along the front of the submarine Cathedral Escarpment. This is evident because of their excellent preservation (all except the *Ogygopsis* assemblage include soft-bodied forms) and their penecontemporaneity (all occur within the *Bathyuriscus-Elrathina* biozone), and because transport before burial was short (all are close to the escarpment and include many articulated specimens). Second, the widespread distribution supports the observation that the Burgess shale faunas may be more representative of Cambrian marine communities than are assemblages of hard-shelled invertebrates (*1*). Moreover, it reinforces the view derived from the discovery of many Burgess shale fossils in Utah, that the Burgess shale includes "a normal Cambrian open-shelf biota" (*21*). This view can now be expanded to that provided by the Stephen Formation, which contains several marine faunas of the Burgess shale type that together make up a normal fore-reef faunal complex.

DESMOND COLLINS
Department of Invertebrate Palaeontology, Royal Ontario Museum, Toronto, Ontario M5S 2C6, Canada
DEREK BRIGGS
Department of Geology, Goldsmith's College, University of London, London SE8 3BU, England
SIMON CONWAY MORRIS
Department of Earth Sciences, The Open University, Milton Keynes MK7 6AA, England

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Expression of Naphthalene Oxidation Genes in *Escherichia coli* Results in the Biosynthesis of Indigo

Abstract. *A fragment of plasmid NAH7 from* Pseudomonas putida *PpG7 has been cloned and expressed in* Escherichia coli *HB101. Growth of the recombinant* Escherichia coli *in nutrient medium results in the formation of indigo. The production of this dye is increased in the presence of tryptophan or indole. Several bacteria that oxidize aromatic hydrocarbons to* cis*-dihydrodiols also oxidize indole to indigo. The results suggest that indigo formation is due to the combined activities of tryptophanase and naphthalene dioxygenase.*

Indigo is one of the oldest dyes known to man. In ancient times it was obtained as a plant extract from several species of the genus *Indigofera* and to a lesser extent from the European woad plant. The dye's brilliant color led to its development as a principal item of commerce between Europe and the Far East. Baeyer's elucidation of the structure of indigo in 1883 was followed by the development of a commercially practical synthesis. Today synthetic indigo has largely supplanted the plant-derived product, and large amounts of indigo are used for dyeing cotton and wool fabrics (*1*). We now report the construction of a strain of *Escherichia coli* that excretes indigo. The organism contains genes from *Pseudomonas putida* that code for enzymes responsible for the conversion of naphthalene to salicylic acid.

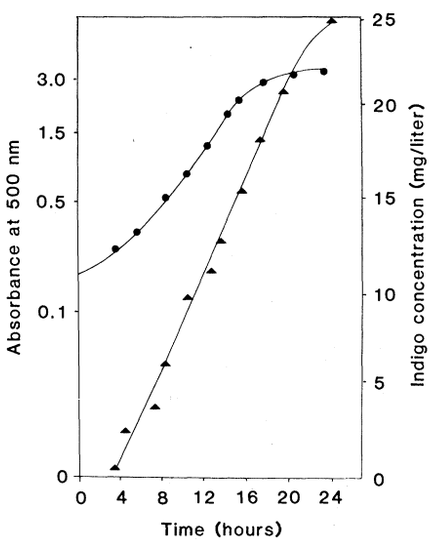

Fig. 1. Synthesis of indigo during growth of an *E. coli* containing pE317. Growth of the organism in Luria broth containing ampicillin (200 μg/ml) was monitored by measuring the absorbance of the culture at 500 nm (●). Indigo synthesis (▲) was measured by removing 1.0 ml of the culture fluid at various time intervals and extracting twice with equal volumes of ethyl acetate. The organic phases were combined and the absorbance of the ethyl acetate solution at 600 nm was determined. The concentrations of indigo were taken from a standard curve for synthetic indigo (Kodak) dissolved in ethyl acetate.

The oxidation of naphthalene by *Pseudomonas putida* PpG7 is catalyzed by enzymes that are encoded by a plasmid. The plasmid, NAH7, carries two gene clusters that enable the organism to grow on naphthalene as a sole carbon source (*2*). Several compounds produced during naphthalene oxidation, including naphthoquinone and salicylic acid (*3*), are widely used in the chemical and pharmaceutical industries. To determine the feasibility of utilizing microorganisms to produce these compounds, we carried out a detailed genetic and physical analysis of the NAH7 plasmid. We found that the entire pathway for the conversion of naphthalene to salicylic acid is encoded by genes that can be expressed in *E. coli*. Our results also led to the unexpected finding that a subset of these genes is responsible for the microbial production of indigo. In addition, we have shown that indigo formation is a property of the dioxygenase enzyme systems that form *cis*-dihydrodiols from aromatic hydrocarbons (*4*).

