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A microbial biomanufacturing platform for natural and semi-synthetic opiates

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Abstract

Opiates and related molecules are medically essential, but their production via field cultivation of opium poppy *Papaver somniferum* leads to supply inefficiencies and insecurity. As an alternative production strategy, we developed baker's yeast *Saccharomyces cerevisiae* as a microbial host for the transformation of opiates. Yeast strains engineered to express heterologous genes from *P. somniferum* and bacterium *Pseudomonas putida* M10 convert thebaine to codeine, morphine, hydromorphone, hydrocodone, and oxycodone. A new biosynthetic branch to neopine and neomorphine was discovered, which diverted pathway flux from morphine and other target products. Strain titer and specificity was optimized by titrating gene copy number, enhancing cosubstrate supply, applying a spatial engineering strategy, and performing high-density fermentation, resulting in total opioid titers up to 131 mg/L. This work is an important step toward total biosynthesis of valuable benzylisoquinoline alkaloid drug molecules and demonstrates the potential for developing a sustainable and secure yeast biomanufacturing platform for opioids.

Introduction

Opiates are a group of natural drug molecules from *Papaver somniferum* (opium poppy) used to treat human disease over more than 3500 years[1]. One of these molecules – morphine (**1**) – is a highly effective and widely prescribed analgesic. Today, satisfying the global medical need for morphine requires industrial processing of tens to hundreds of thousand tons of opium poppy biomass annually[2]. Other extracted natural opiates include the antitussive codeine (**2**), the vasodilator papaverine, and the drug precursor thebaine (**3**) used to chemically manufacture semi-synthetic opioids such as hydrocodone (**4**), oxycodone (**5**), and hydromorphone (**6**). These semi-synthetics have improved properties over natural

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Author contributions

K.T. and C.D.S. conceived of the project, designed the experiments, analyzed the results, and wrote the manuscript. K.T. constructed and tested the strains. S.G. performed NMR analysis, *in vitro* assays, and prepared parts of the manuscript.

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opiates including enhanced gastrointestinal absorption, analgesic strength, and circulatory half-life[3].

Opiates are benzylisoquinoline alkaloids (BIAs), a class of specialized metabolites synthesized from tyrosine in a small number of plant species. Biosynthesis of BIAs proceeds through the simple BIA norcoclaurine. To form morphine from this BIA structural backbone, enzymes catalyze carbon-carbon coupling, NADPH-dependent reduction, hydroxylation, acetylation, methylation, and demethylation tailoring reactions[4]. Because of the complex regioand stereochemistry of morphinan BIAs, chemical synthesis of these molecules is not viable at commercial scale[5]. Currently, the world relies on opium poppy trade to meet the medical demand for opiates and their derivatives.

Knowledge of the opiate biosynthetic pathway informs modern crop breeding and metabolic engineering efforts to improve opium poppy as a plant production host. This understanding increased greatly with the recent discovery of the remaining enzymatic steps in morphine biosynthesis[6], in which the reactions converting thebaine to morphine are catalyzed by codeinone reductase (COR) and the newly-identified dioxygenases thebaine 6-*O*-demethylase (T6ODM) and codeine *O*-demethylase (CODM). Overexpression of *COR* confers enhanced overall morphinan alkaloid content[7], and knockdown of individual pathway genes results in accumulation of morphinan intermediates in transient and stable plant lines[6,8,9]. However, genetic engineering of poppies is slow and challenging due to long generation times, lack of a genome map, and limited tools for genetic manipulation. Agricultural production of drugs generally suffers from susceptibility to climate and disease, a single annual growing season, low alkaloid content in the plant body, need for extraction by chemical processing, and social and political factors related to the potential for illicit use.

Engineering microbial strains provides an alternative strategy for producing opiates. However, the reconstruction of plant biosynthetic pathways in microbial hosts raises many engineering challenges. Metabolic engineers have demonstrated yeast biosynthesis of many microbial natural products, but only a few plant natural products, including terpenoids (e.g., artemisinin) and phenolics (e.g., resveratrol)[10,11]. Plant BIAs in particular present a number of challenges to microbial production platforms due to the complex, branched, multi-step architecture of the biosynthetic pathway. We have previously engineered the yeast *Saccharomyces cerevisiae* as a host for the production of reticuline, a branch point intermediate in the biosynthesis of several subgroups of BIAs, including the morphinan and protoberberine alkaloids, from a fed precursor[12]. Total synthesis of reticuline from tyrosine was later demonstrated in the bacterial host *Escherichia coli*[13-15]. However, downstream modification of reticuline to form further functionalized molecules, including scoulerine, canadine, salutaridine, magnofluorine, and corytuberine, has only been achieved in a yeast host[12,16]. As a eukaryote, yeast is better suited than bacteria to stably express plant tailoring enzymes such as the endomembrane-localized cytochrome P450s.

Standard optimization strategies for enhancing plant natural product biosynthesis in microbial hosts include optimizing codon usage, tuning enzyme expression levels and timing, and redirecting host metabolism to increase supply of precursors and co-factors[17]. In the natural BIA plant host another optimization mechanism is evident: the spatial regulation

of pathway enzymes at the subcellular and cellular level. For example, an enzyme of berberine and sanguinarine biosynthesis localizes to the endoplasmic reticulum and vacuole[18,19], while morphinan enzymes are distributed across cells of the phloem[20]. Such spatial regulation of metabolic pathways has been mimicked in engineered systems to enhance production of target compounds by colocalizing pathway enzymes on nucleic acid- and protein-based scaffolds in bacterial cells[21-24]. Furthermore, in yeast cells heterologous enzymes have been successfully localized to the mitochondria and vacuole to replicate subcellular localization in the native host[25], colocalize enzymes[26], and access precursor and cofactor pools[27,28]. This localization of enzymes in natural and engineered systems may additionally work by providing optimal microenvironments for reactions, concentrating substrates, retaining intermediates, reducing interactions with competing pathways, and preventing export of intermediates from the cell.

Here, we engineer yeast to support the final steps of opiate biosynthesis, yielding strains that can produce naturally occurring opiates and semi-synthetic opioids. Upon reconstructing opium poppy biosynthesis of codeine and morphine in yeast, we observed the production of two additional isomers of these molecules; neopine and neomorphine, respectively. Our results highlight that the loss of native regulation strategies upon transfer of a plant biosynthetic pathway to a microbial host can result in branching pathways that direct flux toward undesired byproducts. To improve pathway specificity for morphine we increased the supply of co-substrate, titrated gene copy number, and delocalized a pathway enzyme. By further incorporating bacterial enzymes within the heterologous pathway, we demonstrated the biosynthesis of a panel of valuable semi-synthetic opiates, including up to 51 mg/L hydrocodone, 70 mg/L oxycodone, and 1 mg/L hydromorphone, which are typically produced by chemical modification of natural opiates. Optimized engineered yeast strains produced 42-131 mg/L total opioid products, demonstrating the potential for development of a microbial biomanufacturing platform to supply natural and semi-synthetic opioids to the pharmaceuticals industry.

