#### Structural insights into hydrolytic defluorination of difluoroacetate by microbial fluoroacetate dehalogenases

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# Supporting information for the manuscript

"Structural insights into hydrolytic defluorination of difluoroacetate by microbial fluoroacetate dehalogenases"

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**Fig. S1.** SDS-PAGE analysis of purified FA dehalogenases used in this study. Coomassiestained SDS gels showing purified proteins and molecular weight markers (M, kDa).

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A		$\rightarrow \rightarrow $	
DAR3835	1	MFDSSYVTRDVDVG ATRIHVRVRENEGRPPLLLLHGYPETHAMWHKVASLLQDRFSLVLPDLRGYGDSGMPASQ	74
NOS0089	1	MFTN FEQTIVDTT EARINL VKAGHG APLLLLHGYPQTHVMWHKIAPLLANNFTVVATDLRGYGDSSRPASV	71
POL4478	1	MTWFDG FESRSFEVNGASIQARFSRVALGDAPRPALLLIHGFPQSHVMWHRVAQRLAQHYFLVMPDLRGYGDS KTPGL	79
RPA1163	1	MPDLADLFPG FGSEWINTS SGRIFARVGGDGP PLLLLHGFPQTHVMWHRVAPKLAERFKVIVADLPGYGWSDMPESD	77
		ווווווווווווווווווווווווווווווווווווו	
Dar3835	75	adagnqskrvmaqdmaelmtalgyqrfhvaahdrgarvlhrlcldhpgriqtacim <mark>d</mark> iaptattfa ltn qalatsyfhw	153
NOS0089	72	phhinyskrvmaqdqvevmsklgyeqfyvvghdrgarvahrlaldhphrvkklall <mark>d</mark> iapthkmyr tid qefatayyhw	150
POL4478	80	pdhsnys <b>krnma</b> hdmvav <b>m</b> sa <b>lg</b> vdr <b>f</b> flcg <b>hdrgarv</b> ah <b>rlald</b> haarviklcvi <mark>d</mark> va <b>pt</b> ldmyegrgmtepymaf <b>a</b> qayy <b>hw</b>	163
RPA1163	78	EQHTPYT <b>KRAMA</b> KQLIEAMEQ <b>lg</b> HVHFALAGH <mark>D</mark> RGARVSYRLALDSPGRLSKLAVL <mark>D</mark> ILPTYEYWQ RMN RAYALKIY <u>HW</u>	156
		י תונותונותווותווותווותווה תונתותו תונותה ת	
DAR3835	154	FFLIQAAPLPENMIAAD PASWLKGCLSRWS MGNEEAFDPAVVSEYVRCFSNPEAIRCSCDDYRAAAGIDLQHDGEDAER	232
NOS0089	151	FFLIQPDNLPETLIGAN PEYYLRKCLEKWG KDFS AFHPQALAEYIRCFSQPAVIHATCEDYRAAATIDLEHDELDMKQ	228
POL4478	164	FHMLQPAPLPEIMMGANHLETAKAYLHAKLGGWG SSGLGYIEPEALAEYERCFCNAEALHTACEDYRASARIDLEHDRESRAH	246
RPA1163	157	SFLAQPAPLPENLLGGD PDFYVKAKLASWTRAGDLSAFDPRAVEHYRIAFADPMRRHVMCEDYRAGAYADFEHDKIDVEA	236
DAR3835	233	RISCPLLVLWGNKGFVGRNYDVVALWREKALD VSGKGLPCG <mark>H</mark> FLPEELPNDVARELVEFIARHSSANSLAG	303
NOS0089	229	KISCPVLVLWGEKGIIGRKYDVLATWRERAID VSGQSLPCG <mark>HF</mark> LPEEAPEETYQAIYNFLTHC	291
POL4478	247	GLKVACDMLVLSGERGVVHRLFNPMALWQEQCSGIVAGHTLQAGHFIPEEQPQAVAQALLEFFALPELDRV	317
RPA1163	237	GNKIPVPMLALWGASGIAQSAATPLDVWRKWASD VQGAPIESG <mark>H</mark> FLPEEAPDQTAEALVRFFSAAP	302

