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Engineering of synthetic, stress-responsive yeast promoters

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ABSTRACT

Advances in synthetic biology and our understanding of the rules of promoter architecture have led to the development of diverse synthetic constitutive and inducible promoters in eukaryotes and prokaryotes. However, the design of promoters inducible by specific endogenous or environmental conditions is still rarely undertaken. In this study, we engineered and characterized a set of strong, synthetic promoters for budding yeast *Saccharomyces cerevisiae* that are inducible under acidic conditions (pH ≤ 3). Using available expression and transcription factor binding data, literature on transcriptional regulation, and known rules of promoter architecture we improved the low-pH performance of the *YGP1* promoter by modifying transcription factor binding sites in its upstream activation sequence. The engineering strategy outlined for the *YGP1* promoter was subsequently applied to create a response to low pH in the unrelated *CCW14* promoter. We applied our best promoter variants to low-pH fermentations, enabling tenfold increased production of lactic acid compared to titres obtained with the commonly used, native *TEF1* promoter. Our findings outline and validate a general strategy to iteratively design and engineer synthetic yeast promoters inducible to environmental conditions or stresses of interest.

INTRODUCTION

Any biomanufacturing application requires a genetically tractable chassis with a large library of genetic parts (e.g. promoters, RNA stabilizing elements, transcription factors, etc.) that can be used to engineer the chassis to produce a desired product (1–3). Such parts can be used to rationally design genetic circuits that should ideally provide sustained or tightly regulated outputs that are well-characterized under conditions of interest. The budding yeast *Saccharomyces cerevisiae* is one of the most widely used chassis for industrial production. While it has many good genetic tools, it lacks promoters tuned for production of chemicals under a variety of industrial processing conditions.

Several successful engineering strategies have been reported for the generation of novel synthetic promoters including generation of randomized libraries (4,5), construction of hybrid promoters (6), manipulation of nucleosome accessibility (7), and total *de novo* synthesis (8). However, most of them have only been tested in standard laboratory glucose-rich or inducible conditions which represent a small part of the environmental conditions yeast can face in industrial fermentations. As yeast is engineered to grow under increasingly diverse conditions or consume feedstocks that are sub-optimal for its native metabolism, the choice of 'constitutive' promoters may be inadequate, to the detriment of strain performance in these conditions (9). It is therefore important to design and test new promoters that perform better under these conditions to allow us to exploit more fermentation conditions for yeast cell factories, making the whole process more economically feasible. Indeed, the selection and application of native promoters responsive to alternate carbon sources (9), stress from metabolite accumulation (10), or by feedback from the end product of a biosynthetic pathway (11) have been employed in eukaryotes and prokaryotes to improve the performance of production strains. However, we cannot rely on native promoters for desirable regulatory output for all relevant stresses. One such stress is low medium pH, which can be imposed on the culture from the start, after sufficient biomass has accumulated, or gradually occur by the accumulation of acidic

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products during fermentation. Yeast tolerates low pH, and improving its performance as a cell factory at low pH could minimize contamination risks, increase product yield, reduce the need for pH control systems, and simplify the recovery of compounds such as organic acids (12).

In this study, we demonstrate the design and engineering of synthetic yeast promoters inducible by low pH using a heuristic approach. From literature searches and data mining of existing low pH studies, we selected a promoter already inducible by low pH, and engineered its upstream activating sequence (UAS). We present a thorough characterization of synthetic promoter performances inferred from both transcript quantification and fluorescent reporter assays. We subsequently show that our engineering strategy can be used to build low-pH induction into native promoters lacking this response. Finally, we demonstrate that best-performing synthetic promoters outperform a commonly used constitutive yeast promoter in a low-pH fermentation for lactic acid production. Based on these findings we propose a general design and engineering strategy for building robust, stress-inducible synthetic promoters.

MATERIALS AND METHODS

Strains and growth media

For plasmid construction and promoter characterization, we used *S. cerevisiae* strain CEN.PK 113-11C (*MATa ura3-52 his3Δ1 MAL 2–8^C SUC2;* from Peter Koetter, Johann-Wolfgang Goethe University, Frankfurt, Germany), while Ethanol Red (Fermentis, A Lesaffre division) was used for low-pH fermentations. All yeast cultures were grown at 30°C in YPD medium (1% yeast extract, 2% peptone, 2% glucose, Sigma-Aldrich). Transformation was done on YPD agar supplemented with 350 mg/l G418 (Sigma-Aldrich), 200 mg/l hygromycin (Life Technologies) or 100 mg/l nourseothricin (clonNAT, Werner BioAgents) for selection. When growing *pdc1*$^-$ *pdc5*$^-$ strains, we used YPE (2% peptone, 1% yeast extract, 2% ethanol) or minimal medium (13) containing 10 g/l glucose and 1 g/l sodium acetate as carbon sources. For bacterial transformations, we used LB broth or agar containing 100 mg/l ampicillin, culturing cells at 37°C.

Promoter design

We used native promoter sequences from the CEN.PK 113–7D reference genome (14) available at the Yeast Genome Database (http://www.yeastgenome.org) as backbones. Native transcription factor binding sites were identified using ChIP-derived data (15), either from visualizing them using the Yeast Genome Database's GBrowse tool with the relevant track, or by searching the promoter sequences in the YeTFaSCo database (16) for the motifs listed in Table 1. When choosing binding sites to screen for or modify, we generally selected motifs from large-scale *in vivo* or *in vitro* data sets (15,17). In a few instances, we also included motifs from individual studies (18,19) when a motif was not identified in the aforementioned studies. All synthetic promoters were ordered as gBlocks from IDT. All promoter sequences are provided as Supplementary Data.

Plasmid and strain constructions

All plasmids were constructed *in vivo* in yeast using gap-repair assembly (20). Transformation was performed by the lithium acetate/PEG method (21). All parts for assembly and transformation were amplified using the Phusion High-Fidelity Master Mix with HF buffer (ThermoFisher), while 2xOneTaq Quick-Load Master Mix with Standard Buffer (New England Biolabs) was used for colony PCR. All primers were ordered from IDT.

A base plasmid (pASR0067) was constructed by assembly of a PCR fragment from plasmid pSH62 (EUROSCARF) containing a bacterial origin, an ampicillin resistance gene, a *CEN6/ARSH4* replication origin, a *kanMX* resistance cassette from pCFB2312 (22), and an expression cassette for yeast-optimized mKate2 (Addgene) (23). mKate2 expression was controlled by the *TEF2* promoter and *ADH1* terminator. For assembly, the *kanMX* cassette and the parts for the mKate2 expression cassette were added in a 40-fold molar excess to 100 ng of the PCR fragment with the origins of replication. Adjacent fragments had 50 bp homology added to them by PCR. Colonies with G418-resistant transformants were screened by colony PCR as described elsewhere (24) and sequence-verified, with the plasmid subsequently isolated from yeast and re-transformed into *Escherichia coli* DH5α (ThermoFisher) for preservation. The plasmid was recovered from yeast spheroplasted with 5 mg/ml β-glucanase (Glucanex, Sigma-Aldrich) with the NucleoSpin plasmid miniprep kit (Macherey-Nagel), following the protocol recommended for low copy-number plasmids. Generally, 10 μl of the prep was used to transform chemically competent *E. coli*.

