博士論文 （要約）

Diversity and evolution of bacterial carbazole-degradative gene clusters

(細菌のカルバゾール分解遺伝子群の多様性と進化)

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論文の内容の要旨

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論文題目

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Chapter 1. Introduction.

Carbazole, a toxic and mutagenic heteroaromatic pollutant structurally similar to dioxins, can be completely converted into tricarboxylic acid cycle intermediates by some bacterial strains. The key enzyme in carbazole degradation is the carbazole 1,9a-dioxygenase, CARDO, a Rieske-type non-heme iron oxygenase (RO), which is composed of the two electron transport components (ETC) CarAd and CarAc and a terminal oxygenase, CarAa, in charge of the initial carbazole dioxygenation reaction. Studies on the carbazole degraders, *Pseudomonas resinovorans* CA10 and *Novosphingobium* sp. KA1, revealed that the RO genes and those encoding subsequent catabolic enzymes are found together forming the *car* gene cluster, which exists in close proximity with genes for the dioxygenation of the intermediate, anthranilate. On the other hand, preliminary research on marine carbazole degraders suggested the existence of novel types of CARDO ETC, hinting to an unexplored diversity of *car* gene clusters in marine bacteria and unknown evolutionary mechanisms that led to such diversification.

Recently, attempts to understand the recruitment process of bacterial catabolic genes have led to the analysis of the genome sequences of related γ-hexachlorocyclohexane (γ-HCH)-degrading strains, resulting in identification of the genetic elements responsible for gene-recruitment and their hypothetical evolution trajectory (Tabata et al., 2016). In the case of carbazole degraders that have been isolated from diverse sources and that are not phylogenetically closely related, such reconstruction of the recruitment trajectory of *car* genes might provide a wider outlook on the evolution of degradative genes in nature. Starting with an exploration of the diversity of carbazole degradative gene clusters using next generation sequencing, I aimed to characterize novel *car* gene clusters and investigate whether there is a common recruitment route with conserved mobile genetic elements (MGE) involved in the acquisition and diversification of known and novel types of *car* gene clusters.

Chapter 2. Diversity of *car* gene clusters, their carriage modalities and their associated downstream pathway genes.

In a previous study, draft genome sequencing of 32 carbazole-degrading strains isolated from soil, seawater, river and activated sludge served to identify the distribution of known and novel types of *car* gene

cluster configurations in different environments (Vejarano 2017, Master's thesis). From these 32 strains, nine were selected as representative carriers of the different types of *car* gene clusters: *Marinobacterium georgiense* IC961 (marine sediments), *Acinetobacter johnsonii* IC001 (river water), *Novosphingobium* sp. KA1, *Sphingomonas* sp. IC081 (both from activated sludge), *Nocardioides aromaticivorans* IC177 (soil), *Nocardioides* sp. S5 (estuarine sediments), *Hyphomonas* sp. KY3, *Erythrobacter* sp. KY5 and *Thalassococcus* sp. S3 (the three from surface seawater). In this work, the genomes of these strains were re-sequenced using Illumina's PCR-free and long insert mate-pair DNA libraries in the MiSeq sequencer, followed by manual in silico contig gap-closing. As a result, the complete genome sequence of eight strains and nearly complete genome sequence of *Sphingomonas* sp. IC081 were determined (Vejarano et al., 2018, 2019). Based on (1) the number of ETC components contained within the *car* gene cluster and the prosthetic groups present in these proteins, as well as (2) the type of genes involved in the degradation of anthranilate, a complete classification of five different types of *car* gene clusters found in nature has been proposed (Figure 1).

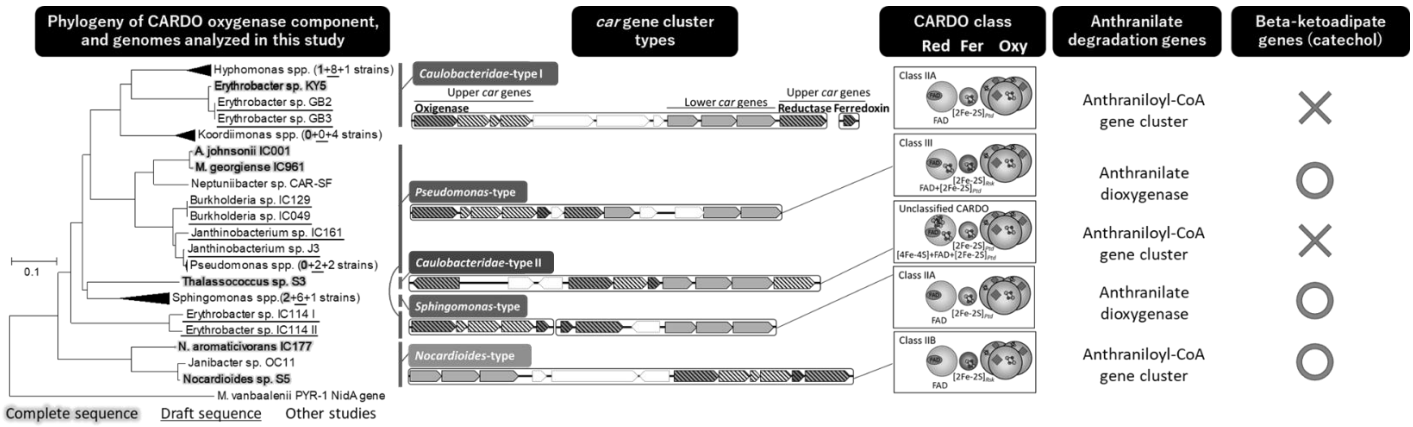

Figure 1. Overview of the five types of *car* gene clusters found in isolated bacteria. CARDO classification follows Batie *et al*. 1991. in *Chemistry and Biochemistry of Flavoenzymes* 3: 543-556.

Two new types of *car* gene cluster were found in bacteria from surface seawater bacteria: *Caulobacteridae*-type I and II *car* gene clusters. These represent the first case of ROs either lacking a ferredoxin gene (type I) or containing a reductase gene encoding a protein with additional 4Fe-4S cluster (type II). Moreover, *Caulobacteridae*-type *car* gene clusters are associated to a previously uncharacterized gene cluster in carbazole degraders for the degradation of anthranilate through a coenzyme A intermediate (anthraniloyl-CoA, *aca* gene cluster), which was also found in Gram-positive bacteria of the genus *Nocardioides*. Carbazole degradation kinetics experiments showed lower degradation rates in strains containing this *aca* gene cluster compared to those carrying typical anthranilate dioxygenase genes.

In addition, novel *car* gene-harboring plasmids were detected: from *A. johnsonii* IC001 plasmids pIC001A and pC001B, carrying lower and upper *car* genes, respectively, and predicted to be maintained via toxin-antitoxin systems, and pIC081A, a 236 kb megaplasmid predicted to be self-transmissible.

Chapter 3. Functional characterization of the *Caulobacteridae*-type *car* gene clusters.

As mentioned, the novel *Caulobacteridae*-type *car* gene clusters have previously unreported compositions of CARDO ETC within them. Heterologous expression in *E. coli*, followed by resting cell transformation and HPLC analysis of the remaining carbazole in culture media were done to investigate if *Caulobacteridae*-type I CARDOs do not require CarAc or if the gene is being co-expressed from a distant locus. With a similar approach, the putative 4Fe-4S cluster-containing *Caulobacteridae*-type II CARDO CarAd of strain *Thalassococcus* sp. S3 is being tested for its ability to catalyze carbazole degradation when co-expressed with *carAa* and *carAc*.

