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ORIGINAL PAPER

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Recombinant carbazole-degrading strains for enhanced petroleum processing

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Abstract Biotechnological upgrading of fossil fuels is of increasing interest as remaining stocks of petroleum show increasing levels of contaminants such as heavy metals, sulfur and nitrogen-containing heteroaromatic compounds. Carbazole is of particular interest as a major petroleum component known to reduce refining yields through catalyst poisoning. In this study, the biotransformation of carbazole was successfully demonstrated in a liquid two-phase system, when solubilized in either 1-methylnaphthalene or in diesel fuel. The effects of solvent toxicity were investigated by expressing the carbazole-transformation genes from MB1332, a rifampicin-resistant derivative of *Pseudomonas* sp. LD2, in a solvent-resistant heterologous host, *P. putida* Idaho [1]. This solvent-resistant strain successfully degraded carbazole solubilized in 1-methylnaphthalene and in the presence of 10 vol% xylenes similar to the non-recombinant strain *Pseudomonas* sp. LD2. Identification of a suitable recombinant host, however, was essential for further investigations of partial pathway transformations. Recombinant *P. putida* Idaho expressing only the initial dioxygenase enzymes transformed carbazole to an intermediate well retained in the oil phase. Partial carbazole transformation converts carbazole to non-aromatic species; their effect is unknown on refinery catalyst poisoning, but would allow almost complete retention of carbon content and fuel value.

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Introduction

Acid precipitation resulting from fossil fuel combustion has driven efforts to develop precombustion technologies for removing contaminants from fossil fuels, including microbial technologies. Aside from certain specialized applications in petroleum recovery (notably deparaffinization), microbial processes have not yet been widely applied to hydrocarbon processing. Microbe-based biorefining applications are difficult due to the complexity of the chemical species present in crude oil and petroleum fractions, to solvent toxicity and substrate specificity [17]. Nevertheless, microorganisms possess the capacity to metabolize the sulfur and nitrogen species in crude oils.

Several nitrogen-containing heteroaromatics are refinery catalyst poisons [9, 15, 19]. The total nitrogen content of crude oils averages around 0.3%, of which non-basic compounds comprise approximately 70–75% [24]. Carbazole is a major non-basic species and is widely used as a model compound in microbial degradation studies [5, 12, 14, 21, 22, 23, 26, 27, 28]. Work on carbazole degradation has primarily focused on carbazole's presence as an environmental pollutant in petroleum and wood-preservation wastes such as creosote [12, 22].

Ouchiyama et al. [22] proposed a probable degradation pathway (Fig. 1) in *Pseudomonas* spp. CA06 and CA10 and identified some metabolites of carbazole degradation. The genes were cloned and expressed in *Escherichia coli* by Sato et al. [26, 27]. The carbazole dioxygenase of *Pseudomonas* sp. CA10 was shown by Nojiri et al. [20] to have a broad substrate range with the capability to catalyze *cis*-dihydroxylations and mono-oxygenations, in addition to the angular dioxygenation observed with carbazole.

Carbazole is a refinery catalyst inhibitor [9], presumably acting by binding to the acid site of the catalyst. The microbial products from carbazole degradation might be expected to possess reduced

