

## Supplementary materials

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# **A step forward to the optimized HlyA type 1 secretion system through directed evolution**

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**Table S1** Primers used for epPCR amplification of fragments

| <b>Fragments</b>         | <b>Forward primer (5' to 3')</b> | <b>Reverse primer (5' to 3')</b> |
|--------------------------|----------------------------------|----------------------------------|
| F <sub>1B</sub> (275 bp) | GCCACATAAGATTGCTATTTTT<br>TG     | CCATCCTCTCTCCAGACTAATG           |
| F <sub>2B</sub> (251 bp) | TCTGGAGAGAGGATGGACGTC            | AACAAGGGTTTCAATAAATATTCTC        |
| F <sub>3B</sub> (283 bp) | GAATATTTATTGAAACCCTTGTT<br>GTG   | CGACTCTCAAAATAAGAGATCGG          |
| F <sub>4B</sub> (256 bp) | CTCTTATTTTGAGAGTCGTCGTG          | CCGTGAAAACCTTATCATCAAGGC         |
| F <sub>5B</sub> (279 bp) | TGATGATAAGTTTTTCACGGAATG<br>CG   | GCAATTAAGTACCAATCGATAAA<br>TCC   |
| F <sub>6B</sub> (291 bp) | CTGGTTATTTCCGGGGATTATC<br>G      | CCCCTGCTTAATACTGAGATTG           |
| F <sub>1D</sub> (279 bp) | CAGTTACAGTCAGACTAACAG            | TTCCACCTGACCTAAAAC               |
| F <sub>2D</sub> (260 bp) | GGTTTCTGGTTATTGCTG               | AATTCAATTGACCTGCTC               |
| F <sub>3D</sub> (289 bp) | CGGTATCAAATTCTGAGCAG             | CCTGTTTATGCAATAAACTCC            |
| F <sub>4D</sub> (290 bp) | GGTTGAAAAAAGCCGTCTG              | AAGCCTGTTGACGCTCTTC              |
| F <sub>5D</sub> (280 bp) | GCATTGAGTTATTAAGTCTGGAG          | CCCACCAGATAACCATATC              |
| F <sub>6D</sub> (287 bp) | CCTTCCTTACACCCGATATG             | CCGGCCGCTCTAGATTAC               |

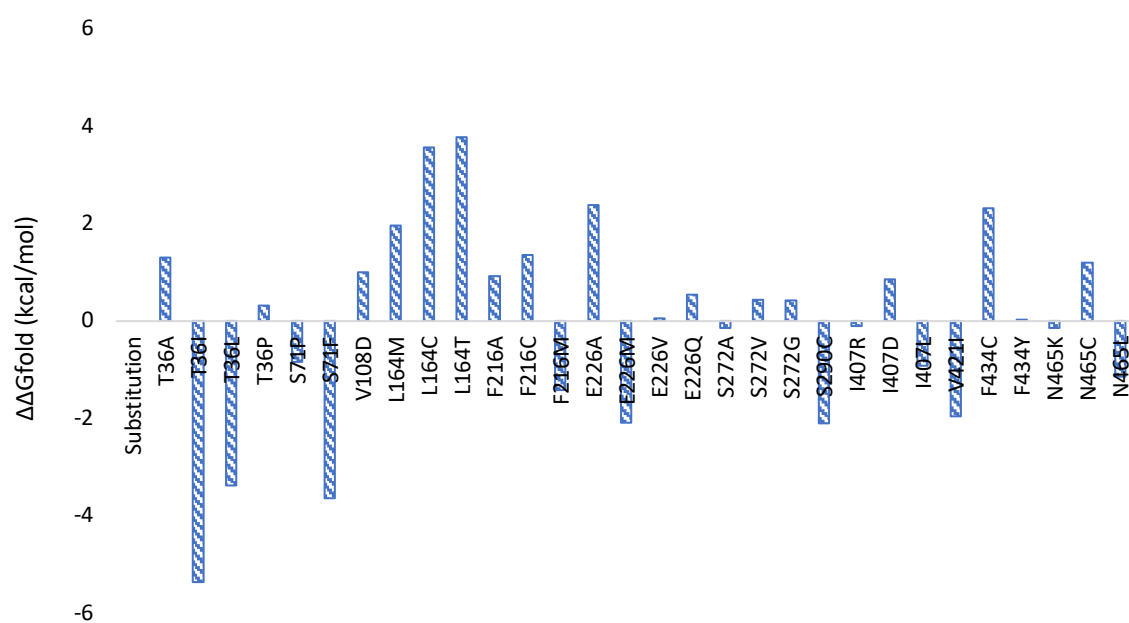
**Table S2** Primers used for site-saturation at potential beneficial positions