The results showed that the *Caulobacteridae*-type I CARDO of *Erythrobacter* sp. KY5 requires the CarAc component, whose gene is located at 465 kb upstream the *car* gene cluster. *In silico* analysis showed that a similar configuration is present in *Hyphomonas* sp. KY3, the other *Caulobacteridae*-type I *car* gene cluster-harboring strain. This is the first report of a bacterium employing a RO whose ferredoxin gene is located in a completely unrelated locus than that of the terminal oxygenase. It is then suggested that these genes haven't been acquired simultaneously by horizontal gene transfer, as were observed in previously studied carbazole degraders. Semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) of identified CARDO genes and key genes of the *aca* gene cluster were done to confirm their inducibility by carbazole when used as sole carbon and nitrogen source in the culture medium. Diverse expression patterns were found between strains KY5, KY3 and S3. Inducibility of all the tested CARDO and *aca* genes were observed in strain KY5, while constitutive expression of the putative *carAc* in strain KY3 was observed which might account for the higher rate of carbazole degradation shown by this strain. Surprisingly, the tested genes seemed to be repressed by carbazole in strain S3, which has the lowest carbazole degradation rate of all the representative strains.

Finally, the construction of a deletion mutant of strain *Hyphomonas* sp. KY3 is underway, to confirm the involvement of the *aca* gene cluster in the degradation of anthranilate.

Chapter 4. Evolutionary scenarios of carbazole degradation gene clusters inferred from comparative genomics analysis.

Previous studies have identified the *Pseudomonas*-type *car* gene cluster in the pCAR1 plasmid as "derived" or "evolved", where most degradative genes have been brought together or "streamlined" into one locus and the IS-mediated recruitment of an inducible promoter led to the enhancement of *car* genes expression. Given the diverse types of *car* gene clusters found in this work, different routes of gene recruitment are expected for each of them. Moreover, several strains carrying *car* gene clusters of the same type can be viewed as carrying intermediate stages of the evolutionary process. Analysis of MGE could offer information on how *car* gene clusters evolved and diversified among these strains. It is possible to identify putative MGEs such as insertion sequences (IS) and conjugative transposons involved in the recruitment of *car* gene cluster by using specialized databases. However, novel genetic elements might be overlooked if they have not been reported and stored in such databases. *In silico* intra-replicon repeats analysis and inter-

replicon comparative genomics analysis were done to locate signature repeat motifs of MGE that serve to identify novel IS (*ab initio* detection of MGE). Several routes for the recruitment of *car* genes are suggested from these analyses, and in each of them, clade-specific MGE seem to be involved. In its ancestral state, the *Pseudomonas*-type *car* gene cluster can be recruited from individual acquisition of *car* gene modules harbored in distinct plasmids. These *car* gene modules seem to be mobilized between plasmids and chromosomes via the conserved insertion sequence (IS) IS*Ppu12*. *Sphingomonas*-type *car* genes are restricted to plasmids predicted to be conjugative, on one conserved transposon-like region that carries all the catabolic genes. The novel IS*Nar1*, identified *ab initio* from repeats analysis, seems to be involved in the recruitment and rearrangement within the chromosome of *Nocardioides*-type *car* gene clusters, and current evidence shows that this IS is exclusive of Gram-positive bacteria. Finally, remains of a conjugative transposon or integrative and conjugative element (ICE) suggest the recruitment mechanism of *Caulobacteridae*-type I *car* gene clusters.

Chapter 5. Conclusions and future prospects

This work underlines the importance of isolation and complete genome determination of environmental strains that might exists in very low population densities and might be overlooked by the current metagenomic approaches. Indeed, *in silico* search of similar *Caulobacteridae*-type *car* gene clusters in metagenomic databases renders no closely related homologs. As the components of these novel CARDOs have been identified in this work, detailed enzyme kinetics studies can be performed to establish a correlation between enzyme activity and relative carbazole degradation rates by each strain. Regarding identification of MGEs, this work shows the feasibility to detect novel IS using repeats analysis. Functionality of these novel elements could be verified using MGE capture plasmids, which will provide additional support to bioinformatics methods.

References

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List of abbreviations

Amp	Ampicillin
ANI	Average nucleotide identity
ATC	Artemis Comparison Tool
CARDO	Carbazole 1,9-dioxygenase
CDS	Coding DNA sequence
CoA	Coenzyme A
COG	Cluster of orthologous groups
CNFMM	Carbon and nitrogen-free mineral medium
Dpd	Dibenzo-*p*-dioxin
DMSO	Dimethyl sulfoxide
DR	Direct repeat
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron transport component(s)
FAD	Flavin adenine dinucleotide
GI	Genomic island
Gm	Gentamicin
HGT	Horizontal gene transfer
HPDA	2-hydroxypenta-2,4-dienoic acid
IS	Insertion sequence
Km	Kanamycin
LB	Lysogeny broth
MGE	Mobile genetic element
ORF	Open reading frame
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
PHA	Polycyclic aromatic hydrocarbons
q-PCR	Quantitative PCR
RT-PCR	Reverse transcription PCR
RO	Rieske-type non-heme iron oxygenases
RPM	Revolutions per minute
TIR	Terminal inverted repeats
Tris	Tris(hydroxymethyl)aminomethane
WGS	Whole genome sequencing

Chapter I

Introduction

1.1. Bacterial degradation of carbazole

1.1.1. Carbazole, its dispersion sources and biological effects

Carbazole is a *N*-heterocyclic aromatic compound that can be found as the major *N*-heterocycle in coal-tar creosote and shale oil, both being complex mixtures of polycyclic aromatic hydrocarbons (PAH), phenolic compounds and *N*-, *S*- and *O*-heterocycles that are produced in the distillation process of tars or the pyrolysis of oil shale for fuel generation (Mueller et al., 1989; Mushrush et al., 1999). Although carbazole is of economic importance in the synthesis of several dyes, insecticides and several polymers (Collin et al., 2006), its dispersion in the environment poses an environmental and public health risk due to its mutagenic and carcinogenic effects. As a mutagen, carbazole exposure in mice results in induction of dominant lethal mutation, abnormal sperm heads, mitotic depression and chromosomal aberrations (Jha and Bharti, 2002; Jha et al., 2002), while as a carcinogenic agent, long term exposure to carbazole leads to an increase in the incidence of hepatocellular carcinomas, pulmonary metastasis and neoplastic lesions in the forestomach of mice (Tsuda et al., 1982).

Historically, carbazole dispersion in the environment has been caused by misuse, accidental spillage or improper disposal of coal-tar creosote and oil shale pyrolysis products by the wood-preserving and oil shale processing industries (Mueller et al., 1989; Mushrush et al., 1999) and among the strategies to ameliorate the effects of carbazole dispersion, the use of biodegradative bacteria represents an environmentally sound approach to successfully treat contaminated sites (Seo et al., 2009). Moreover, as illustrated in Figure 1.1 carbazole is structurally similar to polychlorinated biphenyls, dibenzothiophene, and dioxins, which are by themselves even more toxic and/or mutagenic (McConnell, 1985; Leighton, 1989; Ulbrich and Stahlmann, 2004; White and Birnbaum, 2009). This means that understanding the mechanisms of carbazole biodegradation opens the possibility of understanding the biodegradation of similar xenobiotics.

