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COMMUNICATION

An industrially applicable *Escherichia coli* platform for bioconversion of thebaine to oripavine and codeine to morphine

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A whole cell *Escherichia coli* biotransformation platform converting thebaine to oripavine and codeine to morphine was demonstrated with industrially applicable yields (~1.2×10^{-2} g/(L·h) or ~1.2×10^{-1} g/(L·h)), improving > 13,400-fold upon morphine production in yeast. Mutations enhanced enzyme performance and use of purified substrate with rich raw poppy extract expands applicability.

The biosynthetic production of opioids from simple sugars could reduce agricultural cultivation and provide an alternative to organic synthesis. To this end, complete biosynthesis of two opioids has recently been demonstrated in *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Escherichia coli* (*E. coli*).[1, 2] Whilst representing significant progress, the low yields obtained indicate further optimisation is needed for efficient production.

An alternative application for these same enzymes is the interconversion of different opioids in single step reactions or shorter coupled enzymatic steps. This technology would allow manufacturers to respond more quickly to market demand than the current agricultural cycle and to make larger quantities of opioids such as oripavine,[3-5] which is needed to meet the growing demand for oripavine derived semi-synthetic antagonists (namely, naloxone and naltrexone).[3, 4, 6] Currently, poppies with high oripavine also produce other opiates, particularly thebaine, using a biosynthetic approach could allow opiate production to be balanced with demand, particularly if market demands change post-planting.

Codeine *O*-demethylase (CODM) is a key enzyme for *in planta* generation of morphinan alkaloids, which has potential in both complete biosynthesis or shorter enzymatic transformations. The *O*-demethylation reactions performed by this 2-oxoglutarate/Fe(II)-dependant dioxygenase, which generate oripavine and morphine, are shown in Fig. 1A[7], together with their place within the larger biosynthetic pathway in poppy (see ESI, Fig. S1A). *In vitro,* the purified CODM enzyme displays a high substrate specificity for codeine (k_{cat}/K_M of 785.4 $s^{-1}M^{-1}$) and moderate specificity for thebaine (k_{cat}/K_M of 235.2 $s^{-1}M^{-1}$).[7] Both reactions are of interest, as *O*-demethylations are challenging to perform synthetically due to low yields and the requirement for harmful chemicals.[8]

Despite the potential utility of CODM and key role of CODM in the biosynthetic pathway for production of opiates, including oripavine and morphine (see ESI, Fig. S1A), several studies have reported suboptimal enzyme performance and the potential bottleneck caused by CODM when the pathway is reconstructed in yeast.[9-12] This is likely a result of low enzyme activity and/or poor enzyme expression.[9-12] No morphine was generated when the enzymes for the multi-step conversion of (*R*)-reticuline to morphine were assembled in *S. cerevisiae*, including CODM.[10] Using the same CODM strain, a low conversion yield of 0.5% of codeine to morphine was observed using codeine as the substrate.[10] Conversion of thebaine to morphine was also slightly increased to 1.6%, when the gene copy number of CODM and a second enzyme, thebaine 6-*O*-demethylase (T6ODM), were increased in a separate study, while also supplementing the media with the cofactor 2-oxoglutarate, needed by the two enzymes.[9] These studies show how effective CODM activity is critical to successful bioconversion. A further undesirable CODM property is the formation of by-product neomorphine from neopine[9, 11, 12] CODM is therefore a good target for mutagenesis.

Previous mutations of CODM have increased enzyme activity, with mixed success.[13, 14] A CODM mutant with slightly reduced activity for codeine and much reduced activity for thebaine was created by swapping conserved regions between CODM and T6ODM.[14] In contrast, the CODM mutant N35S+G335V identified through random mutagenesis, produced ~1.4 times more morphine and ~2.6 times more neomorphine from thebaine, than wild type (WT) CODM within a *S. cerevisiae* host, although the enzyme performance is not yet fully described.[13]

Here, we report a whole cell *E. coli* biotransformation system featuring CODM that can efficiently produce either oripavine or morphine from the substrates thebaine or codeine,

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respectively, with yields up to 95-97%. We chose *E. coli*, as this system has successfully been used to increase the titre of other metabolites.[15] We examine the effect of naturally occurring genomic variability in *Papaver somniferum* (*P. somniferum*) CODM sequences on opiate yield and assess how sequence variability in this region impacts on biotransformation performance. Subsequent site-directed mutagenesis was used to generate CODM mutants, which display greater enzyme expression and increased yields of oripavine from thebaine and morphine from codeine.

