

Metabolism of Naphthalene, 1-Naphthol, Indene, and Indole by *Rhodococcus* sp. Strain NCIMB 12038

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The regulation of naphthalene and 1-naphthol metabolism in a *Rhodococcus* sp. (NCIMB 12038) has been investigated. The microorganism utilizes separate pathways for the degradation of these compounds, and they are regulated independently. Naphthalene metabolism was inducible, but not by salicylate, and 1-naphthol metabolism, although constitutive, was also repressed during growth on salicylate. The biochemistry of naphthalene degradation in this strain was otherwise identical to that found in *Pseudomonas putida*, with salicylate as a central metabolite and naphthalene initially being oxidized via a naphthalene dioxygenase enzyme to *cis*-(1*R*,2*S*)-1,2-dihydroxy-1,2-dihydronaphthalene (naphthalene *cis*-diol). A dioxygenase enzyme was not expressed under growth conditions which facilitate 1-naphthol degradation. However, biotransformations with indene as a substrate suggested that a monooxygenase enzyme may be involved in the degradation of this compound. Indole was transformed to indigo by both naphthalene-grown NCIMB 12038 and by cells grown in the absence of an inducer. Therefore, the presence of a naphthalene dioxygenase enzyme activity was not necessary for this reaction. Thus, the biotransformation of indole to indigo may be facilitated by another type of enzyme (possibly a monooxygenase) in this organism.

Naphthalene and its 1-hydroxy derivative, 1-naphthol, are examples of common bicyclic aromatics which are often released into the environment. For example, naphthalene is a major component of coal tar and coal tar products, such as creosote (37). Furthermore, bacteria which degrade this compound under aerobic conditions are widely distributed (13). This has led to extensive studies on the metabolism and enzymology of naphthalene degradation in gram-negative bacteria, such as *Pseudomonas putida* (16, 20, 32, 38, 39). By contrast, the metabolism of naphthalene in the widely distributed gram-positive nocardioform bacteria has not been investigated to the same extent. However, there is some evidence to suggest that both the biochemistry and genetics of naphthalene degradation in gram-positive and gram-negative bacteria do differ. Thus, in gram-negative bacteria, salicylate, a central intermediate in naphthalene metabolism, undergoes oxidative decarboxylation to yield catechol before further metabolism (24). However, during naphthalene metabolism by a *Rhodococcus* sp., a different salicylate hydroxylase enzyme is employed to produce gentisate (25). Furthermore, whereas in the pseudomonads, salicylate acts as an inducer for the naphthalene degradative pathway (3), this was not observed in two *Rhodococcus* spp. (25, 29).

1-Naphthol is also liberated into the environment as a result of the oxidation of naphthalene by certain fungi and bacteria (11, 12, 21). 1-Naphthol is particularly important both as a synthetic precursor of the insecticide Sevin (1-naphthyl-*N*-methylcarbamate) and as a degradation product of this compound via both chemical and biological processes (2, 15). However, despite the environmental significance of 1-naphthol, a complete biochemical pathway specifically for its biodegradation in any one bacterium has not yet been elucidated (5).

The nocardioform bacterium *Rhodococcus* sp. strain NCIMB

12038 (strain 12038) was originally isolated on carbaryl as the sole source of carbon and energy by Larkin and Day (29). This compound spontaneously hydrolyzes to 1-naphthol, methylamine, and carbon dioxide, and strain 12038 was able to utilize these products as carbon and nitrogen sources. Furthermore, it was shown in a later study that this organism could also grow with naphthalene as the sole carbon and energy source (30).

We describe here the independent regulation of 1-naphthol and naphthalene metabolism in strain 12038, the initial products of naphthalene metabolism, and the biotransformation of model compounds indene and indole by cells which express 1-naphthol and naphthalene oxygenases. We show that an enzyme other than a dioxygenase can catalyze the indole to indigo biotransformation in this strain, normally associated with dioxygenases.

MATERIALS AND METHODS

Sources of microorganisms and reagents. *Rhodococcus* sp. strain NCIMB 12038 was obtained from the National Cultures of Industrial and Marine Bacteria Ltd., Aberdeen, United Kingdom.

