Award Review

Structural and Molecular Genetic Analyses of the Bacterial Carbazole Degradation System

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Carbazole degradation by several bacterial strains, including Pseudomonas resinovorans CA10, has been investigated over the last two decades. As the initial reaction in degradation pathways, carboxazone is commonly oxygenated at angular (C9a) and adjacent (C1) carbons as two hydroxyl groups in a cis configuration. This type of dioxygenation is termed “angular dioxygenation,” and is catalyzed by carboxazone 1,9α-dioxygenase (CARDO), consisting of terminal oxygenase, ferredoxin, and ferredoxin reductase components. The crystal structures of all components and the electron transfer complex between the terminal oxygenase and ferredoxin indicate substrate recognition mechanisms suitable for angular dioxygenation and specific electron transfer among the three components. In contrast, the carboxazone degradative car operon of CA10 is located on IncP-7 conjugative plasmid pCAR1. Together with conventional molecular genetic and biochemical investigations, recent genome sequencing and RNA mapping studies have clarified that transcriptional cross-regulation via nucleoid-associated proteins is established between pCAR1 and the host chromosome.

Key words: carboxazone; genome; plasmid; Rieske oxygenase; crystal structure

In the last two decades, many investigators have attempted the isolation of xenobiotic-degrading bacteria. Most such studies have involved environmental aims, such as the isolation and characterization of bacterial biodegradation systems for problematic contaminants followed by their application to the bioremediation of real-world contaminated sites. Efforts in this area have contributed to progress in molecular genomics, enzymology, ecology, and evolution related to the novel metabolic capacities of environmental bacteria. This field can be clearly distinguished from traditional basic microbiology, in which cultivable model microorganisms such as Escherichia coli and Bacillus subtilis are used as research targets. The reasons are as follows: (i) Screenin of xenobiotic degraders has led to the discovery of various types of bacteria of diverse taxonomic ranks; (ii) The targets of these studies include the xenobiotic degradative functions of these bacteria in natural ecosystems consisting of various living organisms (microorganisms, animals, and plants) and abiotic materials under various environmental conditions. Recent developments in the tools and techniques used in the fields of molecular ecology and environmental microbiology have greatly contributed to the discovery of xenobiotic-degrading bacteria.

In 1996, my colleagues and I initiated cloning, sequencing, and functional investigation of the genes involved in carboxazone degradation by Pseudomonas resinovorans CA10 (formerly Pseudomonas sp. CA10). Carbazole is the major nitrogen heteroaromatic in coal-tar creosote and is one of the 13 most common heterocyclic compounds in creosote\(^1\) (the chemical structure of carboxazone is shown in Fig. 1). It is also used in the manufacture of various products, including dyes, reagents, explosives, insecticides, and lubricants, and is used as a color inhibitor in detergents. However, carboxazone itself is also known to be an environmental pollutant, and environmental concerns exist as to its release, because it is both mutagenic and toxic.\(^2,3\) Since the time we succeeded in cloning the carboxazone-degradative car gene cluster from CA10, we have investigated the carboxazone degradation systems of several bacterial strains by means of molecular genetics and structural and genome biology. Here, I want to summarize part of the archives of my research team: the molecular basis of the substrate specificity of and electron transfer in angular dioxygenase, the basic features of a carboxazone-degradative plasmid as a mobile genetic element, and the regulation of host phenotype by plasmid-chromosome interaction.

I. Angular Dioxygenation: A Novel Reaction Involved in Carbazole Metabolism

P. resinovorans CA10 is capable of growth on carboxazone as sole source of carbon, nitrogen, and energy, was isolated from activated sludge of a municipal waste...
water treatment facility in Tokyo in 1993, and is to the best of my knowledge the second-oldest carbazole-degrading strain reported. Anthranilate and catechol accumulated in the culture medium of *P. resinovorans* CA10 as catabolic intermediates of carbazole, and CA10 can grow on anthranilate as sole source of carbon and nitrogen. When anthranilate is supplied, CA10 accumulates catechol, suggesting that carbazole is converted to catechol via anthranilate. In addition, in a culture medium of CA10 grown on carbazole, the production of 2'-aminobiphenyl-2,3-diol and its meta-cleavage product, 2-hydroxy-6-oxo-6-(2'-aminophenyl)hexa-2,4-dienoate (HOADA), was probable. Based on an analogy of the metabolic intermediates of carbazole with those in the dibenzofuran and fluorene degradation pathways, a carbazole degradation pathway was proposed (Fig. 1).

![Fig. 1. Carbazole and Biphenyl Degradation Pathways and Degradative Enzymes in *Pseudomonas resinovorans* CA10 and *Pseudomonas pseudoalcaligenes* KF707](image)

The compound shown in brackets is unstable and has not been detected directly. Solid and broken arrows indicate enzymatic and spontaneous reactions, respectively.

Distinct from angular dioxygenation, this type of dioxygenation is called lateral dioxygenation. Once angular dioxygenation and subsequent spontaneous ring cleavage of carbazole occurs, the 2'-aminobiphenyl-2,3-diol formed is degraded by a pathway analogous to that involved in biphenyl degradation. Together with the dioxygenation of dibenzofuran and 9-fluorenone, the initial dioxygenation of carbazole is a distinct reaction, because hydroxylation occurs at the ring-fused position (Figs. 1 and 2A). This novel type of dioxygenation was termed angular dioxygenation, an original coinage of Engesser et al., during dibenzofuran degradation studies. For various aromatic compounds, including toluene, naphthalene, and biphenyl/polychlorinated biphenyls, initial dioxygenation occurs at the lateral position of the molecules, and both oxygen atoms of molecular dioxygen are incorporated into tandemly linked carbon atoms on an aromatic ring as two hydroxyl groups in a cis-configuration (Fig. 2B). Distinct from angular dioxygenation, this type of dioxygenation is called lateral dioxygenation. Once angular dioxygenation and subsequent spontaneous ring cleavage of carbazole occurs, the 2'-aminobiphenyl-2,3-diol formed is degraded by a pathway analogous to that involved in biphenyl degradation (Fig. 1).
II. Basic Features of the Angular Dioxygenase Involved in Carbazole Degradation

In the carbazole degradation pathway, an initial angular dioxygenation is catalyzed by a Rieske non-heme iron oxygenase (RO) named carbazole 1,9a-dioxygenase (CARDO)\textsuperscript{16–18}. In 1997, Sato et al.\textsuperscript{9,10} succeeded in isolating the carbazole-degradative gene cluster (carAaAaBaBbCAcAd) from the P. resinovorans CA10 genome by shotgun cloning using meta-cleavage activity. That was the first report of genes encoding an angular dioxygenase (carAa, carAc, and carAd genes).

