a pyridoxal phosphate cofactor [59]. Cysteine β-lyase initiates a Michael addition to a halogenated olefin to set up subsequent elimination reactions [60]. While its physiological function is forming a carbon-carbon bond, the set-up reaction generating a carbanion leads to a defluorination in a reaction resembling that catalyzed by propionyl-CoA carboxylase [64].

Isomerases often involve the formation of double bonds, that may or may not be dependent on cofactors [61,62]. Fluoromuconolactone isomerase catalyzes a net elimination of HF, allowing certain bacteria to grow on fluorobenzoic acids [63].

Ligases involved in fluorine biochemistry, but not included in Table 1, catalyze carboxylic acid ligations to Coenzyme A that can set up reductive or hydrolytic defluorination reactions [43,45]. The ligase propionyl-CoA carboxylase generates a carbanion in an activation step for carboxylation, leading to fluoride elimination of a fluorinated analogue (Table 1). Translocases that export fluoride from prokaryotic cells have not been cataloged yet by the Enzyme Commission, but they have been extensively studied, as previously described [29]. For *in vivo* PFAS degradation, fluoride exporters are an essential component to protect against fluoride toxicity. An understudied area is translocation of charged PFAS species into cells. Despite a dearth of dedicated investigations, several studies indicate that the entry of fluorinated carboxylic and sulfonic acid compounds into prokaryotic cells is facile [72,73].

Enzymatic elimination reactions likely figure prominently in fluorotelomer defluorination [74]. A parallel advancement was the reported abiotic defluorination and mineralization of perfluorooctanoic acid in dimethylsulfoxide and NaOH at 40°C [34]. In that study, initial decarboxylation leads to lyase-like chemistry, generating electron deficient fluoroolefins. Subsequent hydration reactions lead to *gem*-elimination of fluoride and further intermediates undergoing extensive defluorination. Considering this chemical knowledge and the biochemical reactions highlighted in Table 1, one should strongly consider elimination reactions to tackle multiply fluorinated PFAS.

Putting the pieces together: enzymes, microbes, and materials engineering

The multi-faceted requirements for the biodegradation of any highly fluorinated compound (fluoride resistance, transport, catalytic enzymes, cofactors) calls for laboratory engineering and evolution. The premise of this review is that natural PFAS-degrading microbes will evolve naturally, eventually, but laboratory evolution can greatly speed up the process.

Engineering a supra-natural fluoride-resistant bacterium

Efficient defluorination of PFAS will require combining different components from naturally evolved organisms to mitigate fluoride stress (Table 2). In nature, prokaryotes contain genes encoding either a CrcB or CLC^F fluoride export protein. Each one singly allows a given prokaryote to withstand tens of millimolar levels of fluoride, a level sufficient for virtually all natural environments. As previously described, the intracellular volume of a typical prokaryote is on the order of 1 femtoliter, such that the displacement of even one fluoride anion from one femtomole of PFAS will generate one thousand-fold the toxic level of fluoride [30]. This indicates that the level of fluoride export for robust PFAS-degraders must exceed natural resistance levels. Fluoride exporters have been exquisitely refined through evolution to achieve recorded rates as high as 10 000–100 000 ions exported per second [32]. However, fluoride resistance may be further enhanced by increasing exporter expression above natural levels or combining both exporters (Figure 1A). Observations of *crcB* and CLC^F-encoding genes co-occurring in the same genome are rare (~3% of genomes) [30] and the physiological consequences of having multiple mechanisms has not yet been examined. CrcB and CLC^F exporters use different mechanisms and so may cover different concentration levels within a cell and act in a complementary fashion.

In addition to previously highlighted natural fluoride abatement functions [30], functions evolved for other purposes may prove beneficial for engineering PFAS degrading organisms. For example, fluoride-binding riboswitches are natural regulatory elements that serve to turn on fluoride stress functions in the presence of fluoride. As such, these 52-mer RNA molecules harbor a Mg^{2+} cation that binds fluoride and changes conformation of the riboswitch to allow expression of fluoride stress genes [20]. We suggest here that riboswitches present an (untested) possibility to engineer cellular resistance solutions. For example, we speculate that fluoride riboswitches, if overexpressed, may also be able to function as protective agents if enough copies were present to

Biochemical Journal (2024) **481** 1757–1770
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Table 2. Several representative fluoride resistance functions shown to protect microorganisms.

