**Supplementary File 3: Fit parameters**

**a: p-values for the difference in lag between each variant and wt.**

|  |  |
| --- | --- |
| AL | 1.16168E-08 |
| EQ | 0.265292515 |
| FW | 2.89942E-05 |
| ∆h1 | 0.488388125 |
| ∆h2 | 2.55424E-05 |
| ∆h3 | 0.00045834 |
| IT | 0.000186505 |
| KQ | 0.00870247 |
| KR | 3.87805E-06 |
| LA | 0.011269789 |
| LXX | 6.15403E-09 |
| ∆p1 | 0.8765962 |
| ∆p2 | 0.158087507 |
| ∆p3 | 0.093423493 |
| QE | 0.002919792 |
| QK | 1.78073E-05 |
| QR | 5.28042E-11 |
| RK | 0.124575186 |
| RQ | 1.08241E-06 |
| TV | 1.52642E-06 |

**b: List of best fit parameters for all pSpyXLX variants**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **variant** | **n** | **kstep,var** | **kblock,var** | **kfail,var** |
| wt | 5.91670 ± 0.19301 | 6.73570 ± 0.24265 | 0.31 (fixed) | 0.06886 ± 0.01291 |
| LA | 4 | 5.07013 ± 0.09586 | 0.31396 ± 0.01771 | 0.24708 ± 0.01415 |
| AL | 5.33333 ± 0.33333 | 2.73244 ± 0.21073 | 0.19021 ± 0.00790 | 0.09855 ± 0.00670 |
| FW | 4 | 2.49783 ± 0.01229 | 0.32322 ± 0.00468 | 0.10616 ± 0.00594 |
| IT | 7 ± 0.57735 | 12.27283 ± 1.05754 | 0.26227 ± 0.02595 | 0.33184 ± 0.04372 |
| TV | 5 | 3.27936 ± 0.05723 | 0.22540 ± 0.00883 | 0.02220 ± 0.00897 |
| EQ | 5.66667 ± 0.33333 | 6.93964 ± 0.83979 | 0.26707 ± 0.02426 | 0.15690 ± 0.02525 |
| QE | 4.66667 ± 0.33333 | 5.66993 ± 0.70510 | 0.19629 ± 0.02956 | 0.53530 ± 0.04485 |
| RQ | ≥20 | ≥64 | 0.25762 ± 0.01797 | 0.39651 ± 0.02940 |
| KQ | 6.66667 ± 0.66667 | 11.35220 ± 2.99614 | 0.28326 ± 0.02741 | 0.11947 ± 0.04241 |
| RK | 6.33333 ± 0.33333 | 8.11439 ± 0.52564 | 0.29334 ± 0.02818 | 0.14943 ± 0.06589 |
| KR | 5 | 3.28732 ± 0.12557 | 0.20297 ± 0.01269 | 0.06205 ± 0.01115 |
| QK | 4.33333 ± 0.33333 | 2.76085 ± 0.24414 | 0.21420 ± 0.00654 | 0.04711 ± 0.02008 |
| QR | 5 | 0.93044 ± 0.06346 | 0.13110 ± 0.00816 | 0.05710 ± 0.01269 |
| ∆h1 | 6 | 6.48068 ± 0.21797 | 0.29663 ± 0.01000 | 0.07904 ± 0.00449 |
| ∆h2 | 7 | 5.32258 ± 0.08994 | 0.22629 ± 0.02141 | 0.07786 ± 0.02570 |
| ∆h3 | 6 | 4.76603 ± 0.29008 | 0.19169 ± 0.00957 | 0.11433 ± 0.02188 |
| ∆φ1 | 4.33333 ± 0.33333 | 4.32668 ± 0.34422 | 0.18862 ± 0.01671 | 0.21557 ± 0.00606 |
| ∆φ2 | 6 ± 0.57735 | 6.13496 ± 1.20729 | 0.21340 ± 0.01078 | 0.27345 ± 0.03692 |
| ∆φ3 | 6 ± 0.57735 | 7.39306 ± 1.17548 | 0.21314 ± 0.02301 | 0.26077 ± 0.05402 |

**c: p-values for bioinformatic analysis of arginine/lysine ratios**

|  |  |  |  |
| --- | --- | --- | --- |
|  | Sec vs unsecreted | Sec vs Tat | Tat vs unsecreted |
| *E. coli* | 1.68728E-47 | 0.071764886 | 0.107526671 |
| *S. meliloti* | 3.444E-143 | 0.009989205 | 1.90911E-24 |
| *B. halodurans* | 0.000433917 | – | – |
| *B. subtilis* | 2.44192E-50 | – | – |

**d: Comparison of the proton-motive force (PMF) generated by whole cells and inverted membrane vesicles (IMVs)**

|  |  |  |  |
| --- | --- | --- | --- |
| **Sample** | **Δψ (mV)** | **ZΔpH (mV)** | **PMF (mV)** |
| **pBAD cells** | 40.7 ± 3.2 | 86.5 ± 3.3 | 127.1 ± 6.6 |
| **pBAD IMVs** | 28.9 ± 24.0 | 52.2 ± 8.1 | 81.1 ± 32.2a |
| **pSEC cells** | 32.8 ± 5.5 | 80.8 ± 5.1 | 113.6 ± 10.6 |
| **pSEC IMVs** | 33.0 ± 22.4 | 70.9 ± 2.5 | 103.9 ± 24.9 |

aStatistically significant vs pBAD cells (*p* = 0.0084, two-way ANOVA, Tukey’s multiple comparison test, 95% C.I.)