CARDO is a three-component dioxygenase system consisting of a terminal oxygenase and electron transport proteins (Fig. 3).\textsuperscript{9,24} The terminal oxygenase component of CARDO, hereafter simply CARDO-O, is a homotrimeric enzyme that contains one Rieske-type [2Fe–2S]\textsuperscript{R} cluster and one active site ferrous iron (Fe\textsuperscript{2+}) in a single subunit (CarAa). Similarly to a number of mononuclear non-heme iron(II) enzymes,\textsuperscript{25–27} RO contains a 2-His-1-carboxylate facial triad motif\textsuperscript{28} for binding of the active site iron. The electron-transport proteins of CARDO from CA10 (CARDO\textsubscript{CA10}), which mediate electron transport from NADH to CARDO-O, comprise ferredoxin (CARDO-F, a monomer of CarAc), and ferredoxin reductase (CARDO-R, a monomer of CarAd), which contains one FAD and one plant-type [2Fe–2S]\textsuperscript{P} cluster. ROs have been classified into five groups (classes IA, IB, IIA, IIB, and III) based on the number of constituent components and the nature of the redox center.\textsuperscript{29} The characteristics of the electron-transport chain suggest that CARDO\textsubscript{CA10} is to be categorized as class III in Batie’s classification.\textsuperscript{24} However, phylogenetic analysis indicates that the amino acid sequence of CarAa\textsubscript{CA10} shares rather low homology (<19% overall length-wise identity) with almost all known catalytic subunits of RO terminal oxygenases. In addition, whereas typical class III ROs contain a terminal oxygenase component consisting of both \( \alpha \) and \( \beta \) subunits in an \( \alpha_3\beta_3 \) configuration, CARDO-O consists only of a catalytic \( \alpha \) subunit in an \( \alpha_3 \) configuration. This homomultimeric structure is typical of class IA ROs, whose terminal oxygenases are believed to have \( \alpha_3 \) configurations.\textsuperscript{16}

With very few exceptions, ROs catalyze lateral dioxygenation to various aromatic compounds. For
example, the lateral dioxygenation of naphthalene and biphenoxy is shown in Fig. 2B. As for other types of oxygenation, the naphthalene 1,2-dioxygenase (NDO) of *Pseudomonas* sp. strain NCIB9816-4 was reported to catalyze lateral dioxygenation, monooxygenation (including sulfoxidation), desaturation, and dealkylation. Studies using *E. coli* expressing the *carAa*, *carAc*, and *carAd* genes from CA10 have indicated that CARDOCA10 catalyzes diverse oxygenation reactions with a broad substrate range, including angular dioxygenation, lateral dioxygenation, and monooxygenation (Fig. 2). Although numerous ROs have been isolated and characterized, only a limited number of these can catalyze angular dioxygenation.

### III. Structural Basis of the Novel Substrate Specificity of CARDO

To clarify the molecular mechanisms governing the ability to catalyze angular dioxygenation, the crystal structure of CARDO-O from *Janthinobacterium* sp. J3 (CARDO-OJ3) was solved at a resolution of 1.95 Å. *CarAaJ3*, a subunit of CARDO-OJ3, contains 384 amino acid residues and is similar to *CarAaCA10*, with only three amino acid residue mismatches. No functional differences have been observed between CARDO-OJ3 and CARDO-OCA10 in terms of substrate specificities or inter-component electron transfer. The overall shape of CARDO-OJ3 resembles that of a 100 Å-wide doughnut, with a 30-Å hole, 45-Å in thickness (Fig. 4A). The monomeric structure of the asymmetric unit facilitates trimeric interactions with neighboring asymmetric units along the crystallographic 3-fold axis. The reported structure of the α-subunits of the terminal oxygenase of NDO (NDO-O) is shown in Fig. 4B. Although some differences are apparent, such as the size of the subunit and centrally located hole, the subunit interactions in CARDO-OJ3 roughly resemble those among the three α subunits in the heterohexameric NDO-O. Based on the mushroom-like overall structures of NDO-O, it is likely that this type of structure is common among the terminal oxygenase components of classes IB, II, and III ROs with an α3β3 configuration. Regardless of the quaternary structure (α3 or α1β3), each (catalytic) subunit can be divided into two distinct domains: the Rieske domain with a [2Fe–2S]3, and the catalytic domain containing the active site iron. Based on the structure of CARDO-OJ3, we were able to determine the structural features responsible for the α3 configuration. The crystal structures of the trimeric oxygenase components of RO have been reported for the terminal oxygenase components of 2-oxoquinoline 8-monoxygenase and *dicamba* monooxygenase. The overall shapes of these trimeric oxygenases are highly homologous to that of CARDO-OJ3, although that of 2-oxoquinoline 8-monoxygenase has an additional small C-terminal trimerization domain consisting of one α helix that forms the contacts in the center of the trimer. Based on these crystal structures, the doughnut-like or ring-like structure is likely to be common to the terminal oxygenase components of an α3 configuration. Although the terminal oxygenase component of phthalate dioxygenase is known to have an α6 homohexameric configuration, this homohexameric structure has itself been proposed to be a stacked form of two α3 trimeric forms.

Substrate soaking and co-crystallization experiments were performed using the CARDO-OJ3 crystal to clarify the manner of docking of the substrate around the active site Fe2+, but all failed, and the molecular mechanism of angular dioxygenation was based only on a predicted three-dimensional structure. However, we succeeded in determining the carbazole-binding and carbazole/oxygen-binding structures under oxidized and reduced conditions using a CARDO-OCA10-CARDO-O complex crystal (see section IV below). Upon carbazole binding, amino acid residues Leu202–Thr214 and Asp229–Val238, which were situated near the entrance of the substrate-binding pocket, adopted different structural conformations (Fig. 5AB). Both regions moved toward the entrance, with shifts of up to approximately 4 Å for the Cα atoms. Especially pronounced were the movements of the side chains of Phe204 and Ile231, which were shifted by about 2.8 to 4.2 Å. Such conformational changes resulted in the closure of a lid over the substrate-binding pocket, thereby apparently trapping carbazole at the substrate-binding site (Fig. 5-AB). Refinedness of the carbazole-bound CARDO-OJ3 structure indicated that carbazole is situated above the non-heme iron. The C1 and C9a carbon atoms were located about 4.3 Å from the iron at the active site, and the water or hydroxyl ligand of the non-heme iron was 2.8–2.9 Å from the two carbon atoms, whose binding characteristics were nearly identical to the predicted docking structure (Fig. 5C). This manner of binding accords with the fact that CARDO-OJ3 catalyzes angular dioxygenation to carbazole. The wall of the substrate-binding site created a flat, elongated compartment in which carbazole was oriented in such a way that the imino nitrogen of its middle ring was situated within the hydrogen-bonding distance of the carbonyl oxygen of Gly178 (approximately 2.9 Å; Fig. 5C).