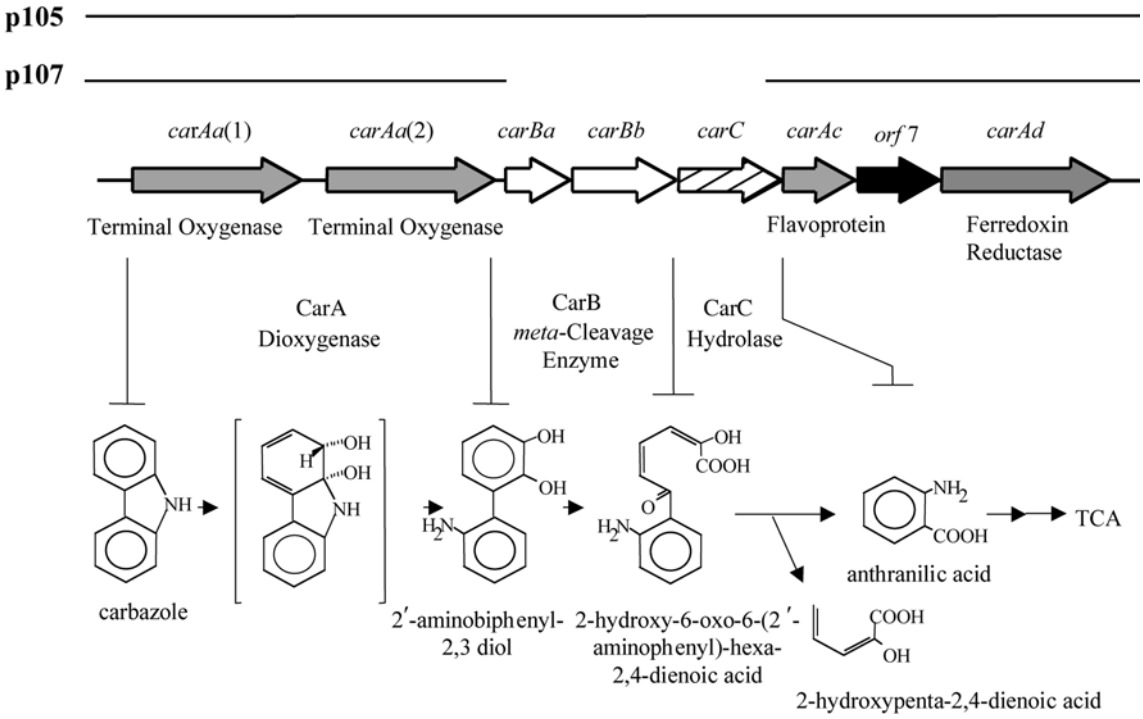

Fig. 1 Genes, enzymes, substrates and products of the carbazole-transforming pathway. *Lines* designate the genes included in the listed plasmids and the *pathway* below shows the gene products and the reactions they catalyze [23, 26, 27]

affinity for the catalyst-active sites. Therefore, microbial transformation of nitrogen heteroaromatics might be useful to alleviate refining catalyst inhibition in several ways. Carbazole, for example, can be completely metabolized to CO_2, cell mass and ammonia or converted to anthranilic acid or other intermediates by appropriately blocked mutant strains. These are likely to cause less catalyst inhibition than their parent compound, and many polar intermediates could readily be extracted from petroleum streams into water. Blocked mutants also have the ability to preserve the carbon content of the fuel being treated, retaining the value of the fuel. It has been reported that carbazole enrichment cultures are capable of degrading a wide range of alkylcarbazoles present in crude oil, generally yielding water-soluble nontoxic metabolites [5]. However, there have been no reports concerning host engineering to address the issue of solvent tolerance in two-phase systems or to investigate partial pathway transformations of carbazole as a possible alternative to its total removal, and the effect of these non-aromatic compounds on catalysts remains to be tested.

Kirimura et al. [12] reported on the continuous degradation of carbazole by resting cells of *Sphingomonas* sp. CDH-7 in a two-phase system and investigated solvent effects on the transformation. The organic solvents *p*-xylene, toluene, and heptanol adversely impacted carbazole degradation and were not capable of dissolving carbazole to a significant extent.

Here we describe the degradation of carbazole by a *Pseudomonas* sp. LD2 derivative (MB1332) in a model aqueous/organic two-phase system [4]. In addition, we demonstrate carbazole transformation using heterologous bacterial strains genetically modified to carry the carbazole degradation pathway. These recombinant strains were chosen for resistance to solvent toxicity, which may be problematic upon exposure to fuels in an actual biorefining process. The concept of a partial pathway transformation of carbazole to an intermediate less likely to act as poison in the refinery was also investigated. In addition, model biotransformation of a real fuel (diesel) containing carbazole was demonstrated.

Materials and methods

Bacterial strains, plasmids and growth conditions

Escherichia coli MB1547 was used for general cloning and plasmid propagation. The genes responsible for carbazole degradation are from MB1332, a spontaneous rifampicin-resistant derivative of *Pseudomonas* sp. LD2 [8] which was selected by plating onto LB plates supplemented with 50 mg l^{-1} rifampicin. Strains for the two-phase system include MB1332, *P. putida* KT2440, *P. putida* Idaho [2] and *E. coli* MB1547. Plasmid vectors used were the broad host range expression vector pMMB66EH [7] and the *E. coli* plasmid pBC(SK+) (Stratagene).

