Burkholderia cepacia Strain G4 (pHG-2) Accumulates *cis*-3-Methyl-3,5-cyclohexadien-1,2-diol While Growing on Toluene

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Received February 18, 2000

***Burkholderia cepacia* strain G4 (pHG-2) containing toluene 2-monooxygenase and toluene dioxygenase, was able to grow on toluene and accumulate *cis*-3-methyl-3,5-cyclohexadien-1,2-diol (*cis*-toluene dihydrodiol) in the liquid culture. The *cis*-toluene dihydrodiol produced was identical to the authentic compound, as judged through mass spectrometry and nuclear magnetic resonance analysis. Our results indicate that pHG-2 provides an economical means to produce chemically-important chiral synthons while growing on toluene.**

Key words: Burkholderia cepacia *G4, cis-toluene dihydrodiol, synthon, toluene dioxygenase, cis-3-methyl-3,5-cyclohexadien-1,2-diol.*

Burkholderia cepacia strain G4, which contains the genes encoding toluene 2-monooxygenase on an approximately 108-kb degradative plasmid TOM,[1] has been known to possess diverse capacities to metabolize on aromatic and aliphatic compounds.[2-5] *B. cepacia* strain G4 uses toluene as the sole source of carbon and energy through meta ring cleavage via *o*-cresol to 3-methyl catechol.[2,3] Toluene 2-monooxygenase is a three- component enzyme system and, along with reduced NADH, carries out consecutive hydroxylations at the ortho and meta positions of toluene under aerobic condition.[6]

Another three-component enzyme, toluene dioxygenase encoded by the *todC$_1$C$_2$BA* genes in *Pseudomonas putida* strain F1 has been shown to simultaneously oxidize toluene at both ortho and meta positions through the addition of a single diatomic oxygen.[7,8] The product of this reaction has an absolute stereochemistry, (+)-(1S, 2R)-3-methyl-3,5-cyclohexadien-1,2-diol (*cis*-toluene dihydrodiol).[9,10] *P. putida* strain 39/D, which contains a defect in the structural gene for *cis*-toluene dihydrodiol dehydrogenase, accumulates *cis*-dihydrodiol products in the culture medium from over 20 monomeric aromatic compounds.[11,12] *cis*-Dihydrodiol compounds are very versatile synthons (chiral building blocks) synthesizing sugars, alkaloids, pharmaceuticals, and chiral compounds.[13] Conventional chemical processes cannot be used to inexpensively synthesize *cis*-dihydrodiols from arenes in significant amounts.[11,14] In addition, there are increasing demands for "green technology" in the area of chemical research and development to minimize or eliminate toxic by-products.[13,15] For these reasons, there have been much interest in the synthesis of homo chiral *cis*-dihydrodiols through biological processes and the utilization of the chiral structure to synthesis valuable products.[15-19]

The objectives of this study were to investigate the metabolism of toluene by both toluene 2-monooxygenase and toluene dioxygenase present in *B. cepacia* strain G4 (pHG-2). The accumulation of *cis*- toluene dihydrodiol, produced by toluene dioxygenase, in the culture medium was also studied, thereby producing chemically important synthons from mixtures of waste solutions containing aromatic rings.

Materials and Methods

Chemicals. *cis*-Toluene-dihydrodiol was purchased from Fluka Chemical Co., (Ronkonkoma, NY, USA). Kanamycin, isopropylthiogalactoside (IPTG), and $CDCl_3$ (99.8%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Indole was obtained from Eastman Kodak Co. (Rochester, NY), and HPLC grade ethyl acetate was obtained from Aldrich Chemical Co. (Milwaukee, WI).

Construction of the recombinant strains. The recombinant *B. cepacia* G4 (pHG-2) strain was constructed through conjugation of a plasmid pHG-2 containing *todC$_1$C$_2$BA* under the control of P_{tac} and *lacI^q* into *B. cepacia* strain G4 as previously described.[20] Transconjugants were selected on the MSB medium[21] containing 20 mM lactate, 50 μg/ml kanamycin, and 5 mM IPTG. The presence and activity of *todC$_1$C$_2$BA* in strain G4 was verified by examining colonies of transconjugants for a blue color on the same medium incubated in the presence of indole vapor. The blue color is caused by the production of indigo from indole.[22] Colonies of wild-type *B. cepacia* strain G4 did not show a blue color.