Results

Constructing a morphine biosynthetic pathway in yeast

The biosynthesis of morphine from thebaine is catalyzed by three enzymes in *P. somniferum*: the 2-oxoglutarate/Fe^{2+}-dependent dioxygenases T6ODM and CODM, and NADPH-dependent aldoketo reductase COR[6,29]. These enzymes form two biosynthetic routes from thebaine to morphine. One route (i) includes a non-enzymatic rearrangement and generates intermediates neopinone (**7**), codeinone (**8**), codeine (**2**), and morphine (**1**) (Fig. 1a). Based on reported substrate affinities of T6ODM and CODM, this is the predominant pathway in poppy[6]. The minor route (ii) generates oripavine (**9**)[30] and morphinone (**10**) as intermediates to morphine (**1**) (Fig. 1a).

To reconstruct a morphine biosynthetic pathway in *S. cerevisiae*, we expressed yeast codon-optimized *T6ODM*, *COR1.3*, and *CODM* each flanked by unique yeast promoters and terminators and assembled into a single yeast artificial chromosome (YAC) vector (pYES1L) (Supplementary Results, Supplementary Table 1). From the four characterized *P. somniferum* COR isoforms, *COR1.3* was selected because it had the highest affinity for

codeinone[29]. After culturing this strain with thebaine for 96 h, we detected codeinone, codeine, and morphine in the culture medium, demonstrating that these heterologous plant enzymes are capable of catalyzing transformations of opiates in yeast (Fig. 1b, Supplementary Fig. 1). However, the detected opiate levels were low, with morphine production as low as 0.2 mg/L, suggesting that optimization efforts would be required to increase conversion efficiencies. Neopinone was not detected in this assay, likely because this intermediate is unstable[31] and rearranges to codeinone over the course of the experiment.

Additional opiates detected in the culture medium indicated differences between the biosynthetic routes observed in the natural and heterologous systems. Intermediates from the minor route to morphine observed in plants – oripavine and morphinone – could not be attributed to the activity of the *P. somniferum* enzymes in our engineered yeast strains. Specifically, a small but equal amount of oripavine was observed in both engineered and no-enzyme control strains (Supplementary Fig. 1) and morphinone was not present at detectable levels under these conditions. However, two other products were observed in similar quantities to codeine and morphine. The first had the same mass/charge (*m/z*) ratio as codeine and was determined to be neopine (**11**) by identity of ^{1}H-NMR spectrum to published spectrum (Fig. 1b, Supplementary Figs. 1-3, Supplementary Table 2). Neopine may be produced by the activity of COR on the direct product of T6ODM, neopinone, before it rearranges to codeinone (Fig. 1a). The second unknown product had the same *m/z* ratio as morphine and was determined to be neomorphine (**12**), produced by the CODM-catalyzed demethylation of neopine (Fig. 1b, Supplementary Figs. 1,3). Therefore, our analysis uncovered a novel, but undesired, opiate pathway (iii) in the engineered yeast strain (Fig. 1a).

Increasing supply of the co-substrate 2-oxoglutarate

We examined whether supply of a key co-substrate, 2-oxoglutarate, was limiting in the heterologous morphine biosynthesis pathway. The dioxygenases T6ODM and CODM require 2-oxoglutarate to accept one oxygen atom in the oxidative demethylation of thebaine and codeine, respectively[6]. 2-oxoglutarate also participates in endogenous yeast nitrogen metabolism, where glutamate dehydrogenase enzymes catalyze its interconversion with glutamate (Supplementary Fig. 4a). Thus, increasing glutamate supply is anticipated to increase the intracellular 2-oxoglutarate pool. We titrated monosodium glutamate (MSG), a common nitrogen source, into the yeast culture medium. Glutamine was included in the culture medium as a nitrogen source to ensure the cultures were not nitrogen-limited and hence prevent positive growth effects due to MSG supplementation. No differences in final cell densities were observed with varying levels of MSG. Upon increasing MSG concentration from 0 to 2.5 g/L we observed an increase in morphine production from 0.24 to 0.45 mg/L after 96 h growth (Supplementary Fig. 4b). We next examined whether direct addition of the co-substrate 2-oxoglutarate to the culture medium would further enhance flux through the pathway. We titrated 2-oxoglutarate into the culture medium up to concentrations of 100 mM and observed increased morphine titers to 2.5 mg/L, a more than 10-fold increase over the titer observed in standard medium (Supplementary Fig. 4b). All

subsequent opiate-producing cultures were grown in this optimized culture medium supplemented with 0.5 g/L glutamine, 2.5 g/L MSG, and 50 mM 2-oxoglutarate.

Balancing enzyme expression to increase morphine titer

We next examined whether optimizing relative enzyme expression levels would increase pathway flux to morphine. COR catalyzes the reversible reduction of codeinone to codeine in morphine biosynthesis[29] and neopinone to neopine in yeast (Fig. 1). The presence of these reversible reactions suggested that pathway flux toward the production of codeine, and consequently morphine, could be further increased by titrating the expression levels of pathway enzymes.

To examine the combinatorial design space around *T6ODM*, *COR*, and *CODM* expression levels, we constructed strains with varied gene copy numbers for each of the enzymes. In all strains, single copies of *T6ODM*, *COR*, and *CODM* were expressed from a YAC vector. Additional copies of one or more genes were integrated with a constitutive *GPD* promoter into the host cell genome at auxotrophic loci (Supplementary Table 3). The strains were cultured with 1 mM thebaine in optimized medium (0.5 g/L glutamine, 2.5 g/L MSG, and 50 mM 2-oxoglutarate) for 96 h in 96-well plates. We compared morphine and neomorphine titers to those of the control strain with only the YAC vector (*T6ODM*:*COR*:*CODM* gene ratio of 1:1:1).

We observed that increasing the copy number of *COR* alone (e.g., 1:3:1) or together with *T6ODM* (e.g., 2:2:1) decreased morphine production while increasing neomorphine production, such that overall opiate production was similar but differed in the ratio of end products (Fig. 2a). For example, the control 1:1:1 strain produced 2.5 mg/L morphine and 3.7 mg/L neomorphine, a total of 6.2 mg/L end product opiates. In contrast, the 1:3:1 strain produced 2.0 mg/L morphine and 4.1 mg/L neomorphine; a different ratio of morphine to neomorphine, but a similar total end product titer. Other gene copy number combinations within the design space provided increases in morphine and total end product titers. For example, we observed that increased *CODM* copy number resulted in higher production levels of both morphine and neomorphine (Fig. 2a). This effect was enhanced by additional gene copies of *T6ODM* such that the most favorable ratio of *T6ODM*:*COR*:*CODM* was 2:1:3, which produced 5.2 mg/L morphine and 4.8 mg/L neomorphine, a total of 10.0 mg/L end product opiates in culture medium (Fig. 2a).