D		$\rightarrow$	
ADE3811	1	MA LAPVRAAVFDAYGTLFDVASAAAEARGALGDRWQPLAELWRAKQLQYTWLRSLAG RHADFWQVTGD	68
POL0530	1	MH AIKAVVFDLYGTLYDVYSVRTSCERIFPGQGEMVSKMWRQKQLEYTWMRTLMG QYQDFESATLD	66
POL4516	1	MEKPRALAPVPIRGVLFDAYGTLFDVYSVGLLAEQLFPGQGQTLGLLWRDKQIEYTRLVTTCNDG AHYQPFWDLTRS	77
RJA0230	1	MAGVPFRSPSTGRNVRAVLFDTFGTVVDWRTGIATAVADYAARHQLEVDAVAFADRWRARYQPSMDAILSGAREFVTLDILHRE	84
		$\rightarrow$	
ADE3811	69	ALDFALEALGLAGPG LRDG LMDAYRRLAAYPEARDALTRLRGAGVRLAVLS <mark>NG</mark> APAMLASAAESAGLAGLLEQVLSVEDV	148
POL0530	67	ALRYTCGSLGLALDA DGEAHLCSEYLSLTPFADVPQALQQLRAAGLKTAILSNGSRHSIRQVVGNSGLTNSFDHLISVDEV	147
POL4516	78	SLRYVCKRLALDLTP EREQRLMNQYRHLSAFPENKGVLQALKKRGIVTGILSNGDPSMLDVAVKSGGLEGLLDHVISVDSI	158
RJA0230	85	NLDFVLRESGIDPTNHDSGELDELARAWHVLTPWPDSVPGLTAIK AEYIIGPLSNGNTSLLLDMAKNAG IPWDVIIGSDIN	165
		$m \circ \leftarrow m m \leftarrow m m \circ \leftarrow m$	
ADE3811	149	GVYKPHPAV <mark>X</mark> RLAVDRLGVPAPEILFVSSNGW <mark>D</mark> AFG <b>A</b> KAF <b>G</b> LQVAWCN <b>R</b> AGQPAERLPAA <b>P</b> DAEIRS LAELPP <b>L</b> LG LP	226
POL0530	148	RLF <b>K</b> PHQKV <b>Y</b> ELAMDTLHLGESEILFVSCNSW <mark>P</mark> ATG <b>A</b> KYF <b>G</b> YPVCWIN <b>R</b> SNGVFDQLGVV <b>P</b> DIVVS DVGV <b>L</b> ASRFSPV	225
POL4516	159	RKYKTHPDAYALGPQHTGLDVRQIAFVSCNGWPALAATWYGYQTLWINR YQLPFEELGTAPTYTGSS LRDVLTLPGMNLPG	239
RJA0230	166	RKY K P D P Q V L C L G L G L A A H A G L A A I L R P V E H G P D L A P V E H G L A P V E H G L A P V E H G L A P V E H G L A P V E H G L A P V E H G L A P V E H G L A P V E H G L A P V E H G L A P V E H G L A P V E H G A A L A A A A A A A A	249
ADE3811	227		
POL0530	226	DEAA	
POL4516	240	AAA	
RJA0230	250	STGFR	

**Fig. S2.** Multiple sequence alignment of hydrolytic dehalogenases used in this study. (A), α/β-hydrolases: DAR3835 from *Dechloromonas aromatica* (Uniprot ID Q479B8), ADE3811 from *Anaeromyxobacter dehalogenans* (Q2IG66), NOS0089 from *Nostoc* sp. (Q8Z0Q1), POL0530 from *Polaromonas* sp. (Q12G50). (B), HADs: RPA1163 from *Rhodopseudomonas palustris* (Q6NAM1), POL4478 from *Polaromonas* sp. (Q123C8), POL4516 from *Polaromonas* sp. (Q122Z0), RJO0230 from *Rhodococcus jostii* (Q0SK70). The catalytic residues are designated by red boxes (Asp107, Asp131, His274 in DAR3835), fluoride coordinating residues are labelled by blue rectangles (His152, Trp153, Tyr215 in DAR3835), and carboxylate binding residues (Arg108 and Arg111 in DAR3835) are indicated by green rectangles, and conserved residues are shown in bold. The secondary structural elements of DAR3835 and RJO0230 (PDB code 3UMG).



**Fig. S3.** Defluorination activity of purified DAR3835 and RPA1163. (A), Formation of glyoxylic acid during DFA defluorination by DAR3835 and RPA1163. The reaction mixtures (200  $\mu$ L) contained 10 mM DFA and 20  $\mu$ g of protein (overnight incubation at 30 °C), and glyoxylic acid was measured using LC-MS under negative ionization (see Materials and Methods for experimental details). (B, C), Defluorinase activity of purified DAR3835 with FA and DFA as a function of time and substrate concentration (20  $\mu$ g of enzyme/assay). (D), Site-directed mutagenesis of RPA1163: defluorination activity with FA or DFA as substrates. The reaction mixtures contained 10 mM substrate, 2  $\mu$ g/ml of phenol red, and 20  $\mu$ g of purified enzyme. All assays were carried out in triplicate, and results are means  $\pm$  SD from at least two independent determinations.





