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Isoprenoid Pathway Optimization for Taxol Precursor Overproduction in *Escherichia coli*

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Abstract

Taxol (paclitaxel) is a potent anticancer drug first isolated from the *Taxus brevifolia* Pacific yew tree. Currently, cost-efficient production of Taxol and its analogs remains limited. Here, we report a multivariate-modular approach to metabolic-pathway engineering that succeeded in increasing titers of taxadiene—the first committed Taxol intermediate—approximately 1 gram per liter (~15,000-fold) in an engineered *Escherichia coli* strain. Our approach partitioned the taxadiene metabolic pathway into two modules: a native upstream methylerythritol-phosphate (MEP) pathway forming isopentenyl pyrophosphate and a heterologous downstream terpenoid–forming pathway. Systematic multivariate search identified conditions that optimally balance the two pathway modules so as to maximize the taxadiene production with minimal accumulation of indole, which is an inhibitory compound found here. We also engineered the next step in Taxol biosynthesis, a P450-mediated 5α-oxidation of taxadiene to taxadien-5α-ol. More broadly, the modular pathway engineering approach helped to unlock the potential of the MEP pathway for the engineered production of terpenoid natural products.

Taxol (paclitaxel) and its structural analogs are among the most potent and commercially successful anticancer drugs (1). Taxol was first isolated from the bark of the Pacific yew tree (2), and early-stage production methods required sacrificing two to four fully grown trees to secure sufficient dosage for one patient (3). Taxol's structural complexity limited its chemical synthesis to elaborate routes that required 35 to 51 steps, with a highest yield of 0.4% (4–6). A semisynthetic route was later devised in which the biosynthetic intermediate baccatin III, isolated from plant sources, was chemically converted to Taxol (7). Although this approach and subsequent plant cell culture–based production efforts have decreased the need for harvesting the yew tree, production still depends on plant-based processes (8), with

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Supporting Online Material
www.sciencemag.org/cgi/content/full/330/6000/70/DC1
Materials and Methods
SOM Text
Figs. S1 to S11
Tables S1 to S4
References

accompanying limitations on productivity and scalability. These methods of production also constrain the number of Taxol derivatives that can be synthesized in the search for more efficacious drugs (9,10).

Recent developments in metabolic engineering and synthetic biology offer new possibilities for the overproduction of complex natural products by optimizing more technically amenable microbial hosts (11,12). The metabolic pathway for Taxol consists of an upstream isoprenoid pathway that is native to *Escherichia coli* and a heterologous downstream terpenoid pathway (fig. S1). The upstream methylerythritol-phosphate (MEP) or heterologous mevalonic acid (MVA) pathways can produce the two common building blocks, isopentenyl pyrophosphate (IPP) and dimethyl-allyl pyrophosphate (DMAPP), from which Taxol and other isoprenoid compounds are formed (12). Recent studies have highlighted the engineering of the above upstream pathways to support the biosynthesis of heterologous isoprenoids such as lycopene (13,14), artemisinic acid (15,16), and abietadiene (17,18). The downstream taxadiene pathway has been reconstructed in *E. coli and Saccharomyces cerevisiae* together with the over-expression of upstream pathway enzymes, but to date titers have been limited to less than 10 mg/liter (19,20).

The above rational metabolic engineering approaches examined separately either the upstream or the downstream terpenoid pathway, implicitly assuming that modifications are additive (a linear behavior) (13,17,21). Although this approach can yield moderate increases in flux, it generally ignores nonspecific effects, such as toxicity of intermediate metabolites, adverse cellular effects of the vectors used for expression, and hidden pathways and metabolites that may compete with the main pathway and inhibit the production of the desired molecule. Combinatorial approaches can overcome such problems because they offer the opportunity to broadly sample the parameter space and bypass these complex nonlinear interactions (21–23). However, combinatorial approaches require high-throughput screens, which are often not available for many desirable natural products (24).

