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Biosynthesis of the Textile Dye Indigo by a Recombinant Bacterium

Burt D. Ensley*

Abstract. The biosynthesis of indigo by microorganisms can be manipulated through molecular biological techniques that alter the stability, activity, and final product of biosynthetic pathways in the cell. Transformed microorganisms may synthesize indigo at commercially significant rates and yields from glucose and other simple precursors. A cluster of five genes comprising the tryptophan biosynthetic operon and containing an altered *trp*B gene causes a host cell to synthesize high levels of indole from glucose after introduction into *Escherichia coli*. The addition of four genes encoding the naphthalene dioxygenase enzyme system enables the organism to rapidly oxidize indole to indigo, which is secreted into the fermentation medium. The synthesis by fermentation of indigo may be a cost-competitive approach if optimization and scale-up efforts are successful.

1. Introduction

The production of indigo from indole by soil bacteria has been observed since the 1920's [1], and the oxygenase-catalyzed formation of indoxyl from indole was measured as early as 1965 [2]. The low conversion rates, absolute requirement for indole, an expensive and toxic starting material, and diminutive final product concentrations characteristic of these microbial syntheses were a barrier to commercialization. The molecular cloning and identification [3] of a naphthalene dioxygenase enzyme system that catalyzes the rapid and efficient oxidation of indole to indigo, presumably through an indoxyl intermediate, offered a new route to the synthesis of this textile dye. In order for this approach to be practical from an economic viewpoint, a route to the *de novo* synthesis of indole from simple carbohydrate starting materials, possibly *via* an amino-acid biosynthetic pathway in the host cell, must also be identified and optimized.

A mutation in the enzyme tryptophan synthetase (*trp*B244) was found by *Yanofsky* and *Crawford* [4] to result in the release of indole by cells into the culture medium. This mutation interrupted normal tryptophan production and caused the pathway to synthesize indole instead. A genetically manipulated tryptophan operon, when introduced into *Escherichia coli*, caused this host to hyperproduce tryptophan from glucose and ammonia salts [5]. The use of site-directed mutagenesis to introduce the *trp*B244 and other mutations into a tryptophan overproducing plasmid resulted in a genetic construct that encodes the high-level synthesis of indole. Rapid oxidation of the indole to indigo was accomplished by the addition of plasmid-borne genes encoding the naphthalene dioxygenase enzyme system under the control of a strong promoter element, resulting in the complete synthesis of indigo from glucose and ammonia salts, as outlined in the *Scheme*.

2. Experimental

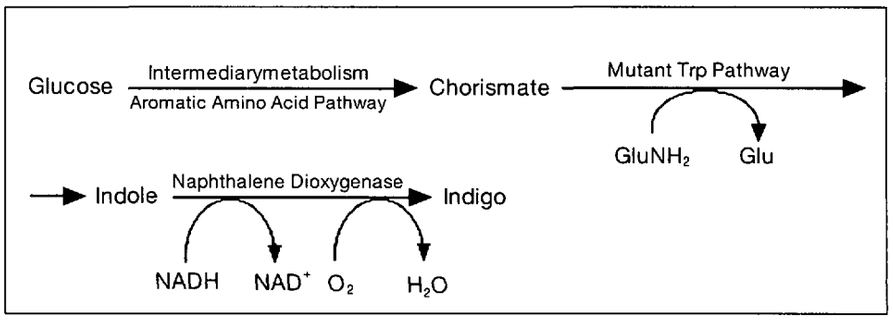
Indole and indigo concentrations in culture media were measured as described by *Starr* and coworkers [6] and *Oshiman et al.* [7]. The specific activity of the naphthalene dioxygenase enzyme system was measured as described previously [8]. *Escherichia coli* strain FM5 [9] is a derivative of *E. coli* K12. Plasmid pAC1 is a derivative of pCFM526 [9], and contains the strong P_L promoter system. Plasmid pD2643 [10] encodes an altered Trp pathway that causes the hyperproduction of tryptophan. Oligonucleotide-directed mutagenesis was carried out according to the methods of *Caruthers* [11], following the protocol described by *Maniatis et al.* [12]. Cultures were grown in 1-l and 10-l of fermentation medium with a fed batch method as described by *Fieshko* and *Rich* [9], and the indigo synthesis enzymes induced by altering the fermentation temperature or the addition of 5 mM IPTG. Samples were removed from the medium at 2–5 h intervals and analyzed for indigo and indole production. Whole cells were harvested from the medium, resuspeded in fresh medium, and analyzed for naphthalene dioxygenase enzyme activity.

3. Results

The organization of the Trp plasmid pD2643-20 is shown in the *Figure, A*. Site-directed mutagenesis at position 382 of the *trp*B gene on plasmid pD2643 produced mutants containing asparagine and glycine replacing lysine at that position and caused the secretion of indole into the culture medium. However, the inadvertent introduction of a proline, replacing an arginine at position 379, surprisingly resulted in a 20-fold increase in the levels of secreted indole over the mutations at position 382 [13]. The plasmid containing the proline mutation at position 379 was designated pD2643-20 and used in subsequent indigo biosynthesis efforts.

