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Supplementary Materials and Methods

Supplementary Material

Different sets of QTLs influence fitness variation in yeast

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Supplementary Materials and Methods

1. The ability to grow at high pH is a complex trait:

1.1. Characterizing the ability to grow at high pH

The ability to thrive under alkali stress varies among different yeast isolates. We examined the capacity of 37 clinically-isolated and laboratory *Saccharomyces cerevisiae* strains (Supplementary 3.1) to grow on media of varying pH, and determined the maximal pH (MP) at which each strain was able to grow. The MP phenotypes of this population exhibited a normal distribution, ranging between 7.4 and 8.6, with a mean around pH 8 (Supplementary Figure 1A). In order to estimate the number of QTLs affecting the ability to grow at high pH we carried a cross between the clinical isolated High MP strain and the laboratory Low MP strain BY4741. The hybrid was subjected to meiosis and the ability of 258 haploid segregants to grow at various pH levels was monitored. The phenotypes of this population also yield a characteristic normal distribution (Supplementary Figure 1B). For a quantitative trait each allele is neither necessary nor sufficient to produce the final phenotype; therefore, prediction of the exact number of loci is impossible. However, from the proportion of segregants exhibiting the most extreme phenotypic characteristic, a minimal estimate of QTLs can be obtained [see (Steinmetz et al., 2002)] Only 1.9% of the segregants were able to grow at pH 8.6, (as their "high MP" parent). This inheritance ratio indicates that at least 5.7 independent unlinked loci determine the variation in the ability to grow at high pH between the two strains analyzed.

1.2. Determining the MP phenotype

A serial ten fold dilutions were spotted on YPD plates at various levels of pH. The MP phenotype was defined as the highest pH solid media at which each strain could grow after five days at 30^{o}C. To avoid inaccuracy resulting from differences between plates we used the same plate batch for every experiment and we put a high MP strain

and a low MP strain with a known phenotype as a control on every plate. We repeated this procedure to verify the MP phenotype of all strains.

1.3. **Fitness measurements**

Strains were inoculated into 2 ml of YPD at pH6 and incubated until exponential growth (~10^7 cells per ml) was reached. Optical density at 600 nm (OD600) of cultures was measured and all strains were diluted to OD 0.1 in 2ml YPD at either pH 6 or pH 8. Cultures were grown to OD 1.5. Optical density of each culture was measured every 30 min, creating a growth curve. For each strain, the interval at which growth was exponential was determined, and the growth rate, i.e. the slope of the exponential curve was calculated. The growth rate of all strains was similar on YPD at low pH (average doubling time of 104 minutes, st.dev. of 5.3 min). The Relative fitness of each strain was defined as the ratio of the growth rates at pH8 and pH6. The Growth Rate ratio was defined as the growth rate of each strain compared to the ancestor, all measured at high pH (see also supplementary 7.).

2. Growth media:

YPD (yeast rich medium) - 1% Bacto yeast extract, 2% Bacto peptone, 2% Glucose. Yeast cells were grown in YPD at pH 6 at 30^{o}C (standard growth conditions). The pH was adjusted and kept constant using 100 mM Tris buffer.

SD (yeast defined medium) - 0.67% Bacto yeast nitrogen base w/o amino acids (DIFCO), 2% Glucose. Amino acids were added according to requirement (sigma).

YPD G418 - YPD containing 200mg/l G418 Geneticin (sigma).

YPD Hygromycin - YPD containing 300mg/l Hygromycin (sigma).

5FOA: 0.67% Bacto yeast nitrogen base without amino acids, 2% glucose, 50mg/l Uracil, 0.8 gr/l 5FOA.

YPA 1% Bacto yeast extract, 2% Bacto peptone, 2% Acetate.

SPO - 1% Potassium acetate, 0.1% Bacto yeast extract, 0.05% Glucose + 10% of all the necessary amino acids.

