**The adaptive landscape of wildtype and glycosylation-deficient populations of the industrial yeast *Pichia pastoris***

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# Additional File 1

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# Supplemental Results

### Growth characteristics of ancestral *P. pastoris* strains

For the purpose of subsequent direct competition assays, *P. pastoris* strains were tagged by the expression two recombinant reporter proteins. The *PGK1* (phosphoglycerate kinase) promoter ($P_{PGK1}$) was applied for fluorescent protein expression (Figure S1). While this promoter is active over a wide range of growth conditions, it results in low recombinant protein expression as compared with other promoters [1]. In this context, metabolic engineering and recombinant protein production have been reported to pose a metabolic burden on microbial host cells [2]. Specifically, in a recent study even moderate recombinant protein production resulted in a detectable metabolic burden in *P. pastoris* [3]. Thus, we analyzed the growth characteristics and fitness of the founding strains in detail.

Subtle growth rate differences between strains BG10 and X-33 on BMD medium indicated minor differences between the ancestral wildtype strains. Furthermore, the DsRed-tagged X-33 strain showed a decreased growth rate on YPDN as compared with the corresponding GFP-tagged strain (Table S2). Similarly, direct competition assays indicated decreased fitness (ω) of DsRed-expressing wildtype strains across all growth conditions (Table S3). Thus, a metabolic burden due to marker protein expression was observed.

In contrast to the ancestral wildtype strains, marker protein-dependent fitness differences were either absent or even reversed in the ancestral BG10 Δ*OCH1* strains (Table S3). Potential differences between the BG10 wildtype and BG10 Δ*OCH1* strain were analyzed. The wildtype only showed a clear growth rate advantage over the GFP- or DsRED-tagged Δ*OCH1* strains on YPD but not on salt stress and minimal growth media (Table S2). Additional competition assays of ancestral BG10 and Δ*OCH1* strains revealed a significant effect of the recombinant protein marker itself; e.g. the DsRed-expressing wildtype strain showed increased competitive fitness over the eGFP-expressing *OCH1* knockout strain during growth on YPD, whereas the *vice versa* combination showed nearly identical fitness (Table S4).

Although the initial fitness differences had to be considered in the calculation of competitive fitness of the evolved populations, it also offered the opportunity to analyze a potential bias during laboratory evolution due to non-neutral markers.

Whereas possible explanations for these phenomena include copy number variations and differences in the amino acid composition of the fluorescent proteins, these data also indicate a more complex correlation of N-glycosylation deficiency, recombinant protein burden and the environmental growth context than previously reported [4, 5] and will merit a detailed analysis in a separate study.

## Supplemental Tables

| name | 5' → 3' sequence | purpose |
|---|---|---|
| Ppgk1__BglII_fw | AAAGATCTAAGTTGGTACCCAGCCGATCAC | reporter plasmid |
| pPpgk1_EcoRI_rv | ATGAATTCTTTCGTAATCAATTGGGCTATG | reporter plasmid |
| eGFP_EcoRI_fw | ATGAATTCATGGTGAGCAAGGGCGAGGA | reporter plasmid |
| eGFP_NotI_rv | ATGCGGCCGCTTACTTGTACAGCTCGTCCAT | reporter plasmid |
| DsRed_EcoRI_fw | ATGAATTCATGGCCTCCTCCGAGAACGT | reporter plasmid |
| DsRed_NotI_rv | ATGCGGCCGCTTACAGGAACAGGTGGTGGC | reporter plasmid |
| ACS1_fw | ACAACTATTTCGAAACGAGGAATTCATGCCATTAGATAACGAACA | cloning pGAPzB |
| ACS1_rv | GTTTTTGTTCTAGAAAGCTGGCGGCCGCTCATTTGCGGGCATCCCTTT | cloning pGAPzB |
| chr3_0669_fw | ACAACTATTTCGAAACGAGGAATTCATGCCGAAACGTAATAAGAC | cloning pGAPzB |
| chr3_0669_rv | GTTTTTGTTCTAGAAAGCTGGCGGCCGCTCAGCCTCTGTGGAAGTTGA | cloning pGAPzB |
| FLC2_fw | ACAACTATTTCGAAACGAGGAATTCATGACCATAGTGACGTGTGC | cloning pGAPzB |
| FLC2_rv | GTTTTTGTTCTAGAAAGCTGGCGGCCGCCTAGTTCCATCTAACGTCTT | cloning pGAPzB |
| TUP1_fw | ACAACTATTTCGAAACGAGGAATTCATGTCGTACAACAGACCATT | cloning pGAPzB |
| TUP1_rv | GTTTTTGTTCTAGAAAGCTGGCGGCCGCTCAAGTCCACTTCCAGATTC | cloning pGAPzB |
| ACS1_qPCR_fw | GAATCCAGACAAACCTGCC | real-time PCR |
| ACS1_qPCR_rv | GTCACCCTTTTTCACACCC | real-time PCR |
| FLC2_qPCR_fw | ACATCCGAAAAATACCACAGAC | real-time PCR |
| FLC2_qPCR_rv | CCATAGAACCTTCCATAGCAC | real-time PCR |
| ACT1_qPCR_fw | CCAAACCACTTACAACTCCATC | real-time PCR |
| ACT1_qPCR_rv | GCATACGCTCAGCAATACC | real-time PCR |

**Table S1. DNA oligonucleotides used in the current study.** List of oligonucleotides that were used for cloning procedures and real-time PCR.

| | strain | |
|---|---|---|
| | **X-33** | |
| **growth medium** | eGFP | DsRed |
| YPD | 0.322 +/- 0.010 | 0.323 +/- 0.005 |
| YPDN | 0.283 +/- 0.006 | 0.233 +/- 0.021 |
| BMD | 0.321 +/- 0.002 | 0.326 +/- 0.022 |
| BMDN | 0.252 +/- 0.003 | 0.256 +/- 0.010 |
| | **BG10** | |
| **growth medium** | eGFP | DsRed |
| YPD | 0.292 +/- 0.013 | 0.289 +/- 0.021 |
| YPDN | 0.199 +/- 0.007 | 0.209 +/- 0.005 |
| BMD | 0.231 +/- 0.010 | 0.223 +/- 0.010 |
| BMDN | 0.225 +/- 0.006 | 0.213 +/- 0.010 |
| | **BG10 *OCH1*** | |
| **growth medium** | eGFP | DsRed |
| YPD | 0.221 +/- 0-030 | 0.233 +/- 0-016 |
| YPDN | 0.196 +/- 0.005 | 0.195 +/- 0.003 |
| BMD | 0.236 +/- 0.004 | 0.230 +/- 0.009 |
| BMDN | 0.204 +/- 0.003 | 0.222 +/- 0.009 |

**Table S2**. **Growth rates, μ [$h^{-1}$] of ancestral GFP-and DsRed-expressing strains in all growth media used for subsequent experimental evolution.** Values represent averages +/- standard error of *n* = 4.

| | fitness [ω] of GFP strain | | | |
|---|---|---|---|---|
| | **YPD** | **YPDN** | **BMD** | **BMDN** |
| **X-33** | 1.07 +/- 0.07 | 1.15 +/- 0.02 | 1.09 +/- 0.13 | 1.11 +/- 0.02 |
| **BG10** | 1.18 +/- 0.07 | 1.16 +/- 0.07 | 1.34 +/- 0.05 | 1.30 +/- 0.09 |
| **BG10 Δ*OCH1*** | 1.04 +/- 0.01 | 0.99 +/- 0.02 | 0.88 +/- 0.01 | 0.95 +/- 0.00 |

**Table S3**. **Fitness [ω] of ancestral GFP-expressing strains**. Fitness is in comparison with the ancestral DsRED-expressing *P. pastoris* strains used in the current study. Values represent average +/- standard deviation of *n* = 4.

| | competition | |
|---|---|---|
| **medium** | **BG10 eGFP vs. Δ*OCH1* DsRed** | **BG10 DsRed / Δ*OCH1* eGFP** |
| **YPD** | 0.956 +/- 0.019 | 2.899 +/- 0.130 |
| **YPDN** | 0.868 +/- 0.030 | 0.854 +/- 0.059 |
| **BMD** | 1.212 +/- 0.014 | 1.007 +/- 0.090 |
| **BMDN** | 1.189 +/- 0.067 | 0.770 +/- 0.029 |

**Table S4 Table**. **Fitness [ω] of ancestral BG10 strains as compared to ancestral BG10 Δ*OCH1* strains.** Values represent the mean of *n* = 3 competitions +/- standard deviation.

| Growth medium | Population | X-33 | BG10 | BG10 Δ*OCH1* |
|---|---|---|---|---|
| **YPD** | Population 1 | 10.45 | 10.45 | 10.38 |
| | Population 2 | 10.45 | 10.46 | 10.34 |
| | Population 3 | 10.45 | 10.44 | 10.38 |
| | Population 4 | 10.45 | 10.45 | 10.36 |
| **YPDN** | Population 1 | 10.39 | 10.42 | 10.27 |
| | Population 2 | 10.37 | 10.42 | 10.22 |
| | Population 3 | 10.37 | 10.42 | 10.25 |
| | Population 4 | 10.37 | 10.37 | 10.23 |
| **BMD** | Population 1 | 10.35 | 10.46 | 10.40 |
| | Population 2 | 10.41 | 10.41 | 10.39 |
| | Population 3 | 10.42 | 10.40 | 10.39 |
| | Population 4 | 10.35 | 10.42 | 10.42 |
| **BMDN** | Population 1 | 10.28 | 10.42 | 10.32 |
| | Population 2 | 10.32 | 10.40 | 10.33 |
| | Population 3 | 10.29 | 10.43 | 10.32 |
| | Population 4 | 10.29 | 10.39 | 10.34 |

**Table S5**. **Estimated number of CCD ($10^x$) for each of the evolved *P. pastoris* population.** CCD was calculated as described in in the Material and Methods sections of the main manuscript.

