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An orthogonal and pH-tunable sensor-selector for muconic acid biosynthesis in yeast

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Abstract

Microbes offer enormous potential for production of industrially relevant chemicals and therapeutics, yet the rapid identification of high-producing microbes from large genetic libraries is a major bottleneck in modern cell factory development. Here, we develop and apply a synthetic selection system in *Saccharomyces cerevisiae* that couples the concentration of muconic acid, a plastic precursor, to cell fitness by using the prokaryotic transcriptional regulator BenM driving an antibiotic resistance gene. We show that the sensor-selector does not affect production nor fitness, and find that tuning pH of the cultivation medium limits the rise of non-producing cheaters. We apply the sensor-selector to selectively enrich for best-producing variants out of a large library of muconic acid production strains, and identify an isolate that produces more than 2 g/L muconic acid in a bioreactor. We expect that this sensor-selector can aid the development of other synthetic selection systems based on allosteric transcription factors.

Keywords: transcriptional activator, biosensor, sustainability, evolution, metabolic engineering, yeast

In order to realize a bio-based economy, metabolic engineering aims to develop microbes that can convert inexpensive, renewable feedstocks into valuable products.[1] Initial genetically-engineered strains, however, regularly need to be further optimized before their performance meets industrial demands on titers, rates and yields. Currently, decreases in DNA synthesis costs and the expansion of genome engineering tools allow for cost-effective building of large libraries of cell factory designs.[2,3] However, since the vast majority of chemicals targeted for overproduction in microbes are not coupled to easy selectable phenotypes, evaluation of individual strains often relies on low-throughput analytical methods, severely challenging the turn-around time of the design-build-test-learn cycle.[4]

In recent years, development within synthetic biology has enabled the design and application of allosterically regulated transcription factors as biosensors.[5,6] Such one-component regulators are abundantly present in prokaryotes,[7] and can convert intracellular concentrations of otherwise inconspicuous chemicals of interest into easily measurable outputs, such as fluorescence (sensor-reporters) and antibiotic resistance (sensor-selectors).[4] Even in the yeast *Saccharomyces cerevisiae,* a well-established biotechnology workhorse, there is a large demand on improving current strains and generating yeasts that incorporate novel biosynthesis routes.[8] To this end, a range of transcription factor-based biosensors that can aid the screening of yeast cell factory variants have been described,[9] including sensor-reporters for detection of xylose,[10] malonyl-CoA,[11] *cis, cis*-muconic acid (CCM) and naringenin.[12] Although sensor-reporters have been used for selection for best-producing cells by fluorescence-activated cell sorting (FACS),[13,14] sensor-selectors can offer high-resolution coupling of chemical abundances with growth-selectable phenotypes in a simple and inexpensive manner.[15] In prokaryotes, sensor-selectors have been widely used to select best-performing microbial strains or to evolve microbes,[16–18] but also in yeast a few examples have demonstrated coupling of production to growth through the expression of auxotrophic marker genes.[19,20]

Previously, we have shown that transcriptional activators belonging to the LysR-type transcriptional regulator (LTTR) family can successfully be

transplanted into yeast and applied as small-molecule sensor-reporters.[12] One of the sensor-reporters, BenM, enabled expression of GFP correlated to *in vivo* CCM production. CCM is a platform chemical that can be converted into adipic acid or terephtalic acid, which can be further polymerized into numerous plastics.[21] Whereas the highest CCM titer to date has been ascribed to *Escherichia coli*,[22] from a process point of view producing CCM in a low-pH tolerant organism such as *S. cerevisiae* is of great interest. Rational engineering as well as evolution have been applied to establish and improve CCM production in yeast.[13,23–25] Notably, Leavitt and co-workers used a synthetic reporter promoter inducible by aromatic amino acids (AAAs) to drive the expression of an antibiotic resistance gene and evolve a strain with an increased pool of endogenous AAAs.[13] Following two consecutive rounds of EMS mutagenesis and adaptive laboratory evolution for approx. 600 h, the authors identified a strain producing 2.1 g/L CCM.

In order to design and apply faster and more simple sensor-selector systems based on small-molecule binding transcriptional activators, we re-engineered our previously identified CCM sensor-reporter design into a sensor-selector. First, we determined the optimal design for the sensor-selector, taking into account parameters such as biosensor expression level and dynamic range. Second, we showed that the sensor-selector does not affect the growth rate nor the performance of yeast engineered to produce CCM. Third, we demonstrated that tuning pH of the medium can be used to minimize the rise of fast-growing, yet low-producing, cheaters. Finally, we applied the sensor-selector to enrich for best-producing strains out of a large library of CCM production strains. From a large library of CCM pathway designs, we also showed biosensor-assisted selection of an isolate able to produce more than 2 g/L CCM, on par with highest reported titers, and with higher productivity. To our knowledge, this is the first report on an orthogonal synthetic selection system in yeast driven by antibiotic resistance, which allows for rapid identification of best-producing cell factory variants from large strain libraries.

Results and Discussion

Design and characterization of a CCM sensor-selector

Previously we carried out a multi-parametric analysis in order to develop a CCM biosensor based on the LysR-type transcriptional regulator BenM transplanted from *Acinetobacter* sp. ADP1 into *S. cerevisiae*.[12] Here, we set out to develop a sensor-selector based in *S. cerevisiae* to couple chemical production to growth by replacing the reporter gene from our previous study with the *kanMX* gene, a widely used marker conferring resistance to the antibiotic G418.[26]

In order to identify an optimal sensor-selector design supporting CCM-dependent growth under selective conditions (i.e. G418), we first compared the growth rates of yeast strains harboring the selector (*kanMX* driven by an engineered CYC1 promoter; CYC1p_*BenO*_T1[12]) combined with no BenM, BenM expressed from the strong *TEF1* promoter, BenM expressed from the weak *REV1* promoter, and a previously identified[12] BenM mutant (BenM*) expressed from the *REV1* promoter. This mutant contains three amino acid substitutions (H110R, F211V and Y286N) and displays an increased dynamic range as compared to wild-type BenM.[12] First, each of the four strains expressing the *kanMX* cassette was pre-cultured in medium with or without CCM, and then subcultured into medium with the same composition with or without G418. Here, we found that all four strains grew well in medium without selection, irrespective of the CCM concentration (Fig. 1A). Contrastingly, without BenM, no growth was observed under selective conditions, whereas BenM expressed from the strong *TEF1* promoter led to constitutive growth, even in the presence of G418 and absence of CCM. Finally, while *BenM* expressed from the weak *REV1* promoter showed modest CCM-dependent growth under selective conditions, the strain expressing BenM* showed pronounced CCM-dependent growth in the presence of G418. Therefore, REV1p-*BenM** combined with the selector was chosen as the optimal design. For this design, we found that when supplemented with 400 mg/L CCM, the growth rate was further increased under selective conditions, but no further increase was observed in the presence of 800 mg/L (Fig. 1B). Finally, we found that introducing the sensor and/or the selector into yeast did not affect the basal growth rate both in medium with and without added CCM (Fig. S1).

