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A Nonconventional Archaeal Fluorinase Identified by In Silico Mining for Enhanced Fluorine Biocatalysis

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ABSTRACT: Fluorinases, the only enzymes known to catalyze the transfer of fluorine to an organic molecule, are essential catalysts for the biological synthesis of valuable organofluorines. However, the few fluorinases identified so far have low turnover rates that hamper biotechnological applications. Here, we isolated and characterized a nonconventional archaeal enzyme from Methanoseta sp. that mediates the fastest S\textsubscript{N}2 fluorination rate reported to date. Furthermore, we demonstrate enhanced production of fluoronucleotides in vivo in a bacterial host engineered with this archaeal fluorinase, paving the way toward synthetic metabolism for efficient biohalogenation.

KEYWORDS: fluorinase, fluorine, organofluorine, synthetic biology, biocatalysis, metabolic engineering, synthetic metabolism

Fluorinated organic compounds (organofluorines), containing at least one fluorine (F) atom, are chemicals of enormous industrial interest\textsuperscript{1,2}—as evidenced by their increasing prevalence in pharmaceuticals (almost one-third of the pharma molecules in the market contain F) and agrochemicals.\textsuperscript{3,4} The unique physicochemical properties of F endow organofluorines with advantageous properties with respect to their nonfluorinated counterparts, e.g., increased chemical stability or improved bioavailability.\textsuperscript{5} However, the abundance of human-made organofluorines contrasts with their relative scarcity in Nature.\textsuperscript{6} S'-Fluoro-S'-deoxyadenosine (S'-FDA) synthase, or fluorinase (FlA), is the only one enzyme known to naturally catalyze the formation of the C–F bond, which requires a high activation energy for desolvation of the fluoride ion (F\textsuperscript{−}). This enzyme, originally identified in Streptomyces cattleya,\textsuperscript{7,10} catalyzes the S\textsubscript{N}2 transfer of F\textsuperscript{−} to the CS' of the essential methyl donor S'-adenosyl-l-methionine (SAM), thereby generating S'-FDA and l-methionine (l-Met) as products\textsuperscript{11} (step I in Scheme 1). Since the discovery of FlA in 2003, only six other fluorinases have been reported in the literature, all of them sourced from actinomycetes.\textsuperscript{12–14} A chlorinase, catalyzing S'-chloro-S'-deoxyadenosine (S'-CIDA) synthesis and closely related to FlAs, has also been identified in the marine actinomycete Salinispora tropica\textsuperscript{15} (step II in Scheme 1). FlA from S. cattleya is capable of catalyzing the chlorination reaction as well, albeit much less efficiently than fluorination.\textsuperscript{16} Conversely, Sall, the chlorinase of S. tropica, cannot catalyze the formation of C–F bonds. This activity difference has been attributed to the presence of a 23-residue loop, present in all known FlAs but absent in Sall.\textsuperscript{17} It was hypothesized that this loop, located near the catalytic site, could influence halide specificity by modifying the architecture of the binding pocket.

Considering the environmentally harsh conditions currently required for the chemical synthesis of organofluorines, FlAs are promising biocatalysts for “green” production\textsuperscript{18} of new-to-Nature, bioderived organofluorines and for the implementation of synthetic metabolism with fluorinated intermediates in living cells.\textsuperscript{19–22} However, all known FlAs are poor biocatalysts,\textsuperscript{23} with turnover rates <1 min\textsuperscript{−1}. So far, the handful of protein engineering efforts aimed at the improvement of FlA activity have had limited success.\textsuperscript{24–26} Furthermore, these studies mostly relied on employing surrogate substrates, for example, S'-CIDA, to select for enzyme variants with improved transhalogenation activity\textsuperscript{25} (see steps III and I in Scheme 1). This strategy hampers the applicability of FlAs in a consolidated, whole-cell bioprocess where only F\textsuperscript{−} and an appropriate carbon substrate would be supplied as feedstock to support de novo biohalogenation.\textsuperscript{27}

Genome-wide databases are a rich source of potentially valuable enzymes, yet their continuous, exponential expansion makes the selection of catalytically attractive candidates challenging. The EnzymeMiner platform\textsuperscript{29} has been recently developed to address this issue as an interactive...
Scheme 1. Fluorometabolite Biosynthesis Pathways and Reactions Catalyzed by Fluorinase/Chlorinase

Reactions catalyzed by fluorinase/chlorinase are indicated in gray: (I) forward fluorination reaction, (II) forward chlorination reaction, and (III) reverse chlorination reaction. The common step in fluorometabolite biosynthetic pathways is shaded in orange. The canonical fluoracetate and 4-fluoro-L-threonine biosynthetic pathway are shown in purple. The 5′-fluoro-5′-deoxyribonucleoside biosynthetic route is indicated in light blue.