Apart from the environmental interest to decontaminate carbazole-contaminated sites, the petroleum refining industry has a particular interest in removing nitrogen containing heterocycles from crude oil, as the presence of such compounds contribute to lowering process yields caused by catalyst poisoning and to the emission of toxic nitrogen oxides, responsible for air pollution and acid rain (Benedik et al., 1998). During the catalytic cracking process (conversion of long-chain hydrocarbons to short-chain ones in the refinery), carbazole can be converted into basic derivatives that absorb to the active sites of the cracking catalyst. Moreover, it is a potent inhibitor of hydrodesulfurization, a process employed to remove sulfur contaminants, which form toxic gases when burnt and also cause catalytic poisoning themselves (Speight and El-Gendy, 2017). In this setting, bacterial degradation of

carbazole has been also evaluated as a plausible alternative to improve crude oil refinement (Kilbane II et al., 2002; Kilbane, 2006; Larentis et al., 2011).

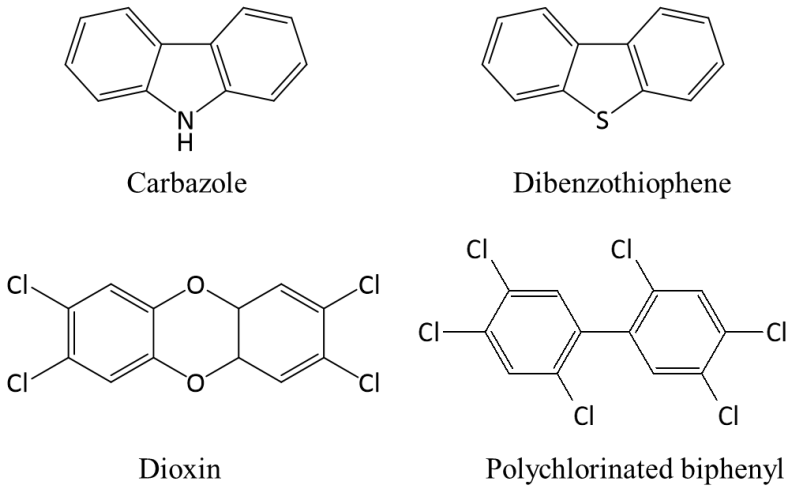

Figure 1.1. Structure of carbazole and the structurally similar xenobiotic compounds

1.1.2. **Carbazole degradation pathway and degradative genes**

The carbazole degradation pathway was first proposed based on the results of experiments performed in the model carbazole degrader *Pseudomonas resinovorans* strain CA10 (Sato et al., 1997b, 1997a; Nojiri et al., 2001, 2002). Figure 1.2 shows the steps in the degradation pathway with the designation of the enzymes in each step. In this pathway, carbazole is dioxygenated at angular (C9a) and adjacent (C1) positions by the carbazole 1,9a-dioxygenase (CARDO, CarAaAcAd) yielding 1-hydro-1,9a-dihydroxycarbazole (not shown), which is unstable and spontaneously cleaved to form 2'-aminobiphenyl-2,3-diol. This compound is then cleaved by the two-subunit *meta*-cleavage enzyme (CarBaBb) to form 2-hydroxy-6-oxo-6-(2'-aminophenyl)-hexa-2,4-dienoate which is subsequently hydrolyzed by a *meta*-cleavage compound hydrolase (CarC) into anthranilic acid and 2'-hydroxypenta-2,4-dienoic acid. Anthranilc acid is converted to catechol by a multicomponent anthranilate dioxygenase (AntDO,), another RO with a reductase (AntC) and a two-subunit terminal oxygenase (AntAB). Catechol is then cleaved in *ortho* position and processed by the three enzymes of the catechol branch of the conserved β-ketoadipate pathway: catechol 1,2-dioxygenase (CatA), *cis-cis*-muconate cycloisomerase (CatB) and muconolactone isomerase (CatC) (Harwood and Parales, 1996). The intermediate formed, β-ketoadipate enol-lactone, is further processed into tricarboxylic acid (TCA) cycle intermediates. On the other hand, 2'hydroxypenta-2,4-dienoic acid is converted to TCA cycle intermediates by three additional conversions (CarDEF) which constitute the *meta*-cleavage pathway (Nojiri et al., 2001)

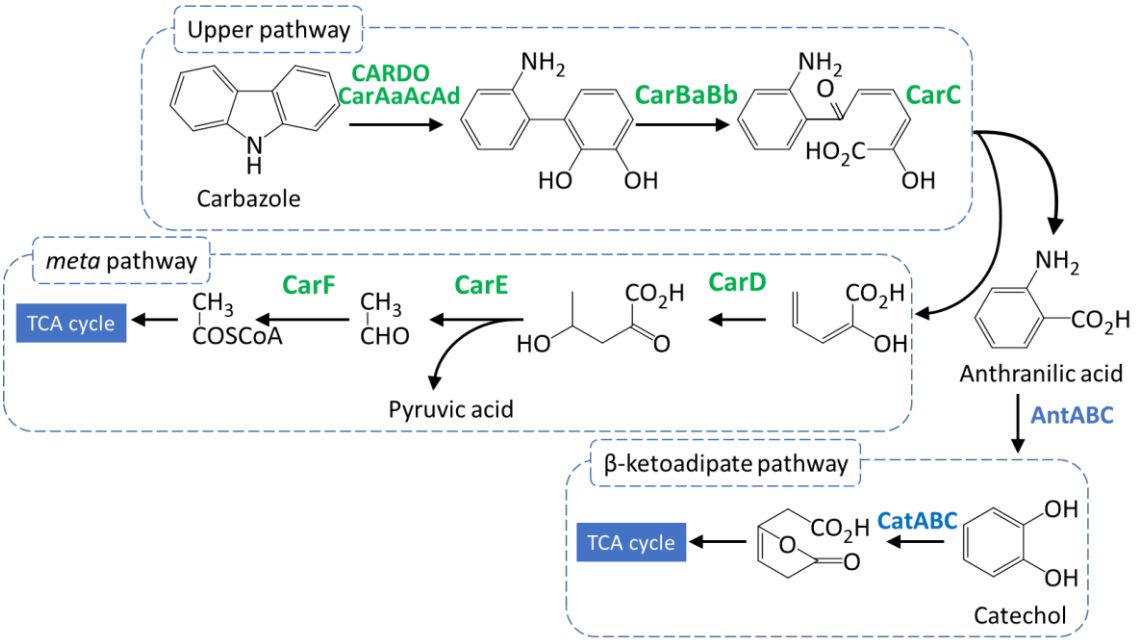

Figure 1.2. Bacterial carbazole degradation pathway in the model carbazole degrader *Pseudomonas resinovorans* CA10 (Nojiri et al., 2001).

The carbazole-degrading genes in *P. resinovorans* CA10 which were found to be organized in the carbazole-degrading *car* gene cluster, with the configuration *carAaAaBaBbCAcAdDFE* (Nojiri et al., 2001), although the additional upstream copy of the *carAa* gene can be spontaneously lost (Shintani et al., 2013) (Figure 1.3). Moreover, the *car* gene cluster is located on the degradative plasmid pCAR1, as well as the genes for anthranilate degradation (*antABC*), while those for catechol dioxygenation (*cat* genes) and the rest of the β-ketoadipate pathway genes are located on the chromosome. Based on these findings it has been proposed that in *P. resinovorans* CA10, the metabolic capacity for carbazole degradation has been horizontally acquired by recruitment of pCAR1 (Nojiri et al., 2001).