**Fig. S4.** Crystal structure of NOS0089: overall fold. (A), Overall fold of the protomer: two views related by a 180° rotation. The protein core domain is coloured in grey, whereas the lid domain is represented in light orange. The protein N- and C-termini are labelled (N and C). (B), Two views of the NOS0089 dimer shown in two views related by a 90° rotation. The protein subunits shown as ribbon diagrams, whereas the core and lid domains of two protomers are coloured in different colours.



**Fig. S5.** DAR3835 active site: amino acid residues selected for site-directed mutagenesis. The protein ribbon is coloured in grey with residue side chains shown as sticks and carbon atoms coloured green, whereas the bound Cl<sup>-</sup> ion is indicated as a magenta-coloured sphere.



**Fig. S6.** Potential catalytic mechanism of DFA defluorination by DAR3835 without tetrahedral intermediates. The reaction involves the DAR3835 catalytic triad (Asp107, His274, and Asp131) and includes the following steps: (A), free enzyme; (B), the DAR3835-DFA (Michaelis) complex; (C), 2-fluoroglycolyl intermediate (acyl intermediate-1); (D), the DAR3835 complex with 2-fluoro-2-hydroxy-acetate (FHA); (E), 2-hydroxyglycolyl intermediate (acyl intermediate-2); (F), the DAR3835 complex with dihydroxyacetate. In organic solvents, DHA is dehydrated to glyoxylic acid, which was detected using LC-MS.

Protein	Protein	Gene	Protein	Uniprot	Microorganism
name	length	name	superfamily	ID	
1. ADE3811	226 aa	Adeh3811	HAD <sup>a</sup>	Q2IG66	Anaeromyxobacter dehalogenans
2. DAR3835	303 aa	Daro3835	$\alpha/\beta$ hydrolase	Q479B8	Dechloromonas aromatica
3. NOS0089	291 aa	Alr0039	$\alpha/\beta$ hydrolase	Q8Z0Q1	Nostoc sp.
4. POL0530	229 aa	Bpro0530	HAD	Q12G50	Polaromonas sp.
5. POL4478	317 aa	Bpro4478	$\alpha/\beta$ hydrolase	Q123C8	Polaromonas sp.
6. POL4516	242 aa	Bpro4516	HAD	Q122Z0	Polaromonas sp.
7. RJO0230	254 aa	RHA0230	HAD	Q0SK70	Rhodococcus jostii
8. RPA1163	302 aa	RPA1163	$\alpha/\beta$ hydrolase	Q6NAM1	Rhodopseudomonas palustris

Table S1. Bacterial hydrolytic dehalogenases purified and screened in this study.

<sup>a</sup> HAD, haloacid dehalogenase

Enzyme	DAR3835	NOS0089	POL4478	RPA1163	POL0530	POL4516	ADE3811	RJO0230
DAR3835 <sup>a</sup>		53.6	41.8	41.5	12.2	11.4	13.5	8.0
NOS0089 <sup>a</sup>	53.6		46.1	45.3	7.8	8.6	10.8	6.4
POL4478 <sup>a</sup>	41.8	46.1		38.9	8.2	9.4	9.9	6.5
RPA1163 <sup>a</sup>	41.5	45.3	38.9		7.0	9.7	12.3	7.9
POL0530 <sup>b</sup>	12.2	7.8	8.2	7.0		39.3	37.1	17.0
POL4516 <sup>b</sup>	11.4	8.6	9.4	9.7	39.3		39.1	23.4
ADE3811 <sup>b</sup>	13.5	10.8	9.9	12.3	37.1	39.1		20.6
RJO0230 <sup>b</sup>	8.0	6.4	6.5	7.9	17.0	23.4	20.6	

Table S2. Amino acid sequence identity of defluorinases used in this study.

