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Novel Carbazole Degradation Genes of *Sphingomonas* CB3: Sequence Analysis, Transcription, and Molecular Ecology

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The degradation of aromatic compounds by bacteria is dependent upon specific catabolic operons. The unique *car* locus isolated from *Sphingomonas* CB3 encodes the first four enzymes involved in the catabolism of the azaarene carbazole. These include a class II three-component dioxygenase enzyme system, a dihydrodiol dehydrogenase, an extradiol (*meta*-cleavage) dioxygenase, and a hydrolase. Homology of deduced amino acid sequences is closer to corresponding biphenyl catabolic genes than to previously characterised carbazole degradation genes. Gene arrangement is also identical to that found in some *bph* loci. The *car* genes are transcribed when carbazole is utilised as a sole carbon source, and although biphenyl does not serve as a growth substrate for *Sphingomonas* CB3 it is able to act as a non-metabolisable inducer of the *car* locus. Ecologically the *car* genes were detected in polycyclic aromatic hydrocarbon (PAH) contaminated soil associated with a former town gas site.

Microbial catabolism of aromatic compounds follows predictable routes which fall into distinct segments: conversion of the growth substrate to an arene diol or substituted arene diol, followed by ring cleavage, with further catabolism of the ring-cleavage product ultimately feeding through to central metabolism. Many of these pathways show a high degree of conservation at the level of gene organization, gene size, and homology of the corresponding enzymes [1].

Catabolic pathways have been described for the microbial degradation of carbazole by *Pseudomonas* sp. strains CA10 [2], and LD2 [3]. Metabolite analysis suggests that carbazole degradation in CA10 and LD2 occurs via an initial angular dioxygenation yielding 2′-aminobiphenyl-2,3-diol which is subsequently cleaved by an extradiol dioxygenase. An alternative pathway via indole acetic acid may also play a role in carbazole degradation by LD2 [3]. Recently the genes involved in the degradation of carbazole by CA10 have been isolated using Tn-5 induced mutational inactivation [4], and subsequently described in detail [5,6]. The characterised genes encode a novel class III initial dioxygenase, carbazole 1,9a-dioxygenase, a divergent extradiol dioxygenase (*meta*-cleavage) enzyme and a *meta*-cleavage compound hydrolase.

To date there is only one other report [5,6] on the molecular biology of catabolic pathways specific for the azaarene carbazole. In this paper we describe 6085-bp of nucleotide sequence of a divergent catabolic locus isolated from *Sphingomonas* (formerly *Pseudomonas*) strain CB3 which is able to utilise carbazole as sole carbon and nitrogen source [7].

MATERIALS AND METHODS

Bacterial strains. *Sphingomonas* CB3 is able to grow at the expense of the azaarene carbazole and has been described previously [7]. Sequencing of the 16S rDNA gene from strain CB3 has subsequently revealed its closest relative, at 99% homology, to be *Sphingomonas subarctica.*

DNA manipulation and sequencing. A clone library was generated in *Escherichia coli* DH5α using *Eco*RI-digested genomic DNA prepared from *Sphingomonas* CB3 [8] ligated into pUC18. All recombinant manipulations were performed according to established methodologies [9] and in accordance with manufacturer's instructions. Colonies harbouring recombinant plasmids were screened for the expression of extradiol dioxygenase activity by the ability to form the yellow ring-fission product 2-hydroxymuconic semialdehyde on spraying colonies with 100 mM catechol [10].

A nested deletion series was generated from the overlapping clones pJO05-01 and pJO12 using a double-stranded Nested Deletion Kit (Pharmacia Biotech). Plasmid DNA was isolated using a Quantum Prep Plasmid Miniprep Kit (Bio-Rad), and clone sequences were determined by the Waikato DNA Sequencing facility using a PRISM Ready Reaction DNA Terminator Cycle Sequencing Kit (Perkin-Elmer). The reactions were resolved using an ABI model 377 sequencer.