Reporter plasmids were constructed by assembling the promoter of interest with YFP and the *VPS13* terminator *in vivo* into pASR0067 linearized by digestion with PacI and XmaI. A Kozak sequence *AAAACA* to enhance translation was added upstream of YFP by PCR (13,25). G418-resistant colonies were screened and selected by colony PCR and sequencing.

To verify the correlation between plasmid-based and chromosomally integrated reporter data, we constructed strains where YFP expression cassettes with different promoters of interest were cloned into the EasyClone site XII-4 (13). A fragment from a plasmid containing the relevant cassette and the G418 resistance marker was amplified by PCR and transformed into yeast with 500bp of homology to XII-4 on either end. G418-resistant colonies were screened by colony PCR; those with the correctly integrated fragment were then streaked out on YPD agar, regrown in YPD with G418 and preserved as glycerol stocks. All primer sequences are listed in Supplementary Table S1, and all plasmids in Supplementary Table S2.

Construction of lactate production strains

Lactate-producing Ethanol Red strains were constructed by *in vivo* assembly in an approach similar to DNA assembler (26). 1 pmol of *L. plantarum* lactate dehydrogenase (*ldhL*), the *ADH1* terminator, the promoter of interest and the *hphMX* cassette for hygromycin resistance with 50bp overlaps were transformed into the Ethanol Red strain for recombination into EasyClone site X-2 (13) with 500

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Table 1. Transcription factor binding sites used to engineer basal and pH-induced promoter output

Binding site	Function	Motif[a]	Reference(s) for motif
Rap1p	Increase overall promoter output	GAACACCCATACATC	(15,47)
Swi5p	Increase overall promoter output	TGCTGGt	(15,17)
Swi4p	Increase overall and low-pH promoter output	aCGCGAAA	(15,17)
Azf1p	Increase overall promoter output	aAAAAGAAA	(17)
Msn2p/Msn4p (STRE)	Improve output at low pH	aAGGGG	(15,17)
Rlm1p	Improve output at low pH	tCTATWWWTAg	(15,18)
Crz1p	Potentially improve output at low pH	GTGGCTG	(15)
Haa1p	Potentially improve overall output at low pH or under stressful conditions	GNNMRGGG	(19)
Nrg1p	Repress output at low pH	GGACCCt	(15)

[a]Lower-case letters are low-information bases flanking the main sequence logos available on YeTFaSCo.

bp homology. Clones with correctly assembled and integrated constructs then had their *PDC1* and *PDC5* genes inactivated using CRISPR/Cas9 (22). First, the Cas9-bearing plasmid pCfB2312 with a G418 resistance marker was transformed into these strains. Following this, the strains were transformed with 1 μg of gRNA-expressing plasmid pCfB2514 targeting an identical site on *PDC1* and *PDC5* and 4 nmol of a double-stranded 90bp oligo which inserted a Stop codon into their sequences. We selected for *pdc1*$^{-}$ *pdc5*$^{-}$ double mutants with YPE agar containing G418 and clonNAT. Mutations in the targeted regions in *PDC1*/*PDC5* were then verified by colony PCR and sequencing. Lactate-producing strains are listed in Supplementary Table S3.

pH- and stress-dependent induction experiments

Strains bearing plasmids expressing YFP under the control of native or synthetic promoters were grown overnight in synthetic drop-out medium minus leucine (SD-leu; Sigma) containing 1.1 g/l monosodium glutamate as a nitrogen source, and 200 mg/l G418. Cells were diluted 20-fold in duplicate in minimal Delft medium (13) with the necessary supplements at pH 6 containing 250 mg/l G418 and grown for 4h to re-enter the log phase. Following this, one replicate was diluted to an OD of approximately 0.1–0.2 in Delft medium, pH 6, while the other was washed and diluted two-fold in Delft medium, pH 2.5. The latter, lower dilution was necessary to have enough cells for fluorescence measurements. In the case of inductions by oxidative stress, cells were diluted two-fold in Delft medium, pH 6, and the oxidative agent diamide (Sigma-Aldrich) was added to a final concentration of 2 mM from a concentrated stock of 500 mM. For inductions by osmotic stress, cells were washed and diluted 2-fold in Delft, pH 6 containing 1 M sorbitol. YFP fluorescence was then measured at 4 or 24 h by flow cytometry, using the base strain without YFP for background correction.

Flow cytometry

Induced cells were diluted 10-fold in phosphate-buffered saline and immediately analyzed on a BD LSRFortessa flow cytometer (BD Biosciences) equipped with a high-throughput screening module for sampling. YFP fluorescence was excited using a 488-nm solid-state laser (Coherent) and detected using an Alexa Fluor 488/FITC filter set with a 530/30 bandpass filter. Ten thousand events per sample were acquired using FACSDiva software and data was exported as FCS files. Data analysis was done using the FCSExtract utility (available at http://research.stowers-institute.org/efg/ScientificSoftware/Utility/FCSExtract/), custom R scripts and Origin 9.1 (OriginLab). Origin's multiple-peak fit for Gaussians was applied on log-transformed fluorescence histograms to retrieve the lowest discernible fluorescent peak, representing the population containing a single reporter plasmid. All fluorescence data presented are the average of at least three biological replicates plotted as mean ± standard deviation. Differences between YFP levels were tested for significance using Welch's two-sample *t*-test when necessary, with $P < 0.05$ taken to be significant.

RNA extraction and two-step RT-qPCR

A set of seven reporter strains with YFP regulated by either the native *YGP1, CCW14, TEF1* or four synthetic promoters integrated as described above were grown in triplicate in deep-well plates at pH 6 and also induced at pH 2.5. After 4h induction, the cultures were transferred to a new plate chilled with ice and recovered by centrifugation at 2272 × *g* for 5 min at 4°C. Following this, we extracted total RNA using a previously described method (27).

RNA samples were diluted to a concentration of 15–30 mg/l and used as templates for cDNA synthesis by the Maxima First Strand cDNA Synthesis Kit (ThermoFisher) following the recommended protocol. The resultant cDNA diluted was five-fold as a template for qPCR with the Maxima SYBR Green Master MIX (ThermoFisher) on an Mx3000P qPCR system (Aglient Technologies). The reaction was set up as recommended by the manufacturer, with 10 nM ROX as reference. We performed two-step qPCR as follows: 10 min denaturation at 95°C, followed by 40 cycles of 15 s denaturation at 95°C, 1 min annealing, extension and data acquisition at 60°C. Melting curve analysis was carried out after amplification. Primers targeting YFP, and *PDC1* (SGD ID S00000434) as a reference gene (28) were designed using the PrimerQuest tool available on IDT's website (http://eu.idtdna.com/primerquest/home/index). Primers and amplicons were checked for secondary structure with mFold (29), and qPCR data analyzed using Q-Gene (30) to calculate fold-induction at low pH.