| | | growth medium | | | |
|---|---|---|---|---|---|
| | **populations** | **YPD** | **YPDN** | **BMD** | **BMDN** |
| **X-33** | **YPD500** | 126.1 +/- 12.6 | 129.5 +/- 24.3 | 87.4 +/- 1.8 | 100.5 +/- 4.6 |
| | **YPDN500** | 112.1 +/- 8.1 | 108.6 +/- 12.9 | 105.6 +/- 5.5 | 116.6 +/- 8.6 |
| | **BMD500** | 87.2 +/- 12.6 | 74.3 +/- 15.5 | 133.7 +/- 27.3 | 122.7 +/- 18.2 |
| | **BMDN500** | 104.5 +/- 16.8 | 74.3+/- 15.5 | 119.8. +/- 6.5 | 136.1 +/- 8.9 |
| **BG10** | **YPD500** | 109.1 +/- 8.3 | 164.1 +/- 52.0 | 118.2 +/- 7.1 | 106.3 +/- 4.5 |
| | **YPDN500** | 108.4 +/- 5.3 | 135.7 +/- 6.1 | 118.7 +/- 11.4 | 111.0 +/- 25.5 |
| | **BMD500** | 102.2 +/- 4.1 | 112.4 +/- 26.0 | 120.9 +/- 20.5 | 142.8 +/- 9.2 |
| | **BMDN500** | 116.1 +/- 15.0 | 94.4 +/- 59.3 | 115.4 +/- 3.5 | 133.7 +/-.9.9 |
| **BG10 Δ*OCH1*** | **YPD500** | 101.6 +/- 10.3 | 101.0 +/- 17.8 | 99.1 +/- 7.0 | 95.1 +/- 2.9 |
| | **YPDN500** | 115.9 +/- 13.6 | 110.3 +/- 5.5 | 99.5 +/- 14.0 | 99.4 +/- 10.2 |
| | **BMD500** | 96.0 +/- 32.6 | 96.8 +/- 20.2 | 96.0 +/- 13.7 | 101.3 +/- 9.5 |
| | **BMDN500** | 74.9 +/- 20.8 | 106.2 +/- 21.9 | 107.5 +/- 15.0 | 105.8 +/- 11.4 |

**Table S6**. **Maximum growth rates during exponential phase [% $\mu_{ancestor}$] of evolved *P. pastoris* populations in adaptive and non-adaptive conditions.** Average growth rates +/- standard deviation from all 4 replicate populations measured in duplicates (*n* = 8).

| | | growth medium | | | |
|---|---|---|---|---|---|
| | **populations** | **YPD** | **YPDN** | **BMD** | **BMDN** |
| **X-33** | **YPD500** | 117.4 +/- 13.3 | 94.1 +/- 33.4 | 106.2 +/- 6.2 | 118.4 +/- 13.7 |
| | **YPDN500** | 87.6 +/- 11.1 | 129.5 +/- 27.5 | 88.7 +/- 36.5 | 141.3 +/- 12.8 |
| | **BMD500** | 104.4 +/- 5.8 | 142.1 +/- 12.3 | 72.2 +/- 41.2 | 61.9 /- 38.9 |
| | **BMDN500** | 120.4 +/- 4.7 | 148.5 +/- 11.7 | 107.8 +/- 29.9 | 67.0 +/- 32.4 |
| **BG10** | **YPD500** | 106.7 +/- 5.4 | 88.2 +/- 13.9 | 103.0 +/- 6.6 | 167.0 +/- 9.9 |
| | **YPDN500** | 94.6 +/- 12.5 | 235.4 +/- 13.6 | 91.3 +/- 20.8 | 62.4 +/- 23.4 |
| | **BMD500** | 86.7 +/- 9.2 | 80.5 +/- 15.6 | 131.7 +/- 11.9 | 150.3 +/- 31.3 |
| | **BMDN500** | 96.0 +/- 20.1 | 108.4 +/- 24.2 | 92.8 +/- 10.3 | 124.9 +/- 4.2 |
| **BG10 Δ*OCH1*** | **YPD500** | 121.9 +/- 8.7 | 73.1 +/- 10.8 | 75.2 +/- 19.2 | 43.6 +/- 36.1 |
| | **YPDN500** | 71.4 +/- 13.1 | 76.7 +/- 7.8 | 132 .0+/- 31.0 | 78.5 +/- 23.7 |
| | **BMD500** | 109.7 +/- 14.7 | 90.4 +/- 12.0 | 66.7 +/- 28.9 | 70.5 +/- 19.7 |
| | **BMDN500** | 120.0 +/- 12.1 | 66.0 +/- 17.9 | 70.9 +/- 11.5 | 122.9 +/-14.2 |

**Table S7**. **Early growth rates [% $\mu_{ancestor}$] from t = 0 to 2 hours after inoculation of evolved *P. pastoris* populations in adaptive and non-adaptive conditions.** Average growth rates +/- standard deviation from all 4 replicate populations measured in duplicates (*n* = 8).

| strain | clone | coverage | mapped | % mapped | properly paired | % properly paired | singletons | % singletons | % genome covered |
|---|---|---|---|---|---|---|---|---|---|
| X-33 | **YPD GFP 1a** | 71 | 2664663 | 81.43% | 2559838 | 78.64% | 60974 | 1.87% | 91,11 |
| | **YPD RED 1b** | 75 | 2820130 | 82.41% | 2710842 | 79.78% | 53594 | 1.58% | 91,12 |
| | **YPD GFP 2b** | 72 | 2771943 | 85.00% | 2611892 | 81.19% | 53112 | 1.65% | 91,09 |
| | **YPD RED 2b** | 81 | 3064606 | 84.26% | 2938400 | 81.43% | 59031 | 1.64% | 91,11 |
| | **YPDN GFP 1c** | 77 | 2893073 | 85.01% | 2783058 | 82.34% | 55823 | 1.65% | 91,12 |
| | **YPDN RED 1c** | 66 | 2510193 | 78.94% | 2420606 | 76.48% | 56242 | 1.78% | 91,09 |
| | **YPDN GFP 2b** | 68 | 2543747 | 78.09% | 2446180 | 75.45% | 64889 | 2.00% | 91,14 |
| | **YPDN RED 2b** | 98 | 3660172 | 79.11% | 3529132 | 76.58% | 90618 | 1.97% | 91,16 |
| | **BMD GFP 1a** | 81 | 3030632 | 79.67% | 2922324 | 77.18% | 64395 | 1.70% | 91,1 |
| | **BMD RED 1b** | 67 | 2553851 | 78.53% | 2464322 | 76.12% | 58431 | 1.80% | 91,16 |
| | **BMD GFP 2b** | 81 | 3084847 | 79.09% | 2998618 | 77.20% | 52575 | 1.35% | 91,11 |
| | **BMD RED 2c** | 54 | 2063370 | 78.31% | 1978066 | 75.54% | 51377 | 1.96% | 91,09 |
| | **BMDN GFP 1a** | 86 | 3273737 | 73.43% | 3148668 | 70.90% | 87233 | 1.96% | 91,09 |
| | **BMDN RED 1a** | 70 | 2631844 | 78.66% | 2540866 | 76.30% | 56574 | 1.70% | 91,12 |
| | **BMDN GFP 2a** | 48 | 1816762 | 79.76% | 1748922 | 77.10% | 45865 | 2.02% | 91,12 |
| | **BMDN RED 2c** | 69 | 2585886 | 81.57% | 2510772 | 79.50% | 49647 | 1.57% | 91,13 |
| BG10 | **YPD GFP1c** | 53 | 2001716 | 91.93% | 1966272 | 90.76% | 16176 | 0.75% | 91,12 |
| | **YPD RED1a** | 60 | 2254029 | 90.80% | 2219370 | 89.70% | 18251 | 0.74% | 91,1 |
| | **YPD GFP2c** | 59 | 2237515 | 91.11% | 2204714 | 90.10% | 16300 | 0.67% | 91,1 |
| | **YPD RED2c** | 61 | 2299644 | 90.71% | 2264960 | 89.64% | 17976 | 0.71% | 91,13 |
| | **YPDN GFP1b** | 56 | 2132540 | 94.01% | 2092680 | 92.66% | 18287 | 0.81% | 91,14 |
| | **YPDN RED1b** | 61 | 2334947 | 90.88% | 2281938 | 89.18% | 27812 | 1.09% | 91,12 |
| | **YPDN GFP2c** | 33 | 1291422 | 91.58% | 1265734 | 90.11% | 14114 | 1.00% | 91,09 |
| | **YPDN RED2c** | 51 | 1965366 | 90.85% | 1919702 | 89.08% | 26562 | 1.23% | 91,1 |
| | **BMD GFP1c** | 49 | 1847041 | 89.99% | 1813780 | 88.74% | 16363 | 0.80% | 91,09 |
| | **BMD RED1c** | 54 | 2078412 | 87.54% | 2032974 | 85.98% | 25341 | 1.07% | 91,16 |
| | **BMD GFP2a** | 48 | 1834157 | 89.81% | 1805502 | 88.72% | 15020 | 0.74% | 91,14 |
| | **BMD RED2c** | 47 | 1792500 | 85.54% | 1750036 | 83.84% | 26212 | 1.26% | 91,13 |
| | **BMDN GFP1b** | 47 | 1775367 | 90.14% | 1746170 | 89.03% | 14386 | 0.73% | 91,12 |
| | **BMDN RED1c** | 46 | 1788519 | 85.47% | 1746294 | 83.78% | 24506 | 1.18% | 91,12 |
| | **BMDN GFP 2c** | 40 | 1511523 | 89.03% | 1486908 | 87.91% | 12534 | 0.74% | 77,6 |
| | **BMDN RED2b** | 47 | 1782214 | 88.48% | 1749532 | 87.21% | 16402 | 0.82% | 91,11 |
| BG10 | **YPD RED 1c** | 48 | 1872063 | 87.64% | 1821760 | 85.61% | 30428 | 1.43% | 91,07 |
| *ΔOCH1* | **YPD GFP 1a** | 42 | 1606320 | 89.43% | 1577670 | 88.19% | 15245 | 0.85% | 91,1 |
| | **YPD RED 2c** | 58 | 2207075 | 89.04% | 2156316 | 87.34% | 26498 | 1.07% | 91,08 |
| | **YPD GFP 2c** | 47 | 1793946 | 88.78% | 1763648 | 87.63% | 15852 | 0.79% | 91,09 |
| | **YPDN RED 1a** | 56 | 2120333 | 89.65% | 2063510 | 87.63% | 30837 | 1.31% | 91,1 |
| | **YPDN GFP 1a** | 55 | 2079675 | 89.97% | 2037092 | 88.55% | 20463 | 0.89% | 91,12 |
| | **YPDN RED 2b** | 38 | 1439110 | 89.10% | 1413830 | 87.85% | 13409 | 0.83% | 91,08 |
| | **YPDN GFP 2b** | 51 | 1941082 | 86.75% | 1899986 | 85.28% | 23164 | 1.04% | 91,08 |
| | **BMD RED 1c** | 28 | 1056749 | 89.62% | 1029048 | 87.58% | 17159 | 1.46% | 91,09 |
| | **BMD GFP 1b** | 55 | 2107001 | 89.30% | 2057074 | 87.50% | 29087 | 1.24% | 91,1 |
| | **BMD RED 2b** | 61 | 2352281 | 86.55% | 2306326 | 85.14% | 27092 | 1.00% | 91,11 |
| | **BMD GFP 2b** | 55 | 2085305 | 91.21% | 2046372 | 89.87% | 18860 | 0.83% | 91,11 |
| | **BMDN RED 1a** | 49 | 1848613 | 92.12% | 1823310 | 91.17% | 12694 | 0.63% | 91,09 |
| | **BMDN GFP 1c** | 52 | 1969505 | 92.31% | 1941602 | 91.33% | 13691 | 0.64% | 91,09 |
| | **BMDN RED 2a** | 53 | 2014884 | 91.72% | 1988122 | 90.82% | 13081 | 0.60% | 91,1 |
| | **BMDN GFP 2b** | 53 | 2007531 | 92.32% | 1977918 | 91.30% | 14321 | 0.66% | 91,1 |

