The Genes Coding for the Conversion of Carbazole to Catechol Are Flanked by IS6100 Elements in Sphingomonas sp. Strain XLDN2-5

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Abstract

Background: Carbazole is a recalcitrant compound with a dioxin-like structure and possesses mutagenic and toxic activities. Bacteria respond to a xenobiotic by recruiting exogenous genes to establish a pathway to degrade the xenobiotic, which is necessary for their adaptation and survival. Usually, this process is mediated by mobile genetic elements such as plasmids, transposons, and insertion sequences.

Findings: The genes encoding the enzymes responsible for the degradation of carbazole to catechol via anthranilate were cloned, sequenced, and characterized from a carbazole-degrading Sphingomonas sp. strain XLDN2-5. The car gene cluster (carRaaBaBbCaC) and fdr gene were accompanied on both sides by two copies of IS6100 elements, and organized as IS6100::IS5sp1-ORF1-carRaaBaBbCaC-ORF8-IS6100-fdr-IS6100. Carbazole was converted by carbazole 1,9a-dioxygenase (CARDO, CarAaAcFdr), meta-cleavage enzyme (CarBbBb), and hydrolase (CarC) to anthranilate and 2-hydroxy-penta-2,4-dienoate. The fdr gene encoded a novel ferredoxin reductase whose absence resulted in lower transformation activity of carbazole by CarAa and CarC. The ant gene cluster (antRacAdAbAa) which was involved in the conversion of anthranilate to catechol was also sandwiched between two IS6100 elements as IS6100-antRacAdAbAa-antRacAdAbAa-IS6100. Anthranilate 1,2-dioxygenase (ANTDO) was composed of a reductase (AntAa), a ferredoxin (AntAb), and a two-subunit terminal oxygenase (AntAcAd). Reverse transcription-PCR results suggested that antRacAdAbAa gene cluster, fdr, and antRacAdAbAa gene cluster were induced when strain XLDN2-5 was exposed to carbazole. Expression of both CARDO and ANTD0 in Escherichia coli required the presence of the natural reductases for full enzymatic activity.

Conclusions/Significance: We predict that IS6100 might play an important role in the establishment of carbazole-degrading pathway, which endows the host to adapt to novel compounds in the environment. The organization of the car and ant genes in strain XLDN2-5 was unique, which showed strong evolutionary trail of gene recruitment mediated by IS6100 and presented a remarkable example of rearrangements and pathway establishments.

Introduction

Carbazole is an N-heterocyclic compound that is known to possess mutagenic and toxic activities [1]. To date, a number of bacterial strains capable of degrading carbazole have been isolated and characterized. Phylogenetically, almost all of these strains belong to pseudomonads and sphingomonads. The degradation of carbazole starts with angular dioxygenation in strains belonging to pseudomonads and sphingomonads. The isolated and characterized. Phylogenetically, almost all of these bacterial strains capable of degrading carbazole have been considered as the key enzyme. In Pseudomonas resinovorans CA10, the most intensively studied pseudomonad, CARDO consists of a terminal oxygenase, a ferredoxin, and a ferredoxin reductase, encoded by carAa, carAc, and carAd, respectively. In pseudomonads, the upper pathway genes, carAaBbBbCaCAd, are in the car cluster, which is transcribed as a single transcriptional unit [6]. Interestingly, unlike the well-organized operons in pseudomonads, the catabolic genes in sphingomonads are often dispersed or

phenyl-2,3-diol at the meta position to generate 2-hydroxy-6-(2’-aminophenyl)-6-oxo-2,4-hexadienoic acid, which is hydrolyzed to produce anthranilic acid and 2-hydroxy-penta-2,4-dienoic acid. The resulting metabolite, anthranilate, is converted to catechol in a single step by anthranilate 1,2-dioxygenase (ANTDO) (Figure 1) [9,10]. In this upper carbazole degradation pathway, CARDO is considered as the key enzyme. In Pseudomonas resinovorans CA10, the most intensively studied pseudomonad, CARDO consists of a terminal oxygenase, a ferredoxin, and a ferredoxin reductase, encoded by carAa, carAc, and carAd, respectively. In pseudomonads, the upper pathway genes, carAaBbBbCaCAd, are in the car cluster, which is transcribed as a single transcriptional unit [6]. Interestingly, unlike the well-organized operons in pseudomonads, the catabolic genes in sphingomonads are often dispersed or

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not coordinately regulated. For example, plasmid pCAR3 contains multiple gene sets, which are involved in the carbazole degradation pathway in a carbazole-degrader *Novosphingobium* sp. KA1 ([previous *Sphingomonas* sp. KA1]) [10]. These unusual organizations of the degradative genes were often observed in other sphingomonads [11,12].