<sup>a</sup>  $\alpha/\beta$  hydrolase superfamily

<sup>b</sup> HAD-like superfamily

Structure	DAR3835	<b>DAR3835 H274N</b> (D107-glycolyl	<b>NOS0089</b> (ALR0039)	
		intermediate)		
PDB code	8SDC	8SDD	3QYJ	
Data collection			-	
Space group	P1	P21	P21	
Unit cell			11 51 99 20	
a, b, c (Å)	45.2, 45.2, 75.2	42.94, 58.49, 138.19	44.54, 88.20,	
$\alpha, \beta, \gamma, (^{\circ})$	87.6, 75.6, 63.0	90, 93.0, 90	90 93 1 90	
Resolution, Å	19.66 - 1.86	46.00 - 2.00	20.00 - 1.78	
$R_{merge}^{a}$	0.049 (0.111) <sup>c</sup>	0.049 (0.098)	0.037 (0.258)	
R <sub>nim</sub> <sup>b</sup>	0.030 (0.061)	0.025 (0.075)	NC <sup>d</sup>	
$CC_{1/2}$	0.997 (0.987)	0.998 (0.986)	NC	
I / σ(I)	17.7 (9.4)	23.3 (11.3)	42.8 (6.3)	
Completeness, %	94.3 (90.6)	94.6 (85.5)	97.4 (94.5)	
Redundancy	3.6 (3.3)	4.8 (4.2)	3.8 (3.6)	
Refinement	· · ·		× /	
Resolution, Å	19.66 - 1.86	30.40 - 2.00	19.86 - 1.78	
No. unique				
reflections:	40567, 2127	43847, 1995	58321, 2021	
working, test				
R-factor/free R-factor <sup>e</sup>	12.5/17.4	21 9/26 2 (24 4/30 8)	14.9/17.9	
R lactor nee R lactor	(13.5/19.0)	21.)/20.2 (24.4/50.0)	(23.4/NC)	
No. refined atoms,				
chains				
Protein	4673,2	4652, 2	4732, 2	
Solvent	2	N/A	N/A	
Water	1018	667	634	
B-factors	15.0	22.1	20.7	
Protein	15.2	32.1	20.7	
Solvent	20.4	N/A 28.7	N/A 20.4	
r m a d	55.0	38.7	30.4	
Rond lengths Å	0.008	0.005	0.025	
Bond angles °	0.008	0.005	1.84	
Ramachandran	0.077	0.730	1.07	
favoured	97 1%	97 1%	99.8%	
allowed	2 9%	2 8%	0.2%	
outliers	0%	0.1%	0%	

Table S3. X-ray crystallographic statistics for the structures of DAR3835 and NOS0089.

 ${}^{a}R_{merge} = \sum_{hkl}\sum_{j}|I_{hkl,j} - \langle I_{hkl}\rangle|/\sum_{hkl}\sum_{j}I_{hk,j}$ , where  $I_{hkl,j}$  and  $\langle I_{hkl}\rangle$  are the *j*th and mean measurement of the intensity of reflection *j*.

 ${}^{b}R_{pim} = \sum_{hkl} \sqrt{(n/n-1)} \sum_{j=1}^{n} |I_{hkl,j} - \langle I_{hkl} \rangle | / \sum_{hkl} \sum_{j} I_{hk,j}$ call values in brackets and  $CC_{1/2}$  refer to the highest resolution shell.

 $^{d}NC = not calculated.$ 

 ${}^{e}R = \Sigma |F_{p}{}^{obs} - F_{p}{}^{calc}|/\Sigma F_{p}{}^{obs}$ , where  $F_{p}{}^{obs}$  and  $F_{p}{}^{calc}$  are the observed and calculated structure factor amplitudes, respectively.

**Table S4.** Structure-based computational analysis of protein tunnels in DAR3835, NOS0089,and RPA1163 using MOLE 2.5ª.

Protein	Lining amino acids	Length,	<b>Charge</b> <sup>b</sup>	Hydrophobicity <sup>c</sup>	<b>Polarity</b> <sup>d</sup>
(subunit/tunnel)	_	Å	_		-
DAR3835	D107, R108, R111, S149,	$21.2\pm0.3$	2	$0.43\pm0.02$	$17.6 \pm 2.4$
(A/1)	Y150, H152, W153, R181,				
	W182, M184, F247, R250,				
	H274				
DAR3835	D107, R111, D131, I132,	$21.4\pm1.2$	2	$0.34\pm0.04$	$18.5 \pm 1.4$
(B/1)	S149, Y150, H152, W153,				
	R181, W182, M184, F247,				
	R250, H274				
NOS0089	D104, R108, D128, I129,	$19.9\pm0.7$	0.5	$0.30\pm0.06$	$22.6 \pm 3.4$
(A/1)	Y147, K178, W179, K181,				
. ,	G242, I243, I244, H270				
NOS0089	D104, R105, R108, D128,	$17.1\pm1.0$	0	$0.20\pm0.08$	$21.3 \pm 0.3$
(B/1)	A146, Y147, W150, K178,				
	W179, G242, I243, H270				
RPA1163	D110, R114, D134, I135,	$18.7\pm0.2$	1	$0.53\pm0.19$	$15.2 \pm 2.6$
(A/1)	Y141, Y149, K152, I153,				
	H155, W156, I253, H280				
RPA1163	D110, R114, I135, Y141,	$15.1\pm0.5$	1	$0.58\pm0.13$	$28.9 \pm 2.1$
(B/1)	K152, I153, H155, W156,				
	W185, H280				
RPA1163	D110, R114, D134, I135,	$16.7\pm1.1$	0	$0.09\pm0.01$	$21.9 \pm 1.7$
(B/2)	L136, Y141, R144, Y149,				
	W156, G252, P260, H280				