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Chromatin immunoprecipitation and quantitative PCR

For assessment of DNA-binding of candidate transcription factors by chromatin immunoprecipitation (ChIP), strains were constructed with the transcription factor of interest C-terminally tagged with multiple copies of the Myc epitope. Cassettes with either 9xMyc (31) (pYM21, EUROSCARF) or 13xMyc tags (32) (pFA6a-13Myc-natMX6, EUROSCARF) and a nourseothricin resistance marker were integrated upstream of the ORF of interest's Stop codon. Strains with the intact, in-frame tag then had the relevant promoter-YFP expression cassette integrated chromosomally at EasyClone site XII-4. These strains were cultivated in Delft medium at pH 2.5 or 6.0 in shake flasks to an OD of 1.5–2.0 (mid-log phase). Formaldehyde cross-linking, quenching by glycine, cell disruption and chromatin shearing were performed as described previously (33). Chromatin fragments were applied to Anti-c-Myc Magnetic Beads (Pierce, Thermo Scientific) for immunoprecipitation at 4°C with gentle agitation overnight. Washing of beads, elution of chromatin, protease K (Thermo Scientific) digestion, reverse cross-linking and DNA extraction were performed as previously described (34). DyNAmo Flash SYBR Green qPCR Kit (Thermo Scientific) was used for qPCR on an Mx3005P qPCR System (Agilent Technologies) as previously described (33). *ALG9* was used as the reference gene for DNA enrichment analysis in precipitated DNA relative to input DNA using the $2^{-\Delta\Delta C}{}_T$ method (35). No enrichment of the targets on *YGP1* promoter was observed in a strain without Myc-tagged protein. All strains used for ChIP are listed in Supplementary Table S4.

Lactic acid fermentation

The *pdc1*$^-$ *pdc5*$^-$ strains expressing *ldhL* were streaked out from a glycerol stock onto YPE agar. Single colonies of each production strain were then grown overnight in YPE with 200 mg/l hygromycin and diluted to a starting OD of 0.5 in 5 ml of minimal medium with 10 g/l glucose and 1 g/l sodium acetate as carbon sources. The pH was buffered to three or six using citrate–phosphate buffer. The fermentation was carried out aerobically for 5 days in an ultra deep-well plate (Enzyscreen) at 30°C with 300 rpm shaking, each fermentation being performed in triplicate. Samples were taken every 24 h with the OD measured and the metabolites analyzed by HPLC as described earlier (22). Data analysis was carried out using Chromeleon 7 (Dionex/ThermoFisher) and Origin.

RESULTS

Engineering the pH-dependent response of the *YGP1* promoter

In order to design synthetic yeast promoters with basal regulatory output similar to common constitutive promoters and with increased or stable output from pH ≤3, we carried out three stages of promoter engineering. We initially tested TF binding site modifications that could independently increase (i) basal and (ii) pH-dependent promoter strength, and then (iii) combined the best modifications and rules of the two approaches to iteratively engineer strong, pH-inducible synthetic promoters.

Experimental set-up for promoter characterization. In order to establish a robust experimental set-up for promoter characterization at low pH we first investigated how pH is affected in short- and long-term yeast cultivations. For this purpose, we sampled pH at several time points throughout cultivation from cultures starting at pH 2.5 or pH 6 and found that medium pH remained above 4 after 24 h in the case of a starting pH of 6, or remained unchanged in the case of a starting pH of 2.5 (Supplementary Table S5). We therefore concluded that cells in the reference state (pH 6) are not subjected to significant stress from decreasing medium pH. Based on this, and the fact that promoters' long-term response to low pH is of more interest in terms of practical applications, our results focus on 24 h inductions by low pH. Furthermore, besides measuring low-pH output relative to pH 6, we also characterized each promoter's output at pH 4.5, 4, 3.5 and 3 to determine how their induction thresholds depend on the TF binding sites. We ultimately scored and selected promoters based on strong basal and low-pH output alone at 24 h, fold-induction at low pH being of secondary importance. Finally, our results from plasmid-based expression correlated well with measurements obtained with the reporter cassette chromosomally integrated (Supplementary Figure S1), and data representations throughout this study are based on outputs from low-copy number plasmid unless otherwise stated.

Selection of YGP1pr as a first candidate promoter backbone. To establish which individual or combined binding sites would give the best response to low pH, we first engineered a native promoter with a known low-pH response. To narrow our search for candidates and minimize screening, we focused on induced genes reported from studies on the response of yeast to pH ≤3.5 (28,36–39). An ideal candidate gene should be strongly induced by low pH at short and long timescales, not tied to any metabolic process to minimize nutrient effects, and have known TF binding sites identified in its promoter (Supplementary Figure S2A). These criteria led us to eliminate promoters for hexose transporters such as *HXT4,* dependent on glucose levels, and the promoters of chaperones such as *HSP26* since expression data suggested that their low pH responses are transient (37,40). Other genes induced by low pH, like *YRO2*, are poorly characterized and were not considered as backbone promoter candidates for this reason (Supplementary Figure S2B and C). Ultimately, we selected the *YGP1* promoter (*YGP1pr*) as a candidate promoter to engineer based on the aforementioned criteria.Ygp1p is a glycosylated, secreted cell wall-associated protein, expressed during conditions of stress or nutrient depletion (41,42), but which has also been reported to be induced by low pH across different strains and conditions (Supplementary Table S6). Therefore *YGP1pr* is a good candidate to be engineered for improved performance at low pH.

Evaluation and selection of pH-responsive regulatory elements. We chose five TF binding sites from mining the available literature and TF binding site data to engineer a promoter's pH-dependent response (Table 1). Yeast's response to low pH is mainly mediated through the HOG kinase and cell wall integrity (CWI) pathways, with

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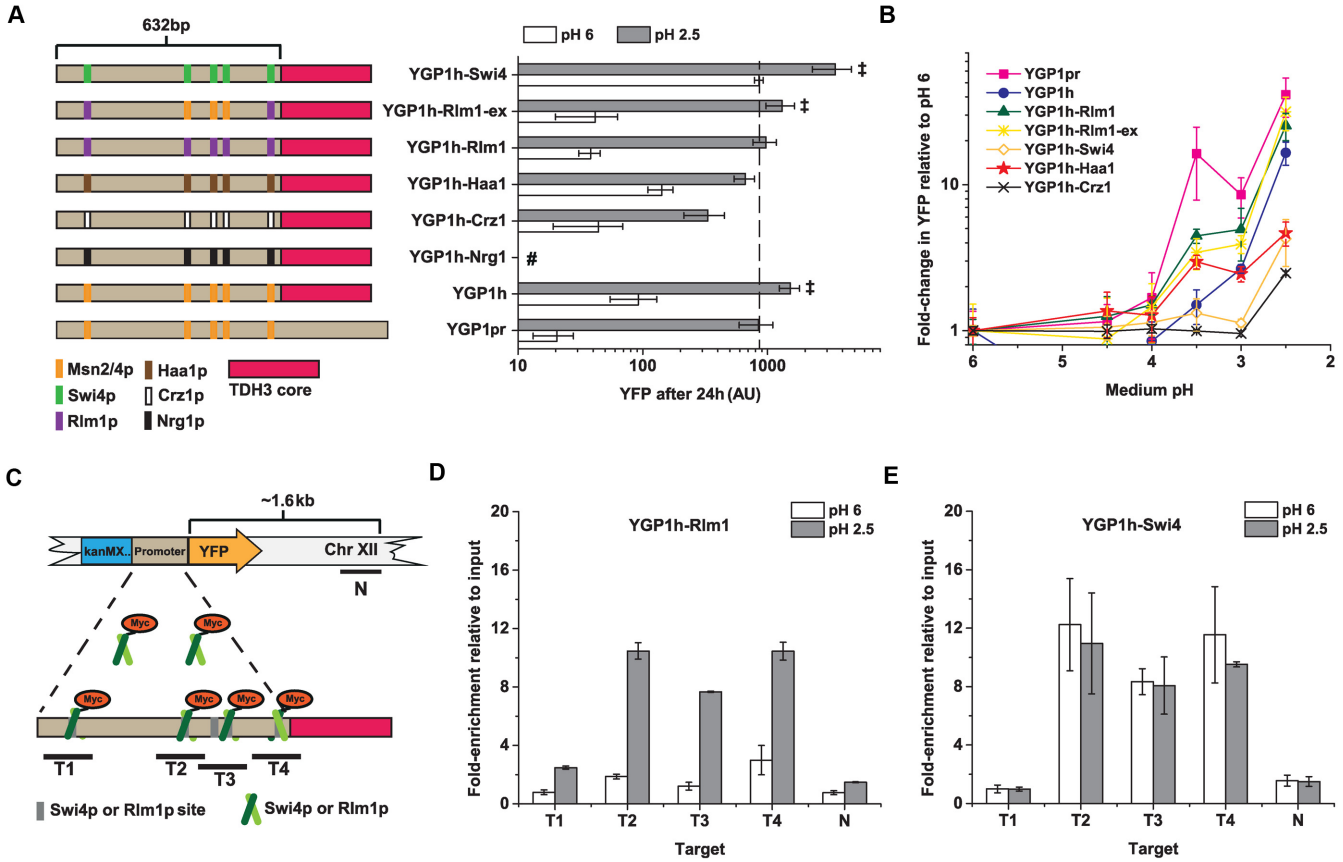