Pseudomonas and *E. coli* cells used for two-phase reactions were initially grown in a modified Pseudomonas Medium 187 containing (per liter of distilled water): 10 g yeast extract, 10 g Bacto tryptone, 5 g K_2PO_4, 10 ml glycerol and 5 ml metal salts solution, where glycerol replaces the standard glucose to avoid catabolite repression and formation of toxic side products with *E. coli* fermentation. The metal salts solution contained (per liter of distilled water): 0.4 g $FeSO_4$, 0.2 g NaCl, 0.4 g $MgSO_4{\cdot}7H_2O$, 0.2 g $MnSO_4{\cdot}4H_2O$ and H_2SO_4 added until pH was below 3.0. The medium was autoclaved for 20 min except for the salts solution, which was

sterilized through a 0.2 μm membrane filter. The cells (250 ml) were cultivated at 30°C at 250 rpm in 1-l shake flasks (Bellco). For the two-phase reactions, *Pseudomonas* and *E. coli* cells were resuspended and cultivated in M9 minimal medium [16]. *E. coli* strains and *Pseudomonas* strains were grown in LB broth to prepare plasmid DNA. Growth medium was supplemented with ampicillin (100 mg l^{-1} for *E. coli* and 1 g l^{-1} for *Pseudomonas*), chloramphenicol (20 mg $l^{-1)}$ or rifampicin (50 mg l^{-1}) as necessary.

DNA manipulations

Restriction digestions, agarose gel electrophoresis, isolation of plasmid and other DNA manipulations were carried out according to standard protocols [16]. Isolation of plasmid DNA from *Pseudomonas* strains was carried out using the method described by Murphy et al. [18] to remove the large amounts of polysaccharides present in *Pseudomonas* species, or by addition of a cetyl-trimethyl ammonium bromide (CTAB) extraction step to the standard protocol. DNA transformation into *E. coli* was performed using the one-step method [1].

MB1332 genomic library

A library was prepared in the cosmid pMAC from MB1332 genomic DNA by partial *Sau*3A digestion. The library was transduced into S17–1 and mated to *P. putida* KT2440. The LD2 genes responsible for carbazole transformation were isolated by screening colonies for their ability to both transform carbazole on a plate as observed by clearing zones, and to transform 2,3-dihydroxybiphenyl (2,3-DHBP) (Wako Pure Chemical Industries, Osaka, Japan) as observed by formation of the yellow meta-cleavage product. This identified cosmids carrying genes for carbazole transformation. A 6.8 kb *Eco*RI fragment containing the carbazole genes was subcloned into pBC(SK +) and the resulting plasmid was named p50.

Construction of plasmids

By deleting the *carB* and *carC* genes from p50, a plasmid that contained only the *carA* and *orf7* genes was constructed. First, a *Pst*I deletion was made by cutting p50 with *Pst*I, blunting the product with T4 polymerase, and ligating it with T4 ligase to yield plasmid p95. Next, p95 was digested with *Bam*HI and *Xba*I, and dephosphorylated with shrimp alkaline phosphatase (SAP) and into this was cloned a gel-purified *Bgl*II-*Xba*I fragment from p50 to create p98. The inserts from p50 and p98 were subcloned into the *Eco*RI site of pMMB66EH [7] and the resulting plasmids were called p105 and p107, respectively.

Detection of phenotypes

Wild type and recombinant strains were tested for carbazole degradation, meta-cleavage enzyme activity and hydrolase activity. Carbazole degradation was visualized as a clearing halo on M9 minimal plates supplemented with 0.5 g l^{-1} carbazole (Sigma) (solubilized in 15 ml dimethylsulfoxide), along with 0.5% (w/v) citrate for *Pseudomonas* or 5% (v/v) glycerol for *E. coli* and the appropriate antibiotic when needed. Meta-cleavage enzyme activity (*carB* genes) was visualized by exposing colonies to an atomized mist of 30 mM 2,3-DHBP substrate and observing the yellow color of the product 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA). Hydrolase activity (*carC*) was visualized by observing the disappearance of the yellow product generated above.