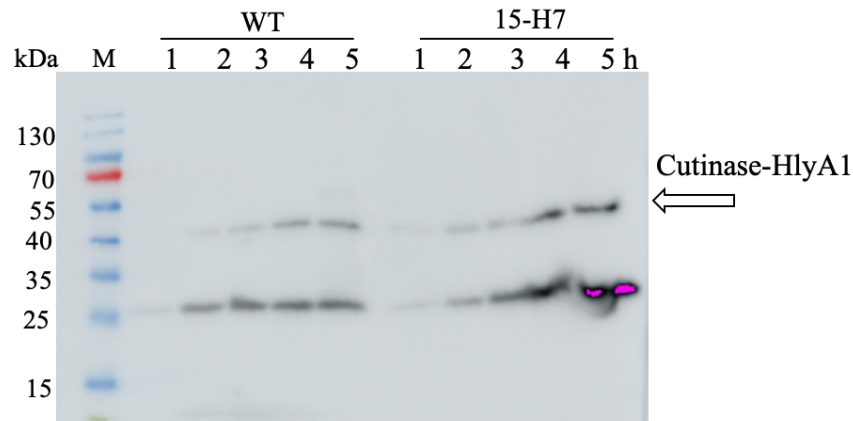
| <b>Beneficial positions</b>                          | <b>Forward primer</b>            | <b>Reverse primer</b>           |
|------------------------------------------------------|----------------------------------|---------------------------------|
| <i>SSM of potential beneficial positions of HlyB</i> |                                  |                                 |
| Ter 36                                               | CATAGATTTGACNNKGACGG             | CCGTCMNNGTCAAATCTATG            |
| Ser 71                                               | GATTAAACTTTATTNNKCTGCCC<br>G     | CGGGCAGMNNNAATAAAGTTTAATC       |
| Val 108                                              | GAAATCCCCGTNNKCTCGAACA<br>G      | CTGTTCGAGMNNACGGGGATTTC         |
| Leu 164                                              | GTCTGTTTTTNNKCAATTATTTG          | CAAATAATTGMNNAAAAAACAGAC        |
| Phe 216                                              | GAACCTACATTNNKGCACATAGT<br>ACAAG | CTTGACTATGTGCMNNAATGTAAG<br>TTC |
| Glu 226                                              | GATTGATGTTNNKTTGGGTG             | CACCCAAMNNAACATCAATC            |
| Ser 272                                              | GACAGGCATTAACANNKGTTC<br>G       | CAGAACMNNTGTTAATGCCTGTC         |
| Ser 290                                              | GTGGTATTACNNKCCAAAG              | CTTTGGMNNGTAATACCAC             |
| Ile 407                                              | GATTTATCGNNKGGTCAGTTAAT<br>TG    | CAATTAAGTACCMNNCGATAAATC        |
| Val 421                                              | GCTGGTCAGATTNNKGCACCG            | CGGTGCMNNAATCTGACCAGC           |
| Phe 434                                              | GCACAAATCTGGCAGGATNNKC<br>AGCAG  | CTGCTGMNNATCCTGCCAGATTGT<br>GC  |
| Asn 465                                              | GGAAATTNNKGGTGATATCACTT<br>TTCG  | CGAAAAGTGATATCACCMNNAATTT<br>CC |
| <i>SSM of potential beneficial positions of HlyD</i> |                                  |                                 |
| Met 6                                                | CATGGTTANNKGGGTTCAG              | CTGAACCCMNNTAACCATG             |
| Ile 49                                               | GGAATTANNKGAAACGCCG              | CGGCGTTTCMNNTAATTCC             |
| Val 69                                               | GTTTCTGNNKATTGCTGTC              | GACAGCAATMNNCAGAAAC             |

