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NOTE

Production of Indigo and Indirubin by *Escherichia coli* Containing a Phenol Hydroxylase Gene of *Bacillus stearothermophilus*

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Escherichia coli recombinants containing the cloned phenol hydroxylase gene of *Bacillus stearothermophilus* BR219 were shown to produce both indigo and its structural isomer indirubin during culture on LB broth. The ratio of indirubin/indigo was highest under conditions of prolonged culture and reduced culture oxygenation.

Indigo is a blue pigment of plant origin utilized extensively for dyeing fabrics. Ensley *et al.* (3) have reported a novel microbial biosynthesis route for indigo which employs the introduction of *pseudomonas putida* napthalene dioxygenase genes into *Escherichia coli*. These investigators suggested that the biotransformation occured via a host-encoded conversion of tryptophan to indole, followed by a dioxygenase-catalyzed oxidation to indoxyl, and spontaneous oxidative coupling of indoxyl to indigo. Since this report, indigo formation has also been observed following cloning into *E. coli* of other aromatic pathway dioxygenase (1, 2), and by a toluene monooxygenase (7). During growth and characterization of an *E. coli* strain expressing a phenol hydroxylase gene cloned from thermophile *Bacillus stearothermophilus* BR219 (6), we observed unusual colonies displaying pink centers and blue peripheries. Since the BR219 phenol hydroxylase is thermostable and structurally simple, use of this enzyme for indigo production might provide certain process advantages and was therefore investigated. In this investigation we report that phenol hydroxylase of *B. stearothermophilus* BR219 catalyzes the formation of both indigo and its structural isomer, indirubin, and that the relative amounts of the pigments produced is influenced by alterations in organism culture conditions.

The recombinant *E. coli* XL-1 containing plasmid pPH222 and the subclones carrying inserts of *B. stearothermophilus* chromosomal DNA encoding a thermostable phenol hydroxylase of 44,000 Da have been described previously (6). Growth of the recombinants was carried out using LB medium or LB plates containing 100 μg/ml ampicillin. To observe pigment production with growth time, 50 ml volumes of LB medium with 100 μg/ml ampicillin in 125 ml Delong flasks with metal closures were utilized. The same flasks and medium were used for studies of pigment production with varied culture volumes. In both cases, cultures were shaken in a New Brunswick G76 gyratory water bath shaker at 37°C.

For pigment analysis, cultures of *E. coli* XL-1 containing pPH222 were extracted with 1/10th volume chloroform and following separation from the aqueous phase, the chloroform layer was analyzed using visible spectroscopy with a double-beam Perkin Elmer 124 spectrophotometer. Analysis of individual pigments was carried out following thin layer chromatography on silica gel plates developed with chloroform.

Chromatographic bands of separated pigments were removed from the plate, extracted with chloroform and analyzed by both visible and mass spectroscopy. The amounts of indigo and indirubin in mixtures were determined utilizing absorption at peak maxima for both indigo and indirubin, and qualitatively confirmed using thin layer chromatography. Molar extinction coefficients utilized were for indigo: ε (605 nm), 4000; ε (540 nm), 2300 and for indirubin; ε (605 nm), 3000; ε (540 nm), 25000. Mass spectroscopy was carried out with the assistance of the Macromolecular Facility of the Biochemistry Department at Michigan State University.

The *E. coli* recombinant containing plasmid pPH222 which demonstrated segmented colored colonies on LB plates was grown in LB culture, where formation of insoluble pigment granules over time was observed. The pigment granules settled easily from cultures upon standing, and were readily dissolved in chloroform. Thin layer chromatography of the dissolved pigments demonstrated

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both a pink pigment with Rf=0.05 and a blue pigment with Rf=0.12, the latter comigrating with authentic indigo (data not shown). Following recovery from plates using chloroform, the pink pigment exhibited a spectral absorption maximum at 540 nm, whereas the blue pigment had an absorption maximum at 605 nm. Although previous studies with aromatic mono- and dioxygenease did not indicate pink pigment formation, Hart *et al.* (4) have reported formation of both blue and pink pigments by the expressed product of a *Rhodococcus* gene of unknown function cloned in *E. coli*. These products were identified as indigo and its structural isomer indirubin, respectively. Similar pigment formed from indolecarboxylic acids have been reported by Eaton and Chapman (2). The visible spectral data for the pigment products are consistent with those reported by these investigators for indigo and indirubin. Although an authentic sample of indirubin was not available, the chromatographic Rf of the pink pigment in chloroform is similar to the 0.07 value reported Hart *et al.* (2). Further confirmation was obtained from mass spectral data, which yielded identical values of 262 for both pigments. To verify that the formation of indigo and indirubin resulted from the action of phenol hydroxylase, pigment formation by subclones containing portions of the 6 kb insert contained in pPH222 was examined. As seen in Fig. 1, pigment production was associated with the 2.1 kb *Hin*dIII fragment of pPH229 which contains only the gene for phenol hydroxylase, and which has significant similarity with the unidentified pigment-producing *Rhodococcus* gene (6).

The unusual color segmentation of pigment-producing colonies suggested that the relative amounts of indigo and indirubin produced might be influenced by growth conditions of the recombinant. To examine this possibility, the amounts of indigo and indirubin during growth were measured. As shown in Fig. 2, indigo was produced optimally at 24 h, and decreased in concentration with further culture. Indirubin formation was first observed at 24 h, and increased in concentration with further culture.