Recombinant *Pseudomonas* strains

Recombinant *Pseudomonas* strains carrying p107 were made by triparental mating with plasmid pRK2013 [13]. Briefly, equal volumes of RR107, HB101(RK2013) and the *Pseudomonas* recipient strain were mixed, spotted onto a LB plate and incubated at 30°C overnight. The cell mass was resuspended in PBS and plated on M9 minimal medium plates supplemented with citrate and ampicillin. Colonies were then transferred to M9 minimal medium plates supplemented with carbazole to test for carbazole degradation. *Pseudomonas* strains with p105 were made by conjugal transfer, utilizing the suicide delivery strain S17–1(*λpir*). Transconjugants were screened for the ability to degrade carbazole on an M9 minimal medium plate. Strains carrying p105 were also tested for the meta-cleavage enzyme by spraying plates with 2,3-DHBP. Colonies demonstrating the correct phenotype were checked for the presence of the plasmid via DNA isolation and restriction enzyme digestion. The strains and plasmids used are listed in Table 1.

Two-phase reaction

Cells for the two-phase reaction were grown in 500 ml *Pseudomonas* Medium 187 supplemented with 1 mM isopropyl-β-D-thiogalactoside (IPTG), 1 g l^{-1} ampicillin and 0.5 ml l^{-1} 1-methylnaphthalene (Fluka). The 500 ml culture was grown for 24 h, centrifuged in a table-top centrifuge and cells were resuspended in M9 medium supplemented with 5% glycerol, ampicillin, metal salts solution and IPTG to an OD_{600} of about 5. The two-phase reaction was carried out by adding 20 ml cell suspension to 10 ml oil/solvent phase in a 300 ml baffled shake flask (Bellco), and incubating the suspension at 30°C in a shaking water bath at 250 rpm.

The solvent used was 1-methylnaphthalene, in which carbazole is soluble to 0.8 wt%. 1-Methylnaphthalene has a low vapor pressure amenable to laboratory studies, and is not metabolized by any of the bacterial strains used in this study. We used a carbazole concentration 4- to 5-fold that found in crude oil as a maximally stringent condition. To model the biotransformation process in real fuels, in some experiments diesel fuel with carbazole added to the solubility limit (500 ppm/0.05 wt%) was utilized in place of 1-methylnaphthalene.

At each time point, an entire reaction flask was analyzed, minimizing error due to sampling of an aqueous/organic emulsion. Emulsions were broken by centrifugation at 18,000 rpm in a Beckman J2–21 centrifuge for 20 min. The oil/solvent layer was removed by aspiration with a disposable polyethylene transfer pipette and transferred to an amber 2 ml GC vial for analysis. As controls, reaction flasks were incubated with and without cells for 24 h as described above. Reaction flasks incubated with cells were measured after incubation with *E. coli* MB1547, MB1332, *P. putida* KT2440, and *P. putida* Idaho.

Analytical methods

Products in the oil phase were analyzed using an HP 6890 gas chromatograph. One microliter of sample was loaded via automatic injector onto a splitless HP-5 column. After injection, the column temperature was held constant at 160°C for 2 min then increased at a rate of 8°C min^{-1} over 15 min to 280°C. This temperature was held for 2 min. The detector was a nitrogen/phosphorus detector (NPD) maintained at 300°C. A standard curve of carbazole in 1-methylnaphthalene was run before and after each set of samples.

Results

Cloning of the genes encoding carbazole degradation enzymes

A genomic library from MB1332 was transferred into *P. putida* KT2440 and colonies were screened for production of the yellow meta-cleavage compound, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA),