Previous studies have provided a catalog of substrates for ROs, with product selectivities and specific yields for individual regio- and stereo-isomers. These studies have demonstrated the general versatility of ROs with respect to a large number of substrates, and at the same time, specificity in product formation for individual substrates. Based on this and structural information, Ferraro *et al.* proposed the hypothesis that while the active sites of ROs are amenable to a wide variety of substrates, the orientation of the substrate in a preferred manner determines the product. The manner of carbazole-binding in the substrate-binding pocket of CARDO-OJ3 was in accord with this hypothesis. In addition, we created CARDO-OJ3 mutants having substrate specificities different from that of wild-type CARDO-OJ3. The crystal structures of CARDO-OJ3 mutants bound to various substrates also accord with Ferraro’s hypothesis (Usami and Inoue *et al.*, unpublished results).

### IV. Structural Basis of the Electron Transfer Interaction among CARDO Components

As described above, we have obtained several CARDO-encoding gene sets from *P. resinovorans* CA10, *Janthinobacterium* sp. J3, *Novosphingobium* sp. KA1, and *Nocardoides aromaticivorans* IC177.
Fig. 5. Carbazole Binding to the Substrate-Binding Pocket of CARDO-O<sub>3</sub><sup>37</sup>. Panels A and B illustrate the closure of the entrance to the substrate-binding pocket (white arrows) upon carbazole binding. Conformational changes are obvious for amino acid residues Leu202–Thr214, shown as cyan (before binding) and orange (after binding) and Asp229–Val238, shown as lime (before binding) and magenta (after binding). Phe204 and Ile231, which underwent large conformational changes, are shown as stick models. In panel B, the molecular surfaces of the CARDO-F:CARDO-O binary complex with/without carbazole from the orientation directed by the white arrows are shown. Panel C shows carbazole binding at the substrate-binding pocket under oxidized conditions. The iron ion ligands and amino acid residues that constitute the substrate binding pocket are shown in stick models and are white in color. Carbazole is also shown as a stick model (salmon for carbon). The iron ions and water ligands are shown as orange and small red spheres respectively. The hydrogen bond interaction between the imino nitrogen of carbazole and the carbonyl oxygen of Gly178 are indicated by a broken red line. The substrate was omitted from the calculation of the difference density map, which is shown in blue and is contoured at 3.0σ.

Fig. 4. Crystal Structures of CARDO-O<sub>3</sub> (A)<sup>31</sup> and the Oxygenase Component of the Naphthalene 1,2-Dioxygenase of <i>Pseudomonas</i> sp. NCIB9816-4 (NDO-<sub>NCIB9816-4</sub>) (B).<sup>32</sup>

In both panels, catalytic (large) subunits are colored red, green, and blue. Structural small subunits are colored gray in the structure of NDO-<sub>NCIB9816-4</sub>. The [2Fe–2S]<sub>]<</sub> and the non-heme iron are shown as spheres (iron ions, orange; sulfide ions, yellow).
Table 1. Characteristics of the Components of CARDOs of Various Origins

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<td>CarAd (M)</td>
<td>[2Fe–2S]</td>
<td>CarAc (M)</td>
<td>[2Fe–2S]_{R}</td>
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<td>CARDO&lt;sub&gt;R&lt;/sub&gt;</td>
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<td>CARDO&lt;sub&gt;R&lt;/sub&gt;</td>
<td>CARDO&lt;sub&gt;F&lt;/sub&gt;</td>
<td>CARDO&lt;sub&gt;O&lt;/sub&gt;</td>
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*Based on the scheme proposed by Batie et al. 29
*The quaternary structure of each component is indicated as follows: (M), monomer; (T), trimer.
*Based on the results of gel-filtration and SDS–PAGE analysis. 29
*Shown by crystal structure. 30
*Based on the results of gel-filtration and SDS–PAGE analyses, although the detailed data have not yet been reported.
*Proposal based on amino acid sequence similarity.
*Based on the results of gel-filtration and SDS–PAGE analysis. 31
*Based on the results of gel-filtration and SDS–PAGE analysis. 40
*Shown by crystal structure. 50

According to Batie's classification system, these four CARDOs belong to three classes based on differences in their electron transport features (Table 1). Both CARDO<sub>CA10</sub> and CARDO<sub>JA1</sub> are classified into class III. The two CARDOs of KA1 are classified into class IIA. Class IIA RO is a three-component oxygenase in which the electron-transfer components comprise a simple flavoprotein and a putidaredoxin-type ferredoxin containing a putidaredoxin-type [2Fe–2S] cluster ([2Fe–2S]<sub>p</sub>). 44, 45 Almost all ROs are classified into class IIB or III. Only a few examples of class IIA ROs are known, including pyrazon dioxygenase from an unidentified bacterium, 3, 5, 6 dioxin dioxygenase from Sphingomonas wittichii RW1, 3, 5, 6 and dicamba-demethylase from P. maltophilia DI-6. 4, 6, 49 The conserved amino acid motifs proposed to bind the [2Fe–2S]<sub>p</sub> and the mononuclear iron, [2Fe–2S]<sub>R</sub>, and FAD were found in the amino acid sequences of CarAa, CarAc, and CarAd of IC177 respectively. On the basis of these results, CARDO<sub>IC177</sub> belongs to class IIB. 20

It is noteworthy that the terminal oxygenase components of the CARDOs of CA10/13, KA1, and IC177 share substantial homology (>45% identity), but their electron-transfer components are clearly different. Investigation of electron transfer selectivity among the components of the three classes of CARDO indicated that the electron transfer interactions between the ferredoxin and oxygenase components are highly specific whereas those between ferredoxin and reductase are not, although class IIA CARDO-F cannot receive electrons from class III or class IIB CARDO-R (Fig. 6). 33, 34, 35 Umeda et al. unpublished results. Similar results have been reported for many other RO members, and strict specificity between the ferredoxin and terminal oxygenase components is a common feature of ROs.

