Supplemental material

6-Hydroxypseudooxynicotine dehydrogenase delivers electrons to electron transfer flavoprotein during nicotine degradation by *Agrobacterium tumefaciens* S33*

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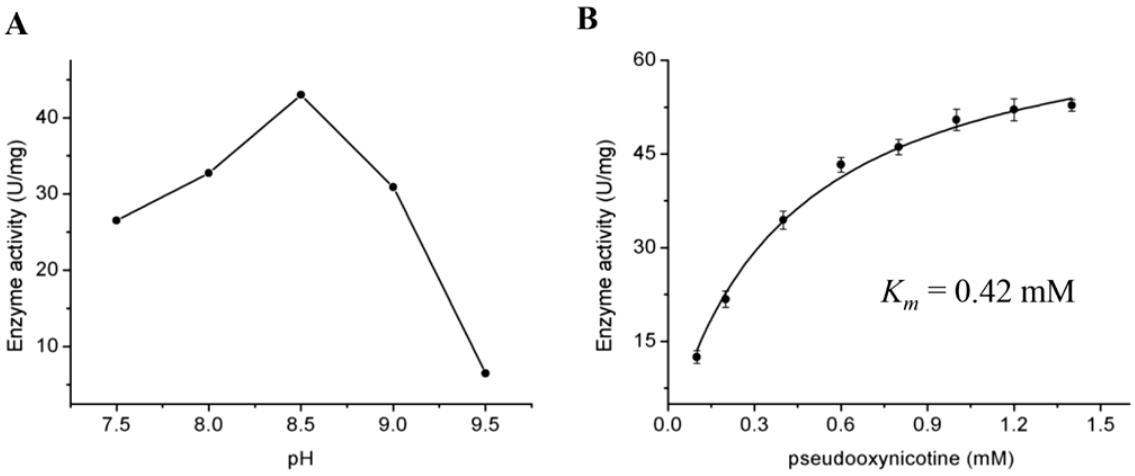


FIG S1 Kinetics of pseudooxynicotine dehydrogenation catalyzed by purified Pno with DCPIP (plus PMS) as artificial electron acceptor. (A) Effects of pH on the activity of Pno. (B) Determination of the apparent *Km* of Pno for pseudooxynicotine.

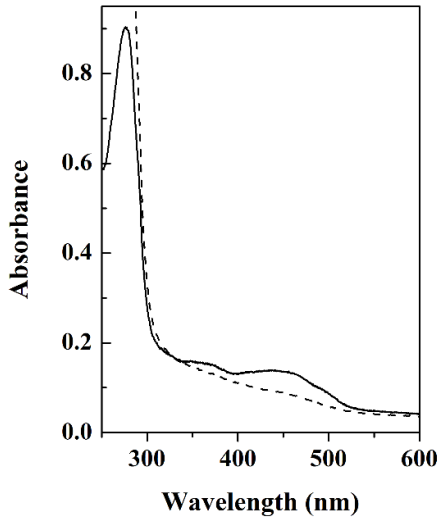
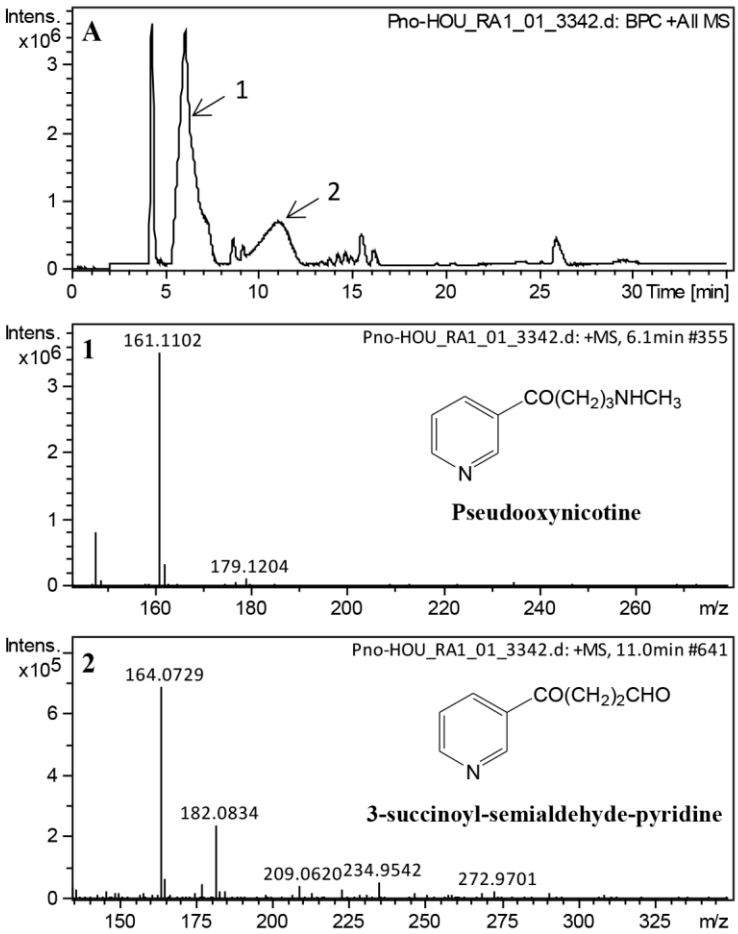


FIG S2 UV–visible absorption spectra of purified Pno as isolated (solid line) and reduced by pseudooxynicotine (dashed line). The sample contained 6 μM purified protein in 50 mM phosphate buffer (pH 7.0). Pseudooxynicotine was added to 1 mM. The light path of the cuvette is 3 mm.