Insertion sequences (ISs) are a subject of increasing interests for biodegradation because of the variety of their structures, modes of action, and the biodegradation abilities they confer bacteria [13]. ISs are small and mobile genetic elements that are ubiquitously distributed within bacterial genomes, and play an important role in evolution by facilitating horizontal gene transfers between bacterial populations, which contribute significantly to the diversity of bacteria by enhancing the organisms’ adaptive and evolutionary capacities. IS6100 is an important IS that flanks a range of catabolic operons, for example, the operons for metabolism of various aromatic substrates [14,15]. In this work, we report that two loci coding for the enzymes that convert carbazole to catechol were found to be flanked by IS6100 elements. Evidence was given for the involvement of these genes in the degradation of carbazole in *Sphingomonas* sp. XLDN2-5.

**Results**

**Screening of the genomic library, DNA sequencing, and genome walking**

The *car* probe, labeled with DIG, was used for screening the genomic library, and a positive clone, designated as pBY13 (Figure 2A), was sequenced and analyzed to contain a DNA insert of 6.8 kb. BLAST search results revealed that there were five intact open reading frames (ORFs), *carR*, *carAa*, *carBa*, *carBb*, and *carC*, which were found to be 99% identical to the corresponding genes of *Novosphingobium* sp. strain KA1 [10]. A closer look at the left region suggested that an IS6100, exhibiting 100% identity to that of *Mycobacterium fortuitum* [16], was interrupted by a novel insertion element IS6100::IS.Ssp1. Therefore, the interrupted IS6100 was designated IS6100::IS.Ssp1. The putative transposase for IS6100::IS.Ssp1 was transcribed in the same direction of the *car* cluster, but in the opposite direction as the interrupted IS6100. IS.Ssp1 belongs to the IS256 family of prokaryotic ISs, a subgroup of the mutator family of transposases (Pfam00872). IS6100::IS.Ssp1 which had imperfect inverted repeats (32-bp in length, one mismatch) was flanked by two copies of 8-bp direct repeat (Figure 2B).

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**Figure 2. Physical maps of car and ant loci.** (A) Physical map of car locus, which is delimited by IS6100 elements. The upstream IS6100 is interrupted by a novel insert element ISSp1, and was designated IS6100::ISSp1. (B) Schematic representation of the main features of the novel ISSp1 sequence in *Sphingomonas* sp. XLDN2-5. The orientation of the ISSp1 is shown by an arrow. The red and blue boxes represent the positions of two direct repeats (DR) and two imperfect, 32 bp, terminal inverted repeats (the left inverted repeat [IRL], and the right inverted repeat [IRRI]) with one mismatch, which is indicated by lowercase letters. The nucleotide sequences of DRs and IRs are also given. (C) Physical map of ant cluster which is flanked by IS6100 elements along a base pair scale.

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At the 3’-end of pBY13, an incomplete ORF (238 bp) showed 100% identity to carA1 of K1. In order to clone the full gene and its flanking sequences, a 1-kb DNA was amplified by genome walking. After ligation of the fragment to pMD18-T followed by transformation to E. coli DH5α, a positive clone, designated p6100-a, was obtained. DNA sequencing indicated that p6100-a contained the left part of carC, an ORF8, and interestingly a partial IS6100 sequence. The presence of the partial IS6100 sequence led to the hypothesis that there was a complete IS6100 and might form a transposon, and therefore triggered further study. The presence of an intact IS6100 copy downstream of ORF8 was successfully validated by PCR using carC-sp3 and IS6100-F2 and IS6100-F2 and sequencing. This transposon-like context (IS6100::ISsp1::ORF1-carRAaBaBbCAc-ORF8-IS6100) was designated TnCar, tentatively.