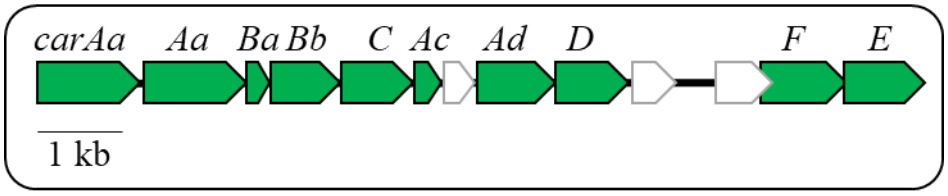

Figure 1.3. The c*ar* gene cluster in *P. resinovorans* CA10 on the degradative plasmid pCAR1. Degradative genes are represented as green arrows while open reading frames (ORF) with unrelated function are shown in white.

1.1.3. **Carbazole 1,9a-dioxygenase (CARDO)**

In the carbazole degradation pathway, the initial reaction is catalyzed by CARDO, a three-component enzymatic complex consisting of a terminal oxygenase (CarAa) and two electron transport components (ETC): reductase (CarAd) and ferredoxin (CarAc). The terminal oxygenase, in charge of adding the two atoms of molecular oxygen into the ring fused position (angular dioxygenation), exists in homo-multimeric form, being composed of three α subunits, each consisting of an N-terminal iron-sulfur cluster domain with a conserved Rieske $[2Fe\text{-}2S]_{2his2cys}$ center and a C-terminal catalytic domain with a conserved mononuclear iron-binding site. This mononuclear iron-binding site is the one responsible for oxygen binding and activation (Chakraborty et al., 2012; Nojiri, 2012). In CARDO, electrons are transferred from reduced nicotinamide adenine dinucleotide (NADH) to the terminal oxygenase via the ferredoxin reductase, which in turn transfers them to the ferredoxin. The terminal oxygenase is then reduced by the ferredoxin and then oxidized by molecular oxygen to trigger its activation and subsequent attack to carbazole (Figure 1.4) (Nojiri, 2012).

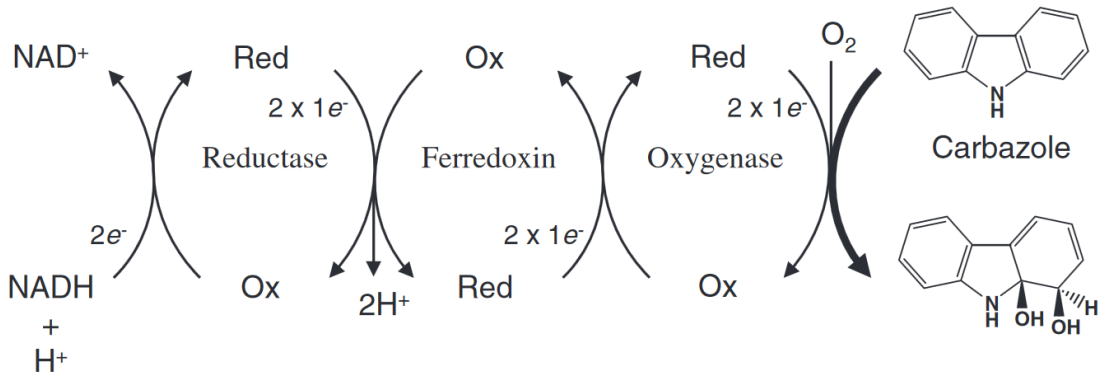

Figure 1.4. Electron transport among the three components of CARDO. The figure shows the reduced (Red) and oxidized (Ox) states of the components (Nojiri, 2012).

CARDO belongs to the family of Rieske-type non-heme iron oxygenases (RO), a group of widely distributed bacterial oxygenases responsible for the generation of *cis*-dihydroxylated metabolites, a common step in the bacterial degradation of many aromatic compounds (Ferraro et al., 2005; Chakraborty et al., 2012). As a signature feature of every RO, the Rieske $[2Fe\text{-}2S]_{2his2cys}$ center of the terminal oxygenase contains two conserved cysteines and two histidines to coordinate the iron ions (Ferraro et al., 2005). Due to their wide distribution and diversity, several RO classification schemes have been proposed based on differing criteria such as the number of the ETC and the nature of their redox centers (Batie et al., 1991), the phylogenetic relationships between different α subunits (Nam et al., 2001), the phylogeny of ETC coupled with that of the α subunit (Kweon et al., 2008) and a combination between the phylogenetic relationships of all the components and the substrate specificity of the terminal oxygenase (Chakraborty et al., 2012).

Although not able to reflect the phylogeny of CARDO components from different organisms due to the limited availability of sequences at the time, Batie's classification system can be used to easily estimate the diversity of CARDO harbored by bacteria. In Batie's classification system, ROs are classified into five groups (classes IA, IB, IIA, IIB and III) and CARDO from different strains exist within classes IIA, IIB and III. Table 1.1 summarizes the general characteristics of ROs according to Batie's system (Ferraro et al., 2005). Following this classification, ferredoxin reductases of Class II CARDOs contain a flavin adenine dinucleotide (FAD) binding site as conserved domain (GR-type reductase), while the ferredoxin can either have a $[2Fe\text{-}2S]_{4cys}$ iron-sulfur cluster (putidaredoxin type ferredoxin) (IIA) or a Rieske-type $[2Fe\text{-}2S]_{2his2cys}$ iron-sulfur cluster (Rieske-type ferredoxin) (IIB). Class III ROs on the other hand contain ferredoxin reductases with a FAD binding site and a $[2Fe\text{-}2S]_{4cys}$ iron-sulfur cluster as conserved domains (FNR-type reductase) with a Rieske-type ferredoxin (Chakraborty et al., 2012).

Table 1.1. Classification of Rieske non-heme iron oxygenases (RO) according to the number of ETC and the nature of their redox centers. The shaded cells correspond to groups in which CARDO from known carbazole degraders are classified.

System	Class	Domains and prosthetic groups		
		Reductase	Ferredoxin	Oxygenase
Two components	IA	FMN- $[2Fe\text{-}2S]_{4cys}$	×	$[2Fe\text{-}2S]_{2his2cys}$-$Fe^{2+}$
	IB	FAD- $[2Fe\text{-}2S]_{4cys}$	×	$[2Fe\text{-}2S]_{2his2cys}$-$Fe^{2+}$
Three components	IIA	FAD	$[2Fe\text{-}2S]_{4cys}$	$[2Fe\text{-}2S]_{2his2cys}$-$Fe^{2+}$
	IIB	FAD	$[2Fe\text{-}2S]_{2his2cys}$	$[2Fe\text{-}2S]_{2his2cys}$-$Fe^{2+}$
	III	FAD- $[2Fe\text{-}2S]_{4cys}$	$[2Fe\text{-}2S]_{2his2cys}$	$[2Fe\text{-}2S]_{2his2cys}$-$Fe^{2+}$

1.1.4. Carbazole-degrading bacteria

Several carbazole degrading strains have been isolated in the past 25 years and among them, *P. resinovorans* CA10 is probably the most extensively studied since its isolation from municipal waste water (Ouchiyama et al., 1993). CARDO in strain CA10 ($CARDO_{CA10}$) is classified as a class III RO, with a Rieske-type ferredoxin and a FNR-type reductase (Nojiri et al., 2001).