SM - 1% Potassium acetate

3. *S. cerevisiae* strains and yeast genetics

3.1. Yeast strains used:

BY4741a: *MAT*a *ura3Δ met15Δ leu2Δ his3Δ.* This strain is of S288c background and was used as a standard for the sequencing project and for the deletion collection (Brachmann et al., 1998).

BY4741α: *MAT*α *ura3Δ met15Δ leu2Δ his3Δ.* Created by transformation with a *URA3*-marked plasmid carrying the HO gene which enabled mating type switch.

BY4741d: Diploid created by mating **BY4741a** and **BY4741alpha.**

EM39: Wild type diploid, parent of S288c (Mortimer and Johnston, 1986).

W303: *MATa leu2-3,112 trp1-1 can1-100 ade2-1 ura3-1 his3-11,15.* (Thomas and Rothstein, 1989).

TBR strains from Sigma 1278b background, kindly supplied by G. Fink (Reynolds and Fink, 2001):

TBR1: *MAT@ ura3-52 his3::hisG leu2::hisG.*

TBR2: *MATa ura3-52 his3::hisG leu2::hisG.*

TBR3:*MATa/MAT@ ura3-52/ura3-52 his3::hisG/his3::hisG leu2::hisG/leu2::hisG.*

TBR5: *MAT@ ura3-52 his3::hisG leu2::hisG flo11del::KanMX***.**

TBR8: *10560-23c flo10 MAT@ ura3-52 his3::hisG leu2::hisG flo10::KanMX.*

TBR12: *MATa ura3-52 his3::hisG leu2::hisG flo11del::KanMX.*

Wild type clinical isolates from immune-compromised patients, kindly provided by G. Fink:

F1411, F1622, F1623 (MKF12), F1624, F1627, F1630, F1632, F1633, F1635, F1637, F1639, F1642, F1644, F1646, F1649, F1651, F1652, F1653, F1657, F1658, F1659, F1660, F1661, F1662.

All strains in this collection are prototrophs. Most are homothallic diploids, although still maintain a high degree of heterozygozity (M.K., unpublished).

Clinical Isolates (McCusker et al., 1994):

YJM128, YJM222, YJM273, YJM309, YJM310, YJM311

3.2. Construction of the High MP GRA2:

GRA2 is a haploid spore of the diploid wild type strain F1623 (MKF12). Natural isolates of yeast are usually diploid, prototrophic and homothallic (self-mating), all of which preclude control crosses. To form a haploid spore from MKF12 we first put MKF12 through meiosis in sporulation medium. Tetrads were separated by micromanipulation. Since wild type isolates are homothallic, the spores switched their mating type and self-mated to create diploid homozygote strains. We tested the ability of these strains to grow at high pH and chose GRA2-dip as the highest MP strain. We then deleted one of the HO alleles in this strain using targeted disruption with a KanMX4 cassette. Since only one of the alleles was deleted, half of the spores were heterotallic. We picked for further analyses *MATa* and *MATalpha* haploid G418R segregants, GRA2a and GRA2alpha, which exhibited a high MP phenotype.

3.3. In-Lab Evolved strains

Strain BY4741 (*MATa Δura3, Δleu2, Δhis3, Δmet15*) grows extremely slowly at pH7.4. Five independent lines were established; line A, line B, line C, line G and line I. When cultures reached saturation, aliquots (5 ul) were transferred to fresh medium (5 ml) at a slightly higher pH (0.1 pH unit steps) and were similarly incubated. This procedure was repeated until populations able to grow at pH 8.5 or 8.6 were obtained. Samples were taken at each passage, the genotype of the strain tested and the population kept in -80^{o}C. At the end of this procedure cells were streaked on YPD at pH 6, and individual colonies were tested for their ability to grow at high pH using a drop assay. For each line, populations were frozen after each selection step, involving increasing pH (starting from pH 7.4, pH7.5, pH7.6, etc., until they were able to grow at pH 8.6 (lines B, C and G) or pH 8.5 (lines A and I).