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Growth of organisms and production of *cis*-toluene dihydrodiol. Starter cultures of *B. cepacia* strain G4 were grown in 50 ml MSB medium containing 20 mM lactate overnight at 27°C with shaking. Lactate-grown *B. cepacia* strain G4 was inoculated in 50 ml MSB liquid medium, in which toluene was added as vapor phase at 27°C until the cell density reached OD 2 at 600 nm. *B. cepacia* strain G4 (pHG-2) was grown in the same manner except that kanamycin (50 μg/ml) was added to the medium. Toluene-grown *B. cepacia* strain G4 and *B. cepacia* strain G4 (pHG-2) were harvested by centrifugation at 10,000×g for 10 min and thoroughly washed with 20 mM phosphate buffer (pH 7.0). *B. cepacia* strains G4 and G4 (pHG-2) were inoculated in 50 ml MSB media in 500 ml flask with and without 5 mM IPTG to make an initial OD at 600 nm of 0.45. Toluene was added in the vapor phase as described above, and cultures were incubated with shaking at 27°C. Aliquots of cultures 0.5 ml were extracted at various times to monitor bacterial growth at 600 nm and the production of *cis*-toluene dihydrodiol at 265 nm.[9] The *cis*-toluene dihydrodiol accumulating in the liquid culture of *B. cepacia* strain G4(pHG-2) was extracted and purified as previously described.[23] To isolate *cis*-toluene dihydrodiol from the cell culture, *B. cepacia* strain G4 (pHG-2) was grown in 500 ml cell culture on toluene vapor and induced with 5 mM IPTG as described above. After reaching an OD of 1.5 at 600 nm, the cell culture was centrifuged at 8,000×g for 15 min, and NaCl was added to reach a final concentration of 160 g/L. The solution was extracted 5 times with one-fourth volume of ethyl acetate. The ethyl acetate extracts were dried under anhydrous sodium sulfate, and the solvent was evaporated by rotary evaporation at 55°C. The residue was crystallized at -20°C for 10 min.

Analytical methods. After measurements of bacterial growth, the 0.5 ml culture aliquots were centrifuged at 15,000 rpm for 5 min, and the supernatants were examined for the presence of *cis*-toluene dihydrodiol. The amount of *cis*-toluene dihydrodiol produced was calculated using a molar extinction coefficient of 5,220 at 265 nm.[9] The identity of the crystalline material was determined using mass spectrometry and NMR. Mass spectrometry was done through direct probe insertion using a Kratos MS25 mass spectrometer with a 70 eV acceleration voltage. The experiment was repeated with commercially available, authentic, *cis*-toluene-dihydrodiol (Fluka Chemical Co. Ronkonkoma, New York). ^{1}H NMR spectra of both the crystals formed from the liquid culture of *B. cepacia* strain G4 (pHG-2) and the authentic *cis*--toluene-dihydrodiol dissolved in $CDCl_3$ were obtained using a Nicolet NT300 NMR spectrometer at 300 MHz.

