



Carolacton





Carylacton



Tetrahydrofolate

Supplementary Figure 1. Chemical structures of compounds mentioned in this paper.

Consensus Frame 1	ATGGCAGCAÄAGATTATTGÄCGGTAAAACĞATTGCGCAGČAGGTGCGCTCTGAAGTTGCŤCAAAAAGTTČAGGCGCGTAŤTGCAGCCG M A A K I I D G K T I A Q Q V R S FELV A Q K V Q A R I A A
1. folD of E. coli∆tolC	ΑͲͼͼϛϫͼϛϫϫϗͼϫϯτϫϯϯͼϫϲͼͼϫϫϫϫϫϛͼϫϯϲͼϛϲϫͼϲϫͼϛϫͼϛϲͼϲϔϲϲϔͼͼϫͼϲϫϲϫͼϫϫ
2. folD of mutant G8S-1	aŤGGÇAGÇAAÂGATTATTGÁC <mark>A</mark> ĞTAÂAA¢GATTGÇGCÄGCÄGGČGCĞCTÇTGÅAGŤTGÇTCÄAAÂAGŤTCÄGGÇGCĞTATTGÇAGÇCG
3. folD of mutant G8S-2	Αϔͼϐϔϲͽϙͼͽϯϫͽϯϫͼϫϲϫͼϫͼϫͼϫͼϫͼϫͼϫͼϫͼϫͼϫͼϫͼϫͼϫͼϫͼϫͼϫͼϫͼϫ
4. folD of mutant K54N	ATGGÇAAAAGATTATTGACGGTAAAAAÇGATTGÇGCAGCAGGTGCGCTÇTGAAAGTTCAAGACTTCAGGÇGCGTATTGÇAGÇCG
5. folD of mutant Q98H	ѧҭ҉ҩҕҫ҄ѧҕҫ҄ѧѧѧ҈ҩѧҭ҅тѧҭ҅тҕѧ҈ҁӄӡҭѧѧ҈ѧѧҁ҈ѳѧҭ҅тҕҫ҄ҩҁѽ҉ҁҫӹ҈ҁӡѽӷӡҭҕѧ҈ѧҕӷ҅ҭҕҫ҄ҭҫѧ҈ѧѧѧ҈ѧҕҭ҅ҭҫӼ҇ҩҫҫ҄ҁҁӹҭҭҕҫ҄ѧҫҫ҄ҫ
Frame 1 6. folD of mutant ΔK54R55 Frame 1	M A A K I I D G K I I A Q Q V K S E V A Q K V Q A K I A A ATGGCAGCAAAAAATTATTGACGGTAAAACGATTGCGCAGCAGGGGGCGCTCTGAAGTTGCTCAAAAAGTTCAGGCGCGTATTGCAGCCG M A K I D G K T I A Q V K S E V A Q K V Q A R I A A
Consensus Frame 1	GACTGCGGGCACCAGGACTGGCCGTTGTGCTGGTGGGTGG
1. folD of E. coli∆tolC Frame 1	GACTGCGGGCACCAGGACTGGCCGTTGTGCTGGTGGGTAGTAACCCTGCATCGCAAATTTATGTCGCAAACGCAAACGCAAGGCTTGTGA G L R A P G L A V V L V G S N P A S O L Y V A S K R K A C E
2. folD of mutant G8S-1 Frame 1	GACTGCGGGGCACCAGGACTGGCCGTTGTGCTGGTGGGTAGTAACCCTGCATCGCAAATTTATGTCGCAAAGCAAACGCAAGGCTTGTGA G L R A P G L A V V L V G S N P A S O I Y V A S K R K A C E
3. folD of mutant G8S-2 Frame 1	GACTGCGGGCACCAGGACTGGCCGTTGTGCTGGTGGGTAGTAACCCTGCATCGCAAATTTATGTCGCAAACGCAAAGGCTTGTGA G L R A P G L A V V L V G S N P A S O I Y V A S K R K A C E
4. folD of mutant K54N Frame 1	GACTGCGGGCACCAGGACTGGCCGTTGTGCTGGTGGGTAGTAACCCTGCATCGCAAATTTATGTCGCAAGCAA
5. folD of mutant Q98H Frame 1	GACTGCGGGCACCAGGACTGGCCGTTGTGCTGGGTGGGTAGTAACCCTGCATCGCAAATTTATGTCGCAAGCAA
6. folD of mutant ΔK54R55 Frame 1	GACTGCGGGGCACCAGGACTGGCCGTTGTGCTGGGTGGGT
	180 190 200 210 220 230 240 250 360
Consensus Frame 1	AGAACCGGGATCGGGATCCCCGCTCTTATGACCTCCCGGAAACCACCAGCGAACCGCGAGCTGCTGGAGCTTATCGATACGCTGAATGCC E V G F V S R S Y D E T S E A E L L E L I D T L N A
Consensus Frame 1 1. folD of E. coli∆tolC Frame 1	<mark>AGAĂGTCGGGTĮCGŢCTÇCCGCTČTTĄTGACCTČCÇGGAAACCĂCCAGCGAAGČGGAGCTGCTĞGAGCTTATCĞATACGCTGAĂTGCC</mark> AGAAGTCGGGTĮCGŢCTÇCCGCTÇTTĄTGACCŢCCÇGGAAACCAÇCAGCGAGCGGAGCTGCŢĞGAGCŢTAŢCGATACGCŢGAĂŢGCC
Consensus Frame 1 1. folD of E. coliΔtolC Frame 1 2. folD of mutant G8S-1 Frame 1	<mark>AGRĂGICGGGTICGTCTCCCGCTCTTATGACCTCCCGGRAACCĂCAGCGRAGCGGAGCTGCTĞGAGCTTATCĞATACGCTGAĂTGCC</mark> AGRAGTCGGGTICGTCTCCCGCTCTTATGACCTCCCGGRAACCACCAGCGRAGCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGRAGTCGGGTICGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGAGGCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC
Consensus Frame 1 1. folD of E. coli∆tolC Frame 1 2. folD of mutant G8S-1 Frame 1 3. folD of mutant G8S-2 Frame 1	AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGAAACCACCAGCGAAGCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGAAACCACCAGCGGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC
Consensus Frame 1 1. folD of E. coliátolC Frame 1 2. folD of mutant G8S-1 Frame 1 3. folD of mutant G8S-2 Frame 1 4. folD of mutant K54N Frame 1	AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGAAGCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGGAGCTTATCGATACGCTGAATGCC
