From Microbes to Painkillers: Genetically Modified *Escherichia coli* Generate Key Morphine Precursor

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In the past twenty-five years, the number of prescriptions for opioids has skyrocketed. Just between the years of 2006 and 2011, opioid prescriptions dispensed by U.S. retail pharmacies witnessed a dramatic escalation from 163 million prescriptions in 2006 to more than 219 million prescriptions by 2011[1]. In the U.S., where opioid medications are widely available, the focus of opiates has largely been on abuse potential. However, the World Health Organization estimates that nearly 5.5 billion people worldwide live in countries with low to nonexistent access to pain medications[2]. Furthermore, while the rapid growth in the number of opioid prescriptions has long been cited as one of the culprits of America's drug epidemic, opiates such as morphine continue to have significant clinical applications, including the treatment of severe chronic pain, myocardial infarction, congestive heart failure, and dyspnea-associated pulmonary edema[3].

Currently, the opium poppy (*Papaver somniferum*) remains the only commercial source for narcotic analgesics such as morphine[4]. However, using plant-extracted starting materials can be challenging due to the long doubling time of the plants, the low levels at which opiate drug intermediates accumulate in the plants, and the difficulty in isolating the desired products from other undesired metabolites[5]. Thus, given morphine's clinical applications and scarcity in lesser-developed countries, it is important to create cost-effective and fast, yet ethical and regulated, opiate production systems. Writing in *Nature Communiciations*, Nakagawa *et al.* demonstrate how four genetically engineered strains of *Escheria coli* can be used as vessels for the microbial biosynthesis of thebaine, a key precursor of other opioid compounds, such as morphine, thereby elucidating an alternate production system with the potential to accelerate the synthesis of opiates[6] (Fig. 1). This breakthrough offers a potentially faster, more low- cost method to produce plant-based medicines.

To create an opiate biosynthetic pathway that improves upon the (R)-reticuline conversion efficiency and total yield of thebaine compared to existing production pathways, Nakagawa et al. genetically engineered four strains of E. coli. The first strain, called AN1126, is a dopamine producer that converts carbon source glycerol into dopamine. The second strain, called AN1005, produces (R,S)-THP from dopamine. The third strain, AN1600, is an (R,S)-reticuline producer that converts (R,S)-THP to (R,S)-reticuline using methyltransferases CNMT and 4'OMT. The R-enantiomer of all reticuline produced in AN1600 was isolated and subsequently converted to thebaine using the fourth strain, AN1829.
Nakagawa *et al.* found during the production system design process that, of all the steps in the biosynthetic pathway of thebaine, the formation of (R)-reticuline, a key thebaine precursor, is the major rate-limiting step. Thus, two different methods of reticuline production were attempted using two different *E. coli* production systems. The first production system expressed an enzyme called STORR that has a P450 enzymatic domain. Since P450 is a haemoprotein, the functional expression of STORR in *E. coli* requires an expensive cofactor that acts as a precursor of haeme called 5-ALA. However, because this strain ultimately demonstrated low activity in the production of (R)- reticuline even with the addition of an expensive cofactor, this production system was not used. Despite the low productivity of this strain,

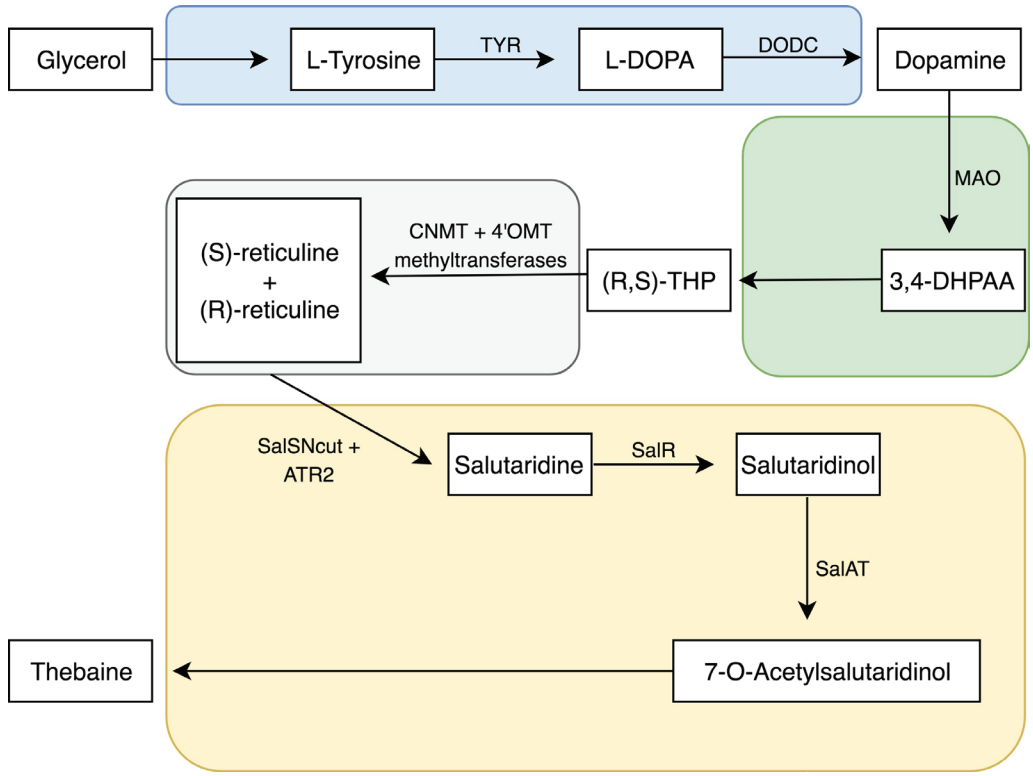

Figure 1: Total biosynthetic pathway of thebaine in E.coli using a step-wise culture

these findings nevertheless demonstrate the limited ability of STORR, an enzyme that has been heralded as the key participant in morphine production[7], in the microbial biosynthesis of opiates for large-scale commercial production.