IS-based PCR

The presence of IS6100 on both sides of the car gene cluster motivated further investigations using IS-based PCR. An IS-based PCR with primers IS6100-F1 and IS6100-R1 targeting the IS6100 element resulted in two distinct PCR products of about 4.3 and 2.8 kb, respectively (Figure S1B, lane 1). The 4.3 and 2.8-kb fragments were ligated to pMD18-T to generate plasmid p6100-1 and p6100-2. DNA sequencing and BLAST search suggested that there were two partial IS6100 at each end as expected in the 4.3 and 2.8-kb fragments. The 4.3-kb fragment contains ORF1-carRAa-BaBbCAc-ORF8 from TnCar.

BLAST search suggested that in the 2.8-kb fragment, there were two truncated ORFs and one intact ORF (Fdr) that showed 62% identity to the FdrI of strain KA1 [10]. A sequence comparison showed that the amino acid sequence of Fdr was homologous over its entire length to other members of the FAD-dependent pyridine nucleotide reductase family, containing a flavin binding domain for FAD (consensus sequence TX₅X₁₇X₁₈X₁₉) and two ADP binding domains (for FAD and NADH, respectively) with the consensus sequence GXXGXXGXXA [17,18,19]. Two truncated ORFs encoded for a putative uncharacterized protein and a transcriptional regulator, were found upstream and downstream of fdr (Table 1). Sequencing of PCR product revealed the existence of entire copies of IS6100 at both ends of the 2.8-kb fragment. These two IS6100 were in the same direction and formed a composite transposon (IS6100-fdr-IS6100) designated TnFdr, tentatively.

TnCar and TnFdr were each flanked by two copies of IS6100 in tail-to-head configuration. Although tail-to-head was the most abundant case for two copies of IS6100, other configurations were also reported [14]. Early attempts to amplify head-to-head and tail-to-tail configurations proved to be unsuccessful. Considering that the amplification of head-to-head and tail-to-tail configurations may be suppressed by intramolecular hybridization between two IS6100 copies during the primer annealing phase of PCR, TaKaRa LA Taq and GC Buffer I were used in the following experiments. One specific fragment (5.2 kb in size, Figure S1C, lane 3) was amplified using IS6100-R1, while no specific fragment was amplified under the same PCR conditions using IS6100-F1 (Figure S1C, lane 2). The 5.2-kb fragment was gel-purified and cloned to pMD18-T to generate p6100-3, and the nucleotide sequence of the 5.2 kb insert was determined. In the sequenced region, five intact ORFs were found to be almost identical (only one bp mismatch) to antR, antK, antId, antAb, and antAc (Figure 2C). Strain XLDN2-5 ANTDO was a three-component dioxygenase and composed of a two-subunit oxygenase (antAcAb), a Rieske-type ferredoxin (antAb), and a ferredoxin reductase (antAc). antR, encoding a putative transcriptional activator, was in the same direction of antKAbAbAk, which was different from that of Burkholderia cepacia DBO1 whose antR is in the opposite direction to its structure genes [20]. In the sequenced region, there were two partial IS6100 at each end as expected. The existence of intact IS6100 elements at both ends was confirmed by PCR and PCR product sequencing. These two IS6100 elements were in the head-to-head configuration and formed a composite transposon designated TnInt, tentatively.

The positional relation of TnCar, TnFdr and TnInt

There were two copies of IS6100 elements at both ends of the three transposon-like entities except that the upstream flanking IS6100 on TnCar was disrupted by a novel insertion sequence ISsp1. It was likely that one IS6100 element was shared by two transposon-like units. In order to analyze the positional relation of TnCar, TnFdr, and TnInt, five primers were designed outside of the IS6100 elements (Figure S2A). If there was a shared IS6100 element, a 0.9-kb DNA fragment containing IS6100 could be amplified by PCR using different primer pairs. As shown in Figure S2B, the expected 0.9-kb fragment could only be amplified using primers TnCar-F1 and TnFdr-R1. These results suggested that TnFdr shared an IS6100 element with TnCar, but not with TnInt. The conclusion was confirmed by second-round PCR (Figure S2C and S2D). Thus, we renamed TnCar and TnFdr to the car locus, and TnInt to the ant locus.