**Table S3** Internal-atomic distance between 12 beneficial positions of the HlyB wildtype measured by YASARA. The distance values were reported with Å unit.

|      | T36 | S71  | V108 | L164 | F216 | E226 | S272 | S290 | I407 | V421 | F434 | N465 |
|------|-----|------|------|------|------|------|------|------|------|------|------|------|
| T36  | 0   | 26.3 | 27.6 | 53.1 | 40.9 | 30.1 | 40.2 | 63.0 | 70.9 | 49.3 | 29.2 | 37.5 |
| S71  |     | 0    | 18.2 | 62.1 | 54.5 | 48.1 | 46.2 | 62.1 | 72.7 | 52.8 | 36.3 | 59.4 |
| V108 |     |      | 0    | 52.3 | 46.2 | 44.1 | 38.5 | 50.6 | 59.4 | 40.5 | 27.7 | 65.0 |
| L164 |     |      |      | 0    | 14.5 | 29.4 | 16.5 | 24.3 | 26.7 | 16.4 | 26.2 | 72.6 |
| F216 |     |      |      |      | 0    | 15.5 | 15.0 | 36.4 | 39.4 | 22.6 | 19.1 | 58.5 |
| E226 |     |      |      |      |      | 0    | 22.8 | 48.3 | 54.1 | 31.0 | 20.8 | 43.2 |
| S272 |     |      |      |      |      |      | 0    | 26.4 | 34.6 | 15.4 | 12.3 | 64.1 |
| S290 |     |      |      |      |      |      |      | 0    | 13.1 | 15.5 | 34.2 | 90.3 |
| I407 |     |      |      |      |      |      |      |      | 0    | 21.7 | 42.3 | 96.9 |
| V421 |     |      |      |      |      |      |      |      |      | 0    | 20.6 | 76.5 |
| F434 |     |      |      |      |      |      |      |      |      |      | 0    | 57.7 |
| N465 |     |      |      |      |      |      |      |      |      |      |      | 0    |

**Table S4**  $\Delta\Delta G_{\text{fold}}$  (kcal/mol) values of amino acid substitutions on the twelve beneficial positions of the HlyB calculated using FoldX method.





**Fig S1** Improved secretion of cutinase-HlyA1 by the 15-H7 variant. a) Western blot analysis of supernatant fractions from the 15-H7 variant the wildtype cultures. The molecular weight (kDa) of the marker proteins (M) is given on the left; xh: cell pellets of culture, where x denotes the number of hours after induction. This experiment performed in triplicate.

## HlyD KnowVolution

**Phase I (Identification):** In the first phase, beneficial positions of HlyD were identified through the cepPCR as already explained above for the HlyB. Based on the cepPCR approach, the gene of *hlyD* was divided into six fragments (F<sub>1D</sub>: 279 bp, F<sub>2D</sub>: 260 bp, F<sub>3D</sub>: 289 bp, F<sub>4D</sub>: 290 bp, F<sub>5D</sub>: 280 bp, F<sub>6D</sub>: 287 bp). All steps of library generation and screening were performed as already explained for the HlyB protein.

For each fragment, a library with a size of 786 was generated. Subsequently, the libraries were screened in a 96-well MTP format using the *pNPB* screening to identify clones with higher secreted lipase-HlyA1. None of the fragments, except of the first fragment, showed promising variants. The promising variants of the first fragment were sent for sequencing. Alignment of the sequence of this promising variants identified three potential beneficial positions, listed as: M6, I49, V69.

**Phase II (determination):** To obtain full diversity on the identified beneficial positions, the two beneficial positions were subjected to individual site-saturation mutagenesis. In this step no promising variant were found after screening of the site-saturation libraries for none of the identified positions. To be sure about the outcome of the KnowVolution of HlyD, a new libraries were generated on the fragments 1-2. But still no positions were found.