*Supplementary file 1 for* Baier at al., Cryptic genetic variation defines the adaptive evolutionary potential of enzymes

**Description of enzymes and directed evolution rounds**

**Supplementary File 1A.** Information about the enzymes characterized in this study.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Enzyme name** | **Uniprot ID** | **Genbank accession** | **Organismal source** | **PDB ID code (resolution)** | **References** |
| FIM1 | K7SA42 | AFV91534.1 | *Pseudomonas aeruginosa* | n.a. | (1) |
| EBL1 | Q2N9N3 | ABC63608.1 | *Erythrobacter litoralis HTCC 2594* | n.a. | n.a. |
| NDM1 | C7C422 | CAZ39946.1 | *Klebsiella pneumonia* | 3spu (2.1Å) | (2) |
| VIM2 | Q9K2N0 | YP\_007509601.1 | *Pseudomonas aeruginosa* | 1ko3 (1.9Å) | (3) |
| VIM1 | Q9XAY4 | CAB46686.1 | *Pseudomonas aeruginosa* | 5n5g (1.3 Å) | (4) |
| VIM7 | Q840P9 | YP\_001427370.1 | *Pseudomonas aeruginosa* | 2y87 (1.9Å) | (5) |

n.a, not available

**Supplementary File 1B.** Catalytic parameters of enzymes characterized in this study for PMH and β-lactamase activities.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **PMH Activity** | | | **β-lactamase Activity** | | |
| **Enzyme** | *k*cat [s-1] | *K*M [μM] | *k*cat *K*M [s-1M-1] | *k*cat [s-1] | *K*M [μM] | *k*cat *K*M [s-1M-1] |
| Fim1 | **0.0008** ± 0.00005 | **1300** ± 200 | **6.0 × 10-1** | **22** ± 1 | **6.5** ± 0.7 | **3.4 × 106** |
| EBL1 | **0.01** ± 0.0003 | **1500** ± 100 | **6.8 × 100** | **13** ± 1 | **6.2** ± 1 | **2.1 × 106** |
| NDM1 | **0.004** ± 0.001 | **13,000** ± 2000 | **3.2 × 10-1** | **39** ± 1 | **17** ± 2 | **2.3 × 106** |
| Vim2 | **0.005** ± 0.0001 | **840** ± 50 | **5.9 × 100** | **16** ± 0.7 | **4.2** ± 0.9 | **3.8 × 106** |
| Vim1 | **0.003** ± 0.0005 | **1400** ± 100 | **1.8 × 100** | **170** ± 3 | **100** ± 3 | **1.7 × 106** |
| Vim7 | **0.005** ± 0.0001 | **1900** ± 100 | **2.4 × 100** | **26** ± 0.4 | **9.6** ± 0.6 | **2.7 × 106** |

**Supplementary File 1C.** Mutation rate in the NDM1-wt and VIM2-wt naive libraries.

|  |  |  |  |
| --- | --- | --- | --- |
|  |  | **NDM1** | **VIM2** |
|  | **No of variants** | 43 | 44 |
| **Non-synonymous** | **No of mutations** | 93 | 143 |
| **mutations / gene** | 2.2 ± 1.4 | 3.3 ± 1.8 |
| **Synonymous** | **No of mutations** | 17 | 44 |
| **mutations / gene** | 0.4 ± 0.6 | 1.1 ± 1.1 |
| **Total** | **No of mutations** | 110 | 187 |
| **mutations / gene** | 2.6 ± 1.6 | 4.3 ± 2.1 |