As a first step in these experiments, we cloned fragments of the NAH7 plasmid in *E. coli*. Plasmid NAH7 DNA was isolated from *Pseudomonas putida* PpG7 that had been digested with Hind III; the fragments were ligated into Hind III–cut plasmid vector pBR322 for transformation into *E. coli* HB101. Ampicillin-resistant colonies of transformed *E. coli*

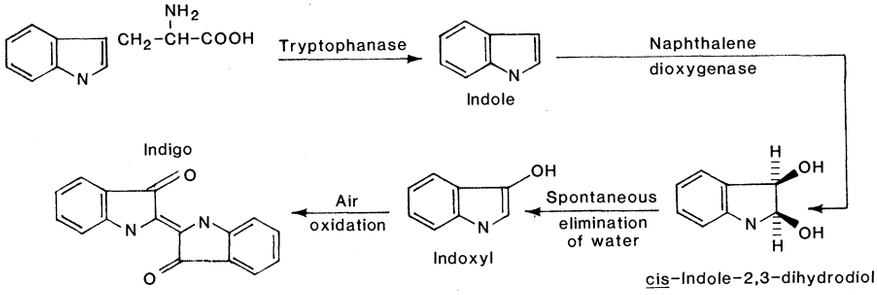

Fig. 2. Proposed pathway for indigo biosynthesis in a recombinant strain of *E. coli*. Indole is formed from tryptophan by tryptophanase, a natural enzyme in *E. coli*. Naphthalene dioxygenase formed by expression of the cloned *Pseudomonas* DNA oxidizes indole to indigo. *cis*-2,3-Dihydroxy-2,3-dihydroindole and indoxyl have not been isolated. Their inclusion is based on the known activities of aromatic hydrocarbon dioxygenases and established mechanisms for the chemical synthesis of indigo.

were selected and screened for tetracycline resistance because the tetracycline resistance gene on pBR322 is inactivated by insertions in the Hind III site (*5*). Of 500 tetracycline-sensitive colonies tested, one colony produced nonvolatile ^{14}C-labeled metabolites from labeled naphthalene (*6*). Plasmid pH625 DNA from this colony contained a 16.6-kilobase insert at the Hind III site of pBR322. The insert was shortened to 10.5 kilobases by further digestion with Eco RI and ligated again to produce plasmid pE317. *Escherichia coli* containing either pH625 or pE317 oxidized naphthalene to a single major metabolite having the chemical properties of synthetic salicylic acid. The enzymes coded for by pH625 and pE317 were expressed constitutively in *E. coli*, whereas the same genes are inducible in *Pseudomonas*. However, *P. putida* PpG7 oxidized naphthalene at a much higher rate than did *E. coli* containing pH625 or pE317. Either the NAH genes in *E. coli* are poorly expressed from a *Pseudomonas* promoter sequence, or a pBR322 promoter initiates only low-level transcription of the cloned genes (*7*).

During the course of experiments with *E. coli* containing pE317, we observed that colonies on agar plates had blue centers and that a dark blue, water-insoluble pigment was formed during growth in liquid medium. The blue pigment was extracted from liquid cultures with chloroform and purified by chromatography over silica gel. The purified pigment was identical to synthetic indigo in its relative mobility on thin-layer chromatography and in its visible, ultraviolet, infrared, and mass spectra. The kinetics of synthesis of indigo during growth of the recombinant *E. coli* is shown in Fig. 1.

The metabolic interactions that produce indigo in the recombinant organism appear to involve the NAH7-encoded naphthalene dioxygenase and indole. Indole is produced during normal metabolic processes of *E. coli* by the activity of the enzyme tryptophanase. The contention that enzymes coded by the cloned genes are responsible for indigo production and that indole is involved is indicated by the following observations. (i) After several passages in ampicillin-free medium, the recombinant organism displayed a simultaneous loss of its ability to oxidize naphthalene and synthesize indigo. (ii) Indigo formation was enhanced if the recombinant *E. coli* was grown in a culture medium supplemented with either 10 m*M* tryptophan or 1 m*M* indole. (iii) Indigo formation was not observed in cultures containing Luria broth and 1 percent glucose. High glucose concentrations can cause catabolite repression of tryptophanase synthesis in *E. coli* (*8*). (iv) Indigo formation occurred when indole was added to cultures of *P. putida* strain PpG7. Unlike *E. coli*, this organism does not produce its own indole. All of these observations suggest that indigo synthesis in the recombinant *E. coli* is catalyzed by naphthalene dioxygenase (*6*). Further support for this hypothesis was provided by the observation that other naphthalene-utilizing pseudomonads also oxidize indole to indigo (Table 1). *Pseudomonas putida* NCIB 9816, strain 11, is a mutant that oxidizes naphthalene to (+)-*cis*-(1*R*,2*S*)-dihydroxyl-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol). This strain lacks the enzyme *cis*-naphthalene dihydrodiol dehydrogenase, which is the second enzyme in the degradative pathway (*9, 10*). The ability of strain 11 to oxidize indole indicates that naphthalene dioxygenase is the enzyme responsible for indigo formation.