Results and Discussion

B. cepacia strain G4 (pHG-2) grown on toluene vapor and induced with IPTG actively accumulated *cis*-toluene dihydrodiol in the culture medium (Fig. 1). The production of *cis*-toluene dihydrodiol was confirmed through mass spectrometry (Fig. 2) and NMR analysis (Fig. 3). Mass spectrometry of the extracted sample showed ions at 51, 65, 79, 90, 97, 108, and 126 *m/z*. (Fig. 2B). These peaks and their relative intensity were in close agreement with the authentic *cis*-toluene dihydrodiol (Fig. 2A). Moreover, the ^{1}H NMR ($CDCl_3$) of the extracted sample showed resonances at d 1.87 (3H, s), 3.56 (2H, br), 3.94 (1H, m), 4.25 (1H, m), 5.68 (1H, m), 5.72 (1H, m), and 5.86 (1H, m) (Fig. 3B). This NMR spectrum was identical to that of authentic *cis*-toluene-dihydrodiol (Fig. 3A). Due to limitations in the resolution of the instrument used, I could not distinguish the hyperfine at the chemical shift resonances of 5.68, 5.72, and 5.86 which were previously reported by Hudlicky *et al.*[14] As compared to *B. cepacia* strain G4 with 5 mM IPTG or *B. cepacia* strain G4 (pHG-2) without IPTG, *B. cepacia* strain G4 (pHG-2) induced with IPTG changed its growth pattern after 4 h of incubation (Fig. 1A). The change of growth pattern, represented by a decrease in growth rate, appeared to be caused by the high expression

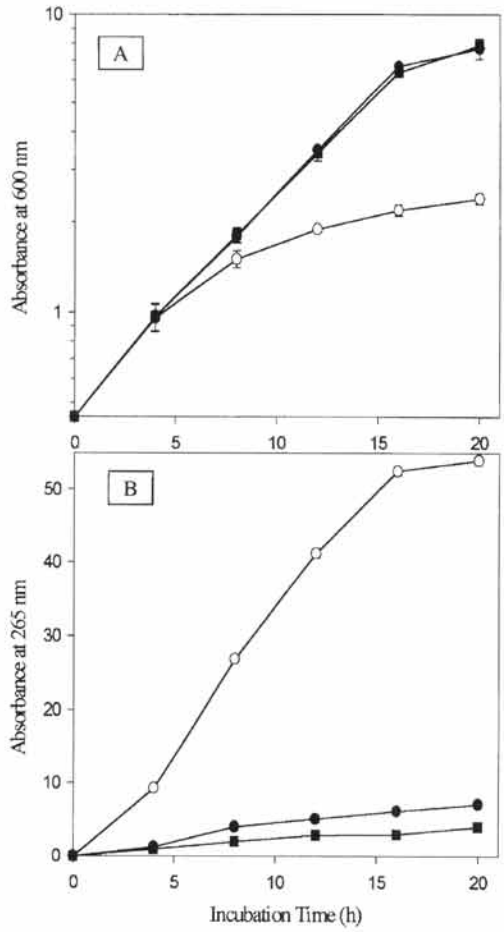

Fig. 1. Growth (A) and *cis*-toluene dihydrodiol production (B). Culture flasks (500 ml) contained 50 ml MSB medium. Toluene was supplied in the vapor phase. *B. cepacia* strain G4 (pHG-2) with 5 mM IPTG (-○-); *B. cepacia* strain G4 (pHG-2) without IPTG (-●-); *B. cepacia* strain G4 with 5 mM IPTG (-■-).

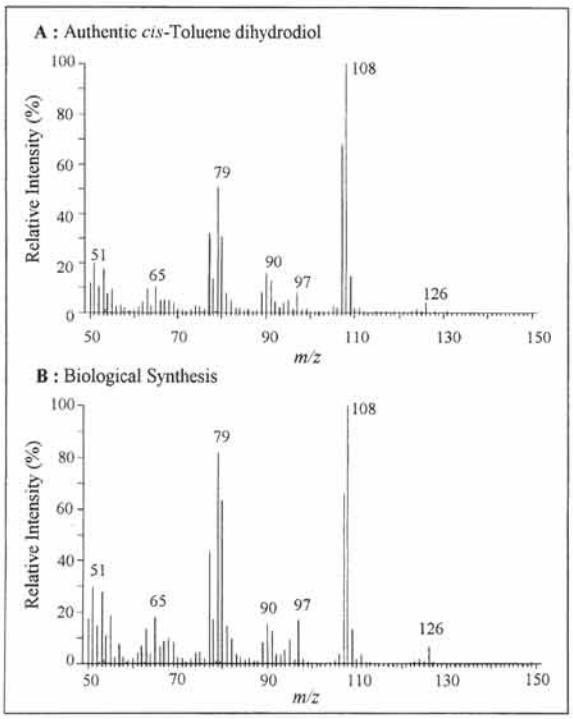

Fig. 2. Mass spectra of *cis*-toluene dihydrodiol of authentic compound (A) and biological synthesis (B).