Table 1 Strains and plasmids

Strain or plasmid	Genotype or relevant features	Source or reference
E. coli strains		
HB101(pRK2013)	*hsd*20 *recA*13 *galK*2 *rpsL20 leuB6 pro*A	[13]
MB1547	*hsd*Δ5 Δ(*lac-proAB*) *supE thi endA*/ F′[*lacI*^{q} *lacZ*ΔM15 *proA*$^+$ *B*$^+$ *traD*36]	Laboratory stock
RR105	MB1547 (p105)	Present work
RR107	MB1547 (p107)	Present work
S17–1 (λ*pir*)	RP4–2-Tc::Mu-1 kan::*Tn*7 integrant *proA hsdR*17 *endA*1 *supE*44 *recA thi*	Laboratory stock
Pseudomonas strains		
LD2	Carbazole-degrading pseudomonad	[8]
Idaho	Solvent-resistant *P. putida* strain	[2]
KT2440	*P. putida* mt–*2 rmo*	[6]
MB1332	Rifampicin-resistant LD2	Present work
RR147	Idaho (p105)	Present work
RR149	KT2440 (p107)	Present work
RR150	KT2440 (p105)	Present work
RR156	Idaho (p107)	Present work
Plasmids		
pBC (SK +)	Cm^R	Stratagene
pMAC	Cosmid: Km^R Tc^R *oriT lacI*^{q} P_{tac}	M. Capage (unpublished)
pMMB66EH +	*lacI*^{q} P_{tac} derivative of RSF1010, Ap^R	[7]
p50	pBC(SK +) [*carA*$^+$ *B*$^+$ *C*$^+$]	Present work
p95	pBC(SK +) [*carA*$^+$ *B*$^+$ Δ*carC*]	Present work
p98	pBC(SK +) [*carA*$^+$ Δ*carBC*]	Present work
p105	pMMB66EH + [*carA*$^+$ *B*$^+$ *C*$^+$]	Present work
p107	pMMB66EH + [*carA*$^+$ Δ*carBC*]	Present work

after being sprayed with 2,3-dihydroxybiphenyl (2,3-DHBP), an analog of 2′-aminobiphenyl-2,3-diol, a catabolic intermediate of carbazole degradation arising from the activity of CarB, a meta-cleavage enzyme. Colonies were concurrently screened for their ability to degrade carbazole suspended in solid media, as visualized by a clearing zone or halo where the carbazole precipitate was degraded. Clones were identified that were capable of forming a halo on carbazole plates and which turned yellow upon exposure to 2,3-DHPB.

Cosmid DNA was extracted and restriction analysis showed it to be identical to that of the carbazole-degrading genes reported by Sato et al. [26]. An *Eco*RI digestion was used to subclone the carbazole-degrading genes into pBC(SK +) and was named p50. In *E. coli*, p50 showed clearing of carbazole on a plate, transformed 2,3-DHBP to HOPDA, and formed a blue indigo color over time (indicative of dioxygenase enzymes).

The *carC* gene was deleted from p50 and the resulting plasmid containing *carAB* was designated p95. In *E. coli* p95 showed clearing of carbazole on a plate and transformed 2,3-DHBP to HOPDA, but remained yellow because p95 does not produce CarC, which is necessary to transform HOPDA to anthranilic acid and 2-hydroxypenta-2,4-dieonic acid. *E. coli* transformed with p95 also formed blue colonies as with p50. A further deletion of p95 resulted in p98, which contains only the *carA* genes. *E. coli* transformed with p98 showed clearing on a carbazole plate and formed blue colonies but did not turn yellow upon exposure to 2,3-DHBP. Subclones of the entire *carABC* operon or only the *carA* genes were transferred into the broad-host-range plasmid pMMB66EH using the *Eco*RI site, and the resulting clones were designated p105 and p107, respectively. In *E. coli*, both p105 and 107 had the same phenotypes as p50 and p98, respectively.

To study the degradation of carbazole by non-carbazole-degrading *Pseudomonas* strains in two-phase reactions, p105 and p107 were conjugated into both *P. putida* KT2440 and the solvent-resistant *P. putida* Idaho. *Pseudomonas* strains demonstrating the proper carbazole-degrading and meta-cleavage enzyme phenotypes were verified by plasmid DNA isolation and restriction enzyme digestion. Restriction analysis indicated that *P. putida* KT2440 and *P. putida* Idaho were harboring p105 and p107.

Sequencing of MB1332 carbazole-degrading genes

To determine whether the carbazole-degrading genes of MB1332 were identical to those of CA10, one end of the MB1332 *Eco*RI fragment of p50 was sequenced. The analysis showed that at least *carAa* through *carBa* was identical to *carA* through *carBa* of CA10. Similarly, the sequence of *carC* from MB1332 matched the sequence determined for *carC* of *Pseudomonas* strain CA10 [27] (GenBank: D89064). This hydrolase was identical to CA10 at the amino acid level and more than 98% identical at the nucleotide level.