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PCR amplification of car genes. A primer pair, C1036 (5′-GGGATCTTTGAACAGGACGA-3′) and C2495 (5′-AGGAGCCTGTCACTGACCAT-3′), was designed to amplify a 1460-bp fragment which contains the *carAa*(partial)/*carAb*/*carAc*/*carAd*(partial) genes. These primers were used to amplify the *car* genes from soil DNA, and also in an RT-PCR amplification experiment using CB3 mRNA as the target.

Total soil DNA was extracted from 0.5 g soil samples using a bead beating method followed by purification through PVPP (polyvinylpolypyrrolidone) spin columns [11]. PCR amplification was carried out in 20 mM Tris-HCl (pH 8.4); 50 mM KCl; 1.5 mM $MgCl_2$; dNTPs at 200 μM each, 2.5 units PLATINUM *Taq* DNA polymerase (GibcoBRL); 0.2 μM forward and reverse primers; and template DNA at 0.1 μg/50 μl reaction. The cycling conditions (Techne Cyclogene Thermal Cycler) were 5 min at 94°C, followed by 25 cycles of 94°C for 2 min, 52°C for 1 min, 72°C for 2 min, maximal ramp rates throughout, with the final 72°C segment of the cycle extended to 10 min before cooling to 4°C. A Southern blot consisting of 20 μl of PCR product amplified from soil DNA resolved on an agarose gel and blotted to a positively charged nylon membrane (GeneScreen Plus (NEN Research Products)) was analysed by hybridisation with ^{32}P-labelled *carAa*(partial)/*carAb*/*carAc*/*carAd*(partial) amplified from CB3 genomic DNA. Membranes were hybridised overnight at 65°C in 5 × SSC, followed by stringency washes with 2 × SSC, 0.1% SDS for 15 min at 65°C, and 0.2 × SSC, 0.1% SDS for 15 min at 65°C.

mRNA was isolated, using an RNA extraction kit (RNeasy Mini Kit Qiagen), from CB3 grown at the expense of different carbon sources. These included 2 mM carbazole, 10 mM acetate, and 10 mM acetate-grown cells induced for 2 h during late log phase, with carbazole and biphenyl added to 1 mM. Purified mRNA was treated with DNase I (GibcoBRL) and RNase inhibitor (Boehringer Mannheim) to ensure complete removal of DNA and to maintain the integrity of mRNA. RT-PCR (Titan One Tube RT-PCR System: Boehringer Mannheim) was used to amplify a fragment of the car locus from mRNA isolated from *Sphingomonas* strain CB3 grown at the expense of different carbon sources. The cycling conditions (Techne Cyclogene Thermal Cycler) for the RT-PCR amplification were: 50°C for 30 min; 94°C for 2 min; followed by 10 cycles of 94°C for 30 s, 52°C for 30 s, 68°C for 1 min; followed by 15 cycles of 94°C for 30 s, 52°C for 30 s, 68°C for 80 s; followed by 15 cycles of 94°C for 30 s, 52°C for 30 s, 68°C for 2 min; followed by 94°C for 30 s, 52°C for 30 s, 68°C for 10 min, and cool to 4°C. PCR amplification controls omitted the initial incubation at 50°C. Optimal $MgCl_2$ concentration was 1.5 mM.

RESULTS AND DISCUSSION

Isolation of the car Catabolic Genes and Characterisation of the Extradiol Dioxygenase