Figure 1. Evaluation of different pH-responsive TF binding sites. (**A**) Partial or total exchange of STREs identified in *YGP1pr* by ChIP with known and putative pH-responsive binding sites generated different low-pH responses, and allowed us to select useful binding sites. Promoters are ordered by their output at pH 2.5, and the dashed line marks the output of *YGP1pr* at pH 2.5. A hash mark indicates no significant fluorescence above the background. Diagrams to the left illustrate modifications to promoters. Bar plots are the mean±S.D of at least three biological replicates, with double daggers indicating significantly higher output than *YGP1pr* at low pH ($P < 0.05$). (**B**) pH-dependent induction of the promoters in (A), normalized to their output at pH 6. Error bars are S.D. of three biological replicates. (**C**) Schematic of ChIP to validate *in vivo* TF binding. Swi4p or Rlm1p is C-terminally tagged with a Myc epitope and binds to its sites in the UAS at pH 6 or 2.5. Enrichments of the sites are confirmed by qPCR targeting four stretches of the UAS which cover all sites, along with a negative control (N) 1.6kb downstream of the promoter-YFP expression cassette. One target (T3) covers two sites due to their proximity. Target sizes are 75–125 bp. ChIP and subsequent qPCR confirmed binding of (**D**) Rlm1p at *YGP1h-Rlm1* and (**E**) Swi4p at *YGP1h-Swi4*. qPCR data are plotted as mean±S.D. of at least two biological replicates.

Msn2/Msn4p and Rlm1p as their transcriptional effectors, respectively (28,36,43). These TFs have been verified to directly induce gene expression at low pH (43,44), making their sites the first choice to add to promoters for a pH-dependent response. We also considered Swi4p sites as potential pH-responsive elements (Table 1), since Swi4p is the DNA-binding component of the SBF transcriptional activator, an effector of the CWI pathway (45). A recent study reported that Crz1p, implicated in alkaline stress responses (46), was upregulated at low pH (28), leading us to include its binding site as a potential pH-inducible element. We also considered modifying *YGP1pr* with Haa1p sites as Haa1p is involved in the response to multiple acid stresses (42).

In addition to selection of pH-responsive elements for engineering low-pH responsiveness, we also aimed to increase basal activity of the synthetic promoters. For this purpose, we considered replacing *YGP1pr*'s core promoter with the strong *TDH3* core promoter (*YGP1h*). We identified this to be 150bp upstream of *TDH3* containing the transcription start site (TSS) and TATA box (47). Based on the selected TF binding sites and core-promoter swapping strategy, we initially outlined the low pH induction of yellow fluorescent protein (YFP) controlled by wild-type *YGP1pr* (Figure 1A). Here we first observed that replacing the *YGP1pr* core with the core of *TDH3pr* strategy resulted in a doubling of both basal and low-pH outputs following 24 h (Figure 1A). Next, we replaced all the Msn2/Msn4p sites, which do not overlap with other TF binding sites (henceforth referred to as STREs) in *YGP1h* (15,48) with each candidate TF binding site (Table 1) and tested the resulting promoter outputs. Crz1p and Haa1p site substitutions made the promoters less sensitive to low pH (Figure 1A), with the former variant even yielding no detectable fluorescence at 4h (*YGP1h-Crz1,* Supplementary Figure S3A). Next, we made Rlm1p site substitutions in regions of low nucleosome occupancy achieving higher low-pH output than *YGP1h* (*YGP1h-Rlm1-ex*, Figure 1A and B). However, replacing all STREs with Rlm1p sites reduced basal and low-pH outputs compared to *YGP1h* (*YGP1h-Rlm1*, Figure 1A), suggesting that both sites are required for an optimal low-pH response.

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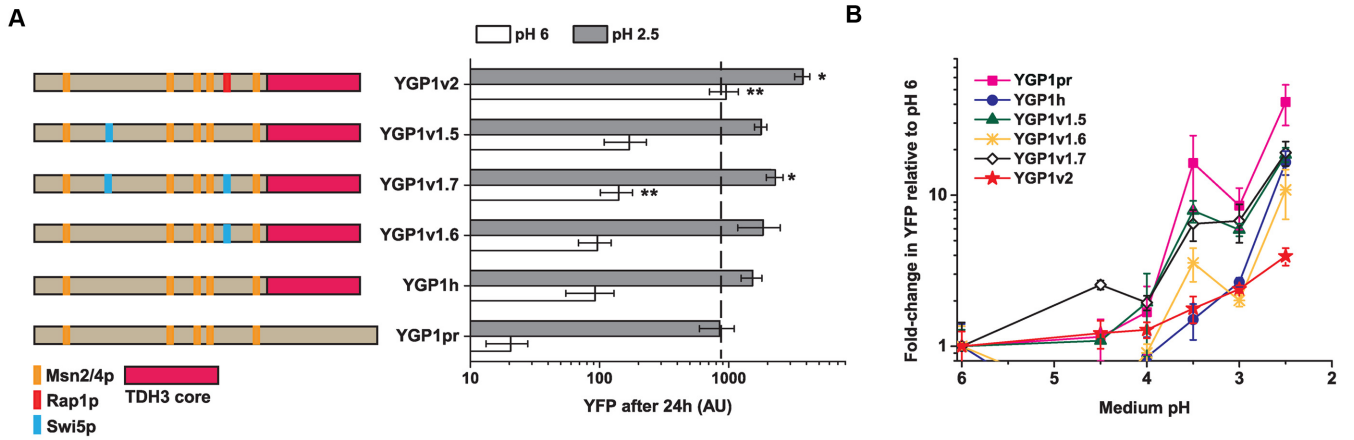

Figure 2. Evaluation of TF binding sites to increase basal output in *YGP1pr*. (**A**) Output of promoter variants with Swi5p or Rap1p sites added to increase basal output. Data are plotted as mean ± S.D. of at least three biological replicates, ordered by basal output. Diagrams to the left illustrate modifications to promoters. Bar plots marked with a single or double asterisk have significantly higher low-pH or basal outputs than *YGP1h*, respectively ($P < 0.05$). (**B**) pH-dependent induction of the promoters in (A), normalized to their output at pH 6. Error bars are S.D. of three biological replicates.