Protein	Function	Microorganism	Mechanism of F^- toxicity mitigation	References
CrcB	Fluoride exporter	Many	Passive, gradient driven	[32]
CLC^F	Fluoride exporter	Many	Antiporter, F^- and H^+	[29]
Enolase	Enzyme inhibited by fluoride	*Streptococcus*	High expression overcomes inhibition by F^-	[26]
Pyro-phosphatase	Enzyme inhibited by fluoride	Multiple	High expression overcomes inhibition by F^-	[26]
Phosphatases	Cleavage of phosphate esters	Multiple	Cytoplasmic acidification	[30]
PO_4^{2-} transporter	Importing phosphate	Multiple	Not well established	[30]
Surface adhesion protein	Promotes biofilm formation	*Pseudomonas putida* KT2440	Not well established	[33]
Divalent cation transporter	Importing Ca^{2+}, Mg^{2+}	Multiple	Not well established	[30]
Universal stress protein	Expressed during multiple stresses	*Enterobacter cloacae* FRM	Not well established	[28]

bind and lessen the amount of cytosolic fluoride (Figure 1A). Although the dissociation constant (K_D) of the fluoride riboswitch is in the high μM range [75], if a high activity enzyme is cleaving C–F bonds intracellularly, riboswitch binding may be able to 'buffer' the high cytoplasmic fluoride levels. Over-expressed riboswitches could prevent inhibition of essential enzymes and ferry enzymatically released fluoride anions to fluoride