31

32 **FIG S3** LC-MS profiles of the reaction catalyzed by Pno with pseudooxynicotine as
33 substrate. (A) HPLC profile of the products monitoring at 260 nm. (1) Mass spectra of
34 the remaining substrate pseudooxynicotine (*m/z*, 179.1204) and its dehydrated product
35 *N*-methylmyosmine (*m/z*, 161.1102). There is a spontaneous hydrolysis equilibrium
36 between pseudooxynicotine and *N*-methylmyosmine. (2) Mass spectra of the reaction
37 product, 3-succinoyl-semialdehyde-pyridine (*m/z*, 164.0729) and its adduct of water (*m/z*,
38 182.0834).

39

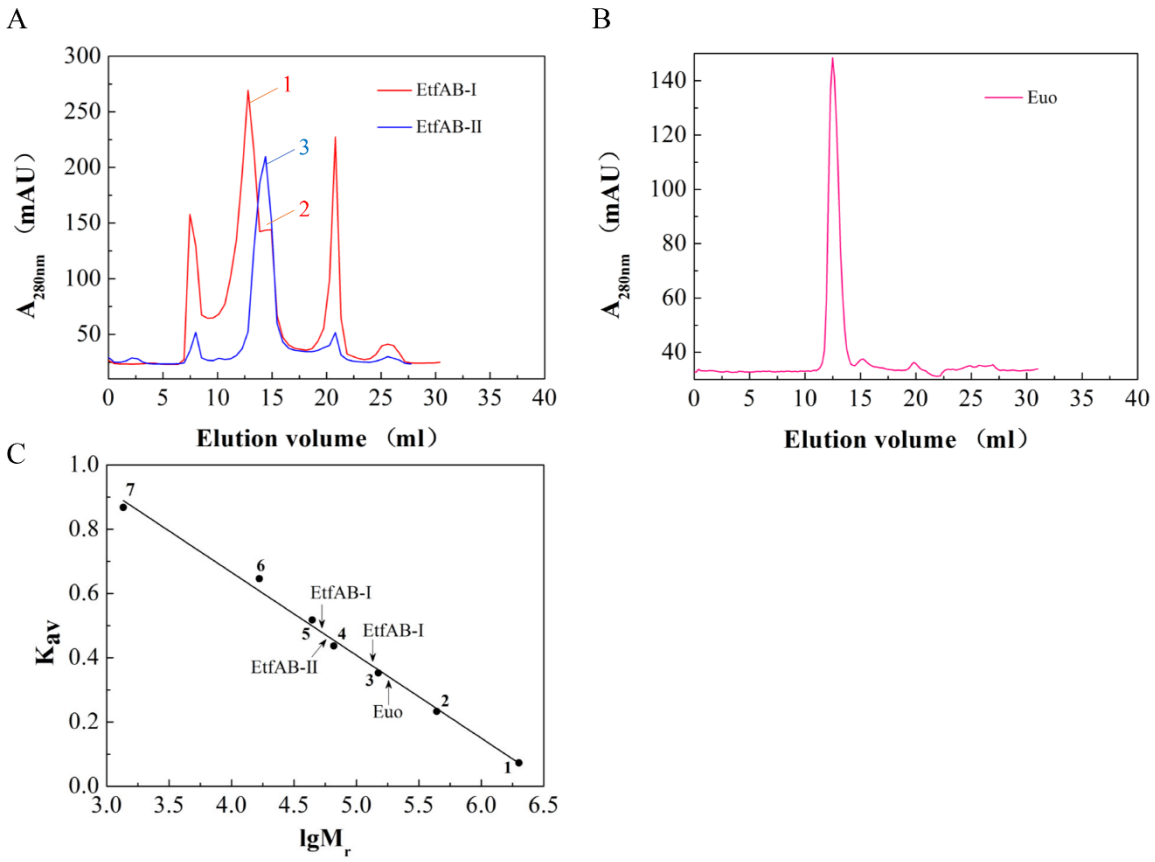


FIG S4 Determination of native molecular masses of EtfAB-I (A), EtfAB-II (A), and Euo (B) by gel filtration on a GE Superdex G200 column (10 mm×300 mm). The buffer used was 50 mM Tris-HCl containing 150 mM NaCl (pH 7.4), and the flow rate was 0.5 ml·min^{-1}. (A) peak 1 and peak 2 in red color are EtfAB-I; peak 3 in blue color is EtfAB-II, other peaks unlabeled are contaminants. (C) Molecular mass calibration curve. 1, Dextran Blue 2000 (2000 kDa); 2, Ferritin from equine spleen (440 kDa); 3, γ-Globulins from bovine blood (150 kDa); 4, Bovine serum albumin (66 kDa); 5, Albumin from chicken egg white (44.3 kDa); 6, Myoglobin (16.7 kDa); and 7, Vitamin B12 (1,360 Da).

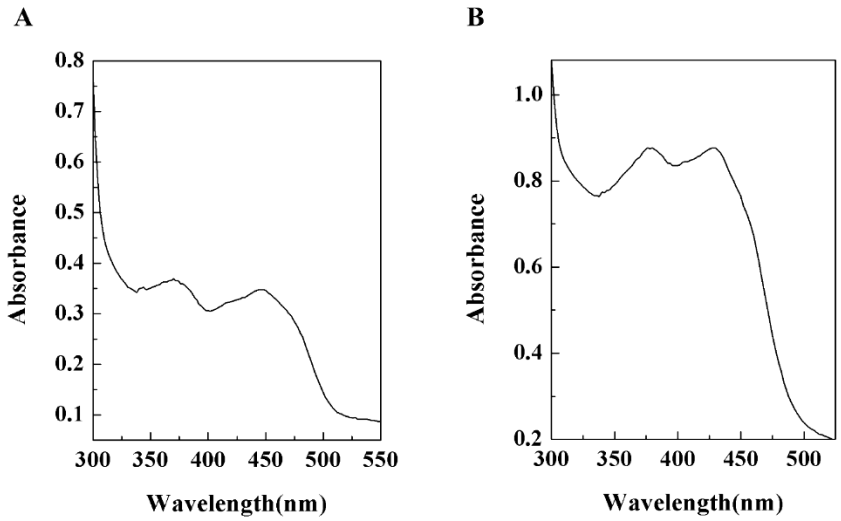


FIG S5 UV–visible absorption spectra of purified EtfAB (A) and Euo (B) as isolated. (A) The sample contained 105 μM EtfAB-I in 20 mM sodium phosphate buffer (pH 7.4). (B) The sample contained 38 μM Euo in 50 mM Tris-HCl (pH 8.5).