**Table S8**. **Illumina MiSeq statistics of the 48 evolved *P. pastoris* genomes.**

| clone | chr | pos | type | ref | alt | gene/locus | effect |
|---|---|---|---|---|---|---|---|
| **YPD GFP1a** | chr. 1 | 1910190 | Indel | CT | C | PAS_chr1-4_0279 (SSK2) | stop at amino acid position 1086 |
| **YPD RED1b** | chr. 1 | 1910860 | Indel | GC | G | PAS_chr1-4_0279 (SSK2) | stop at amino acid position 862 |
| **YPD GFP2b** | chr. 1 | 747966 | SNP | G | A | HOG4/SSK4 MAP Kinase | stop codon |
| **YPD RED2b** | chr. 1 | 1909967 | Indel | GT | G | PAS_chr1-4_0279 (SSK2) | stop at amino acid position 1160 |
| | chr. 3 | 843411 | SNP | G | C | TPS1 related / YDR072C | W233 to S233 |
| | chr. 3 | 1390423 | Indel | C | CTGTTGT | FR839630.1 / XM_002492621 / PUF4-like | pos 571 2 Q insertion (Q repeat region) pumilio-like repeat? |
| **YPDN GFP1c** | chr. 4 | 1096585 | SNP | G | A | FR839631.1 / Pho84 | G159 to D159 |
| **YPDN RED1c** | chr.2 | 2261762 | SNP | C | T | PAS_chr2-1_0073 (RIM21) | S287 to F287 |
| **YPDN GFP2b** | chr. 1 | 1350300 | SNP | T | C | PAS_chr1-1_0428 / ENA2 / P-type ATPase Sodium pump | N93 to D93 |
| | chr. 2 | 1951595 | SNP | C | G | PAS_chr2-1_0235 (Pho89) | G134 to R134 |
| | chr. 4 | 1097181 | SNP | G | A | FR839631.1 / Pho84 | G358 to R 358 |
| **YPDN RED2b** | chr. 1 | 1347944 | SNP | G | A | PAS_chr1-1_0428 (ENA2) | P878 to L878 |
| | chr. 2 | 623956 | SNP | G | T | PAS_chr2-2_0324 (GYL1) | A39 to S39 |
| | chr. 3 | 1058830 | Indel | A | AACTATCT | PAS_chr3_0625 (RIM101) | frameshift and premature stop |
| | chr. 4 | 1096215 | SNP | C | T | FR839631.1 / Pho84 | Q36 to stop |
| **BMD GFP1a** | chr. 3 | 962181 | Indel | GC | G | PAS_chr3_0669 (fungal MHR superfamily) | changed c-terminus and premature stop after 100aa |
| **BMD RED1b** | chr. 1 | 1703984 | SNP | G | A | PAS_chr1-4_0160 (HIS4) | G557 to S557 |
| | chr. 3 | 313639 | SNP | A | G | PAS_chr3_1001 (TUP1) | H467 to R467 |
| **BMD GFP2b** | chr. 1 | 1703984 | SNP | G | A | PAS_chr1-4_0160 (HIS4) | G557 to S557 |
| | chr. 1 | 2682926 | SNP | G | A | PAS_FragB_0008 (PDE2) | W458 to stop |
| | chr. 2 | 174635 | SNP | C | T | XP_002492122.1 / MRPS5 and XP_002492121.1 / ARF1 | non-coding upstream of mitochondrial ribosomal subunit and ARF1 GTPase |
| **BMD RED2c** | chr. 1 | 1704727 | SNP | C | A | PAS_chr1-4_0160 (HIS4) | D804 to E804 |
| | chr. 2 | 1993177 | Indel | GT | G | PAS_chr2-1_0214 cytc reductase complex | changed c-terminus and premature stop after 1042aa |
| | chr. 3 | 2124206 | SNP | C | T | PAS_chr3_0052 | G175 to D175 thiamine pyrophosphokinase |
| **BMDN GFP1a** | chr. 3 | 960331 | SNP | C | A | PAS_chr3_0669 (fungal MHR superfamily) | S700 to I700 |
| | chr. 3 | 1331718 | Indel | AT | A | intergenic PAS_chr3_0480 (XDJ1) and PAS_chr3_0478 (RRN11) | downstream of XDJ1 and upstream of RRN11 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | chr. 3 | 2115746 | Indel | GA | G | PAS_chr3_0061 / TUL1 | frame shift stop codon at position 74 |
| **BMDN RED1a** | chr. 3 | 312675 | SNP | C | T | PAS_chr3_1001 (TUP1) | Q146 to stop codon |
| | chr. 3 | 1178447 | SNP | C | T | PAS_chr3_0560(MSB1) | T1146 to I1146 at protein c-terminus |
| | chr. 3 | 1316342 | SNP | G | A | PAS_chr3_0488 | G729 synonymous mutation |
| | chr. 3 | 1743772 | SNP | G | A | PAS_chr3_0247 (CAC2) | P345 synonymous mutation |
| **BMDN GFP2a** | chr. 1 | 2036266 | Indel | AT | A | PAS_chr1-4_0672 (CYB5) | frameshift stop at pos. 21 |
| | chr. 3 | 959553 | SNP | C | T | PAS_chr3_0669 (fungal MHR superfamily) | premature stop at position 959 |
| **BMDN RED2c** | chr. 1 | 2455746 | SNP | A | G | intergenic PAS_chr1-4_0565 (CUP2) PAS_chr1-4_0567 (PRE4) | downstream of both open reading frames |
| | chr. 2 | 1845389 | SNP | A | G | PAS_chr2-1_0297 (SEC15) | M304 to V304 |
| | chr. 3 | 313098 | SNP | G | T | PAS_chr3_1001 (TUP1) | V287 to F287 |
| | chr. 3 | 1469802 | Indel | AC | A | intergenic g2867 (unknown hypothetical) and PAS_chr3_0397 (CDC11) | noncoding between g2867 (downstream) and g2868 (upstream) |
| | chr. 4 | 526143 | SNP | G | T | PAS_chr4_0649 (UBX3-like) | stop at position 271 |

**Table S9**. **Mutations identified in the evolved *P. pastoris* X-33 clones**. For each mutation the chromosomal location (chr) and nucleotide position (pos) is shown with respect to the CBS 7435 reference genome. The type of mutation (Indel / SNP), reference sequence (ref) and sequence in the evolved clone (alt), as well as the affected locus (gene/locus) and effect are shown. Recurrent mutational targets are highlighted by different colors.

| clone | chr | pos | type | ref | alt | gene/locus | effect |
|---|---|---|---|---|---|---|---|
| **YPD GFP1c** | chr. 1 | 2375201 | SNP | G | T | PAS_chr1-4_0530 (SOK2) | R339 to S339 |
| | chr. 2 | 219784 | Indel | TG | T | intergenic PAS_chr2-2_0111 (GPD1) | upstream of GPD1 |
| **YPD RED1a** | chr. 1 | 1910627 | Indel | GA | G | PAS_chr1-4_0279 (SSK2) | frameshift stop at position 940 |
| | chr. 4 | 1121333 | SNP | T | A | PAS_chr4_0327 (GCN1) | L2712 to I2712 |
| **YPD GFP2c** | chr. 1 | 1911735 | SNP | C | A | PAS_chr1-4_0279 (SSK2) | stop at position 562 |
| **YPD RED2c** | chr. 1 | 2056661 | SNP | C | T | upstream of PAS_chr1-4_0349 (PEX8) and PAS_chr1-4_0350 (DSC2) | - |
| | chr. 2 | 2100084 | SNP | T | A | PAS_chr2-1_0162 (SLN1) | N495 to I495 |
| **YPDN GFP1b** | chr. 1 | 818136 | SNP | T | G | PAS_chr1-1_0139 (SPC7) | L570 to V570 |
| | chr. 2 | 485383 | SNP | C | T | PAS_chr2-2_0258 (EMP70 domain) | P638 to L638 |
| | chr. 2 | 2261762 | SNP | C | T | PAS_chr2-1_0073 (RIM21) | S287 to F287 |
| | chr. 4 | 1097664 | Indel | GC | G | PAS_chr4_0337 (PHO84) | frameshift stop at position 524 |
| **YPDN RED1b** | chr. 1 | 359814 | Indel | T | TGG | upstream of PAS_chr1-3_0192 (RAM2) | - |
| | chr. 2 | 848176 | SNP | C | T | integenic | - |
| | chr. 4 | 1096513 | Indel | GC | G | PAS_chr4_0337 (PHO84) | stop at position 135 |
| | chr. 4 | 1410744 | SNP | C | T | PAS_chr4_0184 (RIM8) | S80 to L80 |
| **YPDN GFP2c** | chr. 2 | 219326 | SNP | A | T | PAS_chr2-2_0111 | L104 to Q104 |
| | chr. 2 | 2261090 | SNP | C | T | PAS_chr2-1_0073 (RIM21) | P63 to L63 |
| | chr. 3 | 1577636 | SNP | G | A | PAS_chr3_0340 (eIF3) | R45 to K45 |
| | chr. 4 | 1096510 | Indel | AGT | A | PAS_chr4_0337 (PHO84) | frameshift stop at position 138 |
| **YPDN RED2c** | chr. 3 | 1058688 | Indel | A | AATTCCAACTC | PAS_chr3_0625 (RIM101) | frameshift stop at position 513 |
| **BMD GFP1c** | chr. 2 | 1760403 | SNP | C | G | PAS_chr2-1_0339, similar to *S. cerevisiae* YLR177W | S20 to stop |
| **BMD RED1c** | chr. 1 | 2682901 | Indel | CG | C | PAS_FragB_0008 (PDE2) | frameshift stopp at position 469 |
| | chr. 3 | 960756 | Indel | GC | G | PAS_chr3_0669 MHR-like | - |
| **BMD GFP2a** | chr. 1 | 2682538 | Indel | T | TTAAA | PAS_FragB_0008 (PDE2) | frameshift stop at position 343 |
| | chr. 3 | 961799 | Indel | GT | G | PAS_chr3_0669 MHR-like | frameshift stop at position 224 |
| **BMD RED2c** | chr. 1 | 1910249 | SNP | A | G | PAS_chr1-4_0279 (SSK2) | L1057 to S1057 |
| **BMDN GFP1b** | chr. 3 | 312675 | SNP | C | T | PAS_chr3_1001 (TUP1 transcriptional repressor) | stop at position 146 |
| **BMDN RED1c** | chr. 4 | 526418 | SNP | C | A | PAS_chr4_0649 (UBX3-like) | stop at position 180 |
| **BMDN GFP2c** | chr. 1 | 2875345 | Indel | - | - | 5582 bp deletion unknown hypothetical orf | - |
| | chr. 3 | 312687 | SNP | C | T | PAS_chr3_1001 (TUP1 transcriptional repressor) | stop at position 150 |
| **BMDN RED2b** | chr. 3 | 2114593 | Indel | G | GA | PAS_chr3_0061 (TUL1) | frameshift stop at position 472 |

