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Authors: Mary C. Andorfer, Ketaki D. Belsare, Anna M. Girlich, and Jared Lewis

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Aromatic Halogenation Using Bifunctional Flavin Reductase-Halogenase Fusion Enzymes

Mary C. Andorfer,[‡] Ketaki D. Belsare,[‡] Anna M. Girlich, and Jared C. Lewis[*]

Department of Chemistry, University of Chicago, Chicago IL 60637

[‡]Authors contributed equally to this work.

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Abstract

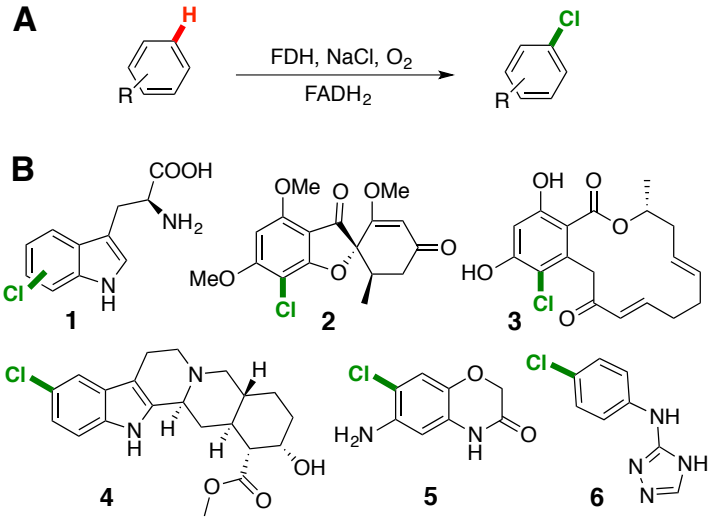
The remarkable site-selectivity and broad substrate scope of flavin dependent halogenases (FDHs) has led to much interest in their potential as biocatalysts. Multiple engineering efforts have demonstrated that FDHs can be tuned for non-native substrate scope and site-selectivity. FDHs have also proven useful as *in vivo* biocatalysts and have been successfully incorporated into biosynthetic pathways to build new chlorinated aromatic compounds in several heterologous organisms. In both cases, reduced flavin cofactor, usually supplied by a separate flavin reductase (FR), is required. Here, we report functional synthetic, fused FDH-FR proteins, containing various FDHs and FRs, joined by different linkers. We show that FDH-FR fusion proteins can increase product titers compared to the individual components for *in vivo* biocatalysis in *E. coli*.

Introduction

Halogenated aromatic compounds often exhibit unique biological activities and are thus commonly used as pharmaceutical drugs and agrochemicals.[1] Aryl halides are also valuable building blocks for synthetic chemistry, particularly due to their centrality to a range of powerful cross-coupling reactions.[2,3] Despite the importance of aromatic halogenation, however, common methods of aromatic halogenation, perhaps most notably electrophilic aromatic substitution, often suffer from poor regioselectivity.[4] More recent efforts have therefore explored the ability

of directing groups to enable selective halogenation of proximal C-H bonds on suitably pre-functionalized substrates.[5,6]

Complementing these traditional synthetic methods, flavin-dependent halogenases (FDHs) have been shown to halogenate a range of electron rich hetero(arenes) with high selectivity (Scheme 1).[7-9] FDH catalysis proceeds via an electrophilic halogen species (both a lysine-derived haloamine[10] and HOX[11] have been proposed), which, due to its orientation relative to bound substrate, can override electronic biases of different substrates to catalyze aromatic halogenation with novel regioselectivity.[12,13] Notably, FDH catalysis proceeds in aqueous solution at ambient temperature and requires only reduced flavin cofactor ($FADH_2$), sodium chloride as a halide source, and oxygen from air as a terminal oxidant. A cofactor regeneration system (CRS) comprised of a flavin reductase, a NAD(P) oxidoreductase (e.g. glucose dehydrogenase), FAD, NAD(P), and a terminal reductant (e.g. glucose, the only stoichiometric reagent in the CRS) can be used to supply $FADH_2$.