When STREs were substituted with Swi4p sites (*YGP1h-Swi4*), a significant increase in both basal and low-pH output was observed, which did not change much between 4 and 24h for this variant (Figure 1A, Supplementary Figure S3A). However, given that Swi4p also regulates genes during the G1/S transition of the cell cycle (49) and that it shifts the induction threshold to pH ≤3 (Figure 1B), its sites alone are unsuitable for engineering pH-dependent responses. Taken together, among the four candidate binding sites used to replace STREs, only Rlm1p and Swi4p TF site exchanges in the *YGP1h* backbone resulted in significant increases in long-term low-pH outputs compared to wild-type *YGP1pr*, though Haa1p also enabled a significant increase in low-pH output at 4h (*YGP1h-Haa1,* Figure 1A, Supplementary Figure S3A). Finally, in addition to the low pH regulatory elements, we also verified that STREs are the main determinants of *YGP1pr*'s response to low pH by replacing them with TF binding sites for Nrg1p, a repressor inactivated by high pH (46). No detectable YFP signal was measured at any pH in this variant (*YGP1h-Nrg1*, Figure 1A, Supplementary Figure S3A).

Next, we validated direct binding of Rlm1p and Swi4p to *YGP1h-Rlm1* and *YGP1h-Swi4* promoters, respectively, by chromatin-immunoprecipitation (ChIP). For this purpose we tagged Rlm1p or Swi4p at their C-termini with Myc epitopes in strains also containing genomically integrated *YGP1h-Rlm1* or *YGP1h-Swi4* promoters controlling YFP expression (Figure 1C). qPCR of immunoprecipitated DNA targeted four regions on the UASes of interest, covering the five Rlm1p or Swi4p sites. For *YGP1h-Rlm1*, qPCR showed enrichment of Rlm1p binding at all four regions analyzed at low pH (2.5–10.5 fold enrichment), while only modest enrichments were observed at pH 6 (0.8–2.9-fold enrichment) (Figure 1D). For *YGP1h-Swi4*, Swi4p enrichment was observed at three out of four regions at both pH 2.5 and pH 6 (8.1–12.2-fold enrichment, Figure 1E). The differences in binding enrichment at different pH values for Rlm1p and Swi4p tally with the high and low-fold induction in their respective promoters. No enrichment was observed at a negative control site 1.6 kb downstream of the YFP expression cassette, sufficiently downstream to not be coprecipitated with bound promoter DNA (Figure 1D and E). Importantly, in addition to validating direct regulation of candidate promoters, the tagged TFs also retained their functionality and ability to induce YFP expression at low pH (Supplementary Figure S4A and B).

In summary, though STREs (Msn2/Msn4p sites) are the principal TF binding sites to use in order to engineer a low-pH response in a yeast promoter, we have successfully engineered low-pH promoter outputs based on addition of Swi4p or Rlm1p sites to the existing *YGP1* UAS. Moreover, the outputs from variant promoters with Swi4p or Rlm1p sites added show enrichments of Swi4p and Rlm1p binding, making these regulatory elements primary candidates for further engineering of strong low-pH inducible promoters.

Selection and evaluation of regulatory elements to increase basal promoter strength. *YGP1pr*'s basal output is low, but was increased nearly 5-fold exchanging its core with the *TDH3* core promoter (Figure 1A and B). We therefore further modified basal output by adding TF binding sites associated with strong promoter output to *YGP1h* (Table 1). In general, new TF binding sites were added without overlap to other binding sites of the *YGP1pr* UAS. If existing binding sites were replaced with new ones, they were replaced with minimal changes to the local sequence context. Based on a recent systematic study of candidate binding sites that were shown to improve promoter outputs (50), we selected Rap1p, Swi4p and Swi5p sites. Having already confirmed the impact of introducing Swi4p sites on basal promoter activity (Figure 1A), we next sought to replace an existing weak Swi5p site (cGGCGGG) identified by ChIP (15) with a consensus site (TGCTGGt), resulting in *YGP1v1.5* (Figure 2). We also added a Swi5p site 270 bp upstream of YFP in a region with no known or predicted TF binding sites (*YGP1v1.6,* Figure 2), but adding a Swi5p site at either location did not increase basal output. Dual substitutions of Swi5p (*YGP1v1.7*, Figure 2) did significantly, yet modestly,

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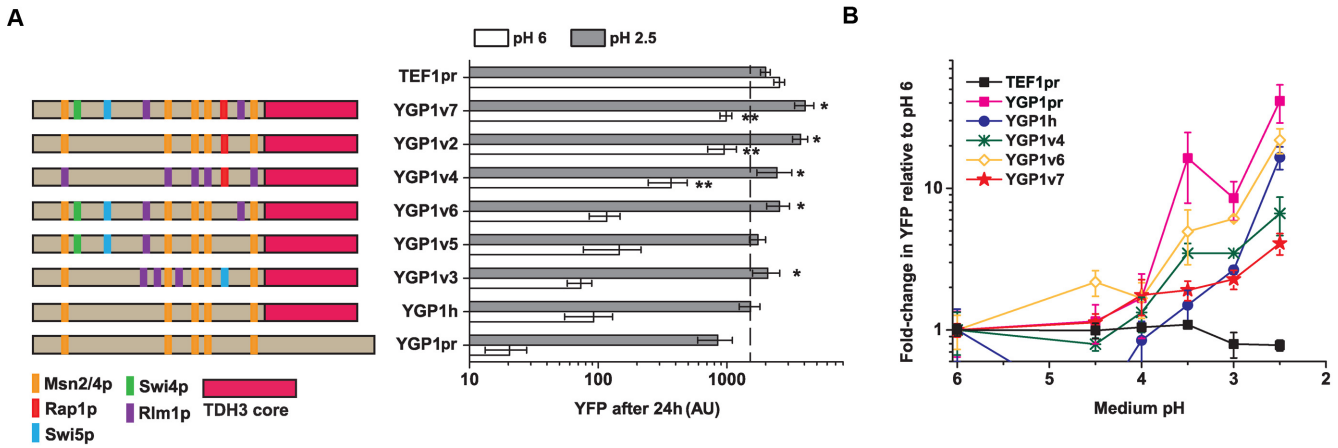

Figure 3. Final engineered *YGP1pr* variants. (**A**) Basal and low-pH responses of promoter variants engineered using sites selected from Figures 1 and 2. Diagrams to the left illustrate modifications to promoters. Data are ordered by overall output and plotted as mean ± S.D of at least three biological replicates. *TEF1pr*'s output is added as a reference for constitutive promoters. Bar plots marked with a single or double asterisk have significantly higher low-pH or basal outputs than *YGP1h*, respectively ($P < 0.05$). (**B**) pH-dependent induction of the best-performing promoters in (A), normalized to their output at pH 6. Error bars are S.D. of at least three biological replicates.

increase basal and low-pH output, but not to a satisfactory level, compared to modifications with Rap1p discussed below. Contrary to their intended use, Swi5p site insertions also increased low-pH output significantly over short time scales (Supplementary Figure S3B). For Rap1p we introduced a binding site in the −270 bp position as for Swi5p in *YGP1v1.6*, creating *YGP1v2* (Figure 2A). This increased basal output by a factor of 10 and low-pH output by a factor of 3, resulting in lower but smoother pH-dependent induction (Figure 2B). Neither Swi5p nor Rap1p sites altered the promoters' induction thresholds, confirming them to be good choices to increase basal output. In summary, in addition to Swi4p, we found both Rap1p and Swi5p sites to be suitable to increase output in general, with Rap1p chosen to be the best modification to strictly increase basal output.