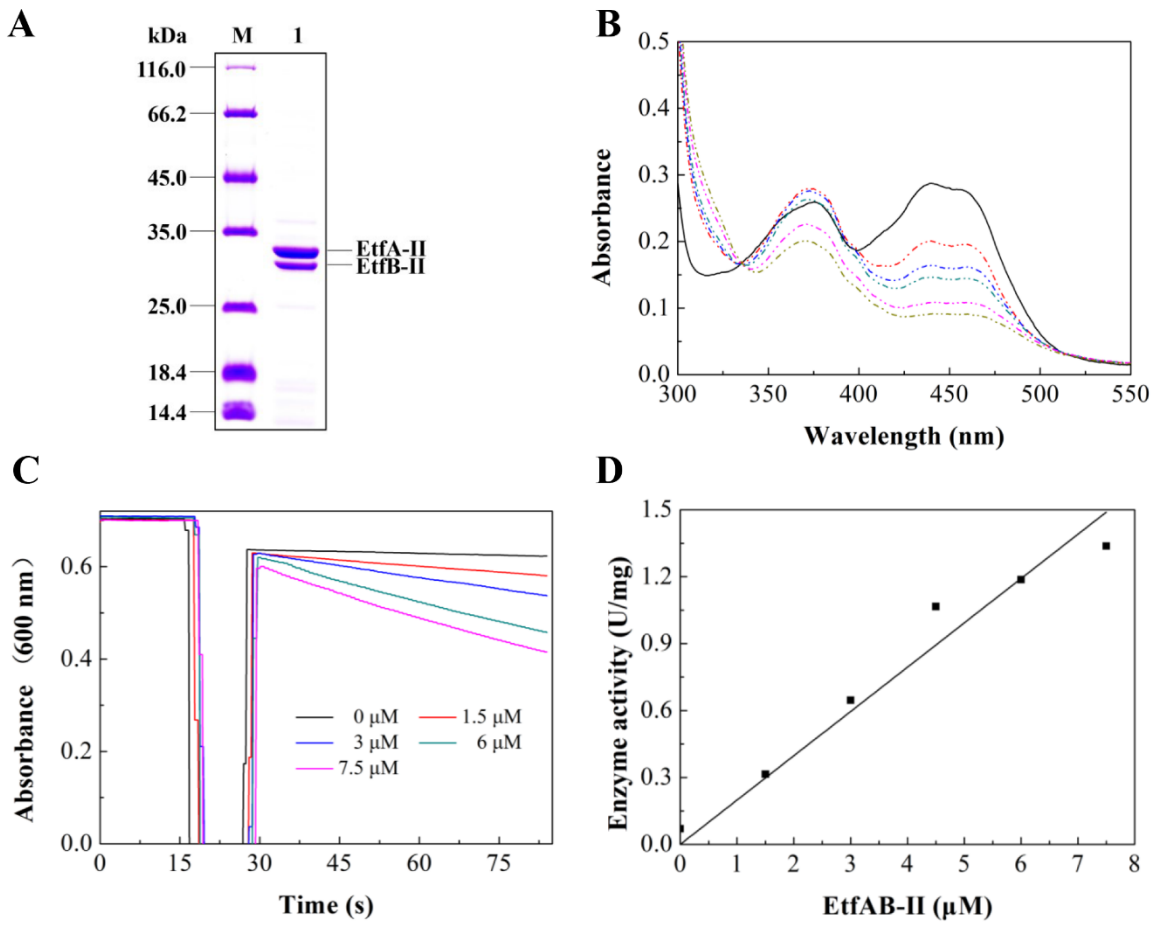


FIG S6 Heterologous expression and purification of EtfAB-II and the pseudooxynicotine oxidation catalyzed by Pno with purified EtfAB-II as the electron acceptor. (A) SDS-PAGE analysis of purified His-tagged EtfAB-II. (B) UV-visible absorption spectra of purified EtfAB-II (solid line) and spectrophotometric changes from 300 nm to 550 nm during the reduction of EtfAB-II (dash dot dot line) with pseudooxynicotine by Pno. Pseudooxynicotine has no absorption in the wavelength window. The reaction mixture contained 100 μM EtfAB-II, 1 mM pseudooxynicotine, and 20 mM sodium phosphate buffer (pH7.4). The reaction was initiated by adding 0.05 μM of Pno. The red dash dot

dot line is the first record after addition of Pno. Reaction time between the first two reaction (between black line and red line) was 45 s. The rest records were done every 45 s, where the absorption continuously decreased. (C) The effect of different concentrations of EtfAB-II (0-7.5 μM) on the reduction of DCPIP by Pno (0.66 μM) in the presence of 1 mM pseudooxynicotine monitored at 600 nm. (D) The relationship between the concentration of EtfAB-II and DCPIP reduction activity (U/mg) catalyzed by Pno. Data were used from Fig. S6C.