Previously published methods were used for the preparation of racemic naphthalene *cis*-diol (31), racemic *cis*-1,2-dihydroxyindan (28), *trans*-1,2-dihydroxyindan (7), indan 1,2-oxide (7), and racemic *trans*-1,2-dihydroxy-1,2-dihydronaphthalene (naphthalene *trans*-diol) (6). Radioactive [¹⁴C]naphthalene (10.1 mCi/mmol; radioactivity was measured in an LKB scintillation counter with PCS scintillation fluid [Amersham International, Amersham, United Kingdom]) was obtained from the Sigma Chemical Company Ltd., Dorset, United Kingdom. All other reagents were either of analytical grade obtained from BDH Chemicals Ltd., Poole, United Kingdom, or otherwise the best purity available obtained from the Aldrich Chemical Company Ltd., Dorset, United Kingdom. Samples of (1*R*,2*S*)- and (1*S*,2*R*)-naphthalene *cis*-diol were obtained from earlier biotransformation studies (1, 8).

Growth of strain 12038 on naphthalene, pyruvate, and salicylate. In all cases, the bacterium was grown on a minimal salts medium (MSM) at pH 6.8 (29), with an appropriate carbon source added to the shake flask or fermenter after sterilization. Solid naphthalene was added directly to the medium (22 mM). Sodium pyruvate (35 mM) and sodium salicylate (3 mM) solutions were filter sterilized prior to addition. In all experiments, the bacteria were grown or incubated at 25°C and were utilized at the late exponential phase of growth.

Catabolism of naphthalene and 1-naphthol by whole cells. All cells were washed twice in a MSM, centrifuged (6,500 × g, 10 min) and finally resuspended in MSM before use. Naphthalene-grown cells were also filtered through glass wool, before centrifugation. Cell suspensions (100 ml, A₆₀₀ = 0.44) were incu-

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bated on an orbital shaker in 500-ml screw-top shake-flasks. Naphthalene (0.16 mM) or 1-naphthol (0.35 mM), were added in 5 ml of methanol. Over 3 h, duplicate samples (1 ml) were removed from each flask and centrifuged for 5 min (MSE Micro Centaur, 12,000 rpm), and the supernatant was stored at -20°C prior to analysis.

Biotransformation of indene, indole, *cis*-1,2-dihydroxyindan, and 1,2-indene oxide. Naphthalene-, pyruvate- and salicylate-grown cells were prepared under the conditions previously described. Sodium pyruvate (35 mM) was used as a cosubstrate and the A_{600} was adjusted to 1.5 using MSM. Indene (2.1 mM) was added directly to the cultures. After 24 h, the cells were removed by centrifugation and supernatants were stored at 4°C . This procedure was repeated for racemic *cis*-1,2-dihydroxyindan and 1,2-indene oxide biotransformations (both substrates dissolved in 0.1% [vol/vol] ethanol) and for indole (1 mM dissolved in 0.2% [vol/vol] methanol).

Extraction, purification, and identification of bacterial metabolites. Biotransformation supernatants were saturated with excess sodium chloride at room temperature and then extracted with three equal volumes of ethyl acetate. The organic extract was subsequently dried over anhydrous magnesium sulfate and filtered, and the ethyl acetate was removed under vacuum on a rotary evaporator at 30°C .

Metabolites were purified by preparative liquid chromatography (PLC) (silica solid phase) and/or thin layer chromatography (TLC) (silica solid phase). *cis*-1,2-Dihydroxyindan, 1-indenol, and 2-hydroxyindan-1-one were prepurified by PLC (0 to 5% methanol in dichloromethane). Before chiral high-performance liquid chromatography (HPLC) analysis, the *cis*-diol and 1-indenol were purified by TLC (1% methanol in dichloromethane). *trans*-1,2-Dihydroxyindan was purified as described above except that 0 to 8% methanol in dichloromethane was used in PLC. Naphthalene *cis*-diol was purified by TLC (1% methanol in dichloromethane). Indigo-containing samples were extracted with chloroform, and no further purification was required.

Standard samples of *cis*-1,2-dihydroxyindan and *trans*-1,2-dihydroxyindan were separated on an RX5 capillary GC column (Restek Corporation, Bellefonte, Pa.) (temperature, 50 to 320°C ; carrier gas flow rate, 1 ml/min) and subsequently detectable by gas chromatography (GC)-mass spectrometry (MS) (VG Quattro mass spectrometer; Fisons Instruments Biotech MS, Loughboro, United Kingdom).