Consensus Frame 1 1, folD of E. coliátolC Frame 1 2, folD of mutant G85-1 Frame 1 3. folD of mutant G85-2 Frame 1 4. folD of mutant K54N Frame 1 5. folD of mutant Q98H Frame 1	AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGAAACCACCAGCGAAGCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGAAACCACCAGCGAAGCGGAAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCGCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGCGGGAGCTTATCGATACGCTGAATGCC
Consensus Frame 1 1. folD of E. coliΔtolC Frame 1 2. folD of mutant G8S-1 Frame 1 3. folD of mutant G8S-2 Frame 1 4. folD of mutant K54N Frame 1 5. folD of mutant ΔK54R55 Frame 1	AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGAAACCACCAGCGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC B V S R Y D C C C G C C C C C C C C C C C C C C C
Consensus Frame 1 1. folD of E. coliΔtolC Frame 1 2. folD of mutant G85-1 Frame 1 3. folD of mutant G85-2 Frame 1 4. folD of mutant K54N Frame 1 5. folD of mutant Q98H Frame 1	AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGAAACCACCAGCGAAGCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGAAACCACCAGCGAAGCGGAACCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGAAACCACCAGCGAAGCGGAACCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGAAGCGGAACCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGAAGCGGAACCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGAGCTGCTGGGAGCTTATCGATACGCTGAATGCC
Consensus Frame 1 1. folD of E. coliΔtolC Frame 1 2. folD of mutant G8S-1 Frame 1 4. folD of mutant G8S-2 Frame 1 5. folD of mutant K54N Frame 1 6. folD of mutant ΔK54R55 Frame 1	AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGAAACCACCAGCGAAGCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGAAACCACCAGCGAAGCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGAAACCACCAGCGAAGCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGAAGCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGAAGCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGAAGCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCACCAGCGAAGCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCACCAGCGAAGCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCCAGCGAAACCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCCGGGAAACCACCAGCGGAACCGGGAGCTGCTGGGACCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCCGGGAAACCACCACCAGCGAAACCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC
Consensus Frame 1 1. folD of E. coliΔtolC Frame 1 2. folD of mutant G8S-1 Frame 1 4. folD of mutant G8S-2 Frame 1 5. folD of mutant K54N Frame 1 6. folD of mutant ΔK54R55 Frame 1 Consensus Frame 1 1. folD of E. coliΔtolC	AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGAAACCACCAGCGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGAAACCACCAGCGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTTTATGACCTCCCGGGAAACCACCAGCGAAGCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCGCTTTATGACCTCCCGGGAAACCACCAGCGAAGCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCGCTTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTTTATGACCTCCCGGAAACCACCAGCGGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCGCTTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGGGCTGCTGGAGCTTATCGGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGGGCTTATCGGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTTTATGACCTCCCGGGAAACCACCAGCGGAACCGGCGGGGCTGCTGGGAGCTTATCGGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGGACCTTATCGGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTTTATGGCTCCCGCGTTACCGGGGGGGTATTGGATAGCGTC MIC for <i>E. coli∆tolC</i> = 0.125 µg/ml
