Supplementary information

One-pot enzymatic synthesis of L-5-methyltetrahydrofolate from folic acid using enzyme cascades

Table S1: Dihydrofolate reductase from 38 different sources

	Strain	Enzyme	GenBank accession NO.	Conversion[a] (%)
Bacteria	*Aminobacter aminovorans*	*Ama*DHFR2855	AMS41780	27.8
		*Ama*DHFR2417	AMS41343	6.4
		*Ama*DHFR0532	AMS39471	6.3
		*Ama*DHFR5858	AMS44763	5.8
	Bacillus subtilis	*Bsu*DHFR168	NP_390064	7.7
	Xanthomonas campestris	*Xca*DHFR	AAY50487	33.5
	Acinetobacter junii	*Aju*DHFR	APU49723	8.4
	Streptococcus mutans	*Smu*DHFR	AAN58651	7.9
	Brevibacterium casei	*Bca*DHFR	QPS35038	7.9
	Citrobacter amalonaticus	*Cam*DHFR	AKE57683	19.5
	Cronobacter sakazakii	*Csa*DHFR	ABU78511	9.9
	Lactobacillus brevis	*Lbr*DHFR0785	ABJ63928	16.7
		*Lbr*DHFR1497	ABJ64593	16.3
	Streptococcus thermophiles	*Sth*DHFR	ALX90712	7.9
	Bifidobacterium bifidum	*Bbi*DHFR	ADO53627	21.9
	Lactiplantibacillus plantarum	*Lpl*DHFR	CCC79138	7.9
	Escherichia coli	*Eco*DHFR	NP_414590	37.6
		*Eco*DHMPR	NP_416123	2.4
		*Eco*DHFR-ANLYF	2D0K_A	13.1
	Ligilactobacillus salivarius	*Las*DHFR	ABD99519	44.6
	Kosakonia cowanii	*Kco*DHFR	APZ03939	23.3
	Bacillus halodurans	*Bha*DHFR	BAB07169.1	37.8
	Alkaliphilus transvaalensis	*Trans*DHFR	WP_026477829.1	12.0
	Oceanobacillus iheyensis	*Oih*DHFR	BAC13695.1	9.4
	Bifidobacterium infantis	*Bin*DHFR	ACJ53202	4.8
	Bifidobacterium longum	*Blo*DHFR	ADH01092	53.7

	Bifidobacterium breve	*Bbr*DHFR	BAR00601	13.7
	Bifidobacterium animalis	*Ban*DHFR	AFI62698	4.9
	Lacticaseibacillus rhamnosus	*Lrh*DHFR	CAR87293	76.1
	Lactobacillus helveticus	*Lhe*DHFR	ABX27049	4.2
	Lactobacillus bulgaricus	*Lbu*DHFR	CAI97615	92.0
	Lactobacillus acidophilus	*Lac*DHFR	AAV42759	13.1
Fungi	*Aspergillus oryzae*	*Aor*DHFR	XP_001826385	9.9
	Kluyveromyces lactis	*Kla*DHFR	XP_453395	19.5
Yeasts	*Saccharomyces cerevisiae*	*Sce*DHFR	NP_014879	7.9
	Pichia pastoris	*Ppa*DHFR	XP_002490391	37.8
Malaria parasite	*Plasmodium falciparum*	*Pf*DHFR-C59R	AAA96491.1	2.6
Human being	*Homo sapiens*	*h*DHFR-35K64F	3GHV_A	51.0

[a]: The cell extract was used for catalyzing the reduction reaction of folic acid (FA) to tetrahydrofolate. Reaction mixture: 2.3 mM FA, 1.0 g/L DTT, 50 μM $NADP^+$, 3.0%(v/v) isopropanol, 0.4 M HEPES buffer (pH=7.5) and cell extract of DHFR and *Lb*ADH. The mixtures were incubated at 37°C for 10 h with a magnetic stirrer.