^1H nuclear magnetic resonance (NMR) spectra (300 MHz) were obtained in deuterated chloroform (*trans*-1,2-dihydroxyindan in deuterated acetone). Spectral data was compared with the literature (7, 28, 44) or authentic standards.

Stereochemical analysis of *cis*-1,2-dihydroxyindan, 1-indenol, and naphthalene *cis*-diol was conducted using chiral HPLC (Diacel Chiralpak AD column [0.46 by 25 cm] [JT Baker BV, Deventer, Holland]) with a 10% isopropanol-*n*-hexane solvent system (0.5 ml/min, 254 nm). Resolution of authentic samples of individual enantiomers (1, 7, 8) was observed. Elution times were as follows: (1*R*)-inden-1-ol, 25.0 min; (1*S*)-inden-1-ol, 13.7 min; (1*R*,2*S*)-*cis*-1,2-dihydroxyindan, 20.6 min; (1*S*,2*R*)-*cis*-dihydroxyindan, 23.0 min; (1*R*,2*S*)-naphthalene *cis*-diol, 31.2 min; and (1*S*,2*R*)-naphthalene *cis*-diol, 27.0 min. For silylation of naphthalene *cis*-diol, *N*-methyl-*N*-trimethyl silyltrifluoroacetamide (MSTFA) was used as a silylating agent in dry diethyl ether. The stereochemistry of *trans*-1,2-dihydroxyindan was found by a published method (7).

Analysis of metabolites by HPLC. The concentration of naphthalene and 1-naphthol in aqueous samples was estimated by HPLC (25-cm reverse-phase HPLC column, 70% methanol-water, 1-ml/min flow rate, Perkin-Elmer LC-235 diode array detector, 210 nm). With indigo, a mobile phase comprising 75% methanol-water was used.

Enzyme assays. Naphthalene dioxygenase, naphthalene *cis*-diol dehydrogenase, and gentisate oxygenase enzyme activity was measured in cell extracts (19, 29, 39). The extract was prepared from frozen cells (10 g [wet weight]) thawed into 20 ml of 50 mM Tris-HCl buffer (pH 7.8), containing 10% (vol/vol) ethanol, 10% (vol/vol) glycerol, and 0.5 mM dithiothreitol (DTT) (TEG buffer). The cells were broken by three passages through a French press at 18,000 lb/in² and 4°C . The suspension was filtered through glass wool and centrifuged ($25,000 \times g$, 1 h) to remove cell debris. 1-Naphthol and salicylate oxygenase activity was measured in whole-cell preparations (29, 30). Protein concentrations were determined by the Pierce BCA method (Pierce, Rockford, Ill.). Total protein present in whole cells utilized for 1-naphthol and salicylate oxygenase assays was found by boiling the cell suspensions used in 1 M NaOH for 10 min, neutralizing with 5 M HCl, and finally centrifugation (MSE microcentaur, 12,000 rpm, 5 min) to remove cell debris. Protein concentration could then be determined in the supernatant.

Autoradiography procedure used. Radioactive naphthalene and 1-naphthol metabolites were detected by autoradiography of TLC plates. Autoradiograph exposure was for 24 h at -70°C . Amersham Hyperfilm- β max autoradiography film was used (Amersham International Ltd.) together with Kodak D-19 developer and Kodak fixer (Eastman Kodak Company, Rochester, N.Y.).

RESULTS AND DISCUSSION

Regulation of naphthalene and 1-naphthol metabolism in whole cells of strain 12038. In order to determine whether the enzyme activities involved in naphthalene and 1-naphthol deg-