Designing strong, low pH-inducible synthetic promoters. As previous modifications established which TF binding sites work best in increasing basal and low-pH output, we next engineered promoter variants iteratively, using the same binding sites to simultaneously optimize basal and low-pH output. Based on the output of *YGP1h-Rlm1-ex*, we initially built *YGP1v3,* by clustering Rlm1p sites near native STREs and a Swi5p site in the *YGP1v1.6* position (*YGP1v3*, Figure 3A), resulting in a stronger promoter than *YGP1h* at low pH. However, the multiple A-T rich Rlm1p sites can alter local nucleosome occupancy and therefore promoter output (51), complicating interpretation of *YGP1v3's* output. Instead, we achieved similar output by shifting the position of the Swi5p site and using fewer Rlm1p sites in conjunction with a Swi4p site (*YGP1v5, YGP1v6*, Figure 3A). For *YGP1v6*, this resulted in a 1.5-fold increase in low-pH output alone compared to *YGP1h*. We finally increased the overall output of *YGP1v6* by adding a Rap1p site in the same location as in *YGP1v2* to create promoter *YGP1v7*. This variant had higher output than *YGP1v2* at low pH over short and long time scales, and was also stronger than the commonly-used *TEF1* promoter at low pH (Figure 3A, Supplementary Figure S3B and C). The increase in basal and low-pH output seen in *YGP1v7* resulted in lower fold-induction (Figure 3B, Supplementary Figure S4), as also observed for *YGP1v2*. To generate a promoter with appreciable overall output *and* fold-induction, we added a Rap1p site to variant *YGP1h-Rlm1,* creating *YGP1v4.* This promoter showed significantly higher basal output than *YGP1h*, higher low-pH output than *TEF1pr*, and higher fold induction than *YGP1v7* (Figure 3A and B).

Our strategy allowed us to improve basal and low-pH promoter output of *YGP1pr*, with a 50-fold increase in basal output and a 5-fold increase in low-pH output in the final variant *YGP1v7* compared to *YGP1pr*. Rlm1p sites and STREs in combination worked best to increase low-pH output, while Swi5p and Rap1p sites increased overall output. We could also alter fold-induction based on the number of STREs or Rlm1p sites used for a low-pH response.

Engineering a pH-dependent response in the *CCW14* promoter

To validate our promoter engineering strategy, we used the same approach and TF binding sites to engineer a strong response to low pH in the *CCW14* promoter. *CCW14* is a cell wall glycoprotein reported to be activated by the CWI pathway (18,52), citric acid stress at pH 3.5 (53), but not by low pH. Accordingly, YFP was not induced by low pH when regulated by *CCW14pr* (Figure 4). As its promoter contains Rlm1p sites and STREs (15,54), this makes it an interesting engineering target to study what modifications are required for it to gain a regulatory response to low pH. Surprisingly, though STREs in the *CCW14* core promoter were lost when it was exchanged with the *TDH3* core, this generated 3- and 8-fold increases in YFP at pH 6 and pH 2.5, respectively (*CCW14h* versus *CCW14pr*; Figure 4). Engineering strategies for *CCW14h* subsequently focused on finding which of the modifications used in *YGP1pr* would best improve basal and low-pH responses in *CCW14h.*

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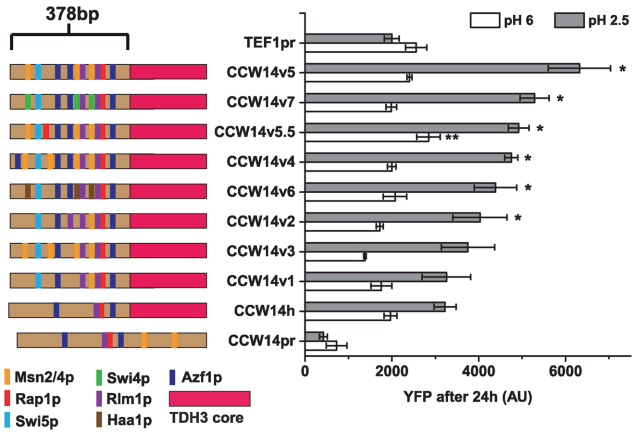

Figure 4. Engineering *CCW14pr* for a response to low pH. The most useful modifications to *YGP1pr* were applied to *CCW14pr* to create strong, pH-inducible synthetic promoters. The modifications in each promoter are illustrated to the left of the graph. Promoter data are ordered by low-pH output, and plotted as mean±S.D of at least three biological replicates. Bar plots marked with a single or double asterisk have significantly higher low-pH or basal outputs than *CCW14h*, respectively ($P < 0.05$).

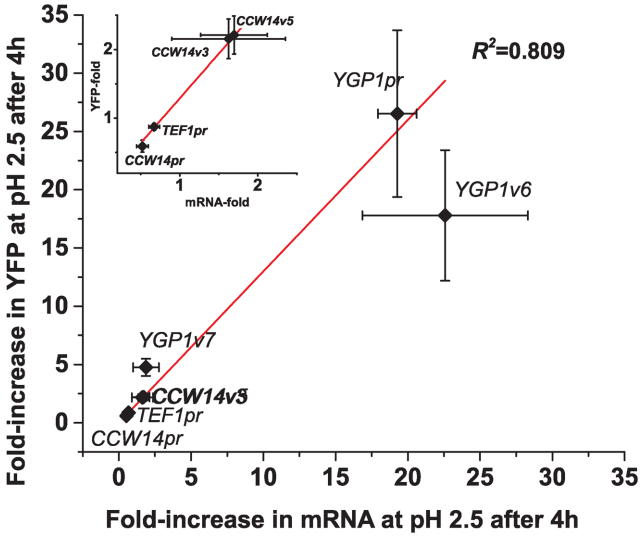

Figure 5. Correlation between fold-increases in YFP fluorescence and mRNA at low pH. YFP data and RNA samples for RT-PCR were taken 4h after induction at pH 2.5 and after 4 h growth at pH 6. YFP and mRNA data were taken using strains with reporter constructs chromosomally integrated as described in the Methods. Data points are labelled after the promoters used, and error bars represent S.D. of three biological replicates. *Inset:* Zoomed-in view of the first half of the plot.