Figure 1. Putative fluorinases identified by genome mining. (a) Residues specified as essential for the EnzymeMiner search, based on the crystal structure of FIA\textsuperscript{MAX} (PDB ID 5B6I). The SAM substrate is shown as a ball-and-stick representation. (b) Phylogenetic tree of retrieved fluorinase sequences obtained using the MEGAX software,\textsuperscript{31} inferred using the Neighbor-Joining method with a bootstrap of 10 000 iterations. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Sequences sourced from Actinomycetes are highlighted as blue squares. Enzymes previously characterized in the literature are indicated in blue bold font. (c-e) 3D structures for FlAMA37 (c), wild-type Salt\textsuperscript{Ray} (d, PDB ID 6RYZ) and FIA\textsuperscript{Sal}\textsuperscript{12} (e, modeled with the SWISS-MODEL Alignment Mode tool using the FIA\textsuperscript{Sal} crystal structure PDB ID 2V7V as template). The loop hypothesized to differentiate fluorinases from chlorinases is circled in a dashed gray line. Two chains from the homotrimer for each structure are shown as cartoon and surface representations, respectively.

Web site (https://loschmidt.chemi.muni.cz/enzymeminer). This user-friendly bioinformatic tool searches through databases upon submitting a sequence of at least one representative member of the target enzyme family, together with the...
identification of essential (i.e., catalytic) residues. EnzymeMiner conducts multiple database searches and accompanying calculations, which provide a set of hits and their systematic annotation based on protein solubility, possible extremophility, domain structures, and other structural information. These collected and calculated annotations provide users with key information needed for the selection of the most promising sequences for gene synthesis, small-scale protein expression, purification, and functional characterization.

With the goal of expanding the FlA toolset for the biological production of organofluorines in engineered bacterial cell factories, here we describe the systematic screening, in vitro characterization, and in vivo implementation of hitherto unknown FlAs retrieved from genome databases. First, in an effort to identify “Nature’s best” biocatalyst, the fluorinase from Streptomyces sp. MA37 (FlA<sup>MA37</sup>) was used as the query sequence (UniProt W0W999), and the amino acid residues D16, Y77, S158, D210 and N215 were specified as essential based on their implication in catalysis and substrate binding in EnzymeMiner (Figure 1a). We selected this enzyme since it is one of the most efficient fluorinases reported in the literature thus far, and it has been used as template for directed evolution experiments.<sup>12,24</sup>

After curing out redundant sequences, 16 unique candidates were obtained (Table 1 and Figure 1b). Some of the retrieved amino acid sequences were found to be missing several N-terminal residues, which were added after manually curating the deposited genome sequences where the fluorinase genes had been predicted (Table S1). Out of the 16 sequences retrieved, five corresponded to fluorinases reported in the literature (thus serving as an internal quality control of the prediction routine), while nine corresponded to new putative fluorinases from different organisms (Figure 1c–e).

Notably, only four of all the retrieved sequences were not sourced from Actinobacteria. These include the putative enzymes from a Chloroflexi bacterium (Chloroflexi), Peptococcaceae bacterium CEB3 (Clostridia), Thermusodorhabdus norvegica (Deltaproteobacteria), and Methanoseta sp. PtaU1.-Bin055 (Methanomicrobia). Phylogenetic analysis of the 16S rRNA sequences of the fluorinase-encoding organisms gave a similar result to that obtained when using the fluorinase amino acid sequences, except that, expectedly, <i>S. tropica</i> groups together with the other Actinomycetes, in a clade separate from the one formed by <i>Streptomyces</i> sp. (Figure S1 and Table S2).