The observed relationship between morphine titers and gene copy number ratios suggested two mechanisms attributed to enhanced CODM expression: an increased forward rate of the reversible COR reaction and improved specificity for the target product morphine over the byproduct neomorphine. Specifically, in the lowest yielding 1:3:1 strain morphine comprised 33% of the total end product opiates, while in the highest yielding 2:1:3 strain morphine comprised 52%. An analysis of pathway conversion efficiencies showed that CODM favors codeine as a substrate and thus biases the pathway for morphine production at high copy number (Supplementary Fig. 5). However, even in the most optimally balanced copy number strains, non-target neomorphine still accounted for almost half the final product.

A localization approach to improve pathway specificity

We examined conversion efficiencies across the engineered pathway and determined that the key cause of the branching from morphine to non-target neomorphine is the intervening spontaneous step between the reactions catalyzed by T6ODM and COR (Supplementary Fig. 5). We hypothesized that an engineering strategy in which T6ODM and COR were spatially separated in the cell may allow additional time for the intervening spontaneous rearrangement of neopinone to codeinone. Specifically, by isolating COR to a yeast organelle we hypothesized that we could restrict access of this enzyme to the neopinone produced by cytoplasmic T6ODM, providing neopinone additional time to rearrange to codeinone and ultimately be converted to morphine.

To address this specificity challenge we selected the 28 amino acid transmembrane domain of integral membrane protein calnexin (CNE1) as an endoplasmic reticulum routing tag (ER1). ER1 was fused to the C-terminus of GFP and GFP-COR1.3 and its ability to direct the subcellular localization of these proteins was confirmed by confocal microscopy (Fig. 2b, Supplementary Fig. 6). We applied the validated tag to COR1.3 and examined whether delocalization of this pathway enzyme could increase flux to the desired product (morphine) and decrease flux to the undesired product (neomorphine). A control strain in which COR1.3 was untagged and thus localized to the cytoplasm with T6ODM and CODM (Supplementary Fig. 6) produced 2.5 mg/L morphine with 44% specificity after 96 h growth (Fig. 2b). In the strain expressing localized COR1.3-ER1 we observed increased specificity (relative production of the desired product) and titer (absolute production levels of the desired product) for morphine relative to the control strain. Specifically, the strain with an ER-localized COR1.3 gave morphine titers of 3.1 mg/L at 86% specificity (Fig. 2b). The results are consistent with the proposed mechanism where delocalizing COR activity from T6ODM activity allows for increased time for the spontaneous conversion of neopinone to codeinone and thus increased specificity for morphine production relative to neomorphine production. However, we cannot rule out the possibility that some of the observed effect of our spatial engineering approach on pathway specificity may be a result of a reduction in COR1.3 activity as a result of its localization to an endomembrane.

Biological synthesis of semi-synthetic opioids

Semi-synthetic opioids are widely prescribed alternatives to the natural opiates codeine and morphine due to their enhanced efficacy, increased solubility, and reduced side effects. For example, the analgesic strength of hydromorphone is 5-7 times greater than that of morphine, and hydrocodone has enhanced oral bioavailability with analgesic strength between that of codeine and morphine[3]. Commercially, semi-synthetic opioids are produced chemically from natural opiates and carry the same dependence on poppy crop cultivation. Therefore, a microbial platform designed both to replace opium poppy as a source of natural opiates and further produce semi-synthetic opioids would constitute a secure and sustainable alternative supply.

Bacterium strain *Pseudomonas putida* M10, identified in waste from an opium-poppy processing factory, performs enzymatic transformations of opioids[32]. Two characterized enzymes from this strain – $NADP^+$-dependent morphine dehydrogenase (morA) and

NADH-dependent morphinone reductase (morB) – catalyze many of these reactions[33,34]. MorA, an aldo-keto reductase, and morB, an α/β-barrel flavoprotein oxidoreductase, have been heterologously expressed in *E. coli* to convert morphine to hydromorphone when the cells were incubated at high cell densities with concentrated morphine in buffer[35].

We examined whether we could use morA and morB to extend the biosynthetic capabilities of our morphine-producing yeast strains to the valuable end products hydrocodone (**4**) and hydromorphone (**6**) (Fig. 3). In a single YAC, we included the *P. somniferum* genes *T6ODM*, *COR*, and *CODM* and the *P. putida* genes *morA* and *morB*. A yeast strain transformed with this YAC and cultured with 1 mM thebaine produced only a trace amount of hydrocodone and no detectable hydromorphone after 96 h growth (Table 1). We suspected that the failure of this strain to produce hydromorphone was likely due to insufficient flux through the morphine branch as a result of the competing neomorphine branch.

We developed an alternative biosynthetic route to the semi-synthetic opioids hydrocodone and hydromorphone. We first determined whether morA could reduce codeinone (8) to codeine (**2**) in place of COR in the morphine biosynthesis pathway by replacing *COR1.3* with *morA* in the YAC encoding morphine production (Fig. 3). The substitution of morA activity for COR resulted in 2.4 mg/L morphine with 69% selectivity (Table 1). We then included morB to generate a four gene YAC with *T6ODM*, *CODM*, *morA*, and *morB*. A strain with this YAC produced 1.3 mg/L hydrocodone and 0.10 mg/L hydromorphone (Table 1). We also tested an alternative morB amino acid sequence (RCSB PDB: 1GWJ_A) with a Glu160Gly mutation relative to the original reported sequence (UniProtKB: Q51990). Substituting morB with morB^{E160G} resulted in 0.9 mg/L hydrocodone and 0.14 mg/L hydromorphone (Table 1). The reduced hydrocodone titer suggested that the morB^{E160G} variant may have reduced activity on codeinone, and thus redirects flux to hydromorphone. Additional control experiments indicated that $NADP^+$/NADPH cofactor supply is limiting to hydromorphone biosynthesis (Supplementary Fig. 7), highlighting strategies to increase cofactor availability as a future direction for increasing opioid production

Several other opioids were detected in trace amounts: dihydrocodeine (**13**) and dihydromorphine (**14**) produced by the activity of morA on hydrocodone and hydromorphone (Fig. 3, Supplementary Fig. 1); 14-hydroxycodeine (**15**) and oxycodone (**5**) presumably resulting from morA and morB activity on 14-hydroxycodeinone (**16**), respectively (Fig. 3, Supplementary Fig. 1). The presence of these 14-hydroxylated products was attributed to a chemical hydroxylation known to occur spontaneously *in vitro* at neutral or slightly alkaline pH[36] although we could not rule out native enzymatic catalysis by endogenous enzymes of *S. cerevisiae*. Based on these results, we engineered a strain expressing only T6ODM and morB to increase pathway flux to the morB products hydrocodone and oxycodone. This two-enzyme strain produced 6.5 mg/L hydrocodone and 2.1 mg/L oxycodone (Table 1, Supplementary Fig. 1).

