SUPPLEMENTARY INFORMATION

A biosynthetic pathway for a prominent class of microbiota-derived bile acids

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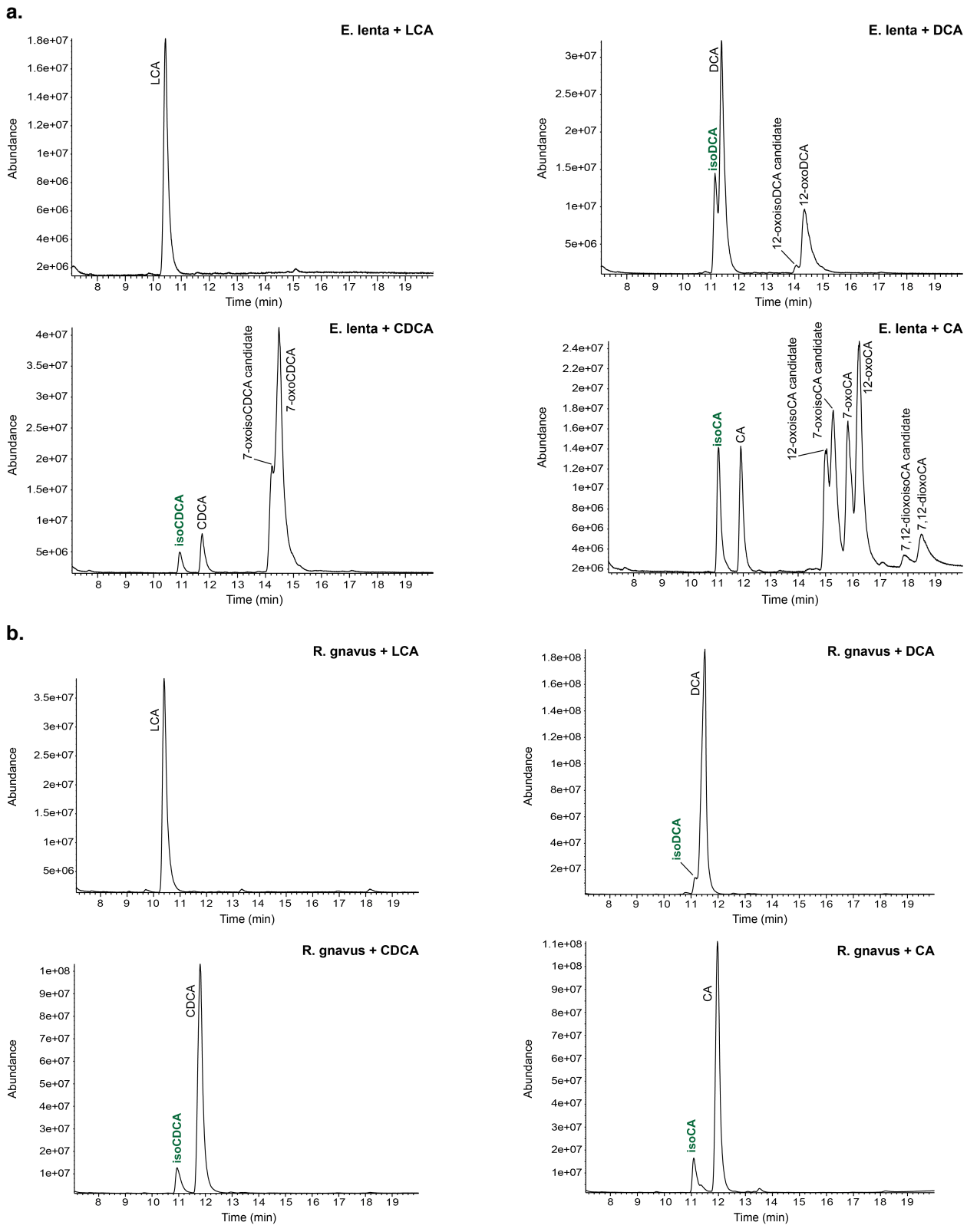
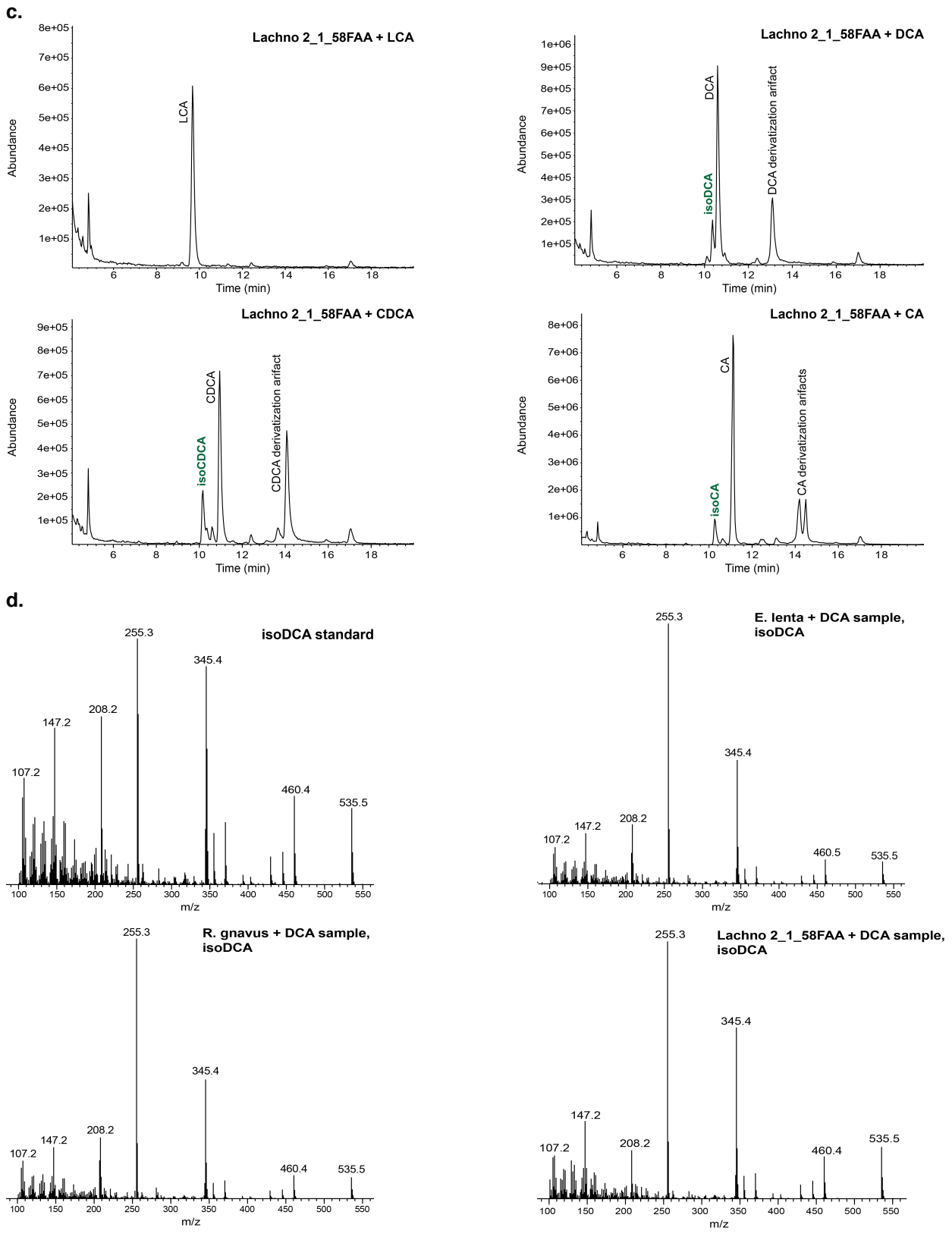
Supplementary Results



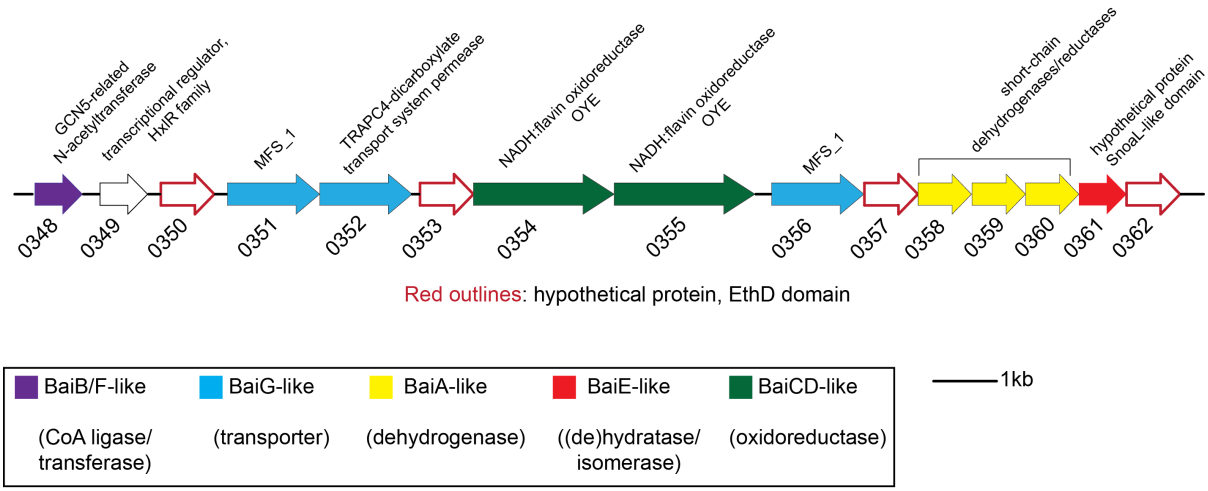
Supplementary Figure 1 (page 1 of 3)



Supplementary Figure 1 (page 2 of 3)

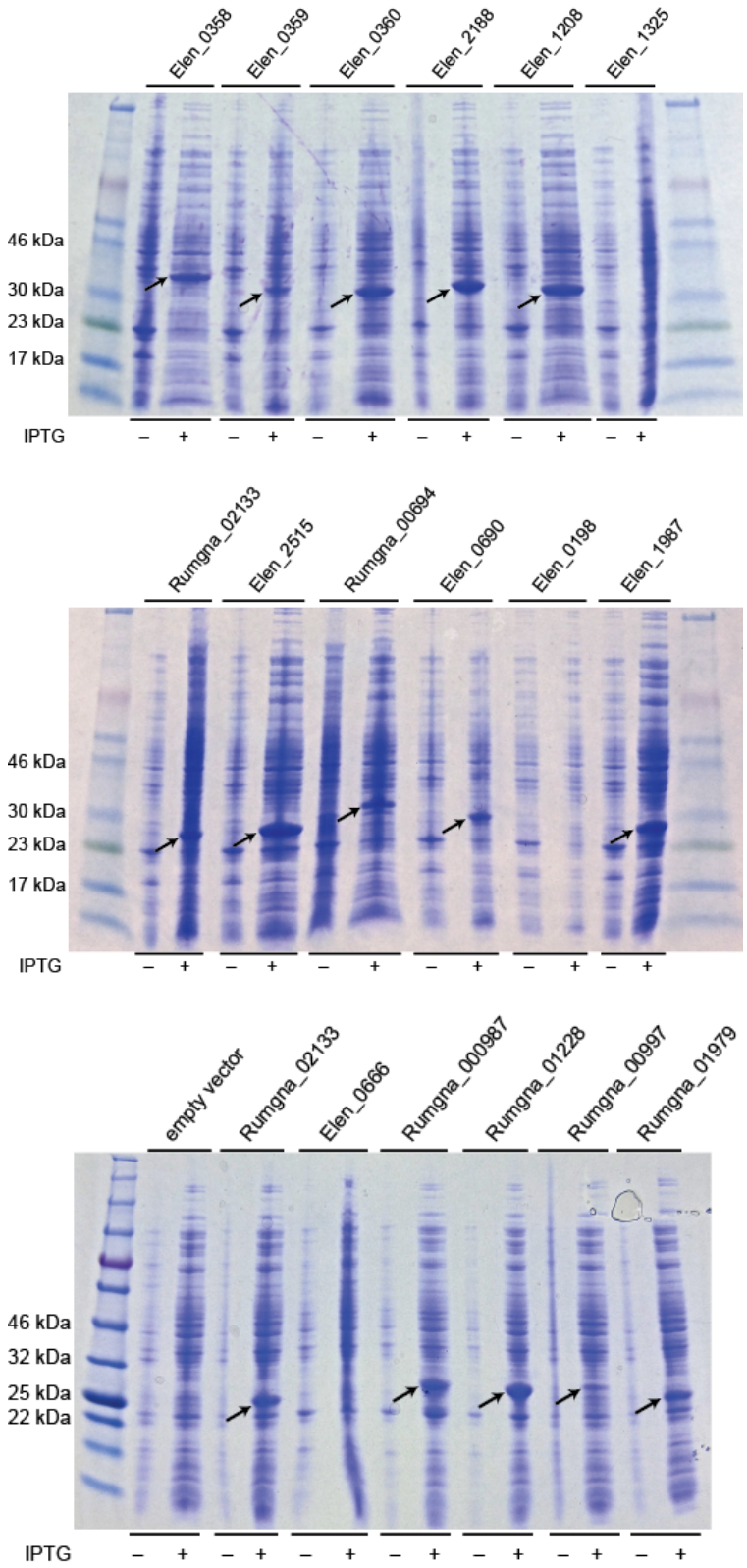
Supplementary Figure 1. *E. lenta*, *R. gnavus, and Lachnospiraceae sp. 2_1_58FAA* produced isobile acids in a whole cell screen.

