

Properties, environmental fate and biodegradation of carbazole

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Abstract The last two decades had witnessed extensive investigation on bacterial degradation of carbazole, an *N*-heterocyclic aromatic hydrocarbon. Specifically, previous studies have reported the primary importance of angular dioxygenation, a novel type of oxygenation reaction, which facilitates mineralization of carbazole to intermediates of the TCA cycle. *Proteobacteria* and *Actinobacteria* are the predominant bacterial phyla implicated in this novel mode of dioxygenation, while anthranilic acid and catechol are the signature metabolites. Several studies have elucidated the degradative genes involved, the diversity of the *car* gene clusters and the unique organization of the *car* gene clusters in marine carbazole degraders. However, there is paucity of information regarding the environmental fate as well as industrial and medical importance of carbazole and its derivatives. In this review, attempt is made to harness this information to present a comprehensive outlook that not only focuses on carbazole biodegradation pathways, but also on its environmental fate as well as medical and industrial importance of carbazole and its derivatives.

Keywords Carbazole · Angular dioxygenation · Environmental fate · Degradative pathways

Introduction

Carbazole: general description

Carbazole (C₁₂H₉N, dibenzopyrrole diphenylenimine, CAS No. 86-74-8) is a non-basic tricyclic aromatic *N*-heteroatomic compound (Fig. 1). It has a molecular weight of 167.21 g/mol, boiling and melting point of 355 and 246 °C (Lide 2003), water solubility of 1.2 mg/l (Johansen et al. 1997), vapor pressure of 1×10^{-4} Pa (Peddinghaus et al. 2012), and octanol/water partition coefficient (log *K*_{ow}) of 3.72 (Blum et al. 2011). It is one of the π -excessive heterocycles (electron-rich rings) and is more recalcitrant than dibenzofuran but less than dibenzothiophene (Balaban et al. 2004). It is a white crystalline solid at ambient temperature. It sublimates, has a scent similar to creosote and exhibits strong fluorescence and long phosphorescence upon exposure to ultraviolet light (Collin and Höke 1986). It is one of the major *N*-heterocyclic aromatic hydrocarbons in fossil fuels (coal, crude oil, oil derived from oil shales pyrolysis) and is also found in cigarette smoke and emitted from coal and wood combustion (Odabasi et al. 2006).

Carbazole is used as a chemical feedstock for the production of dyes, reagents, explosives, insecticides, lubricants and it acts as a color inhibitor in detergents (Nojiri and Omori 2007). It is also widely used as a model compound for the study of biodegradation of aromatic *N*-heterocyclic hydrocarbons (Xu et al. 2006). However, its release into the environment from diverse anthropogenic sources is of serious health and environmental concern, as carbazole is both mutagenic and toxic and classified as “benign tumorigen” (Smith and Hansch 2000; Nojiri and Omori 2007).

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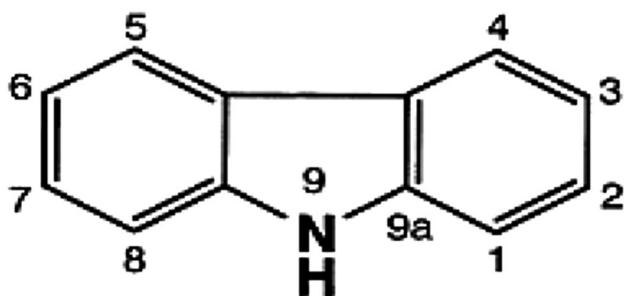


Fig. 1 Molecular structure of carbazole

Properties of carbazole

Solubility

Heterocyclic aromatic compounds are known to exhibit higher polarity and water solubility due to substitution of one carbon atom by nitrogen, sulfur or oxygen (Meyer and Steinhart 2000). These chemical properties lead to increase bioavailability and mobility as compared to the homologous polycyclic aromatic hydrocarbons resulting in various environmental influences of these compounds (Pearlman et al. 1984; Peddinghaus et al. 2012). Carbazole has an aqueous solubility at 25 °C of 1.2 mg/l. It is less soluble than dibenzothiophene (1.5 mg/l) and dibenzofuran (4.8 mg/l) but more soluble than xanthene (1.0 mg/l) in spite of its higher molecular weight (Table 1). It is readily soluble in acetone and dimethyl sulfoxide, slightly soluble in ether and ethanol, and barely soluble in chloroform, acetic acid, carbon tetrachloride, and carbon disulfide (Collin and Höke 1986).

Aromaticity

Aromaticity is a property of planar, cyclic, conjugated molecules that act like unsaturated molecules and undergo

substitution reaction rather than addition due to delocalization of electrons in the ring. It can also be considered a manifestation of cyclic delocalization and resonance (Balaban et al. 2004). The tendency to favor substitution rather than addition suggests that the parent unsaturated ring system has exceptional stability. Aromaticity cannot exist without conjugation (conjugation requires at least three overlapping *p* orbitals in the same plane). This is because aromatic molecules require planarity and overlapping *p* orbitals so that electron can be delocalized for better quality. In the same vein, resonance exists because of electron delocalization and emerges in different patterns based on the structure and arrangement within a molecule. Resonance gives extra stability due to electron delocalization and can be conferred sometimes on a molecule due to cycling double bonds.

Aromaticity in a molecule is premised on possession of four specific qualities (Katritzky et al. 2010). These are (1) Structure must be cyclic with conjugated Pi (π) bonds, (2) each atom in the ring must have an unhybridized *p* orbital, (3) all *p* orbitals must overlap continuously around the ring (planarity) and (4) $4n + 2 \pi$ electrons (n is an integer: 0,1,2,3...) in cyclic conjugation are associated with each ring.

Aromatic heterocyclic compounds electronic structure is in agreement with Huckel's rule, which states that cyclic conjugated and planar systems having $(4n + 2) \pi$ electrons are aromatic. The rings possess diamagnetic currents, react by substitution rather than addition, and bond orders and length tend to be intermediate between single and double (Balaban et al. 2004). Examples of these heterocycles are pyrrole, thiophene, and furan.

Carbazole (dibenzopyrrole) consists of two benzene rings fused together on either side of a pyrrole ring. Pyrrole is a five-membered ring in which the heteroatom has at

Table 1 Properties of some heterocyclic aromatic compounds

Group	Compound	Molecular weight (g/mol)	Aqueous solubility at 25 °C (mg/l)	Log K_{ow}
Nitrogen heteroaromatics	Pyrrole	67.1	58,800	0.75
	Indole	117.0	1875	2.00
	Quinoline	129.2	6718	2.03
	Carbazole	167.2	1.2	3.72
	Acridine	179.2	46.6	3.48
	6-Methylquinoline	143.2	631	2.57
Sulphur heteroaromatics	Thiophene	84.1	3600	1.81
	1-Benzothiophene	134.2	130	3.12
	Dibenzothiophene	184.3	1.5	4.38
Oxygen heteroaromatics	Benzofuran	118.1	678	2.67
	Dibenzofuran	168.2	4.8	4.12
	2-Methylbenzofuran	132.2	160	3.22
	Xanthene	182.2	1.0	4.23

least one pair of non-binding valence shell electrons. Hybridizing this heteroatom to an sp^2 state creates a p orbital occupied by a pair of electrons and oriented parallel to the carbon p -orbitals resulting in a planar ring. Six electrons occupy the π system. Four of the electrons are from two double bonds and two from the heteroatom. Hence, these five sp^2 hybridized atoms form planar six electrons delocalized π -cloud, which is responsible for the aromatic character of pyrrole.