**Supplementary File 1D.** Mutations identified in the naive library of VIM2-wt.

|  |  |  |
| --- | --- | --- |
|  | **VIM-2** |  |
| **Variant** | **Non-synonymous** | **Synonymous** |
| 1 | Y72D, D225G, E242D, G251D, L258W, N264S, N264H, K267P, T270P | p42p, s190s, a224a |
| 2 | D68G, S75F, H122Y, K260Q | g94g |
| 3 | R115C, I235S | g94g, a243a |
| 4 | L86S, V195M, N264D |  |
| 5 | N76S, L194R | l161l |
| 6 | E34G, E45K, V131A, S142P, D223A, Y240S |  |
| 7 | T98P, F121L, S202R | h122h |
| 8 | I54S, D56A, S202R, I210L |  |
| 9 | F121V, H269P | s214s |
| 10 | V38G, Q53P, Q108H, V113G |  |
| 11 | L161P | v80v, y184y |
| 12 | N97D |  |
| 13 | Q108R, S167N, I247T |  |
| 14 | H61Y, I109V, Q238R, H261R |  |
| 15 | D192G |  |
| 16 | E162A, V194L, C208W, N220H, H267R | v58v, p74p, i109i, n271n |
| 17 | W93G, Q238L | t37t, n76n, l148l |
| 18 | Q53R, A141V, V195L, T263A, V265A |  |
| 19 | N97S, T114P, F121L | t37t |
| 20 | Q65R, E106G, I156M, F183C, H189Q | v113v, l258l |
| 21 | Y72D, I79S, E150A, E242A, H269P, N261H | g83g |
| 22 | V43G, L101R, L204R | a243a |
| 23 | Y52S, Q53P, I79M |  |
| 24 | S202G |  |
| 25 |  | a131a |
| 26 | H61Q, V198A | t91t, l132l |
| 27 | S66A, Y184C |  |
| 28 | I109T | d84d, d130d, l194l |
| 29 | I79L, l182R, A187V | g128g, l258l |
| 30 | E40D, D68A, Q108Stop | y35y |
| 31 | S75A, K96Q, R115C, F121L, S214L, L258V |  |
| 32 | L48P, F67S | y35y, l88l |
| 33 | V46D, T262A |  |
| 34 | S144P, Y240D, V266G |  |
| 35 | S160A, V196A | l86l, a141a, a187a |
| 36 | R47W, S75P, V137G, I233V, I236M, Q237P | t37t |
| 37 | E40D, Y52S, G83D, T114P | i41i, a100a |
| 38 | E34A, T37P, I54S | v58v |
| 39 | L86S, L101P, L161P, V246A |  |
| 40 | I156L, F183S, I233S, I236L, T270P, | t37t |
| 41 | K96R, I79M, T158G | a63a |
| 42 | E40G, K267R | y35y, t262t |
| 43 | L161R, Y184S, S202R, Q237H |  |

**Supplementary File 1E.** Mutations identified in the naive library of NDM1-wt

|  |  |  |
| --- | --- | --- |
|  | **NDM-1** |  |
| **Variant** | **Non-synonymous** | **Synonymous** |
| 1 | G219D, L269R |  |
| 2 | A99T, K206R, N166D |  |
| 3 | I203T |  |
| 4 | no mutation |  |
| 5 | Y140F, M154L, N166T |  |
| 6 | T162P, T201A |  |
| 7 | D212G, K214M, N220S | s63s |
| 8 | M154R | s232s |
| 9 | S75P | a116a |
| 10 | I31T, V89A, V247A, S251F |  |
| 11 | Q60STOP |  |
| 12 | no mutation |  |
| 13 | N57K, L102H, F177V, S249I |  |
| 14 | L102P, N103S, E170G |  |
| 15 | K125E, F163V | t260t |
| 16 | L102P, N193K, L218F, F236L |  |
| 17 | H133R, N220K |  |
| 18 | T226I | n220n |
| 19 | F240S, Stop271C |  |
| 20 | I137T, K242R | f183f |
| 21 | T162A, F236L |  |
| 22 | E40A, F70S, T98A, M265T | a172a |
| 23 | N166H, E170D |  |
| 24 | no mutation |  |
| 25 | no mutation |  |
| 26 | I35M, H102P | t41t |
| 27 | R45R, A157V |  |
| 28 | G42A, N166S, K242E, M245T, K268R |  |
| 29 | M67T, V80A, I210T |  |
| 30 | M39I |  |
| 31 | T201A |  |
| 32 | no mutation |  |
| 33 | D223N |  |
| 34 | I79S, K106R, N142K, K242Q, S251P | l148l |
| 35 | L78V, N146H, I203S | f240f |
| 36 | S63A, L78V, L148P | s251s |
| 37 | H61R, K242M | t41t, v117v, l148l |
| 38 | D48G, D199G, K242Q |  |
| 39 | N57S, F70L | a230a |
| 40 | Y184H |  |
| 41 | D199A, F205C, D254E | d43d |
| 42 | S75P, T162A, F183S, D192N, D202G, I210L |  |
| 43 | N103D | e152e |