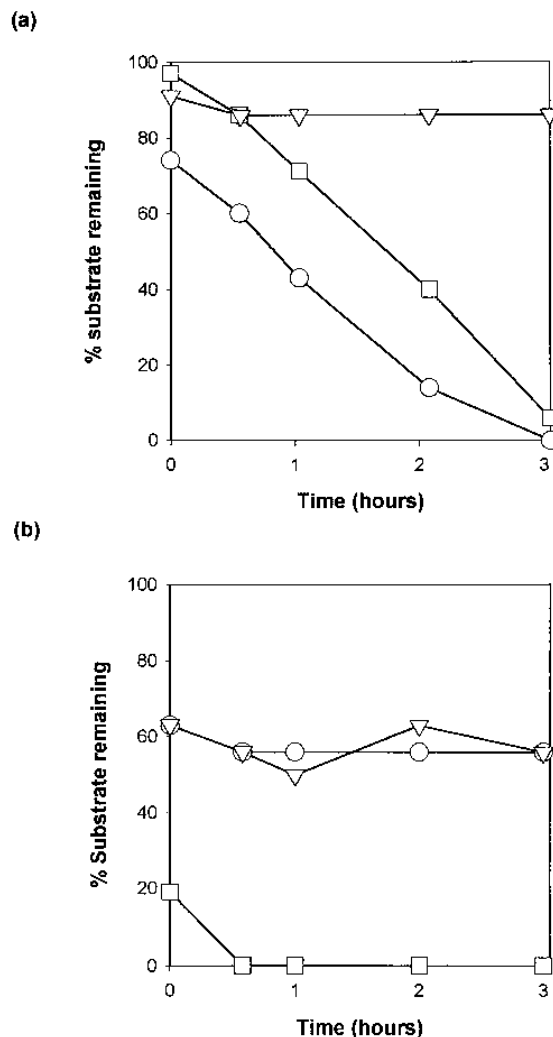


FIG. 1. Degradation of 1-naphthol (a) and naphthalene (b) in cultures of *Rhodococcus* sp. strain NCIMB 12038 grown on pyruvate (○), naphthalene (□), and salicylate (▽) carbon sources, shown as a percentage of initial substrate concentration remaining. Datum points show the means for duplicate samples. Naphthalene was initially added at a concentration of 0.16 mM, and 1-naphthol was initially added at 0.35 mM. Growth conditions are described in Materials and Methods.

radation were inducible or constitutive and also if the expression of these biochemical pathways was coordinated, strain 12038 was grown on one of three different carbon sources, naphthalene, salicylate, and pyruvate. Pyruvate was chosen, as previous studies have shown that 1-naphthol degradation enzymes are present in cells grown under these conditions (29). The cultures were then used for time course studies of 1-naphthol and naphthalene catabolism. Figure 1 shows the change in substrate concentration for each of the three growth conditions over 3 h of incubation.

Both 1-naphthol and naphthalene degradation was evident for naphthalene-grown cells. The degradation of naphthalene was so rapid that even after a few minutes of incubation most of the substrate had disappeared. There is a discrepancy between the initial concentration of naphthalene shown (~ 0.1 mM) and that added (0.16 mM) for the pyruvate- and salicylate-grown experiments. This may be accounted for by the loss

TABLE 1. Enzyme activities of naphthalene and 1-naphthol catabolic pathways detected in *Rhodococcus* sp. strain NCIMB 12038 grown on different carbon sources

Carbon source	Enzyme activity detected (U/mg of protein) ^a				
	NO	SH	GO	NDO	NCD
Pyruvate	0.157	0.023	ND ^b	ND	ND
Salicylate	ND	0.673	0.729	ND	ND
Naphthalene	0.230	0.336	0.746	0.436	0.069 ^c

^a Specific activity of 1-naphthol oxygenase (NO), salicylate hydroxylase (SH), gentisate oxygenase (GO) expressed as micromoles of O₂ · minute⁻¹ · milligram of protein⁻¹. Naphthalene oxygenase (NDO) activity expressed as nanomoles of O₂ · minute⁻¹ · milligram of protein⁻¹. (1*R*,2*S*)-Naphthalene *cis*-diol dehydrogenase (NCD) activity expressed as micromoles of NADH produced · minute⁻¹ · milligram of protein⁻¹.

^b ND, not detected.

^c No activity detected when (1*S*,2*R*)-naphthalene *cis*-diol was used as a substrate for this enzyme assay.

of some substrate to the shaking flask headspace at the very low concentrations used or nonspecific adsorption of the compound to the bacterial cell surface. The aqueous solubility of naphthalene at 25°C is 0.25 mM (35).

Neither 1-naphthol nor naphthalene was degraded by salicylate-grown cells, and only 1-naphthol was degraded by pyruvate-grown cells over 3 h. The induction of naphthalene degradative enzymes occurred during growth on naphthalene by an unknown inducer, and this is in accordance with the observation of Grund et al. (25) for another naphthalene-degrading *Rhodococcus* sp. However, here 1-naphthol degradation appears to be constitutive but also repressed by growth on salicylate.