The resonance energies of pyrrole, thiophene, and furan are 5.3, 6.5 and 4.3 kcal/mol, which gives the order of aromaticity as thiophene > pyrrole > furan. In essence, carbazole is less aromatic than dibenzothiophene but more aromatic than dibenzofuran (Balaban et al. 2004).

Toxicity

Heterocyclic aromatic compounds are highly ubiquitous in the environment and are known to exhibit diverse ecotoxic effects such as acute toxicity, developmental and reproductive toxicity, cytotoxicity, photo-induced toxicity, mutagenicity, and carcinogenicity (Bundy et al. 2001; Barron et al. 2004; Robbiano et al. 2004; Brack et al. 2007; Eisentraeger et al. 2008).

Human exposure to carbazole occurs through tobacco smoking and inhalation of polluted air (IARC 1983), while inhalation of vapors, dust, and dermal contact has been reported as possible routes of carbazole exposure to workers. There are no relevant epidemiological data to the carcinogenicity of carbazole to humans, though limited evidence in experimental animals for the carcinogenicity of carbazole has been reported (IARC 1999).

In a study conducted on groups of 50 male and 50 female, B6C3 F1 mice fed with different concentrations of technical grade carbazole (96% purity) for 96 weeks; neoplastic lesions were found in the livers and fore stomachs of the dead mice. The lesions were classified as neoplastic nodules and hepatocellular carcinomas. However, no such tumor was observed in the respective control groups (IARC 1983).

Carbazole is mutagenic and toxic. Its toxicity to aquatic organisms is well documented (Eisentraeger et al. 2008; Peddinghaus et al. 2012). In a recent study on embryotoxic potential of NSO-heterocyclic compounds using groups of 3-month old zebrafish *Danio rerio*, carbazole displayed a very high embryotoxic potential with LC50 value of 1.07 mg/l, a value preceded only by acridine (0.7 mg/l) (Peddinghaus et al. 2012).

Although carbazole is not a human carcinogen, its hazardous derivatives such as *N*-methylcarbazole and 7-*H*-dibenzo (c,g) carbazole (and its derivatives) found in cigarette smoke and automobile emission are genotoxic and carcinogenic and have been categorized as “IARC

Group 2B carcinogens” (Smith et al. 2000). 7-*H*-dibenzo (c,g) carbazole is a potent multi-site and multi-species carcinogen (Szafarz et al. 1988; Warshawsky et al. 1996) that induces tumor at the site of application and at distant sites, specifically in the liver (Renault et al. 1998).

Synthetic methyl derivatives of 5,9-dimethyl dibenzo (c,g) carbazole, dimethyl-DBC and *N*-methyl-DBC exhibit specific attachment to liver and skin and together with the parent compound (DBC) induce significant levels of DNA strand-breaks, micronuclei, and DNA adducts in immortalized human keratinocytes HaCat cells (Valovicova et al. 2012).

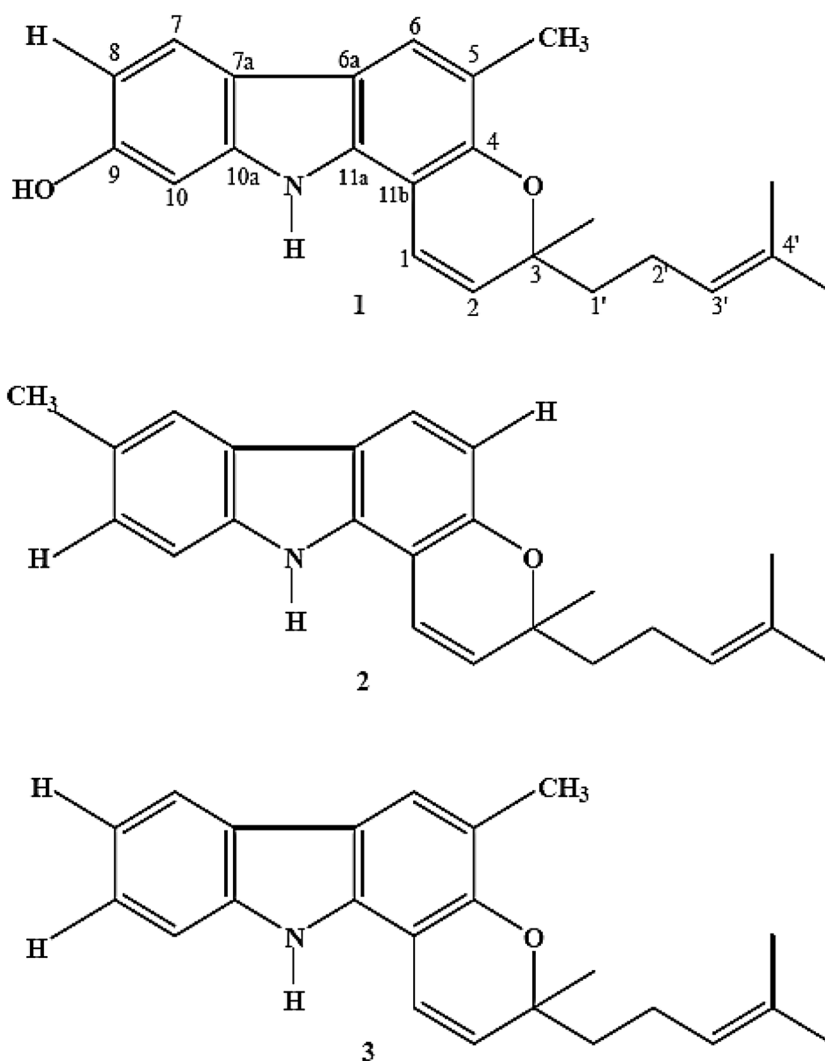
Industrial and medical importance of carbazole

Carbazoles are dominant as structural motifs in various synthetic materials and naturally occurring alkaloids. It exhibits material properties as optoelectronic materials, conducting polymers and synthetic dyes (Roy et al. 2012). Several dyes such as Hydron Blue™, Naphthol™ dyes, anthraquinone vat dyes, styryl dyes, and dioxazine dyes are synthesized from carbazole. Similarly, 1,3,6,8-tetranitro-carbazole (Nitrosan™) is used as an insecticide while reaction of carbazole with phenol and formaldehyde in the presence of acidic catalysts forms Novalacs, which can be cured with hexamethylenetetramine to produce highly heat resistant polymers (Collin and Höke 1986). Carbazole is also used to synthesize the monomer, *N*-vinylcarbazole, which can be polymerized to form polyvinyl carbazole (PVK) (Pearson and Stolka 1981; Collin and Höke 1986).

