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Short Communication

Laccase is a multitasking protein for synthetic gene circuits in the yeast *Saccharomyces cerevisiae*

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ABSTRACT

Laccase is a multicopper oxidase enzyme that oxidizes a variety of substrates, including polyphenols and polycyclic aromatic hydrocarbons (PAHs). It catalyzes the four-electron reduction of molecular oxygen that results in the production of water as a by-product. Thus, laccase can play an important role in environmental care. Previously, we have successfully expressed *Trametes trogii* laccase (TtLcc1) in the yeast *Saccharomyces cerevisiae*. In this work, we have expressed in yeast another laccase, LacA from *Trametes* sp. AH28-2, and tested its function on PAHs. Yeast cells engineered to produce the two laccases performed efficient PAH degradation. Both TtLcc1 and LacA led to the construction of spatiotemporal fluorescence-pulse generators when combined with a benzoate/salicylate yeast biosensor in a two-population system. Moreover, laccases returned a visual output signal in yeast synthetic circuits—upon reacting with ABTS (2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)). Thus, in *S. cerevisiae*, laccases are a powerful alternative to fluorescent reporter proteins.

1. Introduction

Laccases (EC1.10.3.2) are multicopper oxidase enzymes naturally secreted by a variety of organisms such as bacteria, insects, plants, and fungi. They have the capability to degrade complex organic molecules such as lignin and hydrocarbons. Therefore, they represent key components of synthetic circuits aimed at reducing environmental pollution. Fungal laccases from *Trametes* species have been deeply studied because of their high thermal stability. Moreover, they have been expressed in heterologous organisms to enhance their production and meet industrial demands [1]. We have previously described the optimal conditions for expressing *Trametes trogii* laccase (TtLcc1) in the yeast *Saccharomyces cerevisiae* [2] and employed the engineered yeast cells to degrade a mixture of complex hydrocarbons.

Synthetic gene circuits are, usually, completely realized into single cells, even though an elevated number of exogenous genes can increase the metabolic burden on the host organism considerably [3]. As a consequence, cell growth rate slows down and the performance of the same genetic circuits is reduced [4]. An alternative network architecture relies on a cell *consortium*, where a circuit is distributed among a variable number of populations of the same chassis. They carry out different tasks and are wired to each other via the exchange of small molecules [5]. This design was shown to be particularly appropriate to build Boolean circuits [6] and feed forwards loops (FFLs) [7]. Incoherent type 1 FFLs find application as pulse generators, i.e., they return an output signal limited in time or space [8].

In this work, we coupled laccase-expressing yeast strains with a biosensor strain responding to benzoate and salicylate [9], i.e., two metabolites from the degradation of PAHs (polycyclic aromatic hydrocarbons). The overall two-population system represents a novel design for cell consortia where the wiring signal between two synthetic strains is not produced by one strain directly but arises from the interaction of laccase with a specific substrate. Thus, the circuit is switched ON only upon the addition of hydrocarbons in the cell culture and behaves as a spatiotemporal pulse generator. Furthermore, we made laccase expression chemical inducible and showed how to leverage the interaction between laccase and ABTS (2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) to produce a visual output, which can replace fluorescence, for the screening of many engineered yeast colonies. Hence, laccase (together with a proper substrate) also acts as a reporter protein—the output of synthetic gene circuits.

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2. Materials and methods

2.1. Plasmid construction

The plasmids built in this project (listed in Table S1) derive from the pRSII4XY collection—X representing the vector kind (integrative, centromeric, or episomal); Y an auxotrophic marker—available at Addgene (a gift from Stephen Haase) [10].

Laccase-expression-plasmid construction. We dealt with three laccases: TtLcc1 from *Trametes trogii* [11], LacA from *Trametes* sp. AH28-2 [12], and NS/Mg Lcc1-2 from *Termitomyces* sp [13]. *Bam*HI-NS/Mg Lcc1-2/TtLcc1/LacA-XhoI fragments (laccases with their own native secretory signal) were amplified from plasmids via PCR and then inserted into a cut-open acceptor vector pRSII4XY-pGPD/pTEF2-BamHl-ATG-rnd (a short random sequence)-TAA-XhoI-CYC1t (digested with *Bam*HI (NEB-R0136V) and XhoI (NEB-R0146V)) via Gibson isothermal assembly [14]. NS/Mg Lcc1-2 and LacA fused to different signal peptides (from TtLcc1—tt1—or the yeast α-factor) were expressed by a different vector: pRSII403-pGPD-ATG-tt1/α-factor-*Bam*HI-rnd-TAA-XhoI-CYC1t. TtLcc1 expression cassette with pCUP1/pGAL1/pCYC1min_tetOp/2xlex2-Op_pCYC1core and CYC1t was realized via Gibson method inside a cut-open pRSII40Y (digested with Acc65I (NEB-R0599S) and SacI-HF (NEB-R3156S)).

Other-plasmid construction: the LexA-ER-VP64 (TetR-NLS) fragment was inserted, together with DEG1t-pCYC1noTATA (pTEF2) and CYC1t, into a cut-open pRSII40Y (digested with Acc65I and SacI-HF) via Gibson method. crRNA expression cassette (pSNR52-crRNA-SUP4t) on pRSII403 (used in the knockout circuit) was constructed via Gibson method.

All PCRs were touchdown PCRs that were carried out by using Q5 High-Fidelity DNA Polymerase (NEB-M0491S). The PCR products were purified from agarose gel via AxyPrep DNA extraction kit (Axigen-AP-GX-250). PCR products and the cut-open vector were added into 15 μL Gibson isothermal mixture to obtain a final volume of 20 μL. These 20 μL were placed at 50 °C for 1 h. 5 μL were used to transform DH5α (Life Technology—18263–012) *Escherichia coli* cells (30 s heat shock at 42 °C) [15]. New plasmids were checked via Sanger-sequencing at Genewiz Inc., Suzhou (China).