The enzyme activities obtained for key enzymes in these metabolic pathways in strain 12038, when grown on the three different carbon sources is shown in Table 1. The results confirm the observations above. Naphthalene dioxygenase and *cis*-diol dehydrogenase activities were present only in naphthalene-grown cells. 1-Naphthol oxygenase activity was present in both naphthalene- and pyruvate-grown cells. Salicylate-grown cells were clearly metabolically active, as indicated by salicylate and gentisate hydroxylase activities, but unlike *P. putida* (14, 42) were unable to oxidize either naphthalene or 1-naphthol.

The initial stages in the metabolism of naphthalene by strain 12038. Using the radiochemical assay of Ensley et al. (17), with cell-free protein extracts of strain 12038 grown on both naphthalene and pyruvate, it was found that radioactive naphthalene was converted to nonvolatile radioactive metabolites with only the cell extract from naphthalene-grown cells. An autoradiograph of a thin-layer chromatogram which was obtained using these nonvolatile metabolites showed the presence of only one detectable metabolite, which had an *R_f* value identical to that of naphthalene *cis*-diol (0.55) (20% CH₃COCH₃-CHCl₃). It should be noted that a standard sample of naphthalene *trans*-diol comigrated with the *cis*-diol under these conditions, whereas 1-naphthol had a different *R_f* value (0.76).

When the cell extract obtained from naphthalene-grown cells was incubated with unlabelled naphthalene, products were not detectable by fluorescence TLC (254 nm; Sigma Chemical Company, St. Louis, Mo.) or ¹H NMR analysis. However, naphthalene *cis*-diol (retention time, 8.9 min) was detected when the silylated extract was analyzed by GC-MS (*m/zs* [relative intensities] of 306 [2.3%], 275 [1.5%], 231 [0.5%], 203 [5.0%], 191 [21.9%], 147 [12.3%], 128 [5.0%], 103 [2.3%], 73 [41.5%], 45 [7.7%]). The silylated naphtha-

lene *trans*-diol had a different retention time (9.5 min) but an identical mass spectrum. This compound was also detected in the cell extract but with a lower relative yield (<10% of total diols). *trans*-Diol was not detected when an authentic standard of the *cis*-diol was injected onto the column. The origin of the *trans*-diol is therefore uncertain, and its presence warrants further investigation.

The presence of *cis*-diol dehydrogenase enzyme activity in cell extracts was determined with both the purified (1*S*,2*R*)- and (1*R*,2*S*)-enantiomers of naphthalene *cis*-diol (Table 1). These results suggest that the dehydrogenase expressed during naphthalene metabolism was stereospecific and therefore that the (1*R*,2*S*) isomer was produced during naphthalene metabolism. This is the same absolute configuration as is found during naphthalene metabolism by *P. putida* (32).

It was also noted that during naphthalene metabolism by cell extracts both salicylate and gentisate were produced. Both compounds were detected by GC-MS and ¹H NMR analyses of organic extracts. It has previously been shown that salicylate and gentisate accumulate during 1-naphthol metabolism by this organism (30), and this may suggest that the catabolic pathways for the degradation of naphthalene and 1-naphthol converge.

The results suggest that the initial stages in the biochemistry of naphthalene degradation in *Rhodococcus* sp. strain NCIMB 12038 and *P. putida* are identical, even though the genetic regulation of naphthalene degradation is different. Naphthalene degradation is also clearly regulated independently of 1-naphthol degradation.

Biotransformation of indene by strain 12038. Biotransformation studies with whole cells and purified dioxygenases have shown that bacteria which express either toluene or naphthalene dioxygenase enzymes are able to oxidize indene to a mixture of *cis*-1,2-dihydroxyindan and 1-indenol (1, 8, 44). Furthermore, these metabolites accumulated in experiments with both *cis*-diol dehydrogenase-deficient mutant bacteria and wild-type strains, which suggests that the indene biotransformation is a useful method for determining whether any arene-degrading isolates express a similar dioxygenase enzyme during growth on an aromatic substrate (1).