Naturally occurring carbazoles manifest profound biological activities such as antitumor, psychotropic, anti-inflammatory, anti-histaminic, antibiotic and antioxidative activities (Fig. 2) (Lobastova et al. 2004; Roy et al. 2012). The structural features of such carbazole-based natural products are the presence of nuclear hydroxyl groups (major structural feature), quinine functionality and prenyl groups (Roy et al. 2012). In pharmaceutical industry, hydroxylated carbazole derivatives are value-added substances exhibiting strong antioxidant activity and widely used in the treatment of encephalopathy, cardiopathy, hepatopathy and arteriosclerosis (Seto 1991). Furthermore, carbazole moiety is considered as one of the pharmacophores in the cardiovascular pharmaceuticals carvedilol and carazolol, which are used in the treatment of hypertension, ischemic heart disease, and congestive heart failure (Roy et al. 2012).

In the petroleum industry, the removal of nitrogen heteroaromatics is important for several reasons. First, their combustion directly causes the formation of nitrogen oxides (NO_x), which contribute to acid rain and depletion of the ozone layer (Kirimura et al. 1999). Second, nitrogen-containing aromatic compounds presence can cause

Fig. 2 Molecular structures of some natural carbazole alkaloids (1) mahanine (2) mahanimbicine and (3) mahanimbine



poisoning of refining catalysts, resulting in a decrease in yield (Girgis and Gates 1991; Williams and Chisti 2001). Carbazole directly affects the refining process by its conversion into basic derivatives during cracking, which allows it to adsorb to the active sites of the cracking catalyst. It also serves as a potent direct inhibitor of hydrodesulfurization, a property that enables it to be included in the refining process to meet sulfur content criteria (Benedik et al. 1998; Nojiri and Omori 2007). Finally, the presence of nitrogen heteroaromatics promotes corrosion of refining equipment, thereby increasing the refining costs (Benedik et al. 1998).

Environmental fate of carbazole

Atmospheric fate

Carbazole is a semi-volatile organic compound (SOC) found in ambient air in gas phase and sorbed to aerosol

(Odabasi et al. 1999). The fate, transport and removal of carbazole from the atmosphere by dry and wet deposition processes are strongly influenced by its gas-particle partitioning (Bidleman 1988). The vapor pressure of carbazole (1×10^{-4} Pa) suggests that carbazole will exist in the vapor and particulate phases in the ambient atmosphere. Carbazole is released to the atmosphere in emissions from waste incineration, tobacco smoke, aluminum manufacturing, and rubber, petroleum, coal, and wood combustion (Smith et al. 1978; Jacobs and Billings 1985; Pereira et al. 1987). Upon its release into the atmosphere, photochemically produced hydroxyl radicals (estimated half-life of 3 h) rapidly degrade vapor-phase carbazole. In the particulate phase, photodegradation of carbazole is dependent on the adsorbing substrate as substrates containing more than 5% carbon can stabilize photodegradation and permit long-range global transport of the pollutant (Behymer and Hates 1988).

Terrestrial fate

Biodegradation by indigenous carbazole degraders in the soil is the dominant fate process for carbazole even though photolysis of carbazole in soil had been reported (Behymer and Hates 1988; Grosser et al. 1991). However, adsorption of carbazole to environmental substrates will limit or prevent photolysis. The average K_{oc} (organic carbon normalized partition coefficient) value of carbazole is 637 (Ainsworth et al. 1989), which suggests low mobility of carbazole in soil. Sorption of carbazole to soil is non-linear and highly correlated with organic content of soils (Bi et al. 2007).

Aquatic fate

In aquatic environment, biodegradation and photolysis are the dominant fate processes for carbazole. Half-lives of carbazole in a river, pond, eutrophic lake, and oligotrophic lake have been estimated as 0.5, 10, 10, and 3 h, respectively (Smith et al. 1978). The absence of carbazole degraders in the microbial community will foreclose biodegradation as a fate process while adsorption of carbazole to sediment will make

photolysis unattainable (Pereira et al. 1987; Grosser et al. 1991). Volatilization is not a fate process in aquatic environment since carbazole is non-volatile in water (Meylan and Howard 1991). Metabolism of carbazole to its *N*-methyl and *N*-acetyl derivatives by aquatic organisms has been reported. Furthermore, bioaccumulation and acute toxicity of NSO-heterocycles in aquatic organisms such as *Daphnia*, midge, and algae have been documented (Eisentraeger et al. 2008).

Bacterial degradation of carbazole

Diversity of carbazole-degrading bacteria

Various carbazole-degrading bacteria have been isolated from diverse niches by enrichment cultural technique using carbazole as the sole source of nitrogen, carbon and energy or carbon and energy. Majority of carbazole degraders reported in the literature are aerobic, Gram-negative bacteria with the exception of very few carbazole degraders such as *Nocardioides aromaticivorans* IC177 (Inoue et al. 2005), *Gordonia* sp. F.5.25.8 (Santos et al. 2006) and *Microbacterium esteraromaticum* strain SL6 (Salam et al. 2014) that are aerobic, Gram-positive bacteria (Table 2).

Table 2 Some carbazole-degrading bacteria

Bacterial strain	Medium ^a	Products ^b	References
<i>Ralstonia</i> sp. RJGIL.123	Carbon	Anthranilic acid	Grosser et al. (1991); Schneider et al. (2000)
<i>P. resinovorans</i> CA10	Carbon, nitrogen	Anthranilic acid, catechol	Ouchiyama et al. (1993); Nojiri et al. (1999)
<i>P. resinovorans</i> CA06	Carbon, nitrogen	Anthranilic acid, catechol	Ouchiyama et al. (1993)
<i>P. stutzeri</i> ATCC31258	Carbon	Anthranilic acid	Hisatsuka and Sato (1994)
<i>Pseudomonas</i> sp. LD2	Carbon	Anthranilic acid	Gieg et al. (1996)
<i>Burkholderia</i> sp. CB1	Carbon, nitrogen	Not detected	Shotbolt-Brown et al. 1996
<i>Xanthomonas</i> sp. CB2	Carbon, nitrogen	Not detected	Shotbolt-Brown et al. (1996)
<i>Sphingomonas</i> sp. CB3	Carbon, nitrogen	Not detected	Shepherd and Lloyd-Jones (1998)
<i>P. stutzeri</i> OM1	Carbon, nitrogen	Anthranilic acid	Ouchiyama et al. (1998)
<i>Sphingomonas</i> sp. CDH-7	Carbon, nitrogen	Anthranilic acid	Kirimura et al. (1999)
<i>Sphingomonas</i> sp. GTIN11	Nitrogen	Anthranilic acid	Kilbane II et al. (2002)
<i>Sphingomonas</i> sp. KA1	Carbon	None	Habe et al. (2002)
<i>Pseudomonas rhodesiae</i> KK1	Carbon	None	Yoon et al. (2002)
<i>Neptunomonas naphthovorans</i> CAR-SF	Carbon	None	Fuse et al. (2003)
<i>Pseudomonas</i> sp. XLDN4-9	Nitrogen	None	Li et al. (2004)
<i>Achromobacter</i> sp. IC074	Carbon, nitrogen	None	Inoue et al. (2005)
<i>Stenotrophomonas</i> sp. IC193	Carbon, nitrogen	None	Inoue et al. (2005)
<i>Janthinobacterium</i> sp. J3	Carbon, nitrogen	None	Inoue et al. (2004)
<i>Pantoea</i> sp. J14	Carbon, nitrogen	None	Inoue et al. (2004)
<i>Achromobacter</i> sp. SL1	Carbon	Anthranilic acid, catechol	Salam et al. (2014)
<i>Pseudomonas</i> sp. SL4	Carbon	Anthranilic acid, catechol	Salam et al. (2014)
<i>Microbacterium esteraromaticum</i> SL6	Carbon	Anthranilic acid, catechol	Salam et al. (2014)