Novosphingobium sp. KA1 was also isolated from activated sludge, and it is able to grow on carbazole as sole carbon, nitrogen and energy source (Habe et al., 2002). Studies addressing the analysis of its xenobiotics degradation potential, degradative genes and conjugative transfer machinery have been done with this strain (Urata et al., 2006; Shintani et al., 2007), finishing with the determination of the complete sequence of the carbazole degrading plasmid pCAR3, which carries all degradative genes of carbazole and its intermediates, among other genes for the degradation of dibenzofuran/fluorene, protocatechuate and phthalate (Shintani et al., 2007). In pCAR3, *car* genes form two separated *car* gene clusters named *carI* and *carII*, organized as *carRIAaIBaIBbICIAcI* and *carAaIIBaIIBbIICIIAcIIRII* (where CarR is a transcriptional regulator). $CARDO_{KA1}$ reductases are coded in a different locus within pCAR3 (*fdrI* and *fdrII*), close to one more homolog of a functional ferredoxin gene (*fdxI*). Moreover, the genes for the degradation of anthranilate (*and* genes) and catechol (*cat* and other β-ketoadipate pathway genes) are also carried on the plasmid. These *and* genes code for a three-subunit anthranilate dioxygenase, with an additional ferredoxin component, similar to the configuration found in CARDO. $CARDO_{KA1}$ belongs to the class IIA RO, with a putidaredoxin-type ferredoxin and a GR-type ferredoxin reductase.

Inoue et al. reported the isolation of the first Gram-positive carbazole degrader from soil: *Nocardioides aromaticivorans* IC177 (Inoue et al., 2005). In this study experiments revealed the complete *car* gene cluster in this strain, which was organized as *carRcarAaCBaBbAcAd* while *meta*-pathway genes *carDFE* are closely linked and located upstream the main *car* gene cluster. The *car* genes in this strain (car_{IC177}) are more streamlined or "optimized" than those from the Gram-negative CA10 and KA1 strains, as observed by the overlapping of stop and start codons of neighboring genes. $CARDO_{IC177}$ strain belongs to class IIB: it has a Rieske-type ferredoxin and a GR-type reductase. By the time, it was assumed that in strain IC177 anthranilate degradation also occurred by dioxygenation by homologs of previously reported anthranilate dioxygenase, although such genes were not detected. It was also reported that compared to Gram-negative degraders, CARDO of strain IC177 has a stronger preference to carbazole than to other substrates such as biphenyl and dibenzofuran (Inoue et al., 2006).

Other carbazole degraders described to date include *Sphingomonas yanoikuyae* strain XLDN2-5, which is not only capable of degrading carbazole but also can degrade dibenzofuran and dibenzothiophene when carbazole is present as inducer. This strains has a similar *car* gene cluster structure to that of strain KA1, as well as similar anthranilate degradation genes, although genes for the *meta*-cleavage of catechol were also found. (Gai et al., 2007, 2010, 2011). *Sphingomonas* sp.

GTIN11 is another sphingomonad strain isolated from manufactured gas plant soil that was used for the removal of carbazole in petroleum. Interestingly, unlike strain CA10, car_{GTIN11} are expressed constitutively (Kilbane II et al., 2002). Degradation of not only carbazole but of also more toxic carbazole derivatives was conducted using the *Pseudomonas* sp. strain XLDN4-9 (Li et al., 2006). They found that C(1) and C(2) methyl-substituted carbazole derivatives were degraded in the presence of carbazole as a co-substrate (Li et al., 2006). *Pseudomonas stutzeri* strain OM1 is another carbazole degrader with an identical *car* gene cluster structure to that of strain CA10, with the major distinction that it has it on its chromosome (Ouchiyama et al., 1998; Shintani et al., 2003). Other carbazole degraders whose degradation kinetics have been reported are *Sphingomonas* sp. CDH-7, which degraded, in model synthetic petroleum, carbazole to ammonia via anthranilate as an intermediate product (Kirimura et al., 1999) and *Gordonia* sp. strain F.5.25.8, which was originally isolated as a dibenzothiophene degrader that is capable to utilize carbazole as the only source of nitrogen (Santos et al., 2006).

Although most of these studies focused on bacteria isolated from onshore sites, some of them aimed to discover novel components in the *car* gene cluster from marine isolates. For instance, *Lysobacter* sp. OC7 is capable of growing on carbazole, phenanthrene and naphthalene as sole carbon sources. In this strain ETC genes similar to those of previously studied degraders were not found in the *car* gene cluster of this strain (Maeda et al., 2009b). In a follow-up study, *Kordiimonas* sp. OC9 was isolated and it was found that its CarAc had a chloroplast-type [2Fe-2S] center, which is similar to the $[2Fe\text{-}2S]_{4cys}$ but with a characteristic difference in the number of amino acid residues separating the iron-coordinating cysteine residue pairs. This constitutes the first report of a chloroplast-type ferredoxin component in a CARDO system (Maeda et al., 2010a; Ito et al., 2011). Upon cloning the degradative genes into constructed expression plasmids, it was found that $CARDO_{OC9}$ was active towards naphthalene, phenanthrene, biphenyl, fluorene, dibenzofuran and dibenzo-p-dioxin (Maeda et al., 2010a). *Janibacter* sp. OC11 was later reported as the first marine Gram-positive carbazole degrader with a CARDO belonging to the class IIB like the one in strain IC177 (Oba et al., 2014). Considering their diversity, known *car* gene clusters identified using heterologous expression and southern hybridization experiments were grouped into three types (Figure 1.5). This offered a convenient way to visualize the diversity of *car* gene clusters discovered by the time (Maeda et al., 2010b).