Scheme 1. A) General scheme for chlorination by FDHs. B) Representative products from chlorination of native (**1**-**3**) and non-native FDH substrates (**4**-**6**).[14-17]

A number of efforts involving directed evolution and targeted mutagenesis have been used to engineer FDH variants with increased stability,[18,19] expanded substrate scope,[16,20] and altered regioselectivity[21,22]. In all of these efforts, a CRS analogous to that described above was used to ensure maximum product formation, which necessitates the purification of a suitable flavin reductase. Typically, *E. coli* flavin reductase, Fre, or the native RebH (a 7-tryptophan FDH from the native organism *Lechevalieria aerocolonigenes*) partner RebF have been used for *in vitro* halogenation assays.[20] Due to low solubility of RebF when over-expressed in *E. coli*, a fusion of maltose binding protein and RebF (MBPF) is often used in place of RebF.[23] The requirement of flavin reductase (FR) can be tedious for directed evolution efforts, since sufficient reductase for thousands of reactions must be regularly prepared, purified, and quality tested. Of course, this requirement could be eliminated by co-(over)expressing genes for the reductase and halogenase either individually or as fusion enzymes. Genetic fusion of the flavin reductase and halogenase could also improve halogenation efficiency, particularly for *in vivo* applications where a high local concentration of reduced $FADH_2$ cannot necessarily be guaranteed.

The utility of *in vivo* FDH catalyzed halogenation has now been established in a number of different organisms. [15,24-29] For example, Rdc2 has been used to halogenate phenolic compounds in *E. coli* without co-expressing a flavin reductase, since this organism contains naturally occurring flavin reductases.[15,30] Likewise, targeting FDH expression to plant chloroplasts (which have high levels of $FADH_2$) is sufficient to enable FDH catalysis *in planta*, but co-expression of a reductase is required if cytosolic expression is desired.[26] Regardless of whether endogenous reductases may be able to supply $FADH_2$, many studies have shown that increasing the local concentration of enzymes can increase flux through multistep enzymatic pathways.[31,32] Previous work has demonstrated that Baeyer-Villiger monooxygenases can be

genetically fused with $NADP^+$ reductases, simplifying cofactor regeneration.[33] Ferrodoxin and flavodoxin reductase type domains can also be fused to cytochrome P450 heme domains to generate self-sufficient hydroxylation catalysts.[34-38] We therefore envisioned that an FDH-FR fusion enzyme could be useful for a wide range of *in vitro* and *in vivo* applications.

In vitro Characterization of FDH-FR Fusion Enzymes

The genes encoding wild-type RebH and RebF were genetically fused using three linkers based on sequences used to create the functional P450-reductase fusion enzymes noted above.[34,35] These linkers consisted of 10, 16, and 22 amino acid residues (Figure 1A), and the corresponding fusion enzymes are referred to as H-10-F, H-16-F, and H-22-F. The fusion constructs were co-expressed with the pGro7 chaperone system in *E. coli* to afford 10-30 mg L^{-1} soluble protein following purification (Fig. S2). These yields are similar to those observed with the MBP-RebF fusion (33 mg L^{-1}) often used in FDH bioconversions; however, higher yields are observed when expressing RebH with the chaperone system.[39]

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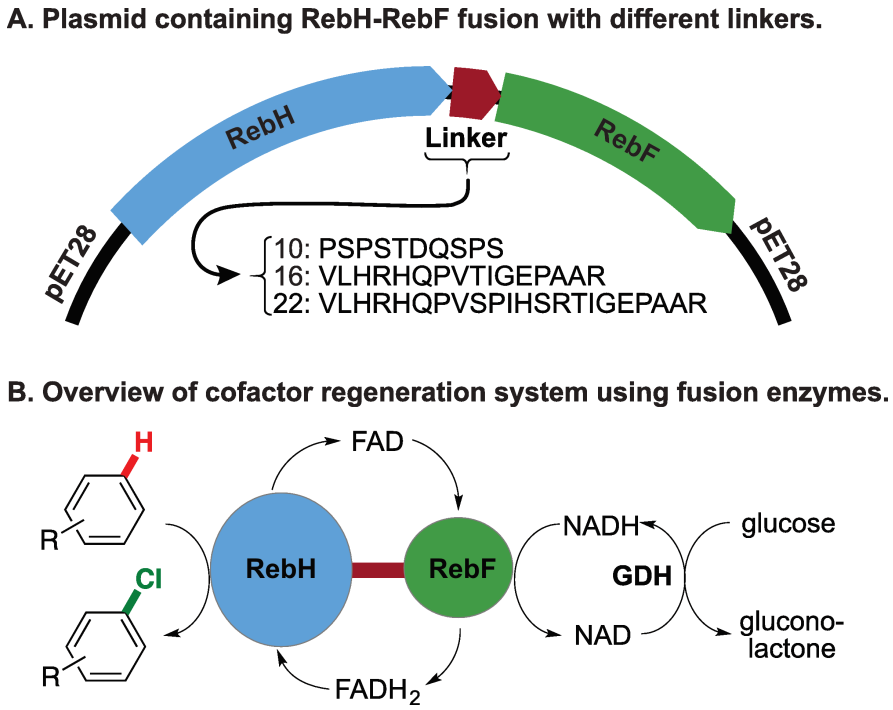