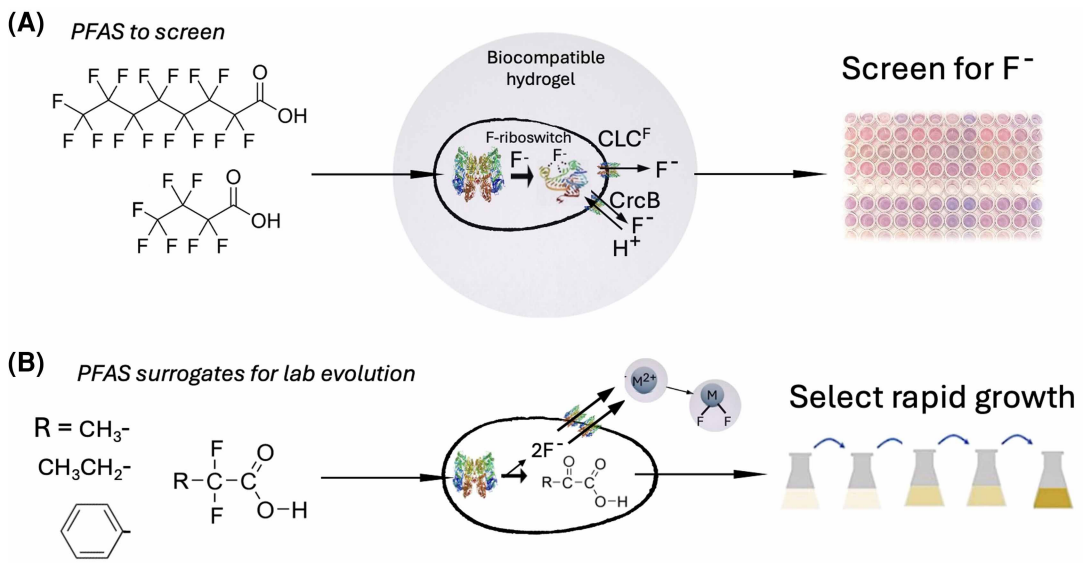

Figure 1. Screening and selecting for enhanced biocatalysts to catalyze defluorination of PFAS.
(**A**) Screening is highlighted with two representative PFAS, perfluorooctanoic acid and perfluorobutanoic acid. A biocompatible hydrogel is shown to encapsulate a bacterial cell containing a defluorinase(s) and membrane fluoride exporters, CLC^F and CrcB. Defluorination may be screened various ways, such as detecting fluoride release in microtiter well plate assays. (**B**) Candidate PFAS 'surrogate substrates' can be used for laboratory evolution. The substrates undergo defluorination to yield organic products such as α-keto acids that can provide carbon and energy for an appropriate host microorganism. The microorganism may be protected against fluoride toxicity by using metal cations that avidly bind fluoride anion after expulsion from the cell. These methods can be used to select for improvements in biological defluorination via adaptive evolution.

Biochemical Journal (2024) 481 1757–1770
https://doi.org/10.1042/BCJ20240283

exporters, hardening cells against fluoride-mediated stress. Since *Pseudomonas putida*, a commonly used synthetic chassis for fluorine chemistry [33] does not have a naturally-encoded fluoride riboswitch, this presents an intriguing orthogonal option separate from normal cell regulatory mechanisms. The cellular consequences of riboswitch overexpression are manifold, organism-dependent [33,76], and not yet tested in this context. Nonetheless, the idea serves as a singular example of how knowledge of biological mechanisms may be tuned for potential PFAS applications.

Creating a 'fluorophile'

Prokaryotes known as halophiles tolerate or require chloride levels of 4 M, and/or grow in high levels of bromide and iodide salts naturally [77]. However, to our knowledge, microorganisms are not designed to tolerate or require even 0.1 M fluoride, for physiological reasons discussed previously. Halophilic microorganisms exclude fluoride from their menu.

The ideal situation would be to create a true 'fluorophile,' an organism benefited by fluoride, but the toxicity of fluoride itself makes this a singularly challenging task. However, it may be plausible to engineer cells that are addicted to fluoride (Figure 1). For example, one could place essential gene(s) under fluoride-sensitive control, such as a modified fluoride riboswitch. In such a synthetic biology scenario, only cells actively generating fluoride in the cytoplasm via C–F bond cleavage would be able to grow. They may additionally be protected by fluoride exporters. In fact, organisms having highly active fluoride exporters will further select for higher level defluorination to maintain sufficient intracellular fluoride concentrations to keep riboswitches 'on.' Alternatively, one could envision engineering fluoride 'addicts' where fluoride down-regulates or turns off a toxic or autolytic function.

In natural environments, biodegradation is best sustained when it brings benefits to the biodegrading organism, and this also could also be applied to fluoride under specific circumstances. For example, there are several metals that co-ordinate with fluoride and form highly insoluble precipitates. For example, moderate concentrations of a metal such as scandium will by itself act as a negative-selective agent in but serve as a neutralizing reagent against fluoride toxicity via co-precipitation of both toxins (Figure 1B). The net effect is to generate a positive selective pressure for fluoride release. In concert with active fluoride exporters, such as CrcB or CLC^F or both, under this scenario the fluoride would leave the cell and protect against metal import and toxification.

Large-scale enzyme library generation and high-throughput screening

In combination with the above-mentioned methods for selection, methods for high-throughput screening would be beneficial for enzyme engineering or metagenomic libraries. In most 'standard' functional metagenomics workflows, enzyme libraries are screened by negative selection. In the case of PFAS, however, fluoride anions formed from defluorination reactions will be more toxic than unmetabolized PFAS. Total defluorination of a standard long chain (C_8) perfluorinated carboxylic acid would produce 15 fluoride anions per single molecule of substrate. Thus, fluoride toxicity would quickly overwhelm any antimicrobial threat posited by the initial screening substrate itself. Hurdles in selecting for the biodegradation of PFAS compounds, such as fluoride toxicity, must therefore be dealt with up front in the selection or evolution of a fluoride-resistant chassis. More hardy alternative hosts to *Escherichia coli* are ideal for such purposes, such as *P. putida* KT2440 [33,78], *Saccharomyces cerevisiae* [79] and other strains with natural or evolved resistance to fluoride [80].