The extradiol dioxygenase activity detected in cell-free extracts of the wild-type strain *Sphingomonas* CB3 grown at the expense of carbazole was isolated and expressed in a clone designated pJO05 which contained a 7400-bp *Eco*RI fragment from CB3. This extradiol dioxygenase activity was induced by growth on carbazole and was not detected in acetate- or succinate-grown cells revealing that this activity is under regulatory control. Southern hybridisation using probe DNA derived from pJO05 confirmed its origin from CB3 genomic DNA, it was not present in digests of the large (>100-kb) plasmid DNA prepared from CB3 (data not shown). The identity of the extradiol dioxygenase enzyme encoded by pJO05 was evaluated by analysing the substrate specificity of the functional enzyme expressed in *Escherichia coli* DH5α. Specific activity (average of four replicates) of the extradiol dioxygenase enzyme towards 2,3-dihydroxybiphenyl (820.6 ± 19.4 μmol/min/mg protein) and catechol (24.0 ± 1.2 μmol/min/mg protein) revealed that the pJO05-encoded dioxygenase activity was characteristic of a multiple-ring extradiol dioxygenase showing a specificity typical of a 2,3-dihydroxy 1,2-dioxygenase (BphC) rather than a catechol-2,3-dioxygenase (C23O) enzyme [12,13].

pJO05 contained an internal *Eco*RI site which allowed the extradiol dioxygenase activity encoded by pJO05 to be further localised, by deletion, to the derivative pJO05-01. A second derivative pJO05-02, which lacked an extradiol dioxygenase activity was also obtained. An additional clone pJO12, located upstream and contiguous to pJO05, was isolated by screening a *Hind*III clone library of CB3 by hybridisation using a 300-bp *Eco*RI/*Hind*III probe derived from pJ005-01.

Nucleotide Sequence Analysis of the Cloned car Genes

The complete sequence of cloned inserts of pJO05-01, pJO12 and 400-bp of the 5′ end of clone pJO05-02 was determined for both strands to yield 6085-bp of sequence (GenBank accession number AF060489). The DNA had a G+C content of 61.1%, close to that reported for established *Sphingomonas* species inclusive of the aromatic compound-degrading sphingomonads (61.7-67.8%) [14].

Computer analysis of the nucleotide sequence revealed the presence of six complete open reading frames (ORFs), and one partial ORF, each transcribed in the same orientation (Figure 1). ORFs were initiated either by the canonical ATG start codon (*carAbAcCD*), or by the rarer and less efficient GTG start codon (*carAdB*) which is not uncommon in operons involved in the degradation of aromatic compounds [15,16,17]. Each complete ORF is preceded by a potential ribosome binding site [18] and ends with TGA as the termination codon, *carAa*, which is incomplete, also ends with the termination codon TGA. By comparison with analogous genes we estimate that the *Eco*RI restriction site utilised for cloning is situated between 21-bp and 78-bp downstream of the start codon of CarAa when compared to the corresponding genes from naphthalene (*Pseudomonas putida* G7 *nah*Ac [29]) or biphenyl catabolic genes (*Burkholderia* LB400 *bph*A [20]). Further efforts to clone this region were unsuccessful. Intergenic regions were absent between three *car* gene pairs; these correspond to *carAb* which overlaps *carAa* by 11-bp, *carAd* overlaps *carAc* by 4-bp, and *carB* which overlaps *carAd* by 14-bp. Similar overlapping genes have also been described in other degradative gene clusters [16,19], and the absence of intergenic spaces between these gene pairs may reflect translational coupling of these genes in CB3, cf. biphenyl dioxygenase of LB400 [20]. The transcript containing *carAd* and

FIG. 1. Nucleotide sequence of 6085-bp from the *car* locus of *Sphingomonas* strain CB3. Start and stop codons are highlighted in bold, and putative Shine-Dalgarno sequences are underlined. Conserved amino acid residues are italicised and highlighted in bold; these correspond to Rieske-type [2Fe-2S] centers, and amino acid residues conserved in extradiol dioxygenases. Positions corresponding to the primers used for RT-PCR are indicated by double underlining.