Considering the lack of a high-throughput screen for taxadiene (or other Taxol pathway intermediate), we resorted to a focused combinatorial approach, which we term "multivariate-modular pathway engineering." In this approach, the overall pathway is partitioned into smaller modules, and the modules' expression are varied simultaneously—a multivariate search. This approach can identify an optimally balanced pathway while searching a small combinatorial space. Specifically, we partition the taxadiene-forming pathway into two modules separated at IPP, which is the key intermediate in terpenoid biosynthesis. The first module comprises an eight-gene, upstream, native (MEP) pathway of which the expression of only four genes deemed to be rate-limiting was modulated, and the second module comprises a two-gene, downstream, heterologous pathway to taxadiene (Fig. 1). This modular approach allowed us to efficiently sample the main parameters affecting pathway flux without the need for a high-throughput screen and to unveil the role of the metabolite indole as inhibitor of isoprenoid pathway activity. Additionally, the multivariate search revealed a highly nonlinear taxadiene flux landscape with a global maximum exhibiting a 15,000-fold increase in taxadiene production over the control, yielding 1.02 ± 0.08 g/liter (SD) taxadiene in fed-batch bioreactor fermentations.

We have further engineered the P450-based oxidation chemistry in Taxol biosynthesis in *E. coli* to convert taxadiene to taxadien-5α-ol and provide the basis for the synthesis of subsequent metabolites in the pathway by means of similar cytochrome P450 (CYP450) oxidation chemistry. Our engineered strain improved taxadiene-5α-ol production by 2400-fold over the state of the art with yeast (25). These advances unlock the potential of microbial processes for the large-scale production of Taxol or its derivatives and thousands of other valuable terpenoids.

The multivariate-modular approach in which various promoters and gene copy-numbers are combined to modulate diverse expression levels of upstream and downstream pathways of taxadiene synthesis is schematically described in fig. S2. A total of 16 strains were constructed in order to widen the bottleneck of the MEP pathway as well as optimally balance it with the downstream taxadiene pathway (26). The dependence of taxadiene accumulation on the upstream pathway for constant values of the downstream pathway is shown in Fig. 2A, and the dependence on the downstream pathway for constant upstream pathway strength is shown in Fig. 2B (table S1, calculation of the upstream and downstream pathway strength from gene copy number and promoter strength). As the upstream pathway expression increases in Fig. 2A from very low levels, taxadiene production also rises initially because of increased supply of precursors to the overall pathway. However, after an intermediate value further upstream pathway increases cannot be accommodated by the capacity of the downstream pathway. For constant upstream pathway expression (Fig. 2B), a maximum in downstream expression was similarly observed owing to the rising edge to initial limiting of taxadiene production by low expression levels of the downstream pathway. At high (after peak) levels of downstream pathway expression, we were probably observing the negative effect on cell physiology of the high copy number.

These results demonstrate that dramatic changes in taxadiene accumulation can be obtained from changes within a narrow window of expression levels for the upstream and downstream pathways. For example, a strain containing an additional copy of the upstream pathway on its chromosome under Trc promoter control (strain **8**) (Fig. 2A) produced 2000-fold more taxadiene than one expressing only the native MEP pathway (strain 1) (Fig. 2A). Furthermore, changing the order of the genes in the downstream synthetic operon from GT (GGPS-TS) to TG (TS-GGPS) resulted in a two- to threefold increase (strains **1** to **4** as compared with strains **5**, **8**, **11**, and **14**). Altogether, the engineered strains established that the MEP pathway flux can be substantial if an appropriate range of expression levels for the endogenous upstream and synthetic downstream pathway are searched simultaneously.

To provide ample downstream pathway strength while minimizing the plasmid-born metabolic burden (27), two new sets of four strains each were engineered (strains **17** to **20** and **21** to **24**), in which the downstream pathway was placed under the control of a strong promoter (T7) while keeping a relatively low number of five and 10 plasmid copies, respectively. The taxadiene maximum was maintained at high downstream strength (strains **21** to **24**), whereas a monotonic response was obtained at the low downstream pathway strength (strains **17** to **20**) (Fig. 2C). This observation prompted the construction of two additional sets of four strains each that maintained the same level of downstream pathway strength as before but expressed very low levels of the upstream pathway (strains **25** to **28** and **29** to **32**) (Fig. 2D). Additionally, the operon of the upstream pathway of the latter strain set was chromosomally integrated (fig S3). Not only was the taxadiene maximum recovered in these strains, albeit at very low upstream pathway levels, but a much greater taxadiene maximum was attained (~300 mg/liter). We believe that this significant increase can be attributed to a decrease in the cell's metabolic burden.