Taken together, these data demonstrate that the sensor-selector design consisting of REV1p-*BenM** and the selector construct has the

potential to couple accumulation of CCM to host growth without affecting the growth rate of yeast under non-selective conditions.

Sensor-selector validation in production strain

Next, we aimed to investigate if the sensor-selector would be functional in yeast engineered to produce CCM from a 3-step heterologous biosynthetic pathway (Fig. 2A).[23,24] We previously introduced this pathway into yeast and measured CCM production of individual variants differing in the number of an integrated cassette containing *KpAroY.B* and *KpAroY.Ciso*, genes encoding subunits of the rate-limiting enzyme AroY, which controls the conversion of protocatechuic acid (PCA) to catechol (see Fig. 2A and Methods).[12] We chromosomally integrated the sensor-selector in a strain (TISNO-11) that stably produced CCM based on a single integrated copy of the expression cassette for *KpAroY.B-KpAroY.Ciso* (Fig. S9). In order to determine the effects of introducing the pathway and the sensor-selector into yeast, we measured growth and CCM production of this strain. To maintain selection for the production pathway (see Methods), these assays were carried out using mineral medium. Whereas the standard nitrogen source of this medium is ammonium sulfate, we replaced this with urea, since G418 is not effective in combination with ammonium sulfate.[27] From this study we found no significant difference in growth rate between the original CCM-producing strain (CCM pathway) and the strain further engineered to express the sensor-selector (CCM pathway + sensor-selector) under non-selective conditions, though the CCM production strain grew significantly slower than wild-type CEN.PK (*t*-test, $p<0.05$), underscoring the growth burden of the production pathway (Fig. 2B). As expected, the production strain without the sensor-selector was not able to grow in selective medium, whereas the production strain with the sensor-selector showed robust growth in the presence of G418 (Fig. 2B). Moreover, there was no significant difference in CCM titer between the two strains (Fig. 2C). Finally, we observed no changes in cell morphology as observed during 72 h cultivations of WT, CCM pathway, and CCM pathway + sensor-selector strains (Fig. S2).

Taken together, these results show that the sensor-selector confers a growth-selectable phenotype when introduced to CCM-producing yeast without affecting CCM production, growth rate or cell morphology.

pH tuning of the sensor-selector system

One of the major considerations for bulk screenings of large diverse populations of cell factory variants is the rise of false-positives; i.e. cells that do not produce the compound of interest but are still able to thrive under selective conditions.[13,17] This is especially relevant for biosynthetic pathways where production confers a growth burden, as observed for CCM (Fig. 2B). Due to the fact that protonated CCM can passively diffuse across the yeast cell membrane,[12] we expected that one prominent way for cheaters to arise and be isolated from large genetic screens, would be for non-producing fast-growing cells to take up CCM secreted by slow-growing producing cells, resulting in sensor-selector activation. We hypothesized that tuning the pH of the growth medium could control the rise of cheaters. CCM is a weak acid with a pKa of 3.57, hence at pH levels close to this value, a higher fraction of the CCM molecules is uncharged and can diffuse into the cell, whereas at higher pH this fraction is smaller.[28] Indeed, by external administration of CCM to the medium, we found that our previously described CCM sensor-reporter (REV1p-*BenM** in combination with the *yEGFP* reporter gene) shows high induction at pH 4.5, but no induction at pH 6.0 (Fig. S3).

In order to further test this hypothesis, co-cultures of the non-producing CCM sensor-reporter strain ('receiver strain') and the CCM production strain TISNO-11 ('sender strain') were performed. To track the relative abundance of the two strains, the receiver strain was also transformed with a plasmid expressing a gene encoding red fluorescent protein (RFP) from the *TEF2* promoter (Fig. 3A). Co-cultures were performed in mineral medium with ammonium sulfate brought to pH 4.5 or pH 6.0 in three different sender:receiver starting ratios: 0:100, 90:10 and 99:1. At the onset of the co-cultures (t = 0) and after 24 h (t = 24) the fraction of the population consisting of the receiver strain (RFP), and the sensor-controlled reporter gene activity (GFP) in those cells was determined using flow cytometry (Fig. S4A). For the sender:receiver 0:100 culture, we found that the percentage of RFP-

expressing cells to be around 80-90% at both t = 0 and t = 24 (Fig. S4B), probably due to expression fluctuations caused by plasmid-based expression (Fig. S4A). Most, importantly, as inferred from percentages of RFP-expressing cells, we found that population distributions for the three different ratios looked similar independent of the pH applied (Fig. S4B). On the contrary, for cells cultivated at pH 4.5, the CCM-inducible biosensor induced GFP expression 2.4-fold, whereas no induction was observed at pH 6.0 (Fig. 3B). Interestingly, quantification of CCM production in co-cultures pointed out that CCM levels were higher in pH 6.0 (approx. 18-49 mg/L) than in pH 4.5 (approx. 8-20 mg/L) (Fig. 3B). By measuring the final pH we found a severe pH drop for all cultures: cultures started at pH 4.5 reached a final pH of 3.0 ± 0.0, whereas the pH of cultures started at pH 6.0 dropped down to pH 4.9 ± 0.1 (Fig. S4C).

These data show that pH of the growth medium can control the degree of passive diffusion of CCM into non-producing cells, and that pH can provide a simple tuning parameter for bulk screening and selection of production strain libraries.

High-throughput screening of a CCM production strain library

In order to determine whether the sensor-selector is able to enrich for high CCM-producing variants when grown in batch, we created a library of CCM-producing strains using a semi-randomized approach. As a starting strain, we used a strain overexpressing the *TKL1* gene encoding transketolase 1 in addition to the CCM biosynthetic pathway consisting of genes coding for *Pa*AroZ, *Kp*AroY.D and *Ca*CatA.[12] This strain does not produce detectable amounts of CCM.[12] We first integrated the sensor-selector into this strain, and following transformation of an expression cassette harboring *KpAroY.B* and *KpAroY.Ciso* for multi-copy integration into Ty4 sites,[29] we obtained a library of approx. 10^4 transformants (see Methods). Next, these transformants were pre-cultured in bulk, followed by inoculation of three different flasks with selective medium (selection, i.e. 200 mg/L G418), as well as three flasks containing non-selective medium (control) (Fig. 4A). Whereas the control cultures grew to saturation within approx. 30 h, the cultures growing under selective conditions needed more than 48 h to reach saturation (Fig. 4B). We found that the pH of the cultures was maintained

around 6 (see Fig. S5 and Methods). Most importantly, whereas no CCM could be detected in the control cultures for any time point, a steady increase in CCM titer in the selective cultures, up to 275 ± 12 mg/L after 96 h, was observed (Fig. 4C), proving the power of the sensor-selector to robustly, and in high-throughput, enrich for CCM-producing variants.