All culture reactions were quenched at t=20 h (stationary phase) by addition of 6N HCl, purified, and prepared for GC-MS analysis as described in the Online Methods. (**a**) *Eggerthella lenta*, (**b**) *Ruminococcus gnavus* and (**c**) *Lachnospiraceae* sp. 2_1_58FAA produced isoDCA, isoCDCA, and isoCA from DCA, CDCA, and CA, respectively. (**d**) Mass spectral fragmentation patterns of isoDCA standard (purchased from Steraloids, Inc.; retention time 11.1 min) and isoDCA (retention time 11.1 min) in experimental samples. Note that bile acid retention times were shifted in (**c**) due to GC-MS column maintenance. Standards were re-run under these conditions to determine retention times and to confirm mass fragmentation patterns.



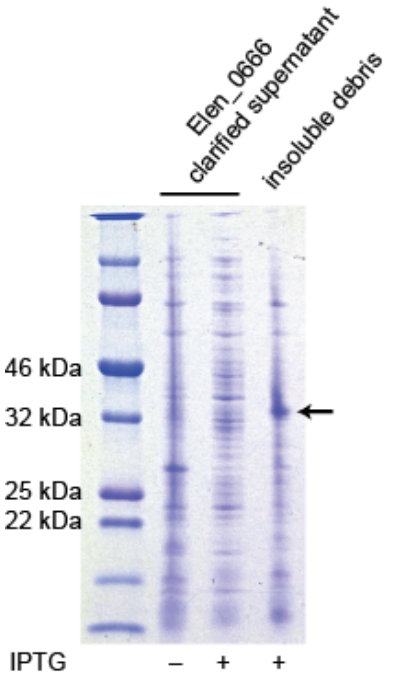
Supplementary Figure 2. Potential bile acid metabolizing cluster in *E. lenta DSM 2243*. Elen_0358, Elen_0359, and Elen_0360 were identified as candidate HSDHs from the initial BLASTP search but were not active in the cell lysate assay.

a.



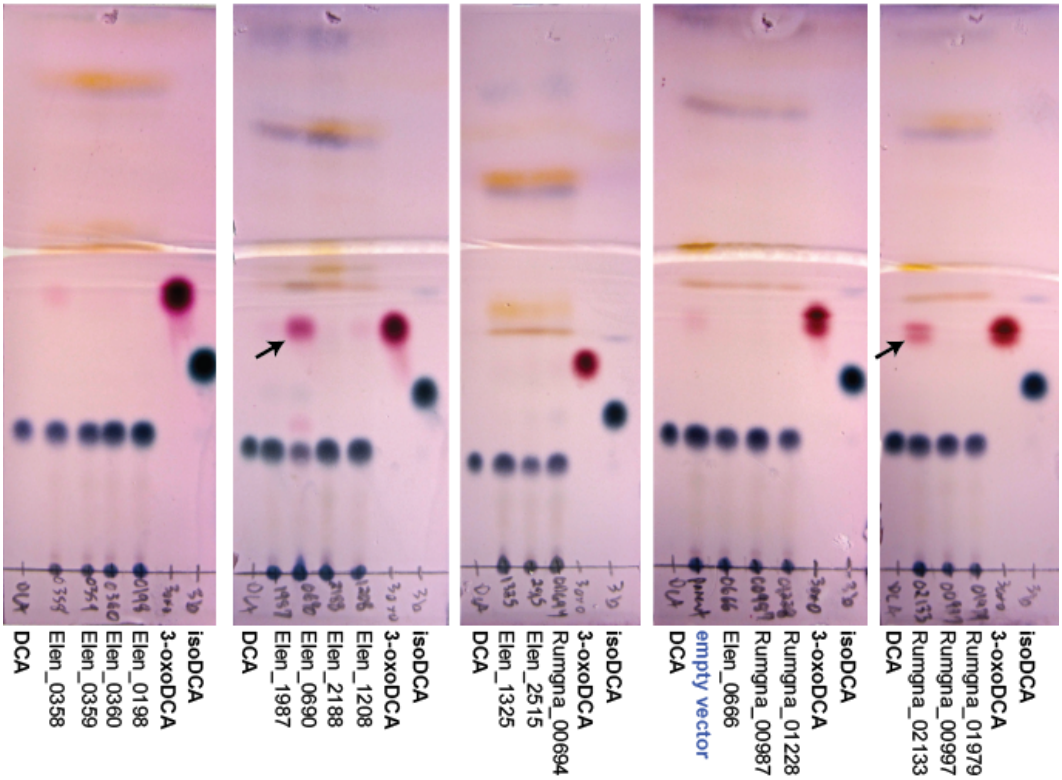
Supplementary Figure 3 (page 1 of 2)

b.



Supplementary Figure 3. Confirmation of heterologous expression of HSDH candidate genes in *E. coli*. (**a**) Clarified supernatant from either uninduced (–) or induced (+) cells was analyzed by SDS-PAGE. Elen_0358: 31.5 kDa, Elen_0359: 27.5 kDa, Elen_0360: 27.0 kDa, Elen_2188: 31.0 kDa, Elen_1208: 29.7 kDa; Rumgna_02133: 26.6 kDa, Elen_2515: 27.1 kDa, Rumgna_00694, 29.0 kDa, Elen_0690: 27.4 kDa, Elen_1987: 26.4 kDa; Rumgna_00987: 26.5 kDa, Rumgna_01228: 27.4 kDa, Rumgna_00997: 28.1 kDa, Rumgna_01979: 26.1 kDa. Expression of Elen_1325 (27.3 kDa) and Elen_0198 (27.6 kDa) was confirmed by Western blot and subsequently by SDS-PAGE analysis of purified protein (Figure S5). (**b**) Elen_0666 (27.5 kDa) was expressed but insoluble.

a.



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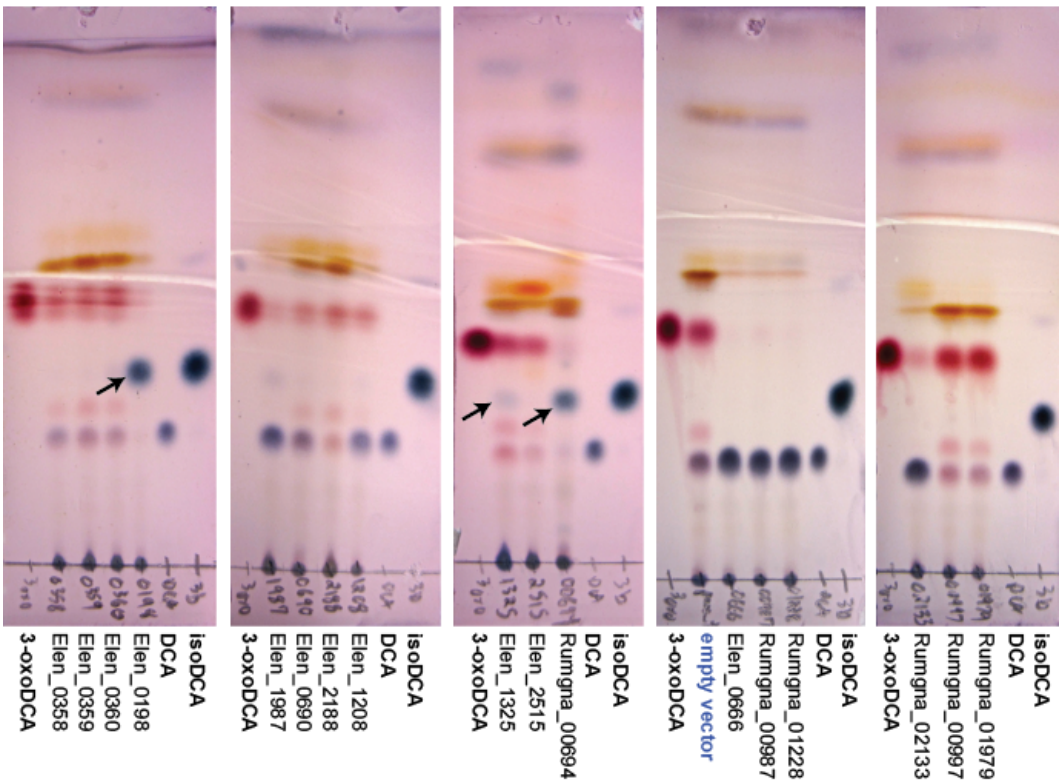
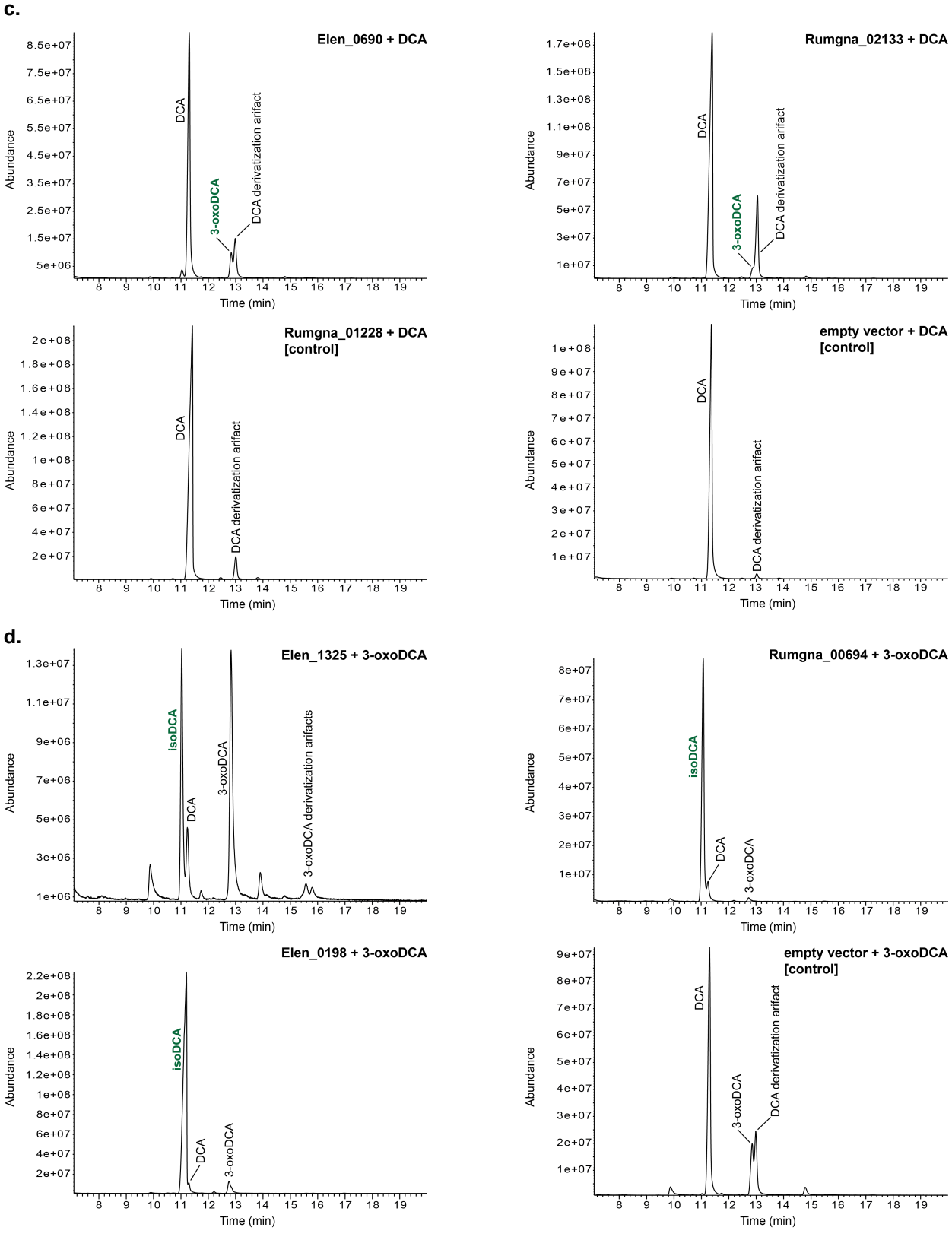
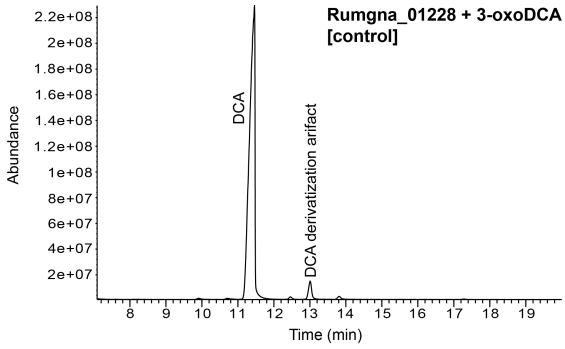