Description of car and ant loci

The structures of car locus and ant locus are depicted in Figure 2 and the ORFs are given in Table 1. Both car cluster and fdr gene were sandwiched between two copies of IS6100. Interestingly, two identical copies of the IS6100 element also flanked the ant gene cluster, possibly making a composite transposon. All five sequenced copies of IS6100 from strain XLDN2-5 were identical over the entire 883 bp. Direct repeats (TGCGCAGG) were found directly upstream and downstream of ISsp1, whereas no direct repeat was found outside of the IS6100 box. Furthermore, the ISsp1 consisted of inverted repeats of 32 bp, of which only one base pair was not identical, and a 1224-bp ORF (mpAmpSsp1), encoding a 407 aa putative transposase that showed similarities to transposases of the IS256 family. In order to illustrate that the genes on these two loci were really working in the degradation of carbazole in strain XLDN2-5, transcriptional and functional analyses were performed.

Transcriptional analyses of car and ant genes

The expression of the genes presenting on the two loci was studied by reverse transcription (RT)-PCR experiments. The primer sets for the carAaBaBbCc, fdr, and antKAbAbAk genes could amplify DNA fragments with the expected sizes (Figure 5). No fragments could be amplified using RNA from glucose-grown XLDN2-5 cells as a template (data not shown). These results revealed that carAaBaBbCc, fdr, and antKAbAbAk genes were expressed in carbazole-grown XLDN2-5 cells, suggesting that the gene products should be involved in the transformation of carbazole to catechol. These results also indicated that carAaBaBbCc and antKAbAbAk gene clusters were operonic. In order to confirm that Fdr was active in the CARDO system, functional analyses were performed.

Functional analyses of putative CARDO and ANTD0

Biotransformation experiments were carried out using E. coli cells expressing putative CARDO components (CarAa, CarAc and...
Table 1. Coding regions of car locus and ant locus.

| Protein | Position (bp) in sequence (direction) | Length (amino acids) | Putative function | Homologous protein |
|---------|--------------------------------------|----------------------|------------------|-------------------|
| car locus |                                      |                      |                  |                   |
| TnpA<sub>6100</sub> | 1116–1263; 2594–3240 (c) | 264 | Transposase of IS6100 | 100 Tnp (Q79A56) Mycobacterium fortuitum |
| TnpA<sub>6100</sub> | 1321–2544 (n) | 407 | Transposase, mutator type | 99 Tnp (A5VHF2) Sphingomonas wittichii RW1 |
| ORF1 | 3272–3400 (n) | 42 | Integrase family protein (truncated, only C-terminal portion) | 100 ORF7 (Q84H11) Novosphingobium sp. KA1 |
| CarR | 3621–4301 (c) | 226 | Transcriptional regulator of car operon, GntR family | 99 CarR (Q84H00) Novosphingobium sp. KA1 |
| CarAa | 4404–5540 (n) | 378 | Terminal oxygenase component of carbazole 1,9a-dioxygenase | 99 CarAa (Q84IG9) Novosphingobium sp. KA1 |
| CarBa | 5489–5821 (n) | 110 | small subunit of meta cleavage enzyme | 100 CarBal (Q84IG8) Novosphingobium sp. KA1 |
| CarBb | 5814–6617 (n) | 267 | large subunit of meta cleavage enzyme | 99 CarBbl (Q84IG7) Novosphingobium sp. KA1 |
| CarC | 6660–7484 (n) | 274 | Meta cleavage compound hydrolase | 99 CarC (Q84IG6) Novosphingobium sp. KA1 |
| CarAc | 7525–7854 (n) | 109 | Ferredoxin component of carbazole 1,9a-dioxygenase | 100 CarAc (Q84IG5) Novosphingobium sp. KA1 |
| ORF8 | 7896–8117 (n) | 74 | TonB-dependent receptor (truncated, only N-terminal portion) | 100 ORF35 (Q84IG4) Novosphingobium sp. KA1 |
| TnpA<sub>6100</sub> | 8172–8966 (c) | 264 | Transposase of IS6100 | 100 Tnp (Q79A56) Mycobacterium fortuitum |
| ORF9 | 8998–9545 (n) | 182 | Putative uncharacterized protein (truncated, only C-terminal portion) | 34 Q74F08 Geobacter sulfurreducens |
| Fdr | 9573–10817 (n) | 407 | Ferredoxin reductase component of carbazole 1,9a-dioxygenase | 62 Fdr (Q2PFP9) Novosphingobium sp. KA1 |
| ORF10 | 10936–11486 (c) | 182 | Transcriptional regulator, TetR family (truncated, only C-terminal portion) | 40 Q1NF20 Sphingomonas sp. SKAS8 |
| TnpA<sub>6100</sub> | 11541–12335 (c) | 264 | Transposase of IS6100 | 100 Tnp (Q79A56) Mycobacterium fortuitum |
| ant locus |                                      |                      |                  |                   |
| TnpA<sub>6100</sub> | 55–849 (c) | 264 | Transposase of IS6100 | 100 Tnp (Q79A56) Mycobacterium fortuitum |
| AntR | 1009–1965 (n) | 318 | Transcriptional regulator, AraC family | 100 AndR (Q0KUJ2) Novosphingobium sp. KA1 |
| AntAc | 2163–3446 (n) | 427 | Anthranilate 1,2-dioxygenase large subunit | 100 AndAc (Q0KUJ3) Novosphingobium sp. KA1 |
| AntAd | 3451–3921 (n) | 156 | Anthranilate 1,2-dioxygenase small subunit | 100 AndAd (Q0KUJ4) Novosphingobium sp. KA1 |
| AntAb | 3935–4243 (n) | 102 | Ferredoxin component of anthranilate 1,2-dioxygenase | 100 AndAb (Q0KUJ5) Novosphingobium sp. KA1 |
| AntAa | 4364–5605 (n) | 413 | Ferredoxin reductase component of anthranilate 1,2-dioxygenase | 100 AndAa (Q0KUJ6) Novosphingobium sp. KA1 |
| TnpA<sub>6100</sub> | 6183–6977 (n) | 264 | Transposase of IS6100 | 100 Tnp (Q79A56) Mycobacterium fortuitum |