The second production system – the one that was ultimately employed by Nakagawa *et al.* in the overall thebaine biosynthetic pathway – expressed a combination of two methyltransferases derived from *Coptis japonica*, CNMT and 4'OMT. While this strain of *E. coli* successfully yielded (R)-reticuline, levels of (R)-reticuline decreased when concentrations of (R,S)-Tetrahydropapaveroline ((R,S)-THP) – the intermediate formed from dopamine that becomes converted to reticuline – increased; conversely, concentrations of (S)-reticuline increased when concentrations of (R,S)-THP increased. Since it has been reported that *C. japonica* methyltransferases demonstrate a preference for the (S)-enantiomer, with limited activity on the (R)-enantiomer[8], this contributes to the inverse relationship between (R)-reticuline levels and (R,S)-THP levels. Furthermore, in this system, half of the reticuline produced was the S-enantiomer and therefore could not be used. Thus, although the CNMT and 4'OMT-expressing strain ultimately successfully generated (R)-reticuline, results of both production systems taken together underscore the necessity for further investigation into the improvement of conversion efficiency from (R,S)-THP into (R)- reticuline before practical production can be achieved.

Currently, one of the most commonly used microbial hosts for experimentation involving the biosynthesis of opiates is yeast[9,10,11]. Nakagawa *et al.*'s system, made up of four strains of genetically engineered *E. coli*, achieved thebaine yields over 300 times greater than the most recently developed yeast system that allows for the complete biosynthesis of opiates: approximately 2.1 milligrams of thebaine was generated by Nakagawa *et al.*'s *E. coli* system compared to only 0.0064 milligrams of thebaine generated by yeast[12]. This result is surprising, given that (R)-reticuline synthesis is more efficient in yeast than it is in *E. coli*. Thus, it seems to be Nakagawa *et al.*'s key use of step-wise cultures, along with their invention of (R,S)-THP, that allowed for the 300-fold improvement in thebaine yield compared to yeast systems.

Nakagawa *et al.*'s use of step-wise cultures instead of single-step cultures in the thebaine production system has several advantages. One advantage of the step-wise culture strategy is that it minimizes any product inhibition that may occur, therefore increasing product yields. Another advantage is that it prevents the propagation of undesired byproducts throughout the biosynthetic pathway[13]. In this case, the

step-wise culture strategy prevents tyrosinase, an enzyme that is necessary for dopamine production, from degrading (R,S)-THP in later steps of the pathway. The prevention from degradation of (R,S)-THP, an early intermediate in Nakagawa *et al.*'s *E. coli* production systems that is absent in yeast systems, is significant because (R,S)-THP is the intermediate that allows for the production of (R)-reticuline without the use of STORR, an enzyme that is necessary for (R)-reticuline production in yeast. Thus, Nakagawa *et al.*'s use of the four-step culture method bypasses additional steps involving cytochrome P450 and STORR that would otherwise be needed in both *E. coli* and yeast systems for the production of (R)-reticuline, allowing for more efficient production of opiates.

Besides the use of (R,S)-THP as an intermediate and the implementation of the four-step culture strategy, Nakagawa *et al.* reported that simply using *E. coli* as a host also contributed to the 300-fold increase in thebaine production compared to yeast. The investigators discovered that the conversion efficiency from (R)-reticuline to thebaine is extremely high, with nearly 80 percent of all (R)-reticuline being converted to thebaine. These findings suggest that SalSNCut, SalR, and SalAT, enzymes used for the conversion of (R)-reticuline to 7-*O*-acetylsalutaridinol, an immediate precursor of thebaine, have more efficient expression in *E. coli*-based systems than in yeast-based systems. This finding further highlights the superiority of using *E. coli* as microbial hosts in opiate biosynthetic instead of yeast, particularly in the (R)-reticuline to thebaine conversion step.

Nakagawa and colleagues' findings raise several objectives for further investigation. How can the conversion efficiency from (R,S)-THP be further improved to increase overall production yield and efficiency? Are there any points in the production pathway in which an *E. coli*-yeast hybrid system could lead to even higher thebaine yields? How can this system be further refined to the point of enabling large-scale industrial production? Apart from increasing the production efficiency and yield of opiate intermediates, it is also important to determine the clinical safety and efficacy for opiates synthesized from microbial systems. How does the biological activity of artificially synthesized opiates compare with that of naturally-occurring, plant-derived opiates? How structurally similar are these microbially-synthesized opiates to U.S. Food and Drug Administration-approved pharmaceuticals?

While there will undeniably continue to be further challenges that must be resolved in Nakagawa *et al.*'s *E. coli* production system, their findings demonstrate that the employment of the step-wise culture strategy, (R,S)-THP as an intermediate, and *E. coli* as a host likely collectively contributed to the superior thebaine yield that was obtained. Currently, twenty-one enzymes have been reconstructed in yeast biosynthetic systems for the production of opioids[14]; the number of enzymes successfully constructed (or even attempted to be constructed) in *E. coli* is comparatively miniscule. Given that these findings not only dethrone yeast-based systems as the primary microbial host for the production of opiates, but also introduce a more efficient and potentially less expensive method of opiate production, maybe it's time to shift gears.

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