3.4. Mating between the ancestor and the evolved strains:

In order to carry out a cross between the ancestor and the evolved strains we first had to switch the mating type of the ancestor. A *URA3*-marked plasmid carrying the HO endonuclease was introduced into this strain. This led to a mating type switch. The plasmid was then lost by growing the transformants in media containing 5-FOA, which selects against Ura+ cells.

3.5. Mating: Two haploid colonies were mixed on YPD plate and incubated overnight at 25ºC. Zygotes were then separated under micro-manipulator.

3.6. Sporulation: Diploids were grown to logarithmic phase at YPD liquid media and then transferred to YPA to 24 hours at 30°C. After 24 hours cells were transferred to SPO or SM for 3 days at 25°C.

3.7. Deletions:

To test candidate genes in the BY4741 background we use the *Saccharomyces* Genome Deletion Project (Giaever et al., 2002) in which each strain was deleted for a single ORF (replaced by the *KanMX4* cassette, which confers G418 resistance).

3.8. Allele swapping:

We carried out allele swapping using the Delito Perfetto methodology (Storici and Resnick, 2003). For each candidate QTL, we deleted the region containing the mutation by insertion of a fragment carrying the *URA3* and *HygB*^{R} genes (which complement uracil auxotrophy and confers resistance to Hygromycin, respectively) and the gene encoding the *I-SceI* nuclease with its restriction site [pCORE plasmid's cassette, a gift from F. Storici (Storici and Resnick, 2003)]. Transformants were selected by resistance to hygromycin and ability to grow on media without uracil. These transformants were confirmed by colony PCR. At least two pCORE transformants were selected for each experiment. We then induced a double strand break at the *I-SceI* site to enhance homologous recombination and replaced the Hyg-*URA3* cassette with a PCR fragment carrying the desired changes (single nucleotide replacements). Replacement of the *Hyg-URA3* cassette with the new allele was selected on 5FOA plates. The sequence of the inserted alleles was confirmed by sequencing of PCR amplicons of the targeted region. Essential genes (*GPI17* and *CDC23*) and the telomeric gene YFR057w were not tested using allele swapping due to technical difficulties.

BY4741 *Mac1* C271W
BY4741 *Mac1* C271S, M386 silent
BY4741 *Mac1* C271Y

BY4741 *Ecm21* 193Nonsense

BY4741 *Ctf3* silent

BY4741 *Oac1* I48F

BY4741 *Rri2* A138P

BY4741 *Sgt2* M1I

BY4741 *Hrd1* L19F

BY4741 *Gtt2/Mmp1* 3'UTR XII 20872

BY4741 *Gph1* 3'UTR XVI 864306, C to A

BY4741 *YFR057w* Base substitution at 3'UTR (+161bp) G to A

BY4741 *Nmd4* Base substitution at promoter (-262bp) C to A

BY4741 *Yhr140w/SPS100* Base substitution at *SPS100* promoter (-384bp) G to T

BY4741 *Ies2* Base substitution at promoter (-65bp) T to C

3.9. Reciprocal Hemizygote strains:

For a particular candidate gene, we first deleted the gene in each parent using a KanMX cassette or the pCORE plasmid's cassette [*URA3*, Hyg, I-Sce-I; (Storici and Resnick, 2003)]. We carried out reciprocal crosses between the ancestor (BY4741) and the evolved strain carrying the deletion of the particular candidate allele. For essential genes, which were impossible to delete, we first carried a cross between the ancestor and the evolved strain and then deleted one of the alleles. We isolated five independent transformants for each gene knockout in both parental backgrounds and confirmed these transformants carried the desired deletion by colony PCR.

