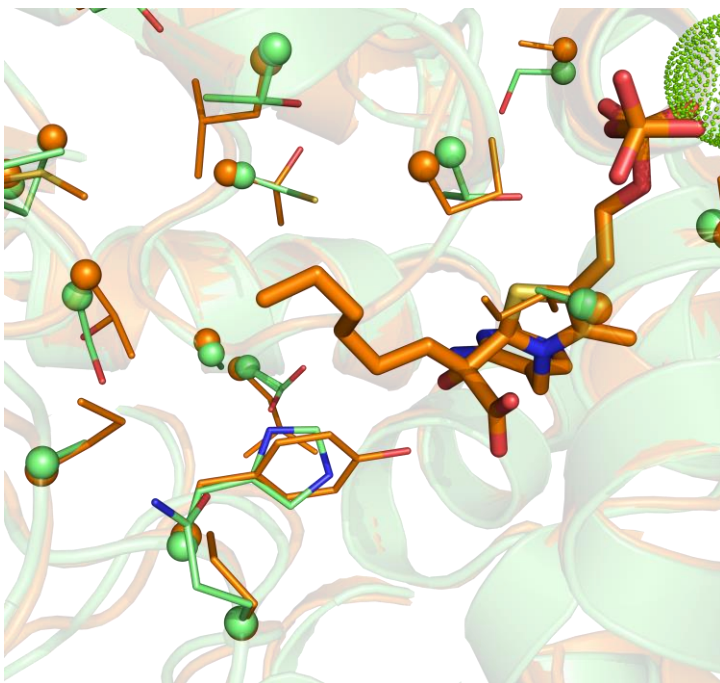
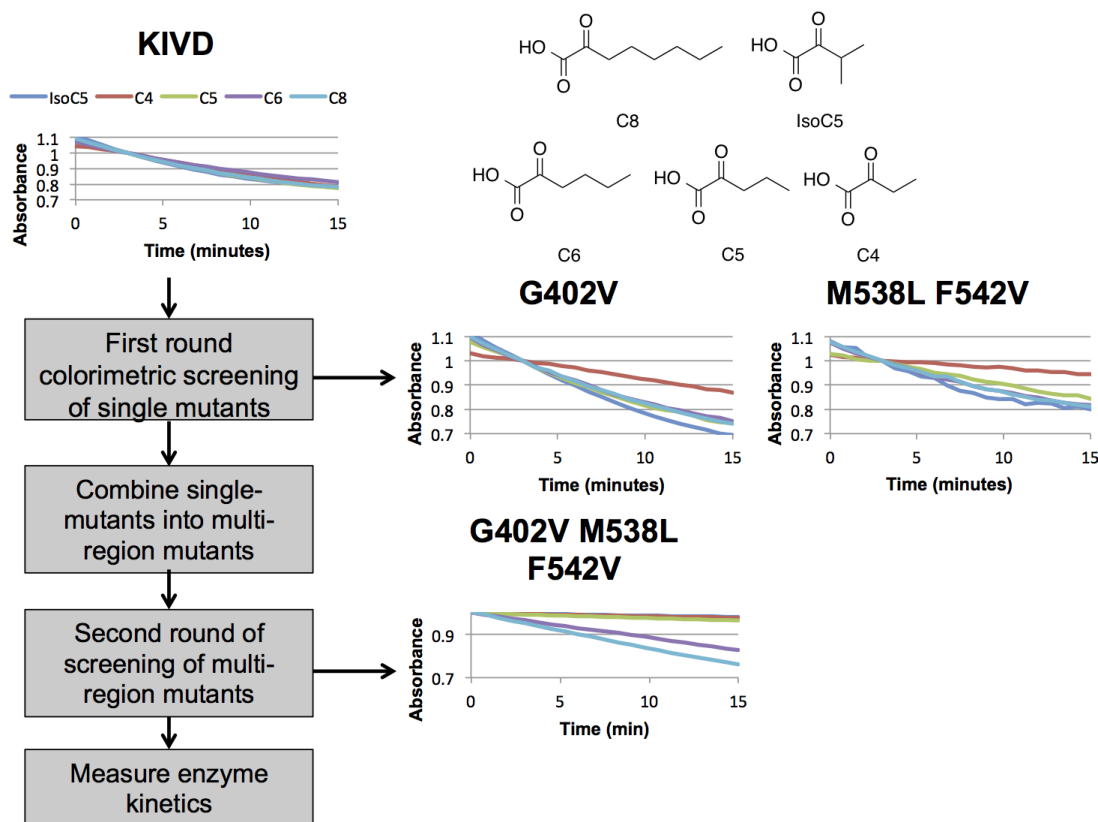
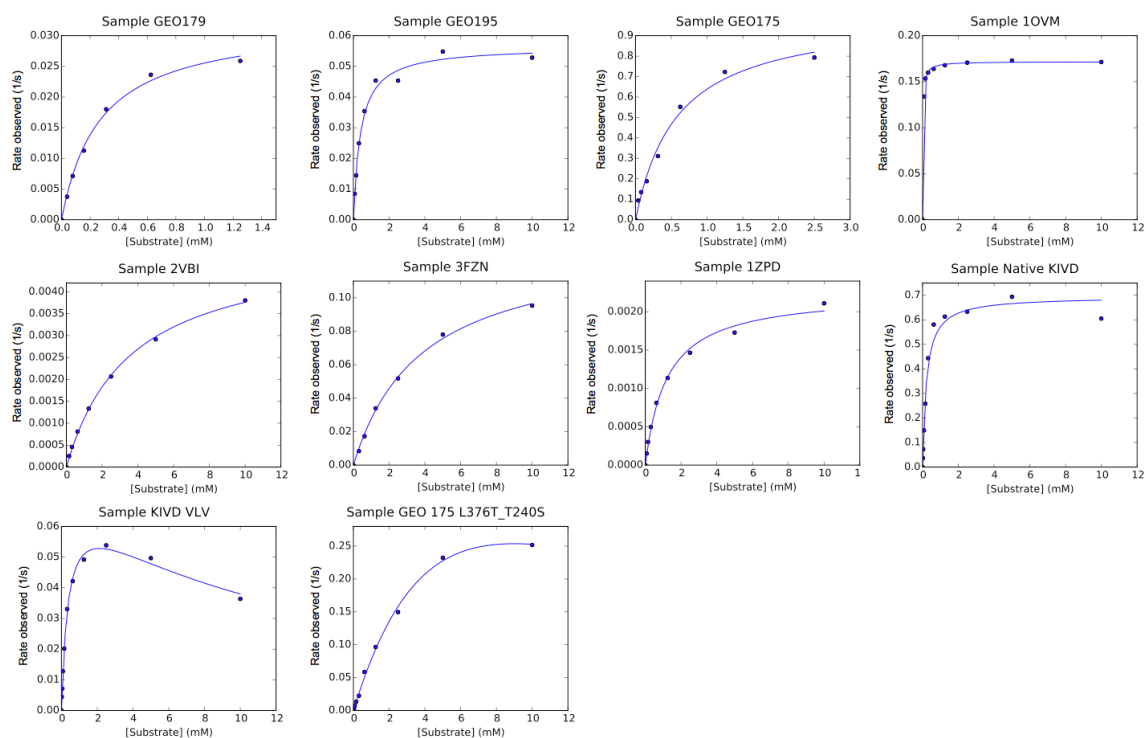




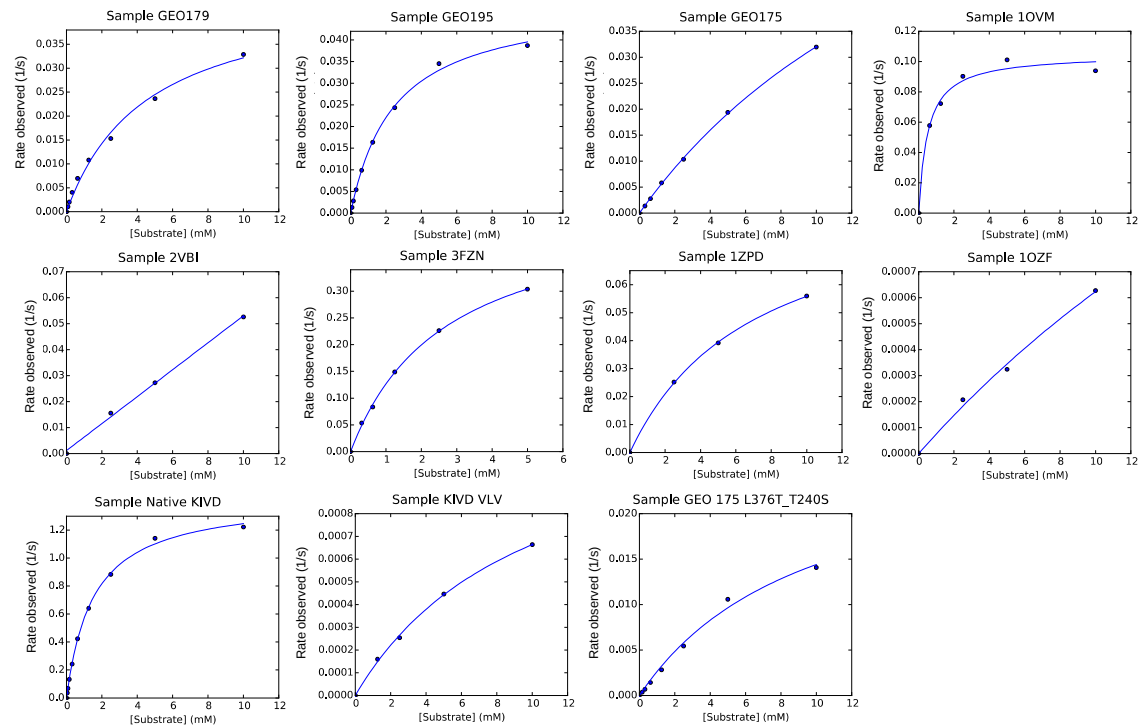
**Supplementary Figure 1.** Overlay of active sites between GEO 175 (orange) and BFD (green). Residues that are different between the proteins are shown in sticks and their corresponding alpha carbons are shown as spheres.