Consensus Frame 1 1. folD of E. coliΔtolC Frame 1 2. folD of mutant G8S-1 Frame 1 3. folD of mutant G8S-2 Frame 1 5. folD of mutant K54N Frame 1 6. folD of mutant ΔK54R55 Frame 1 Consensus Frame 1 1. folD of E. coliΔtolC Frame 1 2. folD of mutant G8S-1 Frame 1	AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGAAACCACCAGCGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGAAACCACCAGCGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTTTATGACCTCCCGGGAAACCACCAGCGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTTTATGACCTCCCGGGAAACCACCAGCGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCGCTTTATGACCTCCCGGGAAACCACCAGCGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCGCTTTATGACCTCCCGGGAAACCACCAGCGAAGCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCGCTTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTTTATGACCTCCCGGGAAACCACCAGCGGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC GGCAACCACCATCGGTTGCCGCTTTATGGCTGCCGCTTACCGGCGGGTATTGGTAAGCGTC GGCAACCACCATCGGATGGCATTCTGGTTCAACTGCCGTTACCGGCGGGTATTGGTAACGCTC MIC for <i>E. coli</i> ΔtolC = 0.125 µg/ml GACAACACCATCGATGGCATTCTGGTTCAACTGCCGTTACCGGCGGGTATTGGTAACGCTC MIC for <i>E. coli</i> ΔtolC = 0.125 µg/ml
Consensus Frame 1 1. folD of E. coliΔtolC Frame 1 2. folD of mutant G8S-1 Frame 1 3. folD of mutant G8S-2 Frame 1 4. folD of mutant K54N Frame 1 5. folD of mutant Q98H Frame 1 6. folD of mutant ΔK54R55 Frame 1 1. folD of E. coliΔtolC Frame 1 1. folD of mutant G8S-1 Frame 1 3. folD of mutant G8S-2 Frame 1 5. folD of mutant G	AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGAAACCACCAGCGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGAAACCACCAGCGAAGCGGGAGCTGCTGGAGCTTATCGATACGCTGAATGCC AGAAGTCGGGTTCGTCTCCCGCTCTTATGACCTCCCGGGAAACCACCAGCGAGGCGGGGGGGG
Consensus Frame 1 1. folD of E. coliàtolC Frame 1 2. folD of mutant G8S-1 Frame 1 3. folD of mutant G8S-2 Frame 1 4. folD of mutant ΔK54N Frame 1 6. folD of mutant ΔK54R55 Frame 1 1. folD of E. coliàtolC Frame 1 1. folD of E. coliàtolC Frame 1 3. folD of mutant G8S-1 Frame 1 3. folD of mutant G8S-2 Frame 1 4. folD of mutant G8S-2 Frame 1 4. folD of mutant K54N Frame 1	AGAACTCGGGTŢCGŢCTÇCCGCTŢTTĂŢGACCŢCCĢGGAAACCACCAGCGAACCGCGGAGCŢGCŢĞGAGCŢTAŢCGATACGCŢGAĂŢGCC AGAACŢCGGTŢCGŢCŢCCCCGCTŢTŢŢGACCŢCCĢGGAAACCACCAGCGAACCACCAGCGAACCŢGCŢGGACCŢTAŢCGATACGCŢGAĂŢGCC AGAACŢCGGTŢCGŢCŢCCCGCTŢTŢŢGACCŢCCĢGGAAACCACCAGCGAAGCGGGAGCŢGCŢGGACŢŢAŢCGATACGCŢGAŢGCC AGAAGŢCGGGŢŢCGŢCŢCCCGCŢŢŢŢĞĞŢCGCĞĞGAACCACCAGCGAAGCGGGAGCŢGCŢGGACŢŢAŢCGAŢACGCŢGAŢGCC AGAAGŢCGGGŢŢCGŢCŢCCCGCŢŢŢŢĞĞŢCGCĞĞGAACCACCAGCGAAGCGGAGCŢGCŢGGACŢŢAŢCGĂŢACGCŢGAĂŢGC AGAAGŢCGGGŢŢCGŢŢŢCGCŢŢŢŢĞĞŢCACŢGCĢĞGAACCACCAGCGAAGCGGAGCŢGCŢGGACŢŢAŢCGĂŢĂŢGCŢGAĂŢĞCC AGAAGŢCGGGŢŢCGŢŢŢCCGCŢŢŢŢŢĞĞŢCACŢGCĢĞGĂAACCACCAĞCGAAGCGGAGCŢGCŢGGAGCŢŢAŢCGĂŢĂŢĠCŢGAĂŢĞCC AGAACŢCGGGŢŢCGŢŢŢCCGCŢŢŢŢŢĞĞŢŢĂĊŢĞĞĞĞŢĂŢŢGĂŢĂĂĊĞĞĞĞACŢGCŢGGĂĞŢCŢGĞĂŢŢĊŢŢĂŢĞĞĊŢĞŢŢĂŢĞĞŢŢĂŢĞĞĞĞŢĂŢŢĠĂŢĊŢGĞĞĞŢĂŢŢĞĂŢĞĊŢĞĂ AGĂACŢCGĞĞŢŢCGŢŢŢCŢĞĞŢŢĊŢĊŢŢŢŢŢĞĞŢŢĂĊŢĞĞĞŢĂŢŢĞĂŢĂĞĊĞĞĞĂŢŢŢĞĂŢĞĞĞĞĂĊŢĞĊŢĞĞĞĞŢĂŢŢĞĂŢĞĊŢĞĂŢĞĞĊŢĂŢĞĞŢĂŢĞĞŢ AĞĂACŢCGĞĞŢŢCGŢŢŢĊĞŢŢŢŢĊĞĞŢŢĂĊŢĞĞĞĞŢĂŢŢĞĂŢĞĞĞĞĂŢŢĞŢĊĞĞĞĞŢĂŢŢĠĂŢŢĊŢĞĂŢĞĊŢĞĂŢĞĞŢĂŢĞĞŢ
Consensus Frame 1 1. folD of E. coliàtolC Frame 1 2. folD of mutant G8S-1 Frame 1 3. folD of mutant G8S-2 Frame 1 4. folD of mutant ΔK54N Frame 1 6. folD of mutant ΔK54R55 Frame 1 1. folD of E. coliàtolC Frame 1 1. folD of E. coliàtolC Frame 1 1. folD of mutant G8S-1 Frame 1 3. folD of mutant G8S-1 Frame 1 3. folD of mutant G8S-2 Frame 1 4. folD of mutant K54N Frame 1 5. folD of mutant K54N Frame 1 5. folD of mutant Q98H Frame 1 5. folD of mutant Q98H Frame 1 5. folD of mutant Q98H Frame 1 5. folD of mutant Q98H	AGAACTCGGGTŢCGŢCŢCCCGCŢŢTĂŢGACCŢCCGGGAAACCACCAGCGAACCGCGGAGCŢGCŢĞGAGCŢTAŢCGATACGCŢGAĂŢGCC AGAACŢCGGTŢCGŢŢCCŢCŢCCGCŢŢŢŢGACCŢCCGGGAAACCACCAGCGAGCG