^a Carbon and nitrogen: carbazole was added to the isolation medium as the carbon, nitrogen and energy sources; *carbon* carbazole was added to the medium as the carbon and energy source, *nitrogen* carbazole was added as the nitrogen source

^b Major metabolic intermediate produced when the bacterium is grown on carbazole

About 23 and 39% of carbazole degraders isolated from activated sludge, soil, and freshwater samples belong to the genera *Pseudomonas* and *Sphingomonas*, respectively (Nojiri and Omori 2007). Recent research on carbazole degraders from marine environments using seawater-based enrichment culture has led to the isolation of novel carbazole degraders with unique carbazole degradative genes and enzymes different from those found in various carbazole degraders from soil, freshwater and activated sludge (Fuse et al. 2003; Maeda et al. 2009a, b, 2010).

Interest in the study of bacterial degradation of carbazole is spurred partly because of the ubiquitous nature, mutagenic and toxic activities, and the fact that it is a structural analog of dioxins and carbazole-degrading enzymes can partly function as dioxin-degrading enzymes (Nojiri and Omori 2007).

Degradation pathways of carbazole

Three major degradation pathways have been reported for carbazole: Lateral dioxygenation at carbon positions 3 and 4, monohydroxylation at carbon positions 1, 2, and 3 and angular dioxygenation at carbon positions 1, and 9a (Grifoll et al. 1995; Lobastova et al. 2004; Nojiri 2012).

Lateral dioxygenation of carbazole

Grifoll and co-workers first suggested lateral dioxygenation of carbazole by fluorene-degrading bacteria *Pseudomonas cepacia* F297 at C3 and C4 carbons yielding 4-(3'-hydroxy-2'-indolyl)-2-oxo-3-butenoic acid as detected by GC-FID and GC-MS. However, strain F297 cannot utilize carbazole as source of carbon and energy (Fig. 3; Grifoll et al. 1995).

Hydroxylation of carbazole

Transformation via hydroxylation appears to be a very common reaction in the metabolism of carbazole by bacteria. Lobastova et al. (2004) were able to identify 1-, 2- and 3-hydroxycarbazoles as the bioconversion products following monohydroxylation of carbazole at position 1, 2,

and 3 by *Aspergillus flavus* VKM F-1024 using TLC, GC, MS and ¹H NMR, respectively. 3-hydroxycarbazole was detected as the major product, while 1-hydroxy- and 2-hydroxycarbazoles were detected as minor products. Yamazoe et al. (2004), and Seo et al. (2006) also reported bioconversion of carbazole to hydroxycarbazoles.

Furthermore, bacterial dioxygenases such as naphthalene 1,2-dioxygenase from *Pseudomonas* sp. NCIB 9816-4 and biphenyl dioxygenase from *Beijerinckia* sp. B8/36 also catalyze the initial oxidation of carbazole to 3-hydroxycarbazole (Resnick et al. 1993). Hydroxylated carbazole derivatives have strong antioxidant activity and are value-added substances in pharmaceutical industry with diverse application in therapies for encephalopathy, cardiopathy, hepatopathy and arteriosclerosis (Lobastova et al. 2004).

Angular dioxygenation of carbazole

Some carbazole degraders reported in the literature degrade carbazole via angular dioxygenation, a novel type of oxidative attack that occurred at the ring-fused position and mediated by a multicomponent enzyme, carbazole 1,9a-dioxygenase (CARDO) with additive preference for angular positions (Nojiri et al. 1999). In contrast to lateral dioxygenation and monohydroxylation, angular dioxygenation result in complete mineralization of carbazole with the resulting catechol converted to tricarboxylic acid (TCA) cycle intermediate (Nojiri and Omori 2002).

Ouchiyama and co-workers isolated a carbazole degrader, *Pseudomonas resinovorans* CA10, from activated sludge of a municipal wastewater treatment facility in Tokyo, Japan. The strain is capable of growth on carbazole as a sole source of carbon, nitrogen and energy and accumulates anthranilic acid and catechol as catabolic intermediates of carbazole. It also grows on anthranilic acid as carbon and nitrogen source and accumulates catechol suggesting carbazole conversion to catechol via anthranilic acid (Ouchiyama et al. 1993). Furthermore, production of 2'-aminobiphenyl-2,3-diol and its meta-cleavage product 2-hydroxy-6-oxo-6-(2'-aminophenyl)-hexa-2,4-dienoate (HOADA) from the culture medium of CA10 grown on carbazole was suggested. Based on

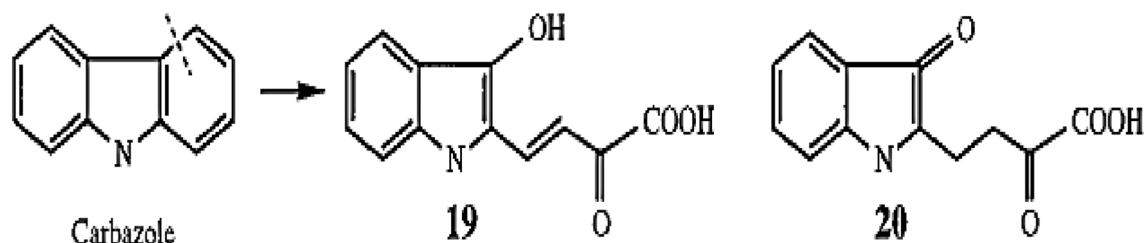


Fig. 3 Lateral dioxygenation of carbazole at C3 and C4. The metabolites detected from the methylated acidic extract are 4-(3'-methoxy-2'-indolyl)-2-oxo-3-butenoic acid (Methylated, 19) and 4-(3'-oxo-2'-indolyl)-2-oxo-3-butenoic acid (Methylated, 20)

these findings and its similarity with dibenzofuran degradation pathway, a carbazole degradation pathway was proposed (Fig. 4). The pathway is divided into upper and lower pathway. The upper pathway encompasses the conversion of carbazole to catechol, while the lower pathway involves catechol mineralization (Nojiri 2012).