**Table S10**. **Mutations identified in the evolved *P. pastoris* BG10 clones.** For each mutation the chromosomal location (chr) and nucleotide position (pos) is shown with respect to the CBS 7435 reference genome. The type of mutation (Indel / SNP), reference sequence (ref) and sequence in the evolved clone (alt), as well as the affected locus (gene/locus) and effect are shown. Recurrent mutational targets are highlighted by different colors.

| clone | contig | pos | type | ref | alt | gene/locus | effect |
|---|---|---|---|---|---|---|---|
| **YPD RED1c** | chr. 2 | 936257 | indel | C | CA | upstream of PAS_chr2-1_0765 (FLC2) and XM_002491656.1 (ACS1) | downregulation FLC2 |
| **YPD GFP1a** | chr. 2 | 937030 | SNP | G | A | upstream of PAS_chr2-1_0765 (FLC2) and XM_002491656.1 (ACS1) | downregulation FLC2 |
| | chr. 3 | 600924 | indel | GT | G | SSK1 | SSK1 frameshift and stop at aa53 |
| **YPD RED2c** | chr. 2 | 936810 | indel | AC | A | upstream of PAS_chr2-1_0765 (FLC2) and XM_002491656.1 (ACS1) | downregulation FLC2 |
| **YPD GFP2c** | chr. 1 | 206601 | indel | C | CCAAACTA | PAS_chr1-3_0106 (NPR1) | frameshift stop at aa300 |
| | chr. 2 | 936817 | indel | GT | G | upstream of PAS_chr2-1_0765 (FLC2) and XM_002491656.1 (ACS1) | downregulation FLC2 |
| | chr. 2 | 1372143 | SNP | T | C | upstream of PAS_chr2-1_0551 (EMC3) downstream of PAS_chr2-1_0552 (ADD66) | - |
| | chr. 3 | 600283 | indel | GC | G | SSK1 | SSK1 frameshift and stop at aa241 |
| **YPDN RED1a** | chr. 3 | 1058511 | SNP | C | T | PAS_chr3_0625 (RIM101) | Q440 to stop |
| **YPDN GFP1a** | chr. 1 | 2779984 | SNP | C | A | PAS_FragB_0063 (PFS2) | D187 to E187 |
| | chr. 3 | 600924 | indel | GT | G | SSK1 | SSK1 frameshift and stop at aa53 |
| **YNPD RED2b** | chr. 2 | 2261762 | SNP | C | T | PAS_chr2-1_0073 (RIM21) | S287 to F287 |
| | chr. 3 | 600794 | indel | AT | A | SSK1 | SSK1 frameshift and stop at aa53 |
| **YPDN GFP2b** | chr. 1 | 1347883 | SNP | C | T | PAS_chr1-1_0428 / ENA2 / P-type ATPase Sodium pump | M898 to I898 |
| **BMD RED1c** | chr. 3 | 1918359 | indel | TA | T | IRA1 | frameshift stop at aa953 |
| | chr. 4 | 1495 | SNP | A | C | PAS_chr4_0879 (SLA2) | |
| **BMD GFP1b** | chr. 3 | 1920040 | indel | TA | T | IRA1 | frameshift stop at aa1504 |
| **BMD RED2b** | chr. 3 | 600794 | indel | AT | A | SSK1 | SSK1 frameshift and stop at aa53 |
| | chr. 3 | 1918078 | indel | TA | T | IRA1 | frameshift stop at aa849 |
| **BMD GFP2b** | chr. 3 | 1918621 | indel | GT | G | IRA1 | frameshift stop at aa1037 |
| **BMDN RED1a** | chr. 1 | 1910362 | SNP | A | T | PAS_chr1-4_0279 (SSK2) | C1019 to stop |
| | chr. 2 | 474950 | SNP | A | T | upstream of PAS_chr2-2_0482 (FLO11) | |
| **BMDN GFP1c** | chr. 1 | 1910843 | Indel | GA | G | PAS_chr1-4_0279 (SSK2) | frameshift stop at aa865 |
| **BMDN RED2a** | chr. 1 | 1910362 | SNP | A | T | PAS_chr1-4_0279 (SSK2) | C1019 to stop |
| **BMDN GFP2b** | chr. 1 | 1909882 | SNP | A | C | PAS_chr1-4_0279 (SSK2) | Y1179 to stop |

**Table S11**. **Mutations identified in the evolved *P. pastoris* BG10 Δ*OCH1* clones.** For each mutation the chromosomal location (chr) and nucleotide position (pos) is shown with respect to the CBS 7435 reference genome. The type of mutation (Indel / SNP), reference sequence (ref) and sequence in the evolved clone (alt), as well as the affected locus (gene/locus) and effect are shown. Recurrent mutational targets are highlighted by different colors.

| Mutation | type | | No. | % |
|---|---|---|---|---|
| **Indels** | insertions | | 6 | **17.1** |
| | deletions | | 29 | **82.9** |
| | | | | |
| **SNPs** | G | A | 11 | **19.6** |
| | G | T | 4 | **7.1** |
| | G | C | 1 | **1.8** |
| | C | A | 5 | **8.9** |
| | C | T | 18 | **32.1** |
| | C | G | 2 | **3.6** |
| | A | G | 4 | **7.1** |
| | A | C | 2 | **3.6** |
| | A | T | 4 | **7.1** |
| | T | G | 1 | **1.8** |
| | T | C | 2 | **3.6** |
| | T | A | 2 | **3.6** |

**Table S12**. **Mutation statistics.** Total number of Insertion-Deletions (Indels) and single nucleotide polymorphisms (SNPs) identified in the 48 sequenced *P. pastoris* clones.

| clone | YPD | YPDN | BMD | BMDN |
|---|---|---|---|---|
| **X33 YPD GFP1a*** | 109.6 +/- 1.5 | 65.2 +/- 4.9 | 98.2 +/- 1.3 | 100.8 +/- 2.2 |
| **X33 YPD GFP2b**[†] | 111.1 +/- 1.1 | 39.7 +/- 8.9 | 97.7 +/- 4.5 | 102.1 +/- 0.3 |
| **X33 BMD GFP1a**[a] | 107.8 +/- 2.0 | 71.7 +/- 2.9 | 115.6 +/- 3.8 | 120.5 +/- 2.1 |
| **Δ*OCH1* YPD RED1c**[b] | 101.7 +/- 4.3 | 66.3 +/- 5.4 | 96.0 +/- 10.6 | 84.1 +/- 4.9 |
| **Δ*OCH1* YPD RED2c**[b] | 111.8 +/- 1.8 | 67.7 +/- 14.8 | 98.7 +/- 5.1 | 71.5 +/- 4.1 |
| **Δ*OCH1* YPD GFP1a**[c] | 170.8 +/- 3.4 | 125.2 +/- 10.6 | 118.4 +/- 1.6 | 117.9 +/- 1.7 |
| **Δ*OCH1* YPD GFP2c**[c] | 155.7 +/- 0.8 | 124.0 +/- 10.0 | 95.2 +/- 3.6 | 109.2 +/- 2.1 |
| **Δ*OCH1* BMD GFP2b**[d] | 103.5 +/- 16.5 | 142.5 +/- 10.2 | 117.1 +- 8.4 | 110.0 +/- 4.3 |
| **Δ*OCH1* BMDN GFP1c**[e] | 149.9 +/- 2.2 | 122.9 +/- 8.4 | 104.6 +/- 4.9 | 117.3 +/- 2.6 |

**Table S13**. **Relative growth rate [% $\mu_{ancestor}$] of selected evolved single clones of *P. pastoris* X-33 and BG10 Δ*OCH1* in all 4 growth environments.** * single SSK2 mutation [†] single *SSK4* mutation [a] single PAS-ch3_0669 mutation [b] single intergenic *ACS1*/*FLC2* mutations [c] *OCH1* deletion clones with an additional mutations; [d] single *IRA1* mutation; [e] single *SSK2* mutation; values represent the mean of *n* = 3 +/- standard deviation.

| clone | YPD | YPDN | BMD | BMDN |
|---|---|---|---|---|
| **X33 YPD GFP1a*** | 93.8 +/- 0.6 | 50.3 +/- 0.4 | 87.1 +/- 1.4 | 84.9 +/- 4.6 |
| **X33 YPD GFP2b**[†] | 90.4 +/- 1.7 | 49.7 +/- 4.6 | 90.5 +/- 2.3 | 85.4 +/- 1.1 |
| **X33 BMD GFP1a**[a] | 102.6 +/- 9.8 | 82.9 +/- 5.2 | 81.5 +/- 4.7 | 90.6 +/- 4.1 |
| **Δ*OCH1* YPD RED1c**[b] | 90.6 +/- 4.2 | 94.0 +/- 0.4 | 95.0 +/- 1.2 | 98.0 +/-2.6 |
| **Δ*OCH1* YPD RED2c**[b] | 91.7 +/- 1.9 | 86.2 +/- 0.0 | 105.2 +/- 2.3 | 109.6 +/- 5.4 |
| **Δ*OCH1* YPD GFP1a**[c] | 124.9 +/- 5.5 | 78.1 +/- 5.3 | 86.7 +/- 2.0 | 89.9 +/- 5.9 |
| **Δ*OCH1* YPD GFP2c**[c] | 121.1 +/- 1.6 | 80.4 +/-4.9 | 105.3 +/- 4.7 | 100.3 +/-2.4 |
| **Δ*OCH1* BMD GFP2b**[d] | 89.1 +/- 5.7 | 88.7 +/- 5.3 | 83.1 +/- 1.7 | 85.1 +/- 2.5 |
| **Δ*OCH1* BMDN GFP1c**[e] | 115.2 +/-7.7 | 74.6 +/- 4.9 | 80.9 +/- 5.2 | 84.8 +/- 1.2 |