Cell preconditioning and medium selection

A comparison was made between reactions carried out in two-phase systems using cells grown in rich medium or in rich medium with 0.1 ml of 1-methylnaphthalene per liter. The cells (RR156) preconditioned by exposure to small

but saturating amounts of 1-methylnaphthalene performed better (44% degraded) than cells that were not preconditioned (29% degraded) in subsequent two-phase reactions.

Rich versus minimal medium

A comparison was made between reactions carried out in two-phase systems using rich medium identical to the original growth medium or M9 minimal medium containing glycerol as a carbon source. When MB1332 was pregrown in rich medium then resuspended in M9 minimal medium, the two-phase reaction was able to remove 64% of the carbazole compared with cells that were resuspended in fresh rich medium, which degraded only 35%. Subsequently, all experiments were conducted with cells pregrown in *Pseudomonas* Medium 187 in the presence of 1-methylnaphthalene and the two-phase reaction was done with cells resuspended in M9 minimal medium with the metal salts solution added.

Reaction controls

Reaction flasks with 0.8 wt% carbazole solubilized in 1-methylnapthalene incubated for 24 h without cells retained 99% of the carbazole content. Reaction flasks with 0.8 wt% carbazole solubilized in 1-methylnapthalene incubated for 24 h with *E. coli* MB1547 retained 98% of the carbazole content. Reaction flasks with 0.8 wt% carbazole solubilized in 1-methylnapthalene incubated for 24 h with *P. putida* KT2440 retained 100% of the carbazole content. Reaction flasks with 0.8 wt% carbazole solubilized in 1-methylnapthalene incubated for 24 h with *P. putida* Idaho retained 99% of the carbazole content.

Carbazole degradation by *E. coli*, solvent-tolerant *P. putida* Idaho, and *P. putida* KT2440

E. coli strain RR105 (*carABC*) with 0.8 wt% carbazole solubilized in 1-methylnapthalene had 88% of the initial carbazole remaining after 24 h. Strain RR107 (*carA*) had 79% of the initial carbazole remaining after 24 h. The presence of 10% xylenes abolished the activity of strains RR105 and RR107 (Fig. 2).

P. putida Idaho strain RR147 (*carABC*) with 0.8 wt% carbazole solubilized in 1-methylnapthalene had 70% of the initial carbazole remaining after 24 h. Strain RR156 (*carA*) had 61% of the initial carbazole remaining after 24 h. Strain RR147 with 0.8 wt% carbazole solubilized in 1-methylnaphthalene plus 10% xylenes had 56% of the initial carbazole remaining after 24 h, while strain RR156 had 63% of the initial carbazole remaining under the same conditions (Fig. 3).

Both *P. putida* Idaho strains, therefore, retained activity in the presence of xylene concentrations sufficient to inactivate *E. coli*. However, *P. putida* KT2440 strains RR149 and RR150 were inactive on 0.8 wt% carbazole solubilized in 1-methylnaphthalene, with or without the addition of 10% xylenes.

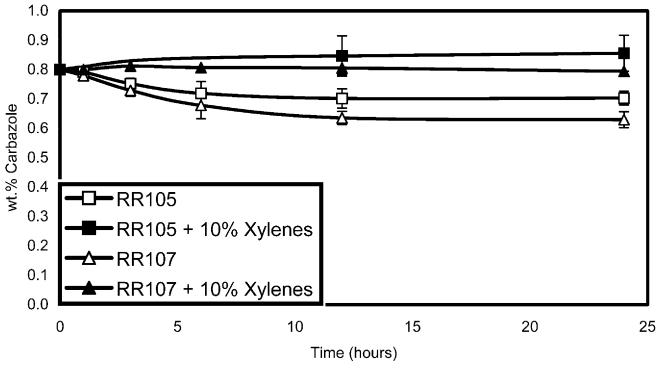

Fig. 2 Transformation of carbazole by recombinant *Escherichia coli* strains. *E. coli* RR105 (*carABC*) and RR107 (*carA*) two-phase reaction with 0.8 wt% carbazole solubilized in 1-methylnapthalene or 1-methylnapthalene + 10 vol% xylenes. *Data points* are the mean of three separate experiments; *error bars* represent the standard deviation