BY4741 Δ*gtt2/mmp1*::core x C8.6

C8.6 Δ*gtt2/mmp1*::core x BY4741

BY4741 Δ*gtt2/mmp1*::core x C8.0

C8.0 Δ*gtt2/mmp1*::core x BY4741

BY4741 Δ*ecm21*::core x C8.6

C8.6 Δ*ecm21*::core x BY4741

BY4741 Δ*ecm21*::*kanMX* x C8.6

C8.6 Δ*ecm21*::*kanMX* x BY4741

BY4741 Δgtt2/mmp1::core x C8.6

C8.6 Δgtt2/mmp1::core x BY4741

BY4741 *Δ nmd4*/*ylrw-a*::core x C8.6

C8.6 *Δ nmd4*/*ylrw-a*::core x BY4741

BY4741 *Δ nmd4*::*kanMX* x C8.6

C8.6 *Δ nmd4*::*kanMX* x BY4741

BY4741 Δ*gpi17*::core x C8.6

C8.6 Δ*gpi17*::core x BY4741

BY4741 Δ*yhr140w/sps100*::core x C8.6

C8.6 Δ*yhr140w/sps100*::core x BY4741

BY4741 Δ*mac1*::core x C8.6

C8.6 Δ*mac1*::core x BY4741

BY4741 Δ*mac1*::*kanMX* x C8.6

C8.6 Δ*mac1*::*kanMX* x BY4741

BY4741 Δ*mac1*::core x A8.5

A8.5 Δ*mac1*::core x BY4741

BY4741 Δ*ctf3*::core x A8.5

A8.5 Δ*ctf3*::core x BY4741

BY4741 Δ*cdc23*::core x A8.5

A8.5 Δ*cdc23*::core x BY4741

BY4741 Δ*oac1*::core x C8.0

C8.0 Δ*oac1*::core x BY4741

BY4741 Δ*rri2*::core x C8.0

C8.0 Δ*rri2*::core x BY4741

BY4741 Δ*gph1*::core x C8.0

C8.0 Δ*gph1*::core x BY4741

BY4741 Δ*ies2*::core x C8.0

C8.0 Δ*ies2*::core x BY4741

BY4741 Δ*sgt2*::core x C8.0

C8.0 Δ*sgt2*::core x BY4741

BY4741 Δ*hrd1*::core x C8.0

C8.0 Δ*hrd1*::core x BY4741

3.10. Congenic strains

A cross between BY4741 and GRA2 was performed, and after meiosis, haploid spores were plated on medium at pH 8.4. Spores that could grow were then re-tested

in a drop assay on media at several pH (as described above) and the best-growing segregants backcrossed to the BY4741 strain. This procedure was repeated for 8 generations, establishing four independent congenic lines.

4. Genotyping

We first applied SNPScanner (Gresham et al., 2006) for detecting SNPs in a single chip. SNPScanner performs model training based on two sets of arrays representing strains with known genomic sequences. In our case, we used as one set the replicates from the reference strain BY4741, and the other set contained the replicate arrays for the strain RM11 [taken from (Gresham et al., 2006)].

Once trained, the SNPScanner model predicts mutations in each chip (and replicate) separately. Nucleotide positions that manifest a significant drop in the intensity of hybridization given the model are predicted to be mutations. This detection takes into account probe nucleotide content and the SNP position within the probe, and integrates input from all ~5 probes that cover each position. Our further analysis focused only on positions that attained a SNPScanner score >=5 in at least 50% of the replicates of the same strain.

In the second phase, we estimated the signal loss at each candidate SNP position using the Hodges-Lehamn estimator for every genomic position (Hollander, 1999), testing whether or not the perfect match probes of a mutated strain have significantly lower signal than their corresponding mismatch probes. This test sacrifices some information as it ignores the probes' content and the position within the probe, which are accounted for in SNPScanner. However, unlike SNPScanner, it takes into consideration repeated measurements and can measure signal loss between any two strains, not requiring that one of them is the original reference strain (BY4741 in our case). In particular, this is important for the congenic lines analysis, as we can compare the High MP parent strain with its congenic descendants.