We hypothesized that before subculturing the cells into selective medium, a proportion of the population would already consist of non-producing, fast-growing cells that have low copy numbers of *KpAroY.B* and *KpAroY.Ciso*. In order to verify this hypothesis, we characterized the starting population by measuring the growth with and without G418 of 89 single colonies isolated at the end of the 48 h pre-culture. From this, we observed a wide variation in growth rates in medium without G418, yet only two isolates were able to grow in the presence of G418 (Fig. S6A). In order to verify whether the 'G418+' phenotype correlated with CCM production, we focused on the isolates from the pre-culture, and measured CCM production for the two G418-positive clones, as well as for five isolates that were not able to grow in the presence of G418 spanning different growth rates (Fig. S6A). Only the two G418-positive clones (isolates 6 and 7) showed CCM production, whereas the remaining isolates (isolates 1-5) did not produce detectable amounts of CCM (Table 1). These data show that right before applying selection, the library indeed consisted of a high proportion of non-producing cells. Contrastingly, scoring G418 phenotypes post-selection, we found all single colonies (90) to be G418-resistant (Fig. S6B). Interestingly, similar to the distribution of growth rates from colonies sampled pre-selection (Fig. S6A), more than 2-fold changes in growth rates were observed between slowest and fastest growing colonies sampled post-selection (Fig. S6B).

We next scaled-up CCM production of the two G418-positive clones (i.e. isolates 6 and 7) in bioreactors in duplicates. In order to reach high titers, additional concentrated medium was spiked as soon as the CO_2 production dropped, indicative of glucose depletion (see Fig. S7). Both strains reached high titers, with isolate 6 reaching on average 1927 mg/L CCM (Fig. 5A), and isolate 7 producing on average 2008 mg/L CCM (Fig. 5B). Isolate 6 displayed an average yield of 11.4 mg CCM/ g glucose with an average productivity of 23.8 mg CCM/ (g glucose.h^{-1}), and isolate 7 showed an average yield of 13.4

mg CCM/ g glucose with an average productivity of 19.9 mg CCM/ (g glucose.h^{-1}) (see Fig. S8 and Methods).

In order to further characterize the two strains, we sequenced the full genomes of these production strains to determine the copy number of the *KpAroY.B-KpAroY.Ciso* expression cassette in each isolate (see Methods). Here we found that copy number correlated with CCM production; with the top-producing isolate 7 bearing seven copies, and isolate 6 displaying six copies (Fig. S9).

In summary, in this study, we designed, characterized and applied a fast and simple sensor-selector system in *S. cerevisiae* that directly couples the concentration of a chemical produced by a single cell to its fitness. Since BenM is part of the LTTR superfamily of small molecule-inducible prokaryotic transcriptional regulators,[30] we envision that the sensor-selector system developed in this study could serve as a blueprint to develop high-throughput synthetic selection systems for a multitude of compounds regulated by LTTR-based transcription factors. Ultimately, this will significantly increase the turn-around time of the design-build-test-learn cycle for engineering future microbial cell factories.

Methods

Strains, chemicals and media

Yeast strains were grown in rich (YPD), Synthetic Complete (SC) or mineral medium with urea (MMU) or ammonium sulfate (MMAS) as a nitrogen source. MMAS was prepared as described previously,[31] for MMU 2.3 g/L urea (Sigma, U1250) was used as a nitrogen source in order for G418 to be effective. Also, the final pH was brought to 4.5 or 6.0. To test the response of non-producing cells to CCM (see further), *cis, cis*-muconic acid (Sigma, 15992) was freshly dissolved in rich or MMAS medium, after which the pH of the medium was brought to 4.5 or 6.0 and filter-sterilized. CCM production strains were grown in mineral medium in order to maintain selection for the destabilized uracil marker. *Saccharomyces cerevisiae* CEN.PK113-5A (MAT*a*, *trp1 his3Δ1 leu2-3/112 MAL2-8^c SUC2*) and CEN.PK113-7D (wild type, *MAT*a *MAL2-8^c SUC2)* strains were obtained from Peter Kötter (Johann Wolfgang

Goethe-University Frankfurt, Germany). CCM production strain TISNO-11 was obtained from an EasyCloneMulti integration of a cassette harboring *KpAroY.B* and *KpAroY.Ciso* as carried out previously,[12] and was in this study found to harbor one copy of this cassette. *Escherichia coli* strain DH5α was used as a host for cloning and plasmid propagation, and was grown at 37°C in Luria-Bertani medium supplemented with 100 µg/mL ampicillin. Phusion® High-Fidelity DNA Polymerase or Phusion U Hot Start DNA Polymerase was used for PCR amplification according to manufacturer's instructions.

Plasmids and strain construction

An overview of plasmids used and constructed in this study is supplied in Supplementary Table 1. The lithium acetate method was used to transform yeast cells,[32] followed by selection of transformants on synthetic drop-out medium (Sigma-Aldrich). For selection of strains transiently expressing *kanMX* and *natMX* markers, 200 µg/mL G418 sulphate (Sigma, G8168) and 100 µg/mL nourseothricin dihydrogen sulfate (WERNER BioAgents, product no. 5.0), respectively, were added to the medium. Genomic integrations were achieved using EasyClone plasmids [31] or marker-free EasyClone plasmids in combination with plasmids containing dominant markers on Cas9 and gRNA plasmids.[33,34] Transformants were genotyped using oligonucleotides described in Supplementary Table 2. The resulting strains are listed in Supplementary Table 3.