2.2. Yeast transformation

S. cerevisiae strain CEN.PK2-1C (MATa; his3Δ1; leu2-3_112; ura3-52; trp1-289; MAL2-8c; SUC2), Euroscarf-30000A (Johann Wolfgang Goethe University, Frankfurt, Germany)—termed byMM584, and *S. Cerevisiae* synthetic chromosome V (syn V)—called byMM562—were used as chassis for all strains engineered in this work. Strain byMM1159 hosting the benzoate/salicylate biosensor comes from a previous publication of ours [9].

Yeast transformation via the lithium acetate-thermal shock method [16] required linearizing 5 μg of integrative plasmids within the auxotrophic marker. Lithium acetate, polyethylene glycol (PEG), dimethyl sulfoxide (DMSO), and salmon sperm DNA (ssDNA) were employed to enhance the plasmid uptake by the cells. Yeast transformation with centromeric and episomal plasmids demanded 800 ng of plasmid DNA. The heat shock was conducted at 42 °C for 15 min. Yeast cells were cultivated on solid synthetic selective media (2 % glucose, 2 % agar) for a maximum of 4 days at 30 °C. All strains utilized and constructed in this study are listed in Table S2.

2.3. Media and reagents

Synthetic defined selective media lacking one nutrient, e.g. SD-URA, were adopted in yeast transformation. If supplied with 2 % galactose and 1 μM β-estradiol, they were used to switch ON the Cre-loxP system. Bushnell Hass (BH) synthetic broth M350 (pH 4.5 ± 0.1) supplied with a specific carbon source (e.g., anthracene) was used for activity tests on PAH degradation by TtLcc1. PAH stock solutions preparation: 6 mM anthracene: 106.938 mg anthracene (CAS number: 120-12-7) were dissolved in 100 mL 99 % ethanol (analytical level); 100 mM naphthalene (Nap): 128.174 mg Nap (CAS number: 91-20-3) were mixed with 10 mL ethanol; 2.37 mM benzo(b)fluoranthene (BbF): 5.98 mg BbF (CAS number: 205-99-2) was dissolved in 10 mL ethanol. SD plate containing 1 mM $CuSO_4$ (Innochem-Cat: 7758-98-7) and 0.5 mM ABTS (CAS number: 9003-99-0) used for laccase activity characterization were prepared as in Ref. [17]. Citrate pH 5.6 preparation: 12.514 g sodium citrate and 10.384 g citric acid were dissolved in 800 mL ddH_2O, pH was adjusted with HCL and NaOH, and finally, the solution was added to 1000 mL ddH_20. 10 mM benzoate/salicylate stock solution: 14.41/16.01 mg sodium benzoate/salicylate (sodium benzoate: CAS number: 532-32-1; sodium salicylate: CAS number: 54-21-7) were dissolved into 10 mL ddH_2O and filtered with a 0.22 μm membrane.

2.4. Flow cytometry

PAH degradation on plate: strains were grown at 30 °C on SDC (synthetic defined complete medium)—with or without copper—supplied with 100 μM anthracene and 0.5 mM ABTS. Some strains were selected and dissolved in 300 μL ddH2O for FACS measurement. PAH degradation characterization in solution: benzoate/salicylate biosensor strains were cultivated at an initial temperature of 30 °C in SDC solution for 16 h. Subsequently, cell solutions were 1:20 diluted in the SDC and added to 1 mL cell-free solution containing metabolites from PAH degradation. Biosensor induction took usually 12 h. 5 μL of this cell culture were then mixed with 300 μL of ddH_2O. Fluorescence intensity evaluation was performed with a BD FACSVerse machine (488 nm; FITC filter of 527/32 nm). Fluorescent beads (BD FACSuite CS&T Research beads 650621) were used to adjust the FITC voltage in different experiments: the difference in peak values between two consecutive beads measurements should not exceed 5 %. Ten thousand cells were collected in each experiment. At least three independent experiments were carried out before calculating fluorescence mean value and standard deviation [18]. Statistical analysis was conducted using two-sided Welch's *t*-test or one-way ANOVA.

2.5. Laccase activity

Laccase activity was quantified by using ABTS as a substrate. Strains expressing laccase were grown in plates supplemented with 1 mM $CuSO_4$ and 0.5 mM ABTS. They were incubated at 30 °C for a maximum of 96 h. Changes in color were photographed with a NIKON digital camera D3200. Laccase activity measurement: strains were cultured in SDC or SD-URA (used for centromeric/episomal vectors) solution for 96 h at 30 °C and 240 RPM. 2 mL cell culture were sampled every 12 h. They were centrifuged at 4000×*g* for 5 min. Cell debris was removed to have a supernatant containing only laccase. 300 μL supernatant was mixed with 15 μL 100 mM ABTS, 15 μL 1 mM $CuSO_4$, and 2670 μL citrate buffer [2]. Laccase activity was measured at an absorbance wavelength of 420 nm via a double beam UV/Vis Hitachi UH5300 spectrophotometer or DS-11 Spectrophotometer/Fluorometer Series. The change of absorbance was determined within the first 3 min. Laccase activity was calculated according to the following formula [19]:

$$\text{Laccase activity (U / L)} = \frac{\Delta E\, V_t}{\epsilon\, d\, V_s\, t}$$

Where ΔE is the changes of absorbance in the first 3 min, V_t is the total volume of the reaction solution (3 mL), ϵ represents the Molar extinction coefficient (36 mM^{-1} cm^{-1}), d is the optical path (1 cm), V_s is the volume of crude laccase (0.3 mL), and t is the reaction time.

One unit (U) of laccase activity is the amount of the laccase required to catalyze the conversion of 1 μmol of ABTS within 1 min.