**Table S14**. **Maximum $OD_{600}$ [% ancestor] of selected evolved single clones of *P. pastoris* X-33 and BG10 Δ*OCH1* in all 4 growth environments**; * single SSK2 mutation [†] single *SSK4* mutation [a] single PAS-ch3_0669 mutation [b] single intergenic *ACS1*/*FLC2* mutations [c] *OCH1* deletion clones with an additional mutations; [d] single *IRA1* mutation; [e] single *SSK2* mutation; values represent the mean of *n* = 3 +/- standard deviation.

| clone | YPD | YPDN | BMD | BMDN |
|---|---|---|---|---|
| **X33 YPD GFP1a*** | 1.201 +/- 0.075 | 0.842 +/- 0.013 | 0.919 +/- 0.006 | 0.748 +/- 0.007 |
| **X33 YPD GFP2b**[†] | 1.373 +/- 0.115 | 0.627 +/- 0.095 | 0.833 +/- 0.011 | 0.837 +/- 0.049 |
| **X33 BMD GFP1a**[a] | 1.010 +/- 0.034 | 0.704 +/- 0.001 | 1.107 +/- 0.019 | 1.300 +/- 0.101 |
| **Δ*OCH1* YPD RED1c**[b] | 1.336 +/- 0.004 | 1.017 +/- 0.009 | 0.890 +/- 0.009 | 0.797 +/- 0.010 |
| **Δ*OCH1* YPD RED2c**[b] | 1.615 +/- 0.039 | 1.066 +/- 0.006 | 0.939 +/- 0.008 | 0.858 +/- 0.009 |
| **Δ*OCH1* YPD GFP1a**[c] | 1.038 +/- 0.058 | 1.203 +/- 0.040 | 1.466 +/- 0.035 | 1.388 +/- 0.021 |
| **Δ*OCH1* YPD GFP2c**[c] | 1.222 +/- 0.003 | 1.282 +/- 0.140 | 1.363 +/- 0.029 | 1.224 +/- 0.038 |
| **Δ*OCH1* BMD GFP2b**[d] | 0.333 +/- 0.031 | 1.193 +/- 0.031 | 1.647 +/- 0.030 | 1.632 +/- 0.007 |
| **Δ*OCH1* BMDN GFP1c**[e] | 0.938 +/- 0.043 | 1.149 +/- 0.031 | 1.310 +/- 0.034 | 1.389 +/- 0.003 |

**Table S15**. **Competitive fitness [ω] relative to the ancestral strains of selected evolved single clones.** *P. pastoris* X-33 and BG10 Δ*OCH1* clones as compared to ancestral strains in all 4 growth environments; * single SSK2 mutation [†] single *SSK4* mutation [a] single PAS-ch3_0669 mutation [b] single intergenic *ACS1*/*FLC2* mutations [c] *OCH1* deletion clones with an additional mutations; [d] single *IRA1* mutation; [e] single *SSK2* mutation; values represent the mean of *n* = 4 +/- standard error.

# Supplemental Figures

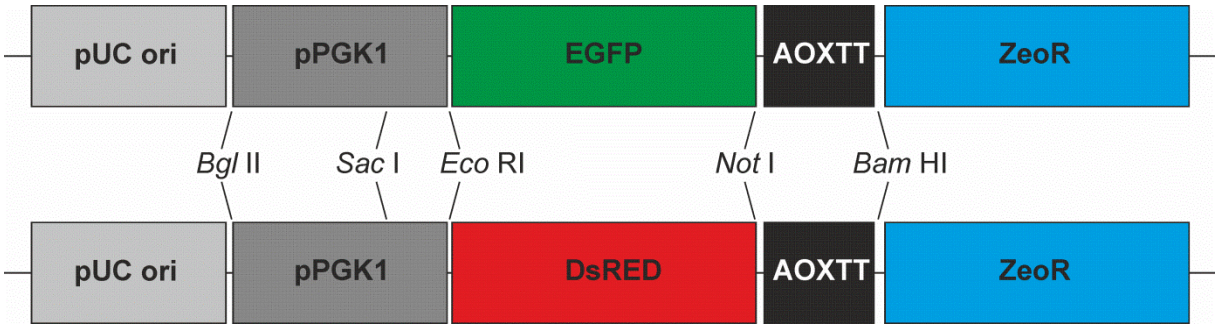


**Figure S1**. **Reporter gene constructs**. Vectors are based on a modified pGAPzB vector backbone, where the $P_{GAP}$ has been replaced with $P_{PGK1}$. See also Table S1 for oligonucleotides used for plasmid construction.

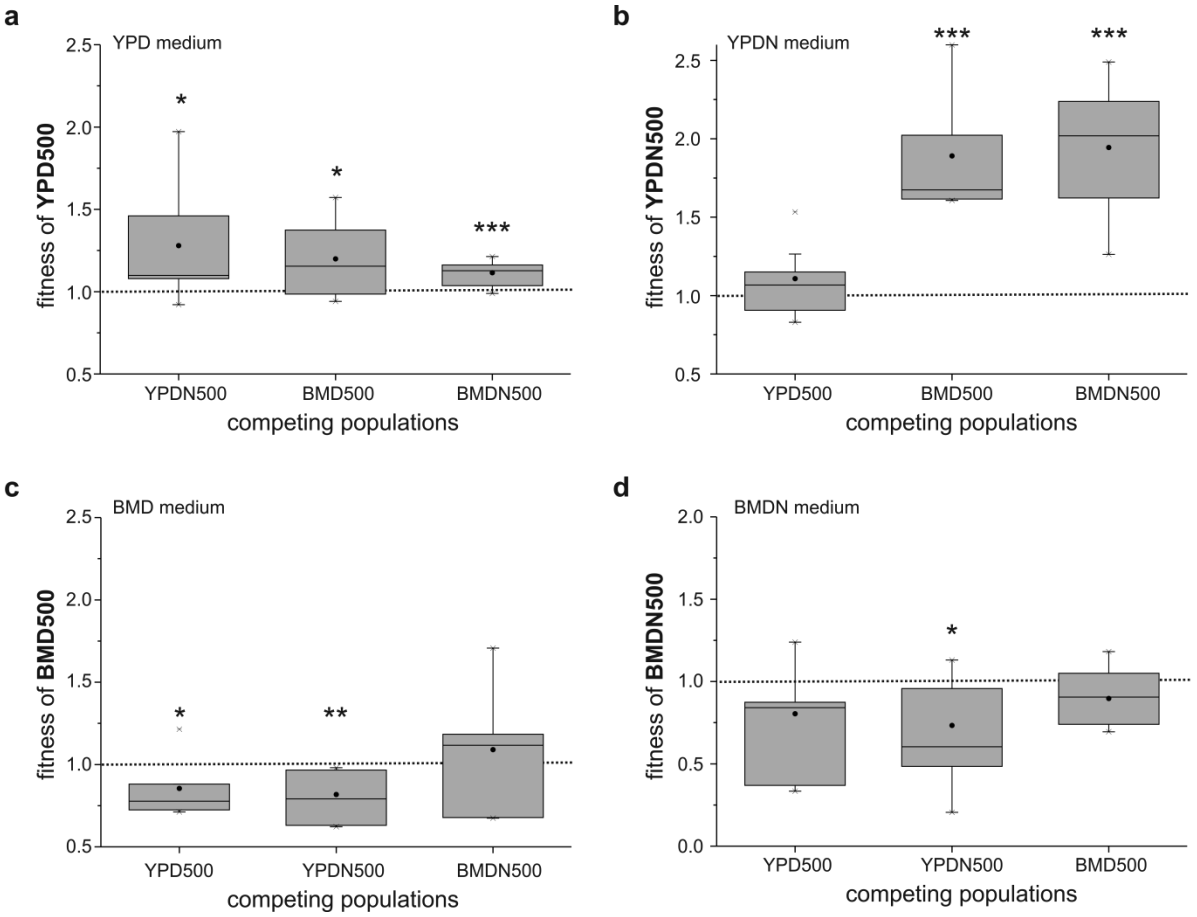


**Figure S2**. **Direct competition assays of evolved *P. pastoris* X-33 populations.** Fitness of YPD-evolved populations on YPD (a), YPDN-evolved on YPDN (b), BMD-evolved on BMD (c) and BMDN-evolved populations on BMDN (d) as compared with the remainder evolved populations. All possible pair-wise competitions were performed in duplicate ($n$ = 8); paired Student's T-test values * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

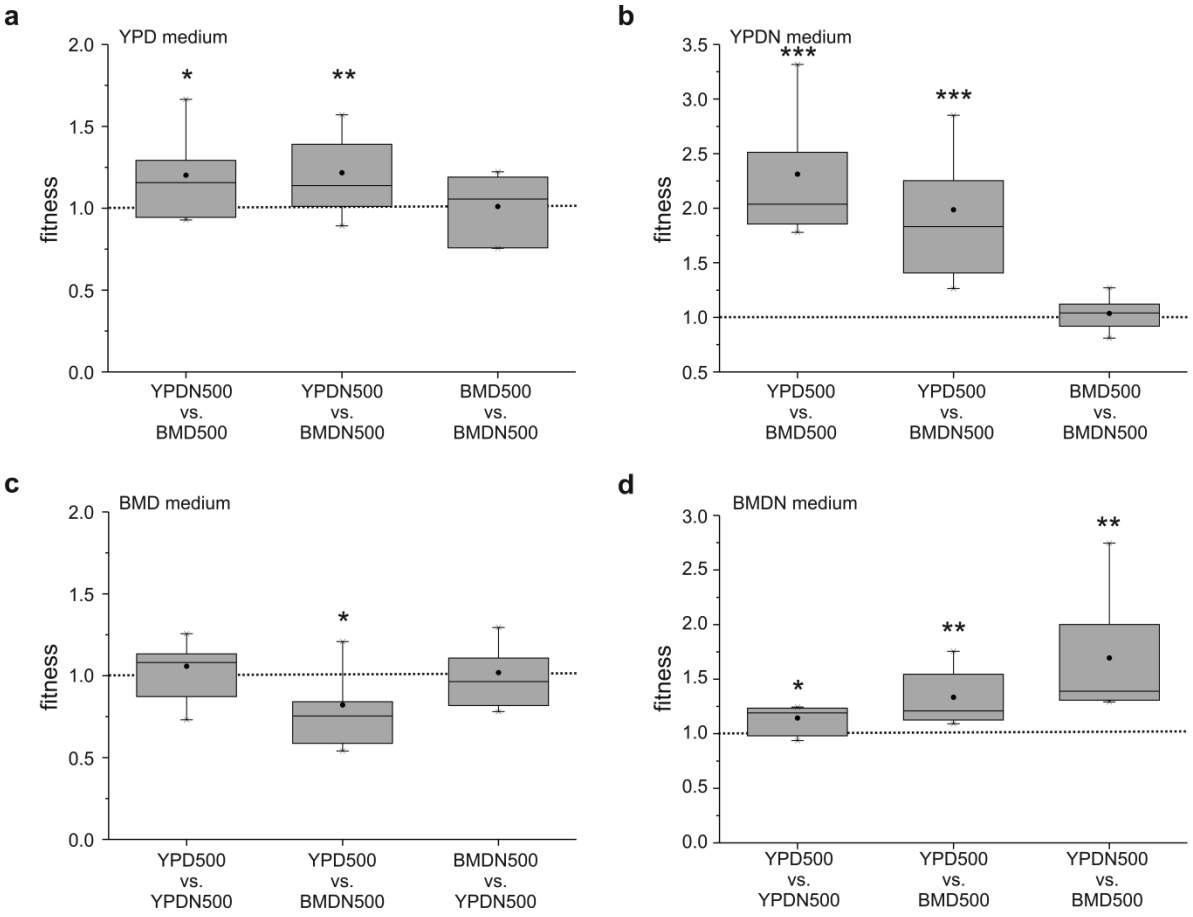


**Figure S3**. **Direct competition assays among evolved *P. pastoris* X-33 populations.** Competitions on (a) YPD, (b) YPDN, (c) BMD and (d) BMDN are shown. All possible pair-wise competitions were performed in duplicate ($n$ = 8); paired Student's T-test values * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