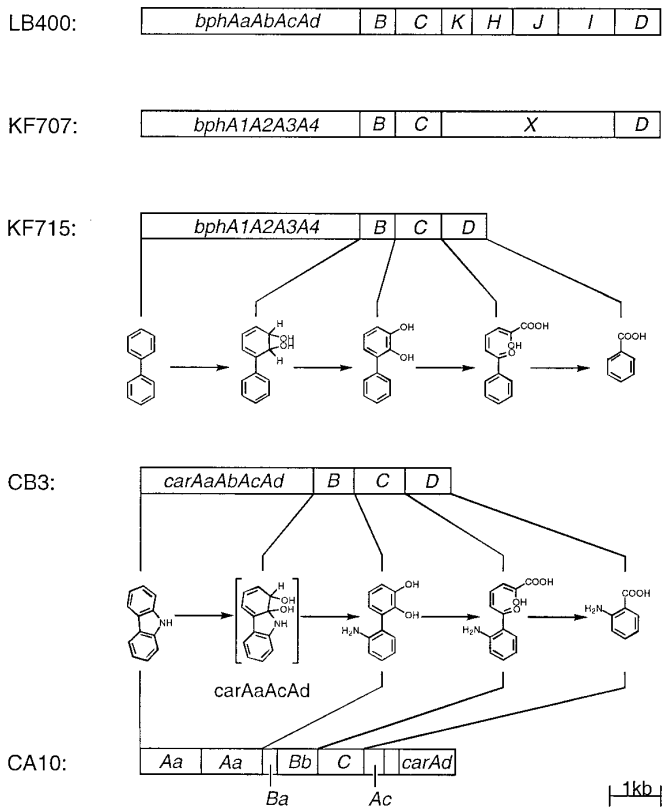

FIG. 2. Comparison of the *car* locus from *Sphingomonas* CB3 with published catabolic pathways. The corresponding operons include the *bph* loci from *Pseudomonas* sp. LB400 [28], *Pseudomonas pseudoalcaligenes* KF707 [27] and *Pseudomonas putida* KF715 [22], and the *car* locus is from *Pseudomonas* sp. strain CA10 [22].

carB may also be subjected to some translational control due to the presence of the less efficient GTG start codons in these genes.

Sequence analysis of the CB3 *car* genes revealed that at the molecular level the deduced amino acid sequences of CarAaAbAcAdBCD showed greatest sequence identity (31-53%) to their homologous counterparts from diverse biphenyl pathways. Homology also extended to the arrangement of these genes which is conserved between some *bph* loci and the CB3 *car* locus (Figure 2). The only other reported *car* sequence shows very low homology and divergent gene arrangement [5,6]. For this reason the gene designations we have adopted use the same nomenclature as that applied to the biphenyl catabolic genes, namely that from carbazole through to anthranilic acid [2](cf. biphenyl through to benzoate [23]) the genes should have the same letter as the isofunctional *bph* genes [22,24,25,26,27], but with a *car* prefix, i.e. *carABCD*. The cloned *car* locus from CB3 therefore encodes, by analogy to previously described *bph* genes, CarAaAbAcAdBCD. Gene organisation is conserved between the *car* locus of strain CB3 and *bph* loci of the type *bphABCD* seen in *Pseudomonas* sp. KKS102 [21] and *Pseudomonas putida* KF715 [22], where the *bphC* and *bphD* genes are closely spaced. This is different to the *bph* locus of *Pseudomonas* strain LB400 where the *bphC* and *bphD* genes are separated by a 3500-bp region containing *bphKHJI* [28].

The properties and characteristics of predicted polypeptides of the CB3 *car* locus are summarised in Table 1. Homology of the predicted amino acid sequences from CB3 to the CA10 *car* gene products is also shown and are particularly low. Sequence analysis reveals that the aromatic ring dioxygenase enzyme from CB3 is a class II type three-component enzyme similar to biphenyl dioxygenase [27] and toluene dioxygenase [17]. This is disparate to the carbazole 1,9a-dioxygenase from CA10 [6] which is a three-component enzyme belonging to the class III type similar to naphthalene dioxygenase [29].