3. Results and discussion

3.1. Expression of a new laccase in S. cerevisiae

Initially, we tried to enlarge the set of working laccases by analyzing LacA from *Trametes* sp. AH28-2 and NS/Mg Lcc1-2 from *Termitomyces* sp. TtLcc1 was used as a positive control. As in the work by Asemoloye and co-authors [2], laccase DNA sequences were codon-optimized for expression in the budding yeast and placed between the strong *GPD* promoter (pGPD) and the *CYC1* terminator (CYC1t) on episomal or centromeric, and integrative vectors [2,10] (all DNA sequences used in this work are reported in Table S3). Moreover, each laccase was secreted to the extracellular matrix via three different secretory signal peptides: the native one, that from TtLcc1 (tt1), and the *S. cerevisiae* α-factor. As reported by Mateljak and co-authors [20], different signal peptides determine higher or lower secretion in yeast. Yeast transformants that produced laccase were identified by the color changes on solid plates supplied with $CuSO_4$ (1 mM) and ABTS (0.5 mM). Laccase activity calculation was carried out by using ABTS as substrate [2]. LacA showed the highest activity—comparable to that of TtLcc1—in conjunction with its native secretory signal upon expression from an integrative plasmid (see Figs. S1A–B). In contrast, NS/Mg Lcc1-2 was not functional (see Fig. S1C). We tried, then, to improve the activity of the three LacA variants through the SCRaMbLE (Synthetic Chromosome Rearrangement and Modification by LoxP-mediated Evolution) technique [21] (see Fig. S2A). Only the weakest among them, αLacA (i.e., LacA carrying the *S. cerevisiae* α-factor), resulted stronger in the newly generated chassis. Its activity, though, was still weaker than that of TtLcc1 expressed in the CEN.PK2-1C genetic background (see Figs. S2B–D).

3.2. A pulse generator based on laccase-driven PAH degradation

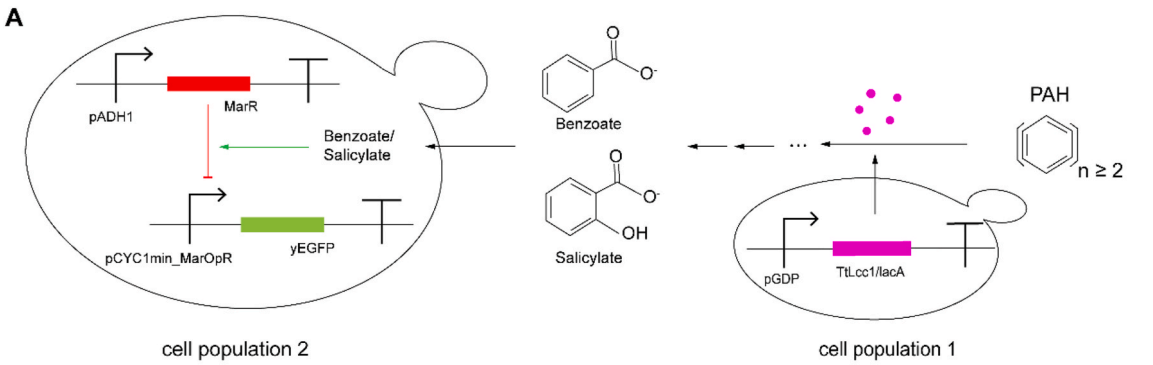
Next, we compared the ability of TtLcc1 and LacA, secreted from yeast cells, to degrade polycyclic aromatic hydrocarbons. To monitor the process, we coupled the laccase-expressing strain with another engineered as a benzoate/salicylate biosensor [9]. In this way, we realized the yeast two-population system in Fig. 1A. Population 1 expresses laccase (TtLcc1 or LacA) constitutively under pGPD. When complex hydrocarbons are supplied to the growth medium, laccase starts their degradation into smaller molecules. Population 2 hosts a benzoate/salicylate biosensor organized into two transcription units (TUs). One is the reporter part, which expresses the yeast enhanced green fluorescent protein (yEGFP) under the synthetic repressed promoter pCYC1min_marOpR (see Fig. S3A). The other is the receptor part, which constitutively expresses the bacterial MarR repressor. In the absence of benzoate/salicylate, MarR binds its DNA binding site (marOpR) and represses transcription from pCYC1min_marOpR (only basal fluorescence is emitted by population 2). In contrast, when benzoate/salicylate are supplied to the growth medium, they diffuse into population 2 cells, bind MarR, and prevent it from further binding the DNA. Consequently, yEGFP production increases (see Fig. S4A). Overall, in the yeast two-populations system, PAHs are degraded into smaller molecules by TtLcc1/LacA secreted by population 1. Then, population 2 returns a fluorescent signal when benzoate/salicylate are among the PAH metabolites in the growth medium (see Fig. 1B). We chose anthracene as a first substrate for laccase. The molecule of anthracene contains three aromatic rings (see Fig. S4B) and it is known to be degraded into benzoate and salicylate by the white-rot fungus *Armillaria* sp. F022 [22]. Upon exposure to anthracene, our two-population system worked as a fluorescence-pulse generator over the degradation time (i.e., population 1 culture time—see Fig. S4C) since yEGFP was expressed only when benzoate or salicylate were present in the cell culture—i.e., for a limited amount of time before being degraded on their turn by laccase. The fluorescence pulse has a lower duration with TtLcc1 (from 6 to 36 h) than with LacA (6–72 h), which implies a stronger metabolic activity of TtLcc1 on anthracene and its second metabolites. The strain expressing TtLcc1 was cultivated in BH solution [2] where the only carbon source was 100 μM anthracene. In contrast, the LacA-expressing strain needed a sugar supplement to grow (due to the weak activity of LacA that demanded too long to generate carbon supplies from anthracene degradation) and was cultured in SDC solution (with 2 % glucose) plus anthracene. We think that the presence of glucose (i.e., a second substrate for LacA with a single aromatic ring) was the cause of the delay in anthracene degradation (see also Fig. 3D below). In these experiments, the induction time (i.e., population 2 culture time) was limited to 12 h (see Fig. S4C). We performed further tests by setting the maximum anthracene degradation time by TtLcc1 and LacA to 36 and 66 h, respectively, and increasing cell population 2 induction time—i.e., the culture time in the supernatant containing the products of anthracene degradation—to 24 h (see Figs. S4D–E). To quantify the response of our two-population system, we calculated, after every fluorescence measurement, the ON/OFF ratio, i.e., the quotient between the fluorescence level in the presence and absence of anthracene (and its metabolites).