<sup>a</sup> Charge, hydrophobicity, and polarity of active site residues were calculated as indicated in Materials and Methods.

<sup>b</sup> Charge: calculated as a sum of charges of amino acid residues along the tunnel.

<sup>c</sup> Hydrophobicity: average hydrophobicity of side chains lining the tunnel (the most hydrophobic amino acid Ile = 1.81, the most hydrophilic amino acid Glu = -1.14).

<sup>d</sup> Polarity: average polarity of amino acids lining the tunnel ranging from nonpolar residues (Ala, Gly = 0) through polar (Ser = 1.67) to charged amino acids (Glu = 49.9, Arg = 52.0) (according to Zimmerman et al., 1968) (83).

Table S5. Structure-based computational analysis of the active site cavities of DAR3835,

NOS0089, and RPA1163 using MOLE 2.5.

Protein	Amino acid residues lining the cavity	Volume
(chain)	Annual residues ming the cuvity	Å <sup>3</sup>
DAR3835	D107, R108, R111, D131, I132, A133, T138, L146, Y150, H152,	733.6
(A)	T153, W182, Y215, G243, G246, F247, V248, G249, Y252, G273,	
	H274	
DAR3835	D107, R108, R111, D131, I132, A133, T138, L146, Y150, H152,	724.9
(B)	W153, W182, Y215, G243, G246, F247, V248, G249, Y252, G273,	
	H274	
NOS0089	Y34, D104, R105, R108, D128, I129, A130, M135, Y147, W150,	1043.3
(A)	L153, Y171, C175, W179, Y211, G239, G242, I244, Y248, V250,	
	Т253, G269, H270	
NOS0089	Y34, D104, R105, R108, L126, D128, I129, A130, M135, F143,	909.3
(B)	Y147, H149, W150, W179, Y211, V236, L237, W238, I244, Y248,	
	D249, V250, T253, W254, H270	
RPA1163	D110, R111, R114, D134, I135, L136, P137, T138, Y141, R144,	668.3
(A)	M145, A150, I153, H155, W156, W185, Y219, A254, V263, H280	
RPA1163	F40, D110, R111, R114, V132, D134, L135, L136, Y141, I153,	1062.3
(B)	Y154, H155, W156, Y219, A246, L247, L261, W264, H280	

**Table S6.** Binding energies for ligand binding in the active sites of DAR3835 and RPA1163

 calculated by docking simulations <sup>a</sup>.

RPA1163						
FA	Free energy of binding (kcal/mol)	DFA	Free energy of binding (kcal/mol)			
Model 1	-3.28	Model 1	-2.93			
Model 2	-3.28	Model 2	-2.82			
Model 3	-3.27	Model 3	-2.81			
Model 4	-3.27	Model 4	-2.80			
Model 5	-3.27	Model 5	-2.80			
Model 6	-3.27	Model 6	-2.78			
Model 7	-3.27	Model 7	-2.78			
Model 8	-3.27	Model 8	-2.78			
Model 9	-3.27	Model 9	-2.77			
Model 10	-3.25	Model 10	-2.77			
	DAF	R3835				
FA	Free energy	DFA	Free energy			
	of binding		of binding			
	(kcal/mol)		(kcal/mol)			
Model 1	-1.73	Model 1	-1.95			
Model 2	-1.73	Model 2	-1.94			
Model 3	-1.72	Model 3	-1.94			
Model 4	-1.72	Model 4	-1.94			
Model 5	-1.70	Model 5	-1.93			
Model 6	-1.70	Model 6	-1.91			
Model 7	-1.70	Model 7	-1.90			
Model 8	-1.66	Model 8	-1.89			
Model 9	-1.64	Model 9	-1.88			
Model 10	-1.63	Model 10	-1.88			

<sup>a</sup> FA- fluoroacetate, DFA, difluoroacetate. The active site models with minimal binding energies are shown in Fig. 8.