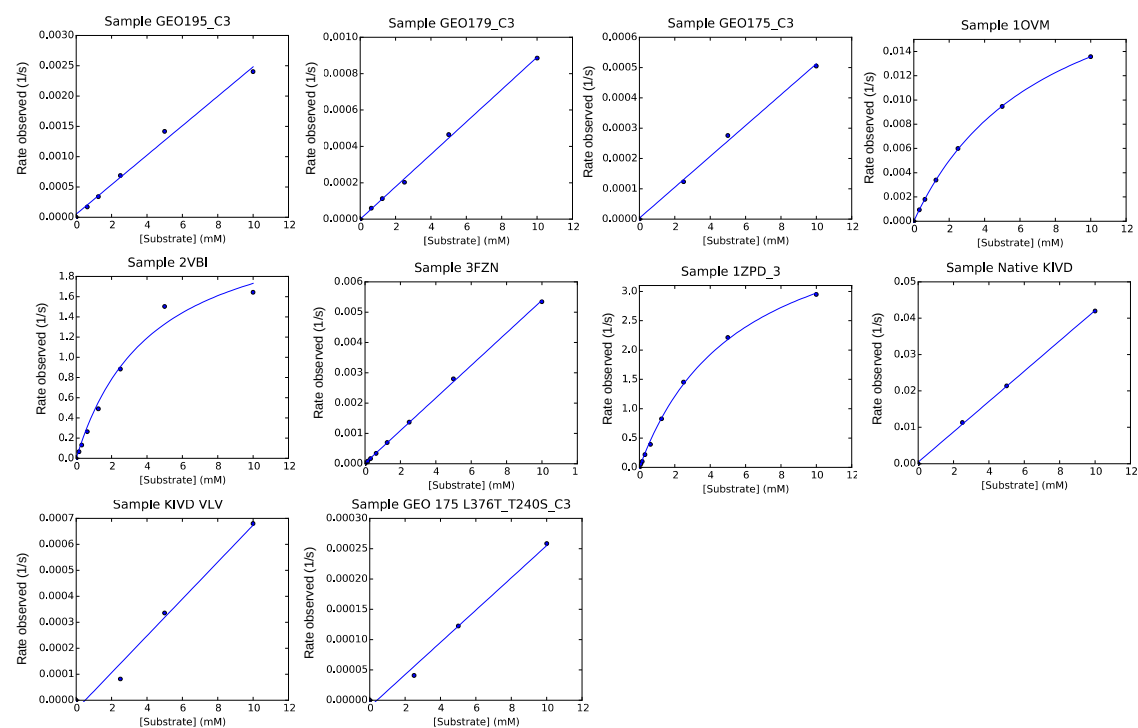
**Supplementary Figure 2.** Screening process for identifying engineered KIVD with altered substrate specificity. Pertinent screening results/candidate mutants are shown (graphs) which contributed directly to finding KIVD\_VLV. Colorimetric assays (graphs) measured approximate enzymatic activity of screened mutants for ketoisovalerate (IsoC5), 2-ketobutyrate (C4), 2-ketovalerate (C5), 2-ketohexanoate (C6), and 2-ketooctanoate (C8). Higher activity is shown by larger downwards slope of [Absorbance]/[Minute]; greater specificity, relative Kivd, was observed in mutants with reduced activity (smaller downward slope) for IsoC5, C4, and C5, and only slightly affected or greater activity (similar or larger downward slope) for C6 and C8. First round high-throughput colorimetric screening of single mutants identified candidates such as G402V and M538L/F542V. Mutations from each region that showed potential in altering the specificity of KIVD were then combined to produce multi-region mutants through PCR-site directed mutagenesis using the plasmids of KIVD single region mutants as templates. These KIVD multi-region mutants, such as G402V M538L F542V (KIVD\_VLV) were then screened a second time with the colorimetric assays. Enzyme kinetics was then measured for mutant KIVD\_VLV.



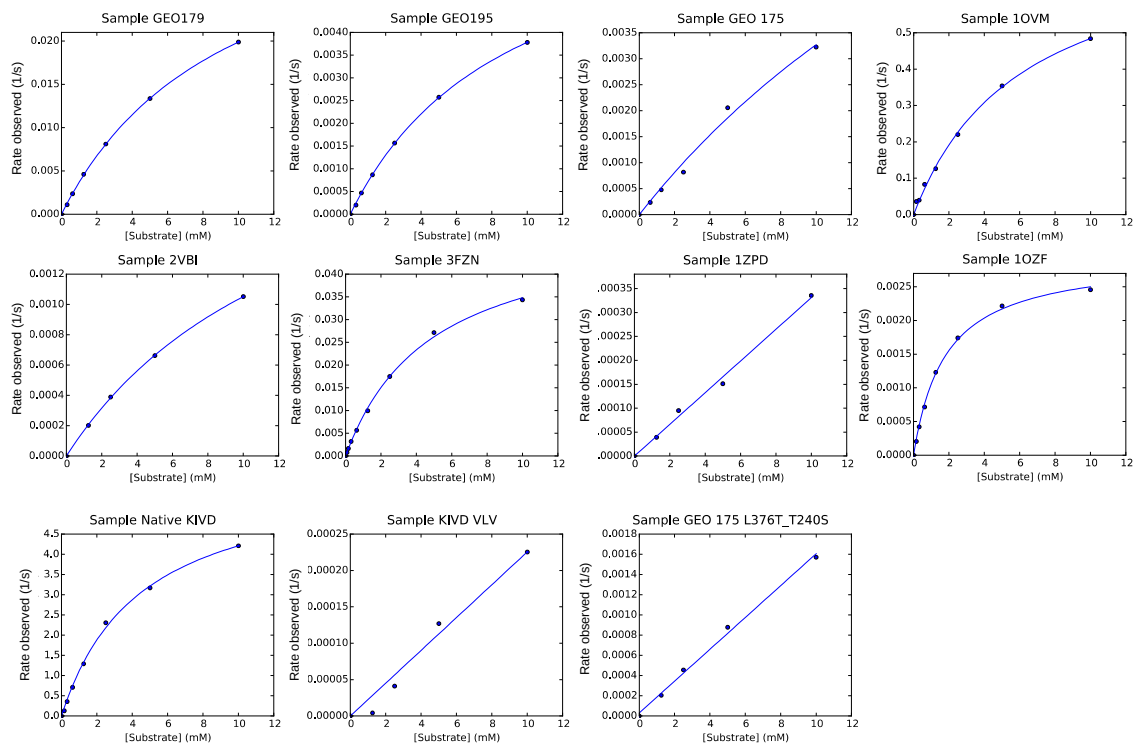
**Supplementary Figure 3.** Michaelis-Menten curve fit of tested enzymes with C8 substrate. The mutant KIVD\_VLV shows inhibition kinetics under high concentration of C8.



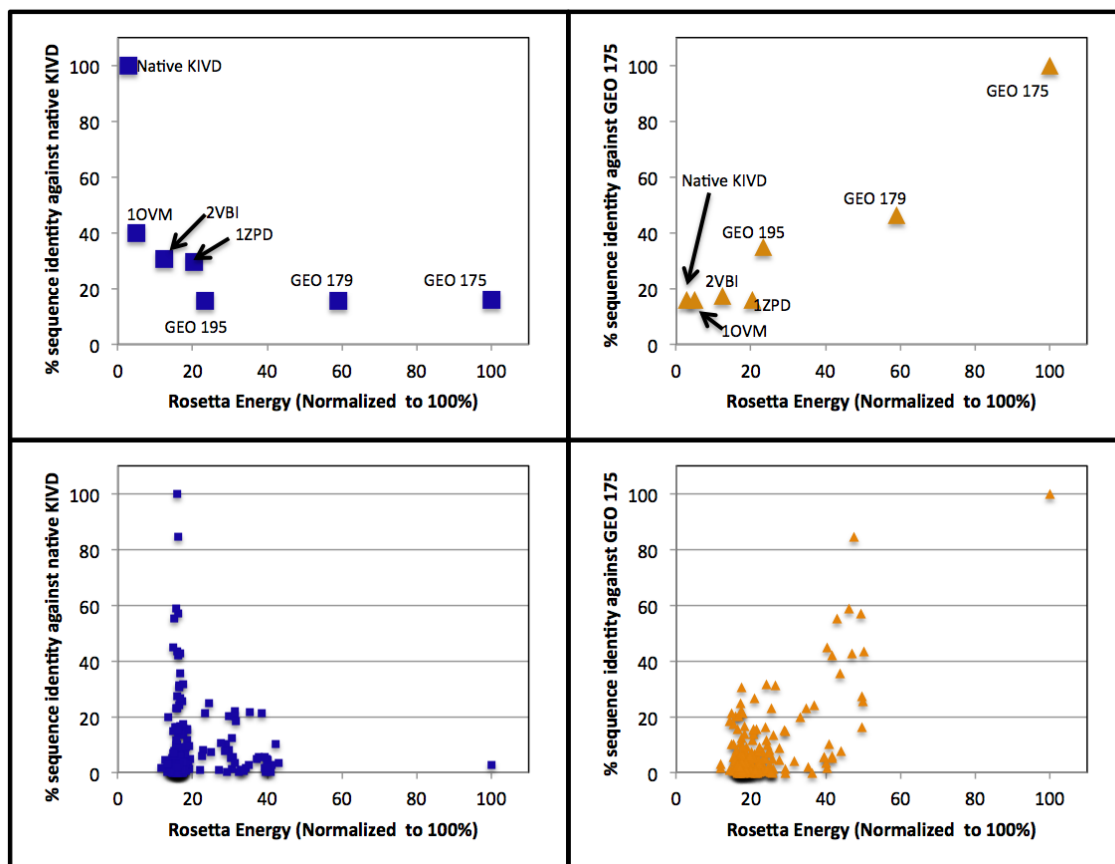
**Supplementary Figure 4.** Michaelis-Menten curve fits of tested enzymes with C5 substrate.