Selection and characterization of binding site modifications. To bottom-up engineer a strong low-pH responsive promoter based on our findings from engineering *YGP1pr*, we first combined Rlm1p sites along with STREs to increase output at low pH. Secondly, as Swi5p sites were also shown to increase low-pH output (Figure 2, Supplementary Figure S3), we also decided to use them in this role. From this we found that the addition of up to two extra Rlm1p sites and a single STRE increased low-pH output significantly above that of *CCW14h* (*CCW14v1, CCW14v2*, Figure 4, Supplementary Figure S5). As further addition of STREs flanking the Swi5p site did not significantly increase output compared to *CCW14v2* (*CCW14v3*, Figure 4), we attempted to increase output using Azf1p sites. Besides resembling imperfect poly(dA:dT) tracts, which increase promoter output by decreasing nucleosome occupancy (51), these sites have been shown to increase promoter output by themselves in synthetic promoters (50). *CCW14pr* is also predicted to have two Azf1p sites, which could also contribute to its basal output. We therefore modified C*CW14v3* to have Azf1p sites flanking the added STREs. The resultant promoter, *CCW14v4*, had not only higher basal output than the previous variant, but also significantly higher low-pH output than previous promoter variants (Figure 4). Assuming that the UAS could be saturated with different binding sites, we removed a STRE and an Azf1p site from *CCW14v4* to create *CCW14v5,* which achieved the strongest low-pH output of our *CCW14pr* variants, lending weight to our hypothesis (Figure 4). Our final modification attempted to increase basal output by adding a second Rap1p site to *CCW14v5* (an existing one being predicted in *CCW14pr*). This increased basal output to a level significantly higher than *CCW14h* and *TEF1pr* compared to other promoters (*CCW14v5.5,* Figure 4), but at the cost of a decrease in low-pH output.

To demonstrate that other TF sites can affect low-pH output in a similar manner across promoters, we replaced all the STREs in *CCW14v5* with Swi4p and Haa1p sites as for *YGP1pr*. While low-pH output of the variant with Swi4p sites (*CCW14v7*) was higher than that of the variant with Haa1p sites (*CCW14v6*), neither had a higher output than *CCW14v5* (Figure 4, *CCW14v6* and *CCW14v7* versus *CCW14v5;* c.f. *YGP1h-Haa1* and *YGP1h-Swi4*, Figure 1B). We suggest that the strong basal output of *CCW14h* compared to *YGP1h* limited the transfer of these sites' functions to low-pH effects alone. While dynamic range was modest compared to *YGP1pr* variants, our engineering strategy nonetheless nearly doubled fold induction from *CCW14h* to *CCW14v5* and *v7* (Supplementary Figure S6), resulting in truly strong promoters inducible by low pH.

In summary, we successfully engineered the *YGP1* and *CCW14* promoters to create a set of strong, synthetic promoters with the potential to outperform commonly used constitutive promoters at low pH. Furthermore, quantitative RT-PCR of YFP mRNA after 4h induction revealed good correlation between fold-induction of mRNA and YFP at low pH for three native and four synthetic promoters covering the range of promoter outputs, confirming that these promoters are indeed transcriptionally upregulated by low pH (Figure 5).

Synthetic promoter applications in low pH fermentations

To demonstrate their value in a practical application, we used a subset of our synthetic promoters to produce lactic acid in low-pH fermentation. Lactic acid production at low pH would simplify product recovery in an industrial setting as lactic acid would exist in its undissociated form, simplifying the chemical recovery of the free acid and minimizing the formation of salt by-products normally formed in this process (12). Furthermore, as the produc-

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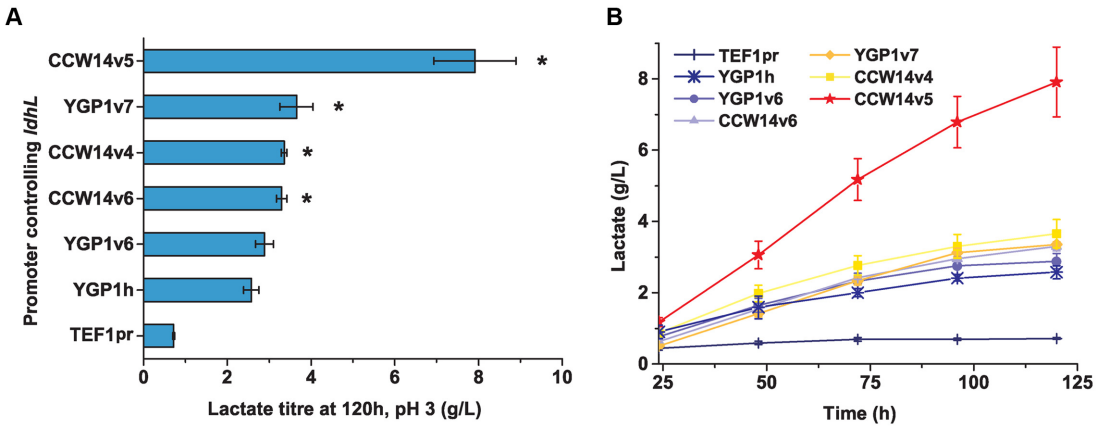

Figure 6. Production of lactic acid at low pH using synthetic, low pH-responsive promoters in an industrial yeast strain. (**A**) Lactate acid titres of seven Ethanol Red strains using either one of five synthetic promoters, *YGP1h* or *TEF1pr* (as baselines) at the end of a 5-day fermentation at pH 3. Bar plots marked with an asterisk have significantly higher lactate titres than *YGP1h* ($P < 0.05$). (**B**) Time-course of lactate production. Error bars represent S.D. of three biological replicates.

tion of lactic acid from pyruvate is a single enzymatic reaction it is easy to evaluate the effectiveness of individual promoters at low pH. We therefore constructed strains bearing the lactate dehydrogenase gene from *L. plantarum* (*ldhL*) under the control of one of six pH-inducible promoters, or the strong commonly used *TEF1pr* (22). Four of these promoters—*YGP1v7, CCW14v4, CCW14v5* and *CCW14v6*—have outputs similar to *TEF1pr* at pH 6 (Figures 3A and 4). *YGP1v6* was also chosen to see how basal output affected production, and *YGP1h* was selected as a baseline promoter. These strains had their pyruvate decarboxylase genes, *PDC1* and *PDC5,* inactivated using CRISPR/Cas9 (22) in order to minimize the conversion of pyruvate to acetaldehyde, thereby increasing its availability for lactate production (55). To demonstrate promoter robustness across strains, we built these strains using the diploid, industrial yeast Ethanol Red. After 120 h fermentation, the strains with *ldhL* controlled by synthetic pH-inducible promoters outperformed the strain with *ldhL* expressed from *TEF1pr,* yielding lactate titres of 2.9–7.9 g/l versus 0.72 g/l for *TEF1pr* (Figure 6A). Significantly, the highest titre was achieved when the promoter with the strongest low-pH output measured, *CCW14v5*, was used to express *ldhL.* Lactate titres for the same strains at pH 6 were higher than those at pH 3, but most of our promoters had similar or superior productivity at pH 3 compared to pH 6 (Supplementary Figure S8). Our promoters' applications are therefore not restricted to low-pH fermentations alone.

Direct comparisons with other reports of lactic acid fermentations in yeast at low or neutral pH are complicated by variations in strain background, medium (especially available glucose), aeration, gene copy number and promoters used. We were nonetheless able to achieve titres which compare favourably to those reported for the same background (22). Given the performance of these promoters in a strain unoptimized or unevolved for performance at low pH, the titres we report are a valid demonstration of our promoters' applications, and of the promoter engineering method employed.