The signal loss at each candidate position was identified using the Affymetrix Tiling Analysis Software, which computes the significance of the hybridization intensity drop using the Wilcoxon signed-rank test. Since the reference genome for the Affymetrix chips is similar to BY4741, a drop in the signal suggests a potential SNP in the given position. We filtered all putative SNPs that did not reach a p-value of 0.01, and also discarded positions that attained $p<0.01$ in the BY4741 strain, implying

that the SNP was present already in our reference strain. We also filtered some additional putative SNPs based on manual inspection of their signals.

An additional analysis was applied to the congenic strains in order to identify regions that originated from the GRA2 strain. For each congenic line, we compared the 8^{th} generation strains to BY4741, using the two-sample-analysis as implemented in the Affymetrix Tiling Array Software. For each nucleotide, the comparison resulted in an estimation of the intensity drop between the congenic strain and BY4741 as a reference. In order to detect regions that are likely to originate from the GRA2 strain, we first selected in each chromosome the top 0.05% nucleotides that showed the highest drop. Next, every two selected nucleotides at a distance <8000bp were defined as an interval that is suspect of originating from the GRA2 strain, and overlapping intervals were merged. Last, isolated intervals of length <1000bp were discarded. The result is a collection of intervals H(1),…H(n).

Using the same method we compared the same congenic strain to the GRA2 strain, resulting with regions L(1),…L(m) that are likely to originate from the BY4741 strain.

If all inference was perfect and complete, the two sets of regions H and L should have formed a complementary cover of the genome, and the endpoints of the intervals in each would give the recombination points. In reality, in order to handle noise, we combined the two information sources as follows. Let H(i)=[r,l], and let L(j)=[r',l'] be the leftmost interval in L s.t. r<r'. If l=<r' we defined the recombination point to be in the middle between the two regions, i.e. (r'-l)/2. If r'<l, and l'<l, we discarded L(j); otherwise, we defined the recombination point to be in the middle of the overlapping region, i.e, (l-r')/2. After applying this method, less than 10% of the genome was found to be inherited from the GRA2 parent.

We repeated this procedure on the 4th and 6th generations of the congenic lines, and reassuringly, virtually every region in a late generation was contained in some region in an early generation of the same line.

Validation of all SNPs acquired during in-lab evolution was done by PCR and sequencing. Some of the regions found by congenic lines were also determined by PCR and sequencing of several SNPs in the genotyped lines and in other independent congenic lines.

5. PCR and sequencing analysis

All SNPs found at the ILE strains were verified by PCR and sequencing. Candidate congenic regions were also verified by sequencing of 1kb inside the region. PCR reaction was performed with an annealing temperature of 52^{o}C, using primers at a final concentration of 0.4µM. Elongation time was 1 minute for each Kb of the longer predicted product.

6. Estimating fixation stage

For each mutation a population of cells from each selection stage was PCR-amplified and sequenced. The sequencing results were then viewed as a chromatogram, in which certain positions contained two overlapping graphs. The surface under these curves was calculated, producing an approximate estimate of the representation of each allele in the PCRed population. For each line, populations were amplified at different stages of the selection procedure. The stage at which only one of the two alleles was detectable was defined as the fixation stage.

7. Estimating the effect of each mutation using allele swapping

In order to evaluate the contribution of each mutation, we used the information from the allele swapping experiment. We divided the growth rate of the strain carrying the swapped allele ($GR_{Allele\ swap}$) by the growth rate of the Low MP ancestor ($GR_{Ancestor}$). We repeated this calculation for three repeats. The result of these calculations can be seen in Figure 4.

$$QTLs'\ effect = \frac{GR_{Allele\ Swap}}{GR_{Ancestor}}$$

In order to correct for multiple testing, we used the standard FDR correction. We first ranked the original p-values, getting a range of values from 1.36e-05 to 0.47. We then corrected the required p-value for every test by their rank, multiplying it by r/n where r is the rank of the test and n is the total number of tests conducted. We terminated the procedure at the first test that did not pass the corrected p-value.