As opposed to negative selection, positive selection can be introduced by using surrogate substrates with some selectable components (Figure 1B). The surrogate concept requires that C–F bond cleavage occurs to unlock the capture of the selectable nutrient. Example surrogate substrates include compounds with CF_2-R groups such as difluorophenylacetate, difluoropropionate, and other difluoroalkanoates and analogs.

Fluoride detection is the preferred method to screen for defluorination over parent compound removal since the latter is subject to sorption artifacts (Figure 1A) [68]. Moreover, background fluoride concentrations in most environmental samples or laboratory materials are low [81]. For enzyme or cell-based libraries with sizes amenable to 96-well plate screening, colorimetric screens are efficient for fluoride detection such as the formation of lanthanum-alizarin complexes or xylenol orange [82] liquid microtiter well plate methods. These 96-well plate detection assays have been adapted to screen alanine scanning libraries of defluorinating enzymes [83] or the *E. coli* ASKA collection [84,85]. A more generalizable screening method, although prone to artifact due to lowered buffering capacity, is the measurement of pH change. These methods are reviewed more thoroughly elsewhere [85].

However, while microtiter-well plate assays are effective for screening hundreds to thousands of samples, several orders of magnitude higher screening capacity is needed for larger enzyme engineering or enzyme design efforts. For screening larger random mutagenesis or saturation (NNN, NNB, NNK, MAX) libraries, 96-well plate screening is not feasible. For randomizing 3 NNN positions, for example, over 10^6 colonies need to be screened to attain a 99% probability of discovering the best variant in variant space [86].

Such libraries require more scalable methods such micro- or nanodroplet-based screening or high-throughput mass spectrometry screening methods, well-reviewed elsewhere [87]. For droplet sorting, standard pH-change assays e.g. with pH-sensitive GFP variants [88,89] may be amenable for the detection of defluorination activity. Biosensors are also effective for fluoride ion detection [90] and have been combined with microfluidic methods [91]. A halide-sensitive green fluorescent protein which binds fluoride as well as other halides was previously reported [92] and may be useful if engineered for better fluoride selectivity and sensitivity.

Interestingly, some microdroplet screening methods themselves use PFAS reagents to build water-in-fluorinated oil drops which are more biocompatible and stable than hydrocarbon alternatives [93]. Perfluorinated reagents are also prized for high-throughput mass spectrometry e.g. nanostructure-initiator mass spectrometry methods [94]. Potential interference, whether from other halides or from fluorinated reagents present in the screening materials, or by phosphates which interfere with colorimetric fluoride detection assays, must be kept in mind with respect to experimental design.

Naturally-evolved and highly-selective, fluoride-specific *crcB* riboswitches have already been applied in combination with various reporter genes or with Cas13a to generate a fluorescent signal for fluoride ion detection based on the Sherlock method [95]. FluorMango [96] is a fluoride-specific RNA-based biosensor combining aptamers from the *crcB* riboswitch [20] and the light-up aptamer Mango-III [97]. Previously, FluorMango proved effective for identification of defluorinating bacterial cells [96]. The method used in combination with fluorescence-based cell sorting was also able to enrich a bacterial population to achieve a 20-fold increase in defluorination activity [98].

Enzymes performing fluorine chemistry, whether C–F bond cleavage or formation, typically suffer from low catalytic efficiency likely due to a combination of factors including the high activation energy and challenging chemical properties of fluorine [71,99,100]. Further techniques are needed to detect or enhance weak phenotypes such as increasing enzyme production levels. For example, a new method for metagenomic library construction [101] enables higher-level expression of captured genes through restriction digestion of DNA at specific 'CATG' restriction cut sites and promotes cloning of start codons in-frame and downstream of strong inducible promoters. Such examples, in combination with more sensitive and better throughput fluoride detection methods, will increase the overall discovery rate.