Table 1. Indigo formation by different bacterial strains. Pseudomonads were grown on a mineral salts medium containing 0.2 percent (weight to volume) L-arginine hydrochloride and a hydrocarbon supplied either as a vapor (toluene and *p*-xylene) or in the growth medium (naphthalene and *p*-cresol). The hydrocarbons selected to induce catabolic enzymes in the respective pseudomonads were those listed under relevant phenotype. The *Beijerinckia* strains were grown on a mineral salts medium containing 0.2 percent (weight to volume) sodium succinate and 0.05 percent (weight to volume) yeast extract. *m*-Xylene was used to induce oxygenase activity. All cultures were grown until the late exponential phase of growth was attained. Cells were harvested by centrifugation and suspended in fresh growth medium that contained 2 m*M* indole. After 3 hours of incubation, the cells were extracted with boiling chloroform. Indigo was identified by migration on thin-layer chromatograms and by visible spectroscopy. Abbreviations: Nah, Tol, Xyl, Cre, and Bp followed by superscript + or − refer to the ability or inability; respectively, of the organism to grow with naphthalene, toluene, *p*-xylene, *p*-cresol, and biphenyl as the sole source of carbon and energy; *tod*, A, B, and C, refer, respectively, to genes coding for ferredoxin$_{TOL}$ reductase, ferredoxin, and ISP$_{TOL}$ components of toluene dioxygenase; *tod*, D and E, refer, respectively, to genes coding for toluene dihydrodiol dehydrogenase and catechol oxygenase activities; CAM, Center for Applied Microbiology, University of Texas, Austin.

Organism	Relevant phenotype	Relevant genotype of plasmid	Reference or source	*cis*-Diol* formation	Indigo formation
P. putida PpG7	Nah$^+$	Wild-type (NAH7)	(*18*)	+	+
P. putida NCIB 9816	Nah$^+$	Wild-type (pTX1)†	(*3*)	+	+
9816-11	Nah$^-$	nddA‡ (pTX1)	CAM	+	+
9816-C2	Nah$^-$		CAM	−	−
P. putida Np	Nah$^+$	Wild-type	(*10*)	+	+
P. putida	Tol$^+$	Wild-type	(*19*)	+	+
39/D	Tol$^-$	*tod*D	(*20*)	+	+
F106	Tol$^-$	*tod*C	CAM	−	−
F102	Tol$^-$	*tod*A *tod*E	CAM	−	−
F26a	Tol$^-$	*tod*A *tod*B	CAM	−	−
P. putida BG	Xyl$^+$	Wild-type (TOL)	CAM	−	−
Pseudomonas sp.	Cre$^+$	Wild-type	(*21*)	−	−
Beijerinckia sp.	Bp$^+$	Wild-type	(*22*)	+	+
B836	Bp$^-$	bddA‡	(*22*)	+	+

*Initial oxidation products derived from aromatic hydrocarbons are *cis*-dihydrodiols. †This strain of *P. putida* contains a single plasmid. The relation of this plasmid to plasmids in different strains of NCIB 9816 (*23, 24*) is unknown. ‡Strains lack active naphthalene diol dehydrogenase (nddA) or biphenyl diol dehydrogenase (bddA).