We next quantified the mRNA levels of 1-deoxy-D-xylulose-5-phosphate synthase (dxs) and taxadiene synthase (TS) (representing the upstream and downstream pathways, respectively) for the high-taxadiene-producing strains (**25** to **32** and **17** and **22**) that exhibited varying upstream and downstream pathway strengths (fig. S4, A and B) to verify our predicted expression strengths were consistent with the actual pathway levels. We found that dxs expression level correlates well with the upstream pathway strength. Similar correlations were found for the other genes of the upstream pathway: *idi*, *ispD*, and *ispF* (fig. S4, C and D). In downstream TS gene expression, an approximately twofold improvement was

quantified as the downstream pathway strength increased from **31** to **61** arbitrary units (a.u.) (fig. S4B).

Metabolomic analysis of the previous strains led to the identification of a distinct metabolite by-product that inversely correlated with taxadiene accumulation (figs. S5 and S6). The corresponding peak in the gas chromatography–mass spectrometry (GC-MS) chromatogram was identified as indole through GC-MS, ^{1}H, and ^{13}C nuclear magnetic resonance (NMR) spectroscopy studies (fig. S7). We found that taxadiene synthesis by strain **26** is severely inhibited by exogenous indole at indole levels higher than ~100 mg/liter (fig. S5B). Further increasing the indole concentration also inhibited cell growth, with the level of inhibition being very strain-dependent (fig. S5C). Although the biochemical mechanism of indole interaction with the isoprenoid pathway is presently unclear, the results in fig. S5 suggest a possible synergistic effect between indole and terpenoid compounds of the isoprenoid pathway in inhibiting cell growth. Without knowing the specific mechanism, it appears that strain **26** has mitigated the indole's effect, which we carried forward for further study.

In order to explore the taxadiene-producing potential under controlled conditions for the engineered strains, fed-batch cultivations of the three highest taxadiene accumulating strains (~60 mg/ liter from strain **22**; ~125 mg/liter from strain **17**; and ~300 mg/liter from strain **26**) were carried out in 1-liter bioreactors (Fig. 3). The fed-batch cultivation studies were carried out as liquid-liquid two-phase fermentation using a 20% (v/v) dodecane overlay. The organic solvent was introduced to prevent air stripping of secreted taxadiene from the fermentation medium, as indicated by preliminary findings (fig. S8). In defined media with controlled glycerol feeding, taxadiene productivity increased to 174 ± 5 mg/liter (SD), 210 ± 7 mg/liter (SD), and 1020 ± 80 mg/liter (SD) for strains **22**, **17**, and **26**, respectively (Fig. 3A). Additionally, taxadiene production significantly affected the growth phenotype, acetate accumulation, and glycerol consumption [Fig. 3, B and D, and supporting online material (SOM) text]. Clearly, the high productivity and more robust growth of strain **26** allowed very high taxadiene accumulation. Further improvements should be possible through optimizing conditions in the bio-reactor, balancing nutrients in the growth medium and optimizing carbon delivery.

Having succeeded in engineering the biosynthesis of the "cyclase phase" of Taxol for high taxadiene production, we turned next to engineering the oxidation-chemistry of Taxol biosynthesis. In this phase, hydroxyl groups are incorporated by oxygenation at seven positions on the taxane core structure, mediated by CYP450-dependent monooxygenases (28). The first oxygenation is the hydroxylation of the C5 position, followed by seven similar reactions en route to Taxol (fig. S1) (29). Thus, a key step toward engineering Taxol-producing microbes is the development of CYP450-based oxidation chemistry in vivo. The first oxygenation step is catalyzed by a CYP450, taxadiene 5α-hydroxylase, which is an unusual monooxygenase that catalyzes the hydroxylation reaction along with double-bond migration in the diterpene precursor taxadiene (Fig. 1).