**Supplementary File 1F.** Information on the directed evolution procedure and mutations that were accumulated during directed evolution.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Round** | **Prescreen1** | **NDM1** | **VIM2** | **EBL1** | **VIM7** |
| 1 | 4 μg/ml AMP | W93G, N166T | V72A | W93L | W93G, K234E |
| 2 | 4 μg/ml AMP | K211R, G222D (S)2 | F67L | M107R, N220H | T232A |
| 3 | 4 μg/ml AMP | Q151R | D223A |  |  |
| 4 | 4 μg/ml AMP | S251F | S202R (S) |  |  |
| 5 | 4 μg/ml AMP | M154V, D96A (S) | G36R, T64A, T263S |  |  |
| 6 | 4 μg/ml AMP | D223E | V41A |  |  |
| 7 | 4 μg/ml AMP | N103K | N154T |  |  |
| 8 | 4 μg/ml AMP | A233V | T191P, V274A |  |  |
| 9 | 250 μM PMH | L49P | E150K, S66P |  |  |
| 10 | 250 μM PMH | V88M | V46D, N264D (S) |  |  |

1 pre-screening conditions for either β-lactam antibiotic resistance selection (AMP) or PMH activity screening on agar plates (PMH).

2 (S) indicates that DNA Shuffling was performed at this round.

**Supplementary File 1G.** PMH fitness values (cell lysate activity) of evolved and designed mutants.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Variant** | **NDM1** | **VIM2** | **EBL1** | **VIM7** |
| PMH fitness (nM/s) | | | |
| WT | **0.9** ± 0.2 | **3.5** ± 0.1 | **0.1** ± 0.02 | **0.3** ± 0.02 |
| R1 | **34** ± 3 | **7.9** ± 0.3 | **4.3** ± 0.3 | **0.9** ± 0.06 |
| R2 | **190** ± 10 | **13** ± 1 | **31** ± 1 | **1.1** ± 0.1 |
| R3 | **410** ± 20 | **21** ± 1 |  |  |
| R4 | **740** ± 120 | **28** ± 1 |  |  |
| R5 | **1300** ± 150 | **50** ± 1 |  |  |
| R6 | **470** ± 60 | **70** ± 2 |  |  |
| R7 | **910** ± 30 | **70** ± 2 |  |  |
| R8 | **1800** ± 250 | **88** ± 1 |  |  |
| R9 | **1500** ± 300 | **75** ± 1 |  |  |
| R10 | **3300** ± 220 | **120** ± 1 |  |  |
| K211R/G222D | **1.2** ± 0.1 |  |  |  |
| Q151R | **1.1** ± 0.1 |  |  |  |
| S251F | **1.5** ± 0.2 |  |  |  |
| W93G | **20.7** ± 2 | **1.1** ± 0.03 |  |  |
| W93A |  | **1.3** ± 0.07 |  |  |
| W93V |  | **0.72** ± 0.06 |  |  |
| W93L |  | **0.45** ± 0.08 |  |  |
| W93F |  | **1.2** ± 0.09 |  |  |
| F67L |  | **6.5** ± 0.1 |  |  |
| D223A |  | **4.9** ± 0.3 |  |  |
| S202R |  | **6.8** ± 0.3 |  |  |

± indicates standard deviation from triplicate measurements.

The cell lysate was diluted 2-fold prior activity measurement with 500 µM p-nitrophenyl-phenylphosphonate substrate. See Material and Methods for a detailed procedure on PMH fitness measurements.

**Supplementary File 1H.** Variants identified in the screening of additional round 1 libraries 1-3.

|  |  |  |
| --- | --- | --- |
| **VIM2 Library** | **Mutations** | **Fitness change in PMH activity1** |
| 1 | D68A, Q108H, P241Q, **T263A (r5)2** | 1.4 | |
| 1 | **V72A (r1)** | 1.5 | |
| 1 | **V41G (r6)**, **S66P (r9)**, D68N | 1.4 | |
| 1 | D68A, Q108H, P241Q, **T263A (r5)** | 1.4 | |
| 1 | K107T, **S202G (r4),** E242K | 1.4 | |
| 1 | **V41G (r6)**, **S66P (r9)**, D68N | 1.9 | |
| 2 | **F67L (r2)** | 1.4 | |
| 2 | **F67L (r2)** | 1.6 | |
| 2 | **F67L (r2)** | 1.6 | |
| 2 | **V72A (r1)** | 1.6 | |
| 2 | **F67L (r2)** | 1.4 | |
| 2 | **F67L (r2)** | 1.4 | |
| 2 | Y73F, I79V | 1.1 | |
| 2 | R50Q, **F67L (r2)** | 1.3 | |
| 3 | P157S | 1.3 | |
| 3 | **S202R (r4)** | 1.4 | |
| 3 | V131A | 0.9 | |
| 3 | D84G | 1.0 | |
| 3 | **F67L (r2),** I156T | 1.3 | |
| 3 | A100V, S144T | 1.1 | |
| 3 | D68N | 1.5 | |

