Simultaneous Biodegradation of S, N, and O Pollutants by Engineering of a Carbazole-Degrading Gene Cassette in a Recombinant Biocatalyst^{∇†}

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The gene cassette encoding enzymes responsible for degrading carbazole to anthranilic acid was introduced into a dibenzothiophene degrader. The resultant strain, *Rhodococcus erythropolis* XPDN, could simultaneously transform the model pollutants dibenzothiophene, carbazole, and dibenzofuran to nontoxic metabolites and may have an application potential for bioremediation.

Sulfur (S), nitrogen (N), and oxygen (O) heterocycles have been found widely in groundwater, seawater, sediments, and soil at sites contaminated with petroleum and wood-preserving wastes (4). Even though their concentrations may be quite small, the influence of these organic, nonhydrocarbon constituents cannot be ignored (30). Petroleum contamination may have a great impact on the organisms that live in the contaminated ecosystems because some petroleum compounds have been reported to be mutagenic and carcinogenic (10, 13). Heterocyclic compounds, particularly dibenzothiophenes (DBTs), can persist for up to 3 years in the environment (6). DBT is the most widely used compound for studies of polycyclic aromatic sulfur-containing hydrocarbon biodegradation and for petroleum biodesulfurization (14, 17, 23, 32, 36, 37). Research has been focused on strains that can selectively remove sulfur by the “4S” pathway, in which DBT is converted to 2-hydroxybiphenyl (2-HBP) (5, 15). Carbazole (CA) is the most abundant nitrogen-containing compound in many petroleum samples and therefore was chosen as a model compound (8). CA and its derivatives have been found to be toxic and mutagenic (11). Moreover, they readily undergo radical chemical reactions to generate the more genotoxic hydroxynitrocarbazoles (1). Microbial degradation of CA by bacteria has been demonstrated, and anthranilic acid (AA) has been shown to be the key intermediate in many isolates (4, 12, 20, 24). Additionally, dibenzofuran (DBF), one of the O heterocycles, has been used as an insecticide. In many studies of DBF biodegradation DBF has been considered a model for chlorinated DBFs and dibenzop-dioxins (DDs), which are of greater environmental concern (2).

These S, N, and O compounds are environmental poisons, and some heterocycles may form persistent toxic substrates or persistent organic pollutants, which may cause serious environmental problems. Eradication of these persisting pollutants

from the environment has attracted a great deal attention from academia, industry, and governmental agencies. Biodegradation has become more popular as an alternative to physical and chemical methods because of its relatively low cost and minimal impact on the environment. The aim of the present study was to investigate the ability of a recombinant *Rhodococcus* strain to simultaneously transform targeted S, N, and O heterocycles.

In previous studies, CA was converted by stepwise degradation to AA by the enzymes encoded by the *carABC* operon (27, 28). Based on the sequence of *Pseudomonas* sp. strain CA10, the following primers were designed: P1 (5'-GCCGACTAGT AAGGAGATGGACGTGGCG-3') containing a SpeI restriction site (underlined) and P2 (5'-GACGAGTACTGCAGC GCCGTCATACGTTGC-3') (ScaI site underlined). Even though *Pseudomonas* sp. strain CA10 and presumably *Pseudomonas* sp. strain XLDN4-9 (16) contain two copies of the *carAa* gene which are adjacent and identical except for a single nucleotide change (28), only one *carAa* gene was amplified in this study. The cloned fragment was digested with SpeI and ScaI and ligated into SpeI-SnaBI-digested pRESQ (33). The structural *carABC* operon (GenBank accession number DQ060076) from *Pseudomonas* sp. strain XLDN4-9 was driven by the *P_{lac}* promoter present in plasmid pRESQ. The recombinant plasmid was introduced into *Rhodococcus erythropolis* XP by electroporation. The resultant recombinant strain was designated *R. erythropolis* XPDN and was capable of transforming CA and DBT simultaneously (data not shown). Whole-cell PCR amplification with specific primers P1 and P2 and Southern blot analysis confirmed the presence of the catabolic genes in the transformant (see Fig. S1 in the supplemental material). These results mean that the carbazole-degrading gene cluster was successfully introduced into strain XPDN. Gas chromatography-mass spectrometry analysis of the metabolites of DBT and CA produced by XPDN revealed 2-HBP, DBT sulfone, and AA (see Fig. S2 in the supplemental material). AA has been detected as a key metabolite of CA produced by other cultures (4, 12), and it is likely that XPDN has a similar pathway, in which CA is converted into AA. CA degraders are also able to attack some O heterocycles, including DBF and DD. Carbazole 1,9a-dioxygenase can transform DBF and DD to 2,2',3-trihydroxybiphenyl and 2,2',3-trihy-