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Discussion

Starting from the carAaBbBaBb fragment isolated from Sphingomonas sp. strain XLDN2-5, a combination of southern blot, genome walking, and IS-based PCR led to the isolation of two loci containing genes involved in the conversion of carbazole from anthranilate to catechol. The DNA fragments obtained are illustrated in Figure 2, and the proposed reaction details are given in Figure 1. All genes were embedded in transposon-like entities, implying the likely involvement of horizontal gene transfer in the evolution of carbazole degradation pathway. Transcriptional and functional analyses suggested that all the genes worked in the degradation of carbazole. The CARDO system catalyzed angular dioxygenation and lateral dioxygenation of carbazole, and ANTDO could degrade sphingomonad strains isolated from geographically dispersed locations, the organization of these genes in strain XLDN2-5 was unique. Figure 5 shows the organization of the known car genes (Figure 5A) and ant genes (Figure 5B) from different evolutionary origins. There were two truncated ORF1 and ORF8 encoding a putative integrase and a TonB-dependent receptor (Table 1), respectively. ORF1 and ORF8 showed 100% identity to ORF42 and ORF35 in strain KA1 [21], respectively. In strain KA1, ORF42 and ORF35 are intact (Figure 5A), whereas the corresponding ORFs were interrupted by IS6100 in strain XLDN2-5. Furthermore, BLAST search revealed that the

Figure 4. Biotransformation of substrates and accumulation of products. (A) Biotransformation of carbazole ( ) and accumulation of 2'-aminobiphenyl-2,3-diol ( ) by E. coli DH5α harboring pUCarAcba. (B) Biotransformation of anthranilate ( ) and accumulation of catechol ( ) by E. coli DH5α harboring pUantAcdb. E. coli DH5α harboring pUC19 ( - - ) served as controls. The initial concentrations of carbazole and anthranilate were 2 mM and 1 mM, respectively. Values are means of three replicates ± SD.

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Figure 3. Electrophoresis results of RT-PCR. carAaBaBb (lane 1), carBbC (lane 2), carCAc (lane 3), fdr (lane 4), antAa (lane 5) and antAcAdAb (lane 6). Samples containing no reverse transcriptase (No RT) are also shown.

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terminal 27-bp region of the reported sequence (1–55 bp in AF442494) in strain GTIN11 is identical to the 27-bp left end of IS6100. This suggested that the carR gene in strain GTIN11 might be disrupted by IS6100 element. However, the entire sequence information outside the car gene in strain GTIN11 was not reported.