In general, functional expression of plant CYP450 in *E. coli* is challenging (30) because of the inherent limitations of bacterial platforms, such as the absence of electron transfer machinery and CYP450-reductases (CPRs) and translational incompatibility of the membrane signal modules of CYP450 enzymes because of the lack of an endoplasmic reticulum. Recently, through transmembrane (TM) engineering and the generation of chimera enzymes of CYP450 and CPR, some plant CYP450s have been expressed in *E. coli* for the biosynthesis of functional molecules (15,31). Still, every plant CYP450 has distinct TM signal sequences and electron transfer characteristics from its reductase counterpart (32). Our initial studies were focused on optimizing the expression of codon-optimized synthetic taxadiene 5α-hydroxylase by N-terminal TM engineering and generating chimera

enzymes through translational fusion with the CPR redox partner from the *Taxus* species, *Taxus* CYP450 reductase (TCPR) (Fig. 4A) (29,31,33). One of the chimera enzymes generated, At24T5αOH-tTCPR, was highly efficient in carrying out the first oxidation step, resulting in more than 98% taxadiene conversion to taxadien-5α-ol and the byproduct 5(12)-Oxa-3(11)-cyclotaxane (OCT) (fig. S9A).

Compared with the other chimeric CYP450s, At24T5αOH-tTCPR yielded twofold higher (21 mg/liter) production of taxadien-5α-ol (Fig. 4B). Because of the functional plasticity of taxadiene 5α-hydroxylase with its chimeric CYP450's enzymes (At8T5αOH-tTCPR, At24T5αOH-tTCPR, and At42T5αOH-tTCPR), the reaction also yields a complex structural rearrangement of taxadiene into the cyclic ether OCT (fig. S9) (34). The byproduct accumulated in approximately equal amounts (~24 mg/liter from At24T5αOH-tTCPR) to the desired product taxadien-5α-ol.

The productivity of strain **26**-At24T5αOH-tTCPR was significantly reduced relative to that of taxadiene production by the parent strain **26** (~300 mg/liter), with a concomitant increase in indole accumulation. No taxadiene accumulation was observed. Apparently, the introduction of an additional medium copy plasmid (10-copy, p10T7) bearing the At24T5αOH-tTCPR construct disturbed the carefully engineered balance in the upstream and downstream pathway of strain **26** (fig S10). Small-scale fermentations were carried out in bioreactors so as to quantify the alcohol production by strain **26**-At24T5αOH-tTCPR. The time course profile of taxadien-5α-ol accumulation (Fig. 4C) indicates alcohol production of up to 58 ± 3 mg/liter (SD) with an equal amount of the OCT by-product produced. The observed alcohol production was approximately 2400-fold higher than previous production in *S. cerevisiae* (25).

The MEP pathway is energetically balanced and thus overall more efficient in converting either glucose or glycerol to isoprenoids (fig. S11). Yet, during the past 10 years many attempts at engineering the MEP pathway in *E. coli* in order to increase the supply of the key precursors IPP and DMAPP for carotenoid (21,35), sesquiterpenoid (16), and diterpenoid (17) overproduction met with limited success. This inefficiency was attributed to unknown regulatory effects associated specifically with the expression of the MEP pathway in *E. coli* (16). Here, we provide evidence that such limitations are correlated with the accumulation of the metabolite indole, owing to the non-optimal expression of the pathway, which inhibits the isoprenoid pathway activity. Taxadiene overproduction (under conditions of indole-formation suppression), establishes the MEP pathway as a very efficient route for biosynthesis of pharmaceutical and chemical products of the isoprenoid family (fig. S11). One simply needs to carefully balance the modular pathways, as suggested by our multivariate-modular pathway–engineering approach.