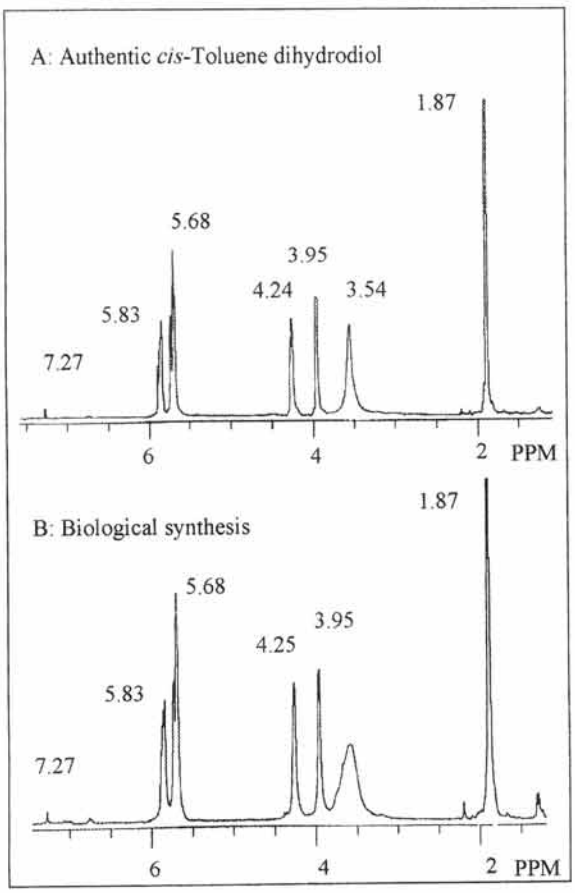

Fig. 3. ^{1}H NMR spectra of *cis*-toluene dihydrodiol of authentic compound (A) and biological synthesis (B).

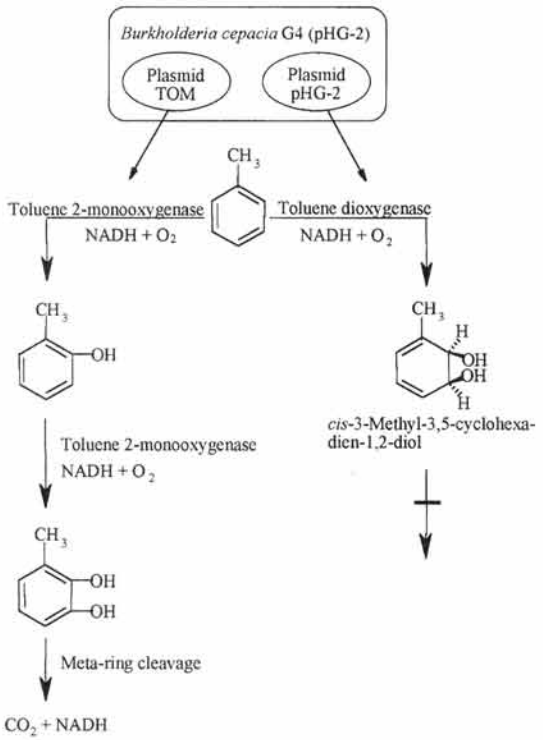

Fig. 4. Proposed metabolism of toluene by *B. cepacia* strain G4 (pHG-2).

level of toluene dioxygenase and related to the active accumulation of *cis*-toluene dihydrodiol in the liquid growth medium (Fig. 1B). Toxic effects of *cis*-toluene dihydrodiol on a gradual deterioration in the state of the cells was previously suggested by Jenkins *et al.*[24]