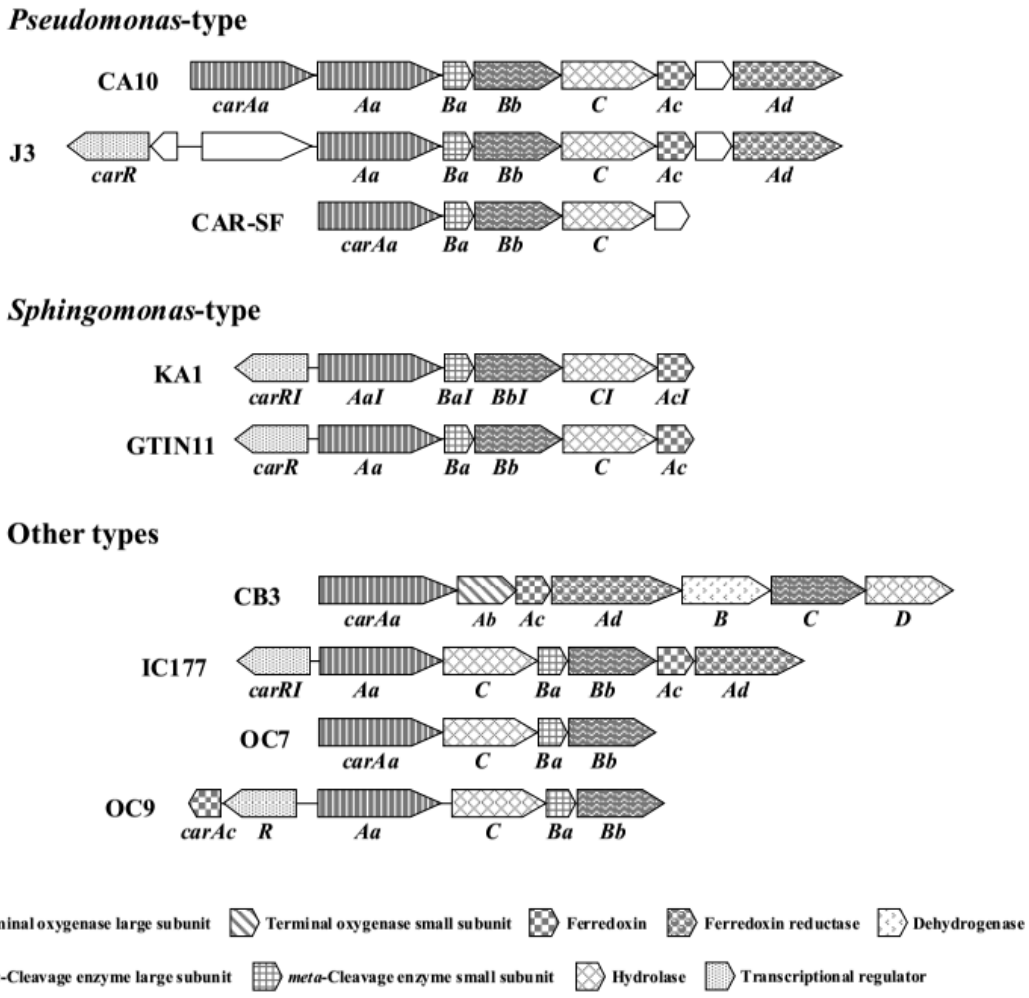

Figure 1.5. Types of *car* gene clusters as found in different strains from onshore and marine environments (Maeda et al., 2010b). The lower part shows the meaning of the fill patterns from each ORF indicated by the pentagons.

In summary, most carbazole degraders studied to date belong to the pseudomonads and sphingomonads, and although there is variation in terms of the genes that code for the individual components of CARDO's ETC and their relative location within the *car* gene clusters, the following characteristics are conserved among carbazole degraders: (i) carbazole is initially degraded by the unique three-component RO CARDO, which is coded by genes located in the *car* gene cluster and (ii) carbazole degradation has as intermediate anthranilate, which is always dioxygenated by the action of conserved Rieske-type anthranilate dioxygenases to produce catechol. Exploration of marine environments suggests that ETC could vary when compared to model carbazole degraders, and this also opens the possibility of alternative pathways for the degradation of intermediates.

1.2. Evolution of xenobiotics-degrading gene clusters

1.2.1. Mobilization and evolution of xenobiotics catabolic genes in the sphingomonads

Recent studies addressing the evolution of xenobiotics degradation genes have underlined the effect of transposable elements and other mobile genetic elements (MGE) in the occurrence of genome rearrangements. Such rearrangements facilitate gene exchange between chromosome and plasmids and ultimately derive in the streamlining and optimization of degradative gene clusters. Insertion sequences (IS) are one kind of transposable elements characterized by being short (between 0.7 and 2.5 kb in length) DNA segments encoding only the enzymes necessary for their transposition and capable of repeated insertion into many different sites within a genome using mechanisms independent of large regions of DNA homology between the IS and target (Berg & Howe, 1989, Craig et al., 2002). Encoding one or two ORF in charge of their transposition, and usually flanked by inverted repeats, they can sometimes carry passenger genes (i.e. xenobiotics degradation or antibiotic resistance genes) as they move across the genomes (Siguier et al., 2014).

Tabata et al, showed in four gamma-hexachlorocyclohexane (γ-HCH)-degrading sphingomonads (*Sphingobium japonicum* UT26, *Sphingomonas* sp. MM-1, *Sphingobium* sp. MI1205 and *Sphingobium* sp. TKS) the hypothetical past genome rearrangement events of replicons that shaped the current configuration of γ-HCH-degrading *lin* genes within chromosomes or plasmids. Sequence analysis of IS and their flanking repeat sequences served to identify IS*6100* as a key factor facilitating dynamic genome rearrangements such as fusion and resolution of replicons carrying *lin* genes and causing the diversification of their flanking regions (Tabata et al., 2016). Their analytical approach was to identify identical pairs of 8-bp repeat sequences flanking IS*6100* copies to infer the most plausible past events of genome rearrangement (replicon fusion, resolution and inversion) caused by IS*6100* transposition (Figure 1.6). As one of their conclusions, IS*6100*, which can be found nearby degradative genes, has served a role of optimizing degradation-related genome loci by "trimming unnecessary regions" and "gathering" specific *lin* genes together (Tabata et al., 2016; Nagata et al., 2019) (Figure 1.7). To provide experimental support for the active transposition of IS*6100* and other

transposable elements identified in their analyses they used an IS entrapment methodology with the pGEN500 plasmid, which retains IS elements that transpose into its *sacB* gene to give rise to sucrose-resistant mutants (Ohtsubo et al., 2005), confirming the transposition of IS*6100* and other IS and transposons in three of the four studied strains (Tabata et al., 2016).