The terminal oxygenase component of the carbazole dioxygenase from CB3 putatively encoded by *carAaAb* consists of two subunits akin to those of biphenyl dioxygenase [27], naphthalene dioxygenase [29] and toluene dioxygenase [17] which is also disparate to the single subunit configuration of carbazole 1,9a-dioxygenase from CA10 [6]. We find, however, that the CarAaAb amino acic sequences show low homology to comparable subunits. Since the specificity of the initial dioxygenases appears to be provided by the subunits of two-component terminal oxygenase of aromatic dioxygenases [30] this may be a reflection of the narrow specificity of the *car* encoded pathway of *Sphingomonas* CB3. *carAa* encodes for a large (α) subunit of the iron sulfur component of the initial dioxygenase. The predicted amino acid sequence contains a Rieske-type [2Fe-2S] motif of conserved $Cys^{75,95}$ and $His^{77,98}$ residues which is a feature of these subunits. *carAb* encodes the small (β) subunit of the iron sulfur component of the initial dioxygenase. CarAc shows homology to the ferredoxin subunits of initial dioxygenases. In common with ferredoxin subunits of this type, CarAc has a conserved binding motif for a Rieske-type [2Fe-2S] centre presented by $Cys^{43,62}$ and $His^{45,65}$. *carAd* encodes the reductase subunit of the initial dioxygenase, the amino acid sequence is predicted to be a membrane-associated protein.

Unlike the *car* genes from CA10, CB3 has a gene encoding a dihydrodiol dehydrogenase which we have designated CarB. *carC* encodes for an extradiol dioxygenase enzyme. CarC contains the amino acid residues - His^{147}, His^{211} and Glu^{262} - which are conserved in extradiol dioxygenases where they are implicated in iron coordination by the active enzyme [31,32]. *carD* encodes for a hydrolase which shows most homology to biphenyl pathway hydrolases.

A closer phylogenetic analysis of the individual CB3 *car* genes consistently places these sequences away from the main groups which represent sequences derived from biphenyl degrading, as well as naphthalene/

TABLE 1

Summary of Predicted Polypeptides from the *car* Locus of *Sphingomonas* Strain CB3

Gene	Protein feature	Nucleotide position	No. of aa	Predicted molecular mass (kDa)	% Similarity to other gene products[b]
*car*Aa	ISP large (α) subunit of ID	1–1296	431[a]	48.2[a]	36 ipbA1 (BD2); 35 bphA1(RHA1); 35 bphC1 (M5); 13 carAa (CA10)
*car*Ab	ISP small (β) subunit of ID	1286–1843	185	21.5	40 ipbA2 (BD2); 40 bphA2 (P6); 40 bphA2 (RHA1); 40 tcbAb (P51)
*car*Ac	Ferredoxin subunit ID	1865–2194	109	11.5	42 bphA3 (P6); 41 ipbA3 (BD2); 40 bphA3 (RHA1); 31 carAc (CA10)
*car*Ad	Reductase subunit of ID	2191–3420	409	43.8	39 cumA4 (IP01); 38 ipbA4 (JR1); 36 bphG (LB400); 10 carAd (CA10)
*car*B	Dehydrogenase	3407–4240	277	28.4	53 bphB (LB400); 52 bphB (B-356); 52 bphB (KKS102); 52 cbpB (OU83)
*car*C	Extradiol dioxygenase	4278–5168	296	33.3	40 bphC (LB400); 40 bphC (KF715); 40 todE (F1); 11 carBb (CA10)
*car*D	Hydrolase	5207–6031	274	30.7	31 bphD (LB400); 31 bphD (KF715)

[a] Predicted size based on incomplete *carA1* gene.