There are three copies of the *CODM* open reading frame (ORF) within the *P. somniferum L.* HN1 genome: *CODM.1, CODM.2 and CODM.3*.[16, 17] (see ESI, Fig. S1B). Such diversity likely arose from duplication and rearrangement of the *P. somniferum* genome.[18] *CODM.1* and *CODM.2* are identical at the amino acid level, with a glutamic acid at position 259,[7] while *CODM.3* contains a lysine at this position (E259K).[16, 17] As *CODM.2* ORF was reported first and is most widely used, we refer to the CODM.2 protein sequence as WT CODM here. We first set out to determine whether WT CODM could demethylate the substrates thebaine and codeine in a whole-cell biotransformation system. Whilst there are some data demonstrating limited enzyme activity in yeast, there are no reports of CODM activity within *E. coli.* The *E. coli* codon optimised WT CODM enzyme was first overexpressed in BL21 (DE3) cells, then a solution of dipotassium phosphate with cofactors and 0.3 g/L (~1 mM) of opiate substrate was used for a biotransformation reaction.[7, 19] Strains were screened by testing after 4 hours for oripavine and after 30 minutes for morphine. Whole cells were selected, using an approach previously taken to screen a tyrosine hydroxylase enzyme for L-3,4-dihydroxyphenylalanine yield,[20] as this is the likely format for commercial use, allowing 2-oxoglutarate to be supplied through host central metabolism and avoiding supplementation.[19]

The WT CODM expressed in *E. coli* successfully *O*-demethylated thebaine and codeine. A thebaine to oripavine yield of 46% ± 2% was achieved after 4 h, while a codeine to morphine yield of 41% ± 6% was achieved after 30 min (see ESI, Table S2, Fig. S5) giving a time space yield (TSY) of 3.3×10^{-2} ± 1.6×10^{-3} g/(L·h) and 2.4×10^{-1} ± 4.0×10^{-2} g/(L·h). The faster demethylation of codeine is consistent with *in vitro* assays using purified WT CODM.[7] The yield is much higher than for WT CODM in *S. cerevisiae*, where only a 0.5% of codeine was converted to morphine after approximately 16 hours[10] and the equivalent TSY was ~8.9×10^{-6} g/(L·h) (i.e., > 20,000 fold increase in TSY). The system examined here, containing just the CODM enzyme, did not produce any by-products.

Position E259 in CODM was selected for mutagenesis, due to the natural variation at this site, specifically the activity of the *CODM.3* enzyme variant, E259K, was assessed to determine if this single amino acid residue alters whole cell biotransformation in *E. coli*. The position of residue 259 within a CODM homology model is shown in ESI, Fig. S1C, which was built using T6ODM,[21] as the structure of CODM has not yet been determined. Residue 259 is located distal from the active site, within a short surface exposed flexible loop between β-sheets 8 and 9.

Although the E259K strain successfully converted thebaine to oripavine and codeine to morphine, conversion was lower than for WT CODM with ~17% decrease and ~11% decreased in mean yield respectively (Fig. 1, and see ESI, Table S2). As whole cells were employed, any changes in yield between strains are the result of changes in both enzyme expression and enzyme activity.[20] In this instance, the amount of soluble E259K CODM enzyme was notably lower than the WT CODM enzyme (see ESI, Fig. S2). Residue 259 occurs within a highly charged flexible loop (see ESI, red box in Fig. S1C), flanked N-terminally by lysine and glutamic acid and on the C-terminal side by arginine (K257-E258-E259-R260).[21] The reduction in solubility indicates the positively charged lysine at residue 259, rather than the negatively charged glutamic acid, is important for CODM expression in *E. coli*. This reduced performance also indicates that the activity of the CODM enzyme could be further optimised using residue 259.

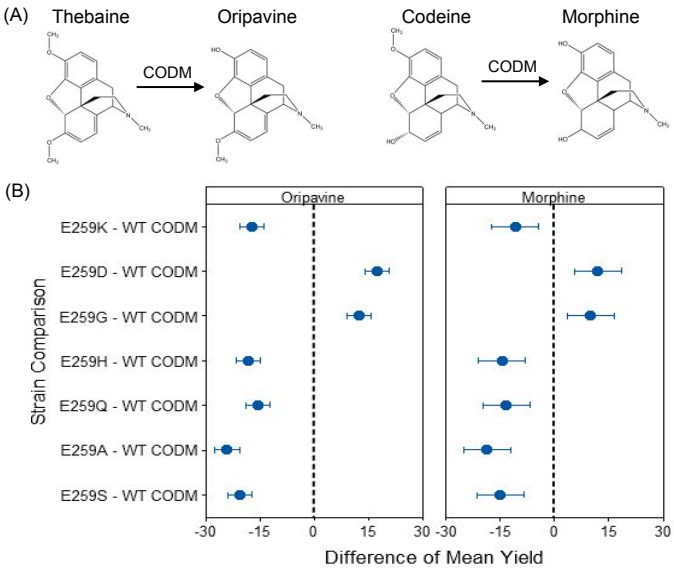

Fig. 1 (A) CODM *O*-demethylation of thebaine to oripavine or codeine to morphine. (B) Difference in the mean yield of oripavine and morphine for *E. coli* strains with CODM mutants at position 259 at 4h or 30 min respectively. Data are mean of three independent replicates ± 95% confidence interval.