Carbazole is dioxygenated at angular (C9a) and adjacent (C1) carbon atoms to produce an unstable hemiaminal (1-hydro-1,9a-dihydroxycarbazole) which is spontaneously cleaved to form 2'-aminobiphenyl-2,3-diol. This metabolic intermediate is converted to anthranilic acid via meta-cleavage and subsequent hydrolysis. Anthranilic acid is converted to catechol by dioxygenation at the C1 and C2 positions followed by spontaneous deamination and decarboxylation reactions (Kobayashi and Hiyashi 1970).

The resulting catechol is converted to a tricarboxylic acid (TCA)-cycle intermediate via *ortho*-cleavage (as in *P. resinovorans* CA10) or *meta*-cleavage (as in *Pseudomonas stutzeri* strain OM1) pathways (Ouchiyama et al. 1993; 1998; Fig. 4).

Anthranilic acid has been detected in the culture extracts of several carbazole degraders and is regarded as the main metabolite of carbazole angular dioxygenation (Ouchiyama et al. 1993; Gieg et al. 1996; Ouchiyama et al. 1998; Kirimura et al. 1999; Schneider et al. 2000; Kilbane II et al. 2002; Inoue et al. 2005). Recently, carbazole degraders with additive preference for angular dioxygenation were also isolated from hydrocarbon-contaminated tropical African soil in Lagos, Nigeria (Salam et al. 2014). The isolates, designated *Achromobacter* sp.

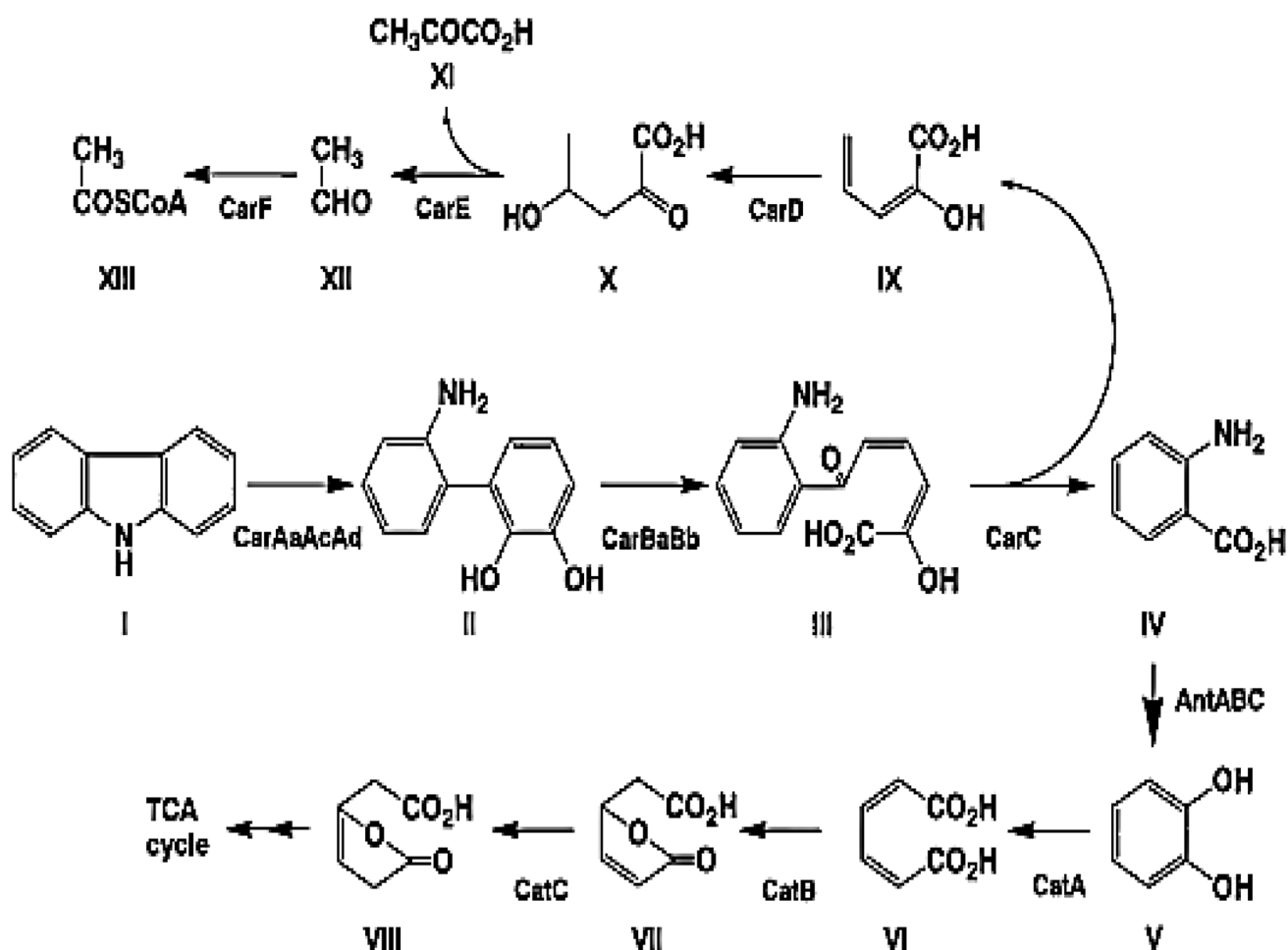


Fig. 4 Carbazole degradation pathway in *P. resinovorans* CA10. Enzymes designations: CarAaAcAd, carbazole 1,9a-dioxygenase; CarBaBb, 2'-aminobiphenyl-2,3-diol 1,2-dioxygenase; CarC, 2-hydroxy-6-oxo-6-(2'-aminophenyl)-hexa-2,4-dienoate hydrolase; CarD, 2-hydroxy-penta-2,4-dienoate hydratase; CarE, 4-hydroxy-2-oxovalerate aldolase; CarF, acetaldehyde dehydrogenase (acylating); AntABC, anthranilate 1,2-dioxygenase; CatA, catechol 1,2-dioxygenase; CatB,

cis,cis-muconate lactonizing enzyme; CatC, muconolactone δ -isomerase. Compounds: I, CAR; II, 2'-aminobiphenyl-2,3-diol; III, 2-hydroxy-6-oxo-6-(2'-aminophenyl)-hexa-2,4-dienoate; IV, anthranilic acid; V, catechol; VI, *cis,cis*-muconate; VII, muconolactone; VIII, β -ketoadipic acid enol-lactone; IX, 2-hydroxy-penta-2,4-dienoate; X, 4-hydroxy-2-oxovalerate; XI, pyruvate; XII, acetaldehyde; XIII, acetyl coenzyme A (Nojiri et al. 2001)

strain SL1, *Pseudomonas* sp. strain SL4 and *Microbacterium esteraromaticum* strain SL6, produce anthranilic acid and catechol as the major metabolites of carbazole angular dioxygenation. Anthranilic acid is a biotic compound and is formed by the degradation of tryptophan in several living organisms (Hayaishi and Stanier 1951). It is an important intermediate in the metabolism of many *N*-heterocyclic compounds and plays an important role in *Pseudomonas* quinolone signal, which is involved in quorum sensing in *Pseudomonas aeruginosa* cells (Calfee et al. 2001).