Interestingly, the ant cluster in strain XLDN2-5 was also bordered by copies of IS6100 elements. The organization of ant gene cluster was clearly different from that of KA1, in which carF, the upstream region of ant cluster, encodes acetyl-CoA acetyltransferase, while the downstream ORF16 encodes a transposase (Figure 5B). Although there are many ISs belonging to different transferase, while the downstream ORF16 encodes a transposase elements. The organization of CARDO from XLDN2-5 and KA1 are identical, the reductase components have a much lower level of similarity (62%). In general, the genes encoding the components of the known Rieske oxygenases were found closely together in tightly regulated transcriptional units, as was observed with the car cluster in strain CA10 (Figure 5A) [26]. However, it is becoming increasingly evident that the genes for catabolic pathways in sphingomonads often locate separately from each other. Recently, it is reported that multiple carbazole degradation genes dispersed on four loci (two car loci, one fdx/fdhI locus and one fdhII locus) on pCAR3 in Novosphingobium sp. strain KA1 [10]. The fdx/fdhI locus located 50 and 85 kb downstream of carI and carII gene clusters, while the fdhII gene located about 80 and 115 kb downstream of carI and carII gene clusters. Additional evidence has also been presented for the genes involved in the degradation of PAH by Sphingobium yanoikuyae B1 and Q1, and Novosphingobium aromaticivorans F199 [11,12,27], dibenzofuran by Sphingomonas wittichii RW1 [28], pentachlorophenol by Sphingomonas chlorophenica ATCC 39723 [29], and γ-hexachlorocyclohexane by Sphingomonas paucimobilis UT26 [30]. These results suggested that loose association with reductase components might be the characteristic for this type of dioxygenase, which became more independent from the reductase component during evolution.

In the course of cloning car genes, we also discovered a novel insertion sequence ISSp1 (see results, IS6100::ISSp1), which insert into the upstream copy of IS6100. ISSp1 can be classified as the mutator family that consists of transposases from prokaryotes and eukaryotes. There was only one example that an IS could insert into another in Comamonas sp. strain JS46 [31]. Thus, it
would be interesting to investigate the distribution of IS6100 in strain XLDN2-5 and its function in the establishment of the carbazole catabolic gene structure.

Materials and Methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2. Sphingomonas sp. strain XLDN2-5 utilizes carbazole as the sole carbon and nitrogen source [2-3]. Strain XLDN2-5 was grown in mineral salt medium (MSM) as previously described [33], and carbazole was added as a 200 mM filter-sterilized stock solution in dimethyl sulfoxide (DMSO). Escherichia coli DH5z was used as the recipient strain in all cloning experiments. E. coli strains were grown in Luria-Bertani (LB) broth at 37°C. Ampicillin (Amp), when required, was added to a final concentration of 100 μg mL⁻¹.

DNA manipulation

Total DNA from pure cultures of Sphingomonas sp. strain XLDN2-5 was extracted using the Wizard® Genomic DNA Purification Kit according to the recommendations of the manufacturer (Promega Corp., Madison, WI). Restriction endonucleases and T4 DNA ligase were used according to the manufacturer (Promega Corp., Madison, WI). Purification Kit according to the recommendations of the manufacturer (Qiagen Corp., Germany). Isolations of DNA fragments from agarose gels were accomplished with the Qiaex II Gel Extraction Kit (Qiagen Corp., Germany). Transformations and agarose gel electrophoresis were carried out using standard methods [34].

Construction of genomic library

The genomic DNA of Sphingomonas sp. strain XLDN2-5 was mechanically sheared. DNA fragments of 6 to 8 kb were gel purified and used for library construction. The fragments were ligated into pUC19 digested with EcoRV, and dephosphorylated with shrimp alkaline phosphatase (Promega). The library was transformed into electrocompetent E. coli DH5z. After incubation at 37°C for 1 h, cells were spread on LB agar plates supplemented with Amp, isopropyl-β-D-galactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Following overnight incubation at 37°C, plates were scored for white colonies. The white colonies were used for southern blotting using a car probe obtained by PCR.