[b] Bacterial strains and Genbank accession numbers of corresponding genes *Rhodococcus erythropolis* BD2 (U24277); *Rhodococcus* sp. RHA1 (D32142); *Rhodococcus* sp. M5 (U27591); *Pseudomonas* sp. strain CA10 (D89064/D89065); *Rhodococcus globerulus* P6 (X80041); *Pseudomonas* sp. P51 (U15298); *Pseudomonas fluorescens* IP01 (D37828); *Pseudomonas* sp. JR1 (U53507); *Pseudomonas* strain LB400 (M86348); *Comomonas testosteroni* B-356 (U57451); *Pseudomonas putida* OU83 (Y07655); *Pseudomonas putida* KF715 (M33813); *Pseudomonas putida* F1 (J04966); *Pseudomonas* sp. KKS102 (M26433).

phenanthrene-degrading strains. Two examples are provided. The first (Figure 3) reveals that CarAa is more closely related to, yet distinct from, the corresponding *bph* sequences than to CarAa from CA10 [5,6]. The second example (Figure 4) is provided by CarC which is also divergent and placed in a position intermediate to that of the corresponding genes from biphenyl of naphthalene degrading strains. Phylogenetic analysis revealed that the remaining CB3 *car* genes all occupy similarly divergent positions.

Pathway Specificity and Induction of the car Genes

Transcriptional analysis has confirmed that this *car* locus is expressed during growth of CB3 at the expense of carbazole, and since no other aromatic hydrocarbon can serve as a growth substrate we propose these are unique *car* genes. By applying RT-PCR using the primer pair C1036/C2495 we were able to amplify *carAa*(partial)/*carAb*/*carAc*/*carAd*(partial) from mRNA extracted from carbazole-grown cells (Figure 5). No RT-PCR product was amplified from mRNA extracted from acetate-grown CB3 cells.

Experiments using biphenyl and carbazole as independent inducers of acetate-grown cells of CB3 revealed that both carbazole and biphenyl were able to act as inducers of the *car* locus (Figure 5). This is particularly interesting as biphenyl does not serve as a growth substrate for CB3 and was not transformed by cells of CB3 growing at the expense of carbazole. As biphenyl is able to induce expression of the *car* operon it would appear that the substrate specificity of the terminal oxygenase component the initial dioxygenase enzyme complex is narrow, and does not extend to biphenyl thus preventing its transformation. This would appear different to the carbazole 1,9a-dioxygenase from CA10 has been shown to readily oxidise biphenyl [6]. Interestingly resting cell assays have been used to show that both naphthalene 1,2-dioxygenase and biphenyl 2,3-dioxygenase enzymes are able to oxidise carbazole [33], however this oxidation does not occur in an angular position as described for the *car* pathway of CA10 [2,5,6].

The lower enzymes of the *car* locus would not constitute a metabolic block to biphenyl catabolism as these are able to transform intermediates of the biphenyl pathway. CarCD-expressing clones transform the arene diol 2,3-dihydroxybiphenyl to the yellow *meta*-cleavage product 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate, which is further metabolised by the hydrolase activity of CarD resulting in the disappearance of the yellow *meta*-cleavage product (data not shown).

The gene sequences of the *car* locus show sufficient homology to isofunctional genes involved in the degradation of other aromatic compounds for common ancestries to be shared [1]. We speculate that this *car* operon is related to ancestral *bph* genes which share a common evolutionary background evolving to degrade biphenyl and its naturally occurring analogues. Evidence to support this supposition includes the following; (i) The ability of biphenyl to act as a non-metabolisable inducer of the *car* genes may signal a recent divergence which allowed the enzymes of this operon to utilise carbazole instead of biphenyl as a growth substrate. This may have occurred through a relaxing of the specificity of regulation, concomitant with a change in the