To understand the molecular basis of the electron transfer interactions between the CARDO components with and without highly specific recognition, we attempted to determine the crystal structures of the components of the three classes of CARDOs. We have to date succeeded in determining the crystal structures of eight components, not including CARDO-R<sub>IC177</sub> and CARDO-F<sub>IC177</sub> (Fig. 6). 33, 34, 35 Ashikawa et al. unpublished results; Umeda et al. unpublished results. To elucidate the manner of binding among the CARDO components, Ashikawa et al. 33 succeeded in determining the crystal structure of the electron-transfer complex between CARDO-O<sub>J3</sub> and CARDO-F<sub>CA10</sub> (Fig. 7A). Three molecules of CARDO-F<sub>CA10</sub> bind to three subunit boundaries of one CARDO-O<sub>J3</sub> molecule. Interactions occurred between four regions (amino acid residues 11–15, 115–119, 210, and 350–363) of CARDO-O<sub>J3</sub> and the cluster-binding domain of CARDO-F<sub>CA10</sub>. This surface in the binary complex structure consisted of 34 residues (19 of CARDO-O<sub>J3</sub> and 15 of CARDO-F<sub>CA10</sub>), and the accessible area buried in the interface was approximately 1,800 Å² for each pairing, which covers 12% and 39% of the total surface area of CARDO-O and CARDO-F respectively. Superposition of the structures of both the binary complex and single state crystals revealed that various conformational changes in both CARDO-O<sub>J3</sub> and CARDO-F<sub>CA10</sub> occur mainly at the component boundary. Most of these conformational changes allow the formation of specific hydrophobic interactions, hydrogen bonding, and electrostatic interactions, which can contribute to stabilization of the complex (Fig. 7B).

To my knowledge, the report of the CARDO-F<sub>CA10</sub>–CARDO-O<sub>J3</sub> binary complex structure 33 was the first report of the position of the binding site in the molecular surface of RO components. Considering the similarity of the three classes of CARDO-O molecules, it is reasonable to assume that CARDO-F can bind to the depression in class IIB CARDO-O, which enables the Rieske cluster in CARDO-F<sub>IC177</sub> to transfer electrons to that in CARDO-O<sub>IC177</sub>. In accordance with this idea, CARDO-F<sub>IC177</sub> was assumed to bind to the depression in the best-scoring complex model. 33 Structural comparison of class III and IIB CARDOs supported the prediction of amino acid residues that are important in the formation of electron transfer complexes in class IIB CARDO and residues important for redox partner selectivity between the terminal oxygenase and Rieske-type ferredoxin 33 (Fig. 7C). For example, His75, Glu71, Phe74, and Pro90 in CARDO-F<sub>IC177</sub> and
**Fig. 6.** Crystal Structures of Three Classes of CARDO Components.

CARDO-Rs and CARDO-Fs are drawn to the same scale, while CARDO-Os are reduced to 0.7 times scale. Red solid and purple broken arrows denote the ability and inability of electron transfer between the components, respectively. The widths of the red arrows indicate relative efficiencies of electron transfer. The detailed crystal structures for CARDO-F\textsubscript{CA10}, CARDO-O\textsubscript{J3}, and CARDO-F\textsubscript{IC177} and CARDO-O\textsubscript{IC177} were published by Nam et al.,\textsuperscript{39} Nojiri et al.,\textsuperscript{31} and Inoue et al.\textsuperscript{43} respectively. Detailed descriptions of the crystal structures of CARDO-R\textsubscript{CA10} and three components of CARDO\textsubscript{KA1} will be published elsewhere (Ashikawa et al., unpublished results; Umeda et al., unpublished results). For class IIB CARDO-R of \textit{Nocardioides aromaticivorans} IC177, the crystal structure determined from the diffraction data to 4.5 Å resolution is shown (Umeda et al., unpublished results).

**Fig. 7.** Overall Structure of the CARDO-F\textsubscript{CA10}:CARDO-O\textsubscript{J3} Binary Complex (A),\textsuperscript{37} and Electrostatic and Hydrophobic Interactions Observed in a Binary Complex of CARDO-F and CARDO-O in a Class III System (B)\textsuperscript{37,43} and Those Proposed for a Class IIB System (C).\textsuperscript{43}

In panel A, the three CARDO-O\textsubscript{J3} subunits (chain A, red; chain B, forest green; and chain C, blue) and the three CARDO-F\textsubscript{CA10} molecules (chain D, orange; chain E, cyan; and chain F, magenta) are shown. The [2Fe–2S]\textsubscript{R} and the non-heme iron are shown as spheres (iron ions, orange; sulfide ions, yellow). In panel B, the amino acid residues involved in binding are mapped on the molecular surfaces of CARDO-F\textsubscript{CA10} and CARDO-O\textsubscript{J3}, based on the CARDO-F\textsubscript{CA10}:CARDO-O\textsubscript{J3} binary complex structure (PDB entry, 2DE5).\textsuperscript{37} In panel C, the residues predicted to be involved in the interactions between CARDO-F\textsubscript{IC177} and CARDO-O\textsubscript{IC177}, based on the results of amino acid sequence alignments with CARDO-F\textsubscript{CA10} and CARDO-O\textsubscript{J3}, are shown.\textsuperscript{43} The basic, acidic, and hydrophobic residues are shown in blue, red, and orange respectively. The residues involved in interactions in the class III system but not conserved in the class IIB system are shown in dark gray. In panels B and C, the acidic and basic residues that form or are predicted to form electrostatic interactions between CARDO-F and CARDO-O are connected with dotted lines.
their corresponding residues in other Rieske-type ferredoxins in classes IIB and III are predicted to be important in interaction with their terminal oxygenase components. Lys20, Trp21, Glu357, Leu359, and Val367 in CARDO-O_{C17} are predicted to interact in both class IIB and III CARDOs. On the other hand, Glu43, His50, and Glu55 of CARDO-F_{C109} and Arg118, Arg210, and Val351 of CARDO-O_{S6}, which are not conserved in class IIB CARDOs, are predicted to determine the specificities of class III CARDO-F and CARDO-O.

