Recruitment of Naphthalene Dissimilatory Enzymes for the Oxidation of 1,4-Dichloronaphthalene to 3,6-Dichlorosalicylate, a Precursor for the Herbicide Dicamba

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_Pseudomonas putida_ expresses plasmid-encoded enzymes and regulatory proteins for the dissimilation of naphthalene through salicylate and the α-keto acid pathway. A strain of _P. putida_ (NAH7/Tn5/G67) defective in salicylate hydroxylase (nahG) was assessed for its ability to oxidize 1,4-dichloronaphthalene. Washed cell suspensions were shown to accumulate 3,6-dichlorosalicylate, which, after further chemical treatment, yields the herbicide dicamba (3,6-dichloro-2-methoxybenzoate). However, the rate of dichlorosalicylate formation from dichloronaphthalene was less than 1% of the rate of salicylate formation from unsubstituted naphthalene.

Recent advances in biotechnology have resulted in the development of microorganisms for the production of chemicals and the detoxification of chemical wastes. Members of the genus _Pseudomonas_ are attractive biocatalysts for the production of chemicals because of their ability to oxidize a wide variety of organic compounds (11, 14–17). The metabolic diversity of many pseudomonads is attributable to plasmid-encoded metabolic sequences (10, 17). In general, plasmid-borne dissimilatory enzymes exhibit relaxed substrate specificity (8, 12) and thus can be used for the oxidation of substrate analogs. The most thoroughly characterized degradative plasmids are the NAH7 plasmid, which specifies naphthalene dissimilation (2, 22), and the TOL plasmid (pWWO), which provides genetic information for the degradation of toluenes, xylenes, and related compounds (7, 20, 21). Genetic manipulations of these plasmids have resulted in the construction of strains with extended metabolic capabilities (8, 12, 17, 23) and the production of chemical products such as indigo (5, 9), catechol (23), cis-dihydrodiosil (1, 13, 23), salicylate (4, 22), and cresols (19).

Naphthalene is degraded by _P. putida_ through catechol and the α-keto acid or _meta_ cleavage pathway (2, 24). The NAH7 degradative plasmid is composed of two gene clusters with an intervening regulatory region (22). The first cluster encodes for the oxidation of naphthalene to salicylate (Fig. 1), and the second cluster encodes for the oxidation of salicylate to pyruvate and acetaldehyde. In this study, we demonstrated the relaxed specificity of the first cluster of NAH-encoded catabolic enzymes for the oxidation of a chlorinated analog to a potentially useful product. More specifically, a _P. putida_ strain harboring a defective NAH plasmid oxidized 1,4-dichloronaphthalene to 3,6-dichlorosalicylate, a precursor for the herbicide dicamba (trade name Banvil; A. M. Robin, U.S. patent 3,335,177, August 1967) (Fig. 1).

_P. putida_ strains containing mutant NAH plasmids were obtained from I. C. Gunsalus and have been described elsewhere (22). Maintenance of cultures, media, and growth

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determinations

Compound separated

transformation

high-pressure chloroimide and

of naphthalene harvested, dried,

and extracted

nati, harvested

conditions

oxidation

30°C

samples

suspensions,

with

agitation

centrifugation,

and

ether. Extracts

were
dried;

products

were

removed

pH 7.0, and suspended in 10 ml

of phosphate buffer in 125-ml baffled Erlenmyer flasks. Crystals of 1,4-dichloronaphthalene were added to the cell suspensions, and the reaction mixtures were incubated at 30°C with agitation (300 rpm). The cell suspensions were harvested, and the supernatants were acidified with 6 N HCl and extracted with 3 volumes of diethyl ether. Extracts were dried, suspended in ethanol, and applied to thin-layer cellulose F254 plates (MC/B Manufacturing Chemist, Inc., Cincinnati, Ohio). Plates were developed in isopropanol, water, and NH4OH (20:2:1, vol/vol/vol) and air dried; the products were visualized under UV light and by spraying chromatographs with 0.4% (wt/vol) 2,6-dichloroquinone-4-chloroimide (Gibbs reagent) followed by 10% (wt/vol) Na2CO3. In addition, oxidation products were resolved by high-pressure liquid chromatography (HPLC) by applying samples of the culture supernatant to a PRP-1 analytical column (250 by 4.1 mm; The Hamilton Co., Reno, Nev.) and eluting them at a flow rate of 1.5 ml/min with a linear gradient of 30 to 100% methanol in 20 mM acetic acid. Detection (230 nm) and spectral scans were performed with a 1040A high-speed spectrophotometric detector (Hewlett-Packard Co., Palo Alto, Calif.).

P. putida GX1005 (NAH::Tn5/G67) uses naphthalene as a carbon and energy source and accumulates salicylate as an oxidation product. Salicylate is not metabolized further because of insertional inactivation of nahG, which encodes for salicylate hydroxylase (22). Strain GX1005 was grown in the presence of salicylate to induce naphthalene catabolic enzymes, and the washed cell suspensions were incubated with 1,4-dichloronaphthalene. Analysis of the oxidation products by thin-layer chromatography revealed a product with an Rf and a color reaction with Gibbs reagent identical to those of authentic 3,6-dichlorosalicylate (Fig. 2). The identity of this oxidation product was further confirmed by HPLC analysis of reaction mixtures. Compound I had an HPLC retention time and UV absorbance spectrum identical to those of authentic 3,6-dichlorosalicylate (Fig. 3).