The naphthalene dioxygenase (NDO) enzyme system was altered by the addition of a gene encoding an extra ferredoxin component to improve enzyme activity and stability [13], and the augmented construct was assembled under the control of the strong lambda phage P_L promoter (*Fig., B*). The two plasmids bearing the altered Trp and NDO pathways, containing a total of ten genes and two regulatory elements, were introduced separately and together into *E. coli* FM5. After growth in a rich fermentation medium containing glucose, ammonium sulfate, and yeast extract, in-

Scheme. *Simplified Indigo Biosynthetic Pathway from Glucose and Ammonia in a Recombinant Bacterium*



*Correspondence: Dr. B.D. Ensley
7 Colt's Neck Dr.
Newtown, PA 18940, USA

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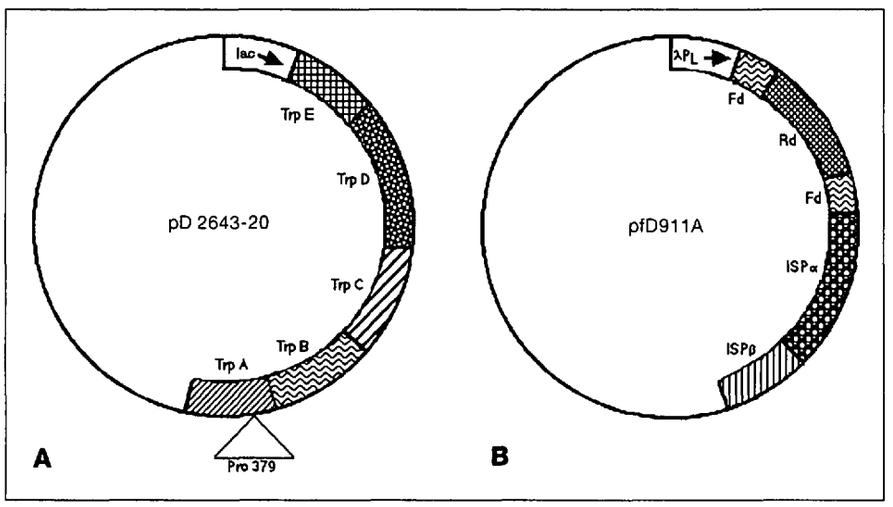

Figure. *Plasmid constructs encoding the enzymes involved in indigo synthesis. A*: Plasmid pD2643-20 carries a Trp operon with altered regulatory elements to enhance synthesis rates. The trpB gene on this plasmid contains an additional mutation at position 379 that causes indole, rather than tryptophan, to be produced. *B*: Plasmid pFD911A bears the four genes encoding the naphthalene dioxygenase enzyme system. A P_L promoter has been introduced to direct overexpression of the genes, and an extra ferredoxin gene has been inserted at the front of the operon to enhance the activity and stability of the enzyme complex.

duction of the appropriate promoter resulted in high levels of indole synthesis and naphthalene dioxygenase activity in the respective cultures [13]. *E. coli* strain FM5 containing both plasmids, after simultaneous induction of the two promoter systems, synthesized indigo at a rate of 0.5–1 g/h/l of growth medium. The indigo was primarily secreted out of the cells into the culture fluid, and precipitated as a dark blue solid. Under most conditions of indigo synthesis, indole accumulation could not be detected.

4. Summary

The results presented here suggest that it is feasible to manipulate the genetic makeup of disparate metabolic pathways in bacterial cells so that new products are formed, possibly in useful quantities. In this case, the output of one altered biosynthetic pathway became *in vivo* the substrate for an unrelated, normally degradative pathway that completed the synthesis of a novel (to the bacterial host) material. In addition to the textile dye used in the present example, biological processes for the manufacture of vitamins, amino acids, chiral intermediates, antibiotics, polymers and other specialty chemicals can be enhanced by this approach. Efforts directed towards the commercial exploitation of specialty and complex chemicals produced through microbial fermentation offer the potential for economically and environmentally improved synthetic processes.

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Neue Chromogene mit verdrillten π-Elektronensystemen – Über Indigo und 'Überindigo'

Albert Beck, **Rudolf Gompper***, **Karsten Hartmann** und **Kazufumi Yokogawa**

Die hohen Energiebarrieren der (*Z*/*E*)-Isomerisierung von Ethylen-Derivaten können grundsätzlich durch eine Stabilisierung des Übergangszustandes oder durch eine Destabilisierung des Grundzustandes, z.B. hervorgerufen durch eine gegenseitige Behinderung voluminöser Substituenten, erniedrigt werden. Die sterische Überfüllung an der (C=C)-Bindung lässt sich durch *syn*- oder *anti*-Pyramidalisierung der C-Atome ('folding') und/oder eine Verdrillung ('twisting') um die Verbindungsachse der olefinischen C-Atome vermindern. Der Energieinhalt der Moleküle steigt mit abnehmender Überlappung der p_z-Orbitale und der HOMO-LUMO-Abstand wird kleiner (Bathochromie) (vgl. [1]).

Es ist unser Ziel, verdrillte und gefaltete Ethylenderivate zu synthetisieren, die

**Korrespondenz*: Prof. Dr. R. Gompper
Institut für Organische Chemie
Universität München
Karlstrasse 23
D-80333 München