Supplementary Information for

Rational Protein Engineering of Bacterial *N*-demethylases to Create Biocatalysts for the Production of Methylxanthines

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Table S1. All plasmids created and tested in this study.

Plasmid Name	Plasmid Characteristics	Source
pACYCDuet-1	P15A origin, two MCS, hexahistidine tag sequence by MCS1, Chloramphenicol resistance	Novagen
pETDuet-1	pBR322 origin, two MCS, hexahistidine tag sequence by MCS1, Ampicillin resistance	Novagen
dDA	pACYCDuet-1 with NdmD and NdmA	(Algharrawi et al. 2015)
dDB	pACYCDuet-1 with NdmD and NdmB	(Algharrawi and Subramanian 2020)
dAA	pACYCDuet-1 with two copies of NdmA	(Algharrawi et al. 2015)
dBB	pACYCDuet-1 with two copies of NdmB	(Algharrawi and Subramanian 2020)
dDA1	pACYCDuet-1 with NdmD and with NdmA mutation N282Q	This work
dDA2	pACYCDuet-1 with NdmD and with NdmA mutation F286L	This work
dDA3	pACYCDuet-1 with NdmD and with NdmA mutations N282Q and F286L	This work
dDB1	pACYCDuet-1 with NdmD and with NdmB mutation L293F	This work
dDB2	pACYCDuet-1 with NdmD and with NdmB mutation Q289N	This work
dDB3	pACYCDuet-1 with NdmD and with NdmB mutations L293F and A289N	This work
pETDuet-NdmA4	pETDuet-1 with NdmA4	(Kim et al. 2019)
dDA4	pACYCDuet-1 with NdmD and NdmA4	This work
pET-28 a (+)	pBR322 origin, Kanamycin resistance	Novagen
pD	pET-28 a (+) with NdmD (previously named pET28-His-ndmD)	(Summers et al. 2012)
pD1	pET-28 a (+) with NdmD mutation C69A	This work
pD2	pET-28 a (+) with NdmD mutations C69A and C50A	This work
pDW1	pET-28 a (+) with NdmD mutations C69A and V541W	This work
pDW2	pET-28 a (+) with NdmD mutations C69A, C50A and V541W	This work
pDR1	pET-28 a (+) with NdmD mutations C69A and V541R	This work
pDR2	pET-28 a (+) with NdmD mutations C69A, C50A and V541R	This work

Table S2. All primer sequences used in the plasmid construction for this study

Primer Name	**Primer Sequence 5′ to 3′**
A-N282Q-F	GATTATCTGCACATTGCATTTCAAGATCTCGTCTTCGCTGAAGAC
A-N282Q-R	GTCTTCAGCGAAGACGAGATCTTGAAATGCAATGTGCAGATAATC
A-F286L-F	GCATTTAATGATCTCGTCTTGGCTGAAGACAAACCAGTAATTG
A-F286L-R	CAATTACTGGTTTGTCTTCAGCCAAGACGAGATCATTAAATGC
A3-N282Q-F	GATTATCTGCACATTGCATTTCAAGATCTCGTCTTGGCTGAAGAC
A3-N282Q-R	GTCTTCAGCCAAGACGAGATCTTGAAATGCAATGTGCAGATAATC
B-L293F-F	GCTTTCCAGAAGCGGGTGTTTGACGAAGACCAGCCTG
B-L293F-R	CAGGCTGGTCTTCGTCAAACACCCGCTTCTGGAAAGC
B-Q289N-F	CACATGCACCTGGCTTTCAACAAGCGGGTGCTTGACGAAG
B-Q289N-R	CTTCGTCAAGCACCCGCTTGTTGAAAGCCAGGTGCATGTG
B3-Q298N-F1	CACATGCACCTGGCTTTCAACAAGCGGGTGTTTGACGAAG
B3-Q289N-R1	CTTCGTCAAACACCCGCTTGTTGAAAGCCAGGTGCATGTG
D-V541W-F	GAAGCTTCTTGTGAGCAGGGTTGGTGCGGGACTTGTATAACTCCAG
D-V541W-R	CTGGAGTTATACAAGTCCCGCACCAACCCTGCTCACAAGAAGCTTC
D-V541R-F	GAAGCTTCTTGTGAGCAGGGTCGCTGCGGGACTTGTATAACTCCAG
D-V541R-R	CTGGAGTTATACAAGTCCCGCAGCGACCCTGCTCACAAGAAGCTTC
D-C50A-F	AATGCTTGGGAGAACCGCGCCCCGCATAGAGGATTGCGG
D-C50A-R	CCGCAATCCTCTATGCGGGGCGCGGTTCTCCCAAGCATT
D-C69A-F	GCTAATACCGGTAACGAGTTGCGAGCTCAGTATCATGGATGGACTTATG
D-C69A-R	CATAAGTCCATCCATGATACTGAGCTCGCAACTCGTTACCGGTATTAGC
Loop-F-NdeI	GCACGGCATATGGAACAGGCAATCATTAATG
NdmA-R-KpnI	CCTCCGGGTACCTTATARGTAGCTCCTATCGCTT

Table S3. All strains created and tested in this study.