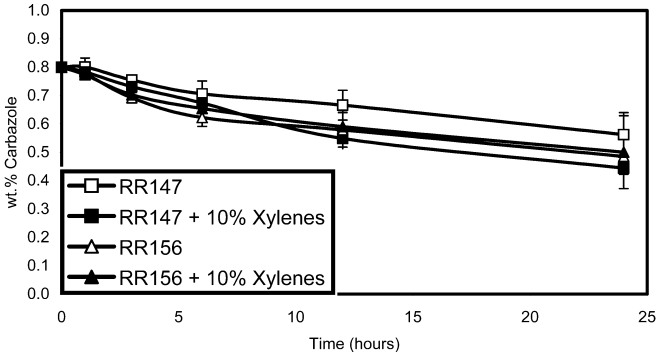

Fig. 3 Transformation of carbazole by recombinant *Pseudomonas putida* Idaho strains. RR147 (*carABC*) and RR156 (*carA*) in two-phase reaction with 0.8 wt% carbazole solubilized in 1-methylnaphthalene or 1-methylnaphthalene + 10 vol% xylenes. *Data points* are the mean of three separate experiments; *error bars* represent the standard deviation

Transformation of carbazole by MB1332

Strain MB1332 (expressing the complete carbazole degradation pathway) with 0.8 wt% carbazole solubilized in 1-methylnapthalene had 1% of the initial carbazole remaining after 24 h. Strain MB1332 with 0.8 wt% carbazole solubilized in 1-methylnaphthalene + 10% xylenes had 7% of the initial carbazole remaining after 24 h (Fig. 4).

Biotransformation of diesel

Both *E. coli* strains RR105 and RR107 were able to remove carbazole solubilized in diesel fuel, removing

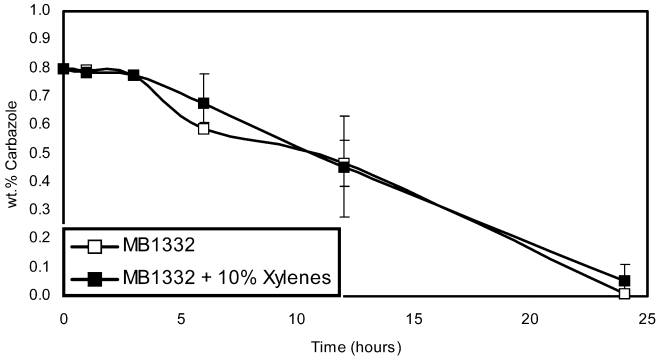

Fig. 4 Transformation of carbazole by MB1332. A two-phase reaction using strain MB1332 with 0.8 wt% carbazole solubilized in 1-methylnapthalene or 1-methylnaphthalene + 10 vol% xylenes. *Data points* are the mean of three separate experiments; *error bars* represent the standard deviation

83% and 70% respectively over a 6-h period. Both *P. putida* Idaho strains RR147 and RR156 were able to utilize all the carbazole solubilized in diesel fuel over a 6-h period. Strain MB1332 was also able to utilize all the carbazole solubilized in diesel fuel over a 6-h period and 84% in only 3 h (Fig. 5). A control study with MB1332 and 1-methylnapthalene with 500 ppm of carbazole showed an identical kinetic profile to the diesel results.

Discussion

Conversion of carbazole to non-aromatic species is promising. Potentially the entire carbon content of the fuel could be preserved, avoiding disposal problems and loss of fuel value. Mutant or recombinant strains capable of carrying out only the first step(s) of the carbazole degradation pathway would be used. A critical need in the development of such processes is the further characterization of the effects on catalysts of carbazole degradation intermediates. Since the presumed mode of catalyst inhibition from carbazole is affinity for acid sites [9], the conversion of carbazole to more acidic intermediates would be expected to alleviate the inhibition.