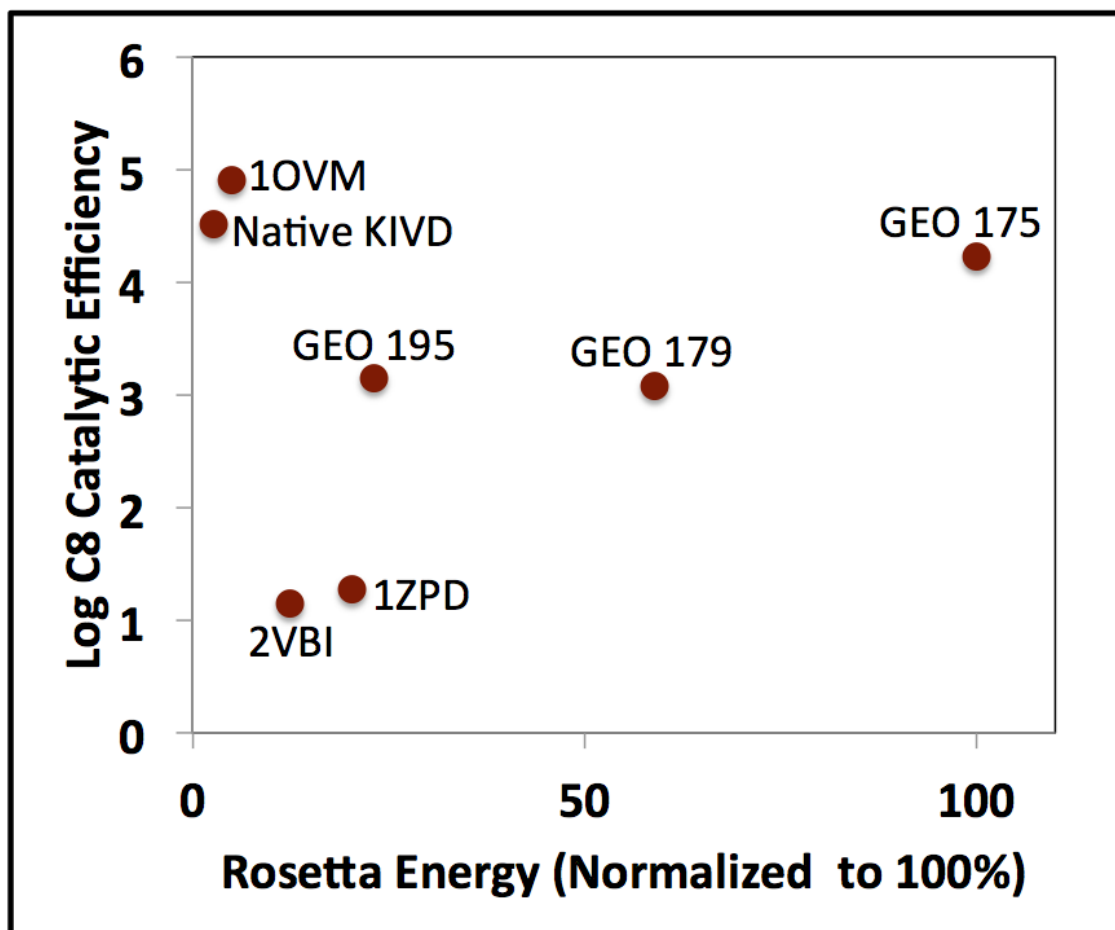
**Supplementary Figure 5.** Michaelis-Menten curve fits of tested enzymes with C3 substrate.



**Supplementary Figure 6.** Michaelis-Menten curve fits of tested enzymes with isoC5 substrate.



**Supplementary Figure 7.** % sequence identity of all 239 KIVDs (against native KIVD on the left; against GEO 175 on the right) plotted against the interface energy, calculated from Rosetta docking simulations.



**Supplementary Figure 8.** Log C8 catalytic efficiency plotted against the interface energy calculated from Rosetta docking simulations.

|    |              |    |              |     |              |     |              |     |              |     |              |
|----|--------------|----|--------------|-----|--------------|-----|--------------|-----|--------------|-----|--------------|
| 1  | 2VBG         | 41 | ZP_08570611  | 81  | YP_001208174 | 121 | ADP12107     | 161 | O53554       | 201 | YP_001070609 |
| 2  | YP_004211875 | 42 | YP_001236700 | 82  | YP_830466    | 122 | YP_004115182 | 162 | YP_003909136 | 202 | YP_004418205 |
| 3  | YP_004506975 | 43 | YP_001208817 | 83  | YP_675840    | 123 | ZP_08465980  | 163 | ZP_08198305  | 203 | YP_675476    |
| 4  | ZP_07949728  | 44 | YP_002353797 | 84  | ZP_06842871  | 124 | ZP_06711201  | 164 | ZP_07042487  | 204 | YP_675461    |
| 5  | ZP_07380174  | 45 | 2Q5Q         | 85  | ZP_05126709  | 125 | ZP_08142152  | 165 | YP_004210411 | 205 | ZP_05074445  |
| 6  | YP_004116674 | 46 | YP_002379932 | 86  | ZP_05094980  | 126 | YP_511062    | 166 | YP_925359    | 206 | YP_004524050 |
| 7  | ZP_08307744  | 47 | O33112       | 87  | ZP_07031890  | 127 | ZP_07657182  | 167 | CAE38656     | 207 | ZP_06822743  |
| 8  | ZP_08461032  | 48 | P0A623       | 88  | YP_004416763 | 128 | ZP_07356713  | 168 | ZP_01901192  | 208 | ZP_01044868  |
| 9  | 1OVM         | 49 | ZP_04998899  | 89  | EGD05600     | 129 | ZP_05100555  | 169 | ZP_07973795  | 209 | ZP_05844198  |
| 10 | ADP13207     | 50 | YP_004291227 | 90  | ZP_08543603  | 130 | YP_001240610 | 170 | CAM60234     | 210 | ZP_07985939  |
| 11 | YP_001281252 | 51 | YP_001353840 | 91  | ZP_06271645  | 131 | YP_001237056 | 171 | ZP_06711620  | 211 | BAJ49000     |
| 12 | YP_001177638 | 52 | 3D7K         | 92  | ZP_06273171  | 132 | AAM12352     | 172 | YP_004020384 | 212 | ZP_01626788  |
| 13 | O53865       | 53 | YP_004519441 | 93  | ABI47993     | 133 | YP_001768986 | 173 | YP_002133221 | 213 | ZP_06710664  |
| 14 | ZP_04562872  | 54 | CAP40738     | 94  | ZP_07282849  | 134 | YP_004216045 | 174 | YP_001378281 | 214 | YP_509362    |
| 15 | AAL18557     | 55 | ZP_08275845  | 95  | YP_001208015 | 135 | YP_001280485 | 175 | ZP_07290467  | 215 | YP_004076523 |
| 16 | Q9CBD6       | 56 | YP_004523976 | 96  | ZP_06370153  | 136 | ZP_00956146  | 176 | BAD32743     | 216 | ZP_08631155  |
| 17 | YP_003731250 | 57 | ZP_07706802  | 97  | ZP_05125236  | 137 | ZP_05033599  | 177 | YP_004522883 | 217 | AAR37826     |
| 18 | AEG26729     | 58 | YP_004081235 | 98  | ZP_06757044  | 138 | ZP_08591801  | 178 | YP_004083736 | 218 | ZP_01302664  |
| 19 | ZP_01623010  | 59 | ZP_08124815  | 99  | YP_830089    | 139 | ZP_06412949  | 179 | ZP_06547677  | 219 | ZP_05001859  |
| 20 | YP_003137934 | 60 | ZP_07949857  | 100 | YP_004230473 | 140 | CAM62618     | 180 | ZP_04607738  | 220 | CAP43929     |
| 21 | ZP_06368393  | 61 | ZP_08303118  | 101 | YP_002536327 | 141 | CAE37944     | 181 | ZP_05135002  | 221 | ZP_03267724  |
| 22 | YP_002380104 | 62 | YP_987677    | 102 | ZP_01130961  | 142 | CAP42719     | 182 | ZP_08119462  | 222 | ZP_06418208  |
| 23 | YP_004674227 | 63 | YP_686086    | 103 | YP_002377567 | 143 | ZP_01999289  | 183 | ZP_07280171  | 223 | ZP_07216338  |
| 24 | YP_004646634 | 64 | ZP_05068817  | 104 | YP_001242472 | 144 | ZP_08124133  | 184 | ZP_07380799  | 224 | ZP_01886096  |
| 25 | ZP_08317680  | 65 | ZP_06549025  | 105 | YP_004075455 | 145 | ZP_01902746  | 185 | YP_641501    | 225 | YP_004695810 |
| 26 | AAM49566     | 66 | YP_003810578 | 106 | YP_004505036 | 146 | YP_003607818 | 186 | YP_004085144 | 226 | ZP_04997132  |
| 27 | YP_004017390 | 67 | YP_004524326 | 107 | ZP_07378926  | 147 | ZP_07280108  | 187 | YP_830320    | 227 | ADI17252     |
| 28 | YP_001224592 | 68 | ZP_07282256  | 108 | ZP_07284792  | 148 | ZP_08317504  | 188 | ZP_04996569  | 228 | ZP_05780727  |
| 29 | 2VBI         | 69 | BAF52673     | 109 | ZP_07278109  | 149 | YP_001769586 | 189 | CAQ52617     | 229 | CAE50484     |
| 30 | ZP_01125207  | 70 | YP_003909474 | 110 | CAK95977     | 150 | ZP_08272344  | 190 | YP_001684628 | 230 | CAC11911     |
| 31 | YP_004210504 | 71 | YP_004212185 | 111 | YP_004490314 | 151 | ZP_05102608  | 191 | AAA17301     | 231 | ZP_06273867  |
| 32 | 1ZPD         | 72 | YP_001279645 | 112 | CAK95978     | 152 | ZP_01306645  | 192 | Q9HUR2       | 232 | YP_001275313 |
| 33 | YP_001806325 | 73 | CAE45665     | 113 | BAC79260     | 153 | YP_001505772 | 193 | YP_004080965 | 233 | YP_001240047 |
| 34 | AAA27685     | 74 | YP_831380    | 114 | ZP_08387358  | 154 | YP_001203581 | 194 | AEG30045     | 234 | ZP_06712511  |
| 35 | YP_730696    | 75 | YP_370152    | 115 | ZP_07278993  | 155 | ZP_07705403  | 195 | ZP_06846103  | 235 | ZP_07033476  |
| 36 | YP_003797329 | 76 | ZP_05101111  | 116 | YP_004230546 | 156 | YP_004416777 | 196 | ZP_07281735  | 236 | YP_004081191 |
| 37 | ZP_08315793  | 77 | AAR05436     | 117 | ZP_06841705  | 157 | ZP_07282273  | 197 | YP_047867    | 237 | ZP_08333578  |
| 38 | ZP_01079084  | 78 | YP_001203568 | 118 | ACR33042     | 158 | ZP_07674873  | 198 | YP_004317430 | 238 | ZP_01725425  |
| 39 | ZP_08074854  | 79 | YP_381143    | 119 | ZP_01749177  | 159 | YP_481591    | 199 | YP_004534047 | 239 | ZP_05285963  |
| 40 | CBE69591     | 80 | CAC11945     | 120 | ZP_08137686  | 160 | NP_484471    | 200 | YP_004077246 |     |              |