Figure 1. A) Fusion constructs encoded on a pET28 vector. B) Overview of cofactor regeneration using fusion enzymes.

The activities of both the reductase and halogenase domains of the fusion enzymes were next examined. Halogenase activity was established by comparing the yield of L-tryptophan chlorination catalyzed by RebH in the presence of MBPF to the yields for the same reaction catalyzed by H-10-F, H-16-F, and H-22-F (Figure 1B). All three fusion enzymes retained substantial halogenase activity (Table 1, entries 2-4, 38-55% yield); however, lower chlorination yields were observed relative to RebH/MBPF (Table 1, entry 1, 90% yield). Chlorination yields were unaffected by the difference in linker length and amino acid composition between linkers 10 and 16 (55% and 56% yield, respectively), but the longest linker, 22, led to a lower yield (38%). Reductase activity was established by measuring NADH oxidation,[40] which occurred at a similar rate for H-16-F and MBPF (k_{cat} = 206 and 197 min^{-1}, respectively, Fig. S3-4).

The apparent melting temperature (T_M) of H-16-F was also compared with that of RebH to determine if decreased stability, in addition to modestly decreased activity, might hinder its performance. Apparent T_m values of 49.5 °C and 44.9 °C were obtained for RebH and H-16-F, respectively, indicating that reduced stability of the fusion could indeed be compromising H-16-F performance (Fig. S5). Previously, a thermostable flavin reductase from *Bacillus subtilis* (Fre) had been found compatible with FDH halogenation systems.[41] This thermostable Fre was therefore fused to RebH using the 16 amino acid linker with the goal of generating a fusion enzyme with increased stability. This resulting fusion enzyme, RebH-16-Fre, also expressed as a soluble protein, but it provided a lower yield for L-tryptophan chlorination (Table 1, entry 5, 30% yield) and had a comparable melting temperature (apparent T_M = 45 °C, Fig. S5) relative to H-16-F.

Three RebH variants previously engineered in our laboratory, 1K,[42] 3SS,[16] and 10S[22] were also fused to RebF via the 16 amino acid linker described above. These variants were engineered for altered substrate scope (1K-E461K+R231K and 3SS-S2P+M71V+G112S+K145M+N467T+N470S) and site selectivity (10S-I52H+L380F+F465C+N470S+Q494R+R509Q). Soluble protein was obtained for all FDH fusion enzymes, and bioconversions were conducted with the purified enzymes. As observed for H-16-F, all three FDH fusion enzymes retained activity for their respective substrates (Table 1, entries 7, 9, 11), but lower conversions were observed relative to the individual enzymes. Bioconversions using H-16-F, 1K-F, 3SS-F, and 10S-F were scaled up, and the site-selectivity of chlorination was found to be the same as the corresponding two-component FDH system in all cases (see supporting information).

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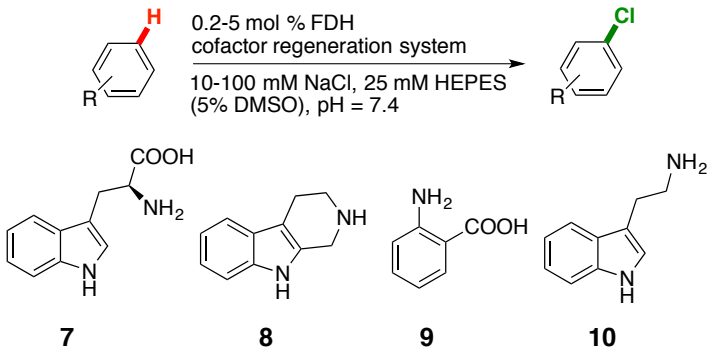

Scheme 2. General reaction scheme for *in vitro* reactions on substrates **7**-**10**.