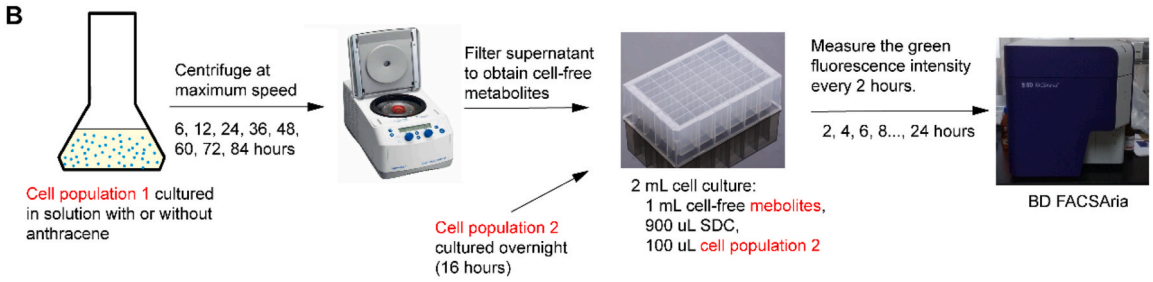
Fig. 1C shows that the yeast two-population system behaves as a pulse generator also over the induction time. Moreover, the system expressing TtLcc1 reached the peak of the pulse via a double 12-h incubation, i.e., the growth time of cell population 1 and 2 were identical. In contrast, LacA demanded 60–66 h for anthracene degradation and 12 h again for the biosensor induction. The peak region of the two impulses is detailed in Fig. 1D as a function of the degradation time, when the induction time is fixed to 12 h.

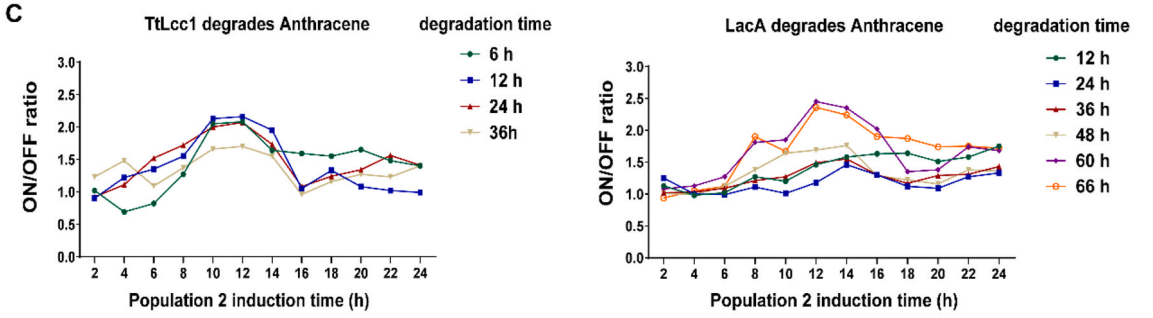
Finally, we tested the two-population system on other PAHs, namely naphthalene (Nap)—which consists of two aromatic rings—and benzo (b)fluoranthene (BbF), whose molecular structure is bigger than that of anthracene and contains four aromatic rings (see Fig. S4B). Nap is reported to be degraded into benzoate/salicylate by the white rot fungus *Armillaria* sp. F022 [23], whereas BbF by laccase from *Pleurotus ostreatus* [24]. We used TtLcc1 only on these two PAHs since its performance in anthracene degradation was higher than that of LacA. Therefore, we incubated cell population 1 in BH solution supplied with Nap/BbF (100 μM) for a time varying between 6 and 36 h and set cell population 2 induction time to 12 h. As shown in Fig. 1E, both Nap and BbF confirmed the pulse generator behavior of the two-population system, with the peaks reached after 12 h of degradation time.

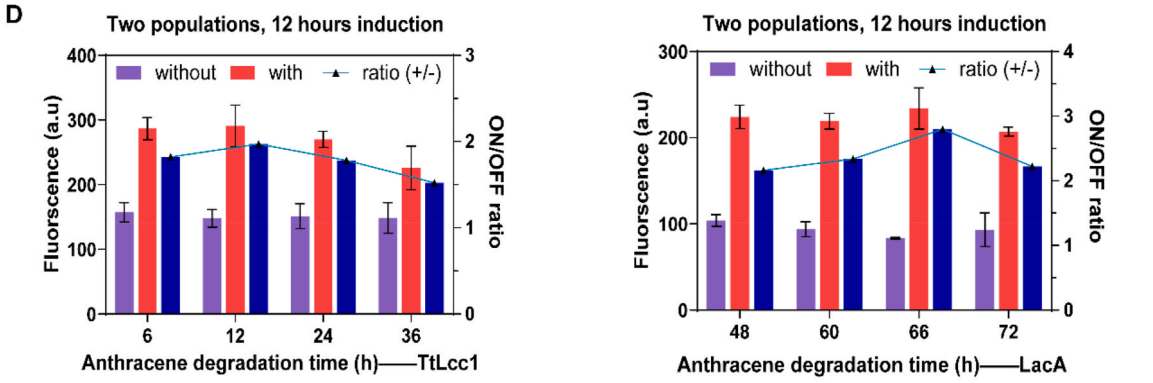
3.3. Anthracene degradation on a solid medium