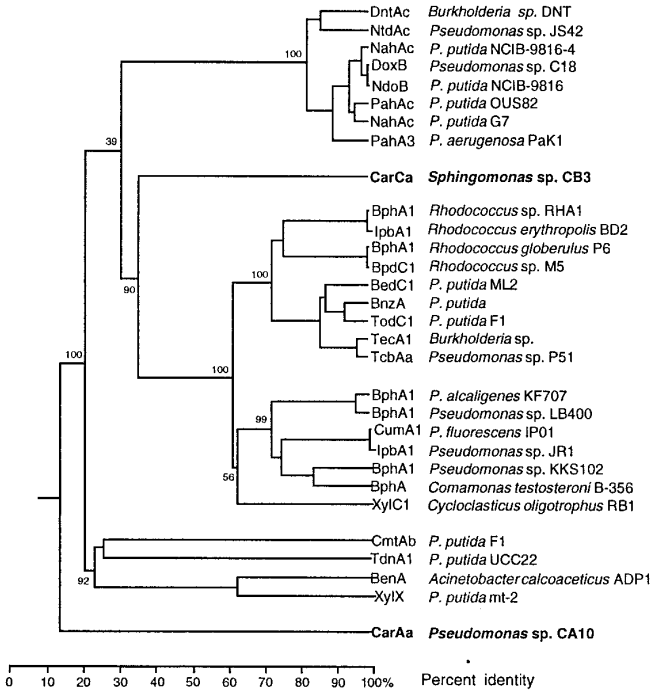

FIG. 3. Phenogram based on an alignment of the deduced amino acid sequences of selected α subunits of initial aromatic dioxygenases. The phenogram was constructed with SEQBOOT (100-bootstrap analysis), and the data set was passed through the PRODIST, NEIGHBOR, and CONSENSE programmes of the PHYLIP package (version 3.2). The final tree was redrawn with branch lengths scaled to reflect percent differences of the aligned sequences. The percentage bootstrap values are indicated.

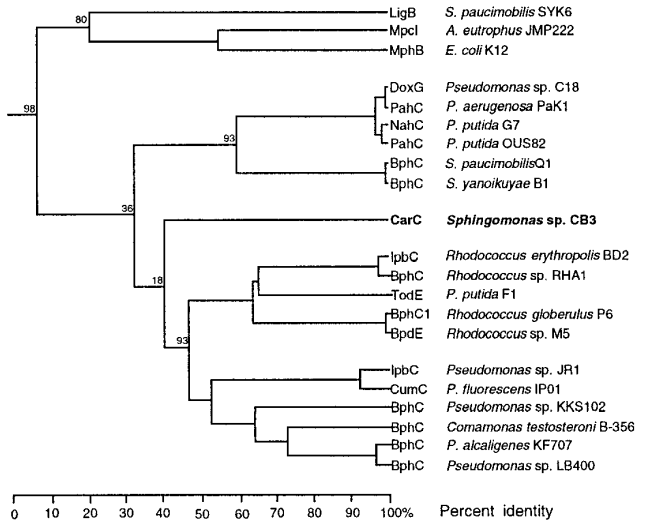

FIG. 4. Phenogram based on an alignment of the deduced amino acid sequences of selected extradiol aromatic dioxygenases. The phenogram was constructed with SEQBOOT (100-bootstrap analaysis), and the data set was passed through the PRODIST, NEIGHBOR, and CONSENSE programmes of the PHYLIP package (version 3.2). The final tree was redrawn with branch lengths scaled to reflect percent differences of the aligned sequences. The percentage bootstrap values are indicated.

specificity of the initial dioxygenase; (ii) That some intermediates of the biphenyl pathway can serve as substrates to enzymes of the *car* locus; (iii) The genes and gene order of the *car* locus from CB3 show greater homology to characterised *bph* loci than to the previously described *car* genes of CA10; (iv) The dihydrodiol dehydrogenase encoded by *carB* may not be required for carbazole degradation. Previous studies have suggested that its product 2′-aminobiphenyl-2,3-diol may form spontaneously from the product of the initial dioxygenase [2,3,5,6], however these studies have yet to be supported by resting-cell assays using a cloned carbazole dioxygenase.