All PCR amplifications were performed with an Eppendorf Authorized Thermal Cycler (Germany) in 50-μl reaction systems containing 5 μl of 10× buffer, 1.5 mM MgCl2, 200 μM dNTPs, 500 pmol of each primer, 10-100 ng of the template DNA, and 2.5 units of DNA polymerase. TransStart FastPfu polymerase (TransGen Biotech Co. Ltd. China) was used in PCRs whose products were used for the construction of plasmids. TaKaRa LA PCR Kit Ver. 2.1 was used for IS-based PCR. Taq polymerase (Generay Biotech Co. Ltd. China) was used in other PCRs. Genome walking was performed using a Genome Walking Kit (TaKaRa) according to the manufacturer’s protocol. The amplified products obtained by Taq polymerase were gel purified and ligated into vector pMD18-T, followed transformation to competent E. coli DH5z. All primers used in this study are listed in Table S1.

Southern blot

Primers pcarF and pcarR were designed according to the carA gene of strain KA1 [21]. Products obtained using primers pcarF and pcarR were gel purified and labeled with DIG-11-dUTP, and the DNAs were transferred following the standard protocols [34]. Southern blot was performed using DIG DNA Labeling and Detection Kit (Roche) according to the manufac-

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Table 2. Bacterial strains and plasmids used.

| Strain or plasmid | Description | source |
|--------------------|-------------|--------|
| **Bacterial strains** | | |
| E. coli DH5z | F- endA1 hisdR17 supE44 thi-1 recA1 gyrA relA1 Δ(lacZYA-argF) U169 deoR | TransGen |
| Sphingomonas sp. strain XLDN2-5 | Aerobic, rod shaped, degrades carbazole | Lab stock |
| **Plasmids** | | |
| pUC19 | Amp’ lacZ, pMB9 replicon, M13IG | TaKaRa |
| pMD18-T | Clone vector | TaKaRa |
| pEASY-Blunt | Clone vector | TransGen |
| pBY13 | Amp’, pUC19 with 7.8-kb fragment that hybridized with car probe | This study |
| p6100-a | Amp’, pMD18-T with 1-kb fragment obtained by genome walking | This study |
| p6100-1 | Amp’, pMD18-T with 4.3-kb fragment obtained by IS-based PCR using pIS6100-F1 and pIS6100-R1 | This study |
| p6100-2 | Amp’, pMD18-T with 2.8-kb fragment obtained by IS-based PCR using pIS6100-F1 and pIS6100-R1 | This study |
| P6100-3 | Amp’, pMD18-T with 5.2-kb fragment obtained by IS-based PCR using pIS6100-R1 | This study |
| pUcarAa | Amp’, pUC19 with 1.2-kb SphI-XbaI fragment containing the carA gene of strain XLDN2-5 | This study |
| pUcarAc | Amp’, pUC19 with 0.3-kb XbaI-KpnI fragment containing the carA gene of strain XLDN2-5 | This study |
| pUfdr | Amp’, pUC19 with 1.3-kb KpnI-EcoRI fragment containing the fdr gene of strain XLDN2-5 | This study |
| pEcArAaAc | Amp’, Kα’, pEASY-Blunt with 1.5-kb fragment containing the carAa and carAc genes of strain XLDN2-5 | This study |
| pUcarAaAcfdr | Amp’, 1.5-kb SphI-KpnI fragment containing carAaAc from pUcarAaAc cloned into SphI-KpnI site of pUC19 | This study |
| pUcarAaAcfdr | Amp’, 1.5-kb SphI-KpnI fragment containing carAaAc from pUcarAaAc cloned into SphI-KpnI site of pUfdr | This study |
| pUantAAdAb | Amp’, pUC19 with 2.1-kb HindIII-EcoRI fragment containing the antAAdAb genes of strain XLDN2-5 | This study |
| pUantAAdAbAa | Amp’, pUC19 with 3.5-kb HindIII-EcoRI fragment containing the antAAdAbAa genes of strain XLDN2-5 | This study |

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amplifications are provided in Table S1. Control experiments
primer sets and the conditions employed for respective gene
kit (Takara) was used for RT-PCR, in which 100 ng of total RNA
harvested and used for extraction of total RNA using E. Z. N. ATM
reciprocal shaking (300 strokes/min) at 30°C for 16 h. Then the cells were
harvested by centrifugation (6,000 g, 10 min, 4°C), washed twice with
MSM, and resuspended in MSM to an OD_{600} of 10. Fifty
microliters of carboxylic acid (200 mM in DMSO) or anthranilic acid
(200 mM in DMSO) was added to 10 ml of cell suspensions. After
incubation on a reciprocal shaker (200 rpm) at 37°C for 20 h, the
mixtures were extracted with an equal volume of ethyl acetate.
After derivatization with N,O-bis(trimethylsilyl)trifluoroacetamide
(BSTFA) (Sigma) the extracts were analyzed by gas chromatog-
raphy-mass spectrometry (GC-MS) as described previously [2,33].