Strain engineering for natural and semi-synthetic opioids

We combined the most favorable genetic design elements from our previous strains to construct three production strains for target opioid biosynthesis. The morphine production

strain (CSY950, Supplementary Table 3) incorporated a genome-integrated copy of *T6ODM*, two integrated copies of *CODM*, and a YAC encoding *COR1.3-ER1*, *T6ODM*, and *CODM*. This strain directed pathway flux to morphine with ER-localized COR1.3 and enhanced overall pathway flux with the optimal 2:1:3 ratio of *T6ODM*:*COR1.3*:*CODM*. The hydromorphone production strain (CSY951, Supplementary Table 3) incorporated a genome-integrated copy of *T6ODM*, two integrated copies of *CODM*, and a YAC encoding *T6ODM*, *CODM*, *morA*, and $morB^{E160G}$. This hydromorphone production strain directed flux to hydromorphone with the $morB^{E160G}$ variant and enhanced overall production with the optimal gene copy number ratio. The hydrocodone/oxycodone production strain (CSY952, Supplementary Table 3) incorporated two integrated copies of *T6ODM* and a YAC encoding *T6ODM* and *morB*. These production strains were grown in parallel 0.25 L closed batch fermentations. Ten key opioid end products - codeine, neopine, morphine, neomorphine, hydrocodone, oxycodone, hydromorphone, dihydrocodeine, 14-hydroxycodeine, and dihydromorphine – were monitored over the course of the fermentation. Target opioids were detected in the culture medium after 24 h, increased in concentration with increasing cell density, and continued to accumulate in stationary phase. Strains CSY950, CSY951, and CSY952 accumulated 42, 74, and 131 mg/L target opioid molecules, respectively, in the culture medium during the course of the fermentation (Fig. 4a, Supplementary Table 4).

Analysis of the total opioid profile across strains at the final time point revealed important differences in pathway flux (Fig. 4a). Strain CSY950 in which COR was localized to the ER produced low titers of neopine and neomorphine relative to codeine and morphine (2.6 and 0.8 mg/L relative to 7.7 and 4.7 mg/L), indicating that the spatial engineering approach remained effective at limiting pathway flux towards non-target byproducts in bench scale fermentations (Fig. 4a). However, the overall yield of morphine from this strain was lower than expected. Examination of the full BIA profile revealed that the side product 14-hydroxycodeine was a large component of the total opioid molecules produced (26 mg/L). In this strain codeinone may form 14-hydroxycodeinone, which is then reduced by COR to 14-hydroxycodeine by an identical reaction to that catalyzed by morA[36,37]. Furthermore, the higher titer of codeine than morphine suggests that factors influencing CODM activity may form a bottleneck in pathway flux to morphine.

Analysis of the CSY951 fermentation culture medium provided more evidence of a bottleneck in pathway flux from codeine to morphine. The products downstream of CODM – morphine, hydromorphone, dihydromorphine – accumulated at low levels (0.5, 1.0, and 0.9 mg/L, respectively) relative to other BIA products, suggesting that activity of CODM may be limiting (Fig. 4a). CSY951 also accumulated low levels of hydrocodone and oxycodone (1.6 and 0.5 mg/L, respectively), consistent with $morB^{E160G}$ limiting production of these side products and routing pathway flux to hydromorphone (Fig. 4b). In this strain, morA activity increased accumulation of side products neopine and neomorphine (21 and 4.4 mg/L, respectively), possibly impacting yields of the target end product hydromorphone. Further optimization of the hydromorphone production strain may be achieved through a dual spatial-temporal regulation strategy for morA to limit flux to the neomorphine branch and switch on morA activity once a suitable level of morphine has accumulated.

The CSY952 strain was engineered for the production of hydrocodone and oxycodone and has a simple pathway architecture. The strain does not incorporate morA/COR or CODM and thus does not lose flux to the neomorphine branch nor encounter the bottleneck between codeine and morphine. CSY952 converted thebaine to hydrocodone and oxycodone with titers of 51 and 70 mg/L, respectively (Fig. 4a). Accumulation of hydrocodone was limited by its conversion to dihydrocodeine over the course of the fermentation, with a final titer of 10 mg/L (Fig. 4b). The reduction of hydrocodone to dihydrocodeine occurs by an unknown mechanism in this strain. CSY952 demonstrates that high flux to target compounds can be achieved through a minimally branched pathway of heterologous enzymes with high activity in yeast.

Discussion

Engineered microbial synthesis of plant-derived therapeutic molecules is a sustainable technology positioned to supplement or replace drug crop cultivation. We have demonstrated *S. cerevisiae* as a production host for the transformation of thebaine to valuable opioids including codeine, morphine, hydrocodone, oxycodone, and hydromorphone. This work adds to the expanding use of yeast as a biosynthetic platform for many valuable BIA target molecules. Gene copy number optimization, co-substrate supply enhancement, and spatial engineering were applied to enhance yeast opiate biosynthesis. Three final engineered strains produced 7.7 mg/L codeine and 4.7 mg/L morphine (CSY950), 1 mg/L hydromorphone (CSY951), and 51 mg/L hydrocodone, and 70 mg/L oxycodone (CSY952) in bench-scale batch fermentations.

In opium poppy, thebaine is converted to morphine via two biosynthetic routes. The initial bifurcation occurs when T6ODM or CODM demethylates thebaine at a distinct position. Since both biosynthetic routes arrive at morphine in opium poppy this bifurcation is unlikely to impact yields *in planta*. However, our engineered yeast strains demonstrate an additional branching due to the activities of COR and CODM on neopinone and neopine, respectively, resulting in production of neomorphine and decreased production of morphine.

We could not find literature reports of COR activity on neopinone to produce neopine, and subsequently neomorphine, *in planta* or *in vitro*. Initial studies on COR purified from *P. somniferum* cell culture lysates reported high substrate specificity for codeinone *in vitro* with no activity on neopinone[38,39]. Researchers later characterized four *P. somniferum* COR variants, recombinantly expressed and purified from *E. coli*, but they did not include neopinone as a substrate[29]. One possible explanation for the observed differences in COR activity is that the plant enzymes function differently when expressed in yeast than in the native plant host, due to the absence of native regulatory mechanisms and altered cellular factors such as protein processing, localization, and microenvironment. However, neopine is a minor constituent of opium[40] and present in urine samples taken from opium users[41]. The production of neopine in our engineered yeast strains may, for the first time, demonstrate the pathway by which opium poppy synthesizes this molecule.