Table S2: DmdAs from 9 different sources

Strain	**Enzyme**	**GenBank accession No.**
Algicella marina	*Ama*DmdA	WP_161862203.1
Candidatus Pelagibacter ubique	*Pub*DmdA	WP_006997663.1
Candidatus Puniceispirillum marinum	*Pma*DmdA	WP_013044947.1
Leisingera methylohalidivorans	*Lme*DmdA	WP_024090329.1
Ruegeria conchae	*Rco*DmdA	WP_260026001.1
Sulfitobacter mediterraneus	*Sme*DmdA	WP_203211332.1
Thalassococcus sp. S3	*Tha*DmdA	WP_130406460.1
Roseibacterium elongatum	*Rel*DmdA	WP_025312765.1
Roseovarius indicus	*Rin*DmdA	WP_057818642.1

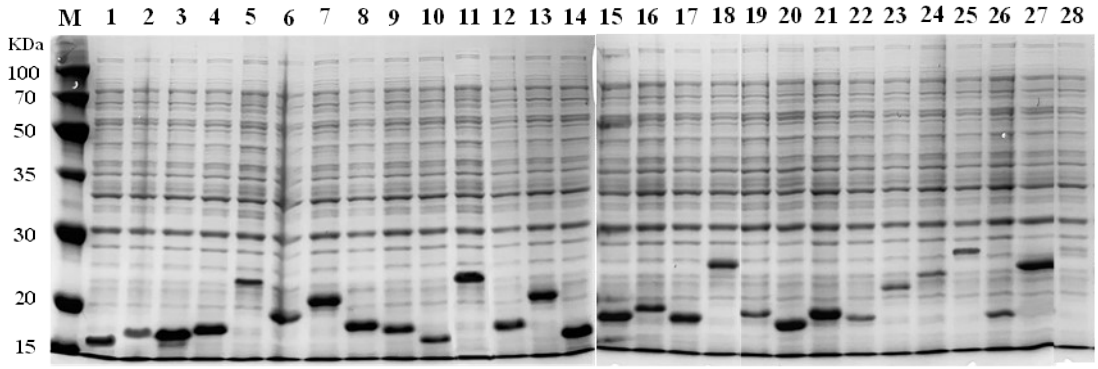

Fig S1 SDS-PAGE profile of partial recombinant DHFRs. DHFRs were produced by *E. coli* BL21(DE3) with the expression vector pET28a. The cell extracts of recombinant DHFRs were used for SDS-PAGE analysis. Lane M: Marker; Lane 1: *Trans*DHFR; Lane 2: *Oih*DHFR; Lane 3: *Eco*DHFR-ANLYF; Lane 4: *Bha*DHFR; Lane 5: *Pf*DHFR; Lane 6: *h*DHFR-35K64F; Lane 7: *Ppa*DHFR; Lane 8: *Lpj*DHFR; Lane 9: *Las*DHFR; Lane 10: *Kco*DHFR; Lane 11: *Eco*DHMPR; Lane 12: *Smu*DHFR; Lane 13: *Sce*DHFR; Lane 14: *Eco*DHFR; Lane 15: *Aju*DHFR; Lane 16: *Bsu*DHFR; Lane 17: *Xca*DHFR; Lane 18: *Bbi*DHFR; Lane 19: *Lbr*DHFR1497; Lane 20: *Lbr*DHFR0785; Lane 21: *Ama*DHFR2417; Lane 22: *Ama*DHFR2855; Lane 23: *Ama*DHFR5858; Lane 24: *Ama*DHFR0532; Lane 25: *Sth*DHFR; Lane 26: *Kla*DHFR; Lane 27: *Kco*DHFR; Lane 28: CK