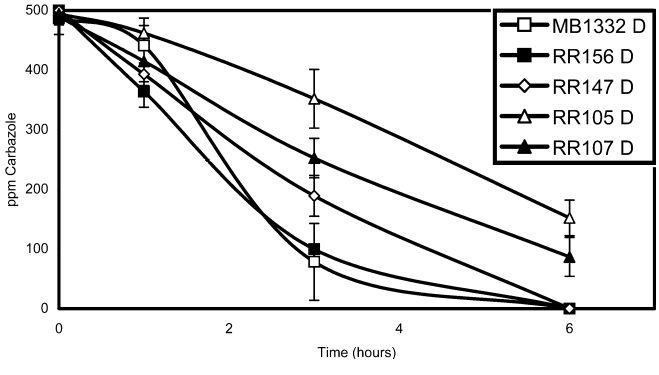

Fig. 5 Transformation of carbazole by recombinant bacterial strains. The parent MB1332 is compared to *E. coli* and *P. putida* Idaho strains carrying the p105 (*carABC*) and/or p107 (*carA*) plasmids in a two-phase reaction with diesel fuel that has carbazole solubilized to its solubilization limit (~500 ppm or 0.05 wt%). *Data points* are the mean of three separate experiments; *error bars* represent the standard deviation

We have demonstrated the ability to biodegrade carbazole in a liquid two-phase system, not only when solubilized in 1-methylnaphthalene, but also when present in diesel fuel. Solvent toxicity issues have been addressed by expressing the carbazole-transforming genes in a solvent-resistant heterologous host, *P. putida* Idaho. Furthermore, retention of carbon content, or fuel value, has been investigated through the construction of strains expressing a partial pathway. In addition, *carA* strains should be able to break down DBT [20], at least the first step, and hybrid pathways could be developed for the breakdown of both compounds.

Denitrogenation in fossil fuels such as diesel will require bacterial strains and enzymes that tolerate solvents. A direct correlation has been observed between enzyme activity and log P, the logarithm of the octanol–water partition coefficient, which serves as a quantitative index of solvent polarity for the correlation of biological activity and chemical structure [10]. In general, the lower the log P value, the more non-polar and lipophilic the solvent is, with aromatic hydrocarbons with a log P between 1.5 and 3.5 being extremely toxic to living organisms [29]. In our two-liquid-phase system 1-methylnaphthalene is just above the toxic range (log P 3.87) while enhancing bioavailability of the water-insoluble carbazole. Xylenes have a log P of 3.1, and therefore adding xylenes to the 1-methylnaphthalene phase provides a robust test for solvent toxicity in the host organisms. The effect of toxic solvent components likely to be present in real fuels was therefore modeled using xylenes; a solvent highly toxic to bacterial cells, especially *E. coli* [3, 11, 30]. *P. putida* Idaho withstands aqueous solutions saturated with toluene and xylenes [2] and is therefore expected to be a good host for the carbazole genes in the two-phase system.

From many examples in the literature, it is known that exposure to a solvent can trigger mechanisms to protect the bacterial cell [29]. Specifically in the case of *P. putida* Idaho, the protective changes induced by solvent exposure have been examined [2]. Following *o*-xylene exposure, *P. putida* showed a decrease in total phospholipid content. In contrast, *P. putida* Idaho demonstrated an increase in phospholipid content 1–6 h after exposure. *P. putida* Idaho has a greater ability than the solvent-sensitive strain to repair damaged membranes through efficient turnover and increased phospholipid biosynthesis. Thus the observation that preconditioning of the strains resulted in better performance is consistent with induction of solvent protection mechanisms [25]. The observation that shifting from rich media in the growth stage to minimal media in the two-phase biocatalysis resulted in higher conversions for MB1332 may indicate a similar influence of a regulated system.

These results indicate that MB1332 was in fact better than *P. putida* Idaho in the two-phase system. While this

result was unexpected, the differences seen between MB1332 and *P. putida* Idaho may be a result of differences in protein expression. However the demonstration that *P. putida* Idaho is a suitable host for the two-phase system opens the way to full control over pathway expression.

We were able to model partial conversion of carbazole using the p107 plasmid, which lacks the genes expressing CarB and CarC activities. Accumulation of a nitrogen-containing intermediate was also apparent in GC chromatograms during carbazole analysis (data not shown). The more polar and acid nature expected of the intermediates was consistent with observed behavior on GC and HPLC separations. Although testing for reduction of refinery catalyst activity was not available, one would expect these intermediates to have a reduced inhibitory potential.

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