It is worthy to note, however, that once angular dioxygenation and subsequent ring cleavage occur for carbazole, the resulting 2'-aminobiphenyl-2,3-diol is degraded via the analogous biphenyl degradation pathways (Furukawa et al. 2004).

The CARDO system in carbazole degraders and its substrate specificity

The extensively studied CARDO system in *Pseudomonas resinovorans* CA10 is a three-component dioxygenase system belonging to the Rieske nonheme iron oxygenase system (ROS) and consist of a terminal oxygenase and electron transport proteins (Sato et al. 1997a; Nam et al. 2002). The terminal oxygenase component of CARDO (CARDO-O) is a homotrimeric enzyme that contains one Rieske [2Fe-2S] cluster ($[2\text{Fe-2S}]_R$) and one active-site iron (Fe^{2+}) in a single subunit (CarAa) (Nojiri and Omori 2007). The electron transport proteins of CARDO, which mediate electron transport from NAD(P)H to CARDO-O, comprise ferredoxin (CARDO-F; a monomer of CarAc), which contains one $[2\text{Fe-2S}]_R$, and ferredoxin reductase (CARDO-R; a monomer of CarAd), which contains one FAD and one plant-type $[2\text{Fe-2S}]$ cluster ($[2\text{Fe-2S}]_P$) (Sato et al. 1997a; Nam et al. 2002).

Phylogenetic analysis revealed a very low homology (<19% overall length-wise identity) of the amino acid sequence of CARDO with almost all known catalytic subunits of ROS terminal oxygenases (Nojiri and Omori 2007). In addition, CARDO-O consists of only catalytic α subunit with the α_3 configuration in contrast to typical class III ROSs whose terminal oxygenase components consist of both α and β subunits with the $\alpha_3\beta_3$ (or $\alpha_2\beta_2$) configuration (Nojiri and Omori 2007). This homotrimeric structure is typical of class IA ROSs, whose terminal oxygenases have α_3 configurations (Ferraro et al. 2005).

CARDO catalyzes diverse oxygenation of aromatic compounds. Aside from angular dioxygenation, which is the most interesting feature of CARDO, biotransformation experiments with *E. coli* cells harboring *carAa*, *carAc*, and *carAd* revealed the ability of CARDO to catalyze lateral dioxygenation and monooxygenation of aromatic

substrates exhibiting broad substrate specificity (Nojiri et al. 1999; Takagi et al. 2002). It was also observed that angular dioxygenation by CARDO occurs effectively at the angular position adjacent to an oxygen or nitrogen atom (due to high electronegativity of oxygen and nitrogen), but not a sulfur or carbon atom (Bressler and Fedorak 2000; Nojiri and Omori 2007).

Carbazole degradative genes

Pseudomonas-type *car* gene cluster

The CAR degradative genes of *P. resinovorans* CA10 have been extensively studied. Sato et al. (1997a, b) first succeeded in cloning the genes involved in upper pathway of carbazole degradation from *P. resinovorans* CA10 genome by shotgun cloning using meta-cleavage activity. The resultant gene fragment contains seven degradative genes, one open reading frame (ORF) that encoded a putative protein or unknown function, and two partial possible genes. Functional analysis of the degradative genes shows two identical copies of *carAa*, *carAc*, and *carAd*, which encode terminal oxygenase, ferredoxin, and ferredoxin reductase components of carbazole 1,9a-dioxygenase (CARDO); *carBa* and *carBb*, which encode structural and catalytic subunits of the meta-cleavage enzyme (2'-aminobiphenyl-2,3-diol 1,2-dioxygenase); and *carC*, which encodes the meta-cleavage compound (HOADA) hydrolase.

Gene walking around the *car*_{CA10} gene cluster revealed the entire gene structure. 2-hydroxypenta-2,4-dienoate (HPD degradative *carDFE* genes (meta-cleavage pathway genes) was found downstream of the *carAd* gene. In addition, *antABC* gene encoding anthranilate 1,2-dioxygenase was found in the 21-kb region upstream from *carAa* (Fig. 5a) (Nojiri et al. 2001). This anthranilate degradative gene cluster is a putative composite transposon flanked by two homologous insertion sequences ISPre1 and ISPre2. Furthermore, *antR* gene encoding a transcriptional regulator of the ant operon was found outside the putative composite transposon containing *antABC*, which regulates the inducible expression of the *car* gene cluster (Urata et al. 2004). Tn5 mutagenesis was used to isolate the β -ketoacid pathway (ortho-cleavage pathway) genes involved in catechol mineralization from strain CA10 genome (Kimura et al. 1996).

Carbazole-degrading bacteria from the genera *Pseudomonas*, *Burkholderia*, and *Janthinobacterium* have been reported that have nearly identical carbazole degradative genes with *car*_{CA10} and are designated *Pseudomonas*-type *car* gene cluster. Even though these carbazole degraders are isolated from different sources, comparison of the gene organization and flanking regions of their *car* gene clusters