Supplementary Figure 2. Partial alignment of *folD* genes and MIC of *E. coli\DeltatolC* wt and four carolacton-resistant mutants. All point mutations and base deletions were highlighted in different colours. The *folD* gene of mutant  $\Delta$ K54R55 has a 6 bp deletion affecting the codons for Lysine 54, Arginine 55 and Lysine 56. The MIC values of carolacton against *E. coli\DeltatolC* wt strain and five carolacton-resistant mutants are shown at the end of alignments.



**Supplementary Figure 3. Alignment of FolD enzymes studied in this paper.** The residues indicated by blue squares are residues mutated in FolD proteins from carolacton-resistant mutants. The residues indicated by red squares are important for carolacton binding. The numbering of residues is according to ecFolD.



Supplementary Figure 4. All purified FolD proteins analyzed by SDS-PAGE and LC-MS. (a) The SDS-PAGE gel of all purified FolDs. M: PageRuler prestained protein ladder; 1: His-Tag fusion FolD of *E. coli\DeltatolC*; 2: FolD of *E. coli\DeltatolC* (His-tag cleaved off) 3: His-Tag fusion FolD of *E. coli\DeltatolC* G8S; 4: His-Tag fusion FolD of *E. coli\DeltatolC* Q98H; 5: His-Tag fusion FolD of *E. coli\DeltatolC* K54N; 6: His-Tag fusion FolD of *E. coli\DeltatolC*  $\Delta$ K54R55; 7: His-Tag fusion FolD of *S. pneumoniae* TIGR4; 8: His-Tag fusion hsMTHFD1\_DC; 9: His-Tag cleaved hsMTHFD1\_DC; 10: His-Tag fusion hsMTHFD2; 11: His-Tag cleaved hsMTHFD2. (b-l) Deconvoluted mass spectra of all FolD proteins. Cal. stands for calculated average neutral mass according to the protein sequence; Obs. indicates the average neutral mass observed by LC-MS measurement. The mass of hsMTHFD1\_DC is 14 Da heavier than its calculated mass, which is possibly the result of methylation on one residue.



**Supplementary Figure 5. Enzyme kinetics of ecFolD.** Data are presented as means  $\pm$  s.e.m of 3 independent replicates. Enzyme specific activity values were calculated based on the  $V_{max}$  values obtained via Michaelis-Menten or Hill fitting. The one-way ANOVA test was used for statistical analysis, *P*<0.01. (a) The determination of 5,10-CH<sub>2</sub>-THF  $K_M$  in the presence of 1 mM NADP<sup>+</sup> and 5,10-CH<sub>2</sub>-THF apparent  $K_M$  in the presence of 1 mM NADP<sup>+</sup> and 10 nM carolacton. (b) The determination of NADP<sup>+</sup>  $K_M$  in the presence of 1 mM 5,10-CH<sub>2</sub>-THF and NADP<sup>+</sup> apparent  $K_M$  in the presence of 1 mM 5,10-CH<sub>2</sub>-THF and 10 nM carolacton. (c) The determination of 5,10-CH=THF  $K_M$  and the determination of 5,10-CH<sub>2</sub>-THF and 10 nM carolacton. (c) The determination of 5,10-CH=THF  $K_M$  and the determination of 5,10-CH=THF apparent  $K_M$  in the presence of 5,10-CH=THF apparent  $K_M$  in t



Supplementary Figure 6. The comparison between ecFolD and ecFolD<sup>Meth</sup> (The lysine methylation processed ecFolD). (a) The column plot for the comparison of DH activity of ecFolD and ecFolD<sup>Meth</sup>, the specific activity calculated based on varied 5,10-CH<sub>2</sub>-THF and NADP<sup>+</sup> both showed. Data are presented as means  $\pm$  s.e.m of 3 independent replicates. DH specific activity values were calculated based on the  $V_{max}$  values obtained via Michaelis-Menten fitting. The one-way ANOVA test was used for statistical analysis, P<0.01. (b) The determination of IC<sub>50</sub> for carolacton inhibition against ecFolD and ecFolD<sup>Meth</sup>. Data are presented as means  $\pm$  s.e.m of 3 independent via logistic dose–response fitting. The one-way ANOVA test was used for statistical analysis, P<0.01.



**Supplementary Figure 7. Superposition of ecFolD crystallized in this study (gray) with the published structure (PDB ID 1B0A, cyan).** (a) Side view. (b) Structures rotated by 90 ° around the horizontal axis towards the viewer.



**Supplementary Figure 8. (a)** Lysine methylation is essential for the ecFolD crystal structures reported in this study. ecFolD protomers are shown as cartoon representations in individual colors. Dimethylated lysine residues involved in crystal contacts are shown as spheres with their color corresponding to the protomer they belong to. (b) Stereo view of the 2Fo-Fc electron density map for carolacton and K54. ecFolD is shown as a yellow cartoon, carolacton and K54 as sticks and the electron density as a blue isomesh.



Supplementary Figure 9. Ligplot diagram showing the detailed interactions of carolacton with ecFoID.