8. Molecular biology techniques

8.1. Yeast genomic DNA extraction (Phenol method) - A 3ml of overnight culture was harvested, and the supernatant was removed. To the tube we added ~0.3g acid-washed glass beads, 300µl lysis buffer (2% Triton, 1% SDS, 0.1M NaCl, 10mM TrisHCl pH 8, 1mM EDTA) and 300µl 25:24:1 phenol:chlorophorm:isoamyl alcohol. Tubes were vortexed for 20', and centrifuged for 10' at 14 krpm. The top phase was carefully transferred to a new tube containing 3 volumes of ethanol in order to precipitate the DNA. Tubes were incubated for 20' at RT and centrifuged for 10' at 14 krpm. The pellet was resuspended in 300µl TE x1 (10mM TrisHCl pH 7.5, 1mM EDTA) with RNase (Sigma) 50µg/ml in 65ºC for 10'. Then DNA was precipitated by adding 0.3 volumes of 10M Ammonium acetate and 3 volumes of ethanol 100% and incubated at -20ºC for 30 min-16 hrs. Tubes were centrifuged for 10' at 14krpm at 4ºC. Pellet DNA was washed with cold ethanol 70%, and resuspended in 50µl H_2O or 10mM Tris-HCL pH 7.5.

8.2. Yeast chemical transformation - Yeast overnight cultures were diluted 1/20 into 50ml of YPD and incubated at 30ºC, 220 RPM for 4hrs. Cells were harvested at a density of $2*10^7$ cells/ml, washed twice with cold H_2O and resuspended at a final volume of 1ml 0.1mM LiAcetate. 100µl of the cell suspension was then added to 360µl transformation mix containing 33% PEG3500, 0.1M LiAcetate, 0.27 mg/ml Salmon Sperm Single Stranded DNA. 1µg of DNA was added and the mixture was vortexed gently and incubated at 42ºC for 40 min. Following heat shock cells were spinned down and resuspended in 1 ml of YPD. Cells were incubated at RT for 30 min and then plated on selective plates.