We tested PAHs degradation and detection on a solid medium via our two-population system. We made use of TtLcc1 and anthracene only. We grew population 1 and 2 on a single solid plate that was supplemented with 100 μM anthracene (the control plate did not contain anthracene), 0.5 mM ABTS, and 1.0 mM $CuSO_4$—see Fig. 2A. The central strain is population 1 (expressing TtLcc1), whereas the surrounding strains are population 2 (benzoate/salicylate biosensors). We studied how the distance between the two populations affected fluorescence expression. As shown in Fig. 2B, fluorescence intensity increased with time at least up to 36 h. The fluorescence ON/OFF ratio (with/without anthracene) however, followed a different trend due to a rather high noise at large time points. Overall, a shorter distance between the two cell populations determined a quicker time to achieve the highest ratio (1 mm: 12 h; 5 mm 24 h; 15 mm: 36 h; 25 mm: 48 h—it should be noted, though, that the highest ratio at 25 mm corresponded to 36 h where, however, the background looks much lower than at the other time points). The background noise also increased with time, which is consistent with the above results (see Figs. S4D–E). Besides glucose, we thought that copper also contributed to the high background noise since it can bind MarR and increase fluorescence emission [9]. To test the copper influence on fluorescence expression, we compared control plates containing different concentrations of copper. Only 1 mM copper determined a high increase in the fluorescence noise at long incubation time (Fig. S5). Our two-population system carried out anthracene degradation and benzoate/salicylate detection on plate. Thus, it reproduced the









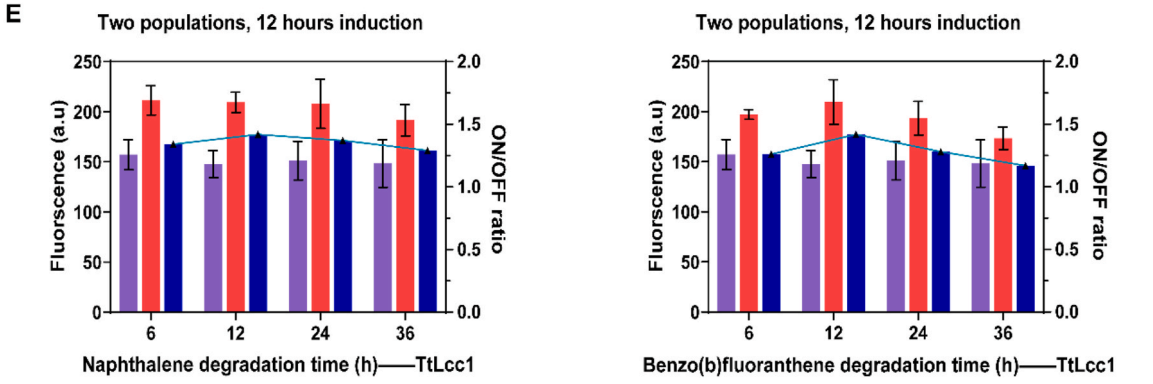


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Fig. 1. PAHs degradation based on a yeast two-population system. (**A**) Yeast two-population system diagram. 'n' refers to the number of aromatic rings. (**B**) Operational procedure. Population 1 is first grown in a solution containing PAHs—SDC or BH only in the control—for a variable amount of time. The solution is centrifuged and filtered to remove population 1 cells. The supernatant, which hosts metabolites from PAH degradation, is mixed with the SDC solution in which population 2—the biosensor—was cultured. The new cell solution is incubated at 30 °C and 240 RPM for a variable number of hours before measuring fluorescence via a FACS experiment. If benzoate/salicylate were among the PAH metabolites, a fluorescence signal is detected. (**C**) The ON/OFF ratio (fluorescence with/without anthracene) at different degradation times over a 24 h induction time. Both diagrams gave the highest ON/OFF ratio when the induction time of population 2 was 12 h. The optimal time interval for PAH degradation by TtLcc1 was 6–24 h, whereas it went from 60 to 66 h for LacA. (**D**) The peak region (in terms of the ON/OFF ratio) as a function of the degradation time for a single induction time corresponding to 12 h. Degradation and induction time were chosen from the results in (C). (**E**) Both Nap and BbF were degraded by TtLcc1 to produce benzoate/salicylate, which is reflected by the pulse in yEGFP expression. ON/OFF ratio in (D) and (E) indicated the optimal time points for benzoate/salicylate generation. The benzoate/salicylate production reached the highest level when PAHs were incubated with TtLcc1 for 12 h, and 66 h with LacA.

pulse-generator trend on a solid medium, with the peak of the pulse moving away from the plate center (population 1) with increasing incubation time.

3.4. Chemical-inducible PAHs degradation

To create a chemical-inducible PAH-degradation tool, we further engineered cell population 1 to express TtLcc1 in response to different chemicals in the cell culture. Overall, we built four inducible systems. The first two were obtained by replacing the constitutive *GPD* promoter with either the *CUP1* (copper inducible—pCUP1) or *GAL1* (galactose inducible—pGAL1) promoter. Each of the other two systems demanded to assemble two new TUs. One TU was for TtLcc1 expression under the control of a synthetic regulated promoter, either 2xlex2Op_pCYC1core [25] or pCYC1min_tetOp [26] (see Fig. 3A and S3B-D). The other TU was for the constitutive expression of the transcription factor proteins: LexA-ER-VP64 (a chimeric activator) and TetR (a bacterial repressor). LexA-ER-VP64 consists of a bacterial DNA binding domain (LexA), the human receptor of β-estradiol (ER), and the strong viral VP64 activation domain. Upon synthesis, LexA-ER-VP64 is kept in cytoplasm by the interaction between ER and Hsp90—the heat-shock protein 90 [27]. When the cells grow in a medium supplied with β-estradiol, the hormone binds ER, which permits the transport of the activator into the nucleus where it binds lex2Op and enhances Ttlcc1 expression. TetR, in contrast, binds tetOp in its wild-type configuration and prevents transcription from pCYC1min. Tetracycline binds TetR and inhibits its capability to bind the DNA. Hence, Ttlcc1 is produced only in the presence of tetracycline in the cell culture. Cell population 1 behaves as a buffer (YES) gate since it returns a high output only in the presence of its sole input. The input (a chemical) triggers the production of Ttlcc1 that, once secreted, interacts with ABTS in the cell-growth plate/solution. If the plate assumes a reddish color (the output), then the presence of the input is visually revealed (see Figs. S6–S9). We characterized our buffer gates by measuring laccase activity at different input concentrations over a time interval of 92 h. As shown in Fig. 3B, the expression of TtLcc1 can be modulated over time via different concentrations of copper or β-estradiol. Tetracycline, in contrast, did not lead to high laccase activity that settled to a plateau after 60 h (the strengths of the promoters expressing Ttlcc1 are reported in Table S4).