In natural BIA metabolism enzyme promiscuity contributes to the large number of diverse molecular structures produced. For example, T6ODM and CODM exhibit broader substrate

specificity than initially thought, catalyzing *O*-demethylation of non-morphinan alkaloid substrates such as scoulerine and allocryptopine[42]. These enzymes also catalyze *O,O*-demethylenation reactions that break methylenedioxy bridges in diverse BIAs, including allocryptopine, cryptopine, and protopine[42]. Other examples of BIA enzyme promiscuity include the *O*- and *N*-methyltransferases, which methylate many BIA substrates[43]. In a heterologous microbial host, the absence of native temporal and spatial regulation mechanisms and the combining of enzymes from different species in a single cell likely further broadens the range of substrates available to the BIA biosynthetic enzymes, leading to newly observed pathway branches and metabolites. This enables the production of increasingly large numbers of natural and previously unobserved BIA molecules from limited starting materials in engineered microbial strains[4]. However, enzyme promiscuity also represents a key challenge in reconstructing targeted microbial BIA biosynthetic pathways: managing flux through highly branching pathways to achieve optimal yields of individual target end products.

Our use of spatial engineering to increase the specificity for morphine production by our strains highlights the importance of understanding natural spatial regulation strategies in plants and their role in directing metabolic flux. In opium poppies morphinan alkaloids accumulate in the laticifer, a specialized addition to the phloem. Upstream enzymes in thebaine biosynthesis are predominantly located in the adjacent sieve element, and downstream enzymes T6ODM, COR and CODM are more abundant in the laticifer[20]. The complete morphine biosynthetic pathway is partitioned between these plant cells types, with the transport of thebaine and other intermediates between the two. The mechanisms by which the cellular environments of the sieve element and laticifer support the activity and regulation of morphinan alkaloid biosynthetic enzymes are not yet understood. Complete determination of whether such enzymes are colocalized within cells or delocalized to subcellular compartments, such as the latex vesicles in the laticifer, will inform further efforts to engineer the heterologous morphine pathway in yeast.

The morphinan pathway construction presented here combined with the previously reported total microbial biosynthesis of upstream BIA reticuline[12-14,16] supports the engineering of a yeast strain capable of producing target opiates from simple sugar sources. Completion of this strain will require functional expression of at least three known enzymes, including one cytochrome P450 to catalyze the conversion of (R)-reticuline to salutaridine. Synthetic biology tools will be key to addressing the many challenges encountered in engineering this and other plant natural product biosyntheses in yeast. Novel molecular tools will be needed to reintroduce regulation and direct pathway flux to the high yielding accumulation of target molecules. Finally, synthetic biology tools will play a critical role in engineering control mechanisms to secure yeast strains for the legitimate production of drugs with the potential for misuse. Genetically encoded safety measures including conditional selection markers, cell cycle controllers, genetic barcodes, and rare auxotrophies can help make engineered yeast a secure, sustainable, and scalable production platform for BIA therapeutics.

Online methods

Plasmid and yeast strain construction

Modern molecular biology techniques were used to construct the plasmids and strains described in this work. The parent *S. cerevisiae* strain from which all strains described here were constructed was haploid W303α (MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15). Yeast synthetic complete (SC) amino acid dropout media with 2% dextrose and complex Yeast Peptone Dextrose (YPD) medium containing 200 mg/L G418 sulfate (Calbiochem) were used for strain construction. Chemically-competent *E. coli* strain TOP10 was used for cloning purposes (Life Technologies) and was grown in LB medium (Becton, Dickinson and Company (BD)) with the indicated antibiotic concentrations. Custom oligonucleotides were synthesized by Integrated DNA Technologies (IDT, Coralville, IA) and the Stanford Protein and Nucleic Acid Facility (Stanford, CA). All heterologous gene sequences (Supplementary Table 1) were downloaded from GenBank, codon optimized for expression in *S. cerevisiae* using the GeneArt GeneOptimizer program (Life Technologies), and synthesized by GeneArt (Life Technologies) or IDT. All yeast endogenous promoters, terminators, and the organelle targeting sequence were amplified from W303α genomic DNA (Supplementary Table 1). Polymerases used for PCR were Pfu Hotstart (Agilent Technologies) for products less than 2 kb and Expand High Fidelity PCR System (Roche Applied Science) for products greater than 2 kb. Plasmids were prepared from *E. coli* using QIAprep columns (Qiagen) and Econospin columns (Epoch Life Science) according to manufacturer's instructions. Sequencing was performed by Elim Biopharmaceuticals, Inc (Hayward, CA).

To express heterologous genes from *P. somniferum* and *P. putida* in W303α, individual expression cassettes were constructed which comprised an open reading frame flanked by a unique promoter and terminator (Supplementary Table 1) and incorporated into the pYES1L vector. In the initial construction of expression cassettes, individual genes were combined with promoters and terminators by Splicing by Overlap Extension (SOEing) PCR and recombined into Gateway vector pDONR221 using BP Clonase II (Life Technologies) for sequence verification and storage. Expression cassettes were then PCR amplified using oligonucleotides designed by the GeneArt High-Order Genetic Assembly online tool (Life Technologies), which adds homology regions for gap repair in yeast. 100 ng of each expression cassette PCR was combined with 100 ng linearized pYES1L (Life Technologies) and transformed into W303α by electroporation. The pYES1L vector contains TRP1 for selection on tryptophan dropout media and an ARS4/CEN5 region such that each newly constructed vector was maintained as a single-copy, episomal plasmid in yeast (Supplementary Fig. 8). pYES1L constructs were verified by PCR screening and sequencing. To propagate the plasmid so that it could be transformed into other yeast background strains, the pYES1L vector was isolated from yeast and transformed into TOP10 *E. coli* (Life Technologies), where it was maintained in single copy and selected on LB media with 50 mg/L spectinomycin dihydrochloride pentahydrate (Sigma-Aldrich). Approximately 2 μg plasmid, enough to transform up to 10 yeast strains, was prepared from 100 mL overnight *E. coli* culture.