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FIG. 1. Simultaneous transformation of DBT, CA, and DBF by resting cells of *R. erythropolis* XPDN. The initial concentrations of DBT, CA, and DBF were 92 mg liter⁻¹ (0.5 mM), 200 mg liter⁻¹, and 200 mg liter⁻¹, respectively. The concentrations of resting cell suspensions were adjusted to 8 g (dry weight) cells liter⁻¹. ■, DBF; ○, DBT; ●, CA; □, 2-HBP. The values are means $\pm$ standard deviations of at least three replicates.

droxydiphenyl ether, respectively (21). Therefore, there is hope that combination of the sulfur and nitrogen degradation genes in one biocatalyst will enable the bacterium constructed to degrade these sulfur, nitrogen, and oxygen heterocycles simultaneously (34).

Recombinant strain XPDN was harvested in the mid-exponential phase of growth and washed twice with 100 mM potassium phosphate buffer (pH 7.0). Portions of the cell suspension (25 ml; 8 g [dry weight] of cells per liter) were incubated at 30°C in 300-ml flasks with shaking at 250 rpm. DBT, CA, and DBF dissolved in ethanol were added to final concentrations of 92 mg liter⁻¹ (0.5 mM), 200 mg liter⁻¹, and 200 mg l⁻¹, respectively. After incubation for 33 h, 82.9% of the CA and 69.8% of the DBF were transformed, and DBT was completely removed, with the majority (96%) transformed in the initial 6 h (Fig. 1). Additionally, salicylic acid was identified as the metabolite of DBF produced by XPDN (see Fig. S3 in the supplemental material). Salicylic acid is a key intermediate in the DBF angular dioxygenation process (35), and the results of this study provided evidence that *R. erythropolis* XPDN does transform DBF.

After incubation for 6 h, 96% of 0.5 mM DBT was removed by the resting cells of XPDN (Fig. 1). The DBT transformation rate was equivalent to that of the host strain *R. erythropolis* XP and the previously described recombinant strain SN8 containing carbazole dioxygenase (38). These results also indicated that the introduced CA-degrading enzymes had no significant effect on the DBT biotransformation rate. However, the CA transformation rate was obviously affected by the addition of DBF, and it decreased from 3.87 mg (g [dry weight] of cells)⁻¹ h⁻¹ to 2.13 mg (g [dry weight] of cells)⁻¹ h⁻¹ (based on the initial 6-h reaction). Furthermore, the DBF transformation rate was also affected by CA, and this rate decreased by 29% in the presence of CA (data not shown). Since CA transformation and DBF transformation were both controlled by the CA-degrading enzymes, substrate competitive inhibition may have been the reason for the decrease in the biotransformation rate.