Table 1. Aromatic chlorination catalyzed by different FDH or FDH-FR fusion enzymes.[a]

Entry	FDH	Substrate	[FDH] (μM)	Yield (%)
1	RebH	**7**	1.5	90.3
2	H-22-F	**7**	1.5	38.3
3	H-16-F	**7**	1.5	56.2
4	H-10-F	**7**	1.5	54.6
5	H-16-Fre	**7**	1.5	30.3
6	3SS	**8**	1	23.4
7	3SS-16-F	**8**	1	14.8
8	1K	**9**	1	38.2
9	1K-16-F	**9**	1	22.5
10	10S	**10**	25	26.3
11	10S-16-F	**10**	25	17.2

[a]0.5 mM substrate, 1-25 μM FDH, 9 U mL^{-1} GDH, 10-100 mM NaCl, 20 mM glucose, 100 μM NAD and FAD, 25 mM HEPES buffer pH = 7.4, 25 °C, 75 μL final reaction volume. 2.5 μM reductase was added to reactions that did not contain a fusion enzyme. Reactions were quenched with one volume MeOH. 0.5 mM phenol (for **7**, **8**, **10**) or 0.5 mM benzoic acid (for **9**) was added as an internal standard, and reactions were analyzed by HPLC.

We sought to further understand the reduced yields of the fusion enzyme *in vitro*. No significant change in reductase activity was observed (see above), and only a slight decrease in

the apparent melting temperature of H-16-F was observed relative to RebH. Although this difference in T_m could be responsible for decreased yield, time courses revealed that decreased catalyst lifetime was not observed. Based on these observations, we hypothesized that reduced kinetic parameters of halogenase activity of H-16-F was primarily responsible for the its reduced yield. We therefore measured the steady state halogenase kinetics for RebH and H-16-F. Because the K_m of RebH-catalyzed chlorination of L-tryptophan is low (~2 μM)[40], low substrate concentrations and correspondingly low FDH concentrations (0.007 μM) were required to ensure that rates were measured at <10% conversion. For preparative FDH-catalyzed reactions, we have generally found that 2.5 μM FR provides satisfactory product yields regardless of FDH concentration on the reaction scales we have investigated.[16-18,22,39] While this concentration represents an enormous excess of FR relative to the FDH concentrations noted above, we reasoned that supplementing bioconversions involving either RebH or H-16-F with 2.5 μM FR would allow for analysis of halogenase activity under conditions in which halogenase activity (rather than $FADH_2$ supply) limits overall product formation.[40,43] Under these conditions, similar K_m values were observed for both halogenases ($K_{m\text{-}RebH}$ and $K_{m\text{-}H\text{-}16\text{-}F}$ = 0.7 μM); however, a nearly two-fold decrease in k_{cat} is observed for the fusion H-16-F compared to that of RebH ($k_{cat\text{-}RebH}$ = 4.26 min^{-1} and $k_{cat\text{-}H\text{-}16\text{-}F}$ = 2.34 min^{-1}, Table 2, entries 1 and 2).

Kinetic parameters were also obtained for H-16-F with no added reductase, conditions under which H-16-F is the only source of $FADH_2$. Interestingly, a much lower k_{cat} was observed under these conditions (0.14 min^{-1}, Table 2, entry 4) relative to those noted above. Presumably, because the concentration of FR was >80-fold lower than previous experiments, the supply of $FADH_2$ to RebH, which involves both the rate of FAD reduction and non-catalyzed $FADH_2$ oxidation prior to RebH binding, could become rate limiting.[40,43] It is possible that the lower

concentration of $FADH_2$ is insufficient to saturate RebH under the conditions used, leading to the lower rates of chlorination observed. To test whether the k_{cat} of the two-component system is also significantly affected by the concentration of reductase, steady state kinetic parameters were obtained for RebH under analogous conditions 1:1 RebH/MBPF. A lower k_{cat} was again observed (0.18 min^{-1}, Table 2, entry 3). Surprisingly, at this lower concentration of reductase (and thus lower concentration of $FADH_2$), there appears to be little difference between the chlorination rates of RebH and H-16-F. This would suggest that as the local concentration of reductase is important in the comparative halogenase activity between the single and two-component systems, however, further detailed analysis will be necessary to fully understand this observation.