Since the centers of colonies on plates are likely to be oxygen-deprived, it appeared possible that indirubin formation in the centers of mature colonies was favored by conditions of decreased oxygen. The influence of available oxygen was tested by variation of the culture surface/volume ratio through utilization of different culture volumes in the same size flask. As seem in Fig. 3, 24 h cultures with higher culture volumes demonstrated sig-

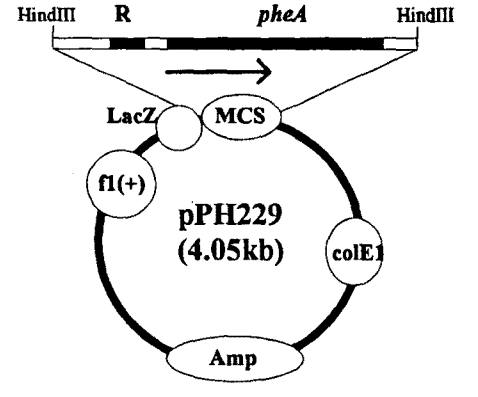

Fig. 1. Structure of the recombinant plasmid, pPH229.

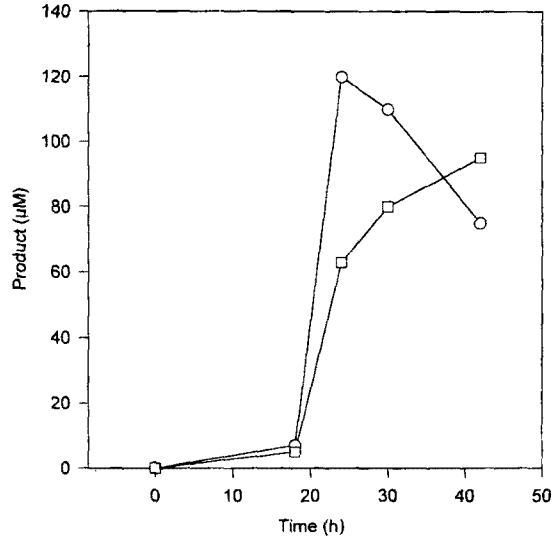

Fig. 2. Production of indigo and indirubin by *E. coli* containing plasmid pPH222.
(○), indigo; (□), indirubin.

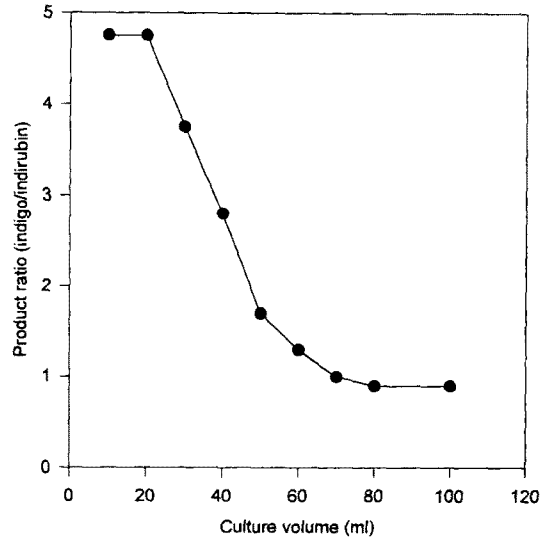

Fig. 3. Product ratio of indigo/indirubin with varied culture volume in 125 ml flask at 24 h.

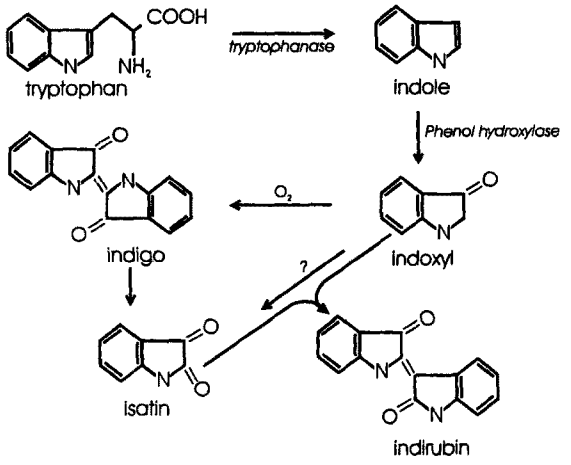

Fig. 4. Proposed routes for formation of indigo and indirubin from tryptophan.

nificantly lower ratios of indigo/indirubin.

The data in these experiments suggests that the BR219 thermostable phenol hydroxylase gene is capable of catalyzing formation of both indigo and its isomer indirubin when expressed in *E. coli*. Although the biotic and agbiotic steps involved in formation of these pigment is not yet understood, the decreased concentration of indigo in older cultures suggests that indirubin may be formed at least in part by conversion from indigo by a non-spontaneous mechanism. One possible route is that isatin produced from either the breakdown of indigo or the further oxidation of indoxyl could react with indoxyl to form indirubin (Fig. 4). Coupling of indoxyl with isatin by dehydration has been implicated previously in the chemical oxidation of indole to indirubin (5).

The differential colony coloration and results from experiments with varied culture oxygenation indicate that indirubin production is favored under conditions of reduced oxygenation. A better understanding of the factors controlling indirubin and indigo bioformation including oxygen concentration will help to achieve optimal production of the desired pigment products.

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