In the solved binary complex, CARDO-F_{C109} binds on the side of the αβ trimer that corresponds to the top of the cap of the mushroom-shaped αββ hexamer (see Figs. 4 and 7A). Friemann et al.\(^5\) suggested that a similar docking site for ferredoxin is possible for the oxygenase in tolulene 2,3-dioxygenase, since there is a depression in the molecule in this area. However, based on the crystal structures of the terminal oxygenase component of tolulene 2,3-dioxygenase, the authors also pointed out that shallow depressions at the interface between αβ heterodimers are most likely docking sites for ferredoxin, because ferredoxin can be positioned so that the His residues of the [2Fe–2S]_{R} cluster of ferredoxin are approximately 12 Å from the [2Fe–2S]_{R} cluster in the terminal oxygenase of the tolulene 2,3-dioxygenase system.\(^5\) To determine the validity of this hypothesis, determination of the crystal structure of binary complex between the αββ heterohexamer oxygenase component and ferredoxin is necessary.

V. A Carbazole-Degradative Plasmid, pCAR1

1. Sequencing of plasmid pCAR1

Genetic analyses of carbazole-degrading bacteria have revealed several types of carbazole-degradative car gene clusters. Of these, that of P. resinovorans CA10 is distributed in various carbazole-degrading strains of the genera Pseudomonas, Burkholderia, and Janthinobacterium.\(^2\) Perhaps because car gene clusters are on mobile genetic elements. In fact, the car gene cluster of CA10 is located on circular plasmid pCAR1.\(^5\) We isolated pCAR1 from CA10 that had been preserved as a glycerol stock since its isolation in 1993. Its entire nucleotide sequence was determined in 2003, whereas other Inc group plasmids were compatible with the test pCAR1 mini plasmid.\(^57\) In addition, Southern hybridization analysis using the pCAR1 repA gene as probe revealed that a single, clear signal was detected with Rms148.\(^57\) These results indicate definitively that pCAR1 belongs to the IncP-7 group. Thus pCAR1 is the first IncP-7 plasmid for which the entire nucleotide sequence has been determined and reported. Although the plasmids from Pseudomonas are classified into at least 14 Inc groups,\(^6\) the degradative plasmids characterized to date belong to the IncP-1, -2, -7, and -9 groups.\(^6\) Among such four Inc group degradative plasmids, the most detailed analyses have been conducted on the IncP-1 and IncP-9 plasmids: IncP-1, pJP4\(^{54,66}\); pADP-1\(^{67,68}\); and pOU1\(^{69,70}\) and IncP-9, pWW0\(^1\)\(^71,74\) NAH7\(^{75,76}\) pDTG1\(^{77,78}\) and SAL1.\(^79\) To date, in addition to pCAR1, complete nucleotide sequences have been reported for three other IncP-7 group plasmids: naphthalene degradative plasmid pND6-1\(^{80}\), toluene degradative plasmids pWW53\(^{81}\) and pDK1.\(^82\) The replication and partition function regions of these plasmids were well conserved with that of pCAR1, and a 53.2-kb IncP-7 plasmid backbone was proposed based on a comparison of the nucleotide sequences.\(^82\) Recently, seven IncP-7 naphthalene and salicylate degradative plasmids were also reported.\(^83\) Thus IncP-7 plasmids are gaining importance as degradative plasmids.

3. Conjugal transfer

The complete nucleotide sequence of pCAR1 suggested the presence of putative transfer genes, designated tra and trh (Fig. 8).\(^5,52,54,55\) The corresponding region was proposed to be a part of the IncP-7 plasmid backbone.\(^52\) According to a classification scheme based on the homology of the conjugative relaxase,\(^84\) pCAR1 belongs to the MOB_{4} group of mobilizable plasmids. Transfers of pCAR1 to P. resinovorans CA10dm4 and P. putida KT2440 were detected experimentally at
frequencies of $3 \times 10^{-1}$ and $3 \times 10^{-3}$ per donor cell,$^{57}$ suggesting that pCAR1 is in fact a self-transmissible plasmid. Mating experiments showed that pCAR1 can transfer from *P. putida* HS01 (*P. putida* DS1$^{85}$) harboring pCAR1 to *P. chlororaphis*, *P. fluorescens*, and *P. stutzeri*, although no transfer of pCAR1 from *P. resinovorans* CA10 to them was detected.$^{86}$ Hence we concluded that the recipient range of pCAR1 was affected largely by the donor cell. A limited recipient range of conjugative transfer is also the case for pDK1.$^{82}$ Using pDK1, Yano *et al.*$^{82}$ reported recently that the host range of IncP-7-specified conjugative transfer was, unlike for other well-known plasmids, narrower than that of its replication, suggesting that the host range of self-transmissible IncP-7 plasmids is in general restricted by the conjugation system rather than the replication system.

On the other hand, monitoring analyses of pCAR1 transfer from a *P. putida* host cell to bacteria in a river water sample suggested that the presence of carbazole promotes the appearance of transconjugants belonging to the genus *Pseudomonas*, and that, intriguingly, pCAR1 was transferred into non-*Pseudomonas*, *Stenotrophomonas*-like bacteria.$^{87}$ Recently, cultivation-independent examination of the conjugal transfer of an IncP-1 plasmid pKJK10 to indigenous consortia in the Barley Rhizosphere was performed to evaluate its natural recipient range.$^{88}$ A similar methodology is now on the point of concluding that pCAR1 is in fact transferable to a wider range of bacterial genera in nature (Shintani *et al.*, unpublished results). These results suggest the possibility of a broader host range of IncP-7 group plasmids, although historically IncP-7 group plasmids have been treated as narrow host range plasmids.$^{61}$ In addition, it is of interest that pCAR1 conjugative transfer events are affected by the divalent cation (Ca$^{2+}$ and Mg$^{2+}$) concentration in the medium.$^{89}$

4. **Class II transposon Tn4676 and ISs**

In pCAR1, *tpnAcCST* genes encode proteins showing $>70\%$ overall length-wise identity with transposition machinery proteins borne in toluene/xylene-degrading transposon Tn4651$^{80}$ of TOL plasmid pWW0. The *car* and *ant* gene clusters, which are involved in the
VI. Behaviors of pCAR1 Host Strains and Fates of pCAR1

1. Structural changes in pCAR1 by host metabolic capacity

The degradation abilities and growth characteristics of pCAR1 on carbazole of Pseudomonas host strains (P. putida KT2440, P. aeruginosa PAO1, and P. fluorescens P0-1) with and without pCAR1 were assessed.\(^92\) The KT2440, Pf0-1(pCAR1) cells results in excessive catechol accumulation after inoculation in all the strains including anthranilate- or catechol-degraders.\(^94\) Genetic analyses followed by comparison of the degradation abilities of the strains that reappeared revealed the generations of pCAR1::rfp derivatives in which the antA gene was disrupted (Fig. 9).