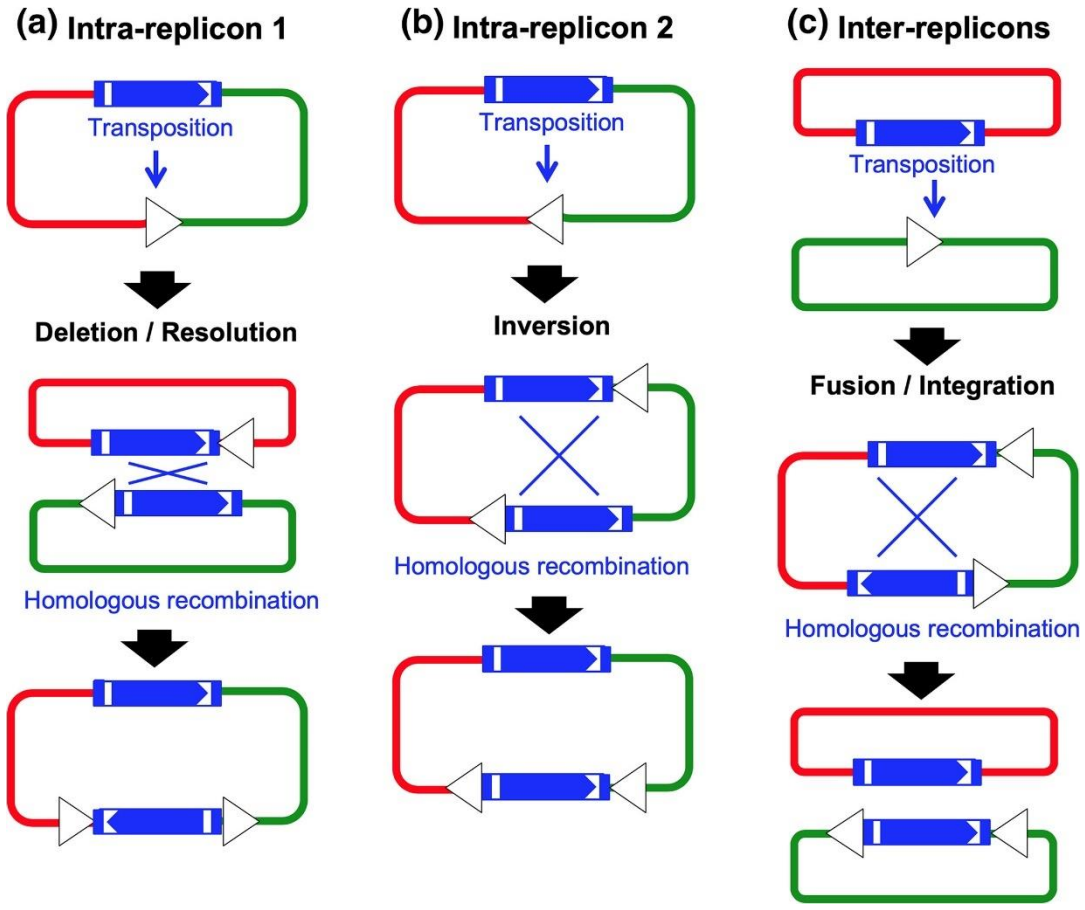

Figure 1.6. Patterns for transposition of IS*6100* in γ-HCH-degrading sphingomonads. Transposition into the same replicon with deletion/resolution (a, intra-replicon 1) or inversion (b, intra-replicon 2) and transposition between two replicons with fusion/integration (c, inter-replicons). Blue pentagons and flanking blue squares represent IS*6100* and the 14-bp inverted repeats, respectively. All these transpositions cause duplication of IS*6100* and generate a 8-bp target duplication (triangle) at the upstream and downstream of IS6100. It should be noted that in most cases the last step of homologous recombination has not occurred in the analyzed strains, so inference of the type of rearrangement is done by tracking the relative location of 8-bp direct repeats around IS copies, right after the transposition (Nagata et al., 2019).

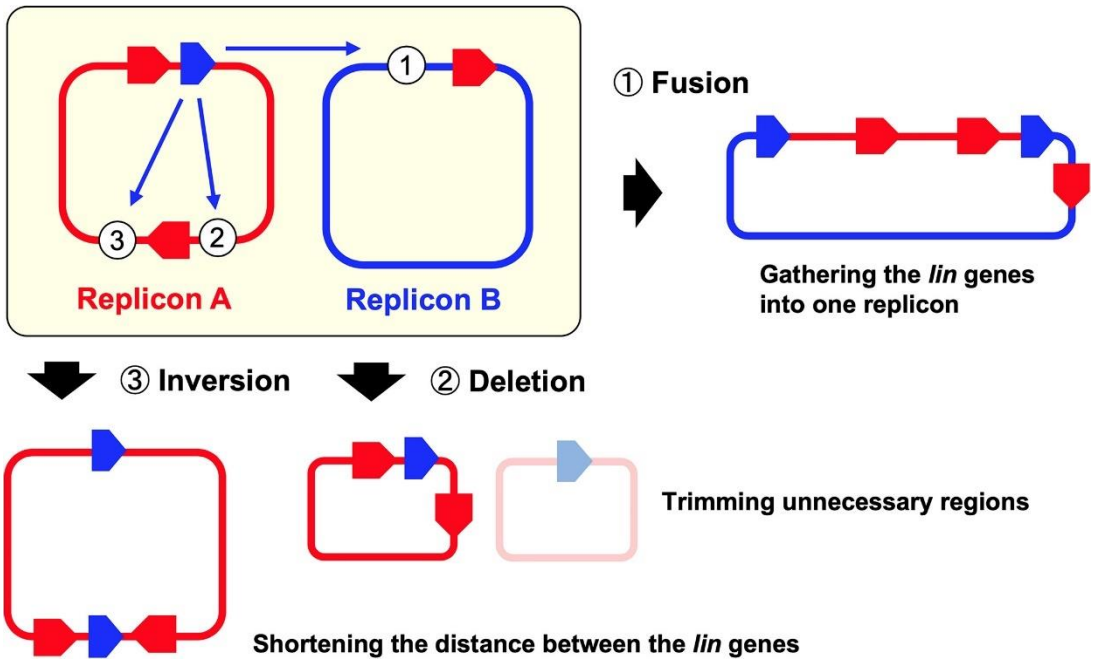

Figure 1.7. Schematic representation of recruitment of *lin* genes and editing role of IS*6100* in γ-HCH-degrading sphingomonads. Blue and red pentagons indicate IS*6100* copies and and *lin* genes, respectively. Transposition of *IS6100* from replicon A (red) to replicon B (blue) produced a fused replicon in which *lin* genes have been gathered together (①); from replicon A to another locus of the same replicon, leading to deletion and trimming of unnecessary regions (②), and again from replicon A to another locus of the same replicon leading to inversion and shortening of the distance between *lin* genes (③) (Nagata et al., 2019).

More recently, the effects of IS-induced genomic rearrangements on the production of more efficient configurations of chlorophenoxy herbicides-degradative gene clusters of several sphingomonad strains were also analyzed. The complete genome sequence of *Sphingobium herbicidovorans* MH and the draft genome sequences of *Sphingomonas* sp. TFD44 were obtained and their degradative genes were compared against homologs and paralogs present in *Delftia acidovorans* MC1, *Sphingomonas* sp. ERG5 and the exogenously isolated plasmids pAKD34 and pAKD16 (Nielsen et al., 2017). Such comparison was done to track the evolution of these gene clusters. To complement this data, a mobilome sequencing approach, which refers to the sequencing of circular DNA unaffected by exonuclease digestion, was used to detect the transiently existing small circular DNA molecules of strain MH. By making use of this sequencing data, it was possible to see how the movement of endogenous MGE affects the structure of degradative gene clusters (Nielsen et al., 2017).

It is worth noting that these analyses have been done on closely related sphingomonad strains given their well-known capabilities for harboring catabolic genes of a wide variety of xenobiotics compounds and the abundant availability of sequencing data for these strains. Analysis on the mobility

of degradative genes in other strains with less widespread degradation potential might offer a more complete view of diversification and evolution of xenobiotics catabolic genes in nature.

1.2.2. **Mobilization and evolution of carbazole-degrading gene clusters**

With respect to carbazole degrading bacteria, it has been demonstrated that the pCAR1 plasmid can work as a vehicle of *car* genes in pseudomonads, and that its Tn*4676* class II transposon can act as a shuttle, mobilizing degradative genes into the chromosomes of new hosts such as *Pseudomonas stutzeri* OM1 (Shintani et al., 2003) and *P. putida* strain HS01 (Shintani et al., 2005).

In CA10 strain, sequence analysis of the *car* genes and their surrounding regions, revealed the presence of four copies of IS elements from the IS5 family, flanking *car* and *ant* genes in the pCAR1 plasmid (Nojiri et al., 2001; Maeda et al., 2003). By analyzing these sequences, a model for the developmental process of the *car* gene cluster in CA10 was proposed (Nojiri et al., 2001). In this model, the IS elements flanking *ant* genes constitute a composite transposon responsible for the mobilization of *ant* genes in the plasmid. Moreover, the transposition of one of these IS caused the replacement of the constitutive *car* operon promoter (P_{carAa}) by that of the inducible *ant* promoter (P_{ant}) (Miyakoshi et al., 2006). In a follow-up study, based on the information compiled regarding IS elements in several Gram-negative carbazole degraders, and the previous model proposed by Nojiri et al., Inoue et al. proposed a model of the differentiation of the *Pseudomonas*-type *car* gene clusters (Inoue et al., 2004) (Figure 1.8). Basically, the model proposes the existence of a prototype *car* gene cluster that by events of duplication, one-ended transposition, and fusion, formed the *car* gene clusters of at least three strains. In this model, IS elements from the families IS*3*, IS*5*, IS*30* and IS*21* were found flanking several *car* gene clusters.

Interestingly, several copies of IS*6100* elements were present in the carbazole degrader *Sphingomonas* sp. XLDN2-5 flanking *car* and *ant* genes (Gai et al., 2010, 2011). As pointed out before, IS*6100* is also responsible for the mobility γ-HCH-degrading genes (Nagata et al., 2011; Tabata et al., 2016). IS*6100* seems responsible for the transfer of *car* genes in XLDN2-5 (Gai et al., 2010), which suggests that *car* gene clusters "take advantage" of the mobilization possibility offered by "endogenous" transposable elements abundant or at least functional in determined bacterial taxa.