The sensor and selector constructs (NotI digested pTS-5 and pTS-7) were integrated into EasyClone sites X-3 and XII-4, respectively, into strain ST2377 and TISNO-11 using pCfB2312 for Cas9 and pTS-9 for gRNA expression, which were subsequently cured off, generating strains DRS16 and TISNO-33, respectively. DRS16 formed the basal strain for the CCM production strain library. ST2377 was described previously[12] and contains the dehydroshikimate DHS dehydratase from *Podospora anserina* (*Pa*AroZ), the D subunit of the PCA decarboxylase from *Klebsiella pneumoniae* (*Kp*AroY.D), and the catechol 1, 2 dioxygenase CDO from *Candida albicans* (*Ca*CatA). As carried out previously,[12] we inserted multiple copies of a cassette containing the genes *KpAroY.B* and *KpAroY.Ciso*, coding for respectively the B subunit and a homolog of the C subunit of the PCA decarboxylase from *K.*

pneumoniae,[23] using the EasyCloneMulti system[25] into DRS16. The library was obtained by adding all the cells post-transformation to a final volume of 25 ml SC-Ura, growing overnight, and storing cells in aliquots at -80 °C. Immediately after transformation, a defined number of cells was plated onto SC-Ura in order to determine the number of transformants as a proxy for library size.

Library enrichment

Ten OD_{600} units of the library (>6600 coverage) were added to a total volume of 25 ml MMU pH 6 (starting OD_{600}= 0.4) in 250 ml-Erlenmeyer flasks and grown for 48 h at 250 rpm, 30°C (pre-culture). After 48 h the pre-culture had reached OD_{600}=7.5. At this stage a small portion of the pre-culture was plated for single colonies, of which 89 random clones were picked for growth rate determination. Biomass was harvested, centrifuged (5 min, 3000 g) and supernatant removed, and used to inoculate three selective cultures (25 ml MMU pH 6 + 200 mg/L G418) as well as three control cultures (25 ml MMU pH 6) to an initial OD_{600}=1.0 and incubated at 30 °C, 250 rpm. The OD_{600} of the six cultures was determined on a daily basis for up to 96 h and every day 1 ml of broth was centrifuged and the supernatant saved for CCM quantification by HPLC and pH measurement. Biomass from the selective cultures was plated for single colonies, after which 90 random clones were picked for growth rate determination.

Growth rate determination

In different experiments the growth rate of yeast strains was determined. In order to assess the response curve to externally applied CCM of different sensor-selector designs, strains were grown overnight in 150 µl YPD per well (pre-culture). The next day, pre-cultures were subcultured 1:150 into either control medium (YPD pH 4.5) or YPD supplemented with CCM (40, 80, 120, 160 or 200 mg/L) at pH 4.5, followed by overnight growth. The next day, saturated cultures were diluted 1:150 into fresh medium with the same composition, with or without addition of 200 mg/L G418. CCM production strains were pre-cultured in SC-Ura medium overnight, and subcultured 1:150 into MMU pH 6.0 with or without addition of 200 mg/L G418. Plates were

sealed with Breathe-Easy® sealing membrane (Sigma Z380059) and incubated at 30 °C in a platereader (BioTek ELx 808) with continuous shaking and OD_{630} measurements every 20 min for 24 h or 72 h. Growth rates were calculated using GATHODE software.[35] For each strain and condition at least three biological replicates were measured.

CCM production assays

For deep-well fermentations, strains were grown overnight in SC-Ura in a microtiter plate. The next day, the OD_{600} of the pre-cultures were measured, and strains were subcultured to starting OD_{600}=1.0 (approximately 10^7 cells/ ml) in 500 µl MMU pH 6.0 in deep-well 96-well plates. After 72 h of incubation at 30°C, 300 rpm, the final OD_{600} was measured, cells were centrifuged (5 min, 3000 g), and the supernatant was used for HPLC quantification of CCM as described previously.[12] Yeast cell morphology was evaluated with a Leica DM4000 B microscope equipped with a DFC 300 FX R2 camera (Leica Microsystems, Wetzlar, Germany).

For bioreactor cultivations, a procedure similar as previously described was followed.[13] Two isolates, TISNO-219 (isolate 6) and TISNO-221 (isolate 7), were pre-cultured in 50 ml SC-Ura in duplicates in 250 ml-Erlenmeyer flasks overnight. The next day, the OD_{600} of each pre-culture was measured, and a portion of biomass was harvested in order to inoculate 1-L bioreactors (Sartorius, Gottingen, Germany) to starting OD_{600} of 1.0. The starting medium of each bioreactor was 500 ml MMU pH 6.0 containing 4% (w/v) glucose. During the cultivation, the stirring speed was maintained at 500 rpm and the dissolved oxygen level was kept above 20% by cascaded mixing of pure oxygen to the air stream (air input flow rate of 0.5 standard liter per minute). The pH was controlled at 6.0 by addition of 7 M NaOH, and the temperature was maintained at 30°C. At regular intervals, samples were withdrawn for OD_{600} measurement, afterwards centrifuged (5 min, 3000 x g, 4°C) and the supernatant kept for HPLC analysis to determine CCM, PCA and glucose levels. During the fermentation, the off-gas CO_2 production was monitored continuously (Thermo Scientific Prima BT MS). Sterile fresh medium (containing 50 or 100 ml of 10X MMU medium) was pulsed after observing a significant drop in CO_2 levels. In total 350 ml 10X MMU was

added.Fermentations were performed for a total period of 5 days. The yield (mg produced CCM / g consumed glucose) for each timepoint was calculated by dividing the CCM concentration (mg/L) by consumed glucose (g/L). The volumetric productivity (mg produced CCM/ (h.L)) for each timepoint was calculated by dividing the CCM concentration (mg/L) by time (h). Subsequently the yield and productivity for each strain replicate was calculated by taking the average of all time points excluding the first batch, i.e. from 31 to 120 h for isolate 6, and from 69 to 120 h for isolate 7 (see Fig. S7).

Flow cytometry analysis of CCM administration assays and co-cultures

To test the effect of external CCM on biosensor-reporter activation, our previously described strains MeLS0138 (reporter-only) and MeLS0284 (biosensor-reporter) were subjected to growth in MMAS pH 4.5 or pH 6.0, +/- supplementation of 200 mg/L CCM. After 24 h of growth the cells were diluted in PBS and analyzed by flow cytometry (MACSQuant® Analyzer 10) with a blue laser (488 nm) to detect yEGFP, FCS files were incorporated into FlowJo, and per replicate the mean GFP intensity of 10 000 single-cell events was determined.

To determine the degree of biosensor-reporter activation in non-producing cells, co-cultures of sender strain TISNO-11 and receiver strain TISNO-31 were set up. Three single colonies of each strain were grown overnight in 3 ml SC-Ura-His-Leu-Trp. The next day the OD_{600} was measured, and co-cultures were started in MMAS pH 4.5 or pH 6.0 with a starting OD_{600} = 0.2. For each medium, sender and receiver strain were mixed in 0:100, 90:10 and 99:1 ratios in triplicates. After approx. 24 h cultures were diluted in PBS and analyzed on a BD Biosciences Aria (Becton Dickinson) with a blue laser (488 nm) to detect yeGFP and a yellow green laser (561 nm) to detect mKate2. FCS files were incorporated into FlowJo, and per replicate the mean GFP intensity of 10 000 RFP+ cells was determined after gating for single-cell events (see Fig. S4A).