For successful microbial production of Taxol, demonstration of the chemical decoration of the taxadiene core by means of CYP450-based oxidation chemistry is essential (28). Previous efforts to reconstitute partial Taxol pathways in yeast found CYP450 activity limiting (25), making the At24T5αOH-tTCPR activity levels an important step to debottleneck the late Taxol pathway. Additionally, the strategies used to create At24T5αOH-tTCPR are probably applicable for the remaining monooxygenases that will require expression in *E. coli*. CYP450 monooxygenases constitute about one half of the 19 distinct enzymatic steps in the Taxol biosynthetic pathway. These genes show unusually high sequence similarity with each other (>70%) but low similarity (<30%) with other plant CYP450s (36), implying that these monooxygenases are amenable to similar engineering.

To complete the synthesis of a suitable Taxol precursor, baccatin III, six more hydroxylation reactions and other steps (including some that have not been identified) need to be

effectively engineered. Although this is certainly a daunting task, the current study shows potential by providing the basis for the functional expression of two key steps, cyclization and oxygenation, in Taxol biosynthesis. Most importantly, by unlocking the potential of the MEP pathway a new more efficient route to terpenoid biosynthesis is capable of providing potential commercial production of microbially derived terpenoids for use as chemicals and fuels from renewable resources.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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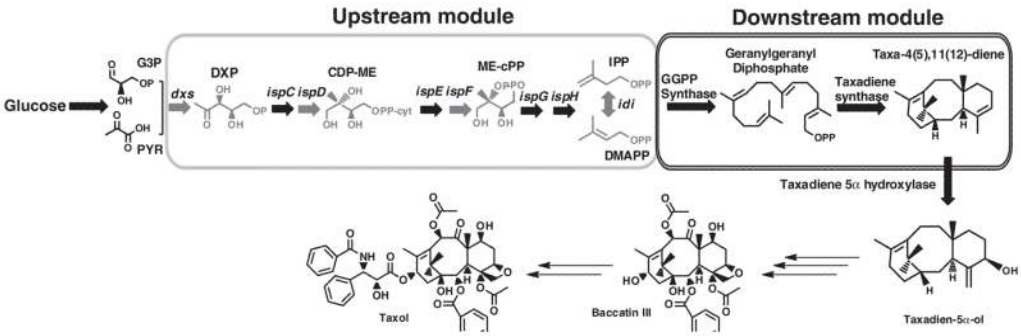

Fig. 1.

Multivariate-modular approach for isoprenoid pathway optimization. To increase the flux through the upstream MEP pathway, we targeted reported enzymatic bottlenecks (dxs, idi, ispD, and ispF) (gray) for over-expression by an operon (*dxs-idi-ispDF*) (21). To channel the overflow flux from the universal isoprenoid precursors, IPP and DMAPP, toward Taxol biosynthesis, we constructed a synthetic operon of downstream genes GGPP synthase (G) and taxadiene synthase (T) (37). Both pathways were placed under the control of inducible promoters in order to control their relative gene expression. In the *E. coli* metabolic network, the MEP isoprenoid pathway is initiated by the condensation of the precursors glyceraldehyde-3 phosphate (G3P) and pyruvate (PYR) from glycolysis. The Taxol pathway bifurcation starts from the universal isoprenoid precursors IPP and DMAPP to form geranylgeranyl diphosphate, and then the taxadiene. The cyclic olefin taxadiene undergoes multiple rounds of stereospecific oxidations, acylations, and benzoylation to form the late intermediate Baccatin III and side chain assembly to, ultimately, form Taxol.