Strain Name	Strain Characteristics	Source
E. coli BL21(DE3)	F^- *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm* (DE3)	Invitrogen
E. coli dDA	BL21(DE3) dDA	(Algharrawi et al. 2015)
E. coli dDA1	BL21(DE3) dDA1	This work
E. coli dDA2	BL21(DE3) dDA2	This work
E. coli dDA3	BL21(DE3) dDA3	This work
E. coli dDA4	BL21(DE3) dDA4	This work
E. coli dDB	BL21(DE3) dDB	(Algharrawi and Subramanian 2020)
E. coli dDB1	BL21(DE3) dDB1	This work
E. coli dDB2	BL21(DE3) dDB2	This work
E. coli dDB3	BL21(DE3) dDB3	This work
E. coli pDdAA	BL21(DE3) pD dAA	(Algharrawi et al. 2015)
E. coli pD1dA	BL21(DE3) pD1 dAA	This work
E. coli pD2dAA	BL21(DE3) pD2 dAA	This work
E. coli pDWdAA	BL21(DE3) pDW dAA	This work
E. coli pDW1dAA	BL21(DE3) pDW1 dAA	This work
E. coli pDW2dAA	BL21(DE3) pDW2 dAA	This work
E. coli pDRdAA	BL21(DE3) pDR dAA	This work
E. coli pDR1dAA	BL21(DE3) pDR1 dAA	This work
E. coli pDR2dAA	BL21(DE3) pDR2 dAA	This work
E. coli pD1dBB	BL21(DE3) pD1 dBB	This work
E. coli pD2dBB	BL21(DE3) pD2 dBB	This work
E. coli pDWdBB	BL21(DE3) pDW dBB	This work
E. coli pDW1dBB	BL21(DE3) pDW1 dBB	This work
E. coli pDW2dBB	BL21(DE3) pDW2 dBB	This work
E. coli pDRdBB	BL21(DE3) pDR dBB	This work
E. coli pDR1dBB	BL21(DE3) pDR1 dBB	This work
E. coli pDR2dBB	BL21(DE3) pDR2 dBB	This work