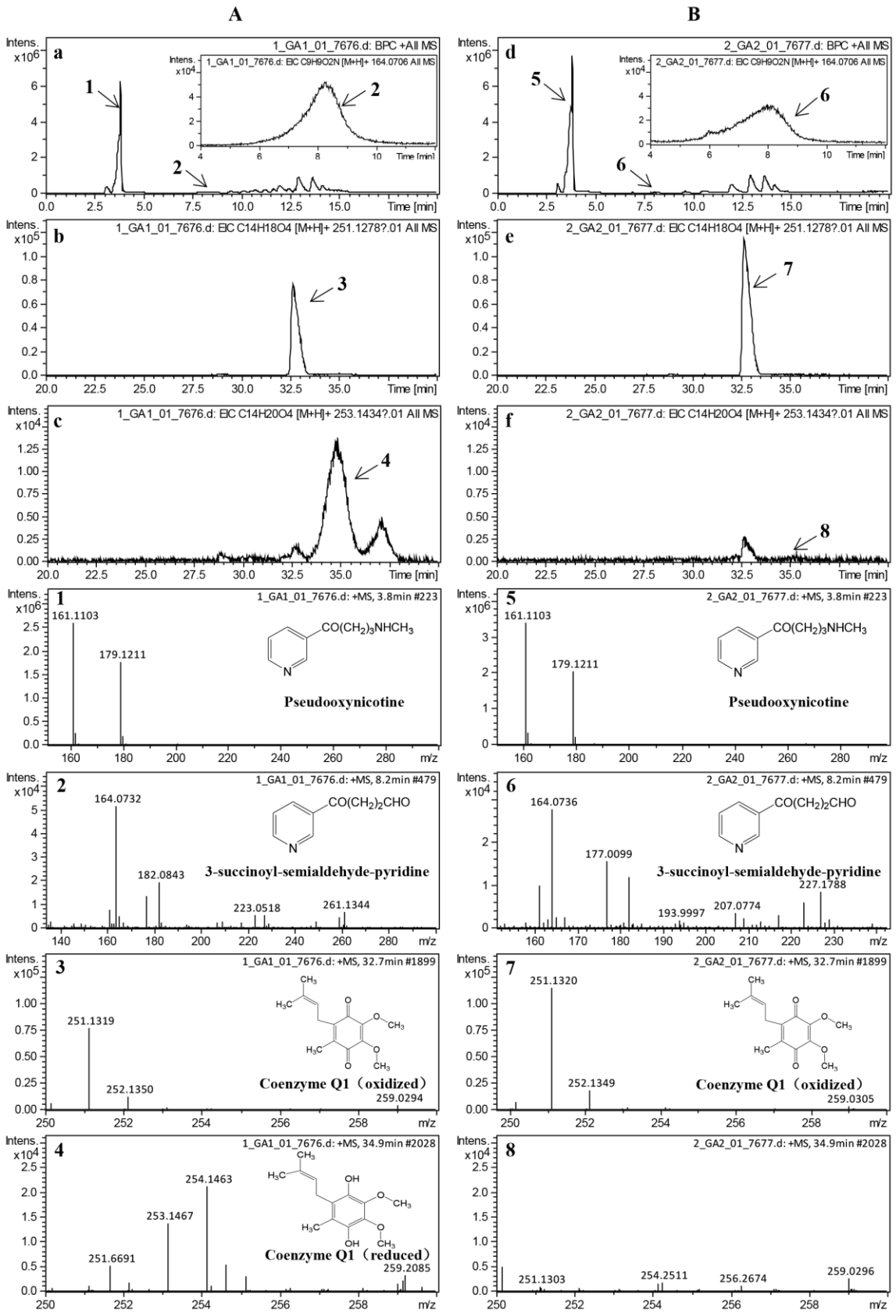
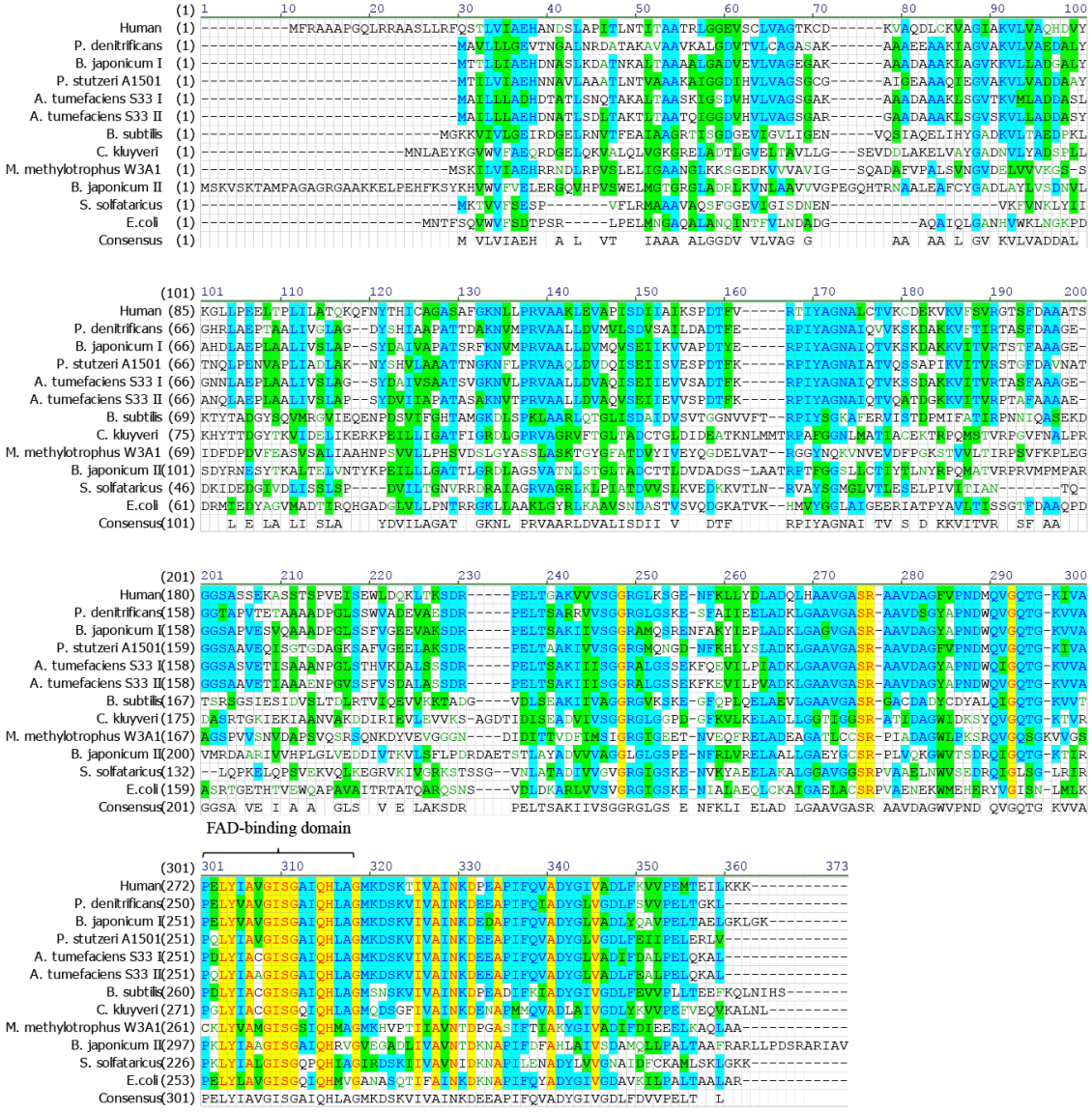