The results presented in Table 1 also suggest that indole oxidation is a property of bacterial dioxygenases that form *cis*-dihydrodiols from other aromatic hydrocarbons. *Beijerinckia* strain B836 is a mutant that oxidizes biphenyl and various polycyclic aromatic hydrocarbons to *cis*-dihydrodiols, whereas *P. putida* strain 39/D oxidizes benzene, toluene, and several monocyclic aromatic hydrocarbons to *cis*-dihydrodiols (*4, 11*). Both of these mutant strains and their wild-type parents oxidize indole to indigo. The ability of both B836 and wild-type *Beijerinckia* strains to oxidize indole to indigo is induced by *m*-xylene. The enzyme system from *P. putida* that catalyzes the formation of (+)-*cis*-(1*S*,2*R*)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol) from toluene has been resolved into three protein components that are essential for enzymatic activity (*11*). This enzyme system consists of a flavoprotein (ferredoxin$_{TOL}$ reductase), a two-iron–two-sulfur ferredoxin (ferredoxin$_{TOL}$), and an iron-sulfur protein (ISP$_{TOL}$). Mutations in any one of the structural genes that code for these enzymes results in a complete loss of toluene dioxygenase activity (*12*). These mutants—strains F106, F102, and F26A (Table 1)—are also incapable of oxidizing indole to indigo.

The reaction sequence shown in Fig. 2 accounts for the formation of indigo by the recombinant *E. coli* and the other bacterial strains used in this investigation. It also provides a possible explanation for previous reports on the bacterial formation of indigo (*13–15*). We have not been able to detect the formation of *cis*-2,3-dihydroxy-2,3-dihydroindole. However, the formation of this intermediate can be inferred from the results presented in Table 1. Elimination of water from the *cis*-dihydrodiol would yield indoxyl, which is a known precursor of indigo. In addition, we have detected the presence of oxindole in culture filtrates after indole transformation by *P. putida*. Oxindole, the keto tautomer of 2-hydroxyindole, is the other expected product after dehydration of *cis*-2,3-dihydroxy-2,3-dihydroindole. The sequence in Fig. 2 differs from the reactions proposed by Fujioka and Wada (*16*) for the oxidation of indole to 2,3-dihydroxyindole by a Gram-positive coccus. However, these authors reported the accumulation of an unidentified blue pigment by indole-grown cells.

Our results illustrate the potential of recombinant DNA technology in the development of innovative microbial methods for the production of useful chemicals. The cloning and expression of naphthalene dioxygenase genes in *E. coli* will facilitate studies on the regulation and expressions of genes involved in the microbial degradation of aromatic hydrocarbons. In addition, the observation that indigo formation is catalyzed by different aromatic hydrocarbon dioxygenases suggests that indole may be a valuable substrate for elucidating the mechanism of action of this class of enzymes.

Note added in proof: Another method for the cloning and expression of naphthalene oxidation genes in *E. coli* has been reported by Schell (*17*).

BURT D. ENSLEY
BARRY J. RATZKIN
TIMOTHY D. OSSLUND
MARY J. SIMON
Amgen, Thousand Oaks, California 91320
LAWRENCE P. WACKETT
DAVID T. GIBSON
Center for Applied Microbiology and *Department of Microbiology, University of Texas, Austin 78712*

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Anticarcinoma Activity in vivo of Rhodamine 123, a Mitochondrial-Specific Dye

Abstract. *Carcinoma cells and normal epithelial cells differ in the mitochondrial retention of a permeant cationic compound, rhodamine 123. The possibility of utilizing this difference in carcinoma chemotherapy was investigated. Rhodamine 123 exhibited anticarcinoma activity in mice, and this activity was potentiated by 2-deoxyglucose.*

Epithelial cancers, particularly those of the breast and colon, are the major causes of death due to cancer in the United States. Most of the anticarcinoma drugs in clinical use are targeted at the DNA of the cell. Carcinoma chemotherapy in which drugs are targeted at the plasma membrane, mitochondria, endoplasmic reticulum, cytoskeleton, or intermediary metabolism unrelated to DNA is largely unexplored. We discovered that a fluorescent dye, rhodamine 123 (Rh123), localizes in the mitochondria of living cells (*1*), probably as a result of high membrane potential across the mitochondrial membrane (*2*). The mitochondria of a variety of carcinomas retain Rh123 for prolonged periods (2 to 5 days), whereas normal epithelial cells release it within a few hours (*3*). This unexpected finding prompted us to investigate whether this difference in the mitochondria of carcinoma and normal epithelial cells can be utilized in cancer chemotherapy. We reported earlier that Rh123 is selectively toxic to carcinoma cells in vitro (*4, 5*). We now report that Rh123 has anticarcinoma activity in mice and that this activity is potentiated by 2-deoxyglucose, an inhibitor of glycolysis.

Ehrlich ascites tumor cells, confirmed to be of epithelial origin by immunofluorescence with antibody to keratin, were injected intraperitoneally (5×10^5 cells) into mice. These tumor-bearing mice had a narrow range of survival times (18 to 22 days; median, 19 days), reflecting consistency in the mortality pattern (Fig. 1