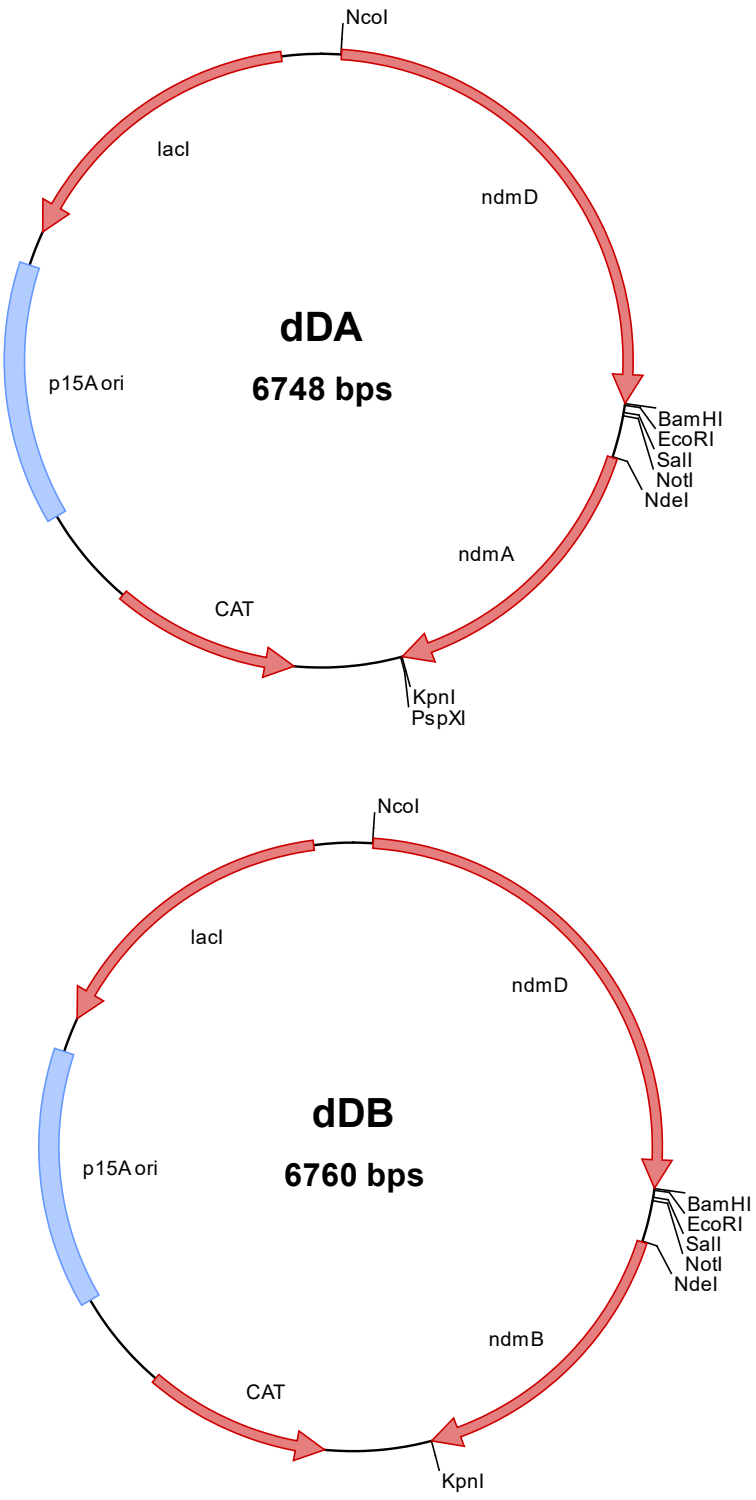

Fig. S1 Plasmid maps of dDA and dDB, which served as the basis for all further plasmids created in this work.

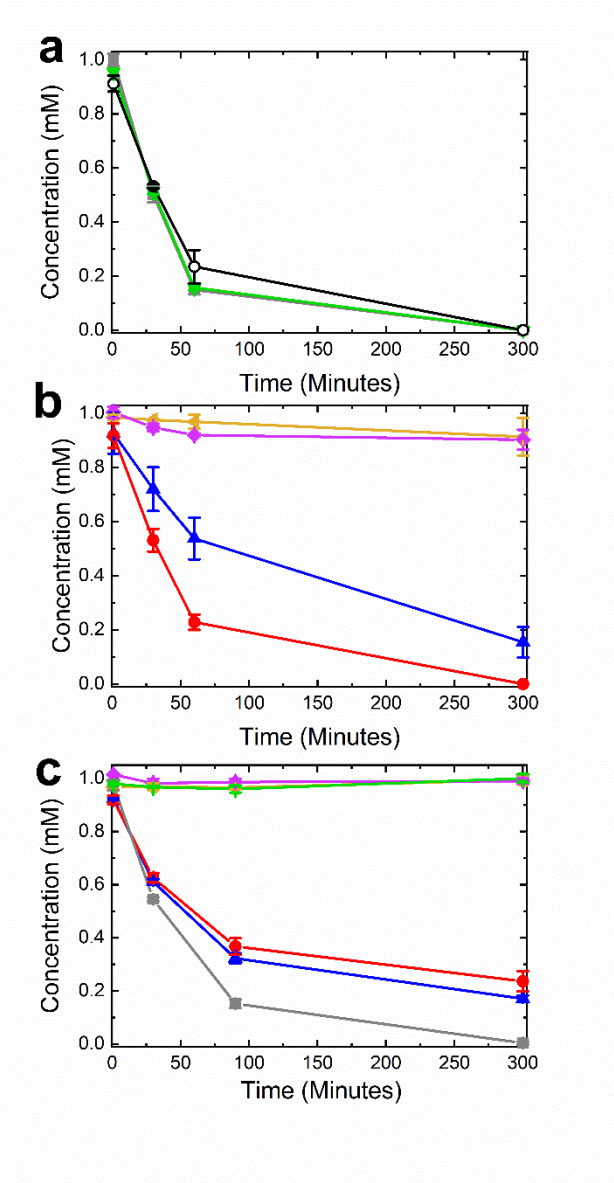

Fig. S2 Substrate conversion by whole cells expressing *ndmA* or *ndmB* with various *ndmD* mutants show varying levels of activity. Caffeine is converted to theobromine by cells expressing *ndmA* with (a) ○, *ndmD*, ■, *ndmD1* and ●, *ndmD2*, or (b) ▲, *ndmDW1*, ◀, *ndmDW2*, ●, *ndmDR1*, and ♦, *ndmDR2*. (c) Theobromine is converted to 7-methylxanthine by cells expressing *ndmB* with ■, *ndmD1*, ▲, *ndmDW1*, ●, *ndmDR1*, ▼, *ndmD2*, ◀, *ndmDW2* and ♦, *ndmDR2*. Cells (OD_{600} = 5.0) were incubated with 1 mM caffeine or theobromine in 50 mM KP_i buffer at 30°C with 200 rpm shaking, and metabolites were quantified by HPLC. Concentrations reported are means with standard deviations of triplicate results.