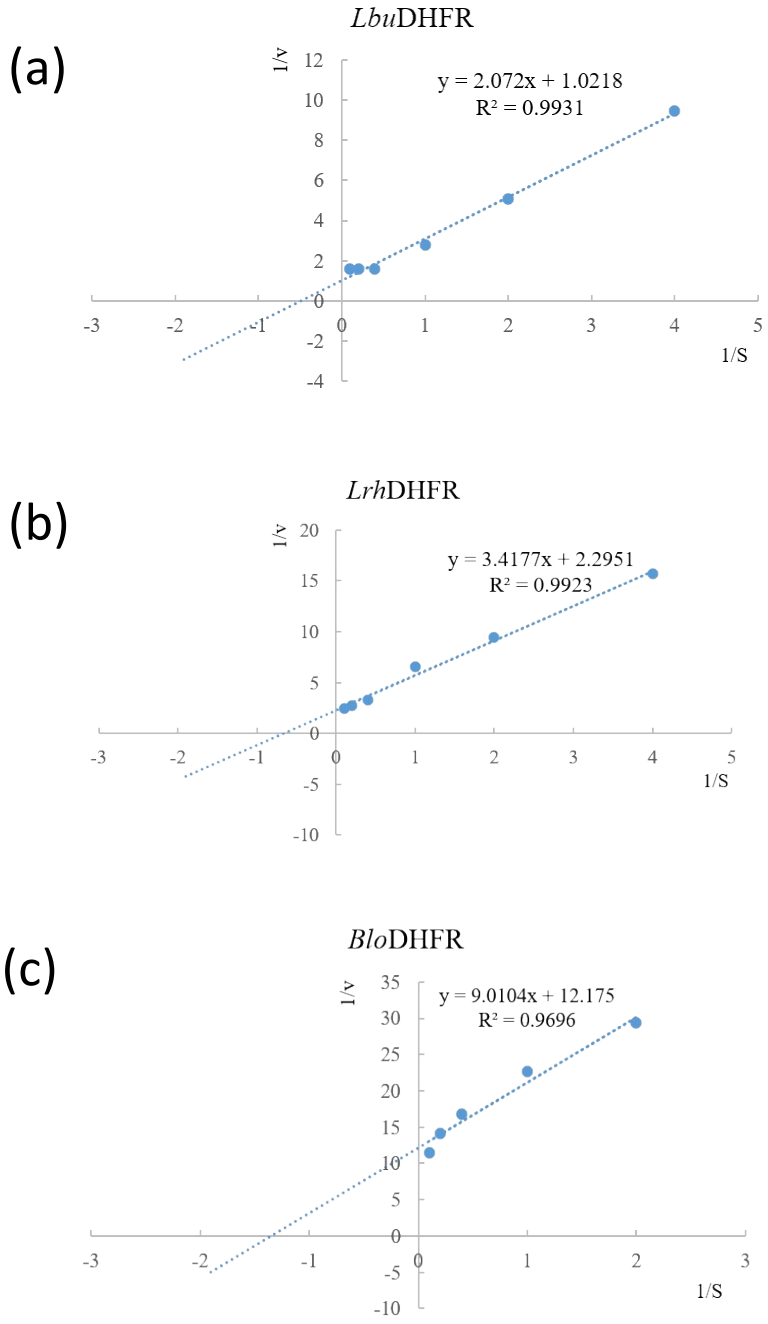

Fig S2 Double reciprocal plots of purified DHFRs based on the data of kinetic parameter assay. (a) *Lbu*DHFR from *Lactobacillus bulgaricus*. (b) *Lrh*DHFR from *Lacticaseibacillus rhamnosus*. (c) *Blo*DHFR from *Bifidobacterium longum*. Reaction mixture for kinetic parameter assay: 0.25 mM-20.0 mM of FA, 20 mM NADPH, 1.0 g/L DTT, 0.4 M HEPES buffer (pH=7.5), and 0.5 mg/mL purified DHFR. The mixtures were incubated at 37°C, 800 rpm for 5 min in a thermomixer. The concentration of FA and THF was determined by HPLC method using an Agilent Technologies 1260 Infinity II LC system with a 1260 DAD WR Detector (USA). HPLC conditions: column, InertSustain® C-18 column (4.6×250 mm) (GL Sciences Inc., Shanghai, China); mobile phase, 50 mM potassium phosphate buffer (pH6.3) : methanol = 92:8 (v/v); flow rate, 1.0 ml/min; and temperature, 40°C; injection volume, 10 µL; absorbance wavelength was set at 290 nm. Each

experiment was carried out in triple.