We degraded PAHs via inducible expression of TtLcc1 and monitor its metabolism with cell population 2. Since copper increases the noise in the benzoate/salicylate biosensor, we recurred only to the β-estradiol-inducible cell population 1. Moreover, since we hypothesized that glucose delayed PAH degradation, we cultivated cell population 1 at different concentrations of glucose, from 0.25 % to 2 % (plus 100 μM anthracene and 1 μM β-estradiol). Our results confirmed that higher concentrations of glucose slowed down the action of TtLcc1 on anthracene. As a consequence, the pulse in fluorescence needed 12 h to reach its peak in the presence of 0.25 % glucose but even 48 h when glucose was at 2 % (see Fig. 3C). We compared the latter peak to the background fluorescence due to a population-1-growth solution without β-estradiol. The pulse peak resulted statistically significantly different from and 1.39-fold higher than the noise signal (Fig. 3D).

3.5. TtLcc1 as a reporter protein

In the pulse generators described so far, laccase is a fundamental circuit component because of its property of degrading PAHs. Laccase can also become a reporter protein due to the color it produces when interacting with ABTS. Since this output signal is visible to the naked eye, the usage of laccase together with ABTS can speed up considerably the screening for strains containing a working synthetic gene circuit. We have already used TtLcc1 as a reporter to evaluate the efficiency of mRNA degradation caused by CRISPR type V proteins [17]. Here, we harnessed TtLcc1 to evaluate gene editing efficiency in a knockout circuit (see Fig. 4A). The LbCas12a:crRNA complex was chosen because it is an effective genome editing tool in *S. cerevisiae* [28]. *Ttlcc1* gene was the target gene for the knockout. To repair the DSBs by exploiting homologous recombination (HR), we transformed the crRNA together with a donor DNA containing part of the *yEGFP* sequence flanked by two 70-nt-long fragments (a1 and a2) homologous to *Ttlcc1*. If knockout were successful, a strain would not change its color on a plate where the selective medium was supplied with 0.5 mM ABTS. Otherwise, the strain would have a reddish halo. We made, as a negative control, a transformation where the crRNA expression plasmid was replaced with an empty backbone. We grew the strains transformed with the full knockout circuit and the control ones on a selective medium (plate) without any ABTS. Then, we randomly selected 8 strains from each plate and grew them on a new plate where the selective medium was supplemented with 0.5 mM ABTS. Every strain containing the full circuit was white on the new plate (Fig. 4B), whereas all control strains looked reddish (Fig. 4C). Hence, TtLcc1 can be used as a reporter to assess the efficiency/working of synthetic gene circuits. This only requires integrating, into the yeast genome, the *Ttlcc1* gene instead of that encoding for a fluorescent protein and growing the engineered cells in a medium containing ABTS. Moreover, compared to fluorescent proteins, the visual screening via TtLcc1 is cheaper and less time-consuming.

4. Conclusions

LacA from *Trametes* sp. AH28-2 is functionally expressed in *S. cerevisiae* and, as TtLcc1, its activity is optimized via genomic integration. Laccases have the important feature of degrading PAHs. This reaction was at the basis of the construction of a new synthetic two-population system (one expressing laccase, the other sensing metabolites of PAHs) that behaves as a spatiotemporal pulse generator—and permits to monitor hydrocarbon metabolites. This novel kind of consortium can be controlled via an external input chemical. Laccase, moreover, can be employed as a circuit reporter because of its interaction with ABTS, which induces a coloration in the cell growth medium. Therefore, laccase is a valid alternative to fluorescent proteins. Overall, laccase has the potential to be largely used, in the future, into new yeast synthetic gene circuits due to its ability to carry out different functions.

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No funding.