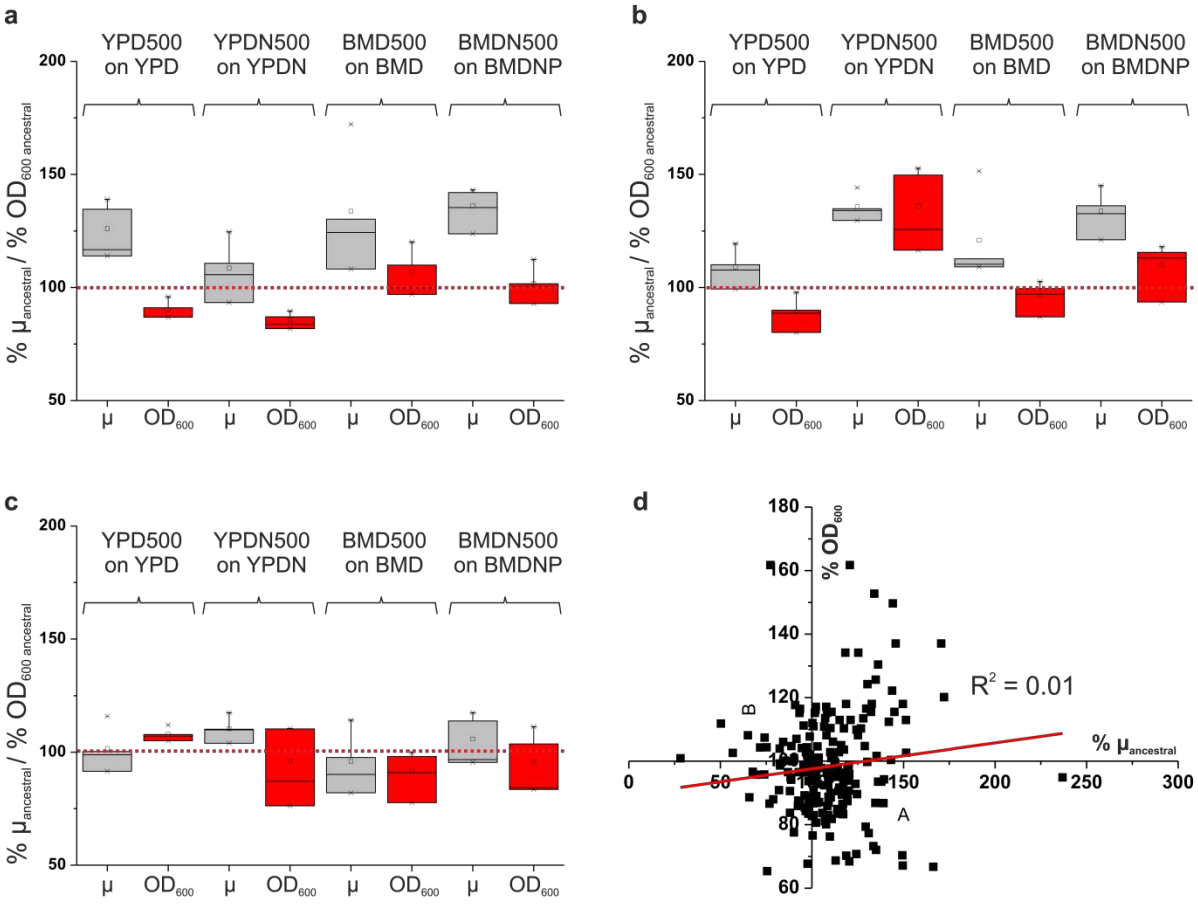


**Figure S4. Growth rates [% $\mu_{ancestral}$, grey boxplosts] and biomass yield [%$_{ancestral}$ $OD_{600}$, red boxplots] of evolved *P. pastoris* populations.** (a) *P. pastoris* X-33; (b) BG10 and (c) BG10 Δ*OCH1* (d) overall correlation of growth rate and biomass yield (final $OD_{600}$) of growth tests performed in this study (*n* = 220).

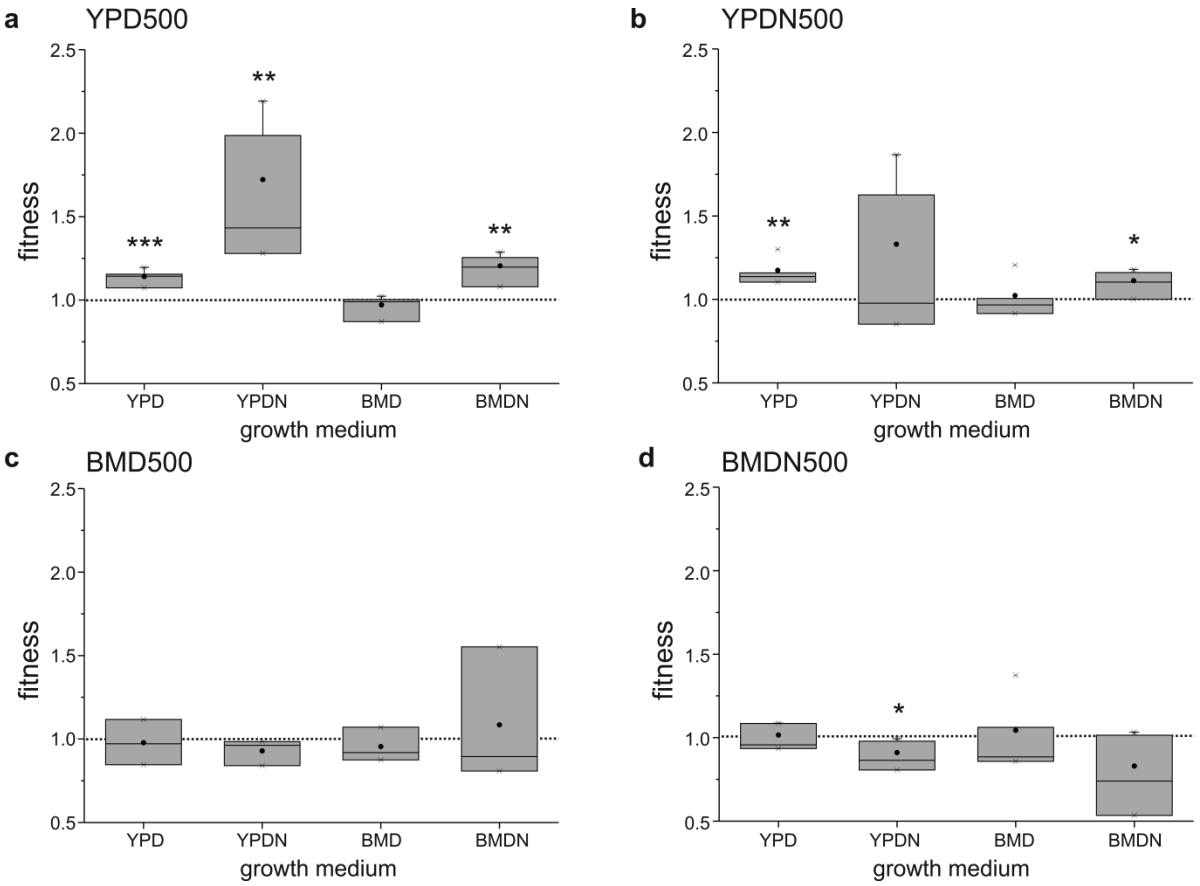


**Figure S5**. **Competitive fitness [ω] of evolved *P. pastoris* X-33 populations in different growth environments as compared with ancestral strains.** Fitness of YPD-evolved (a), YPDN-evolved (b), BMD-evolved (c) and BMDN-evolved populations (d) is shown. All possible pair-wise competitions were performed in duplicate ($n$ = 8); paired Student's T-test values * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