**Supplementary Table 1.** PDB code and accession numbers of GEOs modeled, as listed on the phylogenetic tree depicted in Figure 2. Sequences were obtained from homologous sequence search in the Non-Redundant database using HMMER3. The 10 GEOs chosen for experimental characterization are highlighted (Orange: solubly expressed, blue: did not express).

|                     | Native KIVD<br>(mg/L) | KIVD V_LV<br>(mg/L) | GEO 175<br>(mg/L) |
|---------------------|-----------------------|---------------------|-------------------|
| 1-Ethanol           | 652 ± 32              | n.d.                | n.d.              |
| 1-Propanol          | 693 ± 4               | n.d.                | n.d.              |
| 1-Butanol           | 1964 ± 24             | n.d.                | 19 ± 2            |
| 1-Pentanol          | 594 ± 20              | 118 ± 29            | 88 ± 12           |
| 1-Hexanol           | 75 ± 6                | 341 ± 63            | 160 ± 18          |
| 1-Heptanol          | 20 ± 3                | 269 ± 23            | 274 ± 19          |
| 1-Octanol           | n.d.                  | n.d.                | 10 ± 1            |
| Total Alcohol Titer | 3998 ± 89             | 728 ± 115           | 551 ± 52          |

**Supplementary Table 2.** *In vivo* alcohol production. Cells were incubated for forty hours in microaerobic conditions in a defined media as described in Materials and Methods. The best enzyme in shifting the product profile is GEO 175, with 1-heptanol being the major product. Error measurements (mg/L) show one standard deviation over 3 biological replicates.

**Supplementary Table 3.** DNA sequences for all genes used in this study.

| DNA Sequences                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 3FZN                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
| ATGGCGAGCGTGTCATGGCACCACGTATGAACTGCTGCGTCGCCAGGGTATCGATACCGTGTTTCGGCAACCCG<br>GGTTCAAATGAACTGCCGTTTCTGAAAGATTTCCCGGAAGACTTTCGTTATATCCTGGCACTGCAAGAAGCG<br>TGCGTGGTTGGCATTGCAGACGGTTACGCGCAAGCCTCGCGCAAACCGGCGTTTATTAACCTGCATAGCGCG<br>GCCGGCACCGGTAATGCAATGGGCGCTCTGAGCAACGCGTGGAACAGCCACAGCCCGCTGATCGTGACCGCG<br>GGCCAGCAAACGCGTGCCATGATTGGTGTGGAAGCACTGCTGACGAACGTTGATGCAGCTAATCTGCCGCGC<br>CCGCTGGTCAAATGGTCCCTATGAACCGGCATCAGCGGCCGAAGTGCCGCATGCAATGTCTCGTGCCATCCAC<br>ATGGCAAGTATGGCCCCGCAGGGTCCGGTCTATCTGTCTGTGCCGTACGATGACTGGGATAAAGACGCCGAT<br>CCGCAGAGTCATCACCTGTTTGATCGTCATGTTAGCTCTAGTGTCCGCCTGAACGACCAGGATCTGGATATC<br>CTGGTTAAAGCACTGAACTCTGCTAGTAATCCGGCGATTGTGCTGGGTCCGGATGTTGACGCAGCTAACGCA<br>AATGCTGATTGCGTGATGCTGGCTGAACGTCTGAAAGCGCCGGTTTGGGTGCGACCGTCCGGTCCGCGTTGC<br>CCGTTCCCGACCCGTCACCCGTGTTTTCTGGTCTGATGCCGGCCGGTATTGCAGCAATCAGCCAGCTGCTGG<br>AAGGCCATGATGTCTGCTGCTGCTCATCGGTGCACCGGTGTTCCGCTATCACCAGTACGACCCGGGCCAATATC<br>TGAAACCGGGTACCCGTCGTGTTCTGTTACGTGTGATCCGCTGGAAGCAGCTCGCGCGCCGATGGCGGATG<br>CAATCGTGGCAGACATTGGTGGCATGGCCAGTGCACTGGCTAACCTGGTTGAAGAATCCTCAGCTCAGCTGCG<br>CGACCGCGGCCCGGAACCGGCTAAAGTTGATCAAGACGCAGGTCGTCTGCACCCGGAAACCGTCTTTGATA<br>CGCTGAATGACATGGCCCCGGAACCGCAATTTACCTGAATGAATCCACGTCAACCACGGCCAGATGTGGC<br>AACGTCTGAACATGCGCAATCCGGGTTCTTATTACTTCTGTGCAGCTGGCGGTCTGGGTTTTGCACTGCCCG<br>CGGCAATCGGTGTGCAGCTGGCGGAACCGGAACGTCAAGTATTGCCGTTATCGGCGATGGTAGCGCCAACCT<br>ATTCGATTAGCGCACTGTGGACCGCAGCTCAGTACAATATTCGACGATCTTCGTTATTATGAACAATGGCA<br>CCTATGGTGGCCTGCGTTGGTTTGCAGGTGTGCTGGAAGCTGAAAACGTTCCGGGCCCTGGATGTCCCGGGTA<br>TCGACTTCCGTGCACTGGCAAAAGGCTACGGTGTTCAGGCACTGAAAGCTGATAATCTGGAACAGCTGAAAG<br>GCTCGCTGCAAGAAGCGCTGAGCGCCAAAGGTCCGGTGTGATTGAAGTCTCTACCGTGAGTCCGGTTAAAG<br>GCTCCACGGAATCTGTATTTTCAGTCAGGTGCGCTCGAG                                                                                            |
| 2VBI                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
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| 1ZPD                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |

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Native KIVD

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YP\_381143

ATGGCCCCGTACCGTGCCCCGCACCGTAGCCCGCACTCGTACTTTACCATGAAGGGTCACGAAGCGATTCTG  
CGTCAGTTTTCTGGCGAACGGCATGGATCACATGTTTGGCACGCCGGGCGACGTGCAACAAGGTTTCTTGAT  
GCGCTGGCCGACGTGCCGGAATGAAATATATTCTGACGCTGCAAGAAAGCATCGCTGTGCTGTGTGCGGAT  
GGTTACGCACGTGCTCGCCTGAAGCCGGCGCTGGTTTCAAGTTTACAGCTCTCCGGGCGCTGGGTAACGCCATC  
GGCAATCTGTATCAGGCTATGCGTGGTCAAGCGCCGCTGGTTGTGATTGGCGGTGATGCGGGTATCAAATAC  
CAGGCAATGGATGCTCAAATGGCGGCCGACCTGGTGGCGATGGCCGAACCGGTTACCAAATGGAGCGCGATG  
GTCCAGCATCCGAGTTCCCTGCTGCGCATGGTCCGTGCGGCCATTAAGGTGGCAGCTACGCCGCCGTGCGGTC  
CGGTTTTATCTGTGCTGCGCGAAGATATTCTGGACGCGGAAATCACCGAAAAAATTATCCCGGCCACATT  
CGTCCCTGGAAGTCTCGGGTTCACTGGATCTGGACCGTATGGTGTCTGCCATCCAGAGTGACAAAAACC  
CGATTATCCTGGTGGTGATGGTGTGGCATGGACGGCGGTGTTGAAAAAATTGTGGATCTGGCCGAAACG  
CTGGGCGCAAAGGTTTATTCTGCTGATGGCGGTGAAATCAACTTTCGGATGACCATCTGCTGAATTACGGC  
AGTACCGGTGCCATGTTCCGGTGATCAGTCTCTGCCGATTATGCAAAGTTGCGACCTGTGTCTGACGCTGGGC  
TGCTATCTGCTGCCGGAAGTGTTTCCGCATCTGGGTGATATCTTCAACGAAGACGCAACCATTATCCACGTG  
GATACGAACGTTGACAATATTGCAAAAAATCATCGCGTTGATATCTCGTACGTGCTGAACCGCACAGCGTC  
GTGACCGGTCTGCTGCCGATTCTGAAATCGCTGTATCGAGCTGGCATAATGCGGGCCAGCAACGTGCGAGC  
AAGCTGGAATCTGAAAGTCCGGTTGTCCACAACAATGTTGACCAGAACTATCAAGTCGAACCGCCGTATCCG  
TCTGAAGCGTACGATGGCATTAAATCGTTCCATGCGCTCAGGTTACTTTATCAAAACCCCTGGCAGATAAACTG  
CCGAAGGAAACCATTAATCTTCGACGAAGCTCTGACGAACCTCCCGCCGGTGAATCGTTATCTGCCGGGCCAG  
AAACCGGGTGATCGTATGCTGACCCGCGCGGTTCACTGGGCACGGGTTTTCCGGGCGCGATTGGTGCCAAG

ATCGCATATCCGGATCGCTGCGTTATTGGCTTCTCGGGTGACGGCGGTAGCATGTACACCATCCAGTGTCTG  
TGGACGGCCGTCCGTCATAACGTGGCAGCTAAATTTATTGTGTGCCAGAACCGCAGTTATAAACTGCTGCAA  
TCGAATATTAGCAAGTTCTGGCAGGAACGTGGCATCGAAGGTCGCGAATTTCCGGTTCGGTTCGATCTGTCT  
AAACCGGAAATTTGTTTTAGTGTGATCGCAAATTCCTTCGGCGTTTCAGGTGAACGTGTGGTTCGCCCCGAT  
CAAGTGGGCGAAGCGATTGATCGTATGCTGAACCACGACGGTCCGTATCTGATCAATCTGGTTCGGATGGC  
GACATTTCGTCCGGATCTGATCGGTGTCCGCTGCGGCCAAGGTTCCACCGAAAACCTGTACTTTCAGTCAGGT  
GCGCTCGAG

**Supplementary Table 4.** Protein sequences for all genes used in this study.

| Protein Sequences                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 3FZN                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| MASVHGTTYELLRRQGIDTVFGNPGSNELPFLKDFPEDFRYLALQEACVVGADGYAQASRKPAFINLHSAAGTGNGAMGALSNAWNSHSP LIVTAGQQTRAMIGVEALLTNVDAANLPRPLVKWSYEPASAAEVP HAMSRAIH MASMAPQGPVYLSVPYDDWDKDADPQSHHLFDRHVSSSVRLNDQDL DILVKALNSASNP AIVLGPDVDAAN ANADCVMLAERLKAPVWVAPSAPRCFPFTRHPCFRGLMPAGIAAISQLLEGHDVVLVIGAPVFRYHQYDPGQ YLKPGTRLISVTCDPLEAARAPMGDAIVADIGAMASALANLVEESSRQLPTAAPEPAKVDQDAGRLHPETVFD TLNDMAPENAIYLNSTSTTAQMWQRLNMRNPGSYFFCAAGGLGFALPAAIGVQLAEPERQVIAVIGDGSAN YSISALWTAQAQYNIPTIFVIMNNGTYGALRWFAGVLEAENVPGLDVP GIDFRALAKGYGVQALKADNLEQLK GSLQEALSAKGPVLIEVSTVSPVKGSTENLYFQSGALE                                               |
| 2VBI                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| MTYTVGMYLAERLVQIGLKHHFAVAGDYNLVLLDQLLL NKM KQIYCCNELNCGFSAEGYARSNGAAA AVV TFSVGAISAMNALGGAYAENLPVILISGAPNSNDQGTGHILHHTIGKTDYSYQLEMARQVTC AAESITDAHSAP AKIDHVIRTALRERKPAYLDIACNIASEPCVRPGPVSSLLSEPEIDHTSLKAAVDATVALLEKSASPVMLLGSKL RAANALAA TETLADKLQCAVTIMAAAKGFFPEDHAGFRGLYWGEVSNPGVQELVETSDALLCIAPVFNDYST VGWSAWPKGPNVILAEPDRVTVDGRAYDGTFLRAFLQALAEKAPARPASAQKSSVPTCSLTATSDEAGLTND EIVRHINALLTSNTTLVAETGDSWFNAMRMTLPRGARVELEMQWGHIGWSVPSAFGNAMGSQDRQHVM VGDGSFQLTAQEVAQMVR YELPVII FLINNRGYVIEIAIHDGPYNYIKNWDYAGLMEVFNAGEGHGLGLKATT PKELTEAIARAKANTRGPTLIECQIDRTDCTDMLVQWGRKVASTNARKTTLAGSTENLYFQSGALE               |
| 1ZPD                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| MSYTVGTYLAERLVQIGLKHHFAVAGDYNLVLLDNLLLNKNMEQVYCCNELNCGFSAEGYARAKGAAA AVV TYSVGALSAFDAIGGAYAENLPVILISGAPNNNDHAAAGHVLHHALGKTDYHYQLEMAKNITAAAEAIYTPEEA PAKIDHVIKTALREKKPVYLEIACNIASMPCAAPGPASALFNDEASDEASLNAAVDETLKFIANRDKVAVLVGS KLRAAGAEAAVKFTDALGGAVATMAAAKSFFPEENALYIGTSWGEVSYPGVEKTMKEADAVIALAPVFN DY STTGWTDIPDPKKLVLAEPRSVVNGIRFPSVHLKDYLTRLAQKVS KKTGSLDFFKSLNAGELKKAAPADPSA PLVNAE IARQVEALLTPNTTVIAETGDSWFNAQRMKLPNGARVEYEMQWGHIGWSVPAAFGYAVGAPERR NILMVGDSGFQLTAQEVAQMVR LKLPV IIFLINNYGYTIEVMIHDGPYNNIKNWDYAGLMEVFNGNGGYDSG AAKGLKAKTGGELAEAIKVALANTDGP TLIECFIGREDCTEELVKWGWKRVA AANSRKPVNKVVGSTENLYFQSGALE  |
| ZP_08570611                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
| MSSINSFTVADYLLTRLHQLGLRKVFQVPGDYVANFMDALEQFNGIEAVGDLTELGAGYAADGYARLTGIGA VSVQFGVGTFSVLNAIAGSYVERNPPVVITASPSTGNRKTIKETGVLFHHSTGDLLADSKVFANVTVA AEVLSD PSDARQKIDKALT LAITFRRPIYLEAWQDVWGLACEKPEGELKALPLISEEGALKAMLADSLKLLNSARQPLV LLGVEINRFLQDAVL DLLKASGLPYSTTSLAKT VISENEGIFVGTYADGASFPATVEYIEKADCVLALGVFTD DYLTMLSKQFDQMIVVNDETSRLGHAYYHQYLADFILQLTDEIKKSSLYPRQNSALPLPPQPQITPALLQ QQLSYQNFFDLFYGYLLQHQLQDNISLILGESSLYMSARLYGLPQDSFIADAAWGS LGHETGCVTGIAYASDK RAMA IAGDGGFMMMCQCLSTISRHQLNSVVFVISNKVYAEQS FVDICAF AKGGHFAPFDLLPTWDYLSLAKA FSVEGYRVQNGEELLQALEHIMTQDKPALVEVVIQSQDLAPAMAGLVKSITGHTVEQCAIPTGSTENLYFQSGALE |
| YP_831380                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
| MTTVHAAAYELLRSNRLTTIFGNPGDNELPFLDAMPADFRYLGLHEGVVGMADGFAQASGQA AFVNLHA ASGTGNAMGALTNAWYSHTPLVITAGQQVRPMIGLEAMLSNVDAASLPRPLVKWSAEP AQAPDVPRALSQA IHTATSDPKGPVYLSIPYDDWNQDTGNLSEHLSSRSVS RAGNPSAEQLDDILSALREANPALVFGPDVDAAR ANHHAVRLAEKLAAPVWIAPAAPRCFPFTRHPNFRGVLPASIAGISALLNGHDLIVVIGAPVFRYHQYQPGSY LPENSR LIHITCDAGEAARAPMGDALVADIGQTLRALADIIPQSKRPPLRPRVIPPVPDSQDDLLAPDAVFEVM NEVAPEDVVVYNESVSTVTALWERVELKHGPGSYFFPASGGLGFGMPAAVGVQLANDRRRVIAVIGDGSANYG ITALWTA AQEKIPVVFI LNNGTYGALRAFAKLLNAENAAGLDVPGICFCAIAEGYGEAHRITSLENFKDKLS AALQSDTPTLLEVPSTTSPFGSTENLYFQSGALE                                                |
| ZP_06547677                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
| MKTIHSAAYALLRRHGMTTIFGNPGSNELPFLKSFPEDFQYVVLGLHEGAVVGMADGYALASGKPAFVNLHAA AGTGNGMGALTNSWYSHSPLVITAGQQVRPMIGVEAMLANVDATQLPKPLVKWSYEPANAQDVP RALSQAI                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |