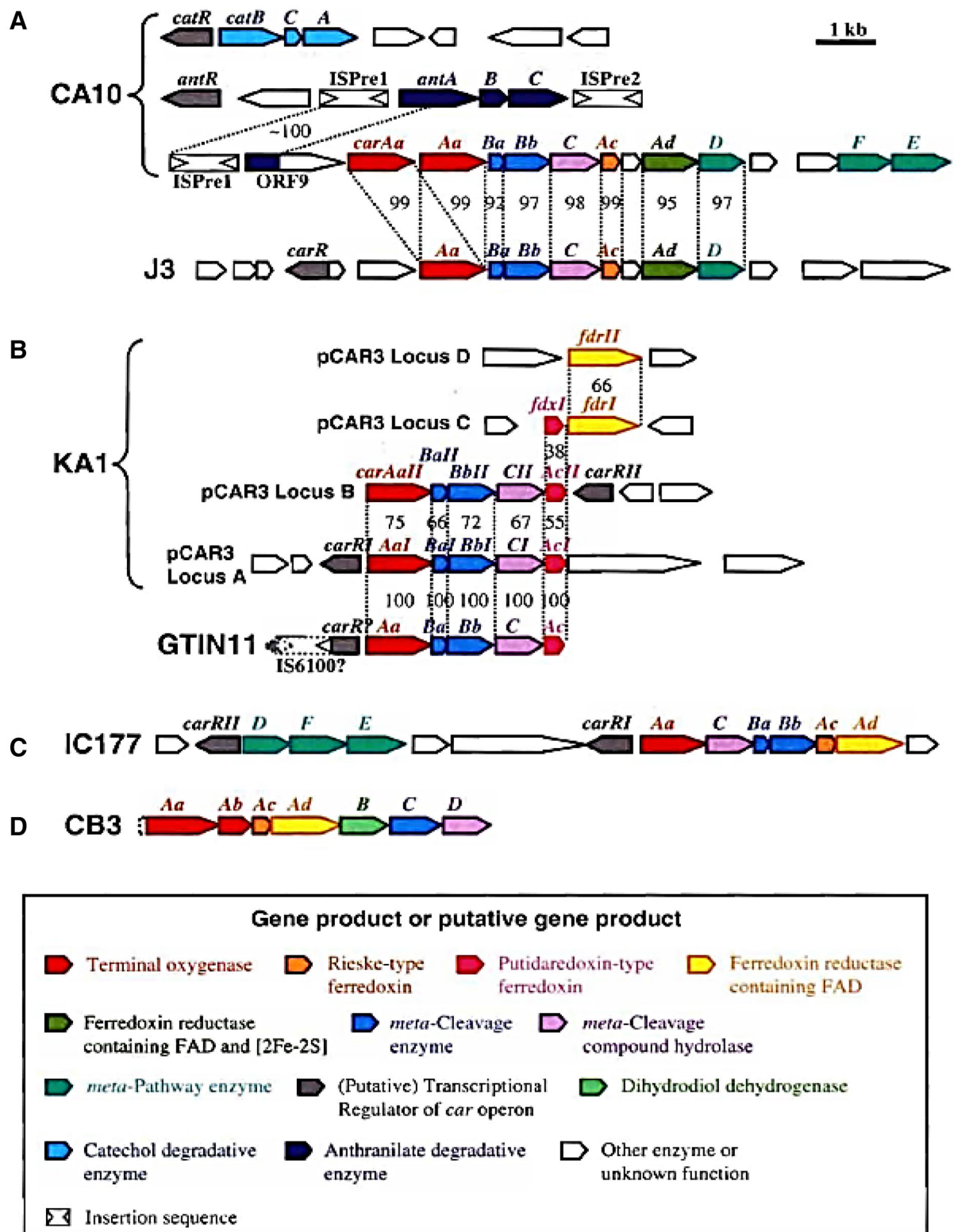


Fig. 5 Genetic structure of the gene clusters involved in carbazole biodegradation by **a** *P. resinovorans* CA10 and *Janthinobacterium* sp. J3, **b** *Spingomonas* (*Novosphingobium*) sp. KA1 and *Spingomonas*

sp. GTIN11, **c** *N. aromaticivorans* IC177, and **d** *Spingomonas* sp. CB3 (Nojiri and Omori 2007)

suggests evolutionary diversity as reflected in differences in copy number of *car* gene cluster among carbazole degraders (Inoue et al. 2004). This phenomenon may arise because *car* gene clusters are sometimes borne on plasmids or transposons and/or flanked by IS (insertion sequence) elements (Inoue et al. 2004).

Sphingomonas-type *car* gene cluster

The genus *Sphingomonas* was found to possess a *car* gene cluster homolog (though relatively low homology, <60% identity) showing similarity in gene organization and phylogeny with the *car*_{CA10} gene cluster. Isolation of *car* gene clusters in sphingomonads was first reported in *Sphingomonas* sp. GTIN11 (Kilbane II et al. 2002) and *Sphingomonas* sp. (reclassified as *Novosphingobium* sp) KA1 (Habe et al. 2002) and the *car*_{KA1/GTIN11} gene cluster homolog have been reported to occur in various carbazole-degrading *Sphingomonas* and related strains (Inoue et al. 2004, 2005).

The *car* gene clusters isolated from these two *Sphingomonas* strain are different from *car*_{CA10} gene cluster in two ways. First, unlike the *car*_{CA10} gene cluster, it does not contain the NAD(P)H:ferredoxin oxidoreductase gene involved in the initial dioxygenase, but contains the genes for terminal oxygenase (*carAa*) and ferredoxin (*carAc*), the meta-cleavage enzyme (*carBaBb*), and HOADA hydrolase (*carC*) (Fig. 5b). Second, though *Sphingomonas* CarAa exhibits significant homology with CA10 CarAa (>55% identity), its ferredoxin (CarAc) is neither related to CarAc_{CA10} nor with other Rieske ferredoxins but shows similarity to the putidaredoxin-type ferredoxins. Because the terminal oxygenase of strain KA1 (CarAa_{KA1}) can receive electrons from strain KA1 ferredoxin (CarAc_{KA1}) and catalyze angular dioxygenation of carbazole, it implies that ferredoxin selectivity differs between strain CarAa_{CA10} and CarAa_{KA1/GTIN11} (Inoue et al. 2004). Furthermore, two copies of *car*_{KA1} gene cluster (*car*-I_{KA1} and *car*-II_{KA1}) were found to be domiciled on a >250-kb circular plasmid pCAR3 in *Novosphingobium* sp. KA1 along with the presence of NAD(P)H:ferredoxin oxidoreductase genes (*fdrI* and *fdrII*) and a third putidaredoxin-type ferredoxin gene. These findings show clearly that the plasmid pCAR3 contains the complete set of genes responsible for carbazole mineralization in strain KA1 (Urata et al. 2006).

The car gene cluster in *Nocardioides aromaticivorans* IC177

Quite distinct *car* gene cluster different from the *Pseudomonas* and *Sphingomonas*-types was found in a Gram-positive bacterium *N. aromaticivorans* IC177 (Inoue et al. 2005, 2006). The *car* gene was clustered in the

carAaCBaBbAcAd and *carDFE* gene clusters encoding the enzymes responsible for degradation of carbazole to anthranilate and 2-hydroxypenta-2,4-dienoate (HPD) (upper pathway) and HPD to pyruvate and acetyl coenzyme A (lower pathway), respectively (Inoue et al. 2006).

However, the position of *carC* relative to *carBaBb* in strain IC177 is the opposite of that in *car* gene clusters of the *Pseudomonas* and *Sphingomonas*-types (Fig. 5c) (Inoue et al. 2006). In the *car* gene operons in strain IC177, the genes overlap each other by 1 or 4 bp with *carDFE* genes closely linked and located upstream of the *carAaCBaBbAcAd* gene cluster. In addition, organization of carbazole catabolic operon in strain IC177 occurred in a more orderly fashion as functional units than those in Gram-negative strains, such as strains CA10, J3, GTIN11, and KA1 (Nojiri and Omori 2007).

The car gene cluster in *Sphingomonas* sp. CB3

Interestingly, the *car* gene cluster of strain CB3 differs from those of the three above-mentioned types in terms of gene organization and phylogeny but showed marked similarity with naphthalene and biphenyl degradative *bph* gene cluster (Shepherd and Lloyd-Jones 1998). The *car* genes of strain CB3 are arranged in the order of *carAaAbAcAdBCD*, and the terminal oxygenase component of strain CB3, unlike those of other CAR degraders, which are composed of a single subunit, is composed of two subunits, CarAa and CarAb, respectively (Fig. 5d) (Shepherd and Lloyd-Jones 1998). Although carbazole metabolic activity of the enzymes encoded in carbazole catabolic operon in CB3 has not been confirmed, its transcription was detected when carbazole was used as source of carbon by strain CB3 (Nojiri and Omori 2007).