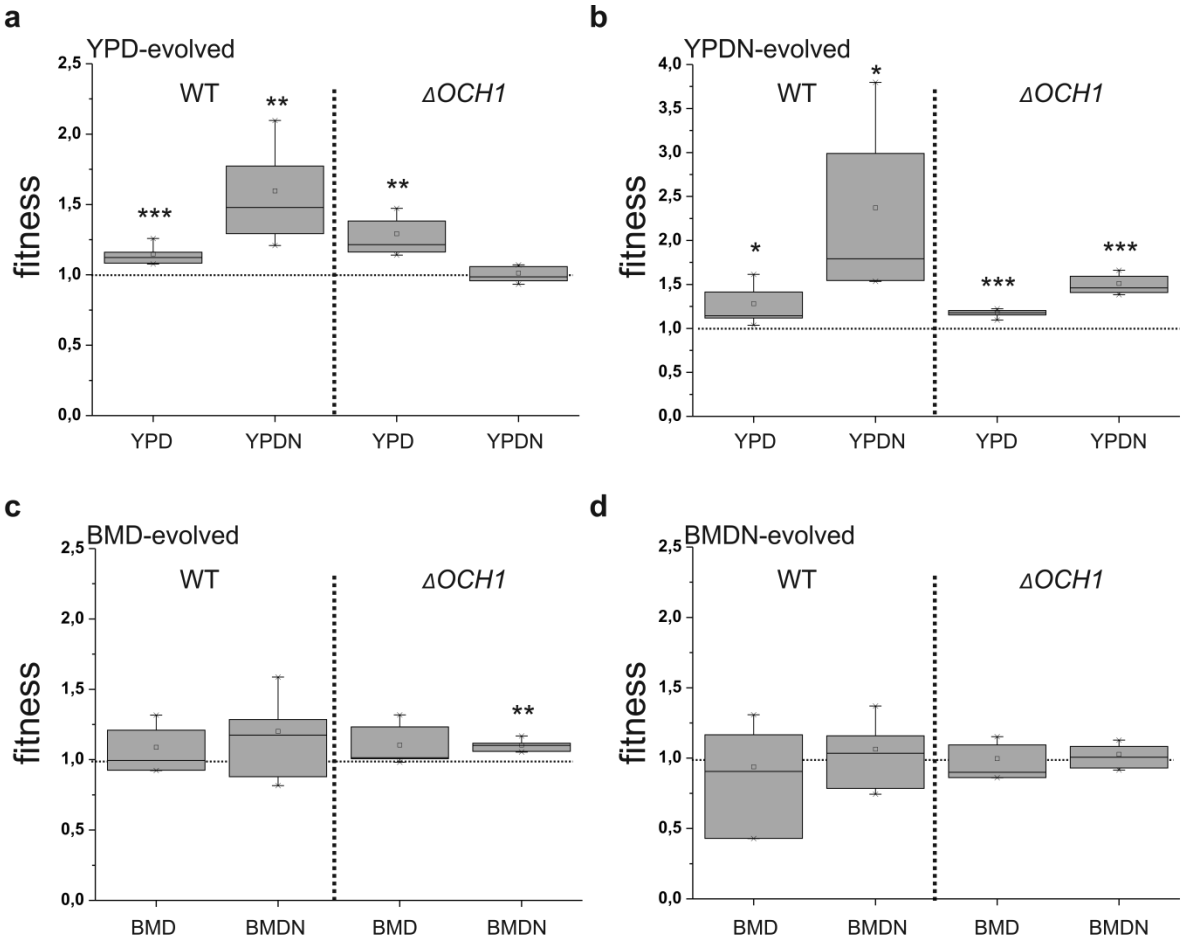


**Figure S6**. **Competitive fitness [ω] of evolved *P. pastoris* BG10 and BG10 Δ*OCH1* populations in different growth environments as compared with the ancestral strains.** YPD-evolved (a), YPDN-evolved (b), BMD-evolved (c) and BMDN-evolved (d); All possible pair-wise competitions were performed in duplicate ($n$ = 8); paired Student's T-test values * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

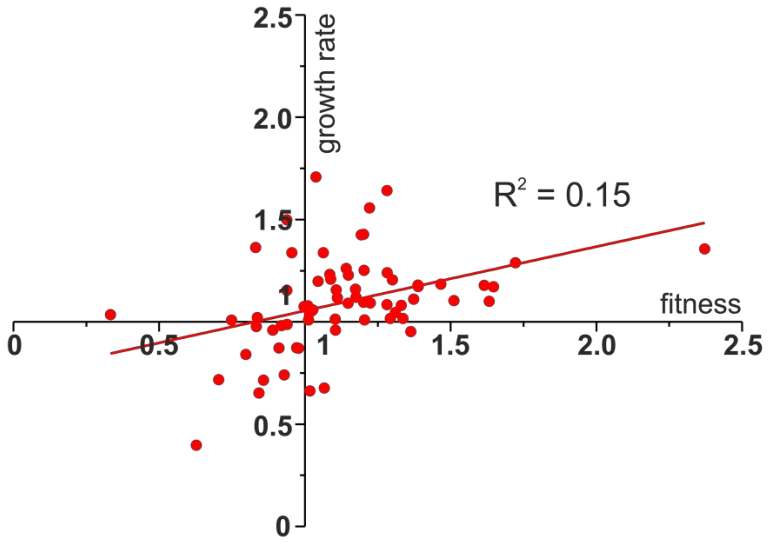


**Figure S7**. **Correlation of maximum growth rate and competitive fitness.** Correlation plot of competitive fitness and relative ancestral growth rates of all tests performed in the current study.

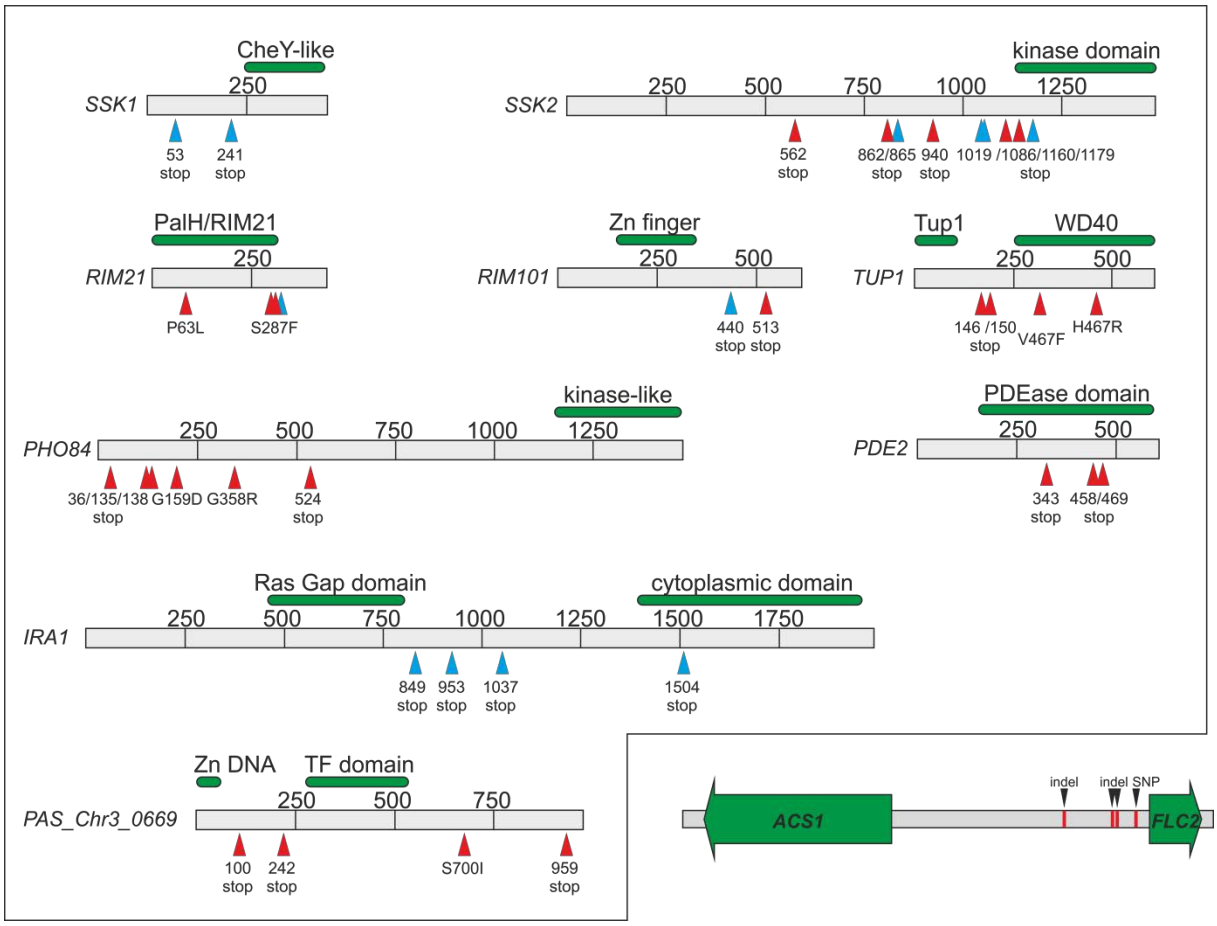


**Figure S8.** Selected recurrent mutational targets isolated from *P. pastoris* populations after 500 generations of laboratory evolution. Linear representations of proteins encoded by the corresponding ORFs are shown. Important protein domains as well as positions and types of mutation are indicated by arrows. Red – mutations in X-33 and BG10 genetic background and blue – mutations identified in evolved BG10 Δ*OCH1* clones. For the non-coding intergenic recurrent mutations in the *ACS1/FLC2* locus (bottom right corner), approximate position and the type of the mutation are shown.

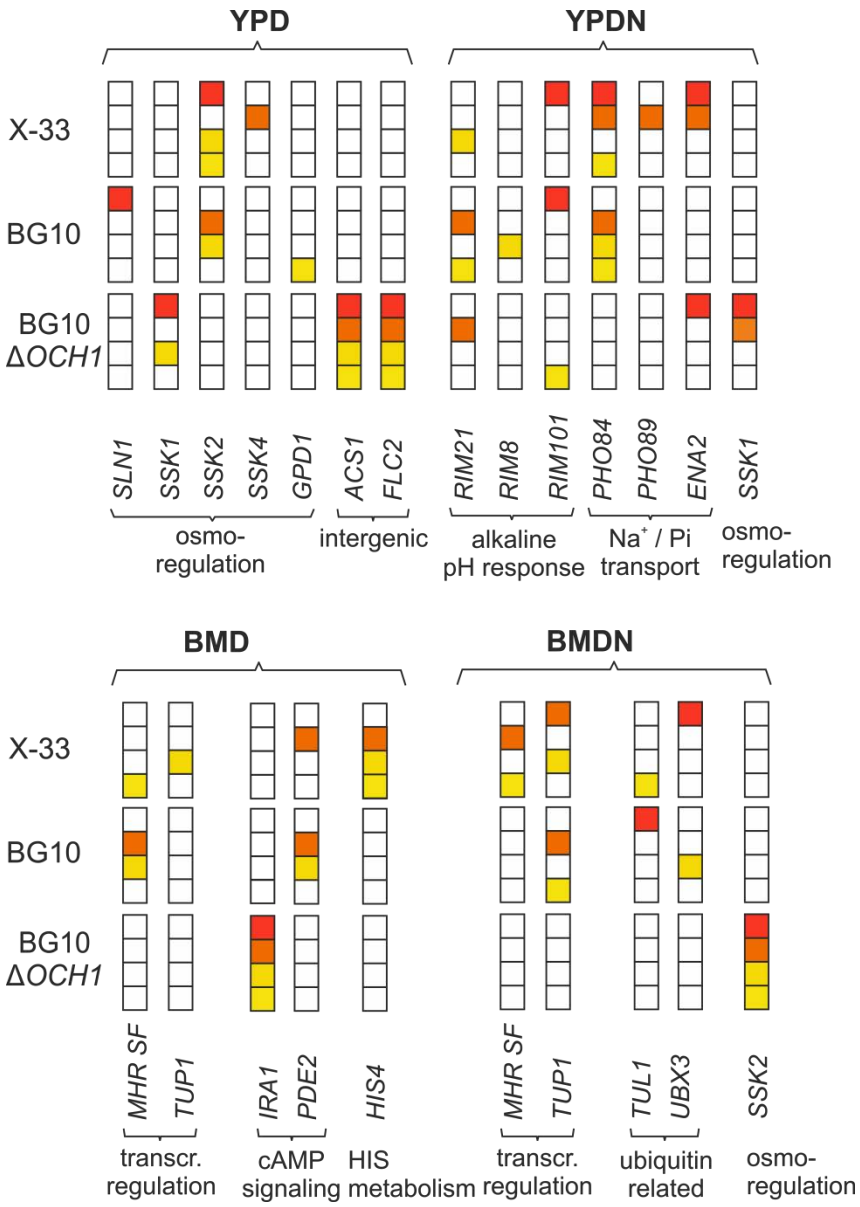


**Figure S9.** Recurrent mutational targets group by their appearance in strain background and growth condition. For each strain and growth condition recurrent functional modules and the affected genes are shown. Since for each growth condition 4 clones were sequenced (represented by four squares), the occurrence of a particular mutation is indicated by color (yellow to red for the presence of a mutation in each individual clone). A blank square indicates the absence of a mutation. MHR-SF: PAS-ch3_0669 middle homology region superfamily GAL4-like transcriptional regulator.