The genomic context of the different <i>flA</i> genes was likewise examined (Table S3). As reported for the fluorination gene clusters of <i>Streptomyces</i> sp. MA37, <i>N. brasiliensis</i>, Actinoplanes sp. N902–109, and <i>S. xinghaiensis</i>, all Actinomycetes harbor gene clusters resembling that of <i>S. cattleya</i>, the most studied source of <i>fl</i> genes described to date<sup>23,32</sup> (Figure 2). The genes <i>flB</i> (encoding a 5'-FDA phosphorylase), <i>flG</i> (encoding a response regulator), <i>flH</i> (encoding a putative cation:H<sup>+</sup> antiporter), and <i>flI</i> (encoding a S-adenosyl-L-homocysteinase) were highly conserved in all actinomycetes. Most of them also presented the genes <i>flF</i> (5-fluoro-5-deoxy-β-ribose 1-phosphate isomerase) and <i>flFT</i> (4-fluoro-l-threonine transaldolase), involved in the synthesis of fluorooacetate and 4-fluoro-l-threonine. These are the two canonical end fluorometabolites described thus far. One also, genes encoding a prolyl-tRNA synthetase-associated protein and an EamA family transporter were usually found in proximity to <i>flF</i>.<sup>26</sup> In <i>S. cattleya</i>, the products of these genes (termed <i>fhB</i> and <i>fhC</i>, respectively) play a role in detoxication by decylation of 4-fluoro-l-threonyl-tRNA and export of 4-fluoro-l-threonine.<sup>33</sup> Interestingly, <i>Amymocolopsis bartoniae</i> and <i>Goodfellowiella</i> sp. AN110305 lacked either <i>flF</i> and <i>flFT</i> orthologues within the <i>fl</i> cluster, presenting instead, orthologues to the <i>flF</i> genes from <i>Streptomyces</i> sp. MA37. The genes are probably involved in the biosynthesis of 5-fluoro-2,3,4-trihydroxypentanoic acid via the fluorosugar intermediate 5-fluoro-5-deoxy-d-ribose.<sup>34</sup> Further biochemical activities encoded in these gene clusters include phosphoesterases, short chain dehydrogenases, dihydroxyacid dehydratases and cyclases, suggesting that the main fluorinated compounds produced by these microorganisms could be different from the canonical fluorometabolites fluorooacetate and 4-fluoro-l-threonine. Similar activities seem to be also encoded by genes in the vicinity of <i>flA</i> in <i>Chloroflexi</i> bacterium and <i>sall</i> in <i>S. tropica</i>.<sup>35</sup> Other genes widely distributed among the different actinomycotal clusters encoded activities related to SAM synthesis (i.e., <i>SAM synthetase</i>) and S-adenosyl-L-homocysteine degradation (i.e., S-adenosyl-L-homocysteinase), a competitive inhibitor of fluorinase activity.<sup>36</sup> As indicated above, the latter gene (<i>flI</i>) was present in all actinomycotal clusters. Since SAM and S-adenosyl-L-homocysteine are involved in essential cellular reactions, it is likely that these enzymes modulate the levels of these compounds during secondary metabolism, when organofluorines are actively produced.<sup>36</sup> Further analysis of the genes found in these <i>fl</i> clusters will provide clues as to what activities are needed to establish robust and efficient biofluorination pathways in heterologous hosts. This prospect is particularly exciting at the light of the need of novel organofluorine biosynthesis enzymes that could be sourced from environmental microbes.<sup>37</sup>

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**Table 1. Putative Fluorinases Retrieved from EnzymeMiner Analysis Using FlA<sup>MA37</sup> as the Query**

| name      | organism and reference | ID (%)<sup>29</sup> |
|-----------|------------------------|---------------------|
| FlA<sup>MA37</sup> | Streptomyces sp. MA37 | query              |
| FlA<sup>MC26</sup>  | Streptomyces cattleyae | 87.6%              |
| FlA<sup>MC1</sup>   | Streptomyces xinghaiensis | 86.0%          |
| FlA<sup>MA11</sup>  | Streptomyces sp. SA15  | 85.0%              |
| FlA<sup>MC2</sup>   | Actinoplanes sp. N902–109 | 80.7%           |
| FlA<sup>MC12</sup>  | Actinopolyspora mazabensis | 78.9%            |
| FlA<sup>MC13</sup>  | Amycolatopsis bartoniae | 79.1%            |
| FlA<sup>MC14</sup>  | Amycolatopsis sp. CA-128772 | 78.6%           |
| FlA<sup>MC15</sup>  | Goodfellowiella sp. AN110305 | 77.7%        |
| FlA<sup>MC16</sup>  | Nocardia brasiliensis IFM 10847 | 75.7%        |
| FlA<sup>MC17</sup>  | Nocardia brasiliensis NCTC 11294 | 75.3%        |
| FlA<sup>MC18</sup>  | Nocardia brasiliensis ATCC 700358 | 75.3%       |
| FlA<sup>MC19</sup>  | Chloroflexi bacterium | 69.3%            |
| FlA<sup>MC20</sup>  | Peptococcaceae bacterium CEB3 | 64.8%          |
| FlA<sup>MC21</sup>  | Thermodesulfuratus norvegica | 54.5%        |
| FlA<sup>MC22</sup>  | Methanoseta sp. PtaU1.Bin055 (FlA<sup>MC12</sup>) | 49.5%          |
| Sall<sup>MC1</sup>  | Salinispora tropica CNB-440 | 35.6%          |

“Sequence identity. References to known FlAs are indicated.”