High performance liquid chromatography was carried out to
analyze the aqueous samples using an Agilent 1200 series
instrument equipped with a variable-wavelength detector and a
reversed-phase C18 column (4.6 mm×150 mm, Hewlett-Pack-
ard). Residual concentrations of carboxylic acid and anthranilic acid
and the formation of 2'-aminobiphenyl-2,3-diol and catechol were
determined using a mobile phase of an 80:20 mixture of methanol
and deionized water at a flow rate of 0.5 ml min^{-1}.

Nucleotide sequence accession numbers
The nucleotide sequences of the car locus and ant locus have been deposited in GenBank under accession numbers GU123624 and GU123625, respectively.

Supporting Information
Figure S1  IS6100-based PCR. (A) Schematics of relative
configurations of two copies of IS6100. The position of primers
IS6100-F1, IS6100-F2, IS6100-R1 and IS6100-R2 are shown by
arrows. (B and C) Gel electrophoresis of DNA fragments amplified
from XLDN2-5 by IS6100-based PCR using IS6100-F1 and
IS6100-R1 (lane 1), single IS6100-F1 (lane 2) and single IS6100-
R1 (lane 3). Two fragments (4.3 kb and 2.8 kb in size, Figure S1B,
lane 1) were amplified using primers IS6100-F1 and IS6100-R1.
One specific fragment (5.2 kb in size, Figure S1C, lane 3) using
IS6100-R1 was amplified, while no specific fragment was
amplified under the same PCR conditions using IS6100-F1 (lane
2). (D) Second-round PCR (lane 4) with primer IS6100-F2 using
the first-round PCR product as a template. Found at: doi:10.1371/journal.pone.0010018.s001 (1.12 MB TIF)

Figure S2  Determination of the positional relation of TnCar,
TnFdr and TnAnt. (A) The positions of primers TnCar-F1,
TnFdr-F1, TnFdr-R1, TnAnt-F1, and TnAnt-R1 with blue
arrowheads showing their directions. (B) Agarose gel electropho-
resis of DNA fragments amplified from the genomic DNA of
XLDN2-5 by PCR using TnCar-F1 and TnFdr-R1 (lane 1),
TnCar-F1 and TnAnt-R1 (lane 2), TnCar-F1 and TnAnt-F1 (lane
3), TnFdr-F1 and TnAnt-R1 (lane 4), TnFdr-F1 and TnAnt-F1
(lane 5), TnFdr-R1 and TnAnt-R1 (lane 6), and TnFdr-R1 and
TnAnt-F1 (lane 7). (C) PCR results using primers carC-sp3 and
fdr-r2 (lane 8-12). (D) PCR results (no specific bands) using primers
fdr-f2 and antAc-r2 (lane 13-17), and fdr-f2 and antAa-f2 (lane
18-22). Found at: doi:10.1371/journal.pone.0010018.s002 (1.06 MB TIF)

Figure S3  Mass spectra for the products of carboxylic acid
transformed by E. coli DH5α harboring pUCarAAacFdr. GC-MS
analysis was performed after trimethylsilylation with BSTFA.
Compound I: 2'-aminobiphenyl-2,3-diol; compound II: hydroxy-
carbazole. Found at: doi:10.1371/journal.pone.0010018.s003 (0.19 MB TIF)
Table S1  Oligonucleotides used in this study for the cloning of genes, genome walking, and the construction of plasmids.  

Found at: doi:10.1371/journal.pone.0010018.s004  (0.05 MB DOC)

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Author Contributions

Conceived and designed the experiments: ZG PX. Performed the experiments: ZG XW XU. CT. Analyzed the data: ZG HT XH PX. Contributed reagents/materials/analysis tools: GW ZD PX. Wrote the paper: ZG PX.

Carbazole-Degrading Genes