Genomic DNA extraction

The genomic DNA (gDNA) of TISNO-11, TISNO-219 (isolate 6) and TISNO-221 (isolate 7) was extracted using the Yeast DNA Extraction Kit

(Thermo Fisher) and final gDNA was eluted in 10 mM Tris-HCl, pH 8.0. The final concentration of each sample was measured by the Qubit 2.0 Fluorometer and Qubit dsDNA HS assay Kit (Thermo Fisher), and afterwards adjusted to a final concentration of 2 ng/μL in 50 μL for genomic library preparation. The genomic libraries were generated using the KAPA HyperPlus Kit (Roche).

Whole genome sequencing and copy number analysis.

Illumina reads were aligned to the reference genome of *S. cerevisiae* CEN.PK113-7D (www.ncbi.nlm.nih.gov/assembly?LinkName=bioproject_assembly_all&from_uid=52955) and to the integrative cassette sequences using bwa (PMID 19451168). Copy numbers for integrated genes (*KpAroY.B* and *KpAroY.Ciso*) were determined by comparing the GC-normalized read coverage of the integrated genes with the average coverage of the *S. cerevisiae* autosome. The read depth was calculated using the mpileup command from samtools [36].

Associated content

Supporting Information: Supplementary Figures and Supplementary Tables (PDF).

Author information

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Author contribution

TS, MKJ and JDK conceived this project. TS and DRS designed the experiments and carried out experimental work. FA performed whole-genome DNA sequencing and NGS data analysis. SS helped designing and carrying out repeated batch fermentations. MLS and JZ provided strains and materials. TS, DRS, MJK and JDK analyzed and interpreted the data. TS and MKJ wrote the paper, all authors assisted in this process.

Notes

JDK has a financial interest in Amyris, Lygos, Constructive Biology, and Demetrix.

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Figure legends:

Figure 1: Characterization of biosensor designs for growth-coupled selection

(**A**) Four different strains harboring the selector construct (CYC1p_*BenO-kanMX*) and no, high (TEF1p), low (REV1p) expression of wild-type BenM, or low expression (REV1p) of a BenM triple mutant (BenM*). Cells expressing either of these four designs were pre-cultured in rich medium pH 4.5 with or without 200 mg/L CCM, followed by subculturing into medium with the same composition with or without addition of 200 mg/L G418. Growth was monitored during 24 h. Growth rates are indicated in the heatmap as mean ± standard deviation from three (n = 3) biological replicates. (**B**) The optimal sensor-selector design was tested in detail to determine the dose-response curve both in the presence and absence of G418. Growth rates are shown as mean ± standard deviation from three (n = 3) biological replicates.

Figure 2: Biosensor-selector does not compromise cell factory performance

(A) A 3-step heterologous CCM pathway was built into yeast, comprising single-copy expression of *Pa*AroZ, *Kp*AroY.D, and *Ca*CatA[21] and multi-copy integration of a cassette containing genes coding for *Kp*AroY.B and *Kp*AroY.Ciso. In addition, Tkl1 is overexpressed. (**B**) The sensor-selector was

integrated into the CCM-producing strain (CCM pathway + sensor-selector) and its growth was compared to the baseline CCM-producing strain (CCM pathway) and wild-type CEN.PK. All three strains were cultured in mineral medium with urea pH 6.0 with or without 200 mg/L G418, and growth was monitored during 24 h. Growth rates are indicated in the heatmap as mean ± standard deviation from three (n = 3) biological replicates.(**C**) CCM titer was measured for the two CCM production strains after 72 h of cultivation in mineral medium with urea pH 6.0. Means and standard deviations from four (n = 4) biological replicates are shown. ns = not significant as evaluated by *t*-test ($p>0.05$).

Figure 3: Safe-guarding selection from cheaters by pH control

(**A**) Outline of experimental set-up. A CCM-producing strain (sender) was co-cultured with a biosensor-reporter strain (receiver, no CCM production) in different inoculum ratios in mineral medium with ammonium sulfate at pH 4.5 or pH 6.0. The receiver strain harbored a plasmid expressing RFP in order to identify biosensor cells by flow cytometry. (**B**) Biosensor activity and CCM production in co-cultures at the onset (t = 0) and after 24 h (t = 24) of culturing. Fold induction indicates the fold-change difference in mean GFP fluorescence intensity of co-cultures relative to the sender:receiver 0:100 co-culture with the same pH of 10 000 RFP^+-cells per co-culture. Fold induction and [CCM] are shown as mean ± standard deviation from three biological replicates (n = 3) per co-culture.

Figure 4: Pathway evolution using synthetic selection

(**A**) Experimental outline of multi-copy AroY library screening using the sensor-selector. The library was based on transformation of a construct consisting of *KpAroY.B* and *KpAroY.Ciso* targeted to Ty4 sites into a strain containing the 3-step CCM production pathway and the sensor-selector. Library screening consisted of pre-culturing, followed by subculturing into mineral medium with urea pH 6 (control) and into mineral medium with urea pH 6 + 200 mg/L G418 (selection). (**B**) OD_{600} values of strain library cultivations with (selection) or without (control) G418 supplemented to the medium. (**C**) CCM titers from library cultivations. For both (B) and (C), means

and standard deviations from three (n = 3) biological replicates per cultivation condition are shown.

Figure 5: Bioreactor fermentations of selected CCM-producing strains
Biomass units (OD_{600}), and titers of CCM and PCA from a repeated batch fermentation of isolate 6 (**A**) and isolate 7 (**B**) during a 120 h cultivation in mineral medium with urea pH 6.0. For both (A) and (B), the mean values and individual data points from two (n = 2) biological replicates are shown. See Figure S7 and Methods for details.