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Next, the coding sequences of all FlA candidates were codon-optimized for production in *Escherichia coli* as N-terminal His-tag fusions (FlAMA37, FlAScat and FlASxin had been previously codon-optimized for expression in Gram-negative hosts; see also Tables S4 and S5). SalLSro was not included in this experimental set since it is reportedly inactive on F−. The expression of the 16 candidate genes was initially evaluated in 96-well microtiter plate cultures. FlATnor, FlAAmza, and FlAPbac could not be obtained as soluble enzymes and were not included in further analyses. Moreover, very faint bands of the expected size were observed in SDS-PAGE of *E. coli* extracts producing either FlATnor or FlAAmza, suggesting limited solubility and were not included in further analyses. However, few faint bands of the expected size were observed in SDS-PAGE of *E. coli* extracts producing either FlATnor or FlAAmza, suggesting limited solubility and were not included in further analyses. Consequently, the enzyme from *Methanosaeta sp.* (FlAPtaU1, predicted to be a chlorinase), was one of the top performers. FlASAJ15 also had high S′-FDA synthase activity in vitro. These two enzymes had specific activities comparable to those of FlAMA37 and FlASxin, with the highest catalytic efficiencies on SAM-dependent S′fluorination reported to date. FlPAUL1 and FlASAJ15 were selected for large-scale shaken-flask production and a more detailed biochemical characterization. Steady-state kinetics assays with 1 μM of the purified protein, varying concentrations of SAM (1.5−800 μM) and 75 mM KF revealed that both of these enzymes presented higher turnover rates (kcat) than FlAMA37. Surprisingly, KM values were consistently <10 μM, much lower than what had been previously reported in the literature for fluorinases. Notably, previous studies used high enzyme concentrations (>10 μM), which impedes reaching a steady state of the reaction for substrate concentrations below 10 μM. We also used a KF concentration that ensures F− saturation without causing any inhibitory effect (previous studies have used KF concentrations >200 mM).

To gain insight on the structural factors that could determine these differences in fluorination activity, we inspected the predicted crystal structures of FlAMA37, FlASxin, FlASAJ15, FlPAUL1, and SalLSro. Examination of the amino acid residues potentially interacting with SAM (at distances <5 Å) revealed important variations between the substrate binding pocket of FlPAUL1 and that of the other fluorinases known to
The alterations could be mapped near to the adenyl moiety of SAM, and involve the substitution of a conserved proline for an arginine residue and an RNAA motif for YYGG. This motif is found in the C-terminal domain of other fluorinases, which is more variable than the N-terminal domain and is presumably also involved in hexamer formation38 (Figure S4). Interestingly, the catalytic features found in FlAPtaU1 do not resemble those of the SalLStro chlorinase, which would place FlAPtaU1 in a different functional group of SN2 halogenases. Evaluating the effect of these amino acid differences in fluorinase activity will be of interest for enzyme engineering efforts.

Since FlAPtaU1 was predicted to be a chlorinase, we evaluated whether it was also active in SN2-dependent addition of Cl\(^{-}\) to SAM. Unexpectedly, no 5′-ClDA accumulation could be detected in enzymatic reactions in which KF was replaced by KCl—in contrast to what has been reported for SalLStro.15 Previous studies have shown that FlAScat can also catalyze the chlorination reaction.16 However, this feature requires the simultaneous removal of L-Met or 5′-ClDA, the reaction products, since the reverse dehalogenation reaction is favored. We could observe transhalogenation on 5′-ClDA (i.e., 5′-FDA production in the presence of L-Met and F\(^{-}\), steps III and I in Scheme 1; Figure 3b). Again, FlAPtaU1 catalytically outperformed all other fluorinases, with a 3-fold higher V\(_{\text{max}}\) value. Although we cannot rule out that FlAPtaU1 could also execute de novo chlorination, the 23-residue loop reportedly found in "conventional" fluorinases is not essential for the activity toward F\(^{-}\).