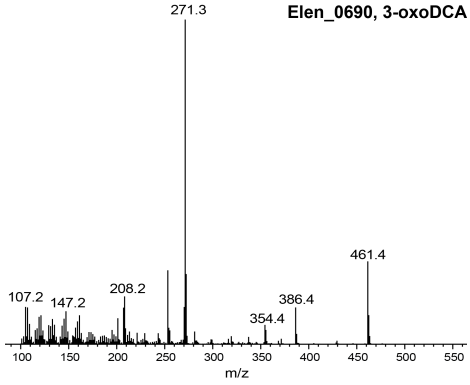
Supplementary Figure 4 (page 1 of 4)

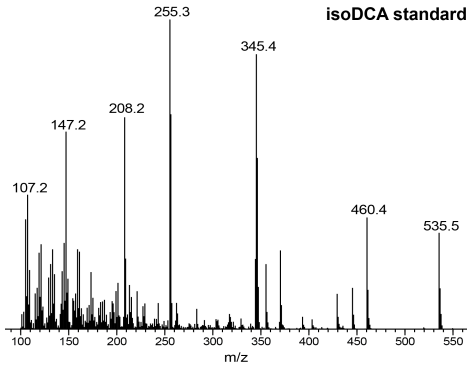


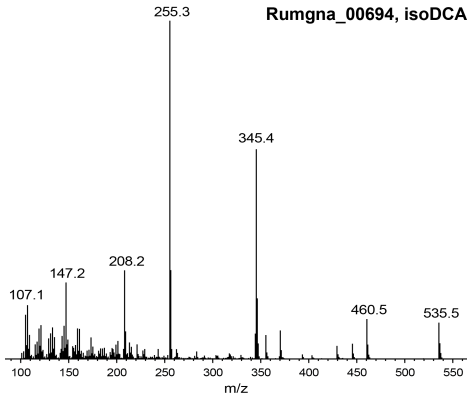
Supplementary Figure 4 (page 2 of 4)

d (cont'd).

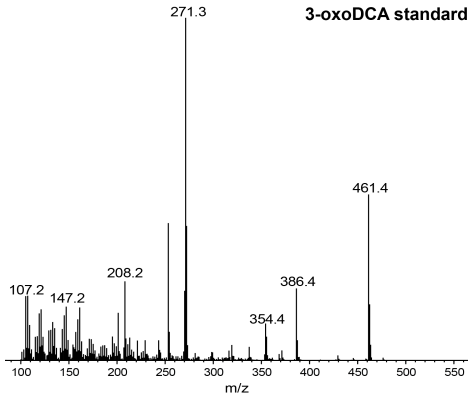


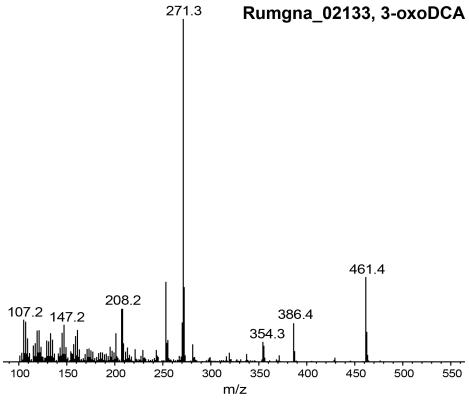


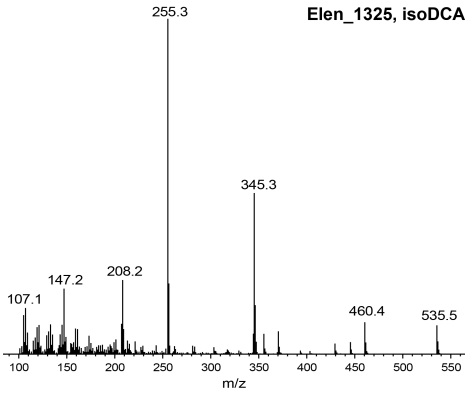


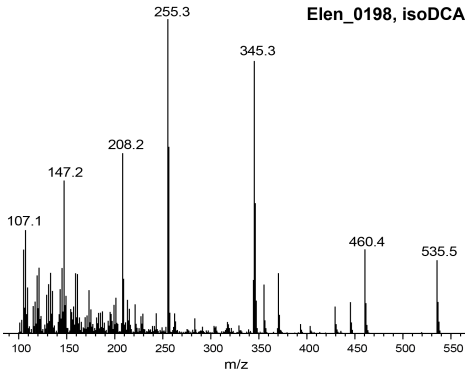


e.



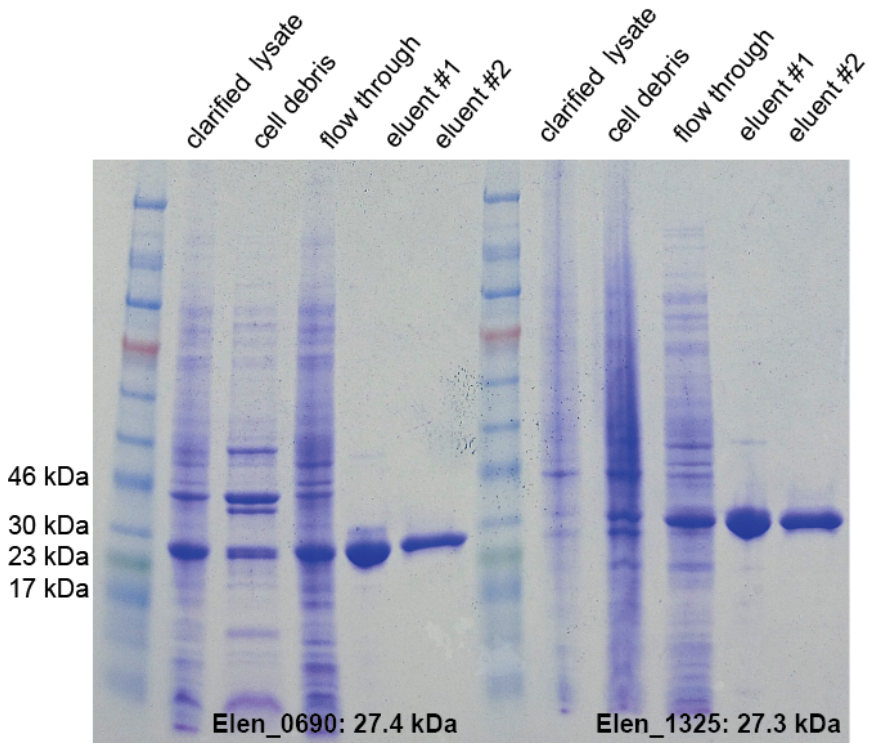


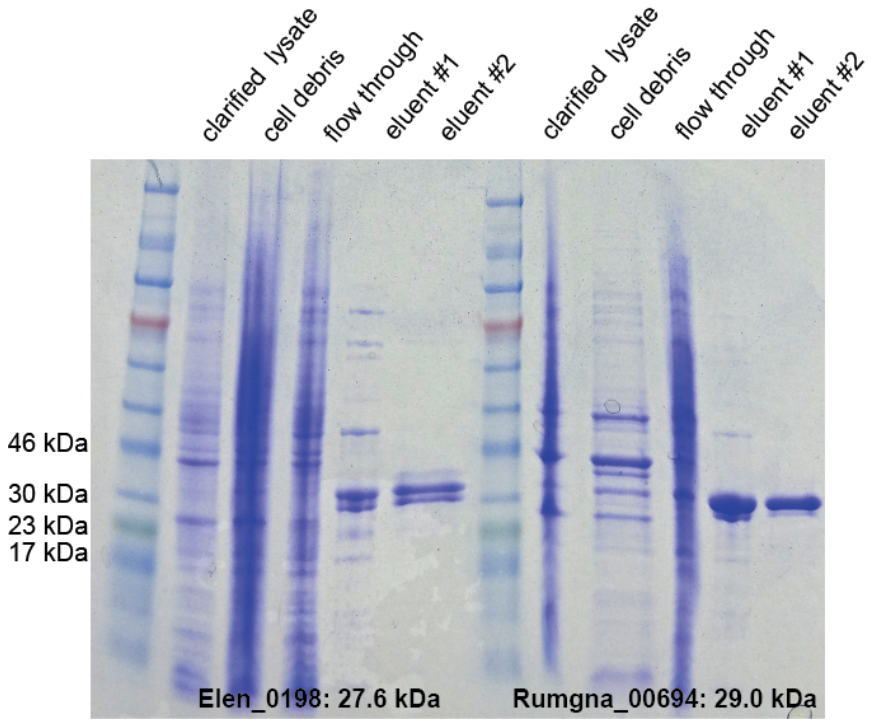




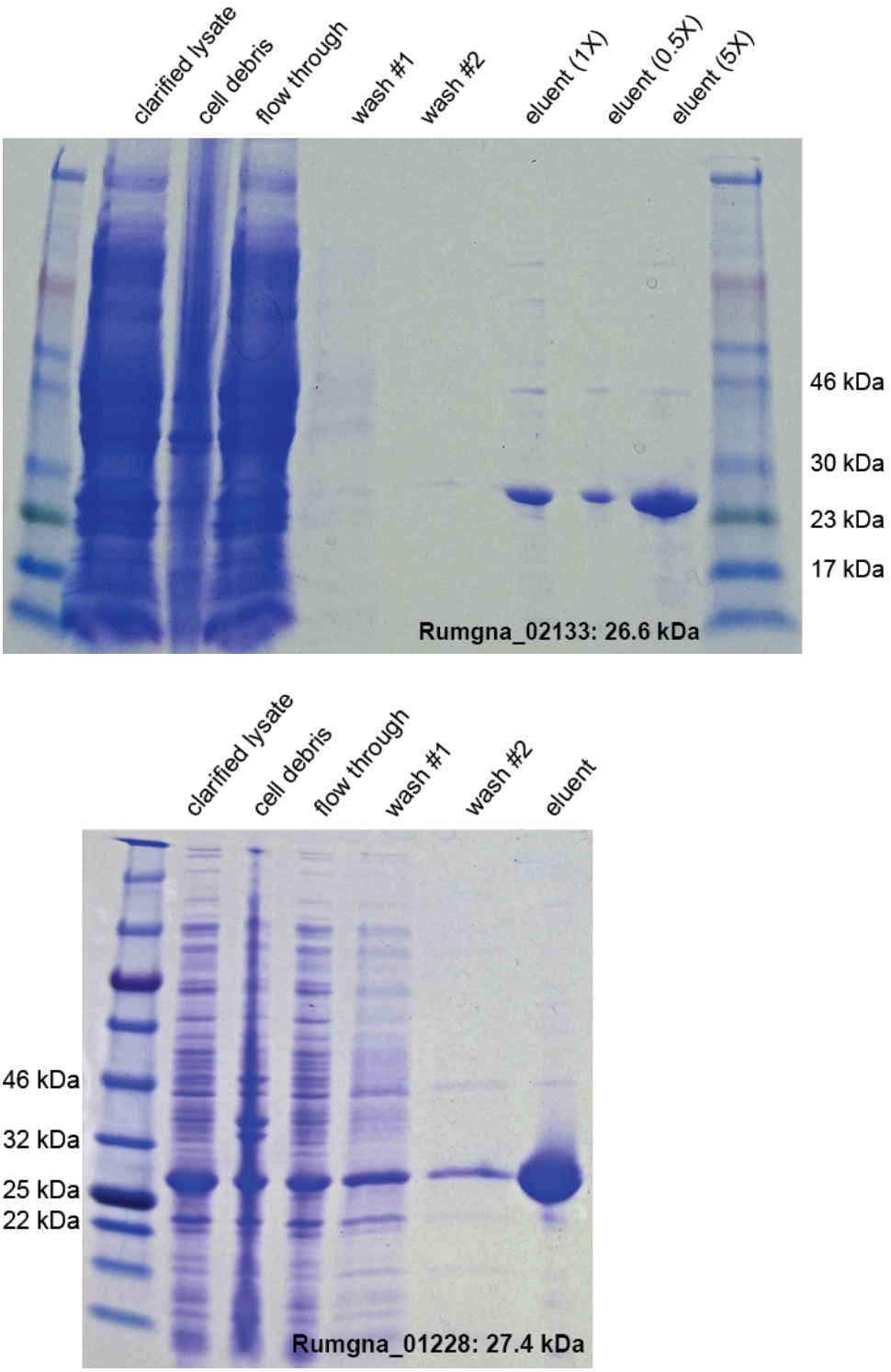
Supplementary Figure 4 (page 3 of 4)

Supplementary Figure 4. Functional 3α- and 3β-HSDHs identified using cell lysate assay. TLC analysis showed that (**a**) Elen_0690 and Rumgna_02133 transformed DCA into 3-oxoDCA and are 3α-HSDHs and (**b**) Rumgna_00694, Elen_1325, and Elen_0198 transformed 3-oxoDCA into isoDCA and are 3β-HSDHs. Because background reactivity (3-oxoDCA into DCA) was observed in the empty vector control condition, reductive 3α-HSDH enzymatic activity could not be assessed using this assay. (**c**) and (**d**) GC-MS confirmed these results. Rumgna_01228 and empty vector traces were included as negative controls for both substrates. (**e**) Mass spectral fragmentation patterns of 3-oxoDCA and isoDCA standards and in experimental samples (retention times 12.9 min, 11.1 min, respectively).