A
B
a
1_GA1_01_7676.d: BPC +All MS
1_GA1_01_7676.d: EIC C9H9O2N [M+H]+ 164.0706 All MS
b
1_GA1_01_7676.d: EIC C14H18O4 [M+H]+ 251.1278?.01 All MS
c
1_GA1_01_7676.d: EIC C14H20O4 [M+H]+ 253.1434?.01 All MS
d
2_GA2_01_7677.d: BPC +All MS
2_GA2_01_7677.d: EIC C9H9O2N [M+H]+ 164.0706 All MS
e
2_GA2_01_7677.d: EIC C14H18O4 [M+H]+ 251.1278?.01 All MS
f
2_GA2_01_7677.d: EIC C14H20O4 [M+H]+ 253.1434?.01 All MS
1
1_GA1_01_7676.d: +MS, 3.8min #223
161.1103
179.1211
Pseudooxynicotine
5
2_GA2_01_7677.d: +MS, 3.8min #223
161.1103
179.1211
Pseudooxynicotine
2
1_GA1_01_7676.d: +MS, 8.2min #479
164.0732
182.0843
223.0518
261.1344
3-succinoyl-semialdehyde-pyridine
6
2_GA2_01_7677.d: +MS, 8.2min #479
164.0736
177.0099
193.9997
207.0774
227.1788
3-succinoyl-semialdehyde-pyridine
3
1_GA1_01_7676.d: +MS, 32.7min #1899
251.1319
252.1350
259.0294
Coenzyme Q1（oxidized）
7
2_GA2_01_7677.d: +MS, 32.7min #1899
251.1320
252.1349
259.0305
Coenzyme Q1（oxidized）
4
1_GA1_01_7676.d: +MS, 34.9min #2028
254.1463
253.1467
251.6691
259.2085
Coenzyme Q1（reduced）
8
2_GA2_01_7677.d: +MS, 34.9min #2028
251.1303
254.2511
256.2674
259.0296
Intens.
Time [min]
m/z

FIG S7 LC-MS profiles of the reaction catalyzed by purified Euo coupling with pseudooxynicotine oxidation by Pno in the presence of Euo (A) or not (B). The reaction mixture in 50 mM Tris-HCl (pH 8.5) contained 1 mM pseudooxynicotine, 0.05 μM purified Pno, 8 μM purified EtfAB and 32 μM CoQ_1. In (A), 30 μM Euo was added. (a)-(f) Chromatographs of the two samples, where the enlargements of peak 2 and peak 6 are shown in the insets, respectively, in (a) and (d), and the peaks in (b), (c), (e), and (f) were identified by searching the masses of the expected compounds. (1) and (5) Mass spectra of the excess substrate pseudooxynicotine (*m/z* 179.1211) and its spontaneously dehydrated product *N*-methylmyosmine (*m/z* 161.1103) with retention time at 3.8 min. There was a hydrolysis equilibrium between pseudooxynicotine and *N*-methylmyosmine. (2) and (6) Mass spectra of the product 3-succinoyl-semialdehyde-pyridine (*m/z* 164.0732 or 164.0736) and its adduct of water (*m/z*, 182.0843 or 182.0842) with retention time at 8.2 min. The peaks of (*m/z* 177.0100 or 177.0099) are possibly caused by the unknown contaminant in the mobile phase (background signal). (3) and (7) Mass spectra of the excess oxidized CoQ_1 (*m/z* 251.1319 or 251.1320) with retention time at 32.7 min. (4) and (8) Mass spectra of the product reduced CoQ_1 (*m/z* 253.1467) with retention time at 34.9 min.

90 A EtfAs



91 (The figure is continued)