The *car* gene cluster in marine carbazole degraders

Carbazole-degrading bacteria from different genera such as *Neptuniibacter*, *Erythrobacter*, *Marinobacter*, *Caulobacter*, *Hyphomonas*, *Lysobacter*, *Sphingosinicella*, *Kordiimonas*, and *Terrabacter* have been isolated from marine environment (Fuse et al. 2003; Inoue et al. 2005; Maeda et al. 2009a, b). Southern hybridization analysis performed under strict conditions at 68 °C (hybridization conditions for similarity of >90%) and 55 °C (hybridization conditions for similarity >60%) using *car*_{CA10} and *car*_{KA1} gene cluster probes for 14 marine isolates showed that they lack *car* genes highly similar to *car*_{CA10} and *car*_{KA1}. This suggests that marine isolates are evolutionarily different from their terrestrial counterpart, with unique *car* gene clusters and CARDO. Furthermore, hybridization analysis at 55 °C showed that eight of the 14 marine isolates have novel *car* gene cluster that are highly different from the *car*_{CA10} and *car*_{KA1} genes.

car gene cluster of *Neptuniibacter* sp. strain CAR-SF

The *car* gene cluster of strain CAR-SF is arranged in the order *carAaBaBbC*, resembling the order of arrangement of the *Pseudomonas* and *Sphingomonas*-type *car* gene clusters showing 48–77% similarity with *car*_{CA10} and *car*_{J3} genes and thus designated as a *Pseudomonas*-type *car* gene cluster (Nagashima et al. 2010). However, in comparison with the *car*_{CA10} and *car*_{J3} gene clusters, the *car*_{CAR-SF} gene cluster lacks the ferredoxin *carAc* and ferredoxin reductase *carAd* genes, though a *carAc*_{CA10}-like gene was revealed by Southern hybridization analysis. This shows that unlike in *car*_{CA10} and related *Pseudomonas*-type *car* gene clusters, ferredoxin gene of CARDO was in a different location in CAR-SF strain and not in the *car*_{CAR-SF} gene cluster (Nagashima et al. 2010).

car gene cluster of *Lysobacter* sp. strain OC7

The *car* gene cluster in strain OC7 is arranged in the order *carAaCBaBb*, with the position of *carC* and *carBaBb* inverted when compared to their positions in *Pseudomonas* and *Sphingomonas*-type *car* gene clusters. However, the genes arrangement followed the same order as in the *car* gene cluster of strain IC177 (Maeda et al. 2009b). The open reading frames (ORFs) containing the *car* gene cluster of strain OC7 share 39–52% similarity with *carAa*, *carC*, *carBa*, and *carBb* genes of strains CA10 and KA1, and showed no similarity with *car* genes of strain CB3, making the *car* genes of strain OC7 phylogenetically distinct from previously reported *car* gene products. Furthermore, southern hybridization analysis shows that only *Caulobacter* sp. strain OC6, a phylogenetically different genus (α -proteobacteria), hybridized with the *car*_{OC7} gene cluster (from strain OC7 belonging to γ -proteobacteria group) probe with more than 90% similarity (Maeda et al. 2009b). This finding is interesting as it reveals the evolutionary diversity of *car* gene clusters and importance of genetic exchange in its distribution across different phylogenetic groups.

The product of *carAa*_{OC7} possessed consensus sequences of a Rieske-type [2Fe-2S] cluster and mononuclear heme iron (Maeda et al. 2009b). However, its ferredoxin and ferredoxin reductase genes are not located near the *car* gene cluster of strain OC7, as in strain CAR-SF. In addition, as in CAR-SF, *E. coli* harboring only *carAa*_{OC7} was unable to convert CAR but *E. coli* cells harboring pBOC77 (*carAa*_{OC7AcAd}_{CA10}) converted CAR to 2'-aminobiphenyl-2,3-diol. However, the transformation ratio of CAR by pBOC77 (*carAa*_{OC7AcAd}_{CA10}) was 32–36%, which is less than 99% recorded for *E. coli* cells harboring pUCARA (*carAaAaAcORFcarAd*) (Sato et al. 1997a) or pSF6 (*carAa*_{CAR-SFAcAd}_{CA10}) used as positive controls, thus

revealing weak electron transfer efficiency of *CarAa*_{OC7}. *AcAd*_{CA10} and suggesting a different electron transfer components and RO class for CARDO_{OC7} (Maeda et al. 2009b).

car gene cluster of novel genus strain OC9

The CARDO system and the arrangement of *car* gene cluster in strain OC9 present a new question in relation to evolution and diversity of *car* genes in bacteria. First, the recovered ORFs of strain OC9 share 35–65% homology with previously reported *car* genes (*carRAaCBaBb*). However, a ferredoxin-like gene (*carAc*) found immediately downstream of *carR* does not show homology with any of the reported ferredoxin component of CARDO as it possesses a chloroplast-type ferredoxin (Maeda et al. 2010). This is a unique type of ferredoxin completely different from the Rieske and putidaredoxin-types reported for strains CA10, KA1, IC177, and CB3 CARDO systems (Sato et al. 1997a; Shepherd and Lloyd 1998; Inoue et al. 2006; Urata et al. 2006). Second, the *car* gene cluster of strain OC9 was arranged in the order *carAcRAaCBaBb* with *carRAc* and *carAaBaBb* having opposite orientation, thus suggesting that the *carAc* and *carAa* genes transcribed within different transcription units.

The product of *carAa*_{OC9} possessed consensus sequences of a Rieske-type [2Fe-2S] cluster and mononuclear heme iron (Maeda et al. 2010). However, unlike *carAa*_{CAR-SF} and *carAa*_{OC7} that could not transform *E. coli* cells without *CarAc*, *E. coli* cells harboring only *carAa*_{OC9} in a resting cell reaction converted CAR to 2'-aminobiphenyl 2,3-diol, though the conversion ratio (12%) is low when compared to that of *E. coli* cells harboring genes for both *carAa* and *carAc* (100%), respectively (Maeda et al. 2010).

Conclusion

In summary, carbazole is a very important compound from the industrial, medical and environmental perspectives. Its use in the industry as chemical feedstock for the production of dyes, reagents, explosives, insecticides, lubricants and color inhibitor in detergent is well documented. The medical importance of naturally occurring carbazole and derivatives of hydroxylated carbazoles and their antitumor, psychotropic, anti-inflammatory, anti-histaminic, antibiotic and antioxidative activities has also been reported extensively. However, environmentally, carbazole is of serious concern as it is recalcitrant, mutagenic and toxic with genotoxic and carcinogenic hazardous derivatives such as *N*-methylcarbazole and 7-*H*-dibenzo[*c,g*]carbazole found in cigarette smoke and automobile emissions categorized as “IARC Group 2B” carcinogens. Carbazole angular

dioxygenation characterized with addictive preference for angular position results in complete mineralization to intermediates of the TCA cycle. The genes involved are evolutionarily diverse and have been detected in various microorganisms cutting across different bacteria phyla.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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