The concentration of the product of DBT in the desulfuriza-

FIG. 2. Curves for transformation of 2-HBP by *R. erythropolis* XPDN and inactive cells of XPDN. The concentrations of resting cell suspensions were adjusted to 8 g (dry weight) cells liter⁻¹. ■, transformation by XPDN; ●, transformation by inactive cells. The values are means $\pm$ standard deviations of at least three replicates.

tion process, 2-HBP, was also determined; this concentration reached only 0.3 mM and then decreased (Fig. 1). These results implied that the introduced CA-degrading enzymes may have also attacked 2-HBP. Further investigation was carried out using resting cells of XPDN to treat 0.25 mM 2-HBP. All the added 2-HBP was transformed by XPDN in 60 h, and no significant decrease was observed with inactive cells of XPDN (Fig. 2). Gas chromatography-mass spectrometry analysis revealed that benzoic acid was the metabolite of 2-HBP (see Fig. S4 in the supplemental material). In the previously described organism *Rhodococcus* sp. strain RHA1, biphenyl, an analog of 2-HBP, could be converted to 2,3-dihydroxybiphenyl (23DHBP) by biphenyl dioxygenase and dihydrodiol dehydrogenase step by step. Then 23DHBP was cleaved by 23DHBP dioxygenase, and the resulting *meta*-cleavage product was hydrolyzed to benzoic acid by a hydrolase (18). The biphenyl-degrading gene sequences were similar to the CA-degrading gene sequences, although the organizations of the clusters were different (29). Biphenyl could also be attacked by carbazole 1,9a-dioxygenase, the first enzyme involved in the carbazole degradation pathway, yielding *cis*-2,3-dihydroxy-2,3-dihydrobiphenyl (21). In our study, the analog of biphenyl, 2-HBP, could be attacked by CA-degrading enzymes of XPDN. It is reasonable to suggest that a similar metabolic mechanism occurred in 2-HBP transformation. 2-HBP has been used as a fungicide for control of postharvest diseases of various fruits since 1937 (31). DBT is converted to 2-HBP by sulfur-specific bacteria, which also increases the possibility of environmental pollution (9). Therefore, transformation of 2-HBP to benzoic acid may partially eliminate the pollution due to this compound.

Additionally, many CA and DBF degraders could also degrade DD with a catabolic pathway similar to that of DBF (22). *R. erythropolis* XPDN could also attack DD, which is a model compound for studying the biodegradation of the more poisonous environmental pollutants polychlorinated dibenzo-*p*-dioxins (7), and 0.1 mM DD could be completely transformed by XPDN within 7 days (Fig. 3).

Gram-positive bacteria, especially *Rhodococcus* strains, have

FIG. 3. Transformation of DD by *R. erythropolis* XPDN in the aqueous phase in the dark to prevent photooxidation. The initial concentration of DD was 0.1 mM. The concentrations of the resting cell suspensions were adjusted to 8 g (dry weight) cells liter⁻¹. ■, transformation by XPDN; ●, transformation by inactive cells of XPDN. The values are means $\pm$ standard deviations of at least three replicates.

a number of advantages for environmental use and therefore were chosen as potential bioremediation organisms (3, 25). *R. erythropolis* XP was capable of transforming sulfur heterocycles with high activity (37). The CA-degrading gene cluster was successfully introduced into this *Rhodococcus* strain, and the resultant recombinant was capable of transforming DBT to benzoic acid, CA to AA, and DBF to salicylic acid simultaneously. Although *R. erythropolis* XPDN could not use AA and the other metabolites as sole nitrogen or carbon sources for growth (data not shown), these compounds are more biodegradation sensitive than their parent compounds, which are easily mineralized in natural environments by other bacterial consortia. *Pseudomonas* sp. strain C3211 was reported to degrade DBT, CA, and DBF with acetone as a cosubstrate at an optimal temperature of 10°C, but the substrate concentrations tested and the biodegradation activities were very low (10). Recently, a *Gordonia* sp. strain was shown to be capable of DBT desulfurization and CA utilization (26). However, no data concerning simultaneous degradation of DBT, CA, and DBF have been published yet. Meade et al. used three marine bacterial consortia to degrade these three compounds separately (19). In our previous study, *R. erythropolis* SN8 was constructed in order to selectively transform the derivatives of carbazole and dibenzothiophene in crude oil, and this strain was suitable only for petroleum biotreatment since no carbon skeletons were destroyed (38). In this study, we found that *R. erythropolis* XPDN has the ability to convert poisonous S, N, and O heterocycles into nontoxic intermediates and may have application potential for bioremediation.

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