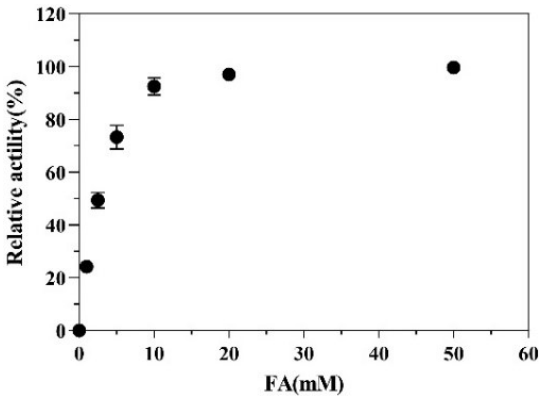

Fig S3 Effect of different adding concentration FA on the reduction activity of *Lbu*DHFR. The mixtures for enzyme activity assay: 0-50 mM FA, 0.5 mg/mL purified *Lbu*DHFR, 0.5 mg/mL purified *Lb*ADH, 50 μM $NADP^+$, 1.0 g/L DTT, 3.0%(v/v) isopropanol, and 0.4 M HEPES buffer. The mixtures were incubated at 40°C, 800 rpm for 10 min in a thermomixer. The maximum value of enzyme activity is defined as 100% of relative activity.