Supplementary Figure 10. Superposition of ecFolD (blue) with hsMTHFD2 (PDB ID 1DIA, magenta). (a) Carolacton (lime) clashes with the bound substrate analog L345899 (cyan). (b) carolacton clashes with co-factor  $NAD^+$  (green). (c) New carolacton analog "carylacton" can engage with Y50 but has a tail which is too long to hydrogen-bond with G261.



**Supplementary Figure 11. Effect of mutation G8S on carolacton binding of ecFolD. Left**: In wt ecFolD (gray) G8 is hydrogen-bonded to G261 (both orange), which in turn forms hydrogen bonds with carolacton (lime). **Right**: In the mutant G8S, the side-chain of the serine clashes with G261 in all conformations. Therefore, the loop containing G261 needs to move to accommodate S8, which results in a clash with carolacton.



Supplementary Figure 12. Comparison of ecFolD CYH activity between wt and the carolacton-resistant mutants.



Supplementary Figure 13. Growth curves of *E.coli*  $\Delta tolC$  and its carolacton-resistant mutant strains.



Supplementary Figure 14. SPR assays of spFolD and ecFolD mutants with carolacton. (a) SPR analysis shows carolacton does not bind to ecFolDK54N. (b) SPR analysis shows carolacton has weak binding to ecFolDQ98H, the dissociation of carolacton is much faster than wt ecFolD, but the  $K_D$  cannot be determined uniquely by SPR kinetics calculations. (c) The affinity calculation based on SPR analysis of carolacton binding to ecFolD Q98H gives a  $K_D$  which is 20  $\mu$ M. (d) SPR analysis of carolacton binding to spFolD.



Supplementary Figure 15. The determination of IC<sub>50</sub> for carolacton inhibition against all wt FolDs in this study. Data are presented as means  $\pm$  s.e.m of 3 independent replicates. IC<sub>50</sub> values were obtained via logistic dose-response fitting. The one-way ANOVA test was used for statistical analysis, *P* < 0.01. (a) IC<sub>50</sub> determination for carolacton against the dehydrogenase activity of FolD from different organisms. (b) IC<sub>50</sub> determination for carolacton against the cyclohydrolase activity of FolD from different organisms.



Supplementary Figure 16. Enzyme kinetics of spFolD. Data are presented as means  $\pm$  s.e.m of 3 independent replicates. Enzyme specific activity values were calculated based on the  $V_{max}$  values obtained via Michaelis-Menten fitting. The one-way ANOVA test was used for statistical analysis, P<0.01. (a) The determination of 5,10-CH<sub>2</sub>-THF  $K_M$  in the presence of 1 mM NADP and 5,10-CH<sub>2</sub>-THF apparent  $K_M$  in the presence of 1 mM NADP and 25 nM carolacton. (b) The determination of NADP  $K_M$  in the presence of 1 mM 5,10-CH<sub>2</sub>-THF and NADP apparent  $K_M$  in the presence of 1 mM 5,10-CH<sub>2</sub>-THF and 25 nM carolacton. (c) The determination of 5,10-CH=THF  $K_M$  and the determination of 5,10-CH=THF apparent  $K_M$  in the presence of 5,10-CH=THF apparent  $K_M$  in the presence of 5,10-CH=THF apparent  $K_M$  in the presence of 25 nM carolacton.



Supplementary Figure 17. Superposition of ecFolD (gray) with human mitochondrial hsMTHFD2 (PDB ID 5TC4, magenta).



Supplementary Figure 18. The enzyme kinetics measurement of hsMTHFD1\_DC and hsMTHFD2. Data are presented as means ± s.e.m of 3 independent replicates. Enzyme specific activity values were calculated based on the  $V_{max}$  values obtained via Michaelis-Menten or Hill fitting. The one-way ANOVA test was used for statistical analysis, P<0.01. (a) The determination of 5,10-CH<sub>2</sub>-THF  $K_{\rm M}$  for hsMTHFD1\_DC in the presence of 0.4 mM NADP and 5,10-CH<sub>2</sub>-THF apparent K<sub>M</sub> for hsMTHFD1\_DC in the presence of 0.4 mM NADP and 30 nM carolacton. (b) The determination of NADP  $K_{\rm M}$  for hsMTHFD1 DC in the presence of 0.4 mM 5,10-CH<sub>2</sub>-THF and NADP apparent  $K_{\rm M}$  for hsMTHFD1 DC in the presence of 0.4 mM 5,10-CH<sub>2</sub>-THF and 25 nM carolacton. (c) The determination of 5,10-CH=THF  $K_{\rm M}$  for hsMTHFD1 DC and the determination of 5,10-CH=THF apparent  $K_{\rm M}$  for hsMTHFD1 DC in the presence of 25 nM carolacton. (d) The determination of 5,10-CH<sub>2</sub>-THF  $K_{\rm M}$  for hsMTHFD2 in the presence of 0.6 mM NADP and 5,10-CH<sub>2</sub>-THF apparent  $K_{\rm M}$ for hsMTHFD2 in the presence of 0.6 mM NADP and 20 nM carolacton. (e) The determination of NADP K<sub>M</sub> for hsMTHFD2 in the presence of 0.6 mM 5,10-CH<sub>2</sub>-THF and NADP apparent  $K_{\rm M}$  for hsMTHFD2 in the presence of 0.6 mM 5,10-CH<sub>2</sub>-THF and 10 nM carolacton. (f) The determination of 5,10-CH=THF  $K_{\rm M}$  for hsMTHFD2 and the determination of 5,10-CH=THF apparent  $K_{\rm M}$  for hsMTHFD2 in the presence of 20 nM carolacton.