A site-directed mutagenesis strategy was next employed to explore how other mutations at residue 259 affect strain performance (see ESI, Table S1 and S2). A group of amino acids (D, G, H, Q, A, S) were selected to rapidly assess the effect of a diverse range of side chain chemistry (size, polarity, hydrophobicity and charge) on performance; an approach recently employed to alter the regio- and stereoselectivity of a P450 enzyme that increases the efficiency of mutant screening.[22]

Strains expressing the CODM variants E259D or E259G, featuring an aspartic acid or glycine at residue 259, displayed significantly improved capacity for biotransformation (Fig. 1B). The E259D strain generated ~17% higher yield of oripavine and ~12% higher morphine yield than the WT CODM strain in the

same timeframe (Fig. 1, and see ESI, Table. S2). The E259G strain also generated ~12% and ~10% higher yields respectively (Fig. 1, and see ESI, Table. S2). This corresponded to an improved TSY of ~4.5×10^{-2} g/(L·h) and ~4.1×10^{-2} g/(L·h) at 4 hours for oripavine and ~3.2×10^{-1} g/(L·h) and ~3.0×10^{-1} g/(L·h) at 30 min for morphine, respectively. The four other variants, with histidine, glutamine, alanine, or serine at residue 259 displayed at least 13% lower yield for both biotransformations (Fig. 1, and see ESI, Table. S2).

Enzyme solubility may contribute to the performance of E259D and E259G expressing strains, as these had a greater concentration of soluble enzyme compared to WT CODM, while the four other strains had less soluble enzyme than the WT CODM (see ESI, Fig. S2). This observation is consistent with the hypothesis that CODM use in biosynthetic systems is influenced by poor heterologous expression of this enzyme.[9-12] The E259D and E259G strains may therefore be superior to the WT CODM strain for opioid biotransformation.

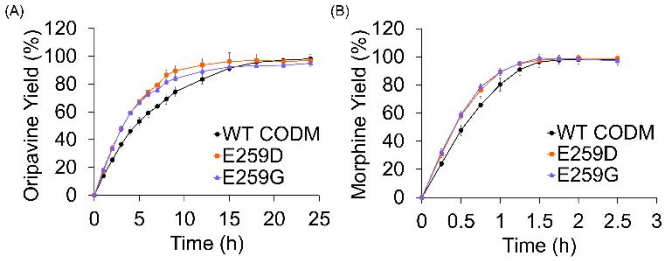

Fig. 2 Oripavine (A) or morphine (B) yield as a function of time using either thebaine or codeine as substrate and *E. coli* strain expressing WT CODM, E259G strain or E259D strain. Data are mean ± standard deviation of three independent replicates.

The effect of mutagenesis at site 260 of CODM was assessed next, by generating strains R260T and R260K, which introduced threonine or lysine. Residue 260 was selected, as the *P. somniferum* paralogous enzymes papaverine 7-*O*-demethylase (P7ODM), T6ODM and protopine *O*-dealkyase (PODA)[7, 23] contain threonine or lysine at this equivalent site, as well as aspartic acid or glycine residues at equivalent residue 259 (see ESI, Fig S1B), suggesting that threonine or lysine at residue 260 alone and in combination with the mutations at residue 259 may improve CODM activity. The double mutant strains; E259G+R260T, E259D+R260K, E259G+R260K were also assessed.

Although strains R260T and R260K did not outperform the WT CODM strain, including double mutants (see ESI, Fig. S3, Fig. S4, Table. S3), they illustrate how non-conserved residues can be used to identify amino acids impacting performance and further site-directed mutagenesis may improve performance.

Having identified the strains E259D and E259G with better capacity for biotransformation, we next investigated their performance over the course of a biotransformation reaction. Near complete conversion of thebaine (≥ 95%), was achieved for all strains, including the WT CODM after 24 hours (Fig. 2). Earlier timepoints revealed a difference in kinetics between strains. By 9 hours, E259D generated 90 ± 4 % oripavine, E259G 84 ± 2 % and the WT CODM 75 ± 4 % (Fig. 2A, and see ESI, Table S4). All strains produced similar high conversions (≥ 97%) for morphine within 2.5 h, with earlier timepoints such as 0.75 h again showing a difference, with E259D generating 76 ± 4 % morphine, E259G 79 ± 4 % and WT CODM 66 ± 5 % (Fig. 2B, and see ESI, Table S5). These observations are consistent with measurements made at four hours or 30 minutes in earlier assays (Fig. 1B). With optimised downstream processing, the faster kinetics observed for the E259D and E259G strains could contribute to improved economic feasibility relative to the WT CODM strains.