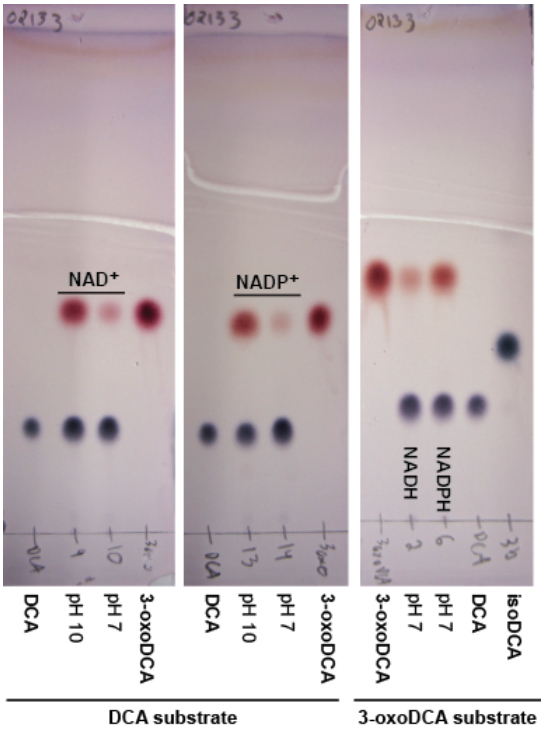


Supplementary Figure 5 (page 1 of 2)

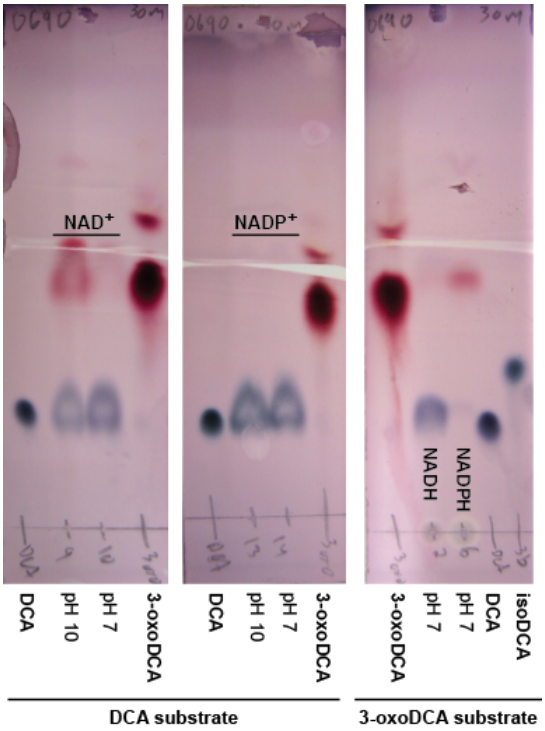


Supplementary Figure 5. **SDS-PAGE of purified enzymes**. L to R, top to bottom: Elen_0690, Elen_1325, Elen_0198, Rumgna_00694, Rumgna_02133, and Rumgna_01228 (purified as a negative control to use in enzymatic assays).

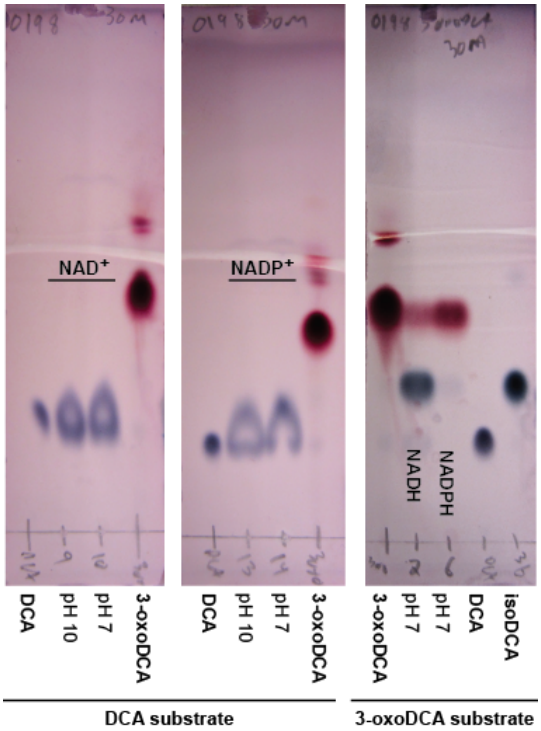
a. Rumgna_02133



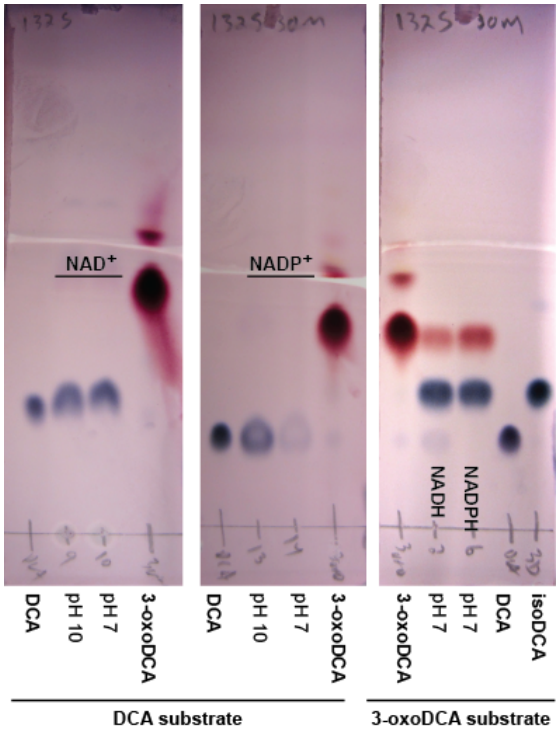
b. Elen_0690



c. Elen_0198



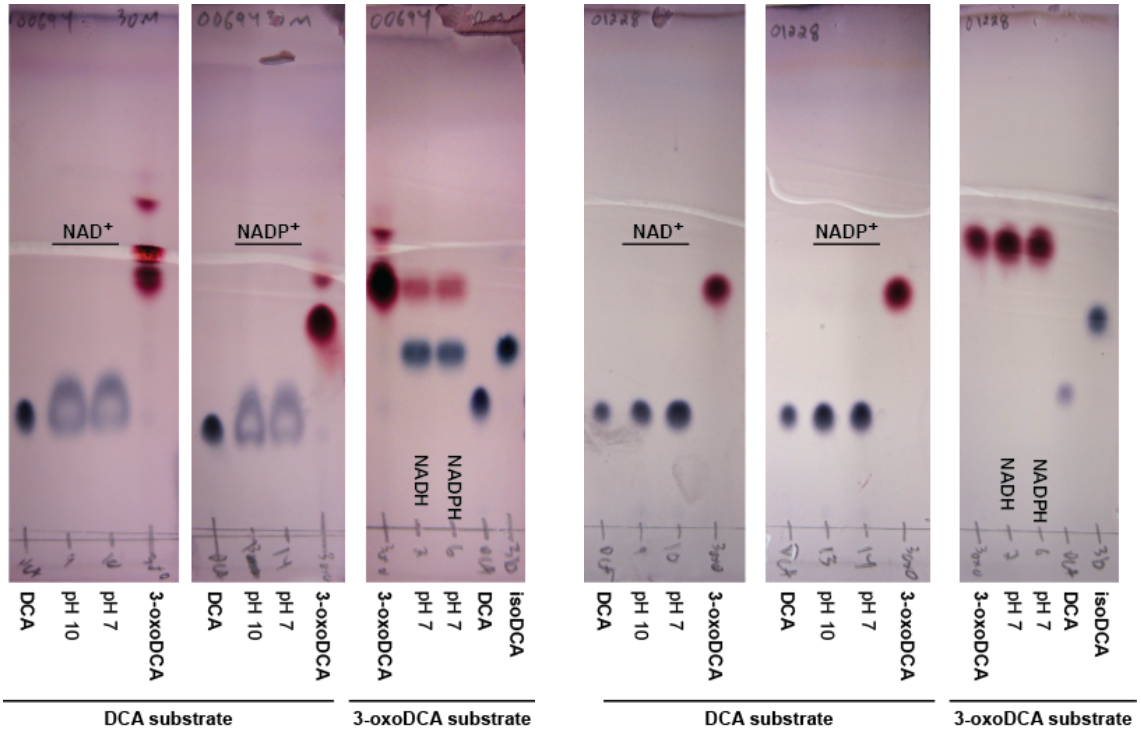
d. Elen_1325



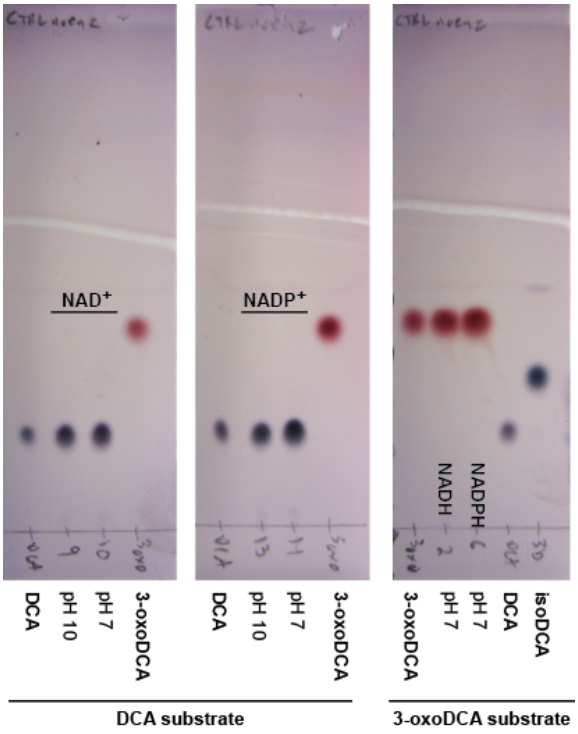
Supplementary Figure 6 (page 1 of 2)

e. Rumgna_00694

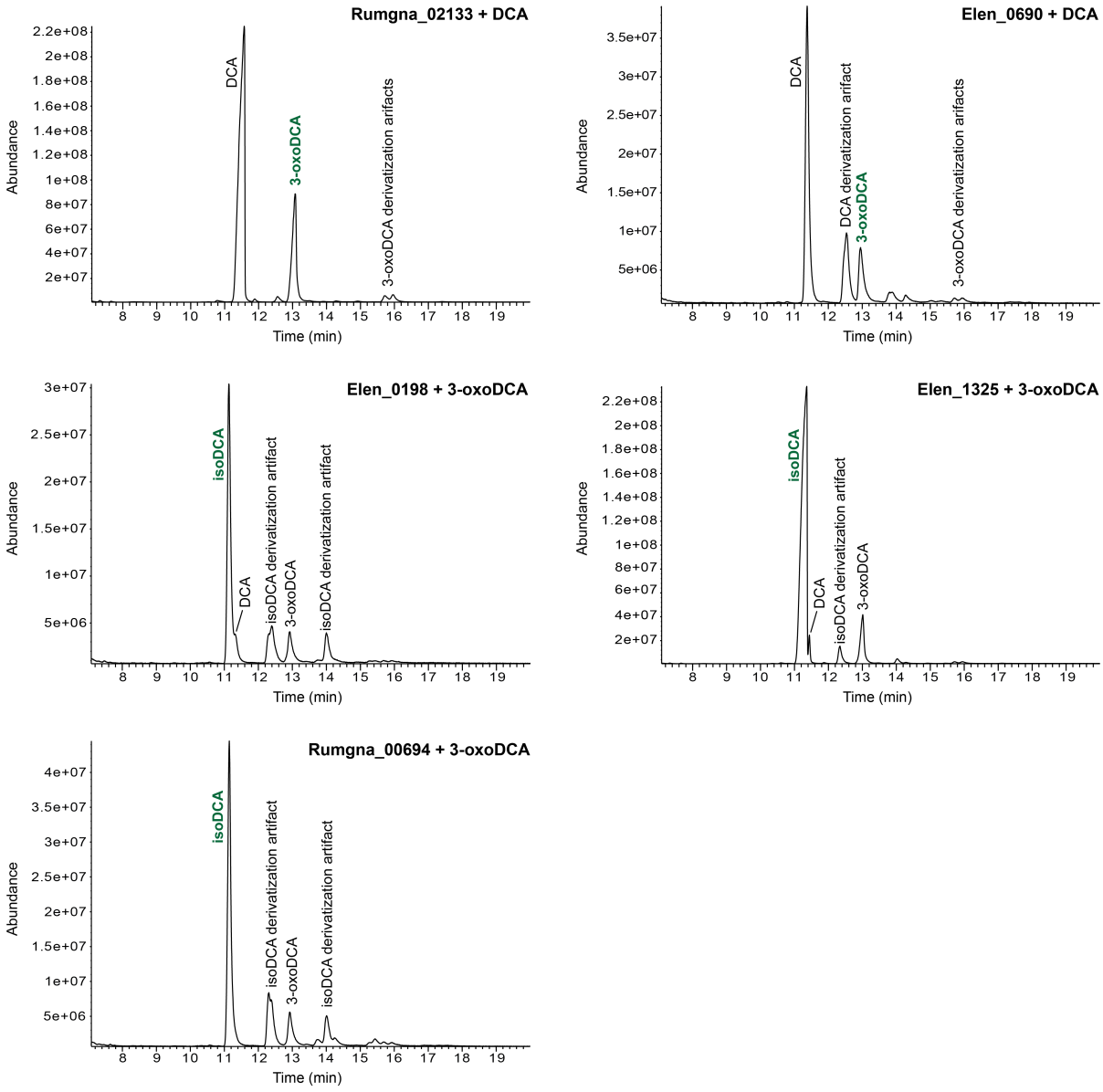
f. Rumgna_01228 [control]



g. No enzyme [control]