HYANTTPKAPVYLSIPYDDWDQPSGPGVEHLIERDVQTAGTPDARQLQVLVQQVQDARNPVLVLGPDVDAT  
LSNDHAVALADKLMPVWIAPAASRCFPFTRHPSFRGVLPAAIAGISKTLQGHDLIIVVGAPVFRYLQFAPGD  
YLPVGAQLLHITSDPLEATRPMGHALVGDIRETLRVLAEEVVQSRPYPEALAAPECVTDEPHHLHPETLFD  
VLDAVAPHDAIYVKESTSTVTAFWQRMNLRHPGSYYFPAAGGLGFGLPAAVGVQLAQPPRRVVALIGDGSAN  
YGITALWTAQAQYRIPVVFILKNGTYGALRWFAGVLKAEDSPGLDVPGLDFCAIAKGYGVKAVHTDTRDSFEA  
ALRTALDANEPTVIEVPTLTIQPHGSTENLYFQSGALE

1OVM\_P23234

MRTPYCVADYLLDRLTDCGADHLFGVPGDYNLQFLDHVIDSPDICWVGCANELNASYAADGYARCKGFAALL  
TTFGVGELSAMNGIAGSYAEHVPVLHIVGAPGTAAQQRGELLHHTLGDGEFRHFYHMSEPITVAQAVLTEQN  
ACYEIDRVLTTMLRERRPGYLMLPADVAKKAATPPVNALTHKQAHADSACLKAFRDAAENKLAMSKRTALL  
ADFLVLRHGLKHALQKWVKEVPMAHATMLMGKGFDERQAGFYGTYSGSASTGAVKEAIEGADTVLCVGRTR  
FTDTLTAGFTHQLTPAQTIEVQPHAARVGDVWFTGIPMNQAIETLVELCKQHVHAGLMSSSSGAIFFPQPDG  
SLTQENFWRTLQTFIRPGDIILADQGTSAFGAIDLRLPADVNFIVQPLWGSIGYTLAAAFGAQTACPNRRVIVL  
TGDGAAQLTIQELGSMRLDKQHPILVLNNEGTYVERAIHGAEQRYNDIALWNWTHIPQALSPLDPQSECWRV  
SEAEQLADVLEKVAHHERLSLIEVMLPKADIPLLGALTKALEACNNAGSTENLYFQSGALE

ZP\_06418208

MGAHYPMMSGESTVHDVTYQLLRSLGITTTFGNPGSTEQTFLQDFPSDFTYVLGLQEASVMAMADAFQVTR  
RPALVNLHSSAGVGHSIGNLVSAFDAHTPLIVTAGQQHREM VIGEPALSNREATNLPRPVVWKSYPEPARAQD  
VPEAFMRACAIATQPPAGPVFLSIPLDDWNAPMTGPAVVRVSSTVCAPETERLRGFARRIRASQRPVLFVGP  
VDRSGGWHAALAEENLGVVFGAAGPDRVSFPEDHPLFQGRLGMSQKSVSDRLTGYDLVVVIGAAVFRYYP  
HVPGDILPAGTELLHITGDPVAVAGAAARVGDVSLGDARLAIELLTELLDAEAAHSPRRPQHQPHPQPQHV  
VQHQQPPQTRFQARAQPRPRRHADPRPETAGGPLCADEVHAVINASRPRNAALVYESTSTIGEQQVEWLPVIE  
PASFFANASGGLGWAVPAAVGVALGDRDRGVHRPVIIGDGAQYQSVQALWTATQHSPLPIVFFVLRHEYSIL  
KSFAELERTAGVGSTENLYFQSGALE

ZP\_07290467

MRTVRESALDVLRLARGMTTVFGNPGSTELPMLKQFPDDFRYVLGLQEAVVVGMAADGFALASGTTGLVNLHT  
GPGTGNAMGAILNARANRTPMVVTAGQQVRAMLTMEALLTNPQSTLLPQPAVKWAYEPPRAADVAPALAR  
AVQVAETPPQGPVFSVSLPMDDFDVVLGEDEDRAAQRAAARTVTHAAAPSAEVVRRLLAARLSGARS  
NDVDASGAWDAVVELAERTGLPVWSAPTEGRVAFPKSHQYRGMLPPAIAPLSRCLEGHDLVLVIGAPVFCY  
YPYVPGAHLPELTELHTRDADEAARAPVGDAVVADLALTVRALLAELPAREAAAPAARTARAESTA  
EVDGVLTPLAAMTAIAQGAPANTLWVNESPSNLGQFHDATRITPGSFLFTAGGGLGFLGAAVGAQLGAPDRPV  
VCVIGDGSTHYAVQALWTAAYKVPVTFVVLNQRYSAILQWFAQVEGAQAGAPGLDIPGLDIAAVATGYGVRA  
HRATGFGLSKLVRESALQQDGPVLIDVPVTTELPTLGSTENLYFQSGALE

Native\_KIVD

MYTVGDYLLDRLHELGIIEIFGVPGDYNLQFLDQIISRKDMKWVGNANELNASYMADGYARTKKAAAFLTTF  
GVGELSAVNGLAGSYAENLPVVEIVGSPTSKVQNEGKFVHHTLADGDFKHFMKMHEPVTAARTLLTAENAT  
VEIDRVLSALLKERKPVYINLPVDVAAAKAEKPSLPLKKENSTSNSTSDQEILNKIQESLKNAKKPIVITGHEIIF  
GLEKTVSQFISKTKLPITTLNFGKSSVDEALPSFLGIYNGKLSEPNLKEFVESADFILMLGVKLTDSSTGAFTHH  
LNENKMISLNIDEGKIFNESIQNFDSESLISLLDLSEIEYKGYIDKKQEDFVPSNALLSQDRLWQAVENLTQS  
NETIVAEQGTSSFFGASSIFLKPCKSHFIGQPLWGSIGYTFPAALGSQIADKESRHLLFIGDGSLLQTVQELGLAIRE  
KINPICFIINNDGYTVEREIHGPNQSYNDIPMWNYSKLPESFGATEERVVSKIVRTENEFVSVMKAEQADPNR  
MYWIELILAKEDAPKVLKKMGKLFQAEQNK

CAK95977

MKTVHGATYDILRQHGLTTIFGNPGDNELPFLKGFPEDFRYILGLHEGAVVGMADGYALASGQPTFVNLHAA  
AGTGNGMGALTNAWYSHSPLVITAGQQVRSMIGVEAMLANVDAQAQLPKPLVKSHEPATAQDVPRALSQAI  
HTANLPPRGPVYVSIPYDDWACEAPSGVEHLARRQVSSAGLPSAQLQHLCLERLAAARNPVLVLGPDVDGSA  
ANGLAVQLAEKLRMPAVVAPSASRCFPFTRHACFRGVLPAAIAGISHNLAGHDLLIVVGAPVFRYHQAQFAPGN  
YLPAGCELLHLTCDPGEAARAPMGDALVGDIALTLEAVLDGVPQSVRQMPTALPAAEPVADDGGLLRPETVF  
DLLNALAPKDAIYVKESTSTVGAFWRRVEMREPGSYFFPAAGGLGFGLPAAVGVQLASPGRQVIGVIGDGSAN  
YGITALWTAQAQYNIPVVFILKNGTYGALRWFADVLVDNDAPGLDVPGLDFCAIARGYGVQAVHAATGSAFA

QALREALESDRPVLIEVPTQTIEPGSTENLYFQSGALE

ZP\_07282849

MARFGVRKAFGVVGSNGFHTNGLIQGGAEFVAARHEGGATTMADAYARCSGEVAAVSVHQCGGLGNATTG  
IGEAAKSRTPLVVVTAEATDPLSNFHIDQDALARSVGASSILVRSAKTALADVRRRAFTQARQDRRTVLLRLPL  
EVQAEPFDESLLLEGLTAIEALPQPRAAEAEVQALASILQRAERPVFLCGRGSRAARAELVALADRCGALLAEGA  
VAKGLFAGEPWAIGVSGGFSSPLTTELIQGADVVGWGSALNDWTTAHGRLLSPETTLVQVDLESAALGRNR  
PVDLGIVGDVGGTALAVAELLEHVHNGYRSAELKTRIAREIRWRDNEYDDVSTGEVIDPRTLAADELDPANR  
VVGVDSGNFMGYPTMYLDVPDENGFCFTQAFASIGLGLATAIGTALARPDPRFPVAACGDGGFLMSIAELETVV  
RLKLPLMILVVYNDHAYGAEVYFFEPGGHPADTVTFPDTDLAAIARGYGCDAVTVRTKEDLAEVATRVAAGLD  
RPLVVDAKIAGFSAWWLQAAMTHHGSTENLYFQSGALE