To integrate additional gene copies into the yeast genome, expression cassettes comprising a gene flanked by a promoter and terminator were produced by PCR amplification of pUG vectors, which were modified to enable Gateway Cloning (Life Technologies). Vectors pUG6 and pUG73[44] containing KanMX and *Kluyveromyces lactis* LEU2 selection markers, respectively, were modified to include a GPD promoter and CYC1 terminator flanking the Gateway cassette attR1-ccdB/Cam^R-attR2 to generate two new pDEST vectors called pCS2643 and pCS2644 (Supplementary Fig. 9). Individual pENTR vectors each containing a gene open reading frame prefixed by a Kozak sequence were recombined with the pDEST vectors. From the resulting vectors, the gene expression cassette and adjoining selection marker (KanMX or LEU2) flanked by *loxP* sites was PCR amplified with oligonucleotides that added 103 bp of homology to the target integration site in the yeast genome (Supplementary Table 5). The PCR product was transformed into W303α by standard lithium acetate transformation. Integration events were selected by growth on G418 or leucine-dropout media and confirmed by PCR screening of both integration borders and by sequencing. The *loxP* sites were used to remove the selection marker by expression of Cre recombinase[45].

Other plasmids were constructed from the Lindquist suite of destination vectors, especially pAG416GPD-ccdB[46]. Vectors for microscopy were constructed with the ER1 targeting 28 amino acid sequence ILEQPLKFVLTAAVVLLTTSVLCCVVFT from CNE1 (Genbank ID#: AAA65967.1)[47]. ER1 was fused to the C-terminus of GFP and COR1.3 separated by a ProGly$_6$ linker. The targeting sequence, linker and protein were combined by SOEing PCR and cloned into pDONR221 using BP clonase II (Life Technologies). The resulting entry vector was recombined into pAG416GPD-ccdB destination vector using LR clonase II (Life Technologies) to create a shuttle vector for expression in yeast. The established endoplasmic reticulum marker Kar2-DsRed-HDEL was subcloned from YIPlac204TKC-DsRed-Express2-HDEL (Addgene plasmid 21770)[48] into a high-copy TRP vector (YCplac22 backbone).

Culture and fermentation conditions

To assay for opioid production, yeast strains were cultured in 96-well plates (BD Falcon) with 0.4 mL SC growth medium (tryptophan-dropout, 2% dextrose) per well and incubated in a Kuhner LT-X plate shaker (Kuhner AG) at 30°C, 480 rpm agitation, 1.24 cm orbital diameter, with 80% humidity. Strains were initially inoculated into SC medium with 0.5 g/L glutamine (Calbiochem) substituted for ammonium sulfate as the nitrogen base and grown for 16 h. Cultures were then back-diluted 40× into SC medium with 0.5 g/L glutamine, 2.5 g/L monosodium glutamate (Sigma-Aldrich), 50 mM 2-oxoglutarate (Sigma-Aldrich), and 1 mM thebaine (Sigma-Aldrich). As thebaine has reduced solubility in aqueous solution, a 50 mM stock solution was first prepared in methanol. Strains were grown 96 h or until morphine production by the control strain reached approximately 2.5 mg/L. To determine cell density the final OD_{600} (after 10× dilution) was measured on a TECAN Safire microplate reader (Mäanedorf, Switzerland).

For enhanced closed-batch culture conditions, strains were cultured in a Biostat Q-plus bioreactor with 0.5 L univessel size (Sartorius Stedim Biotech). Initial medium volume was

250 mL and contained 10× SC tryptophan dropout medium components supplemented with 5 g/L glutamine, 25 g/L MSG, 100 mM 2-oxoglutarate, and 1 mM thebaine. Glucose concentration was 10% and the medium was further supplemented with 2 g/L adenine hemisulfate. Each vessel was inoculated with a 10 mL overnight culture grown in selective medium which was pelleted and the cells resuspended in the fermentation medium before addition to the univessel. Process parameters were kept constant during the fermentation at 30°C, 200 rpm stirring, and 2 L/min compressed air flow rate. At appropriate time points cell density was recorded from diluted samples measured in a cuvette on a Nanodrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA) and additional samples were taken for metabolite analysis.

Analysis of opiate production

Opiates secreted into the culture medium by the engineered yeast strains were identified and quantified by high performance liquid chromatography mass spectrometry (HPLC-MS). Cultures were pelleted by centrifugation and 5 μL of the supernatant separated on a Zorbax SB-Aq column (3.0 × 50 mm, 1.8 μM particle size) (Agilent Technologies). The column was equilibrated with water, 0.1% acetic acid, and 0.1% methanol (Solvent A) and samples were eluted with a mobile phase of methanol and 0.1% acetic acid (Solvent B) in the following sequence: 0-1 min at 100% A, 1-4 min 0-25% B, 4-7 min at 25% B, followed by steps to clean the column with 100% B then re-equilibrate in A. The flow rate was held constant at 0.6 mL/min. Eluted opiates were identified on an Agilent 6320 Ion Trap mass spectrometer (Agilent Technologies) operated in scan mode for total ion monitoring to identify peaks with *m/z* and retention time consistent with commercially available standards spiked into spent yeast culture medium (Supplementary Fig. 1). Commercially acquired standards were thebaine (Sigma-Aldrich, >99% purity), codeine sulfate (US Pharmacopeia, 100% purity), morphine sulfate pentahydrate (Sigma-Aldrich, >98% purity), hydrocodone bitartrate (Sigma-Aldrich, >98% purity), oxycodone hydrochloride (Sigma-Aldrich, >97% purity), hydromorphone hydrochloride (Sigma-Aldrich, >98% purity), dihydromorphine (Sigma-Aldrich, 1.0 mg/mL in methanol), codeinone (Noramco, 95.5% purity), oripavine (Noramco, 98.6% purity), morphinone (Noramco, 93.3% purity), dihydrocodeine (Noramco, 95.8% purity), and 14-hydroxycodeine (Noramco, 98.8% purity). The identity of each compound was confirmed by comparing MS2 spectra of samples and coeluting standards (Supplementary Fig. 1). Compounds were quantified by comparing the peak area of the extracted ion chromatograms to standard curves for each molecule. Where standards were unavailable (neopine and neomorphine), MS2 spectra and, when sufficient yields were obtained for isolation, ^{1}H-NMR spectra were compared to published spectra (Supplementary Figs. 1-3 and Supplementary Table 2)[49,50] .

To obtain an ^{1}H NMR spectrum of neopine, the molecule was isolated by extraction and HPLC and analyzed on a Varian Inova 500 MHz spectrometer (Varian, Inc). CSY960 was cultured in 5 1-L flasks with 100 mL of uracil dropout media, 2% dextrose, and 1 mM thebaine for 1 week at 30°C. Yeast cells were separated from the media by centrifugation for 5 min at 6000xg. The supernatant was extracted three times with one-third volume of dichloromethane (DCM). The resulting organic extracts were combined and evaporated *in vacuo*. The crude residue was then dissolved in 500 μL 1:1 HPLC grade acetonitrile

(CH_3CN)–water with 0.1% formic acid (FA). Some insoluble material remained. The residue was purified by reverse-phase HPLC (Pursuit XRs-C18, 5 μm, 50 mm ×10 mm) with a linear gradient of 5 to 25% acetonitrile with 0.1% FA (CH_3CN, v/v in water) over 5.5 min and 100% CH_3CN (v/v) for 4.4 min with a flow rate of 5 mL/min and injection volume of 30 μL. Peak-based fractions were collected at approximately 1.35 min – 1.75 min. Fractions were pooled and freeze dried. The residue was dissolved in 1 mL DCM, washed three times with 300 μL pH 8 0.5 M phosphate buffer, and dried *in vacuo*. 1D proton NMR analysis was performed on a Varian Inova 500 spectrometer with $CDCl_3$ as the solvent and residual $CHCl_3$ as reference (Supplementary Fig. 2 and Supplementary Table 2).