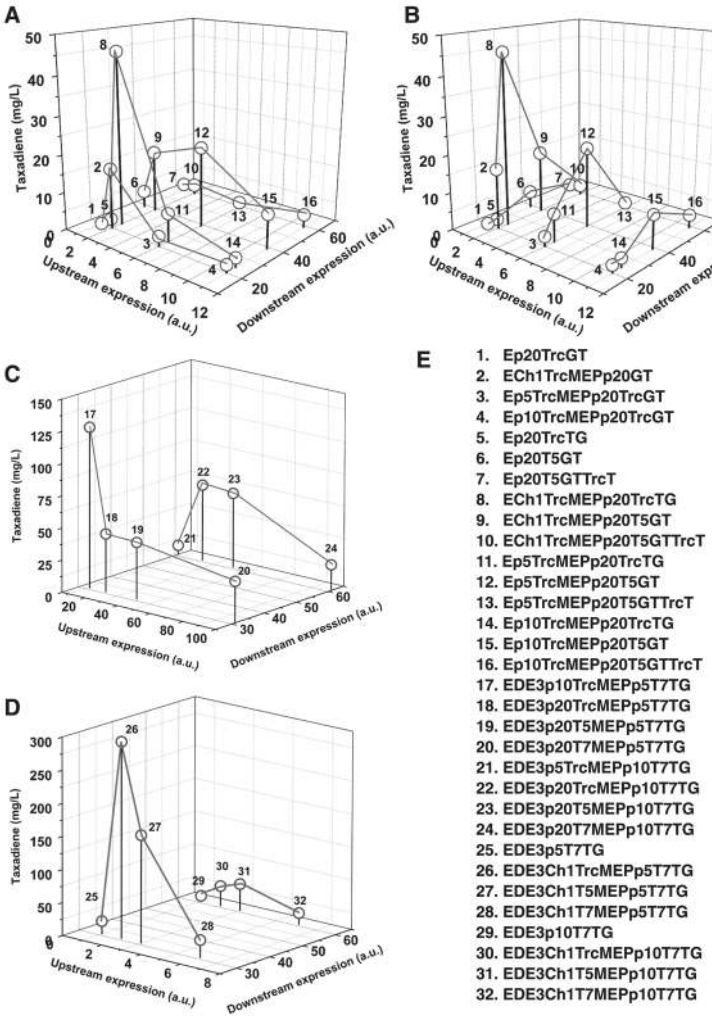

Fig. 2.

Optimization of taxadiene production through regulating the expression of the upstream and downstream modular pathways. (**A**) Response in taxadiene accumulation to changes in upstream pathway strengths for constant values of the downstream pathway. (**B**) Dependence of taxadiene on the downstream pathway for constant levels of upstream pathway strength. (**C**) Taxadiene response from strains (**17** to **24**) engineered with high upstream pathway overexpressions (**6** to **100** a.u.) at two different downstream expressions (**31** a.u. and **61** a.u.). (**D**) Modulation of a chromosomally integrated upstream pathway by using increasing promoter strength at two different downstream expressions (**31** a.u. and **61** a.u.). (**E**) Genotypes of the 32 strain constructs whose taxadiene phenotype is shown in Fig. 2, A to D. E, *E. coli* K12MG1655 Δ*recA*Δ*endA*; EDE3, *E. coli* K12MG1655 Δ*recA*Δ*endA* with DE3 T7 RNA polymerase gene in the chromosome; MEP, *dxs-idi-ispDF* operon; GT, GPPS-TS operon; TG, TS-GPPS operon; Ch1, 1 copy in chromosome; Trc, Trc promoter; T5, T5 promoter; T7, T7 promoter; p5, pSC101 plasmid; p10, p15A plasmid; and p20, pBR322 plasmid.