Thin-layer chromatography analysis revealed another major oxidation product with an Rf of 0.89 (Fig. 2). Furthermore, a major oxidation product (compound II) other than dichlorosalicylate, with a retention time of 24 min (Fig. 3), was also apparent after HPLC analysis. By comparison, P. putida GX1005 (nahG) did not accumulate intermediates other than salicylate with unsubstituted naphthalene as the substrate (Fig. 2 and 4D).

To identify compound II, P. putida strains containing NAH plasmids (22) with Tn5 insertions in various nah structural genes were examined. Mutant strains were induced as described above and exposed to dichloronaphthalene, and reaction products were analyzed by HPLC. Strain GX1002 (NAH::Tn5/B11), a nahB (naphthalene cis-dihydriodiol dehydrogenase) mutant, accures an oxidation product from dichloronaphthalene with an HPLC retention time (Fig. 4B) and UV absorbance spectrum (not shown) identical to those of compound II. P. putida GX1001 (NAH::Tn5/A1), which is defective in nahA (naphthalene dioxygenase), did not form any discernible oxidation products (Fig. 4A).

Strain GX1002 accumulates cis-1,2-dihydroxy-1,2-dihydronaphthalene from naphthalene (6, 21). This finding was confirmed by showing that the UV absorption spectrum of the oxidation product formed from naphthalene by GX1002 conform to the published spectrum (6) for cis-1,2-dihydroxy-1,2-dihydronaphthalene (Fig. 5A). Further confr-
data, we conclude that compound II is 5,8-dichloro-

cis-1,2-dihydroxy-1,2-dihydronaphthalene (Fig. 1). (The relative

stereochemistry in this molecule was not established, but is

based on studies by Jeffrey et al. [6].)

The rate of 3,6-dichlorosalicylate formation was measured by

HPLC analysis of supernatants of reaction mixtures as a

function of time. For comparison, washed cell suspensions of

P. putida GX1005 were incubated with naphthalene. The

rates of 3,6-dichlorosalicylate and salicylate formation were

5.1 and 560 μg/h per g of cell dry weight, respectively. This

large disparity in reaction rates may be due to one or more of

the following events: toxic intermediate or toxic product

formation, limited availability of substrate due to insolubility,

inability of cells to transport and thus further oxidize

intermediates, or ineffective catalysis of chlorinated inter-

mediates by specific enzymes of the pathway due to either

steric effects or deactivation of the aromatic nucleus as a

result of the halogen substituents. None of these possibilities

has been excluded. The significant accumulation of the

cis-dihydrodiol intermediate by GX1005 (nahG) during me-
			
tabolism of dichloronaphthalene indicates one dispro-

portionately slow step in the dissimilation of the chlorinated

analog. GX1005 appears to oxidize naphthalene to salicylate

without accumulation of an intermediate (Fig. 4D). Although

other oxidation products are observed during the oxidation of

dichloronaphthalene (Fig. 4C). It is significant that all of the

carbon flow is directed toward the end product, dichlorosalicylate.

This process is an example of how a biological route can be more

advantageous than a chemical synthesis, namely, by making efficient use of the starting

material by precluding isomeric side products.

FIG. 4. Oxidation products of 1,4-dichloronaphthalene from P. putida mutant GX1001 (NAH:Tn5/A1) (A), GX1002 (NAH:Tn5/B1) (B), and GX1005 (NAH:Tn5/G67) (C) and oxidation products of naphthalene from P. putida mutant GX1005 (D). Strains were induced and biotransformation experiments were performed as described in the text. Clarified reaction mixtures (at 9 h, 100 μl was injected for dichloronaphthalene reaction mixtures [A to C], and at 3 h, 20 μl was injected for naphthalene reaction mixture [D]) were resolved by HPLC. I and II, Compounds I and II.

FIG. 5. Absorption spectra of cis-1,2-dihydroxy-1,2-dihydronaphthalene (A) and compound II (B) formed by P. putida GX1002 from naphthalene and dichloronaphthalene, respectively. (A) ———, cis-1,2-Dihydroxy-1,2-dihydronaphthalene; ———, 1-naphthol formed after acid treatment; ———, 2-naphthol formed after acid treatment (18). (B) ———, Compound II; ———, compound II after acid treatment. Acid treatment was done as described elsewhere (18). λ max, Maximum wavelength.
In summary, the data presented here represent another example of how microorganisms can be used to make useful chemicals. *P. putida* was shown to use naphthalene dissimilatory enzymes to oxidize 1,4-dichloronaphthalene to 3,6-dichlorosalicylate; further chemical treatment results in the formation of 3,6-dichloro-2-methoxybenzoate, which is an herbicide sold commercially under the trade name Banvil or dicamba (Fig. 1). The slow rate of conversion of chlorinated naphthalene relative to that of naphthalene with the strain used in this study is exemplary of many biological transformations of substrate analogs (D. R. Durham, unpublished data); no attempts were made to increase the rate of 3,6-dichlorosalicylate formation. Whether further genetic manipulations such as amplification of the *nah* gene products or alteration(s) in the catalytic sites of rate-limiting enzymes via protein engineering would result in an economical biotransformation is not known.

We thank Robert Butz of Velsicol Chemical Corp. for providing 1,4-dichloronaphthalene and 3,6-dichlorosalicylate. We also thank D. Anderson for critical review of the manuscript, L. Lorenz for typing, and D. Hopkins for proofreading the manuscript.

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