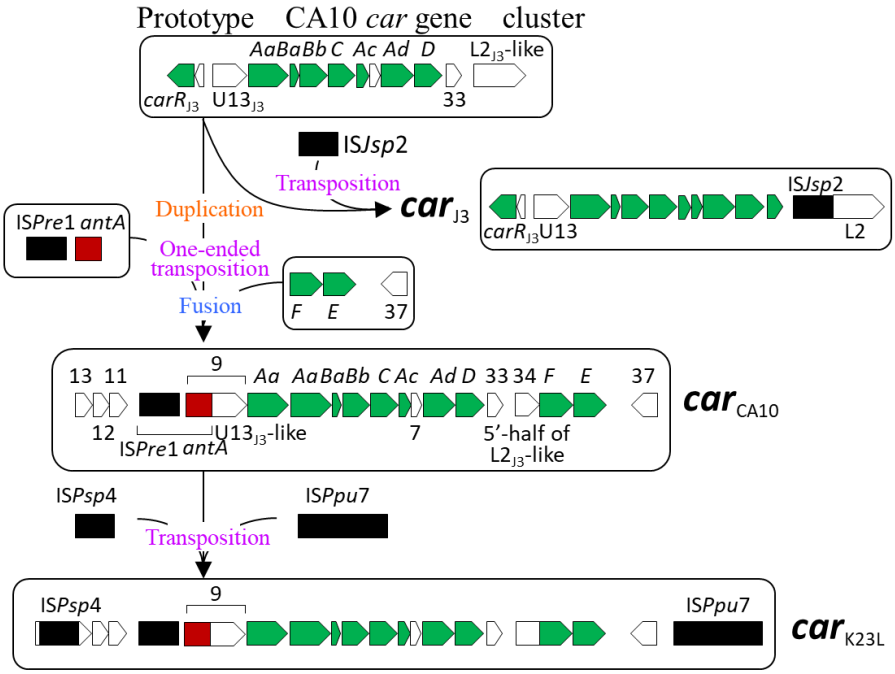

Figure 1.8. Proposed model for the diversification of the *car* gene cluster in *Pseudomonas resinovorans* CA10, *Pseudomonas* sp. K23 and *Janthinobacterium* sp. J3. Strains K23 and J3 were isolated to explore the diversity of *car* gene clusters in Gram-negative bacteria. Strain K23 contains two *car* gene clusters, one long (*car*$_{K23L}$, shown here) with two tandemly repeated copies of *carAa* and one short (*car*$_{K23S}$), with one copy. Green pentagons represent putative transcriptional regulator genes for the *car* operon and other *car* genes. Insertion sequences are indicated as IS followed by the initials of the strains. Black boxes represent the ISs observed in the flanking regions of *car* gene clusters. Red boxes represent the 5'-part of the *antA* gene transposed with IS*Pre*1. Image modified from (Inoue et al., 2004).

The aforementioned models for *car* gene cluster differentiation and the identification of transposable elements likely responsible for recruitment of *car* genes, were obtained by using molecular cloning techniques to determine the sequence and organization of degradative genes from several carbazole degraders. With the current availability of massive parallel sequencing technologies, it should be possible to devise equivalent models of *car* gene clusters differentiation from a wider selection of phylogenetically unrelated carbazole degraders isolated from diverse environments.

1.3. Objective of this study

As carbazole degradative genes have been found in phylogenetically unrelated bacterial strains from diverse environments, sequence analysis of their carbazole-degrading genes offers a unique opportunity to understand the extent to which these genes have diversify in nature. The studies

mentioned in sphingomonads have provided an interesting approach to reconstruct to some extent the evolutionary trajectory of degradative genes. A similar reconstruction of the recruitment history of *car* genes might provide a wider outlook on the evolution of degradative genes in nature. As diversification can be interpreted as one of the outputs of ongoing evolution, the objectives of my thesis are (i) to study the diversity of *car* gene clusters and genes for the mineralization of degradation intermediates by obtaining the complete genome sequences of bacterial isolates from diverse environments across Japan, (ii) to functionally characterize novel genes involved in the mineralization of carbazole and (iii) to analyze the newly sequenced genomes for mobile genetic elements (MGE) associated to the *car* gene clusters, which could provide information about their recruitment, evolution and diversification.

Chapter II

Diversity of *car* gene clusters, their carriage modalities and their associated downstream pathway genes

2.1. Introduction

This chapter describes the complete sequence determination of nine representative carbazole-degrading strains based on the types of *car* gene clusters and the environments from where the strains were isolated. Draft genome sequences for these strains have been determined previously, but in many cases downstream analysis was not possible due to the fragmentation of the draft assemblies. Therefore, the objective was to obtain complete genome sequences to locate catabolic gene clusters, which might be fragmented in previous draft genome sequences. Also, this was done with the purpose of determining the type of replicon carrying such catabolic genes, as discovery of novel catabolic plasmids could provide information on the distribution of such genes, or if they are carried along antibiotic resistance genes, which is a critical aspect to be considered in when bacteria with bioremediation potential is released to the environment. Complete genome sequences also permitted the determination of the catabolic potential of the analyzed strains. This chapter also presents the experimental confirmation the carbazole degradation ability for these representative strains.

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Chapter III

Functional characterization of the novel *Caulibacteridae*-type *car* gene clusters

3.1. Introduction

In this chapter a functional characterization of the newly described *Caulobacteridae car* gene clusters types I and II are presented. Unlike previously described carbazole degraders, strains *Hyphomonas* sp. KY3 and *Erythrobater* sp. KY5 (*Caulobacteridae*-type I *car* gene cluster) don't carry a CARDO ferredoxin gene *carAc* inside or the vicinity of the *car* gene cluster. This might mean either that both use a novel two-component CARDO that only requires CarAa and CarAd or that unlike any other strains harboring a functional RO, the gene for the ferredoxin component is located in a distant locus from that of the terminal oxygenase. On the other hand, *Thalassococcus* sp. S3 (*Caulobacteridae*-type II *car* gene cluster), harbors a putative CARDO ferredoxin reductase gene *carAd* in the vicinity of the *car* gene cluster, but unlike any other RO reductases, this has an additional iron sulfur dicluster of 4Fe-4S configuration. Identification of the CARDO components is presented by means of heterologous expression in *E. coli* in a resting cell biotransformation assay. Expression analysis of key carbazole and anthranilate degradation genes for these three marine strains when carbazole is supplied as the only carbon and nitrogen source is also presented using semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR).

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Chapter IV

Evolutionary scenarios of carbazole degradative gene clusters inferred from comparative genomics analysis

4.1. Introduction

Complete genome sequences facilitated the study of insertion sequences and related repeat elements, which are difficult to analyze in draft genome sequences as they appear isolated within short contigs. Analysis of IS and their related repeats elements can only be properly interpreted with genomic context data which is only provided in complete genome sequences. Bioinformatic analysis of genome-wide repeat elements was done to for the *ab initio* detection of novel MGE that have been replicated within the genomes. Interpretation of the location of such newly detected MGE in relation to the *car* gene clusters from the representative strains is done to provide hypothesis for the recruitment and evolution of *car* genes in diverse bacteria.

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Chapter V

Conclusions and future prospects

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