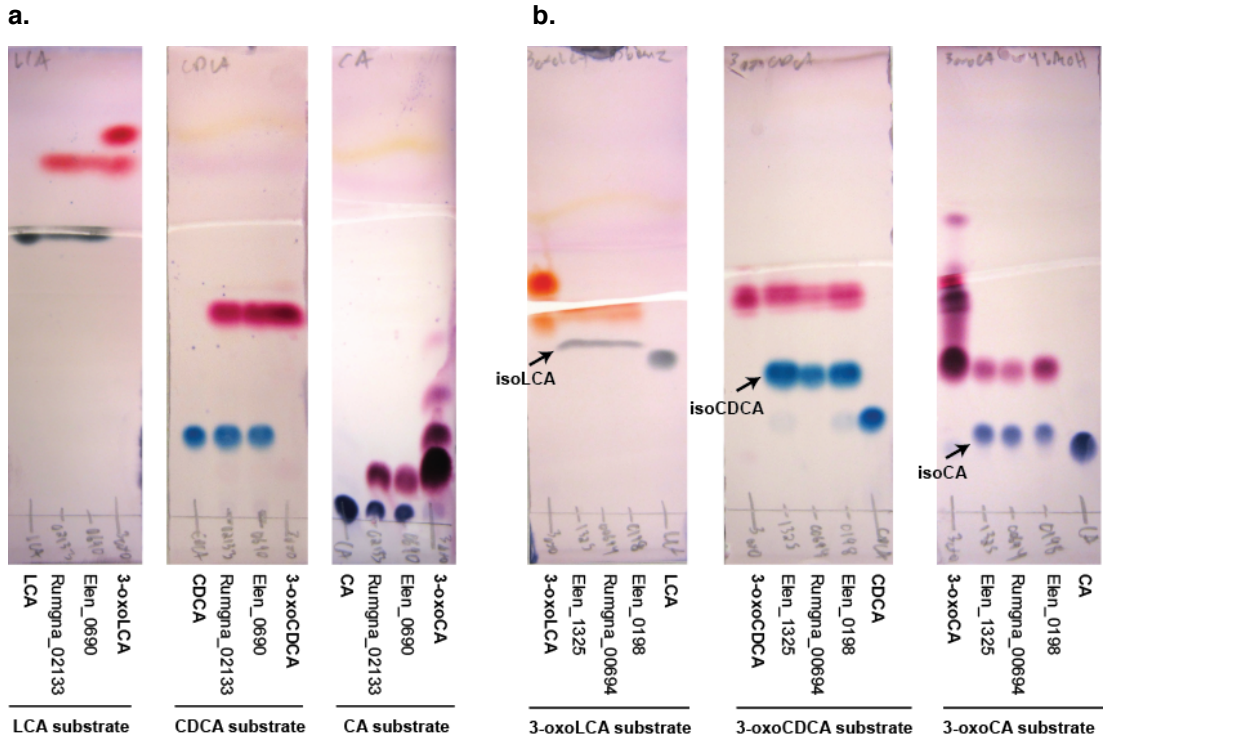


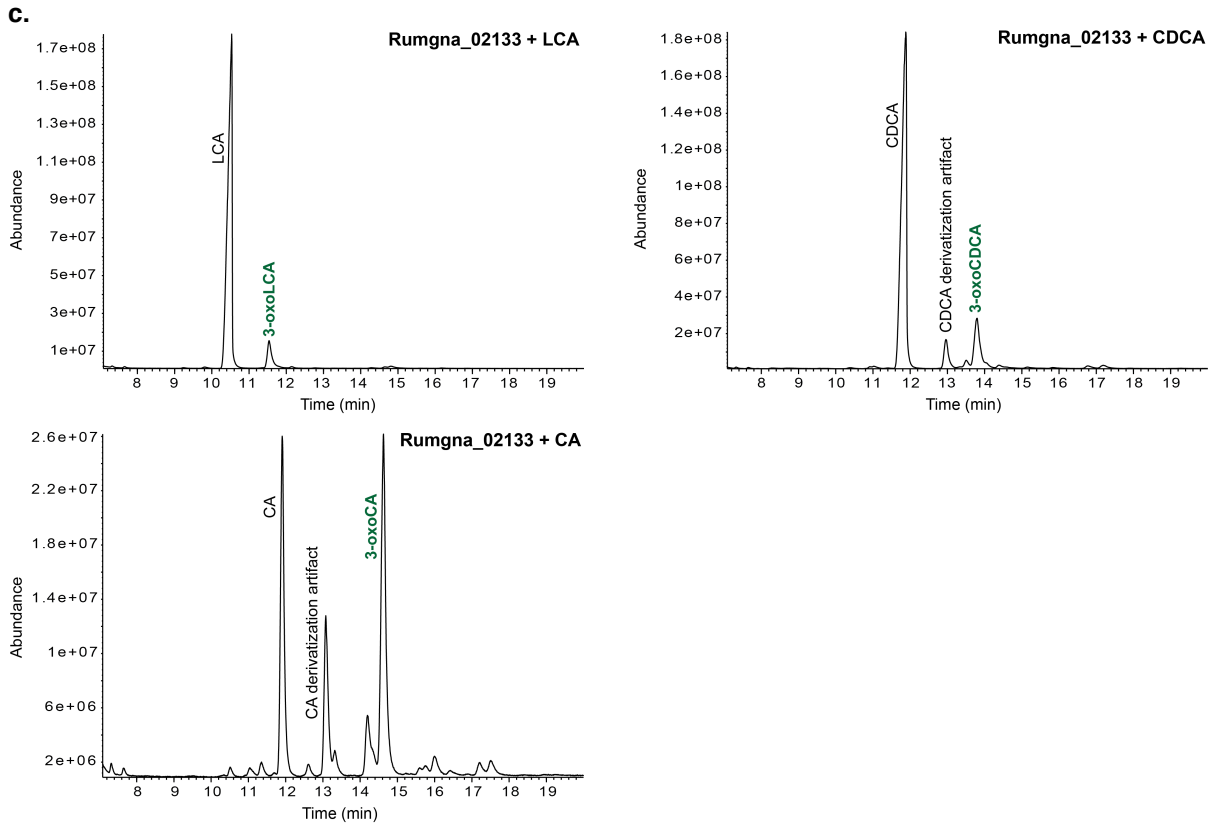
Supplementary Figure 6. Biochemical activity of Elen_0690, Rumgna_02133, Elen_0198, Elen_1325, and Rumgna_00694 with DCA and 3-oxoDCA as substrates: **TLC analysis**. Reactions were quenched by the addition of HCl after 30 min. (A) Rumgna_02133 is a 3α-HSDH, converts DCA into 3-oxoDCA, converts 3-oxoDCA into DCA, and accepts both NADH and NADPH as cofactors. Transformation of DCA is more efficient at pH 10 than pH 7. (B) Elen_0690 is a 3α-HSDH, converts DCA into 3-oxoDCA, converts 3-oxoDCA into DCA, and prefers NADH as cofactor. Transformation of DCA is more efficient at pH 10 than pH 7. (C) Elen_0198 is a 3β-HSDH, is unreactive toward DCA, converts 3-oxoDCA into isoDCA, and prefers NADH as cofactor. (D) Elen_1325 is a 3β-HSDH, is unreactive toward DCA, converts 3-oxoDCA into isoDCA, and accepts both NADH and NADPH as cofactors. (E) Rumgna_00694 is a 3β-HSDH, is unreactive toward DCA, converts 3-oxoDCA into isoDCA, and accepts both NADH and NADPH as cofactors. (F) and (G) Lack of transformation of either DCA or isoDCA in Rumgna_01228 and no enzyme controls confirmed that conversion to product was due to enzymatic activity.



Supplementary Figure 7. Biochemical activity of Elen_0690, Rumgna_02133, Elen_0198, Elen_1325, and Rumgna_00694 with DCA and 3-oxoDCA as substrates: **GC-MS confirmation**. GC-MS traces of purified TLC samples from Figure S6 showing conversion of DCA to 3-oxoDCA by Rumgna_02133 and Elen_0690 (pH 7) and conversion of 3-oxoDCA to isoDCA by Elen_0198, Elen_1325, and Rumgna_00694 (pH 10). The relative ratio of 3-oxoDCA to (iso)DCA in these traces is lower than the ratio apparent by TLC due to the difficulty of derivatizing 3-oxo bile acids for GC-MS analysis.[1]

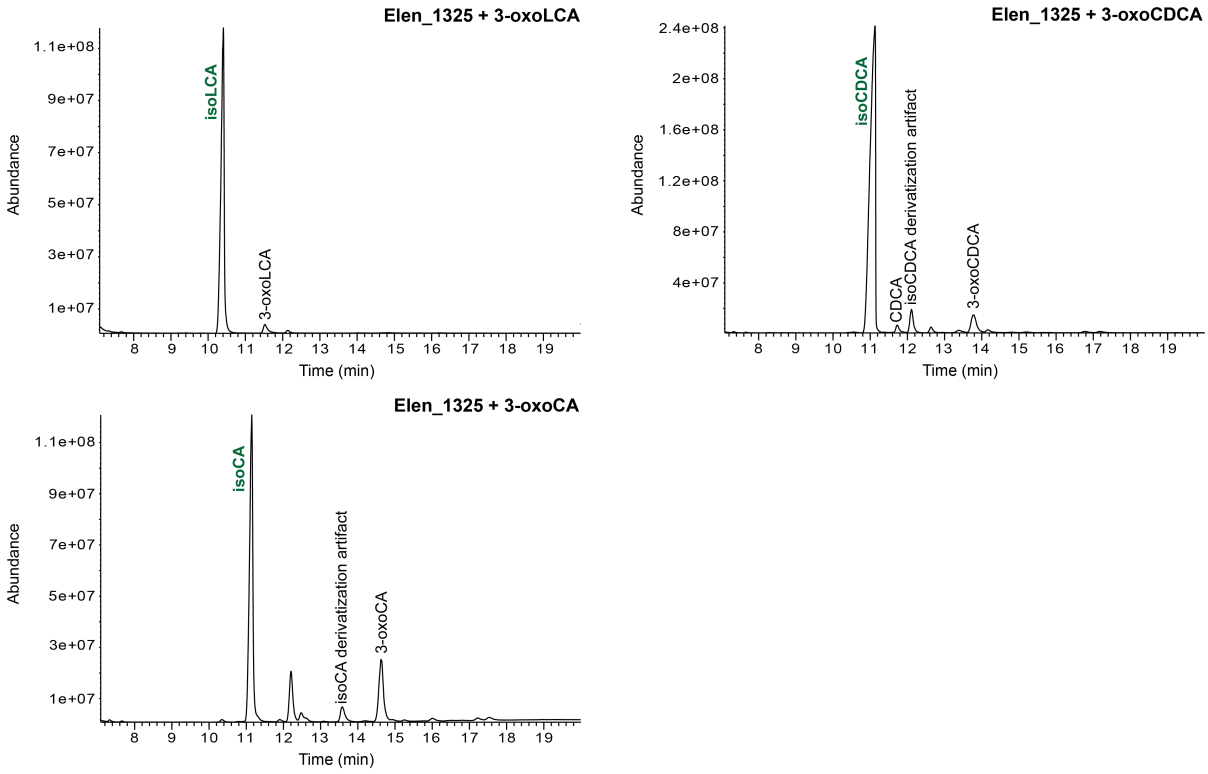
[1] Lawson, A. M. & Setchell, K. D. R. Mass spectrometry of bile acids. In *The Bile Acids: Chemistry, Physiology, and Metabolism* Vol. 4 (eds. Setchell, K. D. R., Kritchevsky, D. & Nair, P. P.) 167–267 (Plenum Press, New York, USA, 1988).





Supplementary Figure 8 (page 1 of 3)

d.