Table 1: Overview of growth rates (see Fig. S6A), phenotype in selective medium (mineral medium with urea pH 6.0 + 200 mg/L G418) and CCM production in seven library isolates.

isolate	phenotype	growth rate mineral medium (h^{-1})	CCM titer (mg/L)
1	G418-	0.34	0.3 +/- 0.1
2	G418-	0.29	0.3 +/- 0
3	G418-	0.33	0.3 +/- 0
4	G418-	0.19	0 +/- 0
5	G418-	0.21	0 +/- 0
6	G418+	0.11	379 +/- 3
7	G418+	0.14	470 +/- 41

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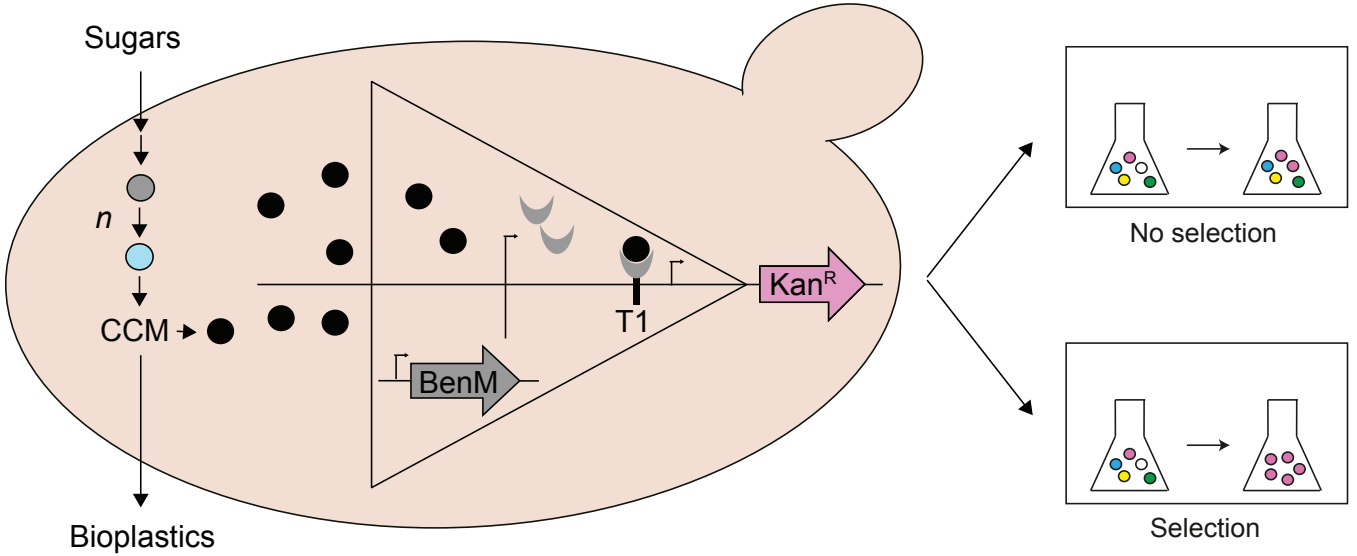
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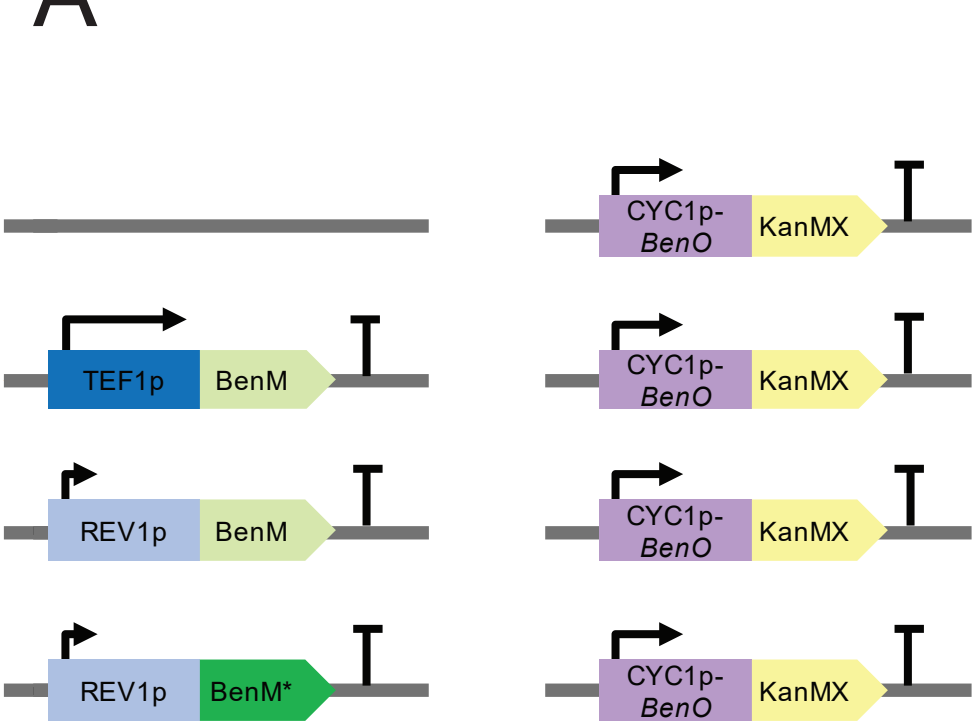
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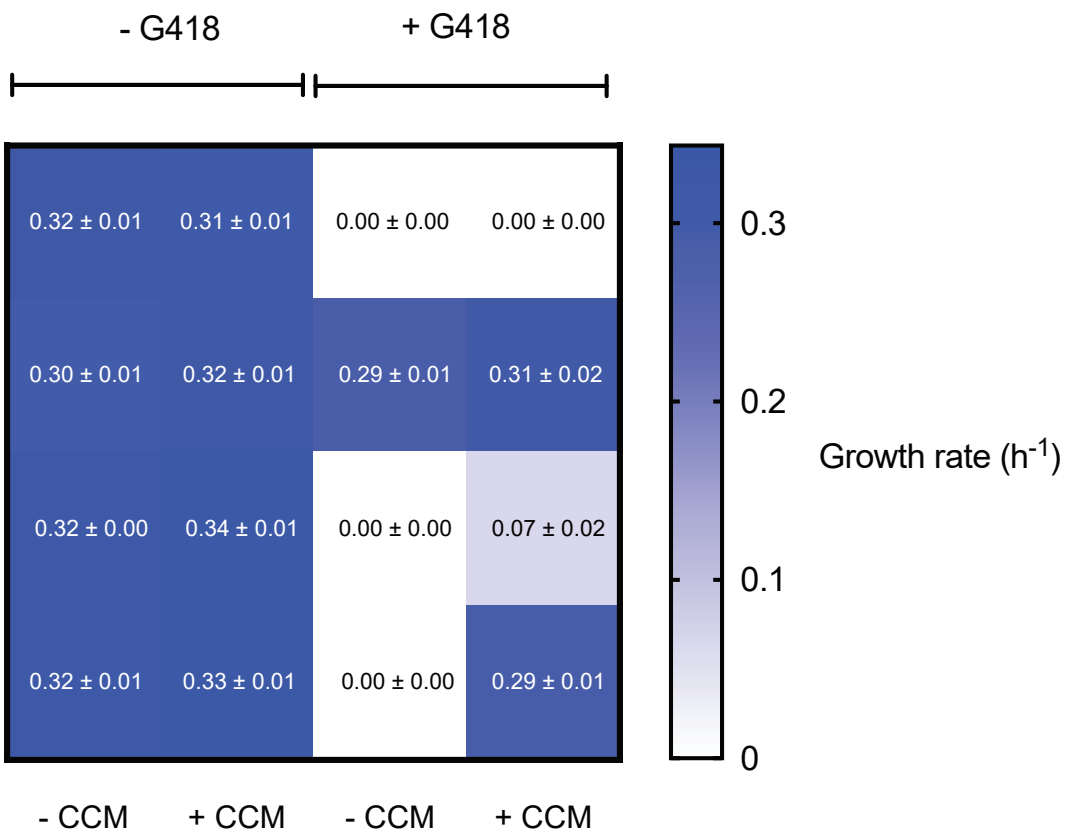
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+ G418
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0.29 ± 0.01
0.31 ± 0.02
0.32 ± 0.00
0.34 ± 0.01
0.00 ± 0.00
0.07 ± 0.02
0.32 ± 0.01
0.33 ± 0.01
0.00 ± 0.00
0.29 ± 0.01
- CCM
+ CCM
- CCM
+ CCM
0.3
0.2
0.1
0
Growth rate (h-1)