ZP\_06846103

MTRSSSFSPPSASEQRGADIFAEVLQCEGVRYIFGNPGTTTELPLLDALTDITGIHYVLGLHEASVAMADGYAQ  
ASGKPGFVNHLHTAGGLGNAMGAILNAKMANTPLVVTAGQQDTRHGVTDLLHGDLTGIARPNVKWAEIEH  
HPEHIPMLLRRALQDCRTGPAGPVFLSLPIDTMERCTSVGAGEASRIERASVANMLHALATALAEVTAGHIAL  
VAGEEVFTANASVEAVALAEALGAPVFGASWPGHIPPTAHPQWQGTLPKASDIRETLGPFDAVLILGGHSL  
ISYPYSEGAIPPHCRFLQLTGDGHQIGRVHETTLGLVGDQLSLRALLPLLARKLQPQNGAVARLRQVATLKR  
DARRTEAAERSAREFDASATTPFVAAFETIRAIGPDVPIVDEAPVTIPHVRACLDASARQYLFTRSAILGWGM  
PAAVGVSGLDRSPVCLVGDGSAMYSPPQALWTAHERLPVTFVFNNGEYNALKNFARAQTNYSARANR  
FIGLDISDPAIDFPALASSLGVPARRVERAGDIAIAVEDGIRSGRPNLIDVLISSSSGSTENLYFQSGALE

ZP\_04996569

MLRTAGEESGVKVRDAFFEVLRSHGITTTFGNPGSNELPLLRDFPDFFRYVLALHEGAAIAMADGYALATGR  
PSLVNLHAAAGTGAMGNLTNTQSGHVPVVVTSQQARRYTALNALLTNVDATALAEPLVKWSCEPLRPED  
VPQALSQGILLAGSAPAGPVYLSPLDDWDHQADPGALKHLKARTVQGDPPVSEPALDLLRRRLTGAANPV  
MVVGPGLDDATGWDGACRLADRLALPVFVAPSPSRCPFTRHPGYRGVLPDIPAVARHFDGHDLVVAFGAA  
IFRYFAFEEGDYLPPTGTELWAVTSDPDEATRAPFGRILVGNPSDALARLTETVPARHRPPPPPLERTSRLNEA  
GPAFSAEAIVDALDAKDESTVLAHEWTSVLTTWDRFDRPGSLYFPASGGLGWGLPAAIGLQLGDPSSRRVL  
AMLGDGALHYTVSALWTAARYRVPVVFVARNGEYGALKKFTQAMQAPGVPGLELPGIDITGIASAYGISAT  
RIDTLDALTAAVTAALATDEPHLIEVPQQPLTASGSTENLYFQSGALE

YP\_381143

MAPYRAPHRSPHSYFTMKGHEAILRQFLANGMDHMFGTGPDVEQGFLDALADVPEMKYILTLQESIAVLCA  
DGYARARLKPALVQIHSSPGLGNAIGNLYQAMRGQAPLVVIGGDAGIKYQAMDAQMAADLVAMAEPVTKWS  
AMVQHPSLLRMVRRRAIKVAATPPCGPVYLCPLPEDILDAEITEKIIPAHIPSLETCPGSLDLDRMVSAIQSAQNP  
IILVGDGVAWTGGVEKIVDLAETLGAKVYSADGGEINFPDDHLLNYGSTGAMFGDQSLPIMQSCDLCLTLGCY  
LLPEVFPHLGDIFNEDATIIHVDTNVDNIAKNHRVDISYVAEPHSVVTGLLPILKSLSSSWHNAAQRRSKLES  
ESPVVHNNVDQNYQVEPPYPSEAYDGINRSMRSGYFIKTLADKLPKETIIFDEALTNSPPVNRYLPGQKPGDR  
MLTRGGSLGTGPGAIGAKIAYPDRCVIGFSGDGGSMTYIQCLWTAVRHNVAKFIVCQNRSYKLLQSNISKF  
WQERGIEGREFPVPFDLSKPEICFSVANSFGVSGERVVRPDQVGEAIDRMLNHDGPYLINLVLDGDIDRDLIG  
VRCCQGSTENLYFQSGALE

| Library | Native               | Mutations                                                                 |
|---------|----------------------|---------------------------------------------------------------------------|
| 1       | D285<br>S286         | ADLF<br>FMSTWY                                                            |
| 2       | Q377<br>F381<br>F382 | AFMWQ(ILR*) <sup>a</sup><br>ALF(SV) <sup>a</sup><br>AFMW(IL) <sup>a</sup> |
| 3       | G402                 | AILMTV(S) <sup>a</sup>                                                    |
| 4       | V461<br>I465         | AMWTV<br>IV                                                               |
| 5       | M538<br>F542         | AHLMV(T) <sup>a</sup><br>AFHIMTVWY(L) <sup>a</sup>                        |

**Supplementary Table 5.** KIVD Mutants with Altered Specificity for Long-chain 2-Ketoacids from the Results of High-throughput Kinetic Assays. \*(a) Potential mutations in parenthesis were included due to degenerate codon used for mutagenesis.

**Supplementary Table 6.** Partially degenerate oligonucleotides used to generate libraries of *kivd* mutants (with N-terminal histidine tags) by PCR.

| Primer Sequence                                           | Description                                                          |
|-----------------------------------------------------------|----------------------------------------------------------------------|
| GAGTTAAACTCACATTNTHYTCAACAGGAGCCTTCACTCATC                | primer for KIVD D285, S286 mutants D285LF S286SFY                    |
| GAGTTAAACTCACATTNAYGTCAACAGGAGCCTTCACTCATC                | primer for KIVD D285, S286 mutants D285LF S286TM                     |
| GAGTTAAACTCACATTNTGGTCAACAGGAGCCTTCACTCATC                | primer for KIVD D285, S286 mutants D285LF S286W                      |
| GAGTTAAACTCACAGMYTHYTCAACAGGAGCCTTCACTCATC                | primer for KIVD D285, S286 mutants D285AD S286SFY                    |
| GAGTTAAACTCACAGMYAYGTCAACAGGAGCCTTCACTCATC                | primer for KIVD D285, S286 mutants D285AD S286TM                     |
| GAGTTAAACTCACAGMYTGGTCAACAGGAGCCTTCACTCATC                | primer for KIVD D285, S286 mutants D285AD S286W                      |
| CAATCGTTGCTGAAGCNGGGACATCAKYNGCNGGCGCTTCATCAATTTTCTTAAAAC | primer for KIVD Q377, F381, F382 mutants Q377A F381LAFVS F382A       |
| CAATCGTTGCTGAAGCNGGGACATCAKYNWTKGGCGCTTCATCAATTTTCTTAAAAC | primer for KIVD Q377, F381, F382 mutants Q377A F381LAFVS F382MFIL    |
| CAATCGTTGCTGAAGCNGGGACATCAKYNTGGGGCGCTTCATCAATTTTCTTAAAAC | primer for KIVD Q377, F381, F382 mutants Q377A F381LAFVS F382W       |
| CAATCGTTGCTGAAWTKGGGACATCAKYNGCNGGCGCTTCATCAATTTTCTTAAAAC | primer for KIVD Q377, F381, F382 mutants Q377MFIL F381LAFVS F382A    |
| CAATCGTTGCTGAAWTKGGGACATCAKYNWTKGGCGCTTCATCAATTTTCTTAAAAC | primer for KIVD Q377, F381, F382 mutants Q377MFIL F381LAFVS F382MFIL |
| CAATCGTTGCTGAAWTKGGGACATCAKYNTGGGGCGCTTCATCAATTTTCTTAAAAC | primer for KIVD Q377, F381, F382 mutants Q377MFIL F381LAFVS F382W    |
| CAATCGTTGCTGAAYRGGGGACATCAKYNGCNGGCGCTTCATCAATTTTCTTAAAAC | primer for KIVD Q377, F381, F382 mutants Q377WQR* F381LAFVS F382A    |
| CAATCGTTGCTGAAYRGGGGACATCAKYNWTKGGCGCTTCATCAATTTTCTTAAAAC | primer for KIVD Q377, F381, F382 mutants Q377WQR* F381LAFVS F382MFIL |
| CAATCGTTGCTGAAYRGGGGACATCAKYNTGGGGCGCTTCATCAATTTTCTTAAAAC | primer for KIVD Q377, F381, F382 mutants Q377WQR* F381LAFVS F382W    |
| GTCAACCCTTATGGNTRTCAATTGGATATACATTCCCAGCAG                | primer for KIVD G402 mutants G402VILM                                |
| GTCAACCCTTATGGRSYTCAATTGGATATACATTCCCAGCAG                | primer for KIVD G402 mutants G402GATS                                |
| AATGATGGTTATACARYGGAAGAGAAARTHCAATGGACCAAATCAAAGCTACAATG  | primer for KIVD V461, I465 mutants V461MTAV I465IV                   |
| AATGATGGTTATACATGGGAAAGAGAAARTHCAATGGACCAAATCAAAGCTACAATG | primer for KIVD V461, I465 mutants V461W I465IV                      |
| AAAGTACTGAAAAACWYGGCAAACCTAYWYGTGAACAAAATAAATCATAAGCATG   | primer for KIVD M538, F542 mutants M538LH F542YHFL                   |
| AAAGTACTGAAAAACWYGGCAAACCTARYNGCTGAACAAAATAAATCATAAGCATG  | primer for KIVD M538, F542 mutants M538LH F542MITAV                  |