Table 2. Kinetic parameters for aromatic chlorination catalyzed by RebH and H-16-F.[a]

Entry	FDH	[H-16-F] (μM)	[RebH] (μM)	[RebF] (μM)	k_{cat} (min^{-1})	K_m (μM)
1	RebH	-	0.007	2.5	4.26±0.13	0.7±0.1
2	H-16-F	0.007	-	2.5	2.34±0.07	0.7±0.1
3	RebH	-	0.03	0.03	0.18±0.01	0.7±0.2
4	H-16-F	0.03	-	-	0.14±0.01	0.9±0.3

[a]0.5-15 μM L-tryptophan, 0.007-0.03 μM FDH, 0.03-2.5 μM reductase, 9 U mL^{-1} GDH, 10 mM NaCl, 20 mM glucose, 100 μM NAD and FAD, 25 mM HEPES buffer pH = 7.4, 25 °C, 75 μL final reaction volume. Reactions were quenched with MeOH 5-20 minutes after reaction initiation. 0.5 mM phenol was added as an internal standard, and reactions were analyzed by HPLC. Saturation plots used to calculate values can be found in the supporting information (Fig. S8-11).

In addition to using FDH-FR fusion enzymes to facilitate directed evolution efforts, we also envisioned that they could provide higher product yields in whole cell bioconversions. Our kinetic data support the hypothesis that as reductase concentration decreases, the use of fusion to generate higher local concentration of $FADH_2$ becomes more relevant. Within a cell, the local concentration of $FADH_2$ could greatly impact FDH activity. To test whether the fusion would outperform the two-component system *in vivo*, we examined the halogenase activity of *E. coli* BL21 pGro7 cells transformed with H-16-F, RebH, RebH+RebF, and RebH+MBPF (note that RebH+RebF and RebH+MBPF cells contain these two enzymes on two separate plasmids). Cultures were grown to an OD_{600} of 0.85-0.9, IPTG (100 μM) and L-arabinose (2 mg/mL) were added to induce halogenase expression, and solutions of L-tryptophan (**7**, 1 mM) and NaCl (100 mM) were added to initiate the halogenation reactions. High titers of 7-chlorotryptophan were obtained following incubation at 30 °C for 24 h, and a 2.5-fold increase in product concentration was observed for cells containing H-16-F relative to RebH, RebH+RebF, or RebH+MBPF (Figure 2). Excited by this result, we sought to demonstrate *in vivo* chlorination on a non-native substrate. Because *E. coli* cells contain significant quantities of L-tryptophan, we used an engineered FDH that does not halogenate L-tryptophan. Variant 1K was found to have greatly reduced activity on L-tryptophan, and in competition reactions between L-tryptophan and anthranilic acid (**9**), no conversion of tryptophan is observed (see supporting information). Significantly higher titers (55.01 mg L^{-1} with 1K-16-F, 15.21 mg L^{-1} with 1K+MBPF) were obtained with this enzyme *in vivo* (Figure 2).