B


REV1p
BenM*
CYC1p-
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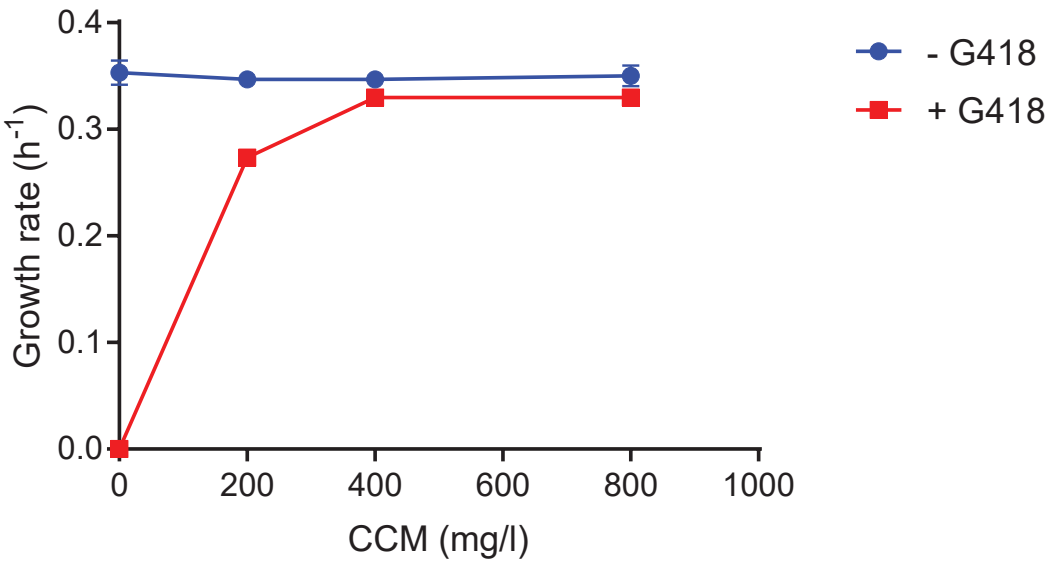
KanMX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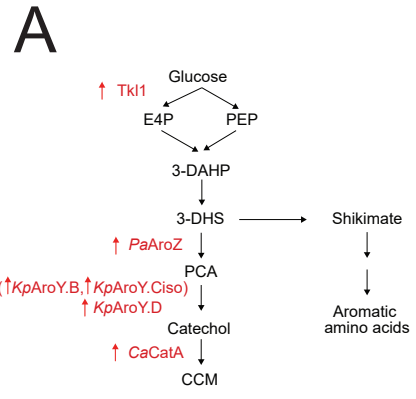
600
800
1000
CCM (mg/l)
- G418
+ G418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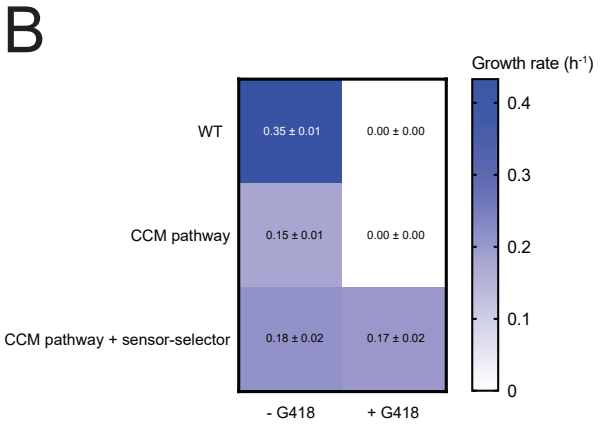
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Growth rate (h-1)
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0.3
0.2
0.1
0
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CCM pathway
CCM pathway + sensor-selector
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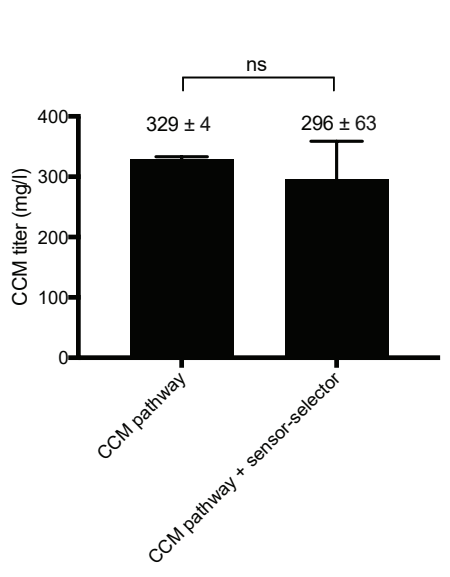
- G418