B EtfBs

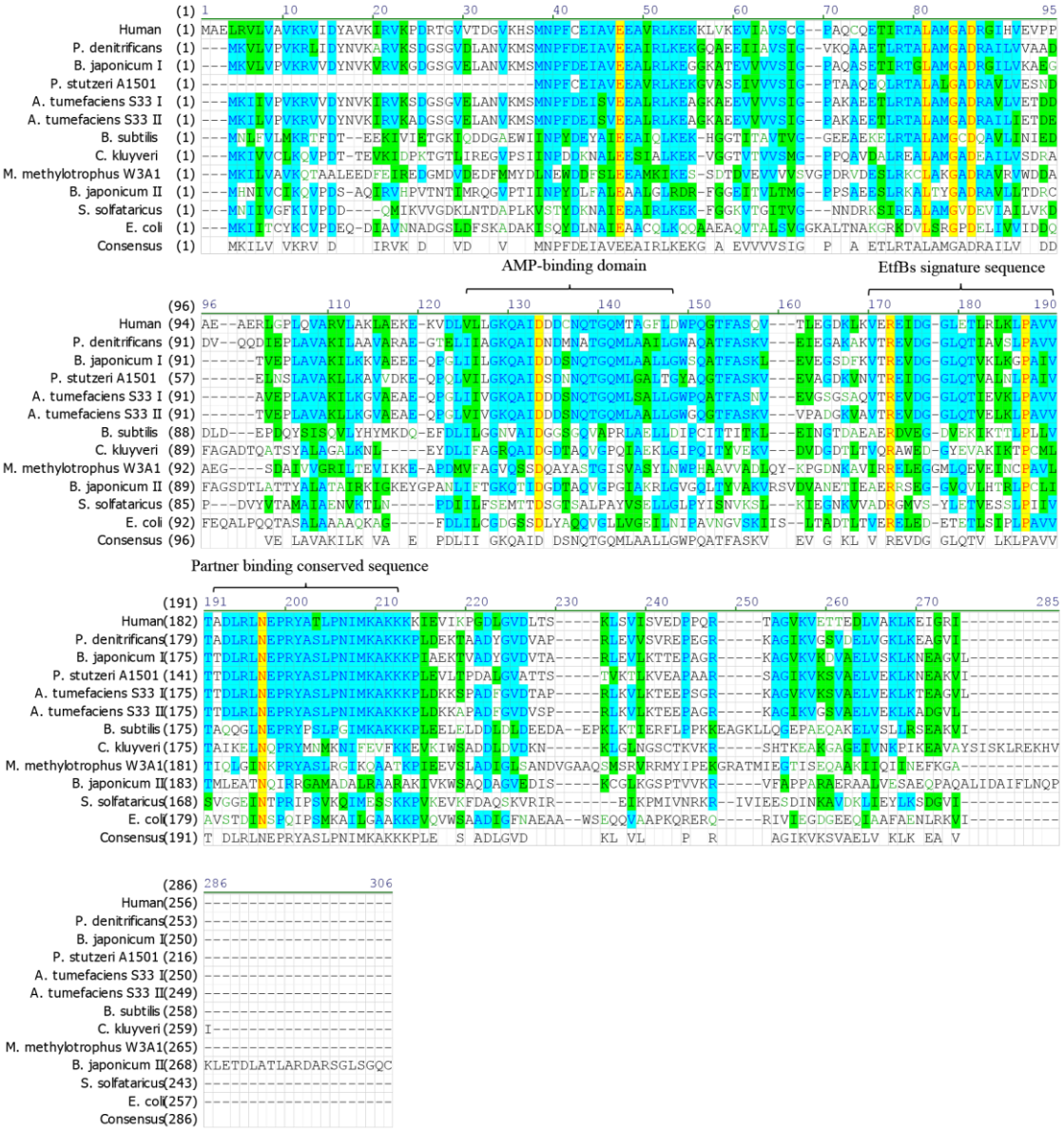
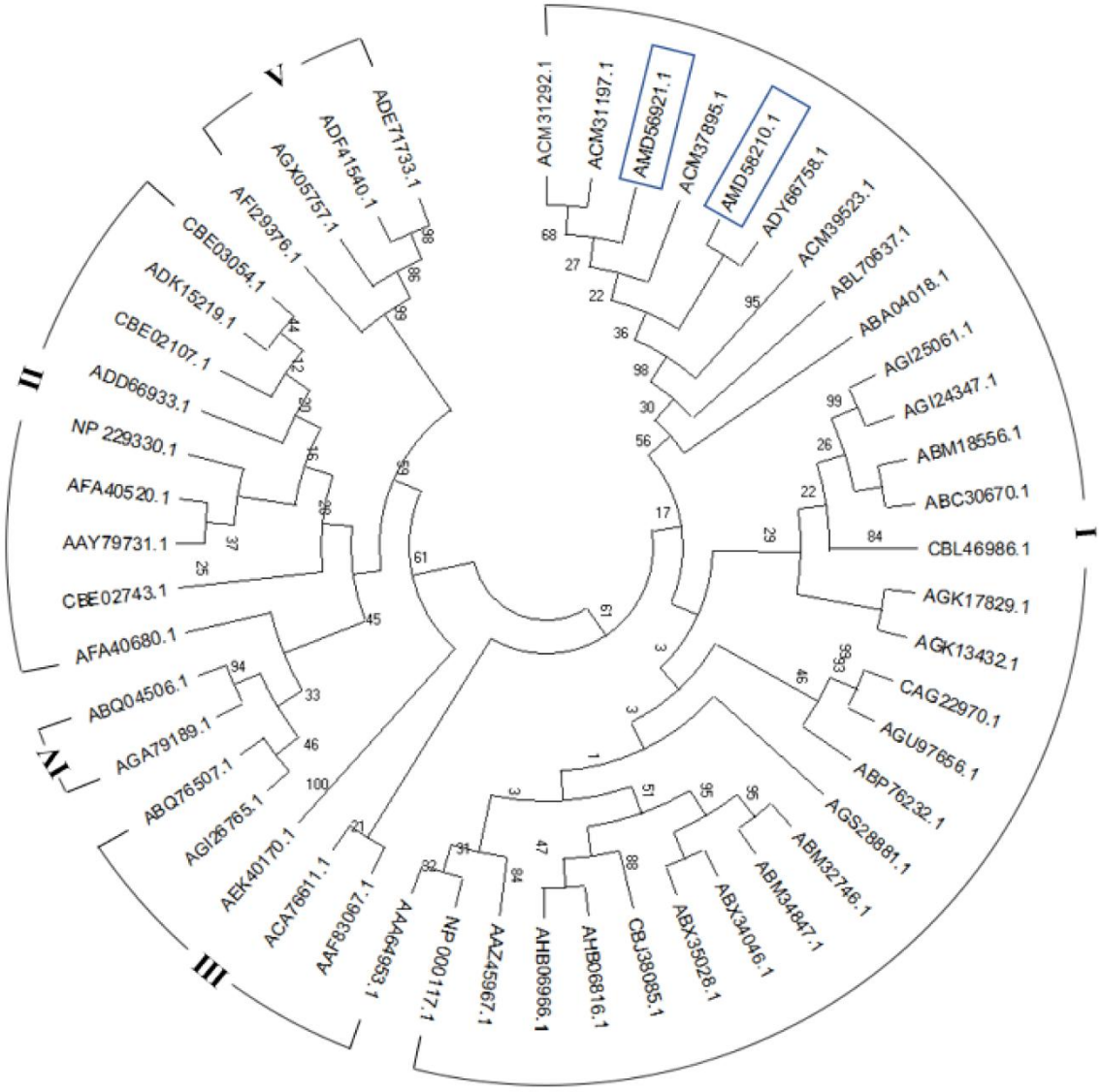