**Supplementary Table 1.** Characteristics of data obtained during whole genome sequencing and analysis.

Sample	Mutant 1	Mutant 2	Mutant 3	Mutant 4	Mutant 5	WT		
Technology		MiSeq, Illumina, Paired-End						
Number of reads	2,426,032	2,423,998	2,376,598	2,513,800	2,314,528	2,372,346		
Read Length, bp			2:	51				
Genome size, bp			4,641,652	(expected)				
Coverage (theoretical)	131	131	128	135	125	128		
Coverage (post-assembly)	108	108	108	116	107	109		

**Supplementary Table 2.** List of mutations found in carolacton-resistant mutants (CaroM1-CaroM5) when compared to the control sample (WT).

Gene	Mutation	CaroM1	CaroM2	CaroM3	CaroM4	CaroM5	WT
bifunctional 5,10-	G8S		+	+			
methylenetetrahydr							
ofolate	K54N				+		
dehvdrogenase/							
5.10-	Q98H					+	
methenvltetrahvdr							
ofolate							
cyclohydrolase	K54 K56delinsK	+					
(FolD)	-						
(101D)							

Bacteria	FolD	DH activity (specific activity µmol min <sup>-1</sup> mg <sup>-1</sup> )	CYH activity (specific activity µmol min <sup>-1</sup> mg <sup>-1</sup> )	Reference
E. coli	ecFolD	$173.57 \pm 6.32$	298.81 ± 37.87	This study
Acinetobacter baumannii	abFolD	$161.4 \pm 5.7$	$350.2 \pm 4.4$	Eadsforth et al. <sup>1</sup>
E. coli	ecFolD	200	33	D'Ari et al. <sup>2</sup>
E. coli	ecFolD	31.2	6.12	Dev et al. <sup>3</sup>
E. coli	ecFolD	19	39	Sah et al. <sup>4</sup>
Peptostreptococcus productus	ppFolD	627	ND	Wohlfarth et al. <sup>5</sup>
Clostridium formicoaceticum	cfFolD	ND	469	Clark et al. <sup>6</sup>

**Supplementary Table 3.** The specific activity of dehydrogenase and cyclohydrolase of bacterial FolD reported in current and previous studies.

	carolacton inhibition of	on DH activity	carolacton inhibition on CYH activity
enzyme	$K_i$ (nM)	$K_i$ (nM)	$K_i$ (nM)
	(5, 10-CH <sub>2</sub> -THF)	(NADP <sup>+</sup> ) <sup>a</sup>	(5, 10-CH=THF)
ecFolD	21.31	10.91	31.60
spFolD	42.18	34.21	38.07
hsMTHFD1 DC301	5.93	58.69	20.59
hsMTHFD2	6.42	16.69	12.67

**Supplementary Table 4.** The inhibition constants of carolacton against all wt FolD in this study.

 $a^{a}$  it is nicotinamide adenine dinucleotide (NAD<sup>+</sup>) in the case of hsMTHFD2

Enzyme	k <sub>on</sub> (1/Ms)	$k_{off}(1/s)$	$K_D$ (M) (by kinetics)	$K_D$ (M) (by affinity)
ecFolD	1.043 x 10 <sup>6</sup>	0.01003	9.62 x 10 <sup>-9</sup>	8.76 x 10 <sup>-9</sup>
spFolD	1.188 x 10 <sup>6</sup>	0.03156	26.57 x 10 <sup>-9</sup>	18.74 x 10 <sup>-9</sup>
hsMTHFD2	1.170 x 10 <sup>6</sup>	0.02220	18.98 x 10 <sup>-9</sup>	14.44 x 10 <sup>-9</sup>
ecFolD Q98H	5.815 x 10 <sup>4</sup>	0.6084	1.05 x 10 <sup>-5</sup>	1.95 x 10 <sup>-5</sup>

**Supplementary Table 5.** Binding kinetic information ( $k_{on}$  and  $k_{off}$ ) and  $K_D$  calculated by kinetics or affinity.

Supplementary Table 6. Data collection and refinement statistics

	Apo-FolD <sup>Meth</sup>	FolD <sup>Meth</sup> -caro	FolD <sup>Meth</sup> Q98H
Data collection	_		
Space group	P1 2 <sub>1</sub> 1	P1 2 <sub>1</sub> 1	P1 2 <sub>1</sub> 1
Cell dimensions			
<i>a</i> , <i>b</i> , <i>c</i> (Å)	99.6, 79.8, 101.4	99.7, 81.1, 101.0	100.1, 79.4, 101.8
α, β, γ (°)	90.0, 113.3, 90.0	90.0, 112.9, 90.0	90.0, 113.8, 90.0
Resolution (Å)	1.89 (1.94-1.89)	2.10 (2.21-2.10)	1.90 (2.00-1.90)
R <sub>merge</sub>	8.4 (79.80)	9.1 (60.6)	6.1 (54.1)
Ι/σΙ	17.9 (2.4)	10.4 (2.5)	22.1 (4.4)
Completeness (%)	99.8 (98.9)	98.9 (99.8)	98.9 (99.2)
Redundancy	6.7 (6.5)	4.4 (4.5)	9.7 (9.9)
Refinement			
Resolution (Å)	36.59 - 1.89	45.88 - 2.10	46.56 - 1.90
No. reflections	116,659	85,476	113,624
$R_{\rm work}$ / $R_{\rm free}$	0.190/0.216	0.175/0.206	0.186/0.198
No. atoms			
Protein	8640	8441	8658
Ligand/ion		132	
Water	1014	588	876
B-factors	36.22	42.23	34.75
Protein	35.90	41.65	33.97
Ligand/ion		69.76	
Water	38.92	44.16	42.51
R.m.s. deviations			
Bond lengths (Å)	0.003	0.005	0.013
Bond angles (°)	0.65	0.70	1.11