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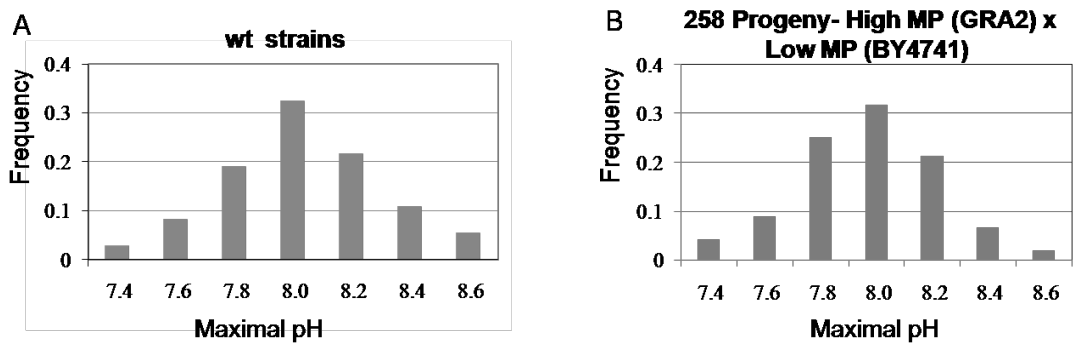
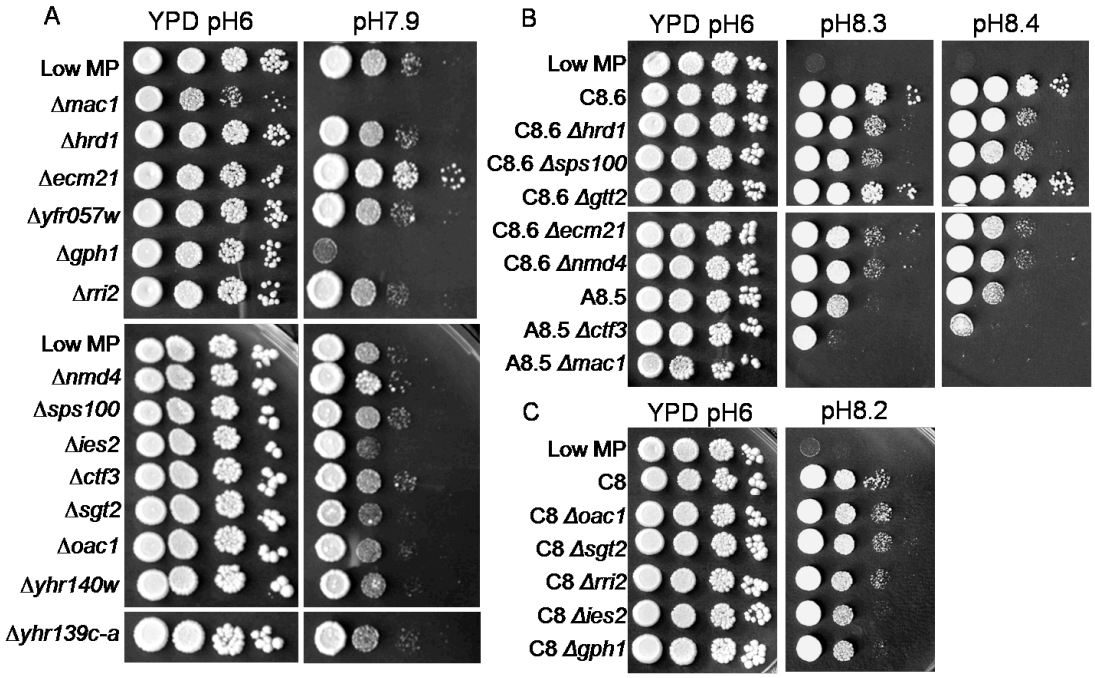
Supplementary Figures

Suppl. Figure 1



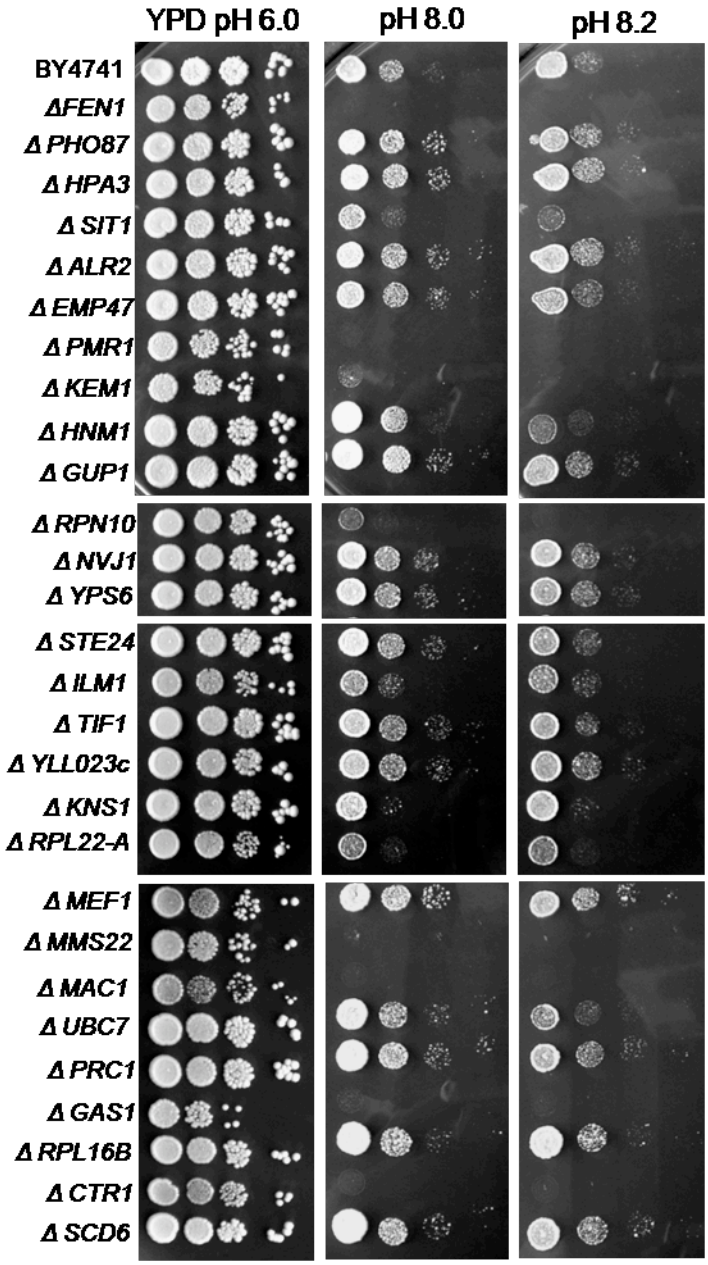
Suppl. Figure 1: Normal distributions of the MP phenotype in two populations. **A.** A mixed population of wild type laboratory and clinical isolated strains. **B.** A population of 258 progeny from a cross between the high MP clinical isolated strain (GRA2) and the Low MP laboratory strain BY4741.