For analysis of bioreactor culture medium, samples were diluted between 2- and 10-fold and separated on a Zorbax SB-Aq column (3.0 × 250 mm, 5 μM particle size) (Agilent Technologies). The column was equilibrated with water, 0.1% acetic acid, and 0.1% methanol (Solvent A) and samples were eluted with a mobile phase of methanol and 0.1% acetic acid (Solvent B) in the following sequence: 0-10 min at 100% A, 10-30 min 0-90% B followed by steps to clean the column with 100% B then re-equilibrate in A. Flow rate was held constant at 0.8 mL/min. Eluted opioids were identified and quantified on an Agilent 6320 Ion Trap mass spectrometer as described above.

Confocal microscopy

Yeast cells harboring the appropriate plasmids were grown in SC dropout media then 1.5 mL was pelleted and resuspended at high cell density (in <100 μL medium). Slides were prepared by placing a 2% low-melting point agarose pad combined with yeast media on a microscope slide, spotting 1 μl of yeast cells on the agarose pad, covering with a No. 1. coverslip, and sealing. Cells were imaged on a Leica TCS SP5 confocal microscope (Leica Microsystems) with a 63.0x glycerine immersion objective, 1.30x numerical aperture, and up to 8x digital zoom. Hybrid detector (HyD) smart gain was adjusted from 30-200% depending on sample fluorescence intensity. As an example of the fluorescence settings, for single-color imaging of GFP the sample was excited with laser line 488 nm and emitted fluorescence was recorded by a HyD channel in the range 500-550 nm (dichroic mirror = DD 488/594). Images were recorded with an airy1 pinhole size (108.4 μm), a minimum of 2x line averaging, a pixel size of 30-100 nm, and optical section thickness of 0.856 μm. Scale bars of 4 μm were added within the Leica Application Suite Advanced Fluorescence (LAS AP) software. Images were exported from LAS AP and brightness and contrast were adjusted in ImageJ.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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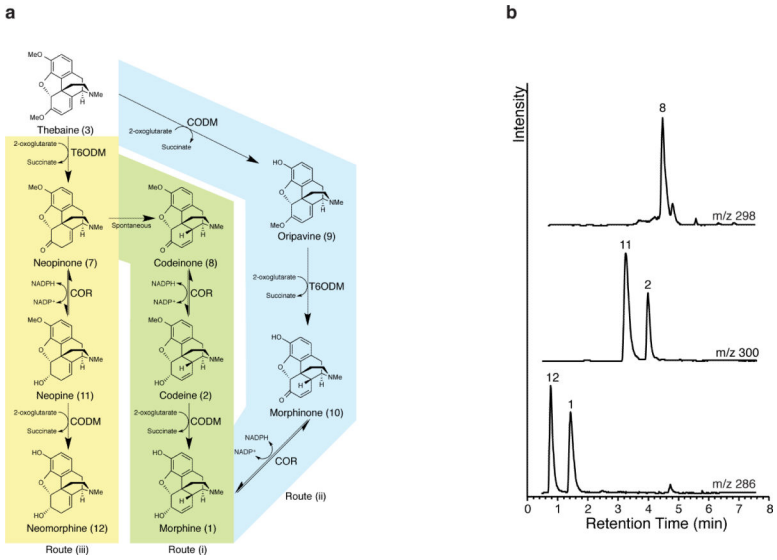

Figure 1. Engineering a heterologous morphine biosynthesis pathway in yeast

(a) The schematic depicts observed transformations of thebaine by the morphine biosynthesis enzymes - thebaine 6-*O*-demethylase (T6ODM), codeine *O*-demethylase (CODM), and codeinone reductase (COR) from opium poppy *P. somniferum*. Two routes to morphine which pass through intermediates codeinone and codeine (route i) and oripavine and morphinone (route ii) occur in opium poppy. Our work demonstrates that route (i) and a newly-identified route to neomorphine (iii) occur in the heterologous context of a yeast cell, revealing a broader substrate range for COR and CODM than previously reported. (b) LC-MS analysis of opiates produced from an engineered yeast strain (CSY907) expressing *T6ODM*, *COR*, and *CODM* from a pYES1L vector. Cells were cultured with 1 mM thebaine for 96 h in deep-well plates prior to analysis of the culture medium. Labeled peaks corresponding to opiate molecules are shown on the extracted ion chromatograms as: 8, codeinone (*m/z* 298, RT=4.5 min); 2, codeine (*m/z* 300, RT=4.0 min); 11, neopine (*m/z* 300, RT=3.2 min); 1, morphine (*m/z* 286, RT=1.4 min); 12, neomorphine (*m/z* 286, RT=0.8 min). The identities of opiates detected in the culture medium were confirmed by comparison to the MS2 spectra and retention times of purchased standards where available, or by NMR analysis (Supplementary Figs. 1-3, Supplementary Table 2). Empty vector control strains did not perform transformations of thebaine to other opiates (Supplementary Fig. 1).

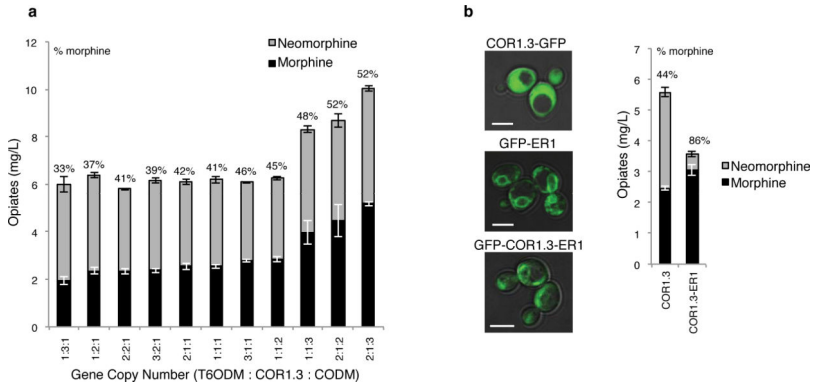

Figure 2. Altering gene copy number and localizing COR1.3 to the endoplasmic reticulum increases pathway specificity for morphine