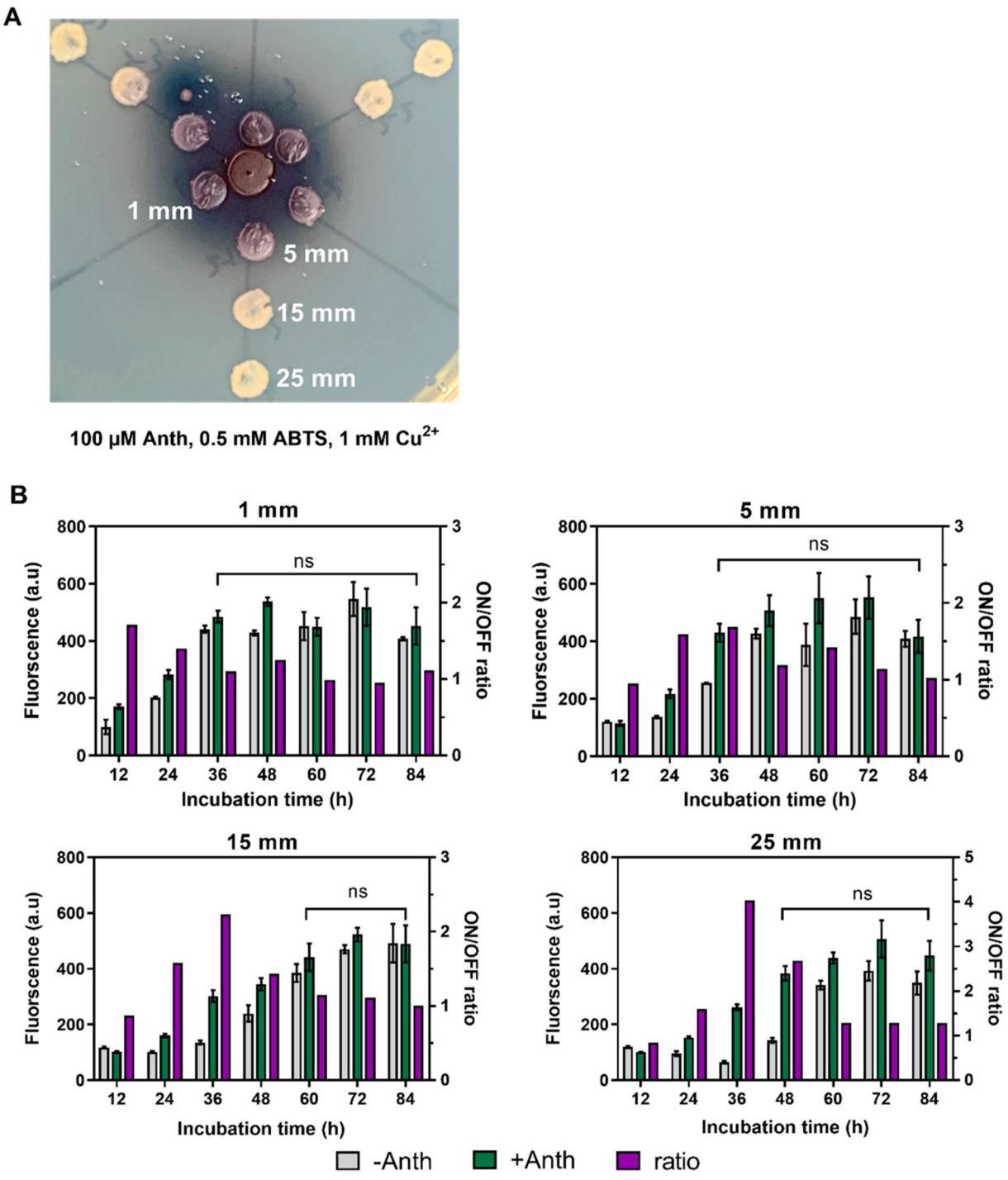

Fig. 2. Anthracene degradation by the two-population system (expressing TtLcc1) on a solid plate. (**A**) A plate supplied with 100 μM anthracene after 84 h of incubation at 30 °C. The central strain expresses TtLcc1, the others are benzoate/salicylate biosensors. The color reflects TtLcc1 production and its effect on ABTS. (**B**) Fluorescence from population 2 at varying distances from population 1. The shorter the distance, the lower the time necessary to reach the highest ON/OFF ratio. 'ns': no significant statistical difference among the green bars, one-way ANOVA.

Notes

The authors declare no competing financial interest.

Data availability

All FCS files from FACS experiments are available at the Flow-Repository (https://flowrepository.org/). Accession ID: FR-FCM-Z76C.

CRediT authorship contribution statement

Lifang Yu: Conceptualization, carrying out experiments, Formal analysis, Writing – original draft. **Michael Dare Asemoloye:** Conceptualization, carrying out experiments, Formal analysis, Writing – original draft. **Mario Andrea Marchisio:** Conceptualization, Supervision, writing the manuscript.