With this background, we tested the biosynthesis of fluorometabolites in vivo by engineering selected fluorinases in the bacterial platform Pseudomonas putida, a robust chassis for engineering complex chemistries using synthetic biology tools.39−43 We have designed a fluoride-responsive genetic circuit that enabled biofluorination in this Gram-negative host.27 Here, this system was adapted to express either FlAPtaU1 or FlA	ext{Sxin}, the best-performing fluorinases according to the kinetic parameters in Table 2. FlAMA37 and FlASxin were

![Figure 3](https://doi.org/10.1021/acscatal.2c01184)  

**Table 2. Michaelis–Menten Kinetic Constants of Selected Fluorinases**

| fluorinase    | K\(_{\text{M}}\)\(_{\text{SAM}}\) (μM) | k\(_{\text{cat}}\) (min\(^{-1}\)) | k\(_{\text{cat}}\)/K\(_{\text{M}}\)\(_{\text{SAM}}\) (mM\(^{-1}\) min\(^{-1}\)) |
|---------------|-------------------------------|-------------------------------|---------------------------------|
| FlAMA37      | 4.42 ± 0.58                   | 0.16 ± 0.01                   | 36.36 ± 4.82                   |
| FlASxin      | 3.76 ± 0.15                   | 0.22 ± 0.01                   | 58.63 ± 2.63                   |
| FlASAJ15     | 9.62 ± 1.43                   | 0.34 ± 0.01                   | 35.81 ± 5.43                   |
| FlAPtaU1     | 6.99 ± 1.06                   | 0.41 ± 0.01                   | 57.54 ± 8.85                   |

Assays conducted in 50 mM HEPES, pH = 7.8, with 75 mM KF and varying SAM concentrations incubated at 37 °C. Average and standard deviations are given for triplicate independent measurements.
included in control experiments, as we have previously used them for engineering in vivo fluororination. Upon inducing gene expression with NaF (which is also the substrate of the reaction of interest) and producing the fluorinases for 20 h at 30 °C, S'-FDA biosynthesis was determined by LC-MS to evaluate de novo fluororination activity (Figure 4a). Production of S'-FDA by engineered P. putida could be detected in all cases (Figure 4b). Notably, the S'-FDA content, indicative of in vivo fluororination, was 12-fold higher in cells expressing fIA\textsuperscript{PtaU1} with respect to any other fluororinase gene. Fluororination activity in cell-free extracts of P. putida incubated for 20 h at 30 °C in the presence of exogenously added 200 μM SAM and 5 mM NaF was similar for the fluorinases tested (Figure S5), with a higher activity detected in cell-free extracts carrying fIA\textsubscript{PtaU1}, the Archaeal fluororinase. In the cell-free extract assay, the final S'-FDA concentrations detected were within the ranges previously reported.\textsuperscript{27,38} Interestingly, no other fluorometabolites than S'-FDA could be detected in these assays.

In conclusion, out of the 10 newly identified enzymes, the nonconventional fIA from the archaea Methanosaeta sp. PtaU1.Bin055 (fIA\textsuperscript{PtaU1}) was found to present turnover rates superior to those of all FAs reported to date. Surprisingly, this enzyme lacks the loop that was so far hypothesized to be a challenging the hypothesis that this loop is required for activity toward F'. Engineering this nonconventional fluororinase in P. putida mediated the highest in vivo production of S'-FDA described to date—and, for that matter, the highest fluorometabolite levels reported for any biological system, either natural or engineered. This work highlights the importance of systematic and efficient biocatalyst selection across the ever-expanding genomic databases, followed by careful characterization in vitro and cell factory engineering in vivo. This study also expands the known sequence diversity for fluororinase enzymes, helping in the identification of other nonintuitive sequence features. Interestingly, when the mining run was repeated with either fIA\textsubscript{AMA37} or fIA\textsuperscript{PtaU1} as query, the number of putative fluororinase sequences retrieved (24 hits) was essentially the same as obtained with the enzyme from S. cattleya as the template. These features will be useful for predicting protein function(s) from genomic databases annotations. Additionally, this fundamental knowledge will inform future engineering endeavors of fluorinases by rational and semirational design. Taken together, our results open avenues for the implementation of neo-metabolic pathways to incorporate F in bacterial hosts by synthetic biology approaches.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.2c01184.

Materials and methods and supplementary figures and tables (PDF)

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Author Contributions
I.P. performed most of the experimental work and phylogenetic analysis, interpreted the data, and wrote the manuscript draft. P.C. and C.D.V. performed experimental work and contributed to manuscript writing. D.B. and J.D. performed in silico work and contributed to manuscript writing. P.I.N. acquired funding, conceptualized the study, supervised the work and finalized the manuscript. All authors have approved the final version of the manuscript.

Notes
The authors declare no competing financial interest.

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