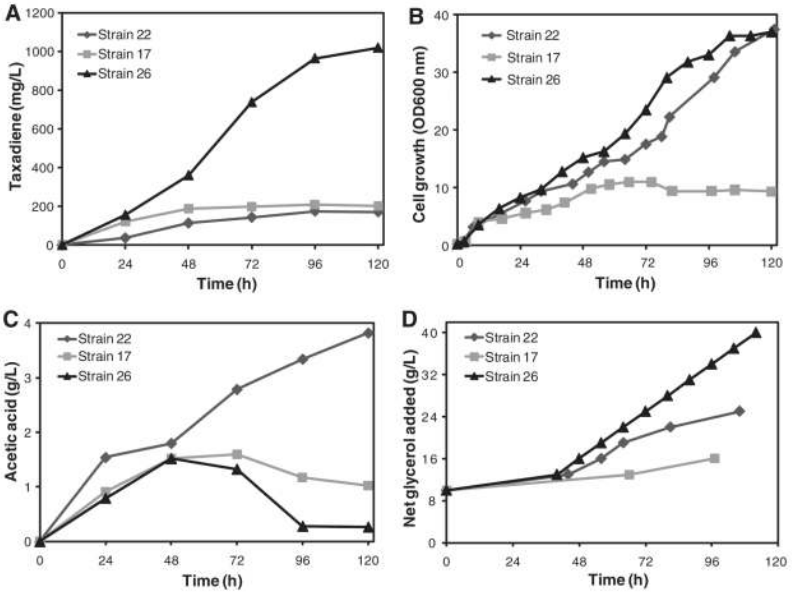

Fig. 3.

Fed-batch cultivation of engineered strains in a 1-liter bioreactor. Time courses of (**A**) taxadiene accumulation, (**B**) cell growth, (**C**) acetic acid accumulation, and (**D**) total substrate (glycerol) addition for strains 22, 17, and 26 during 5 days of fed-batch bioreactor cultivation in 1-liter bioreactor vessels under controlled pH and oxygen conditions with minimal media and 0.5% yeast extract. After glycerol depletes to ~0.5 to 1 g/liter in the fermentor, 3 g/liter of glycerol was introduced into the bioreactor during the fermentation. Data are mean of two replicate bioreactors.

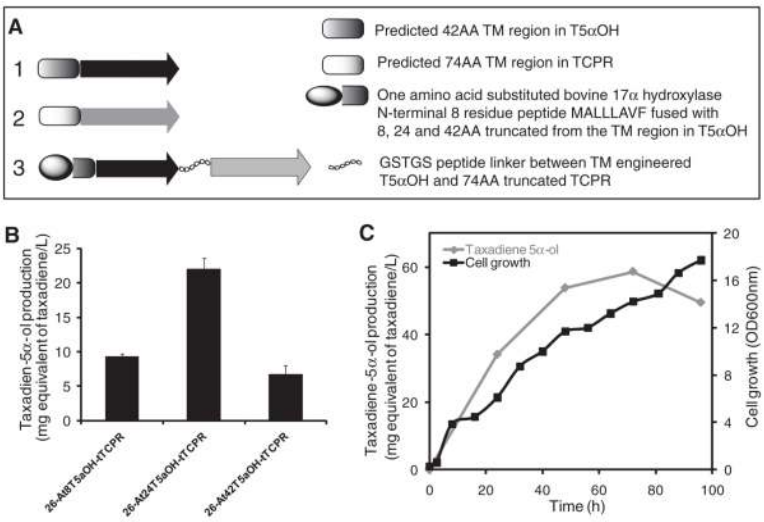

Fig. 4.

Engineering Taxol P450 oxidation chemistry in *E. coli*. (**A**) TM engineering and construction of chimera protein from taxadien-5α-ol hydroxylase (T5αOH) and *Taxus* cytochrome P450 reductase (TCPR). The labels **1** and **2** represent the full-length proteins of T5αOH and TCPR identified with 42 and 74 amino acid TM regions, respectively, and **3** represents chimera enzymes generated from three different TM engineered T5αOH constructs [At8T5αOH, At24T5αOH, and At42T5αOH constructed by fusing an 8-residue synthetic peptide MALLLAVF (A) to 8, 24, and 42AA truncated T5αOH] through a translational fusion with 74AA truncated TCPR (tTCPR) by use of linker peptide GSTGS. (**B**) Functional activity of At8T5αOH-tTCPR, At24T5αOH-tTCPR, and At42T5αOH-tTCPR constructs transformed into taxadiene producing strain **26**. Data are mean ± SD for three replicates. (**C**) Time course profile of taxadien-5α-ol accumulation and growth profile of the strain **26**-At24T5αOH-tTCPR fermented in a 1-liter bioreactor. Data are mean of two replicate bioreactors.