Computational methods for *in silico* screening or 'design-of-experiments' may additionally narrow the search space [101]. Data-driven approaches typically combine *in silico* deep-learning approach with activity screening, exemplified by a recent microfluidics-based study that yielded a high hit rate in the discovery of nuclease variants [102,103]. Such methods are widely applied in drug discovery [104] and are well-poised to contribute to the screening of enzymes against a large fluorochemical library for a relatively rare phenotype such as defluorination. However, drug discovery largely focuses on small molecule candidate drugs binding to structurally defined protein targets. Binding is only the first step in enzyme catalysis and predicting catalytic functionality remains a significantly more challenging task.

Materials-microbe co-engineering

Fluorine in the earth's crust exists largely as fluoride anion bound to cations of aluminum, calcium, magnesium and other metals. Industrial fluorine used for PFAS synthesis is largely mined from fluorite (CaF_2) ores with strong metal-to-fluorine bonds. The propensity of fluoride to bind avidly to metals can be leveraged for PFAS biodegradation, specifically by mitigating fluoride toxicity (Figure 1B). Intracellularly, Mg^{2+} bound in a fluoro-riboswitch may help lower fluoride toxicity until it can be exported, as described previously. Following export, fluoride can re-enter cells in an aqueous environment, particularly if the pH is <7. Various hydrogel materials containing metals have been prepared and characterized exclusively for the removal of fluoride anions from water [105–107]. Many hydrogel materials can be prepared under mild conditions, making them potentially biocompatible. Such materials include silica, polyacrylamide, polyvinyl alcohol, and alginate. Alginate beads are typically prepared via inducing gel formation with di- or trivalent metal cations. Calcium, magnesium, and aluminum are typically combined with alginate, but many others have been used for different

Biochemical Journal (2024) **481** 1757–1770
https://doi.org/10.1042/BCJ20240283

purposes. Porous, biocompatible, metal-laced gel materials would be ideal to harbor biodegrading microorganisms and sequester fluoride after enzymatic defluorination (Figure 1).

An additional benefit to a material-centered biocatalytic system is the option to include materials to concentrate PFAS from a water sample to enhance biodegradation rates. Independent of catalysis, various materials have been developed to specifically adsorb PFAS. Adsorbent materials for this purpose include modified cyclodextrins and graphene oxide materials [108,109]. Most current methods for treating water use adsorbents to sequester PFAS. Contaminated adsorbents may be land-filled or treated thermally at temperatures that are EU-mandated to exceed 850°C [110]. Optimal microbe-material interfaces would both adsorb and degrade PFAS *in situ*. Previous research has shown the capability of gels containing specific microorganisms to bind, concentrate and biodegrade *s*-triazine herbicides, hydrocarbons and phenols [111–113].

Conclusion

There is no silver bullet to solve the PFAS crisis. Phasing in the use of non-fluorinated PFAS alternatives where possible as well as applying synergistic combinations of chemical, physical, and biological PFAS removal methods will all be required. This review provides a suite of 'prescriptions' for how to envision, build, or enhance PFAS biodegradation with the aid of enzymes and microorganisms. One could envision a future, for concentrated PFAS waste streams where biocontainment is possible, where combinations of components from different organisms can create inspired synthetic biology 'FluoroBrick' platforms [75]. While numerous evolutionary hurdles must be overcome, the ever-growing toolbox for enhancing and evolving enzymes provides optimism even for these truly 'new-to-nature' substrates such as PFAS. Regardless, with or without our own meddling in response to PFAS, biological evolution will continue tinkering.

Data Availability

Additional metadata and sequences associated with Table 1 are available at: https://github.com/serina-robinson/PFAS-biodegradation-toolkit or the shortcut: z.umn.edu/pfas-biodeg.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRediT Author Contribution

Lawrence P. Wackett: Conceptualization, Data curation, Formal analysis, Writing — original draft, Writing — review and editing. **Serina L. Robinson**: Conceptualization, Data curation, Formal analysis, Writing — original draft, Writing — review and editing.

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Abbreviations

LMS, laccase mediator systems; PFAS, polyfluorinated alkyl substances.

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