FIG S8 Protein sequences alignment of EtfAs (A) and EtfBs (B) from different organisms. GenBank/NCBI accession numbers: Human (NP_000117.1/NP_001976.1), *Paracoccus denitrificans* (ABL70638.1/ABL70637.1), *Bradyrhizobium japonicum* I (NP_768018.1/NP_768017.1), *Bradyrhizobium japonicum* II

(NP_768413.1/NP_768678.1), *Pseudomonas stutzeri* A1501 (ABP80254.1/ABP80255.1), *Agrobacterium tumefaciens* S33 I (AMD56921.1/AMD58724.1), *Agrobacterium tumefaciens* S33 II (AMD58210.1/AMD58211.1), *Methylophilus methylotrophus* W3A1 (AAA64953.1/AAA64952.1), *Sulfolobus solfataricus* (AAK42873.1/AAK42874.1), *Clostridium kluyveri* (EDK32511.1/EDK32510.1), *Escherichia coli* (NP_414584.1/NP_414583.2), *Bacillus subtilis* (NP_390730.1/NP_390731.1). The sequence alignment was performed with Vector NTI 10. The last line refers to the consensus sequence. Color code: dark blue foreground and light blue background, conservative residues; black foreground and light green background, block of similar residues; red foreground and yellow background, identical residues; dark green foreground and white background, weakly similar residues; black foreground and white background, non-similar residues. The conserved AMP-binding sites (responding to V120-D143 of EtfB from Human) only exist in Group I Etfs from human, *P. denitrificans*, *B. japonicum* I, *P. stutzeri* A1501, and *A. tumefaciens* S33.



114

115 **FIG S9** Phylogenetic analysis of the EtfABs from different organisms (for details, please

116 refer Table S1). The connected protein sequences of EtfA and EtfB were used for analysis.

117 Only the GenBank accession numbers of EtfAs are shown. EtfABs from *A. tumefaciens*

118 S33 are indicated in blue boxes, where AMD56921.1 is for EtfA-I, and AMD58210.1 is

119 for EtfA-II. This tree was inferred by using the Neighbor-Joining method through

120 MEGA-X-10.0.5. The evolutionary distances were computed using the JTT matrix-based

121 method and are in the units of the number of amino acid substitutions per site. The rate
122 variation among sites was modeled with a gamma distribution (shape parameter = 1).
123