	Dehydrogenase						
enzyme	substrate	$K_{\rm m}$ ( $\mu$ M)	$k_{\rm cat}$ (s <sup>-1</sup> )	$\frac{k_{\text{cat}}/K_{\text{m}}}{(\text{s}^{-1} \ \mu\text{M}^{-1})}$	Specific activity (µmol min <sup>-1</sup> mg <sup>-1</sup> )		
F - ID	5,10-CH <sub>2</sub> -THF	$76.47 \pm 8.27$	$90.34 \pm 3.29$	1.18	$173.57 \pm 6.32$		
ecroid	NADP	$186.51 \pm 21.02$	$89.64 \pm 3.54$	0.48	$172.22 \pm 6.81$		
asEalD C8S	5,10-CH <sub>2</sub> -THF	$179.44 \pm 26.56$	$20.27 \pm 1.75$	0.11	$35.67 \pm 3.08$		
ecroid G85	NADP	$510.80 \pm 35.67$	$20.74\pm0.71$	0.04	$36.50 \pm 1.25$		
asEalD K54N	5,10-CH <sub>2</sub> -THF	$27.03 \pm 1.85$	$18.19 \pm 0.39$	0.67	$32.06 \pm 0.39$		
ecroid K34N	NADP	$173.31 \pm 21.66$	$16.00 \pm 0.40$	0.09	$28.20 \pm 0.68$		
asEalD AV54D55	5,10-CH <sub>2</sub> -THF	$61.75 \pm 7.29$	$0.81 \pm 0.03$	0.01	$1.44 \pm 0.06$		
ecroid AK34K33	NADP	$675.63 \pm 86.00$	$1.16 \pm 0.06$	0.0017	$2.06 \pm 0.11$		
asEalD 008H	5,10-CH <sub>2</sub> -THF	$160.57 \pm 15.87$	$8.28 \pm 0.34$	0.05	$14.58 \pm 0.59$		
ecFoID Q98H	NADP	$311.53 \pm 55.44$	$6.67 \pm 0.45$	0.02	$11.75 \pm 0.79$		
anEalD	5,10-CH <sub>2</sub> -THF	$76.52 \pm 9.25$	$68.10 \pm 3.11$	0.89	$118.69 \pm 5.42$		
sproid	NADP	$70.33 \pm 5.53$	$62.30 \pm 1.02$	0.89	$108.59 \pm 1.77$		
haMTHED1 DC201	5,10-CH <sub>2</sub> -THF	$5.54 \pm 0.66$	$4.32 \pm 0.35$	0.78	$7.15 \pm 0.59$		
lisivi i HFD1 DC301	NADP	$27.13 \pm 6.55$	$4.85 \pm 0.58$	0.18	$8.04 \pm 0.96$		
heMTHED2	5,10-CH <sub>2</sub> -THF	$37.29 \pm 8.05$	$15.04 \pm 1.14$	0.40	$25.70 \pm 1.95$		
IISIVI I HF D2	NADP	$171.67 \pm 9.28$	$33.12 \pm 0.43$	0.19	$28.29 \pm 0.74$		
0.000		0	Cyclohydrolase				
enzyme	substrate	$K_{\rm m}$ ( $\mu$ M)	$k_{\rm cat}({\rm s}^{-1})$	$\frac{k_{\rm cat}/K_{\rm m}}{({\rm s}^{-1}\mu{\rm M}^{-1})}$	Specific activity (µmol min <sup>-1</sup> mg <sup>-1</sup> )		
ecFolD	5,10-CH=THF	$26.63 \pm 6.77$	$155.53 \pm 19.71$	5.84	$298.81 \pm 37.87$		
spFolD	5,10-CH=THF	$41.08 \pm 8.38$	$91.\overline{33 \pm 7.97}$	2.22	$159.17 \pm 13.88$		
hsMTHFD1 DC301	5,10-CH=THF	$42.61 \pm 9.39$	$137.56 \pm 13.93$	3.23	$227.79 \pm 23.07$		
hsMTHFD2	5,10-CH=THF	$16.14 \pm 3.69$	$242.44 \pm 22.41$	15.02	$414.23 \pm 38.29$		

Supplementary Table 7. Enzyme kinetic parameters for all FolD enzymes in this study.

	carolacton inhibition on DH		carolacton inhibition on CYH		
enzyme	act	ivity	activity		
	IC <sub>50</sub> (nM)	$IC_{50} (nM)$ $IC_{80} (nM)$		IC <sub>80</sub> (nM)	
ecFolD	15.49	52.22	49.83	180.01	
ecFolD G8S	86.49	736.59	ND	ND	
ecFolD K54N	ND <sup>a</sup>	ND	ND	ND	
ecFolD ΔK54R55	ND	ND	ND	ND	
ecFolD Q98H	313.21	1931.66	ND	ND	
spFolD	36.80	168.90	38.09	101.34	
hsMTHFD1 DC301	38.05	77.58	19.45	67.54	
hsMTHFD2	6.50	31.71	85.73	201.78	

Supplementary Table 8. The  $IC_{50}$  and  $IC_{80}$  values determined for carolacton inhibition on different FolD enzymes in this study.