|  |  |  |
| --- | --- | --- |
| **NDM1 Library** | **Mutations** | **Fitness change in PMH activity1** |
| 1 | **W93G (r1)**2 | 55 |
| 1 | **W93G (r1)** | 45 |
| 1 | **W93G (r1)** | 40 |
| 1 | **W93C (r1)**, K181Q | 25 |
| 1 | **M154T (r5)**, K276R | 2.1 |
| 1 | **M154T (r5)**, K276R | 1.9 |
| 1 | **M154V (r5)** | 1.6 |
| 2 | **W93L (r1)**, N57D | 28 |
| 2 | V113A, H133R | 1.7 |
| 2 | V133A, H133R | 1.5 |
| 2 | V133A, H133R | 1.5 |
| 2 | **M154V (r5)**, D212N | 1.5 |
| 3 | **M154T (r5)** | 2.7 |
| 3 | **M154T (r5)** | 2.3 |
| 3 | **M154T (r5)** | 2.3 |
| 3 | **M154T (r5)** | 2.3 |
| 3 | **M154T (r5)** | 2.2 |
| 3 | **M154T (r5)** | 1.9 |
| 3 | L148F | 1.6 |

1 indicates the PMH fitness of the variant relative to that of the wild-type, measured in cell lysate.

2 Bolded mutations indicate positions that were also mutated in the original trajectory. The round at which the position was mutated is indicated in between brackets.

**Supplementary File 1I.** Changes in catalytic activity of purified MBL mutants compared to their respective wild-type enzymes, and melting temperature.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Enzyme** | **Variant** | **Catalytic activity**  **relative to WT** | | ***T*m50 (°C)** |
| PMH | β-lactamase |
| **FIM1** | WT | **1.0** ± 0.05 | **1.0** ± 0.05 | **53.5** ± 0.7 |
| **FIM1** | H72V | n.d. | n.d. | **54.0** ± 0.9 |
| **FIM1** | W93G | **6.5** ± 0.30 | **0.9** ± 0.03 | **46.2** ± 1.3 |
| **EBL1** | WT | **1.0** ± 0.05 | **1.0** ± 0.05 | **66.2** ± 0.5 |
| **EBL1** | W93G | **5.0** ± 0.20 | **1.6** ± 0.08 | **64.0** ± 0.8 |
| **EBL1** | P72V | **0.4** ± 0.02 | **1.3** ± 0.06 | **64.8** ± 0.6 |
| **EBL1** | P72A | **0.5** ± 0.02 | **1.8** ± 0.08 | **65.9** ± 0.6 |
| **NDM1** | WT | **1.0** ± 0.09 | **1.0** ± 0.02 | **55.0** ± 0.4 |
| **NDM1** | W93G | **104** ± 7.55 | **2.1** ± 0.05 | **47.4** ± 0.4 |
| **NDM1** | A72V | **0.8** ± 0.15 | **1.3** ± 0.05 | **54.8** ± 0.5 |
| **VIM2** | WT | **1.0** ± 0.01 | **1.0** ± 0.01 | **58.7** ± 0.6 |
| **VIM2** | V72A | **1.7** ± 0.03 | **1.1** ± 0.01 | **57.3** ± 0.6 |
| **VIM2** | W93G | **0.1** ± 0.01 | **0.1** ± 0.01 | **62.0** ± 1.0 |
| **VIM1** | WT | **1.0** ± 0.03 | **1.0** ± 0.01 | **59.0** ± 0.3 |
| **VIM1** | V72A | **3.0** ± 0.15 | **1.1** ± 0.02 | **55.1** ± 0.5 |
| **VIM1** | W93G | **0.3** ± 0.01 | **0.1** ± 0.01 | **55.4** ± 1.8 |
| **VIM7** | WT | **1.0** ± 0.03 | **1.0** ± 0.03 | **56.8** ± 1.0 |
| **VIM7** | V72A | **1.1** ± 0.04 | **0.8** ± 0.05 | **54.1** ± 0.5 |
| **VIM7** | W93G | **3.2** ± 0.14 | **0.2** ± 0.01 | **53.2** ± 0.8 |

n.d, not determined

± indicates standard deviation from triplicate measurement.

All MBL mutants were purified using Strep-tactin affinity chromatography. The catalytic activities of the purified enzymes were measured at single enzyme (5 μM for PMH and 1 nM for β-lactamase activity) and substrate (500 μM for PMH and 100 μM for β-lactamase activity) concentrations.

**Supplementary references**

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