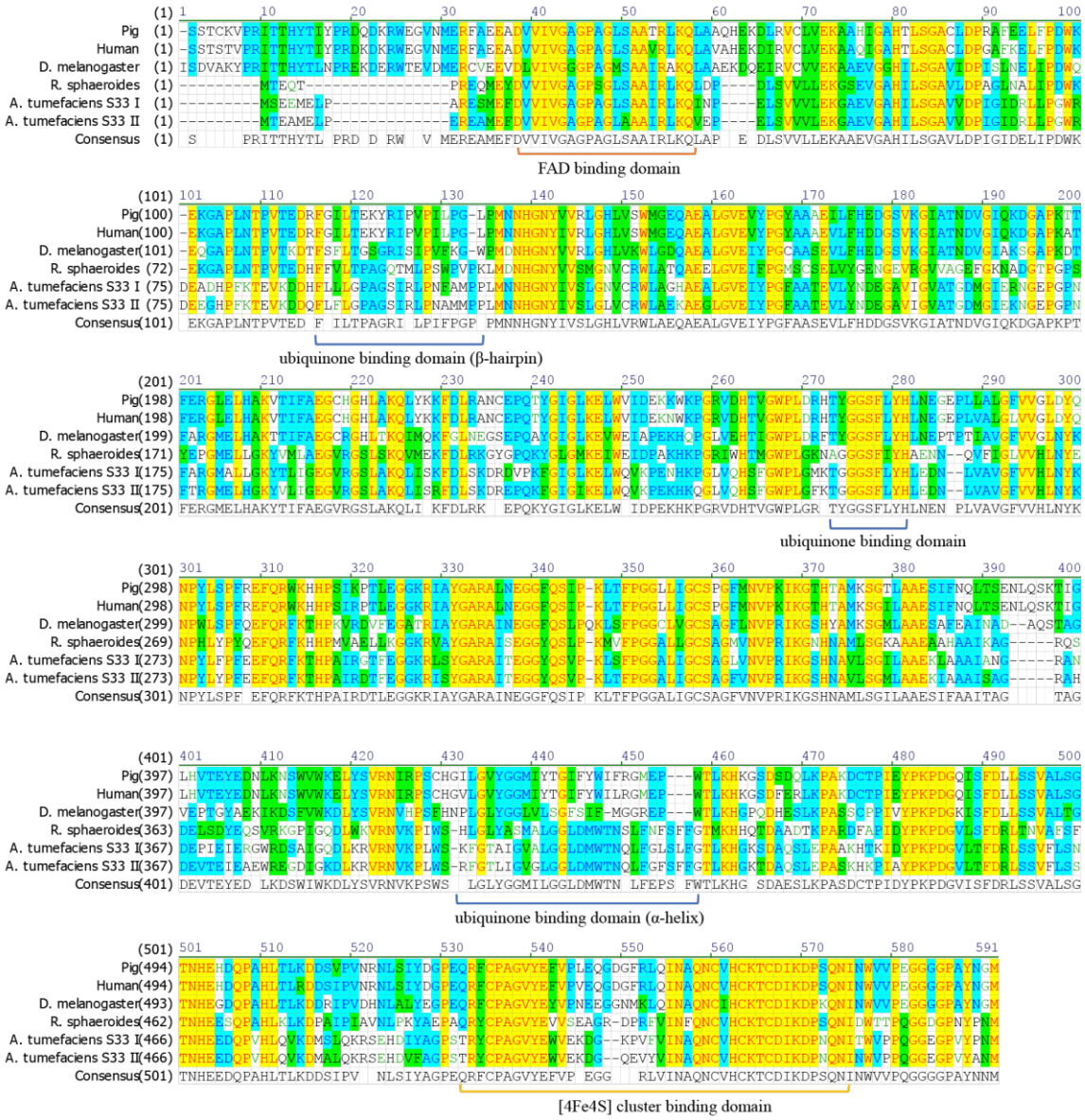


FIG S10 Protein sequences alignment of Euo from different organisms. GenBank accession numbers: Pig (AAB30031.1), Human (AAN03724.1), *Drosophila melanogaster* (AFH07986.1), *Rhodobacter sphaeroides* (ATN62075.1), *Agrobacterium tumefaciens* S33 I (AMD56919.1), *Agrobacterium tumefaciens* S33 II (AMD61690.1). Sequences were obtained from NCBI (https://www.ncbi.nlm.nih.gov/) with GenBank

accession numbers. The mitochondrial targeting sequences of eukaryotic sequences were removed as Watmough and Frerman (2010) described. The alignment of the six sequences was performed with Vector NTI 10.

Table S1 EtfABs from different organisms used for phylogenetic analysis in FIG S9.

Group	GenBank Accession Numbers [a]	Organisms
1	AAF83067.1	*Xylella fastidiosa* 9a5c
1	AAW60643.1	*Gluconobacter oxydans* 621H
1	AAZ45967.1	*Dechloromonas aromatica* RCB
1	ABA04018.1	*Nitrobacter winogradskyi* Nb-255
1	ABC30670.1	*Hahella chejuensis* KCTC 2396
1	ABL70637.1	*Paracoccus denitrificans* PD1222
1	ABM18556.1	*Marinobacter aquaeolei* VT8
1	ABM32746.1	*Acidovorax avenae citrulli* AAC00-1
1	ABM34847.1	*Acidovorax avenae citrulli* AAC00-1
1	ABM94258.1	*Methylibium petroleiphilum* PM1
1	ABP76232.1	*Shewanella putrefaciens* CN-32
1	ABX34046.1	*Delftia acidovorans* SPH-1
1	ABX35028.1	*Delftia acidovorans* SPH-1
1	ACM31197.1	*Agrobacterium radiobacter* K84
1	ACM31292.1	*Agrobacterium radiobacter* K84
1	ACM37895.1	*Agrobacterium vitis* bv. III S4
1	ACM39523.1	*Agrobacterium vitis* bv. III S4
1	ADY66758.1	*Agrobacterium* sp. H13-3
1	AGI24347.1	*Pseudomonas denitrificans* ATCC 13867
1	AGI25061.1	*Pseudomonas denitrificans* ATCC 13867
1	AGK13432.1	*Azotobacter vinelandii* CA
1	AGK17829.1	*Azotobacter vinelandii* CA6
1	AGS28881.1	*Salmonella enterica* sv. Newport USMARC-S3124.1
1	AGU97656.1	*Vibrio campbellii* ATCC BAA-1116
1	AHB06816.1	*Pandoraea pnomenusa* 3kgm
1	AHB06966.1	*Pandoraea pnomenusa* 3kgm
1	CAG22970.1	*Photobacterium profundum* SS9
1	CBJ38085.1	*Ralstonia solanacearum* CMR15
1	CBL46986.1	*gamma proteobacterium* sp. HdN1
1	NP_000117.1	*Homo sapiens*
4	ABQ04506.1	*Flavobacterium johnsoniae* UW101, ATCC 17061
4	AGA79189.1	*Echinicola vietnamensis* KMM 6221, DSM 17526
5	ADE71733.1	*Bacillus megaterium* QM B1551
5	ADF41540.1	*Bacillus megaterium* DSM 319
5	AFI29376.1	*Bacillus* sp. JS
5	AGX05757.1	*Bacillus infantis* NRRL B-14911

(The table is continued.)

Group	GenBank Accession Numbers	Organisms
2A	CBE02107.1	*Clostridioides difficile* R20291
2A	CBE02743.1	*Clostridioides difficile* R20291
2A	CBE03054.1	*Clostridioides difficile* R20291
2B	AAY79731.1	*Sulfolobus acidocaldarius* 98-3, DSM 639
2B	ADK15219.1	*Clostridium ljungdahlii* PETC, DSM 13528
2C1	AFA40520.1	*Pyrobaculum oguniense* TE7, DSM 13380
2C1	AFA40680.1	*Pyrobaculum oguniense* TE7, DSM 13380
2D1	NP 229330.1	*Thermotoga maritima* MSB8
2E	ADD66931.1	*Denitrovibrio acetiphilus* N2460, DSM 12809
3A	ABQ76507.1	*Pseudomonas putida* F1
3A	ACA76611.1	*Escherichia coli* C ATCC 8739
3A	AGI26765.1	*Pseudomonas denitrificans* ATCC 13867
3C	AAA64953.1	*Methylophilus methylotrophus* W3A1
3D	AEK40170.1	*Amycolatopsis mediterranei* S699, ATCC 13685
1	AMD56921.1	*Agrobacterium tumefaciens* S33(EtfAB-I)
1	AMD58210.1	*Agrobacterium tumefaciens* S33(EtfAB-II)

[a] Only GenBank accession numbers of EtfAs are listed.