+ G418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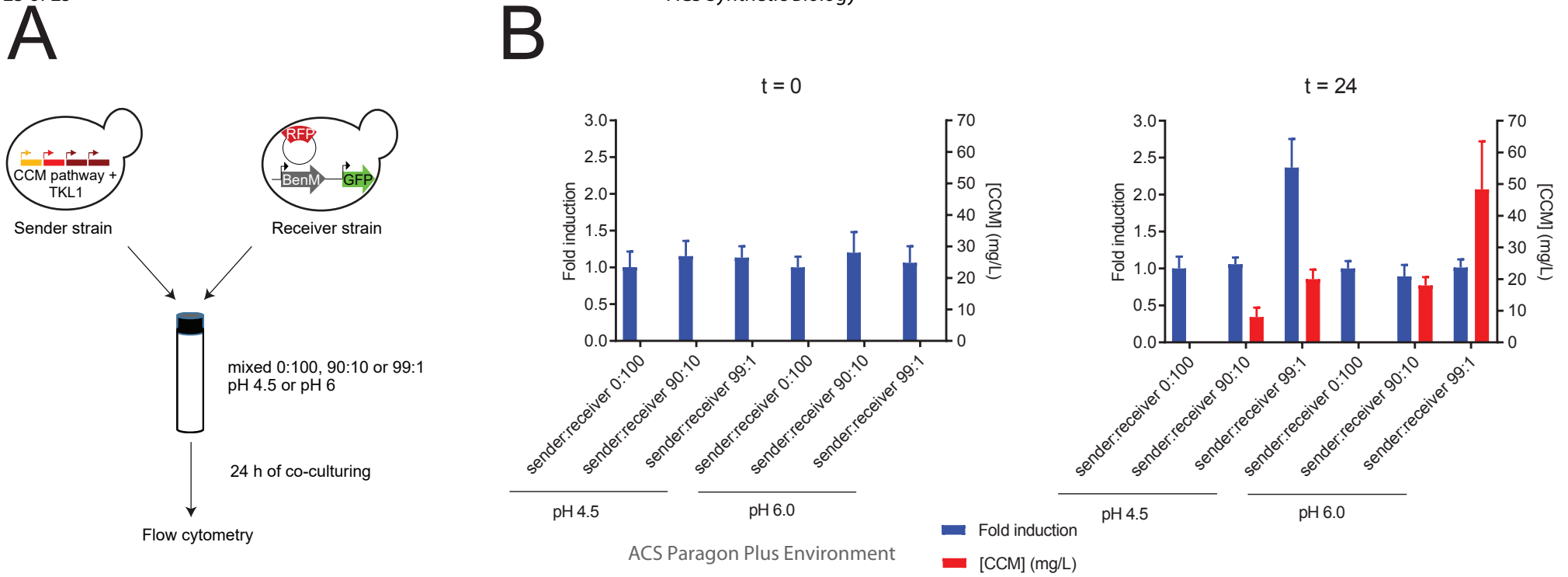
CCM pathway
CCM pathway + sensor-selector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CCM pathway + TKL1
Sender strain
RFP
BenM
GFP
Receiver strain
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pH 4.5 or pH 6
24 h of co-culturing
Flow cytometry
B
t = 0
t = 24
Fold induction
[CCM] (mg/L)
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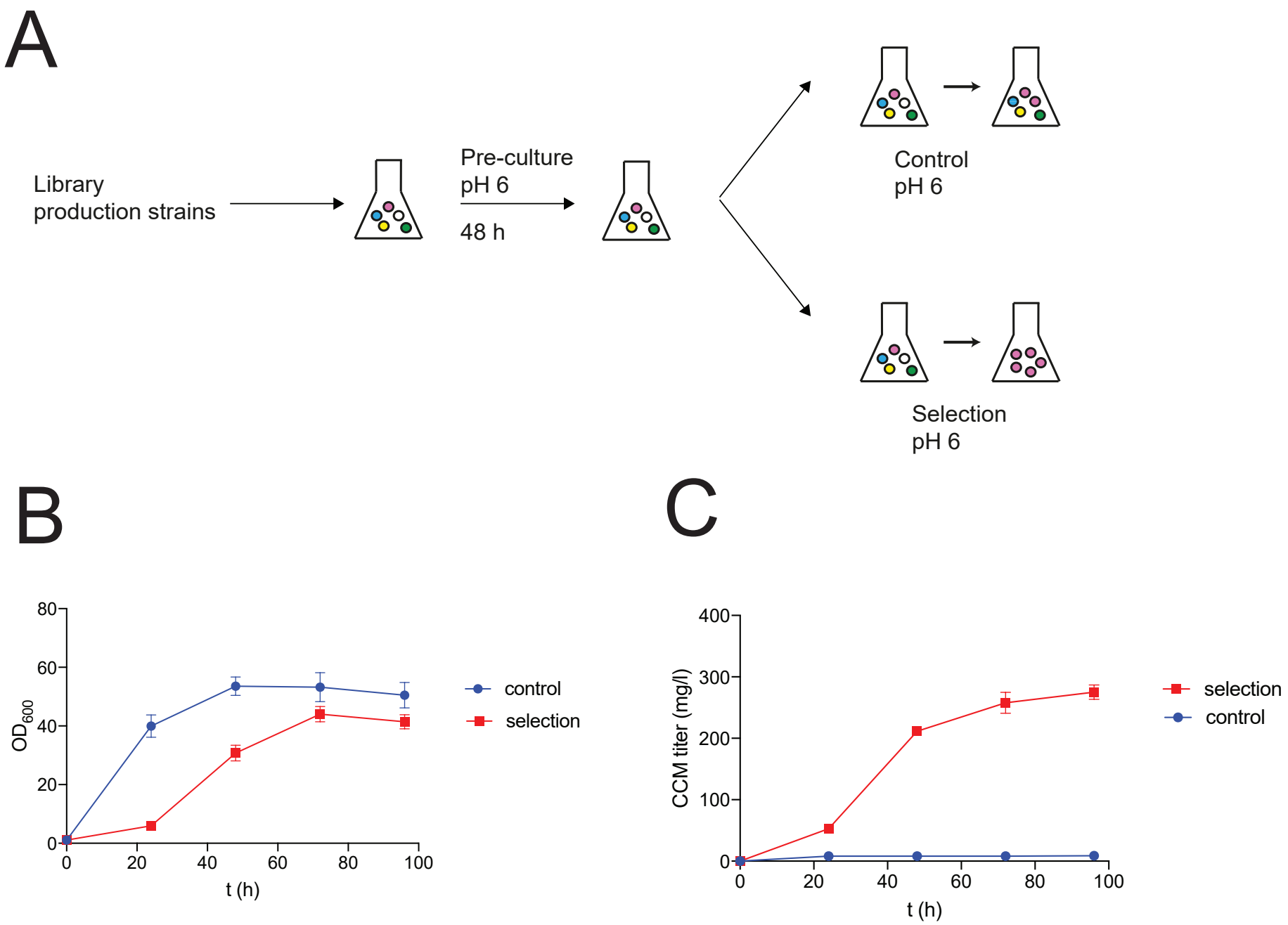
Fold induction
[CCM] (mg/L)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48 h
Control
pH 6
Selection
pH 6
B
OD600
t (h)
control
selection
C
CCM titer (mg/l)
t (h)
selection
control