<sup>a</sup> ND means not determined, because either the enzymatic activity is totally abolished or too low or the enzymatic activity is not affected.

Plasmid	Relevant genotype	Reference
pEHISTEV	kan <sup>R</sup>	Liu, 2009 <sup>7</sup>
pEHISTEV::ecfolD	pEHISTEV, kan <sup>R</sup> , <i>folD</i> <sub>Eco</sub>	This work
pEHISTEV::ecfolD G8S	pEHISTEV, kan <sup>R</sup> , <i>folD</i> <sub>EcoG8S</sub>	This work
pEHISTEV::ecfolD Q98H	pEHISTEV, kan <sup>R</sup> , <i>folD</i> <sub>EcoQ98H</sub>	This work
pEHISTEV::ecfolD K54N	pEHISTEV, kan <sup>R</sup> , <i>folD</i> <sub>EcoK54N</sub>	This work
pEHISTEV::ecfolD AK54R55	pEHISTEV, kan <sup>R</sup> , <i>folD</i> <sub>EcoAK54R55</sub>	This work
pEHISTEV::spfolD	pEHISTEV, kan <sup>R</sup> , <i>folD</i> <sub>Spn</sub>	This work
pEHISTEV::mthfd1_DC	pEHISTEV, kan <sup>R</sup> , <i>mthfd1_DC</i>	This work
pEHISTEV::mthfd2	pEHISTEV, kan <sup>R</sup> , <i>mthfd2</i>	This work

**Supplementary Table 9**. Plasmids used in this study.

11 0	U	
Target gene	Primer	Sequences (5' to 3') <sup>a</sup>
E. $coli\Delta tolC$ folD, Q98H,	ecFolD_NcoI_F	ATA <u>CCATGG</u> GCGCAGCAAAGATTATTGACGGTAAAAC
κ54Ν, ΔΚ54Κ55 <i>JoiD</i> –	ecFolD_HindIII_R	GTC <u>AAGCTT</u> TTACTCATCCTGTGGATCATGATAT
E. coli∆tolC G8S folD	G8S_NcoI_F	ATA <u>CCATGG</u> GCGCAGCAAAGATTATTGACAGTAAAAC
	ecFolD_HindIII_R	GTC <u>AAGCTT</u> TTACTCATCCTGTGGATCATGATAT
S. pneumoniae TIGR4	spFolD_EcoRI_F	TCC <u>GAATTC</u> ACACAGATTATTGATGGGAAAGCTTTA
j0iD	spFolD_XhoI_R	GCA <u>CTCGAG</u> TTATTTTCTATCCAATGTCCTAAGTG
Mthfd1 <sup>b</sup>	hsMTHFD1_NcoI_F	GCG <u>CCATGG</u> CTCCAGCAGAAATCCTGAA
	hsMTHFD1_HindIII_R	CGC <u>AAGCTT</u> TCACTGAATCATCCACTTTCCT
mthfd2 (without signal	hsMTHFD2_EcoRI_F	TCC <u>GAATTC</u> ATGGAAGCTGTTGTCATTTCTGGAAG
	hsMTHFD2_HindIII_R	CGC <u>AAGCTT</u> TTAATTAGTGGCTACCCCAA

Supplementary Table 10. Oligonucleotides used for constructing FolD expression plasmids.

<sup>a</sup>The underlined characters indicate the restriction sites introduced; <sup>b</sup>only dehydrogenase and cyclohydrolase encoding region

## Supplementary Table 11. The buffers for protein purification

Protein	Lysis buffer	Elution buffer	Desalting buffer	Gel filtration buffer
				10 mM HEPES
ecFolD				150 mM NaCl
	20 mM Bis-Tris pH 6.8	20 mM Bis-Tris pH 6.8	20 mM Bis-Tris pH 6.8	1mM TCEP
ecFolDG8S	150 mM NaCl	150 mM NaCl	150 mM NaCl	
ecFolDQ98H	20 mM imidazole	250 mM imidazole		
ecFolDK54N	-			
ecFolD∆K54R55	-			
spFolD	20 mM Bis-Tris pH 6.8	20 mM Bis-Tris pH 6.8	20 mM Bis-Tris pH 6.8	
	200 mM NaCl	200 mM NaCl	200 mM NaCl	
MTHFD1_DC	10% glycerol	10% glycerol	10% glycerol	
	20 mM imidazole	250 mM imidazole	1 mM TCEP	
	1 mM TCEP	1 mM TCEP		10 mM HEPES
				150 mM NaCl
	20 mM Tris-HCl pH 8.0	20 mM Tris-HCl pH 8.0	20 mM Tris-HCl pH 8.0	1mM TCEP
MTHFD2	200 mM NaCl	200 mM NaCl	200 mM NaCl	
	10% glycerol	10% glycerol	10% glycerol	
	20 mM imidazole	250 mM imidazole		
1	1			1

**Supplementary Table 12.** Ramachandran statistics for the ecFolD strutcures after final refinement.

	ecFolD <sup>Meth</sup>	ecFolD <sup>Meth</sup> -carolacton	ecFolD <sup>Meth</sup> Q98H
Ramachandran	95.75	97.14	96.77
favored (%)			
Ramachandran	3.97	2.77	3.05
allowed (%)			
Ramachandran	0.27	0.09	0.18
outliers (%)			

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