Naphthalene-, pyruvate-, and salicylate-grown cells of strain 12038 were used as catalysts in whole-cell biotransformations of indene. After extraction and purification of metabolites, it was found that the naphthalene-grown cells oxidized indene (compound 1; X = CH₂) to *cis*-(1*S*,2*R*)-dihydroxyindan (compound 2; X = CH₂, 6.5% yield; 80% enantiomeric excess [e.e.]) and (1*S*)-indenol (compound 3; 1.8% yield; >97% e.e.) (Fig. 2). In addition, a trace of 2-hydroxyindan-1-one (compound 4) was tentatively identified (stereochemistry not determined, for ¹H NMR data see reference 4). The natures of these products are very similar to the indene metabolites isolated from the naphthalene-degrading organism *P. putida* NCIMB 8859 (1) and from the purified naphthalene dioxygenase enzyme from *P. putida* NCIB 9816 (23), suggesting further similarities between the naphthalene dioxygenase enzymes in the gram-positive and gram-negative organisms. From the pyruvate-grown cells, however, only *trans*-(1*R*,2*R*)-dihydroxyindan (compound 6; X = CH₂, 2% yield; 8% e.e.) was recovered (Fig. 2). *trans*-1,2-Dihydroxyindan (1% yield; <5% e.e.) was also purified from indene biotransformation with salicylate-grown cells.

To determine the origin of the *trans*-1,2-dihydroxyindan, a sample of racemic *cis*-1,2-dihydroxyindan was incubated with pyruvate-grown cells under the same conditions used for the indene biotransformation. In this reaction, no *trans*-1,2-dihydroxyindan was detected by either method of anal-

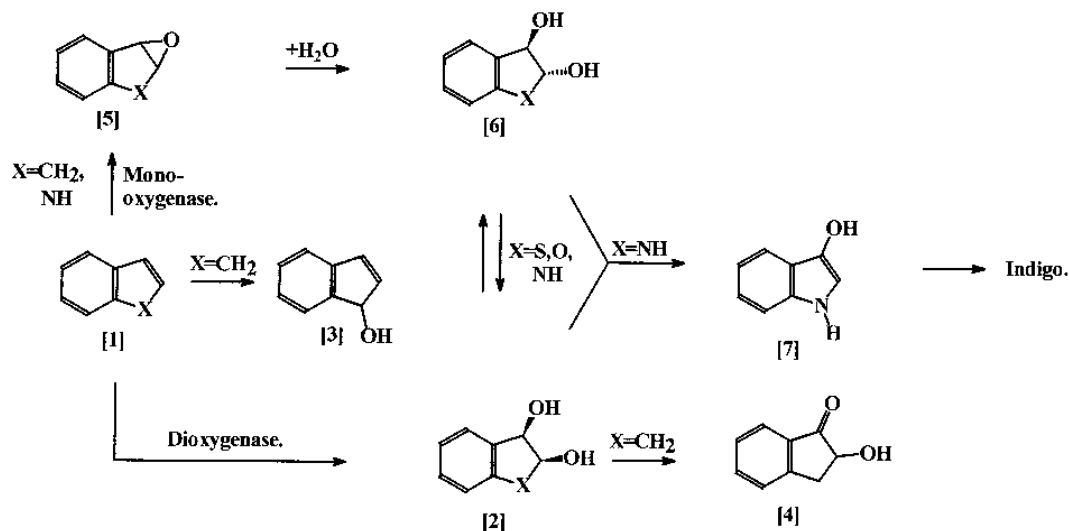


FIG. 2. Metabolism of indene and heterocyclic analogs by *Rhodococcus* sp. strain NCIMB 12038. Methods used for the identification of metabolites are described elsewhere. Naphthalene-grown cells oxidized indene (compound 1) (X = CH₂) to *cis*-(1*R*,2*S*)-dihydroxyindan (compound 2) (X = CH₂), (1*S*)-indenol (compound 3), and 2-hydroxyindan-1-one (compound 4). Both pyruvate- and salicylate-grown cells oxidized indene to *trans*-1,2-dihydroxyindan (compound 6) (X = CH₂). Possible biotransformation routes to indigo (X = NH) are shown. Ensley et al. (18) have proposed the action of a dioxygenase enzyme to produce the *cis*-diol (compound 2) (X = NH) with subsequent dehydration to yield indoxyl (compound 7) and indigo. Recent studies (9, 10) have shown that for benzothiophene (X = S) and benzofuran (X = O) dioxygenase attack may also yield a *cis*-diol (compound 2), which may then undergo rearrangement to the *trans*-diol (compound 6). Thus, it is feasible that the action of a monooxygenase enzyme on indole (compound 1) (X = NH) may also yield indigo via an epoxide (compound 5) (X = NH).

ysis. This would suggest that the formation of the *cis*- and *trans*-diols described above was as a result of different processes.