pCAR1 derivatives obtained from mutants in pure cultures of P0-1(pCAR1), P01d or P01Δ2, had commonly lost the antR-antABC genes necessary for anthranilate conversion and induction capability of the ant and car operons (Fig. 9). AntR is an AraC/XylS family regulator that induces the transcription of both car and antABC operons by the P\(_\text{car}\) promoters in the presence of anthranilate.\(^96\) In the absence of anthranilate, the car operon is constitutively transcribed from the P\(_{\text{carA}}\) promoter, although its transcriptional level is lower than that of an anthranilate-inducible P\(_{\text{car}}\) promoter.\(^97\) The absence of antR renders the host cell unable to express the large amount of upper pathway enzymes in the carbazole degradation pathway and slows carbazole degradation. Because of the absence of strains capable of metabolising catechol efficiently, it can be important to delay carbazole degradation (the conversion of carbazole to catechol) in pure cultures of P0-1(pCAR1) to avoid excessive catechol accumulation. In contrast, systems for anthranilate-inducible higher transcription are retained in the mutants from the artificial water microcosms\(^95\) (Fig. 9). Many other anthranilate- (and/or catechol-) metabolising bacteria were present in the microcosms.\(^89,94\) Although these strains were able to degrade catechol, excessive catechol was toxic to them (the final concentration was >0.1% w/v, data not shown). Thus, carbazole and anthranilate degradation rates and their concentrations can be the key factor in determining whether catechol accumulates or is degraded. These strains are also able to metabolise anthranilate efficiently and this can have prevented catechol from accumulating excessively. Perhaps there was no requirement to slow the rate of carbazole degradation in the artificial water microcosms due to the presence of other anthranilate- or catechol-metabolising bacteria. On the other hand, the presence of antA gene mutants only and no other strains including anthranilate- or catechol-degraders cannot be beneficial from the viewpoint of carbon utilization, because they made use of only a portion of the available carbazole. It is thus possible that deletion of the antA gene does not occur in pure cultures of P0-1(pCAR1), but only in artificial microcosms. The direction of evolution of pCAR1 in host P0-1 might have been affected by the presence of other anthranilate-metabolising bacteria due to differences in catechol metabolism in the culture. This highlights the importance of comparing the behavior of plasmids and their hosts when grown on different carbon sources and in different environments, and in the presence of other bacteria.

2. Structural changes in pCAR1 due to the metabolic capacities of co-existing bacterial strains

We have monitored how the pCAR1 host behaviors in artificial microcosms inoculated with 15 other bacterial taxa.\(^89,94\) In that study, we monitored the behavior of a derivative strain of P0-1(pCAR1), P. fluorescens P0-1L(pCAR1::rfp). As expected, P0-1L(pCAR1::rfp) disappeared immediately after inoculation in all the artificial water microcosms employed, but carbazole degraders became detectable after approximately 14 d in some microcosms.\(^84\) Notably, carbazole degradation was detected coincidentally with the recovery of the degraders.\(^93\) Genetic analyses followed by comparison of the carbazole degradative abilities of the strains that reappeared revealed the generations of pCAR1::rfp derivatives in which the antA gene was disrupted (Fig. 9).

3. Behaviors of host strains affected by pCAR1 carriage

In the above-mentioned study monitoring pCAR1 host behaviors in artificial model environments, we
observed that behavior varied between hosts. In terms of carbazole degradation capacity, *P. putida* is an appropriate host in soil environments, and *P. resinovorans* in water environments. Conjugal transfer of pCAR1 was detected only in a water environment inoculated with *P. putida* and *P. chlororaphis* as hosts. It is noteworthy that the transconjugants obtained were all *P. resinovorans* for both types of donor cell, suggesting that *P. resinovorans* is a good recipient of pCAR1 under the conditions imposed.

In addition, we have observed that the pCAR1 carriage affects host primary metabolism, motility, biofilm formation, and plasmid fitness cost (in words, load to the plasmid fitness), and that such effects vary between hosts (data not shown). It has been reported that the carriage of plasmids other than pCAR1 affects host cell behavior, including cell motility and biofilm formation. Although the mechanisms underlying the effects of plasmid carriage are not well understood, considering that many natural plasmids are conjugative, it is important to determine the deviations in host phenotype caused by plasmid carriage in order to understand the fate of the plasmid itself and host bacterial behaviors in natural environmental systems.

**VII. Response of the Host Transcriptome to the Carriage of pCAR1**

To elucidate the impact of pCAR1 carriage on the host, we compared RNA maps of three pCAR1-free and pCAR1-containing *Pseudomonas* strains, *P. putida* KT2440, *P. aeruginosa* PAO1, and *P. fluorescens* Pf0-1, using tiling arrays. We found 121 KT2440, 73 PAO1, and 125 Pf0-1 genes whose expression was affected by pCAR1 carriage. Similarly, we identified the differential transcription of 95 KT2440, 40 PAO1, and 48 Pf0-1 transcriptional units caused by the carriage of pCAR1. The number of upregulated genes and units was greater than that of downregulated genes in each strain, suggesting that pCAR1 carriage might have invoked the transcription of chromosomal genes rather than repressing them (Fig. 10). The ORFs in the various host strains were categorized into 23 groups based on Clusters of Orthologous Groups of Proteins (COG) analysis, and the up- and downregulated genes in each strain were categorized as shown in Fig. 10. "Amino acid transport and metabolism" and "inorganic ion transport and metabolism" were common COG categories among the differentially transcribed genes in each strain, although no other common feature was detected. These facts indicate that the impact of pCAR1 carriage on host cells differs between hosts.