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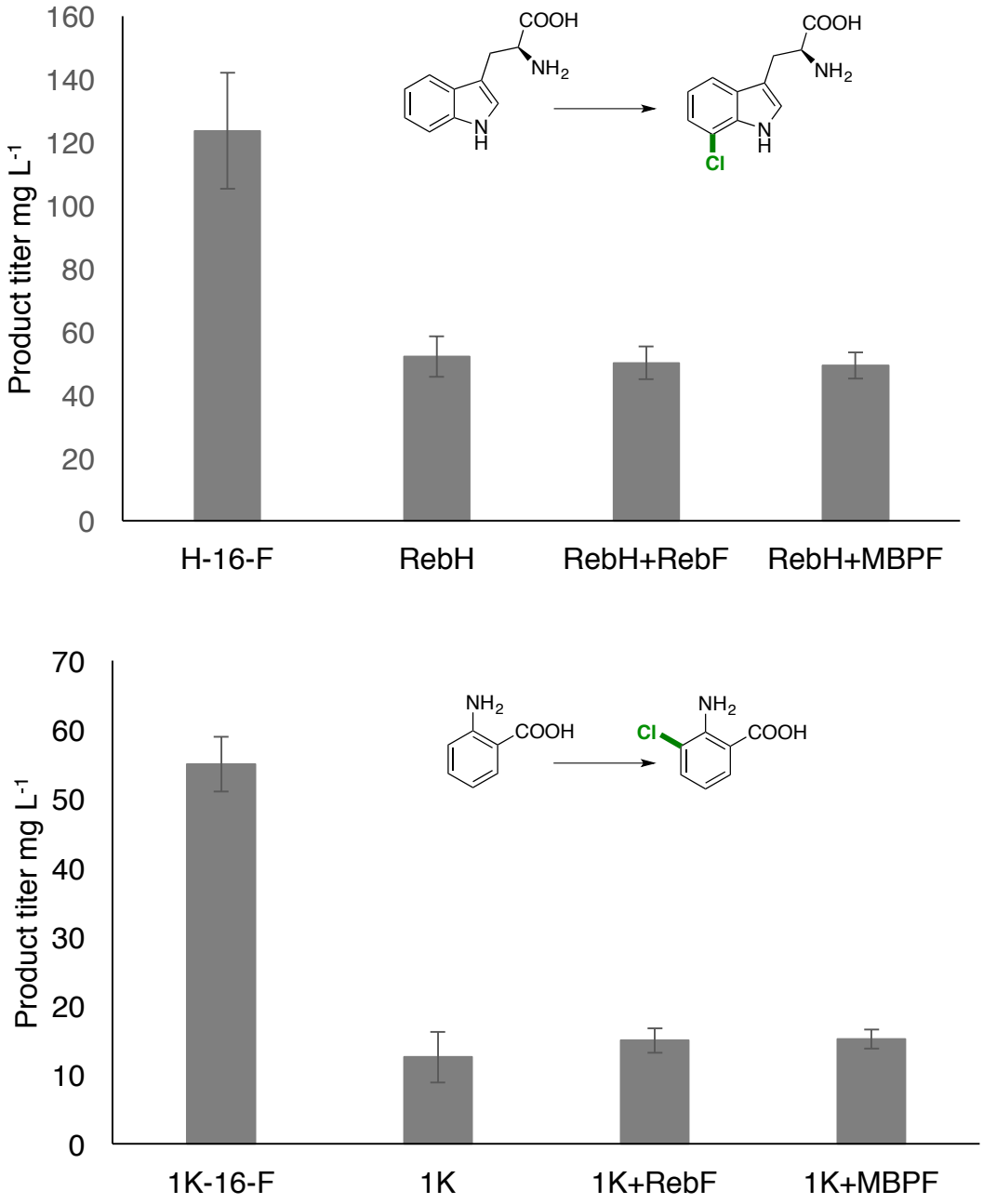

Figure 2. *In vivo* biocatalysis with H-16-F and 1K-16-F to afford chlorinated L-tryptophan (**7**) and anthranilic acid (**9**), respectively. Upon induction of expression of 50 mL cultures in TB media, 1 mM substrate and 100 mM NaCl were added. Cultures were expressed for 24 hours at 30 °C, and aliquots of the supernatant were analyzed by HPLC. Three independent trials of

triplicate cultures were performed for each cell line, and resulting standard deviations are shown as error bars (n = 9).

Because lower yields of purified, soluble enzyme were produced for *in vitro* studies using the fusion FDH-FRs, we anticipated that the observed increase in product formation *in vivo* was the result of higher local concentration of $FADH_2$, not increased fusion expression relative to the corresponding FDHs. An SDS-PAGE gel and a western blot for the soluble fractions of *in vivo* bioconversions confirmed low levels of soluble fusion expression for H-16-F compared to RebH (see supporting information, Fig. S12-13). While this does not unequivocally confirm our hypothesis, it does qualitatively suggest that increased enzyme expression is not responsible for high product titers *in vivo* using the fusion system.

In summary, we have demonstrated that functional FDH-FR fusion enzymes can be engineered using different linkers, FDHs, and reductases. Although a slight reduction in activity is observed for these enzymes compared with their corresponding two-component systems *in vitro*, the use of fusion enzymes could simplify FDH engineering efforts by eliminating the need for added FR. In addition, higher product titers are observed when FDH-FR fusion enzymes are used for *in vivo* biocatalytic transformations. These systems could therefore serve as valuable tools for *in vivo* chlorination in several different organisms, and efforts are currently underway in our laboratory to engineer systems that provide increased product titers for large-scale halogenation in *E. coli*.

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