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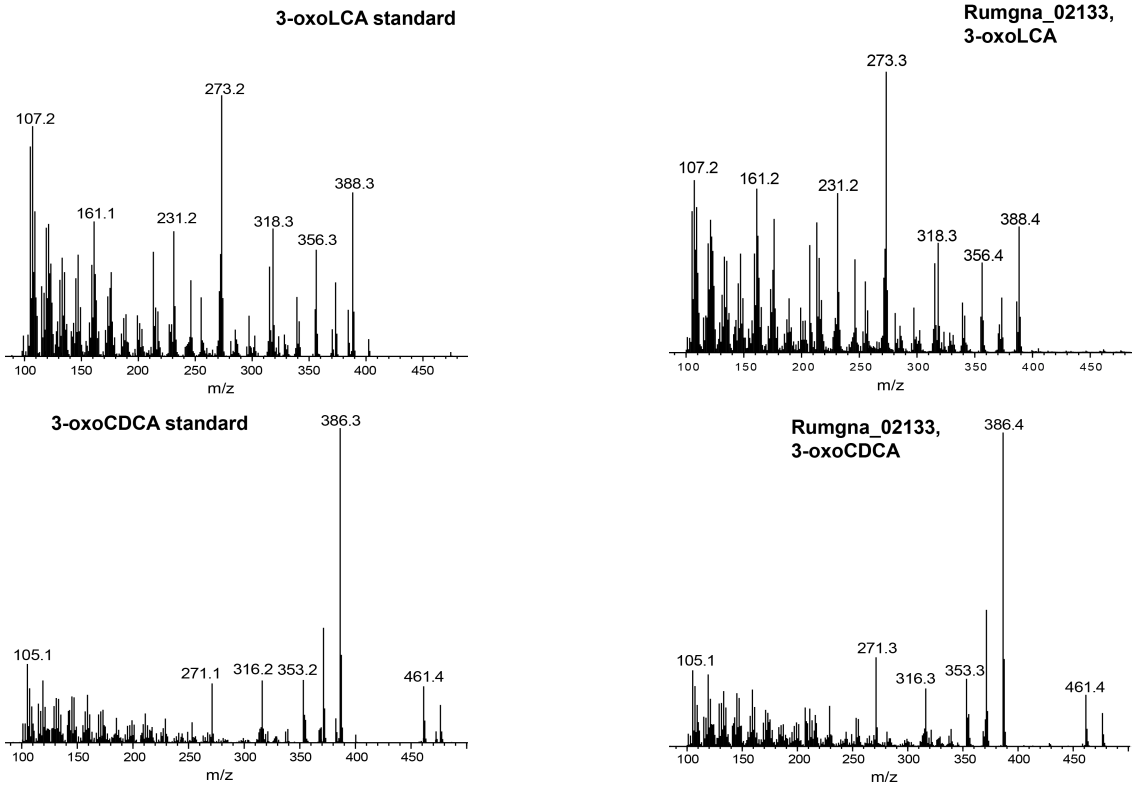
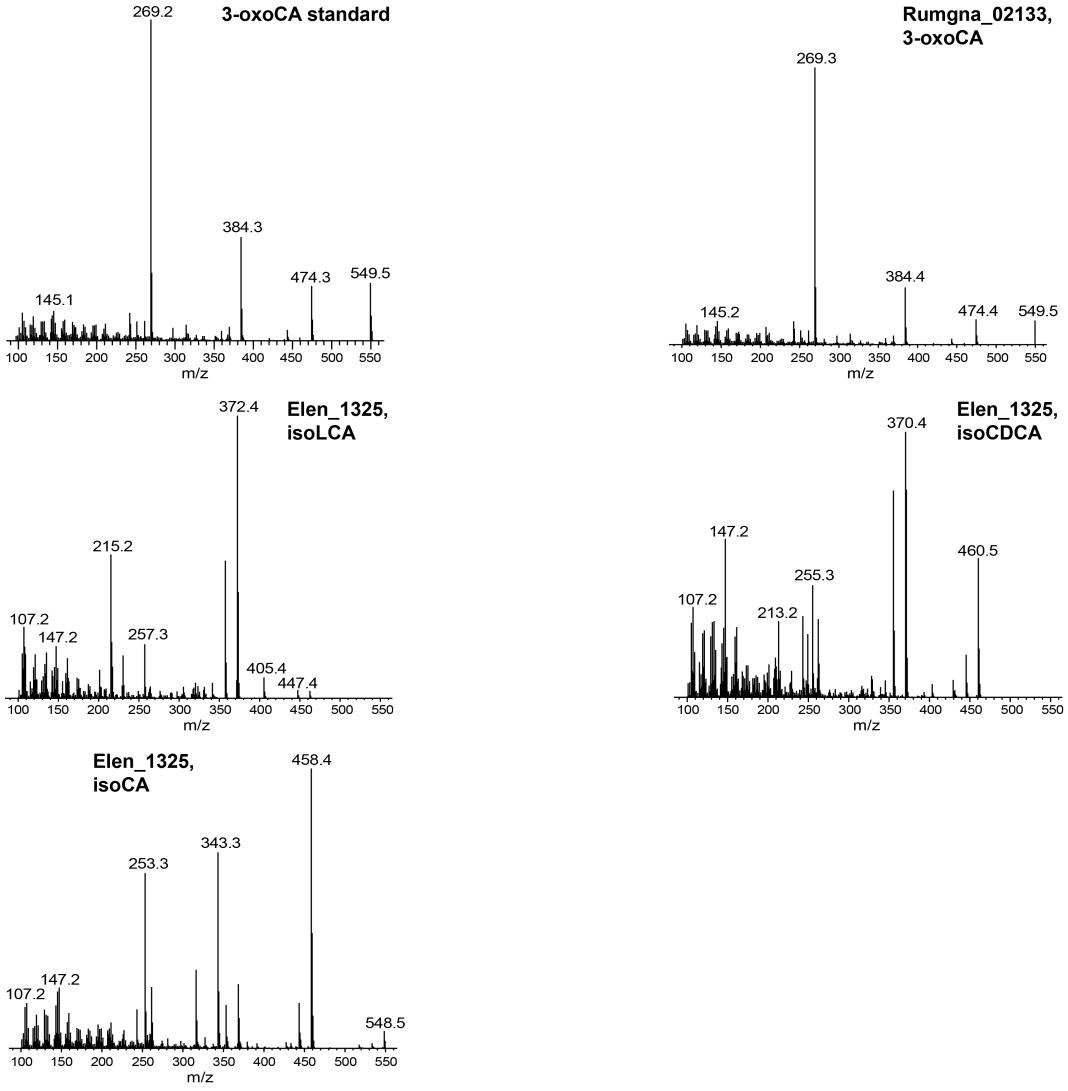
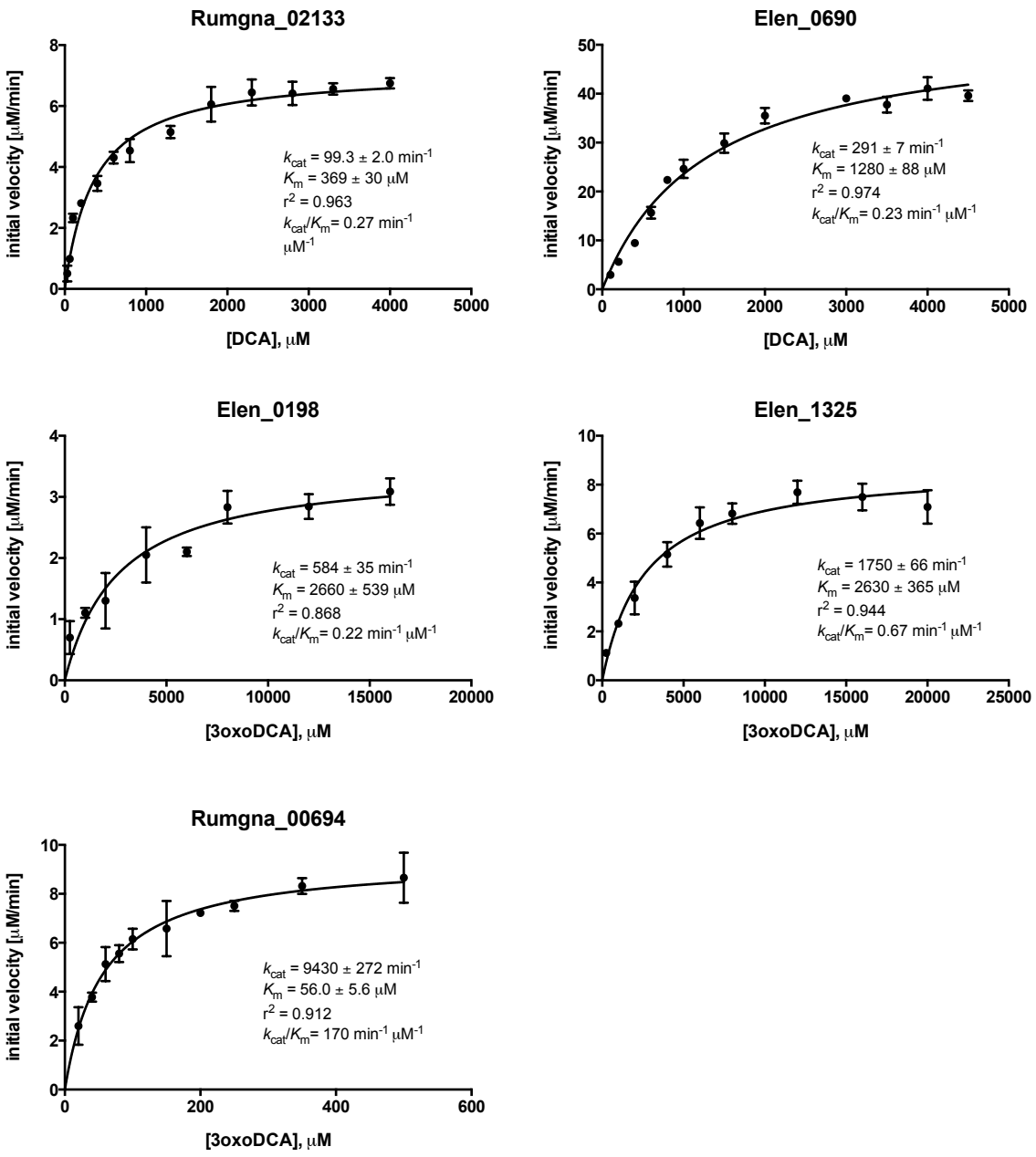


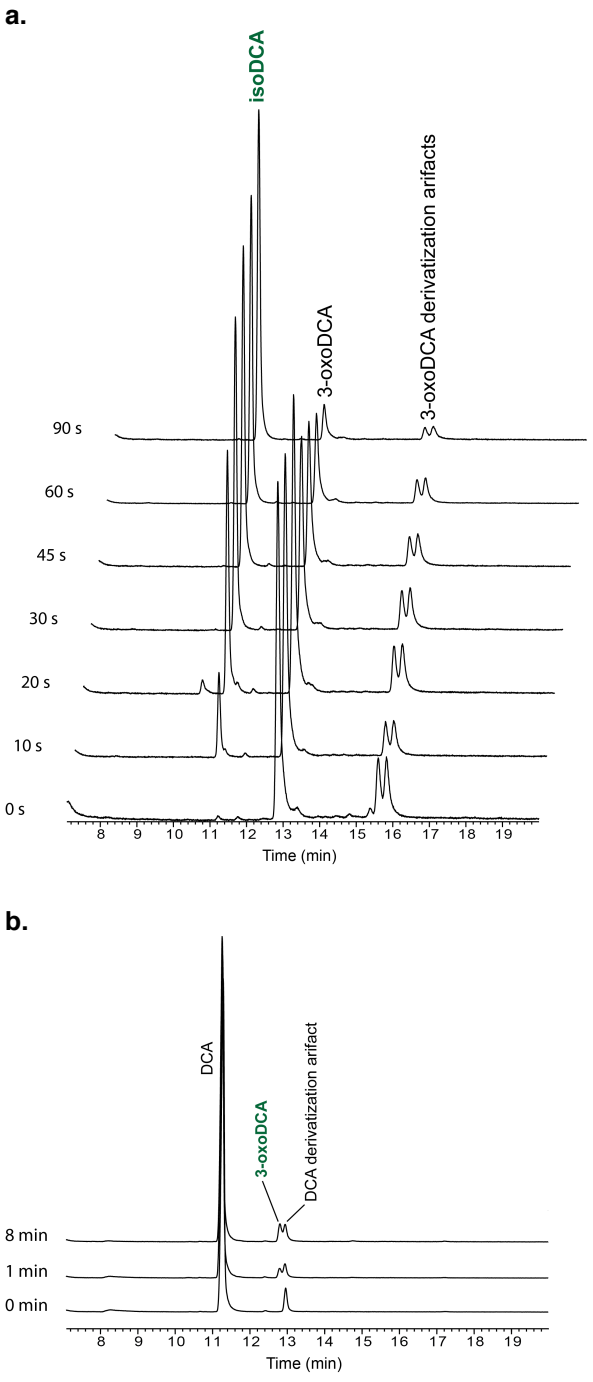
Supplementary Figure 8 (page 2 of 3)