Among the genes commonly regulated in two host strains by pCAR1 carriage (12 genes in KT2440 and PAO1, four in PAO1 and Pf0-1, and two in KT2440 and Pf0-1), most were related to iron acquisition and transport systems. Iron is an essential element for organisms, including bacteria, and the genus *Pseudomonas* produces a wide variety of ferric-specific siderophores that mediate the acquisition of insoluble ferric iron via specific receptor and transport systems. In the case of iron deficiency, the genes related to siderophore production and transport were upregulated, especially for the major siderophore pyoverdine. Expression of the genes involved in pyoverdine synthesis and transport was greater in plasmid-containing *P. putida* KT2440 and *P. aeruginosa* PAO1 than in the plasmid-free host strains (Fig. 11). Hence it is possible to conclude that possession of pCAR1 altered the portion of the iron acquisition system mediated by siderophores. Indeed, pCAR1-containing strains show transiently reduced growth rates during the early-log phase, and
that transient growth delay is clearly observed at low iron concentrations. Considering that our transcriptome comparisons were done during early log-phase growth, iron requirements might have been partially responsible for the transient growth delays. The expression of carbazole degradation enzymes from pCAR1 was one reason for this reduction in host growth rate. Moreover, iron requirements and thus increased pyoverdine production might have occurred in the pCAR1-containing host strains, especially KT2440 and PAO1. As explained above, the carbazole degradative car gene cluster is constitutively transcribed from the P_{carha} promoter under un-induced conditions, for example, in succinate-grown culture.\(^5\) Transcription of it was detected in the pCAR1 tiling array during early log-phase growth.\(^5,6\) Considering that some Car enzymes contain iron,\(^4,10\) it is likely that the iron requirement for expression of Car enzymes reduces the growth rate of pCAR1-containing host cells during early log-phase growth.

On the other hand, the mexEFoprN operon, encoding an efflux pump of the resistance-nodulation-cell division family,\(^105\) was specifically upregulated by pCAR1 carriage in \(P.\) putida KT2440 (Fig. 12A), whereas the expression of orthologous operons in other species remained unaltered.\(^101\) Induction of the mexEFoprN genes increased the resistance of pCAR1-containing KT2440 to chloramphenicol, as compared to pCAR1-free KT2440.\(^101\)

VIII. A Key Regulator of pCAR1 and Host \textit{Pseudomonas} Functions

1. Nucleoid-associated proteins (NAPs) on pCAR1

pCAR1 carries three genes encoding NAPs: Pmr, a histone-like protein H1 (H-NS)-family protein; Phu, a histone-like protein from \(E.\) coli strain U93 (HU)-like protein; and Pnd, an NdpA-like protein. H-NS is an oligomeric DNA-binding protein identified in \(E.\) coli through its effect on transcription \textit{in vitro}.\(^109,110\) It acts as a global repressor and binds to horizontally-acquired DNA regions.\(^111\) HU is a 9-kDa protein that shows DNA-binding activity as both homo- and heterodimers.\(^112\) The DNA binding of HU is not nucleotide sequence-specific, but it prefers to bind to bending regions of DNA, such as cruciform and nicked and gapped regions of DNA, and is involved in homologous recombination and DNA repair.\(^113\) NdpA-like proteins constitute a group well-conserved in Gram-negative bacterial genomes, one of which, YejK, is involved in the \(E.\) coli nucleoid,\(^114\) but the details of the physiological functions of NdpA-like proteins remain unknown.

2. H-NS family proteins in \(P.\) putida KT2440 (pCAR1)

Because NAPs have both architectural and regulatory functions in bacterial cells,\(^110\) they might be responsible for regulating the transcriptional changes caused by pCAR1 carriage. In fact, a plasmid-encoded H-NS, Sfh, can function as a “stealth” protein that switches off gene expression on chromosomes or plasmids and maintains host cell fitness.\(^112\) As the first step in clarifying the role of NAPs encoded on pCAR1, we assessed the function of Pmr in \(P.\) putida KT2440, which carries pCAR1.\(^123\) KT2440 has five genes encoding H-NS family proteins, and recently Renzi et al.\(^123\) named them as follows: PP\_1366 (turA), PP\_3765 (turB), PP\_0017 (turC), PP\_3693 (turD), and PP\_2947 (turE). Quantitative reverse transcription-PCR combined with RNA mapping analyses using tiling arrays suggested that pmr and turA are the primary transcribed H-NS family genes during early log-phase growth, whereas turB is transcribed in the late log and stationary growth phases in KT2440 (pCAR1).\(^123\) Gel filtration chromatography and protein-protein cross-linking analyses indicated that Pmr forms homo-oligomers consisting of its homodimers.\(^125\) In addition, pull-down assays followed by
Western blots indicated that the strength of the interactions between Pmr and itself and between Pmr and TurA, TurB, or TurE is higher than those between Pmr and TurC or TurD. On the other hand, atomic force microscopic analysis revealed that Pmr has DNA-bridging capacity. Based on these results, we conclude that Pmr has features common to H-NS family proteins, and that it functions as a DNA-binding protein constituting homomeric and heteromeric oligomerizations with Pmr itself and at least two chromosomal

Fig. 11. RNA Maps of ORFs for Pyoverdine Production and Transport in the Various pCAR1-Free (blue) and pCAR1-Containing (red) Hosts: *P. putida* KT2440, *P. aeruginosa* PAO1, and *P. fluorescens* Pf0-1. The x-axis indicates the positions of the chromosomes, and the y-axis indicates the signal intensities of hybridization with single-stranded cDNA. Bars denote signal intensities of probes. Pentagons represent the directions and locations of annotated genes, and their colors correspond to the homologous genes in the various host strains. The transcription of these ORFs in KT2440 and PAO1 was upregulated by carriage of pCAR1 in some instances.

Bacterial Carbazole Degradation System
3. Effects of Pmr disruption on the transcriptomes of the pCAR1 and the KT2440 chromosomes

Transcriptome comparison of pmr disruptant KT2440(pCAR1Δpmr) and wild-type KT2440(pCAR1) revealed that the transcription of 31 genes on pCAR1 was altered by pmr disruption.123) For example, the constitutive transcription level of the carbazole-degradative car operon under succinate-growth conditions was decreased by pmr disruption (Fig. 13A).123) Similarly, the transcription level of the parAB genes was also reduced by pmr disruption (Fig. 13B). Because ParAB proteins are required for the stable maintenance of pCAR1 in the host strain,57) downregulation of these genes might cause instability in pCAR1, but we did not detect changes in the stability of pCAR1 or pCAR1Δpmr in KT2440 cells.

As for chromosomally-encoded genes, comparison of the transcriptomes of KT2440, KT2440(pCAR1), and KT2440(pCAR1Δpmr) indicated that pmr disruption had a greater effect on the host transcriptome than did pCAR1 carriage.123) Of the 5,398 genes of KT2440 (after rRNA and tRNA removal), the transcription of 5,146 genes was unaffected by either pCAR1 carriage or pmr disruption. Of the remaining 252 genes, 93 were affected by pCAR1 carriage but not pmr disruption, suggesting that Pmr is not involved in their regulation, but 17 genes were upregulated by pCAR1 carriage and downregulated by pmr disruption, and for one gene it was the reverse. These 18 genes are considered to be regulated primarily by Pmr itself, directly or indirectly.

paralogs, TurA and TurB.