Pyruvate-grown cells were also incubated with 1,2-indene oxide (compound 5; X = CH₂, Fig. 2) as a substrate. In this experiment, *trans*-1,2-dihydroxyindan was identified as the predominant metabolite, along with a trace of the *cis*-diol. A similar result was also observed when killed cells were used in the experiment. Thus, it is possible that the biological product of indene oxidation by pyruvate-grown cells is actually 1,2-indene oxide, which is then subject to nonenzymatic hydration to the observed *trans*-diol under these reaction conditions. The absence of indan *cis*-diol and inden-1-ol accumulation by pyruvate-grown cells indicates that no naphthalene dioxygenase activity was present under these conditions. The formation of *trans*-1,2-dihydroxyindan suggests that a monooxygenase is involved in the degradation of 1-naphthol (22, 41).

Biotransformation of indole with strain 12038. The metabolic pathway leading to indigo formation in dioxygenase-expressing bacteria is thought to proceed through an unstable *cis*-diol intermediate, with spontaneous elimination of water to yield indoxyl (compound 7 [Fig. 2]) (18, 33). The metabolism of indole to indigo was also used to test for the expression of oxygenase enzymes produced during growth of strain 12038 on the various substrates.

A blue pigment was observed in both naphthalene- and pyruvate-grown liquid cultures to which indole had been added after 2 to 3 h. Conversely, no blue pigment was observed with the salicylate-grown culture, even after 24 h of incubation. However, when the bacteria were grown on agar plates with the same salicylate MSM and drops of 250 mM indole (in methanol) were applied to the colonies, some blue pigment formation was observed. Cells growing slowly on salicylate on agar plates may not be subject to catabolite repression to the same extent as in liquid culture, and this may explain the presence of an enzyme capable of producing indigo.

It was noted that the pigment isolated from pyruvate-grown

cultures had the same *R_f* value (0.78; solvent, 20% diethyl ether in chloroform) as that of an authentic indigo standard. Analysis of the retention time and UV spectrum of the isolated pigment using reverse-phase HPLC with a diode-array detector also indicated that the metabolite had identical properties to the authentic standard (retention time, 5.44 min; λ_{max}, 202, 243, 287, 336, and 600 nm in 190- to 600-nm range).

The ability of pyruvate-grown cells of strain 12038 to transform indole to indigo would suggest that certain enzymes, other than the toluene and naphthalene dioxygenases previously reported, may be able to catalyze this reaction. Boyd et al. (9, 10) have shown that *cis*-diols arising from the biotransformation of certain unsaturated heterocyclic compounds (such as benzothiophene and benzofuran) undergo a reversible spontaneous solvent-dependent rearrangement reaction to corresponding *trans*-diols (Fig. 2). It is probable that the *cis*-diol of indole proposed by Ensley et al. (18) will also undergo isomerization to a *trans*-diol. Furthermore, if indole was converted to a 2,3-oxide intermediate via monooxygenase-catalyzed epoxidation, this arene oxide could yield a 2,3-*trans* diol upon spontaneous or enzyme-catalyzed hydration. Thus, indigo may be produced as a result of both mono- and dioxygenase enzyme activities (Fig. 2). If a monooxygenase was required for the initial oxidation of 1-naphthol (as would be suggested from the isolation of indan *trans*-diol from indene transformation with pyruvate-grown cells), then this might suggest similarities to the biochemistry of phenol metabolism in bacteria (40). Furthermore, the oxidation of indole to indigo by monooxygenase enzymes has been indicated in earlier studies (34, 36, 45). There has been some interest in the use of the indole transformation as a marker for genetic engineering (26, 27, 43). These results suggest that a cloned indigo-producing enzyme need not necessarily be an arene dioxygenase. The indene biotransformation pathway may however provide a convenient method for establishing if arene dioxygenase enzymes are expressed in wild-type bacteria.

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