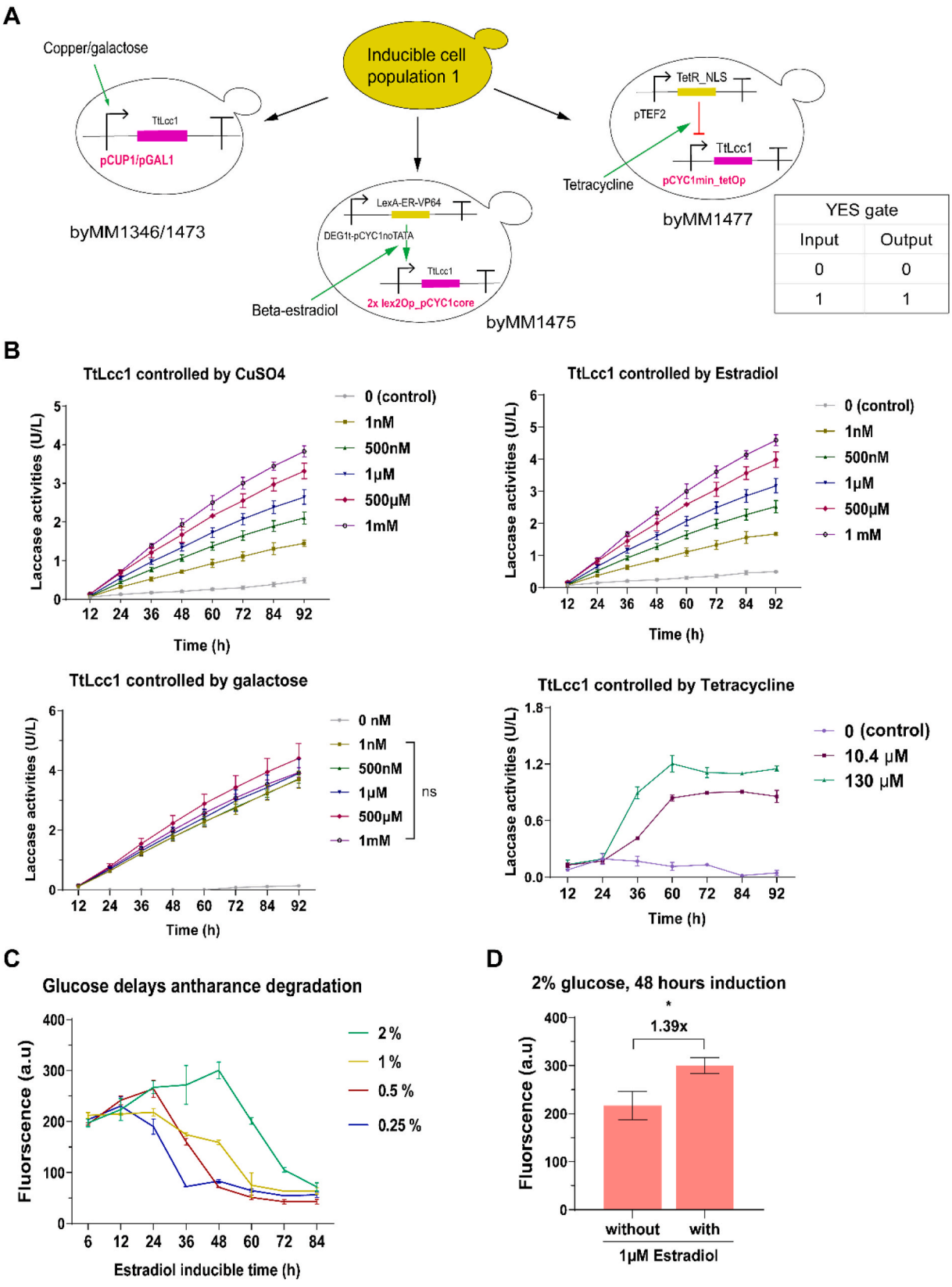

Fig. 3. PAH degradation based on inducible expression of TtLcc1. (**A**) Scheme of the four YES gates based on population 1. (**B**) TtLcc1 activity was measured over 92 h under variable concentration of the four chemicals. 'ns' (one-way ANOVA) means that there was no significant statistical difference among the laccase activities due to five concentrations of galactose. (**C**) The influence of glucose concentration on anthracene degradation by TtLcc1. The higher the glucose concentration, the longer the time necessary to reach the fluorescence peak. (**D**) byMM1475 cultured for 48 h in SDC with and without β-estradiol. The cell culture supernatant was collected and used for a 12-h induction of cell population 2.

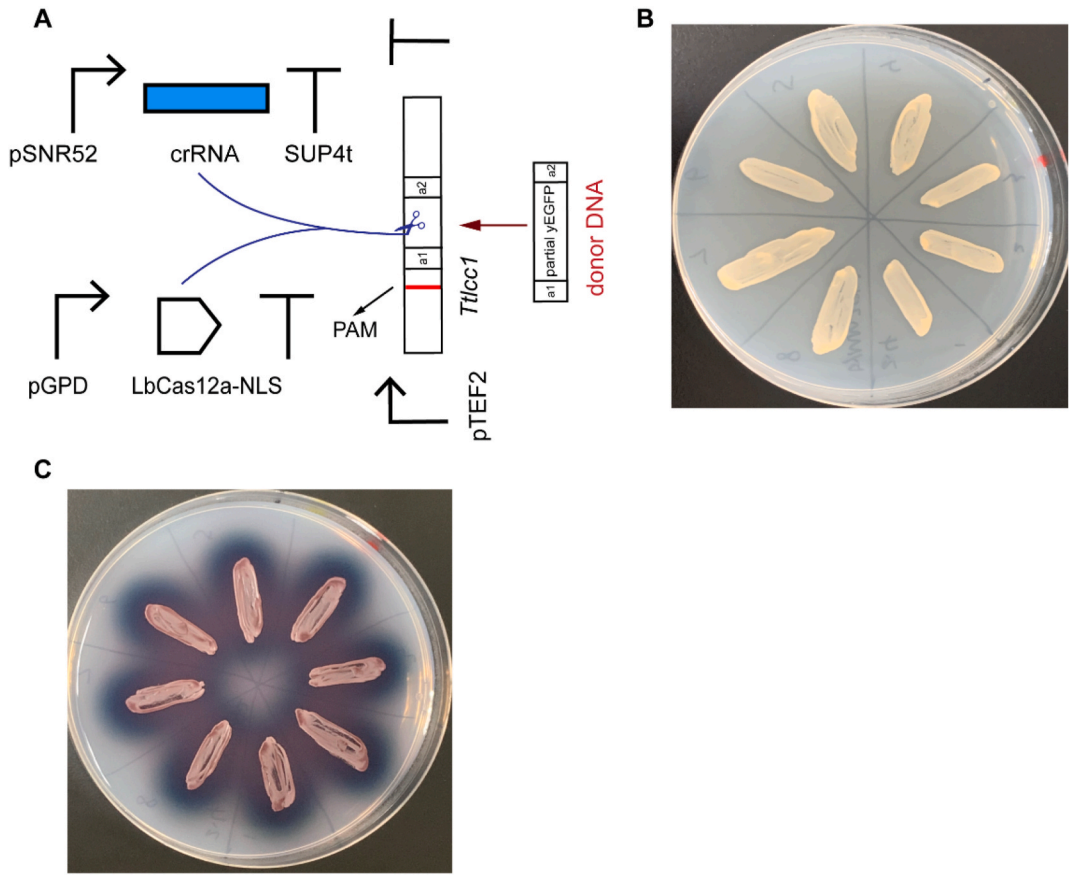

Fig. 4. TtLcc1 as a reporter. (**A**) The knockout circuit. LbCas12a:crRNA complex binds *Ttlcc1*, nearby the PAM, and induces a double-strand break that triggers the self-repair yeast pathway. (**B**) Gene editing was successful in every chosen strain transformed with the full circuit. (**C**) The strain transformed with the control circuit expressed TtLcc1 that reacted with ABTS to produce the typical reddish color on plate.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2024.05.007.

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