When growing on toluene vapor, IPTG-induced *B. cepacia* strain G4 (pHG-2) accumulated *cis*-toluene dihydrodiol even after cultures had reached stationary phase. In contrast, the control strain, *B. cepacia* strain G4 induced with IPTG, did not accumulate *cis*-toluene dihydrodiol in the liquid culture medium. After 20 h incubation of IPTG-induced *B. cepacia* strain G4 (pHG-2), the optical density at 265 nm reached 54 which amounted to a *cis*-toluene dihydrodiol concentration of 1.3 g per liter (Fig. 1B). This yield is comparable to the amount (1.3-1.5 g per liter) presented by Gibson *et al.*[9] of a mutant strain, *Pseudomonas putida* strain 39/D, but is much less than the amount (18-24 g per liter) reported by Jenkins *et al.*[24] of a mutant strain, *Pseudomonas putida* NG1 of Imperial Chemical Industries (ICI). Jenkins *et al.*[24] used fed-batch, non-growing cell cultures, which were previously grown on glucose, under restricted supply of nitrogen and an excess supply of co-substrate for NADH regeneration. In a related study to enhance biotransformation of toluene to *cis*-toluene dihydrodiol using fed-batch cell cultures of a mutant strain, *P. putida* UV4, Brazier *et al.*[25] examined the effect of concentration of toluene and oxygen, and ethanol which is a co-substrate for NADH regeneration. They found that the maximum reaction rate (1 g/h per gram of cells) was

achieved at the concentration of toluene above 2.5% aqueous saturation with dissolved oxygen tension at 20% air saturation or above, and ethanol concentration in the range of 2-18 g per liter. In another approach to produce *cis*-arene dihydrodiol, van den Tweel *et al.*[26] reported the continuous production process by a mutant strain, *Pseudomonas* sp Mt 92. After 40 h incubation with benzene, production of *cis*-benzene dihydrodiol reached a maximum of 0.9-1.5 g per liter. However, the mutant strains used faced a common problem of reversion to the wild type causing a loss in biotransformation ability.

The metabolic pathway in *B. cepacia* strain G4 (pHG-2) may provide a fortuitous biological mechanism to produce *cis*-toluene dihydrodiol (Fig. 4). *B. cepacia* strain G4 (pHG-2) seems to efficiently supply reducing cofactor, NADH, using TOM pathway of wild type *B. cepacia* strain G4 for synthesis of *cis*-toluene dihydrodiol. Therefore, *B. cepacia* strain G4 (pHG-2) may be used for the continuous production of *cis*-toluene dihydrodiol from toluene based on growing cells. Because *B. cepacia* strain G4 can grow on benzene and ethylbenzene, and its constitutive mutant *B. cepacia* strain G4/PR1 can additionally grow on propylbenzne,[2,4,5] these strains may prove valuable for the production of other *cis*-arene dihydrodiols. Taken together, the metabolic system of *B. cepacia* strain G4 (pHG-2) suggests a possibility to produce expensive chemical synthons from the industrial waste chemicals while growing on them or cleaning them up. It should be noted, however, that since IPTG is very expensive, other gene induction systems need to be investigated.

In order to increase the yield of *cis*-toluene dihydrodiol by *B. cepacia* strain G4 (pHG-2), more thorough investigation will be needed in terms of effects of toluene concentration on toxicity and NADH regeneration, toxic effects of *cis*-toluene dihydrodiol, dissolved oxygen tension, and stability of the plasmid. The experimental results, however, suggest the possibility that with a suitable promoter to actively induce toluene dioxygenase, valuable and versatile synthons, such as *cis*-toluene dihydrodiols, can be easily synthesized in an environmentally friendly manner through the creation of a novel bacterial metabolic pathway.

Acknowledgments. I offer my deep gratitude to Professors Michael J. Sadowsky and Lawrence P. Wackett at the University of Minnesota for their critical review of this manuscript and Dr. Thomas P. Krick at the University of Minnesota for his technical help in mass spectrometry and NMR experiments. Also thanks are due to Mr. Dae Soon Kim for his help in manuscript preparation.

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