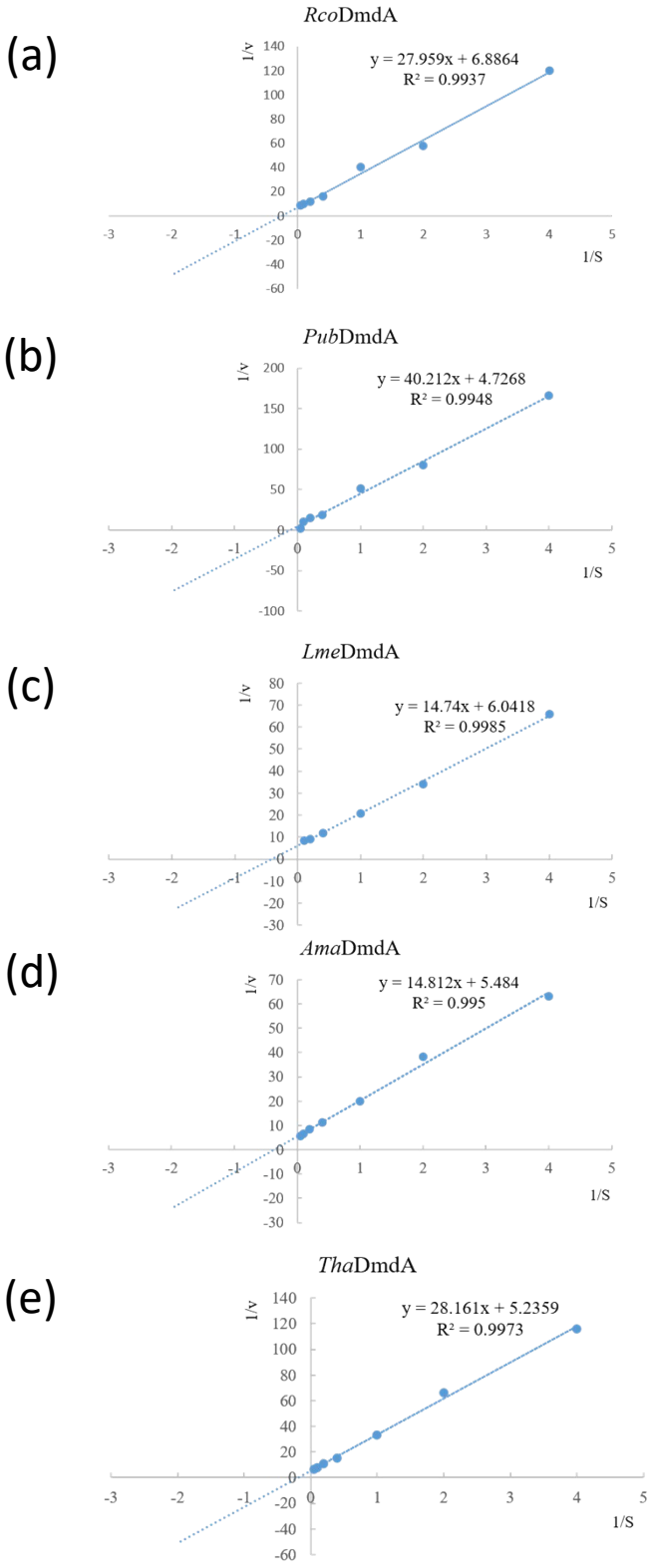

Fig S4 Double reciprocal plots of purified DmdAs based on the data of kinetic parameter assay. (a) *Rco*DmdA from *Ruegeria conchae*. (b) *Pub*DmdA from *Candidatus Pelagibacter ubique*. (c) *Lme*DmdA from *Leisingera methylohalidivorans*. (d) *Ama*DmdA from *Algicella marina*. (e) *Tha*DmdA from *Thalassococcus sp.* S3. Reaction mixture contained 0.25-20.0 mM of THF, 200.0 mM of MSDS, 1.0 g/L of DTT, 0.4 M of HEPES buffer (pH=7.5), and 0.7 mg/mL purified

DmdAs. The mixtures were incubated at 37°C, 800 rpm for 5 min in a thermostatically shaken metal bath. The concentration of FA, THF, and *L*-5-MTHF was determined by HPLC method using an Agilent Technologies 1260 Infinity II LC system with a 1260 DAD WR Detector (USA). HPLC conditions: column, InertSustain® C-18 column (4.6×250 mm) (GL Sciences Inc., Shanghai, China); mobile phase, 50 mM potassium phosphate buffer (pH6.3) : methanol = 92:8 (v/v); flow rate, 1.0 ml/min; and temperature, 40°C; injection volume, 10 μL; absorbance wavelength was set at 290 nm. Each experiment was carried out in triple.