Suppl. Figure 2



Suppl. Figure 2: Ten-fold dilutions of haploid yeast cells on optimal (pH 6) and high pH solid media. **A.** Deletion of candidate genes at BY4741 background. **B**. Deletion of candidate genes at C8.6 and A8.5 background. **C**. Deletion of candidate genes at C8.2 background.

Suppl. Figure 3



Suppl. Figure 3: Ten-fold dilutions of haploid yeast strains on optimal (pH 6) and high pH solid media. Each strain carries a single deletion of a gene found at the congenic regions and was predicted by our algorithm to be a good candidate to effect the ability to grow at high pH. Out of 29 deletions tested, thirteen have an MP phenotype.

Supplementary Table 1: Sequencing verification of regions inherited from the High MP parent in the congenic lines.

"+": region inherited from the High MP parent. "-": the information in the region is from BY4741. Regions identified in strains N4HA, N8HA (fourth and eight generations), N6HB and N8HB (sixth and eith generations) by hybridization to tiling arrays, were also sequenced in independent lines O and C at the sixth and seventh generation (N6HO and N7HO, N6HC and N7HC).

Ch.	Position	N	N4HA	N8HA	N6HB	N8HB	N6HO	N7HO	N6HC	N7HC
III	168966-200800	13	+	+	-	-	+	+	+	+
V	1-29961	7	+	+	-	-		-		-
VI	30356-51606	8	+	+	-	-		-		-
VII	143903-200324	26	+	-	+	-		-		-
VII	325256-396816	31	+	-	+	+	+	+		-
VIII	478122-517418	20	-	-	+	+		-		-
IX	424859-431838	1	+	+	-	-		-		-
X	633129-683951	20	+	+	+	+	+	+	+	+
XI	533219-574982	18	-	-	+	+	+	+		-
XII	92342-114724	7	+	+	-	-		-		-
XII	262946-280320	8	+	+	+	-		-		-
XII	345291-374049	7	+	+	+	+	+	+		-
XII	606720-775305	67	+	+	-	-	+	+		-
XII	937245-949420	2	+	+	-	-	-	-	-	-
XIII	307000-355700	24	-	-	+	-	-	-	-	-
XIV	453937-509171	28	+	+	-	-		-		-
XVI	785313-804496	12	-	-	+	+		-	+	+