The bioconversion of thebaine by CODM described here may also be applied alongside the recently described thebaine to codeine *E. coli* cell compartmentalization process,[19] offering greater flexibility in the management of opiate stock by using thebaine as a common starting material to produce oripavine, codeine or morphine to meet market demands.

A second attractive substrate for biotransformation is raw poppy extract. From a thebaine poppy, this feedstock contains extracted thebaine alongside other opiates and plant impurities (Fig. 3A).[24] Successful biotransformation of crude feedstock would be cheaper than using purified thebaine, due to reduced processing steps. To investigate this possibility, raw poppy extract from a high thebaine poppy cultivar with an equivalent thebaine concentration (0.3 g/L) was used in proof-of-concept experiments with WT CODM, E259D and E259G.

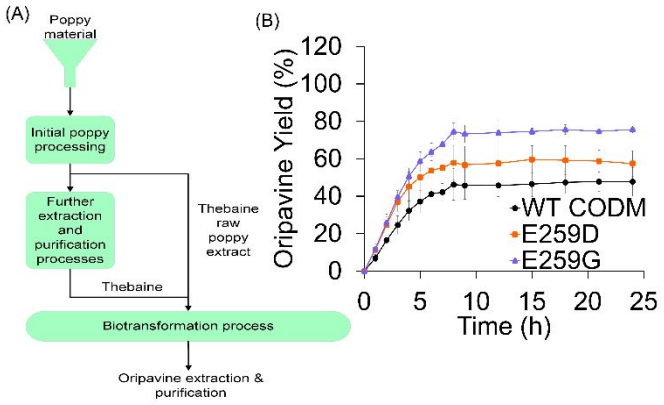

Fig. 3 Flow diagram with the origins of biotransformation substrates (A). Oripavine yield (B) as a function of time using thebaine raw poppy extract as substrate for *E. coli* strains expressing WT CODM or mutants E259D or E259G. Data are mean ± standard deviation of three independent replicates.

Oripavine was successfully produced from the thebaine in raw poppy extract by whole cell biotransformation for all three strains (Fig. 3B, and see ESI, Table S6, Fig. S5), although the reaction slowed after ~8 hours, leading to a 30-60% lower yield compared to using pure thebaine (Fig. 2). This incomplete conversion suggests poppy straw impurities may inhibit biotransformation.[25-27] The greater yield observed for E259G using raw poppy extract (Fig. 3B), could also arise from altered enzyme stability, as computational predictions using PremPS or INPS-Seq [28-30], indicate E259G could be more stable than E259D (see ESI, Table. S7). Both mutants had greater yield than the WT CODM. Further experiments could identify the mechanism of inhibition and compare the activities of purified mutant CODMs to assess catalytic rate and stability, as it would be interesting to understand why these mutants behave differently with

poppy straw extract compared to pure thebaine substrate. The biotransformation of raw poppy extract containing codeine would also be worthy of further study.

The reduced number of processing steps using raw poppy extract (Fig. 3A), may make this approach worthy of further investigation, even with the small reduction in TSY ($2.7\times10^{-2} \pm 9.6\times10^{-4}$ g/(L·h)) compared to pure thebaine ($2.9\times10^{-2} \pm 6.0\times10^{-4}$ g/(L·h)) using E259G at the end of reaction (8 h) (Fig. 3B, Fig. 2A, and see ESI, Table S6, Table S4).

In this work, CODM was placed in an *E. coli* host to create a whole cell biotransformation platform that could successfully convert ≥ 95% of thebaine to oripavine and ≥ 97% of codeine to morphine with a high productivity (TSY of ~1.2×10^{-2} g/(L·h) at 24 hours and ~1.2×10^{-1} g/(L·h) at 2.5 hours), advancing on the low yield previously reported for WT CODM in yeast. This research expands the diversity of opiates that can be effectively produced at high yield in parallel or sequential reactions in *E. coli* and facilitates a more sustainable and flexible route for production of essential opiates. Residue 259 in CODM was found to impact significantly on enzyme expression and performance, with two mutants, E259D and E259G, leading to improved soluble expression within *E. coli,* a faster biotransformation process and higher TSY at earlier timepoints. The CODM enzymes described may have broader application within multistep or complete pathways for opioid biosynthesis. The successful use of raw poppy extract will also enhance flexibility for manufacturers, whilst potentially increasing the economic viability of enzymatic biotransformation.

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Author contributions

G.W.K. Spencer performed experiments and drafted the manuscript. X. Li assisted with experiments, drafting and co-supervised together with A. Jarrold and S. L. Gras. S.L. Gras acquired funding and wrote/revised the manuscript.

Conflicts of interest

There are no conflicts to declare.

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