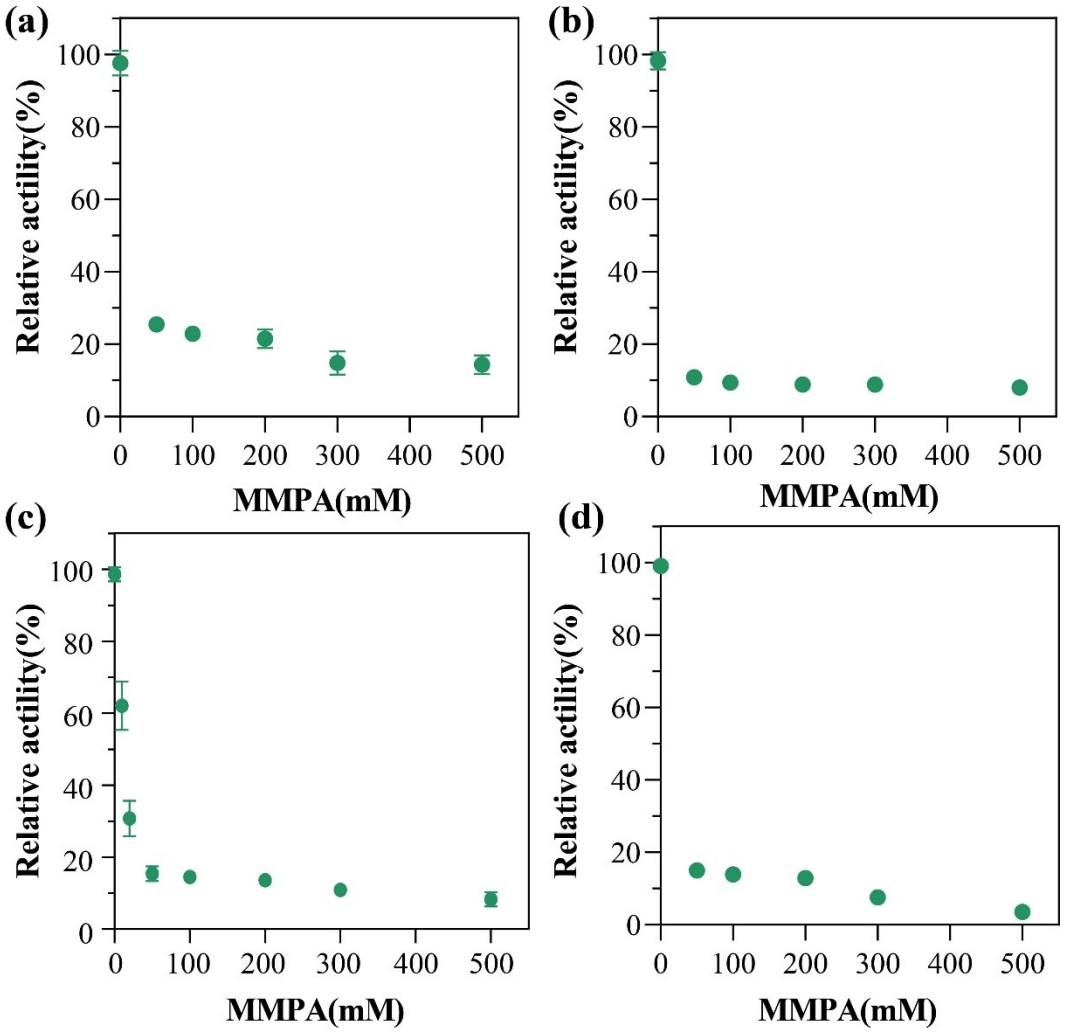

Fig S5：Effect of the by-product MMPA on the transmethylation activity of *Ama*DmdA (a), *Lme*DmdA (b), *Pub*DmdA (c) and *Tha*DmdA (d). 0-500 mM of by-product MMPA was added to the reaction mixture for evaluating the inhibitory effect on methylation of THF. Reaction mixture contained different concentration of MMPA, 2.3 mM THF, 10 mM MSDS, 1.0 g/L DTT, 0.6 mg/mL of purified DmdAs, and 0.4 M HEPES buffer (pH=7.5). The mixtures were incubated at 800 rpm for 15 min in a thermomixer. The enzyme activity without the addition of MMPA was defined as 100% of relative activity.

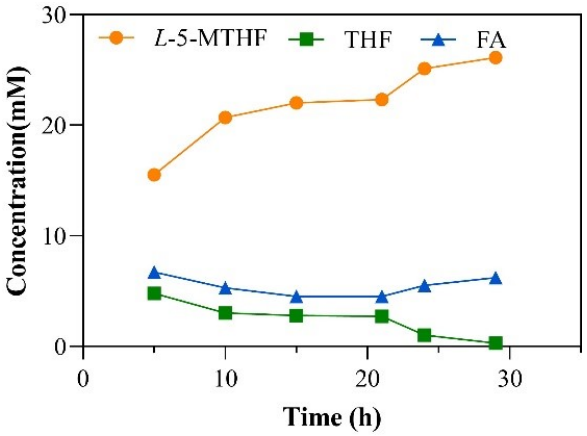

Fig S6：Monitoring the process of three-enzyme cascade reaction with the adding of 34 mM of FA. Reaction mixture: 34 mM FA, 150 mM MSDS, 50 μM $NADP^+$, 1.0 g/L DTT, 3.0 % (v/v) isopropyl alcohol, cell extract equivalent to 60 of OD_{600}. The ratio of cell density was 1:1:4 (*Lb*ADH: *Lbu*DHFR: *Rco*DmdA). The pH was controlled at pH7.5 by an automatic titrator. The mixtures were incubated at 35°C for 29 h in a thermostatic water bath with a magnetic stirrer.