|                                                            |                                                       |
|------------------------------------------------------------|-------------------------------------------------------|
| AAAGTACTGAAAAAACWYGGCAAACCTATGGGCTGAACAAAATAAATCATAAGCATG  | primer for KIVD M538, F542 mutants M538LH F542W       |
| AAAGTACTGAAAAAARYGGGCAAACCTAYWYGCTGAACAAAATAAATCATAAGCATG  | primer for KIVD M538, F542 mutants M538VAMT F542YHFL  |
| AAAGTACTGAAAAAARYGGGCAAACCTARYNGCTGAACAAAATAAATCATAAGCATG  | primer for KIVD M538, F542 mutants M538VAMT F542MITAV |
| AAAGTACTGAAAAAARYGGGCAAACCTATGGGCTGAACAAAATAAATCATAAGCATG  | primer for KIVD M538, F542 mutants M538VAMT F542W     |
| GTCAACCCTTATGGTCTTCAATTGGATATACATTCCCAGCAG                 | KIVD G402S mutation primer                            |
| GTCAACCCTTATGGGTGTCAATTGGATATACATTCCCAGCAG                 | KIVD G402V mutation primer                            |
| AATGATGGTTATACATGGGAAAGAGAAATTCATGGACCAAATCAAAGC           | KIVD V461W mutation primer                            |
| AATGATGGTTATACAGCGGAAAGAGAAGTTCATGGACCAAATCAAAGCTACAATG    | KIVD V461A I465V mutation primer                      |
| AATGATGGTTATACATGGGAAAGAGAAGTTCATGGACCAAATCAAAGCTACAATG    | KIVD V461W I465V mutation primer                      |
| AATGATGGTTATACAATGGAAAGAGAAGTTCATGGACCAAATCAAAGCTACAATG    | KIVD V461M I465V mutation primer                      |
| AATGATGGTTATACAATGGAAAGAGAAATTCATGGACCAAATCAAAGC           | KIVD V461M mutation primer                            |
| AAAGTACTGAAAAAAcatGGCAAACCTActgGCTGAACAAAATAAATCATAAGCATG  | primer for KIVD M538, F542 mutants M538H F542L        |
| AAAGTACTGAAAAAAatgGGCAAACCTAgtgGCTGAACAAAATAAATCATAAGCATG  | primer for KIVD M538, F542 mutant F542V               |
| AAAGTACTGAAAAAAcatGGCAAACCTAtttGCTGAACAAAATAAATCATAAGCATG  | primer for KIVD M538, F542 mutants M538H              |
| AAAGTACTGAAAAAAActgGGCAAACCTAaccGCTGAACAAAATAAATCATAAGCATG | primer for KIVD M538, F542 mutants M538L F542T        |
| AAAGTACTGAAAAAAcatGGCAAACCTAtggGCTGAACAAAATAAATCATAAGCATG  | primer for KIVD M538, F542 mutants M538H F542W        |
| AAAGTACTGAAAAAAcatGGCAAACCTAaccGCTGAACAAAATAAATCATAAGCATG  | primer for KIVD M538, F542 mutants M538H F542T        |
| AAAGTACTGAAAAAAActgGGCAAACCTAgtgGCTGAACAAAATAAATCATAAGCATG | primer for KIVD M538, F542 mutants M538L F542V        |
| AAAGTACTGAAAAAAcatGGCAAACCTAgcgGCTGAACAAAATAAATCATAAGCATG  | primer for KIVD M538, F542 mutants M538H F542A        |
| GAGTTAAACTCACACTGTTTTCAACAGGAGCCTTCACTCATC                 | primer for KIVD mutant D285L S286F                    |
| GAGTTAAACTCACAGACTGGTCAACAGGAGCCTTCACTCATC                 | primer for KIVD mutant S286W                          |
| CAATCGTTGCTGAAATGGGGACATCATTCCTGGGCGCTTCATCAATTTTCTTAAAC   | primer for KIVD mutant Q377M F382L                    |
| CAATCGTTGCTGAAATTGGGACATCACTGTGGGCGCTTCATCAATTTTCTTAAAC    | primer for KIVD mutant Q377I F381L F382W              |
| AAAGTACTGAAAAAAcatGGCAAACCTAatgGCTGAACAAAATAAATCATAAGCATG  | primer for KIVD M538, F542 mutants M538H F542M        |
| AAAGTACTGAAAAAAcatGGCAAACCTAcatGCTGAACAAAATAAATCATAAGCATG  | primer for KIVD M538, F542 mutants M538H F542H        |
| AAAGTACTGAAAAAAgtgGGCAAACCTAgtgGCTGAACAAAATAAATCATAAGCATG  | primer for KIVD M538, F542 mutants M538V F542V        |
| TCCGACTTACTCTGAAGACGCGTCTGGCATCACCGCGAAATAATCAAATG         | Left primer for rhtC knockout                         |
| GTGCCAGTATGAAGACTCCGTAAACGTTTCCCCGCGAGTCAAATGTATG          | Right primer for rhtC knockout                        |
| ATGCGACCCTTGTGTATCAAACAAGACGATTAAAAATCTTCGTTAGTTTC         | Left primer for recA knockout                         |
| CAGAACATATTGACTATCCGGTATTACCCGGCATGACAGGAGTAAAAATG         | Right primer for recA knockout                        |

|                                                    |                                |
|----------------------------------------------------|--------------------------------|
| CCGTTTATGTTGCCAGACAGCGCTACTGATTAAGCGGATTTTTTCGCTTT | Left primer for adhE knockout  |
| CGAGCAGATGATTTACTAAAAAAGTTTAACATTATCAGGAGAGCATTATG | Right primer for adhE knockout |

**Supplementary Table 7.** All *E. coli* strains and plasmids used in this study.

| Name                           | Relevant Genotype                                                                                                                                                  | Source                           |
|--------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------|
| <i>Strains</i>                 |                                                                                                                                                                    |                                  |
| XL-1 Blue                      | <i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i><br>[F' <i>proAB lacIq</i> Δ <i>M15</i> Tn10 (Tetr)].                                                       | Stratagene, La Jolla, California |
| KS145                          | <i>rrnB<sub>T14</sub> ΔlacZ</i> WJ16 <i>hsdR514 ΔaraBAD<sub>AH33</sub> ΔrhaBAD<sub>LD78</sub> ΔilvB ΔilvI</i><br>[F' <i>proAB lacIq</i> Δ <i>M15</i> Tn10 (Tetr)]. | (Atsumi et. al. 2008a)           |
| ATCC98082 Δ <i>rhtA</i>        | <i>ilvA<sub>442</sub> supE spoT thrC<sub>1010</sub> Sac<sup>+</sup> ThrR ΔrhtA</i>                                                                                 | Zhang et. al. 2010               |
| EcRJM191                       | ATCC 98082 Δ <i>rhtA</i> Δ <i>recA</i>                                                                                                                             | This study                       |
| ST128                          | ATCC 98082 Δ <i>rhtA</i> Δ <i>adhE</i> Δ <i>recA</i>                                                                                                               | This study                       |
| ST139                          | ATCC 98082 Δ <i>rhtA</i> Δ <i>adhE</i> Δ <i>recA</i> pZS_thrO,<br>pZAlac_ilvA <sub>BS</sub> leuA, pZE_LeuA*BCDKA6_kivd_wt                                          | This study                       |
| ST140                          | ATCC 98082 Δ <i>rhtA</i> Δ <i>adhE</i> Δ <i>recA</i> pZS_thrO,<br>pZAlac_ilvA <sub>BS</sub> leuA,<br>pZE_LeuA*BCDKA6_kivd_G402V/M538L/F542V                        | This study                       |
| ST141                          | ATCC 98082 Δ <i>rhtA</i> Δ <i>adhE</i> Δ <i>recA</i> pZS_thrO,<br>pZAlac_ilvA <sub>BS</sub> leuA,<br>pZE_LeuA*BCDKA6_kivd_F381L/V461A                              | This study                       |
| <i>Plasmids</i>                |                                                                                                                                                                    |                                  |
| pQE9                           | colE1, <i>bla</i> , P <sub>T5/lac</sub> MCS                                                                                                                        | Qiagen                           |
| pQE_hiskivd_wt                 | colE1, <i>bla</i> , P <sub>T5/lac</sub> <i>his<sub>6</sub>kivd</i>                                                                                                 | Zhang et. al. 2008               |
| pZS_thrO                       | pSC101, <i>aadA</i> , P <sub>Llac01</sub> <i>thrA<sup>fbr</sup>BC</i>                                                                                              | Zhang et. al. 2008               |
| pZE_LeuA*BCDKA6                | colE1, <i>bla</i> , P <sub>Llac01</sub> <i>leuA*BCD kivd adh6</i>                                                                                                  | Marcheschi et. al. 2012          |
| pZAlac_ilvA <sub>BS</sub> leuA | p15A, <i>aphA</i> , <i>lacI<sup>q</sup></i> , P <sub>Llac01</sub> <i>ilvA<sub>BS</sub> leuA<sup>fbr</sup></i>                                                      | Marcheschi et. al. 2012          |