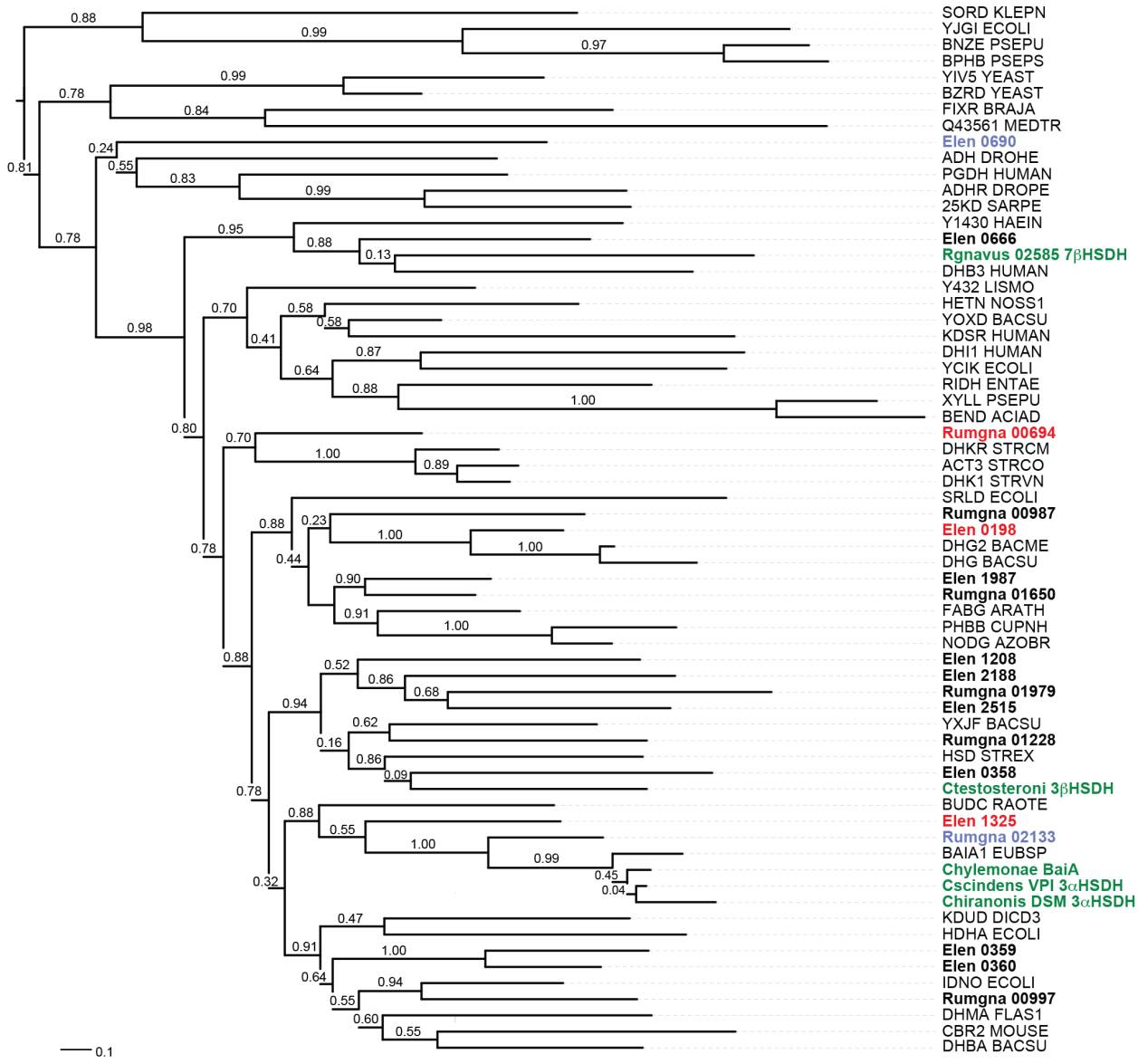
Supplementary Figure 8. Biochemical activity of Elen_0690, Rumgna_02133, Elen_0198, Elen_1325, and Rumgna_00694 with alternative bile acid substrates. TLC analysis showing conversion of (**a**) LCA, CDCA, and CA into 3-oxoLCA, 3-oxoCDCA, and 3-oxoCA, respectively, by Rumgna_02133 and Elen_0690 and (**b**) 3-oxoLCA, 3-oxoCDCA, and 3-oxoCA into isoLCA, isoCDCA, and isoCA, respectively, by Elen_1325, Rumgna_00694, and Elen_0198. The 3-oxoCA substrate TLC plate was run at a higher polarity (70:20:4 benzene/dioxane/AcOH) to obtain better separation of the isoCA product from the CA standard. These results were confirmed by GC-MS, and representative traces for (**c**) one 3α-HSDH (Rumgna_02133) and (**d**) one 3β-HSDH are shown. (**e**) Mass spectral fragmentation patterns of 3-oxoLCA, 3-oxoCDCA, and 3-oxoCA standards and in experimental samples (retention times 11.6, 13.8 min, and 14.7 min, respectively); isoLCA, isoCDCA, and isoCA in experimental samples (retention times 10.4, 11.0, and 11.1 min, respectively) (identities determined as described in the Methods section).



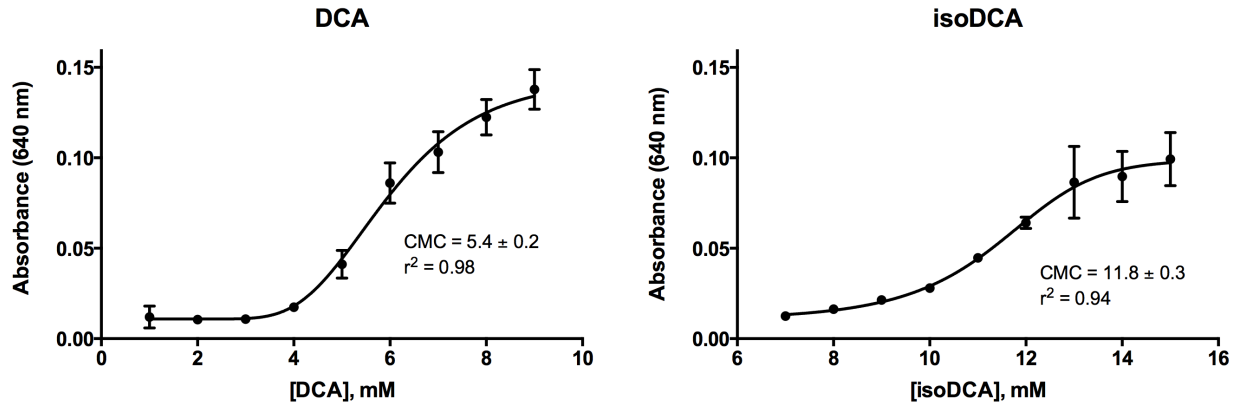
Supplementary Figure 9. **Michaelis-Menten analysis of kinetic data**. Rate vs substrate concentration curves for Rumgna_02133 (72.4 nM, DCA substrate, n=4 experiments), Elen_0690 (185 nM, DCA substrate, n=5), Elen_0198 (6 nM, 3-oxoDCA substrate, n=4), Elen_1325 (5 nM, 3-oxoDCA substrate, n=3), and Rumgna_00694 (1 nM, 3-oxoDCA substrate, n=4). Each data point for all five enzymes was obtained under initial velocity conditions in which less than 10% starting material was consumed. Graphpad was used to fit the Michaelis-Menten equations. Note: previously reported oxidative HSDH kinetic parameters have been determined at the optimal pH of the enzymes (8.5–10), as the reaction rate was observed to decrease significantly at lower pH. We observed similar pH-dependent behavior for DCA oxidation by Rumgna_02133 and Elen_0690. As a result, because all kinetic experiments herein were performed at physiological pH (7.0), lower k_{cat} values were obtained for these two enzymes than typical literature values for similar enzymes.



Supplementary Figure 10. Representative time courses for 3α-HSDH and 3β-HSDH activity. Purified protein was added to a solution of 2.5 mL of buffer, substrate, and cofactor at 37 ˚C. Aliquots of 250 μL were removed and quenched with 250 μL 6N HCl. Samples were purified and analyzed by GC-MS as described in the Materials and Methods. Aliquots for t=0 timepoints were removed immediately prior to addition of enzyme. Note that the relative amount of 3-oxoDCA in these traces is suppressed in comparison to (iso)DCA due to the difficulty of derivatizing 3-oxo bile acids. (**a**) 10 nM Rumgna_00694, 10 mM MOPS buffer, pH 7, 3-oxoDCA substrate, 400 μM final concentration, NADPH, 400 μM final concentration. (**b**) 100 nM Elen_0690, 0.2N glycine-NaOH buffer, pH 10, DCA, 400 μM final concentration, NAD^+, 400 μM final concentration.



Supplementary Figure 11. Phylogenetic tree of candidate and query HSDH genes. Numbers next to the branches represent the percentage of replicate trees in which this topology was reached in a bootstrap test of 1,000 replicates. Green text indicates query genes from the BLASTP search, blue text indicates 3α-HSDHs identified from the cell lysate assay, red text indicates 3β-HSDHs, and bold black text indicates HSDH candidates that were not active in the cell lysate assay.



Curves fit to the following 5-parameter logistic growth equation:

$$y = F + \frac{D - F}{(1 + 10^{(G-x)B})^C}$$

where $G = A + \frac{1}{B}\log_{10}(2^{1/C} - 1)$

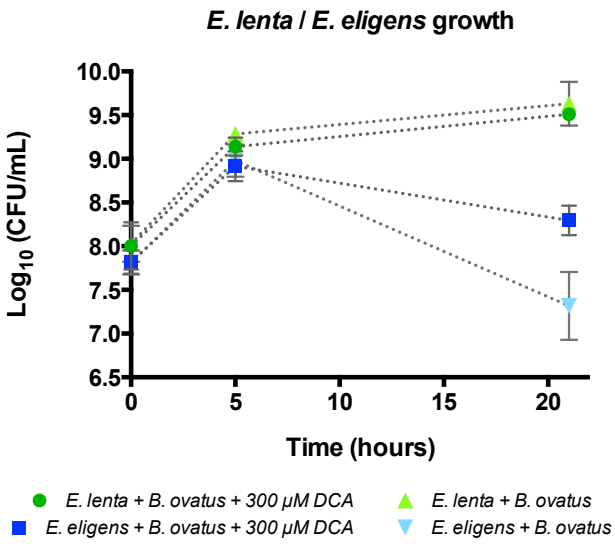
Inflection point = $A + \frac{1}{B}\log_{10}((2^{1/C} - 1)C)$

Curve parameters	DCA curve	isoDCA curve
A	5.9	11.5
B	0.33	0.54
C	1.1 x 10^6	0.54
D	0.14	0.10
F	0.011	0.011
Inflection point	5.4 ± 0.2	11.8 ± 0.3

Supplementary Figure 12. Critical micelle concentrations (CMCs) of DCA and isoDCA.
The critical micelle concentrations of DCA and isoDCA were determined to be ~ 5 mM and 12 mM, respectively. Absorbance data from three independent experiments was plotted versus detergent concentration for each compound. A standard five-parameter logistic curve fit was performed using Graphpad, and the critical micelle concentration was determined by computing the associated inflection point of each curve.[1] Inflection point values represent mean ± s.e.m.

[1] Gottschalk, P. G. & Dunn, J. R. The five-parameter logistic: a characterization and comparison with the four-parameter logistic. *Anal. Biochem.* **343**, 54–56 (2005).

a.