DISCUSSION

The development of regulatory parts with controllable and predictable behavior goes hand-in-hand with integrating them into practical applications, as we better understand the regulatory elements involved in transcriptional responses to different signals. The work presented here, and the promoters designed and engineered through it, represent an attempt to identify yeast regulatory elements that respond to an environmental signal by designing regulatory parts responsive to such a signal—low extracellular pH in this case. Instead of large-scale design and extensive screening, our attempts to rationally select regulatory elements that respond to low pH represent a heuristic strategy that can streamline screening and eventually provide an alternate path to rational, rule-based design of synthetic parts for gene expression with a desired function (Figure 7).

Recapitulating our findings, our approach found that three TF binding sites—Msn2/Msn4p, Rlm1p and Swi4p—can directly and individually induce a transcriptional response to low pH, as evidenced from reporter assays and ChIP. Each of these sites can impart different fold-inductions and pH induction thresholds to promoters. By combining them, we can engineer synthetic promoters inducible by low pH with different induction thresholds and low-pH outputs. In this study, we also demonstrated that four binding sites earlier reported to increase overall promoter output in short, synthetic promoters (50)—Rap1p, Swi5p, Swi4p and Azf1p – also could do the same in larger, more complex promoters such as those engineered in this study. Moreover, these 'strong' TF binding sites can make the pH-dependent induction smoother, albeit at the cost of fold-induction. Moreover, a concise, pH-dependent characterization of promoters bearing different combinations of these sites allows promoters to be assigned different applications based on their relative outputs under inducing and non-inducing conditions. Finally, by successfully using our promoters in a low-pH fermentation in an industrial yeast strain, we demonstrate that they exhibit a degree of interoperability between strains and plug-and-play

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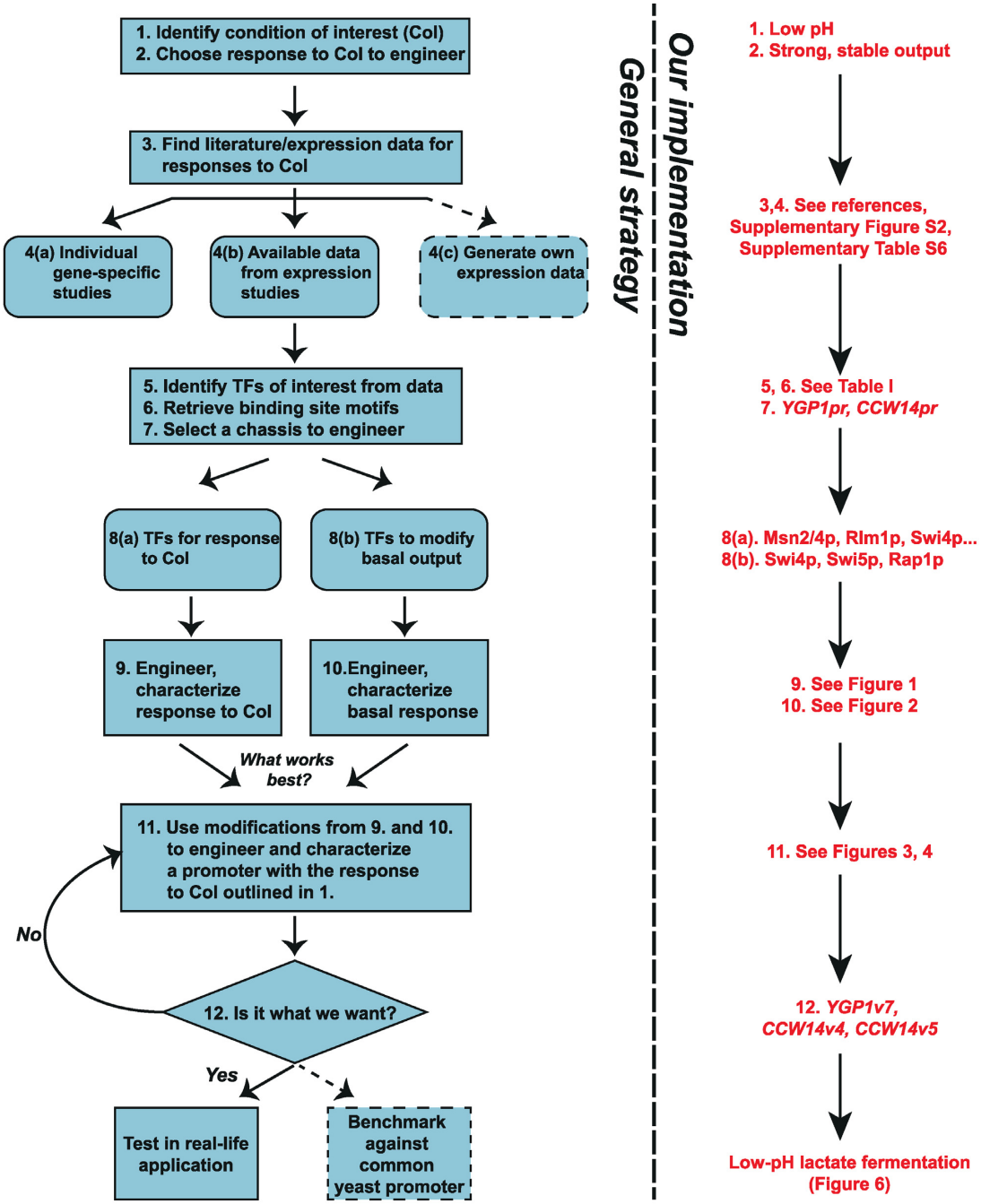

Figure 7. Our heuristic promoter engineering strategy summarized. The overall procedure is outlined to the left, with examples from this work listed to the right in red referring to the relevant figures and tables when necessary.

functionality, traits desirable for synthetic biology parts (56).

The low pH-responsive TF binding sites chosen in this study are also the effectors of other stresses (28,45). We therefore expected our promoters to show some degree of cross-reactivity. Indeed, two such stresses—osmotic and oxidative—can induce the synthetic promoters (Supplementary Figure S9). As compared to low pH, however, both stresses induce similar but noisier responses in the promoters. While such cross-reactivity is at odds with synthetic biology's design goal of precisely controlled output, it could be a positive attribute in an industrial setting where yeast is likely to face multiple stresses at the same time. In fact, it was recently reported that low pH, or even acid accumulation, can induce an oxidative stress response in yeast (39,57). Therefore, two stresses activating the same TF may act synergistically, but this cannot be guaranteed to always be the case.

To summarize, the promoter engineering approach we present here lends itself well to the design of new yeast pro-

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moters sensitive to particular stresses whose response mechanisms may not be fully understood, or whose stress response is affected through multiple pathways. In principle, all that would be needed for such a task would be expression data for yeast exposed to the condition of interest under relevant culture conditions. From such a set, the pipeline adopted in this study can identify TF binding sites overrepresented in up- or down-regulated genes and use these to heuristically design hybrid or wholly synthetic promoters with a desired response. While not a true implementation of the ideal modular approach to building parts espoused by synthetic biology, our approach provides a working solution bridging this principle and the use of endogenous yeast promoters in metabolic engineering applications irrespective of their *a priori* suitability for the given task.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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