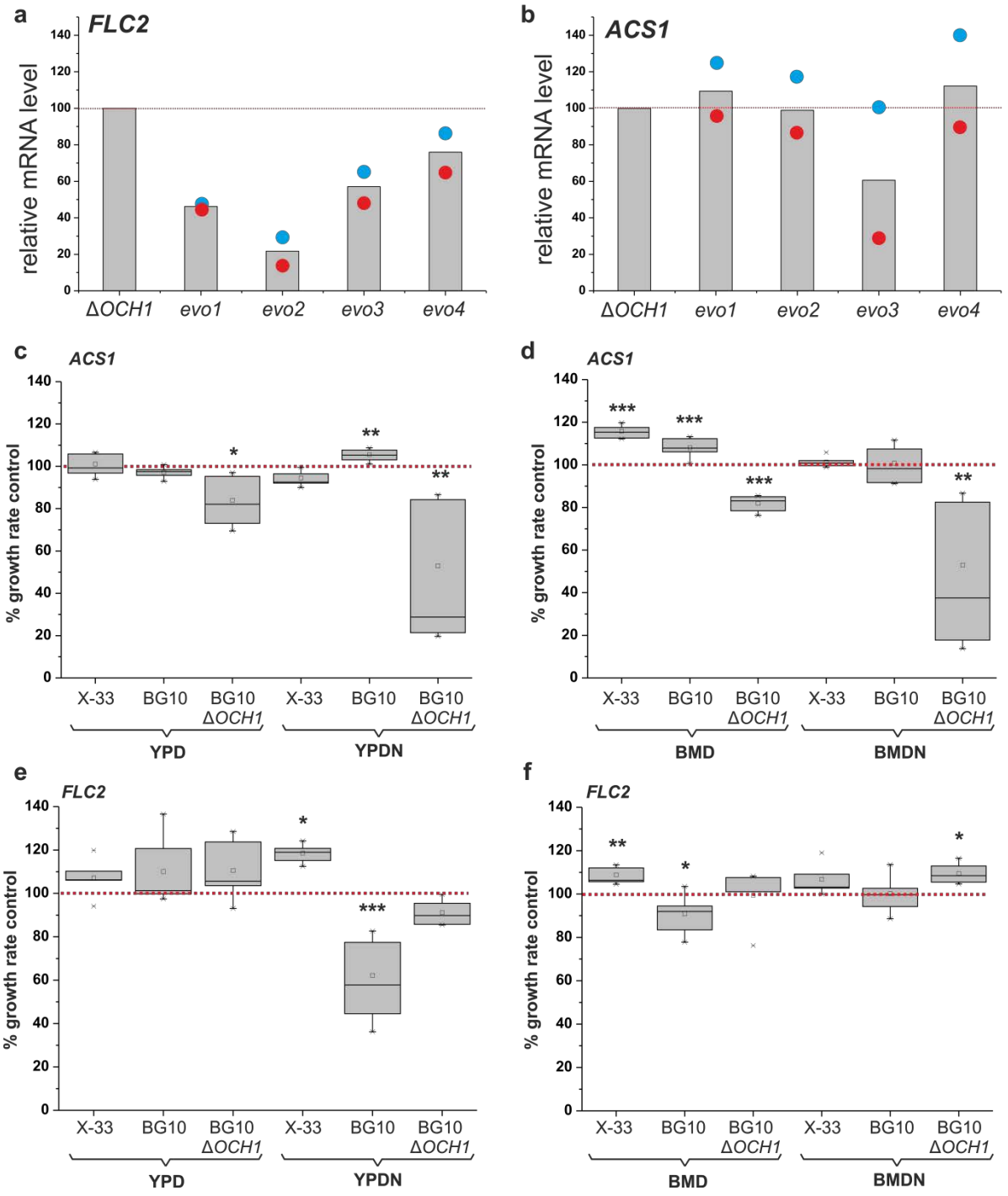


**Figure S10.** Relative *FLC2* (a) and *ACS1* (b) mRNA expression levels (*ACT1*-normalized) in evolved clones (evo1-4) as compared to the ancestral Δ*OCH1* clone during growth on YPD. The average mRNA level for each strain as well as the individual value for each biological replicate ($n$ = 2) is shown. (c, d) Effect of *ACS1* overexpression in the ancestral strains relative to an empty vector control. (e, f) Effect of *FLC2* overexpression in the ancestral strains relative to an empty vector control. $n$ = 6; paired Student's T-test values * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

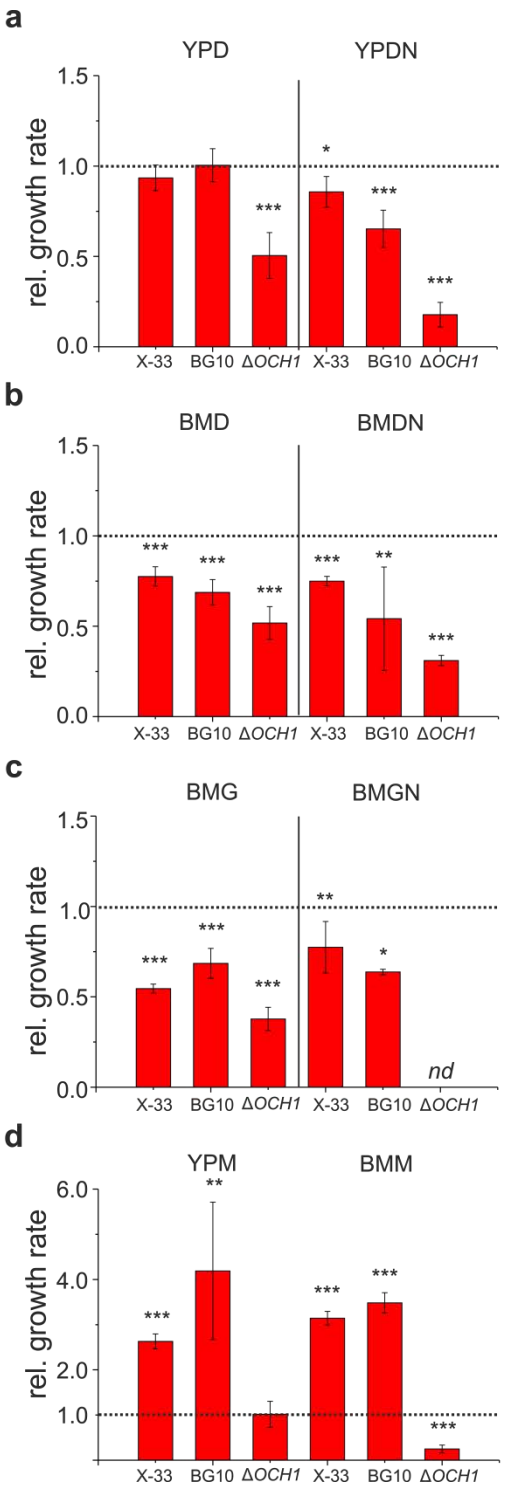


**Figure S11.** Effect of *TUP1* overexpression in all ancestral *P. pastoris* backgrounds. Growth rates relative to an empty-vector control are shown in various growth media (a) YPD and YPDN (b) BMD and BMDN (c) BMG and BMGN minimal media with glycerol as carbon source and (d) YPM and BMM rich and minimal media with methanol as carbon source. Values represent averages of $n$ = 6 +/- standard deviation; paired Student's T-test values * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. *nd* - no growth detected.

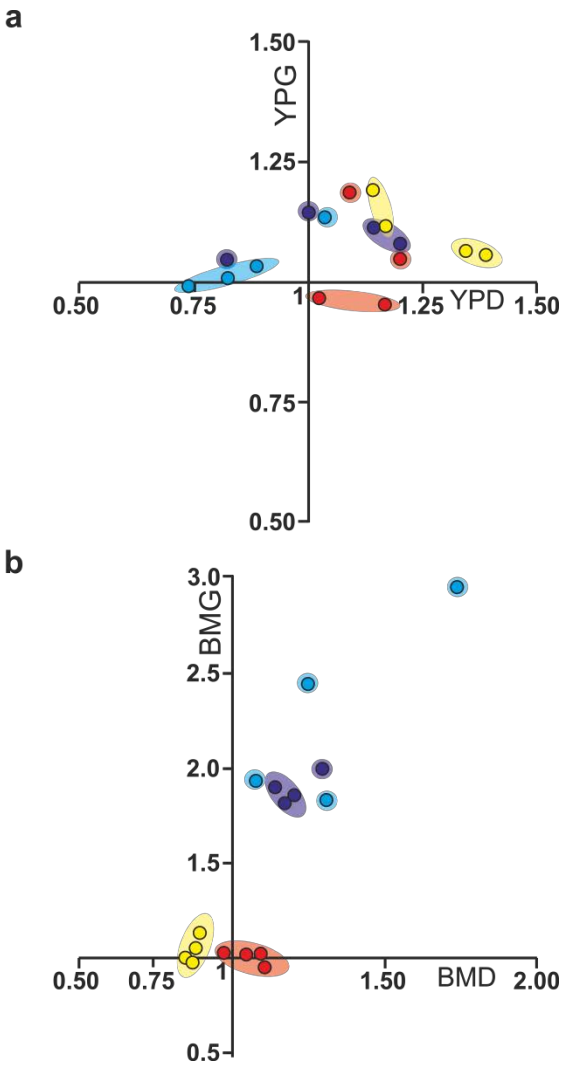


**Figure S12.** Growth rates of evolved X-33 populations on glucose (x-axis) and glycerol-containing (y-axis) growth media. (a) YPD and YPG growth rates (b) BMD and BMG growth rates. Plots show growth rates of all 4 replicate populations evolved on: YPD (yellow), YPDN (red), BMD (blue) and BMDN (dark blue).

## References


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