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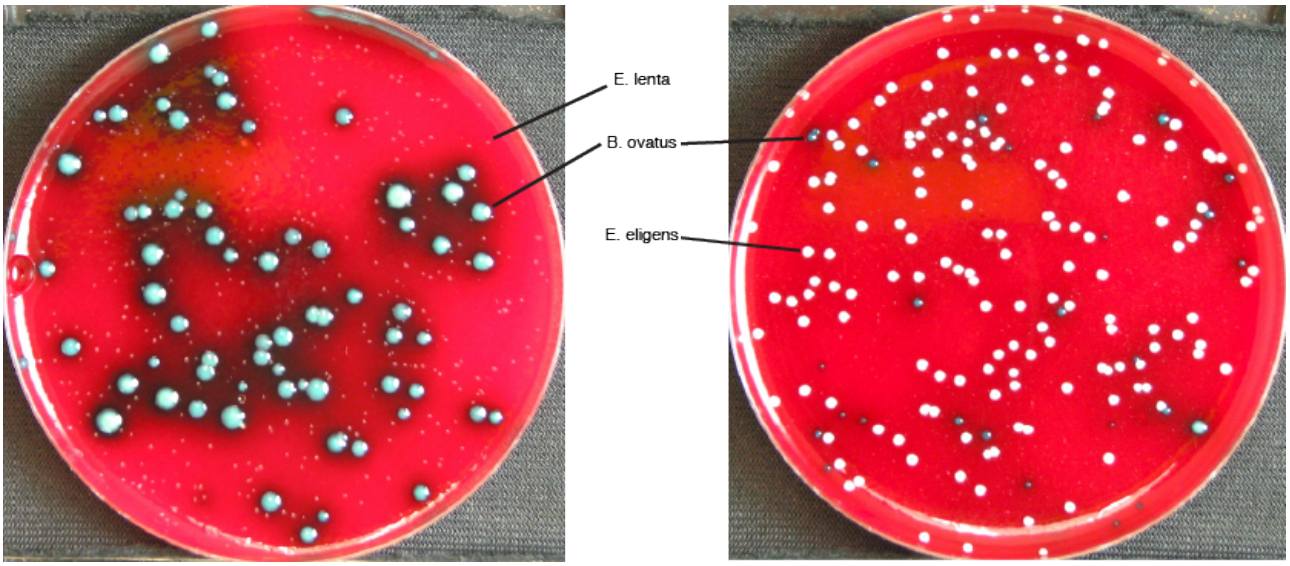
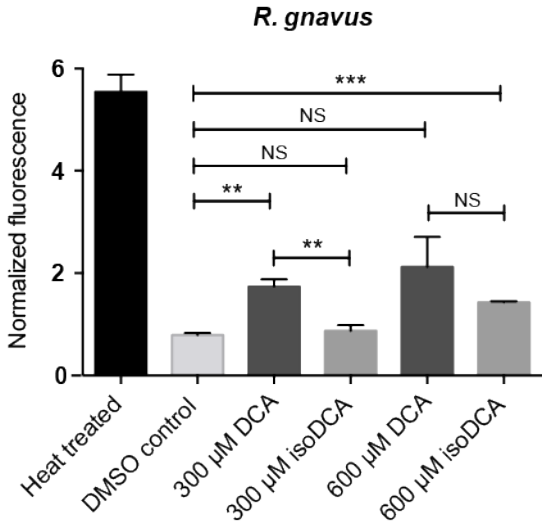


Supplementary Figure 13. Co-culture experiment.
(**a**) Growth of either the isoDCA producer *Eggerthella lenta* or the common gut commensal and non-producer *Eubacterium eligens* in co-culture with *Bacteroides ovatus* with or without 300 μM DCA (starting concentration). *E. lenta* growth (green triangles, green circles) in *E. lenta* + *B. ovatus* co-cultures is largely unaffected by the addition of DCA, while *E. eligens* grows more robustly (blue squares, blue triangles) in the presence of DCA, likely due to the growth inhibition of *B. ovatus* by DCA.. Data shown are from three biological replicates. Error bars represent standard deviation. (**b**) Representative examples of BHI + 10% horse blood + X-gal plates showing the difference between translucent *E. lenta* colonies (left), white *E. eligens* colonies (right), and blue *B. ovatus* colonies (both).



Supplementary Figure 14. Effect of DCA and isoDCA on R. gnavus membrane integrity.
Cell membrane damage caused by exposure of actively dividing *R. gnavus* cells to either DCA or isoDCA was assessed using a propidium iodide (Pi) fluorescence assay. At ½ MIC (300 μM), DCA caused significantly more fluorescence (indicating membrane damage) than isoDCA (P = 0.0016), which was comparable to a DMSO control. Importantly, 300 μM DCA falls within the range of physiologically relevant DCA concentrations. (DMSO vs. 300 μM DCA, P = 0.0046; DMSO vs. 600 μM isoDCA, P = 0.0001). Error bars represent standard deviation. Asterisks represent significant differences between groups, Welch's t test, n = 3 per group.

Supplementary Table 1. Summary of bacterial strains.

Bacterial strain	Source	Growth Medium	Assay
Clostridium innocuum ATCC 14501	ATCC	RCM	Whole cell screen[a]
Clostridium paraputrificum ATCC 25780	ATCC	RCM	Whole cell screen
Clostridium scindens VPI 12708	JCM	BHI-F	Whole cell screen
Clostridium hiranonis DSM-13275	DSMZ	BHI-F	Whole cell screen
Clostridium hylemonae DSM-15053	DSMZ	BHI-F	Whole cell screen
Blautia producta ATCC 27340	ATCC	EG	Whole cell screen
Lachnospiraceae sp. 1_4_56FAA[1]	BEI	EG	Whole cell screen
Lachnospiraceae sp. 2_1_58FAA[2]	BEI	PYF	Whole cell screen
Eubacterium dolichum ATCC 29143	ATCC	EG	Whole cell screen
Eubacterium eligens ATCC 27750	ATCC	PYF	Whole cell screen, Co-culture[b]
Ruminococcus gnavus ATCC 29149	ATCC	PY-F, BHI-F	Whole cell screen, Growth inhibition[c], Cell membrane integrity[d]
Eggerthella lenta DSM-2243	DMSZ	BHI-F + 1% Arg w/v[3] + 0.5 mg/mL Cys-HCl	Whole cell screen, Growth inhibition, Co-culture
Clostridium sporogenes ATCC 3584	ATCC	BHI-F	Whole cell screen, Growth inhibition
Enterococcus faecalis ATCC 70082	ATCC	BHI-F	Growth inhibition
Bacteroides thetaiotaomicron ATCC 29148	ATCC	TYG or BHI-F	Growth inhibition
Bacteroides vulgatus ATCC 8482	ATCC	TYG or BHI-F	Growth inhibition
Bacteroides ovatus ATCC 8483	ATCC	TYG or BHI-F	Growth inhibition, Cell membrane integrity, Co-culture
Bacteroides uniformis ATCC 8492	ATCC	TYG or BHI-F	Growth inhibition

[a]Whole cell screen for bile acid metabolism by gut commensal bacteria.
[b]Co-culture of *B. ovatus* with *E. lenta* or *E. eligens* with or without DCA.
[c]Determination of minimal inhibitory concentrations (MICs) for DCA and isoDCA; Growth curves of *B. ovatus* at sub-minimal inhibitory concentrations of DCA and isoDCA.
[d]Effect of DCA and isoDCA on cell membrane integrity.

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[1] This reagent was obtained through BEI Resources, NIAID, NIH as part of the Human Microbiome Project: *Lachnospiraceae* sp., Strain 1_4_56FAA, HM-161.

[2] This reagent was obtained through BEI Resources, NIAID, NIH as part of the Human Microbiome Project: *Lachnospiraceae* sp., Strain 2_1_58FAA, HM-154.

[3] Arginine (at least 0.5%) is necessary for robust growth. Macdonald, I.A., Mahony, D.E., Jellet, J.F. & Meier, C.E. NAD-dependent 3α- and 12 α-hydroxysteroid dehydrogenase activities from *Eubacterium lentum* ATCC No. 25559. *Biochim. Biophys. Acta* **489**, 466–476 (1977).

Supplementary Table 2. Primers for cloning candidate 3α- and 3β-HSDHs from genomic DNA.

Gene	Forward (F) Primer (5' to 3')	Reverse (R) Primer (5' to 3')	Restriction Enzymes
Elen_0358	CCTGGTGCCGCGCGGCAGCCATATGAAGTCAAAGCGAGCAGAGTT	GGTGCTCGAGTGCGGCCGCAAGCTTTTACTTTGCGTCGGCCGTC	*Nde*I (F), *Hind*III Hf (R)
Elen_0359	CCTGGTGCCGCGCGGCAGCCATATGGCTCGGTTCGATAACAAGATC	GGTGCTCGAGTGCGGCCGCAAGCTTCTAGAGGCTCTGCCCACCATC	*Nde*I (F), *Hind*III Hf (R)
Elen_0360	CCTGGTGCCGCGCGGCAGCCATATGGAAAGAGAGCGGTTTGTTGAC	GGTGCTCGAGTGCGGCCGCAAGCTTCTAGGTCAAGCTGAAACCGCC	*Nde*I (F), *Hind*III Hf (R)
Elen_1987	CCTGGTGCCGCGCGGCAGCCATATGACCAACGAAACCGAAACTCC	GGTGCTCGAGTGCGGCCGCAAGCTTTCATAGCGACATACCTCCGTCG	*Nde*I (F), *Hind*III Hf (R)
Elen_0198	CCTGGTGCCGCGCGGCAGCCATATGTACGACGATCTCAAGGGCAAG	GGTGCTCGAGTGCGGCCGCAAGCTTCTAGCCCTGCCCGAACTGG	*Nde*I (F), *Hind*III Hf (R)
Elen_1325	CCTGGTGCCGCGCGGCAGCCATATGATGTCAGAAGCACGCCACAAC	GGTGCTCGAGTGCGGCCGCAAGCTTCTAGTACGCGCTCCAGCCG	*Nde*I (F), *Hind*III Hf (R)
Elen_2188	CCTGGTGCCGCGCGGCAGCCATATGTCGGAACTCGTAGCATTGG	GGTGCTCGAGTGCGGCCGCAAGCTTCTACCCGTTCACCACGGTG	*Nde*I (F), *Hind*III Hf (R)
Elen_2515	CCTGGTGCCGCGCGGCAGCCATATGGACATGGGCTTGAAGGAC	GGTGCTCGAGTGCGGCCGCAAGCTTTTACGGCTTCATGCCGGAAC	*Nde*I (F), *Hind*III Hf (R)
Elen_1208	CCTGGTGCCGCGCGGCAGCCATATGGTCGATTACGGATTGGACG	GGTGCTCGAGTGCGGCCGCAAGCTTCTACATCGTGTCCCAGGGGT	*Nde*I (F), *Hind*III Hf (R)
Elen_0690	CCTGGTGCCGCGCGGCAGCCATATGGGCATCTACGTCATCACC	GGTGCTCGAGTGCGGCCGCAAGCTTCTAGTAGACTTTCTCGGAATTGAGCAG	*Nde*I (F), *Hind*III Hf (R)
Rumgna_00694	CCTGGTGCCGCGCGGCAGCCATATGAATTTTGGAGGTTTTATTATGGGACG	GGTGCTCGAGTGCGGCCGCAAGCTTTTAGTATGTAGTAAATCCACCATCACTTG	*Nde*I (F), *Hind*III Hf (R)
Rumgna_02133	CCTGGTGCCGCGCGGCAGCCATATGTTTATGATGTTAAAAAATAAAGTAGCAATTG	GGTGCTCGAGTGCGGCCGCAAGCTTCTAGCTTCTTGCAGCTCCATC	*Nde*I (F), *Hind*III Hf (R)
Rumgna_00997	TGCCGCGCGGCAGCCATATGGCTAGCATGGTAAATTTTTCACTGGAAGGTAAAATTG	GGTGCTCGAGTGCGGCCGCAAGCTTTTATGGCTGTTTTCCAATGTATGCC	*Nhe*I Hf (F), *Hind*III Hf (R)
Rumgna_01979	CCTGGTGCCGCGCGGCAGCCATATGTTTCACAACAAAGTAGTTGTCATC	GTGGTGGTGCTCGAGTGCGGCCGCTCAATTATTTTCCTGATTCAGAGTCCATC	*Nde*I (F), *Not*I Hf (R)
Rumgna_01228	TGCCGCGCGGCAGCCATATGGCTAGCATGACATTTGAAGGAAAAG TTGTGGCAG	GTGGTGGTGCTCGAGTGCGGCCGCTTAGCCAAGTGTCATTCCGCCATC	*Nhe*I Hf (F), *Not*I Hf (R)
Elen_0666	CCTGGTGCCGCGCGGCAGCCATATGGCACAAACCGCATTGGTC	GTGGTGGTGCTCGAGTGCGGCCGCCTACCAGCGATCCCAGCTGATG	*Nde*I (F), *Not*I Hf (R)
Rumgna_00987	CCTGGTGCCGCGCGGCAGCCATATGGAGTATGTTATGAACCAGAAATATG	GTGGTGGTGCTCGAGTGCGGCCGCTTAAATATAACCGCCGTCCAGG	*Nde*I (F), *Not*I Hf (R)

Supplementary Table 3. Assay conditions for determination of kinetic parametics for 3α- and 3β-HSDHs.

Enzyme (concentration)	Substrate (concentration range)	Cofactor (concentration)
Rumgna_02133 (72.4 nM)	DCA (30 – 4,000 μM)	$NADP^+$ (10 mM)
Elen_0690 (185 nM)	DCA (100 – 4,500 μM)	NAD^+ (6 mM)
Rumgna_00694 (1 nM)	3-oxoDCA (20 – 500 μM)	NADPH (1 mM)
Elen_0198 (6 nM)	3-oxoDCA (250 – 16,000 μM)	NADH (1 mM)
Elen_1325 (5 nM)	3-oxoDCA (250 – 20,000 μM)	NADH (1 mM)

Supplementary Table 4. Assay conditions for determination of minimal inhibitory concentrations (MICs).

Strains	**Compound**	**Concentrations**
Bacteroides spp.	DCA	50 μM, 100 μM, 150 μM, 200 μM, 250 μM, 300 μM, 350 μM, 400 μM, 450 μM
Bacteroides spp.	isoDCA	75 μM, 125 μM, 250 μM, 500 μM, 750 μM, 1 mM, 1.5 mM
E. faecalis, *C. sporogenes, R. gnavus, E. lenta*	DCA	300 μM, 400 μM, 500 μM, 600 μM, 700 μM, 800 μM, 900 μM, 1 mM
E. faecalis, C. sporogenes, R. gnavus, E. lenta	isoDCA	500 μM, 1 mM, 1.5 mM, 2 mM