Fig. 12. RNA Maps of the Flanking Regions of mexEFoprN (A), parl (B), hemB (C), and PP_3332 (D) on the P. putida KT2440 Chromosome.101,123) Blue, red, and green bars represent the signal intensities of the probes in the RNA maps of KT2440, KT2440(pCAR1), and KT2440(pCAR1Δpmr). Pentagons indicate the directions and locations of annotated genes.

Fig. 13. Effects on RNA Maps of the Flanking Regions of the car Operon (A) and the parAB Genes (B) on pCAR1 Caused by pmr Disruption.123) Red and green bars represent the signal intensities of probes in the RNA maps of KT2440(pCAR1) and KT2440(pCAR1Δpmr). Pentagons indicate the directions and locations of the annotated genes.
Typical examples are the \textit{mexEFoprN} and \textit{parL} genes, whose transcriptional levels increased with pCAR1 carriage but reverted after \textit{pmr} disruption to levels similar to those in pCAR1-free KT2440 (Fig. 12AB). On the other hand, 122 and 18 genes were up- and downregulated by \textit{pmr} disruption respectively, but neither group was affected by pCAR1 carriage, suggesting that Pmr has a stealth function minimizing the effect on host strain fitness, as has been reported for the \textit{psf}-R27-encoded H-NS, Sfh.\textsuperscript{122} Figure 12C and D show examples of alternations in the transcription of which appeared to be repressed by the Pmr stealth function.

Identification of genome-wide Pmr binding sites by ChAP-chip (chromatin affinity purification coupled with high-density tiling chip) analysis indicated that Pmr preferentially binds to putative horizontally acquired DNA regions with a low G+C content.\textsuperscript{123} Pmr-binding sites overlapped with the locations of those genes differentially transcribed with \textit{pmr} disruption on both the plasmid and the chromosome, although not every gene near a binding-site region was affected by \textit{pmr} disruption. Our findings indicate that Pmr is a key factor optimizing gene transcription on pCAR1 and the host chromosome.

4. Mode of cooperative function of H-NS family proteins

We also performed ChAP-chip analysis to identify the binding sites of TurA and TurB, and compared them to those of Pmr, but the TurA- and TurB-binding sites were almost identical to those of Pmr.\textsuperscript{123} These results were similar to those observed for \textit{E. coli} and \textit{P. aeruginosa}, in which two chromosomal H-NS or H-NS family proteins (H-NS and StpA or MvaT and MvaU) bound to the same regions of the respective chromosomes.\textsuperscript{126,127} Recently, Dillon \textit{et al.}\textsuperscript{128} reported that the DNA binding sites of plasmid-encoded H-NS, Sfh, of \textit{Salmonella} overlapped with those of chromosomally-encoded H-NS. Sfh does not bind uniquely to any site, and the number of binding sites of Sfh in Sfh is smaller than in H-NS. Although Sfh binding sites are located within H-NS, the DNA binding sites greatly expand in its absence, suggesting that Sfh plays a backup role for H-NS.\textsuperscript{128} This suggests that Pmr, TurA, and TurB function cooperatively as global regulators in \textit{P. putida} KT2440(pCAR1) cells, and that Pmr also performs backup functions for TurA and/or TurB in a way different from Sfh, because the Pmr, TurA, and TurB binding sites are identical.

On the other hand, disruption of \textit{turA} or \textit{turB} in the KT2440(pCAR1) genome affected the transcription of not only the common genes but also genes different from those affected by \textit{pmr} disruption (Yun \textit{et al.}, unpublished results), suggesting that their functions are non-equivalent. This result appears not to accord with the fact that these proteins bind near-identical regions of both the pCAR1 and the KT2440 chromosome.\textsuperscript{123} To interpret this discrepancy, we hypothesized that binding of the C-termini (DNA-binding domains) of Pmr, TurA, and TurB at respective preferred sites, followed by homo- and heterooligomerization of the N-termini (oligomerization domains) of DNA-bound and free soluble dimers, determines the genome-wide distribution of the regions bound by H-NS family proteins. That is, it is plausible that DNA-bound oligomeric forms contain all the H-NS family proteins, although the proportions of the various proteins remain unknown.

Further investigation is needed to clarify the heteromeric oligomerization process of H-NS family proteins in the nucleoid of KT2440(pCAR1). As of now, we have identified some amino acid residues of Pmr that are important for its self-oligomerization (Suzuki \textit{et al.}, unpublished results). Many of these are well conserved with TurA but not TurB. Such residues might play important roles in the unequal oligomerization of H-NS family proteins.

IX. Prospects

In the last two decades, we have extensively investigated carbazole metabolism in several bacteria. Some of these studies focused on the novel genetic structures of degradative gene clusters and their diversity and the molecular mechanisms underlying the novel substrate specificity of degradative enzymes, especially in CARDO. In terms of CARDO research, we have succeeded in interpreting the novel substrate specificity by X-ray crystallography. The molecular basis of the electron transfer mechanism among CARDO components remains to be clarified. There are three types of CARDO system, which have different types of electron transport chain (classes IIA, IIB, and III), as mentioned above, and these are good tools for clarifying the electron transfer mechanisms in a class-specific manner. Recently, a new CARDO system with a chloroplast-type ferredoxin as an electron donor to the terminal oxygenase was isolated from the marine carbazole degrader \textit{Kordimonas gwangyangensis} OC9.\textsuperscript{129} This should also assist in clarifying the molecular mechanism that accords the recognition of the appropriate electron transfer partners. In addition, redox-specific association/dissociation mechanisms are indispensable for effective electron transfer among components. They are future targets of studies aiming to increase our understanding of basic and general RO function.

Another future research target is the interaction between the plasmid and host chromosome that modulates host cell functions and affects the behavior of host cells in a natural ecosystem. In connection with such issues, plasmid-encoded NAPs are of great importance. Since many NAPs have the capability to form homo- and/or heterooligomers with themselves and paralogous proteins,\textsuperscript{121} investigation of both plasmid-encoded NAPs and host chromosomal paralogous proteins should be carried out. A significant number of natural plasmids are self-transmissible or mobilizable, and so the host chromosome can be swapped upon conjugation. In fact, the distribution of NAP genes in plasmids is heterogeneous, and might thus be important to bacterial plasmid behavior and evolution.\textsuperscript{130} In addition, heterologous interactions among NAPs should be investigated. The studies I propose should provide basic information on the behavior and the fate of both the plasmid itself and its host bacteria in natural environments.

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