(a) Titers of the target product morphine (black bars) and non-target neomorphine (grey bars) were analyzed from strains harboring different numbers of copies of *T6ODM*, *COR1.3*, and *CODM*. The culture medium was analyzed by LC-MS for opiate production after 96 h growth in deep-well plates with 1 mM thebaine. Each strain expressed one copy of *T6ODM*, *COR1.3*, and *CODM* (Supplementary Table 1), on a pYES1L vector (Supplementary Fig. 8). Additional gene copies were integrated into the host cell genome at the *ura3*, *his3* and *leu2* loci (Supplementary Table 3). (b) A spatial engineering approach was applied to delocalize COR1.3 from T6ODM activity. A COR1.3-GFP fusion protein was observed to localize to the yeast cytoplasm, similar to T6ODM and CODM (Supplementary Fig. 6). An endoplasmic reticulum localization tag (ER1) was fused to the C-termini of GFP (GFP-ER1) and a GFP-COR1.3 fusion protein (GFP-COR1.3-ER1). Confocal microscopy confirmed ER-localization of these ER1-tagged proteins. Cytoplasmic, untagged COR1.3 and ER-localized COR1.3-ER1 were each expressed together with T6ODM and CODM from a pYES1L vector. Strains were cultured in optimized media with 1 mM thebaine, grown for 96 h, and the culture medium analyzed for morphine (black bars) and neomorphine (grey bars) by LC-MS. Bars represent mean values ±1 s.d. of three biological replicates. The percentage of morphine (out of the sum total of morphine and neomorphine) is displayed above each bar. Image scale bars, 4 μm.

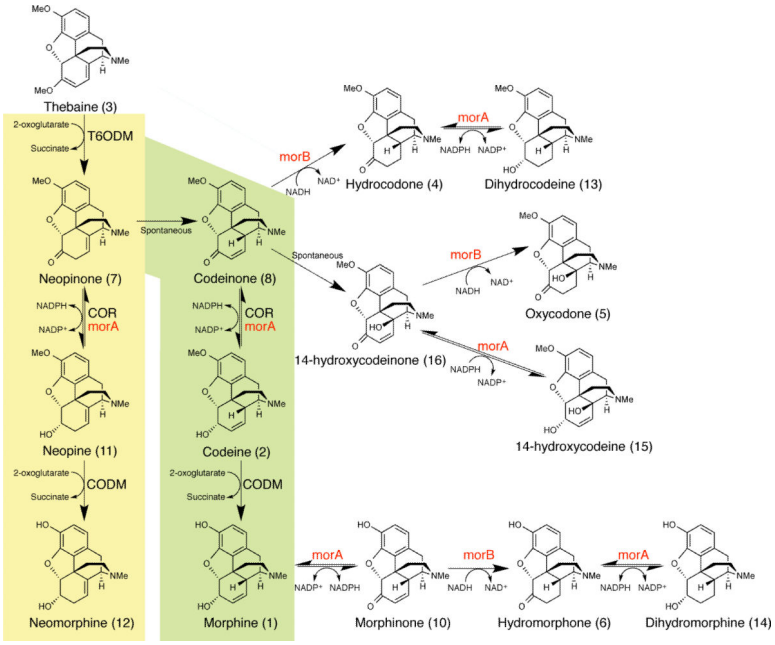

Figure 3. Incorporating bacterial enzymes allows for the biological synthesis of semi-synthetic opioids

Schematic depicting the extended transformations of thebaine in yeast by incorporating morA, morphine dehydrogenase, and morB, morphine reductase, from *Pseudomonas putida* M10 into the heterologous pathway.

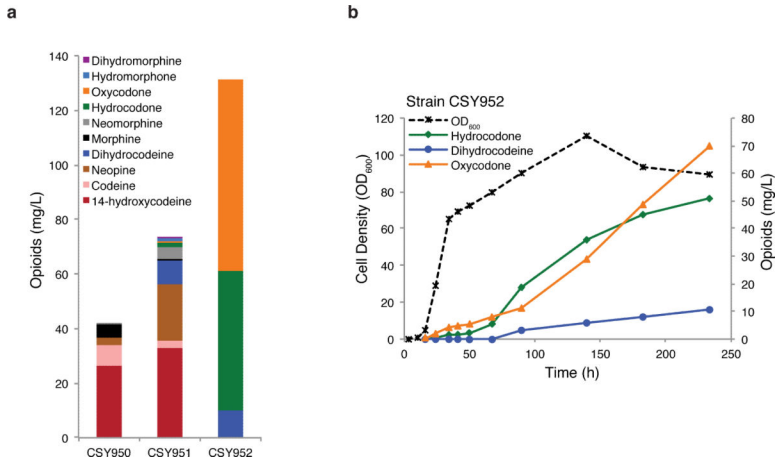

Figure 4. Optimized yeast strains for the production of diverse opioids

(a) Total opioid molecule concentration in the culture medium after closed-batch fermentation. Yeast strains CSY950, CSY951, and CSY952 (Supplementary Table 3) were optimized for the production of morphine, hydromorphone, and hydrocodone/oxycodone, respectively. The indicated strains were cultured in closed batch fermentations in media supplemented with 1 mM thebaine (equivalent to 311 mg/L). Culture medium was analyzed at the end of the fermentation for a panel of opioids through LC-MS. (b) Cell density and concentrations of key opioids (hydrocodone, dihydrocodeine, and oxycodone) as a function of time for the fermentation of yeast strain CSY952. At indicated time points, samples were taken, diluted, and analyzed for cell density through spectrometry and opioid production through LC-MS.

Table 1

Opioids produced by yeast strains incorporating bacterial enzymes morA and morB

Engineered yeast strains expressing different combinations of *P. somniferum* and *P. putida* M10 enzymes from a pYES1L vector produced different titers of target opioids. Cells were cultured with 1 mM thebaine for 96 h and the final culture medium was analyzed by LC-MS. Data represent mean values ±1 s.d. of three biological replicates.

	Opiates (mg/L) in Culture Medium				
Genes Expressed:	**Morphine**	**Neomorphine**	**Hydrocodone**	**Oxycodone**	**Hydromorphone**
T6ODM, CODM, COR1.3, morA, morB	0.21 ± 0.01	2.08 ± 0.15	trace	-	-
T6ODM, CODM, morA	2.36 ± 0.04	1.04 ± 0.02	-	-	-
T6ODM, CODM, morA, morB	0.26 ± 0.01	1.68 ± 0.15	1.34 ± 0.12	trace	0.10 ± 0.01
T6ODM, CODM, morA, morB^{E160G}	0.39 ± 0.06	1.79 ± 0.28	0.91 ± 0.14	trace	0.14 ± 0.01
T6ODM, morB	-	-	6.48 ± 0.23	2.12 ± 0.15	-