Environmental Occurrence of the car Genes

As with all sequencing studies it is important to bear in mind the significance of the characterised genes not only from the prespective of the host wild type strain, but also from an environmental or ecological perspective. Given that azaarenes such as carbazole have been detected in ground water at industrial locations such as town gas sites [34], coal gasification plants [35] and creosote contaminated soils [36] we were interested in determining whether *car* genes analagous to those present in Sphingomonas CB3 were also present in soils associated with these industries. The *carAa*(partial)/*carAb*/*carAc*/*carAd*(partial) was therefore selected as a target for PCR amplification directly from soil

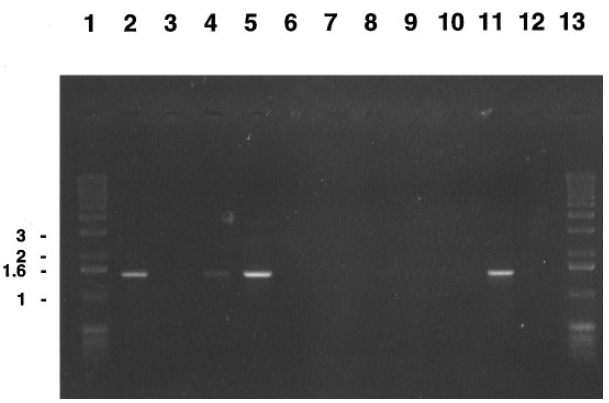

FIG. 5. DNase-treated RNA extracts from *Sphingomonas* strain CB3 were amplified by RT-PCR (plus PCR controls) using primers specific for a 1460-bp fragment partially encompassing four components of the carbazole dioxygenase (*carAa*(partial)/*carAb*/*carAc*/*carAd*(partial)). Aliquots of 10 μl were run on a 0.9% agarose gel. Lanes correspond to: 1 and 13, 1-kb DNA ladder (BRL); 2, RT-PCR carbazole-grown CB3; 3, RT-PCR acetate-grown CB3; 4, RT-PCR acetate-grown CB3 induced with carbazole; 5, RT-PCR acetate-grown CB3 induced with biphenyl; 6, RT-PCR blank (no target nucleic acid); 7, PCR carbazole-grown CB3; 8, PCR acetate-grown CB3; 9, PCR acetate-grown CB3 induced with carbazole; 10, acetate-grown CB3 induced with biphenyl; 11, PCR 0.1μg CB3 genomic DNA (5 μl aliquot); 12, PCR blank (no target nucleic acid).

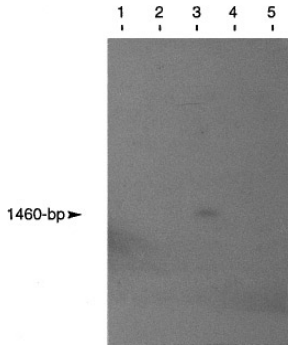

FIG. 6. Southern blot of *carAa*(partial)/*carAb*/*carAc*/*carAd*(partial) amplified from soil DNA. Aliquots of 20 μl were run on a 0.9% agarose gel. Lanes correspond to: 1, Town gas site soil LS; 2, Town gas site soil HS; 3, Town gas soil HHS; 4, Uncontaminated soil from maize pasture; 5, Uncontaminated native forest soil.

DNA extracts. Pristine soils and PAH contaminated soils were collected from the site of a former town gas site in Hamilton, New Zealand. Using a 25 cycle PCR amplification we were able to detect by hybridisation of a Southern blot the presence of *carAa*(partial)/*carAb*/*carAc*/*carAd*(partial) in only the most contaminated soil sample HHS, but not in less heavily contaminated and uncontaminated soils (Figure 6). These results indicate that the *car* genes are indeed present as a component of the microbial population present in some contaminated soils. The development of additional strategies for environmental probing will ultimately benefit from the continued characterisation of diverse catabolic genes such as the *car* genes we have described in this study. Further studies would be useful to determine whether these *car* genes are enriched in environments contaminated with azaarenes such as carbazole or are broadly distributed.

ACKNOWLEDGMENTS

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