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Cloning and Nucleotide Sequences of Carbazole Degradation Genes from Marine Bacterium *Neptuniibacter* sp. Strain CAR-SF

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Abstract The marine bacterium *Neptuniibacter* sp. strain CAR-SF utilizes carbazole as its sole carbon and nitrogen sources. Two sets of clustered genes related to carbazole degradation, the upper and lower pathways, were obtained. The marine bacterium genes responsible for the upper carbazole degradation pathway, *carAa*, *carBa*, *carBb*, and *carC*, encode the terminal oxygenase component of carbazole 1,9a-dioxygenase, the small and large subunits of the *meta*-cleavage enzyme, and the *meta*-cleavage compound hydrolase, respectively. The genes involved in the lower degradation pathway encode the anthranilate dioxygenase large and small subunit AntA and AntB, anthranilate dioxygenase reductase AntC, 4-oxalocrotonate tautomerase, and catechol 2,3-dioxygenase. Reverse transcription-polymerase chain reaction confirmed the involvement of the isolated genes in carbazole degradation. *Escherichia coli* cells transformed with the CarAa of strain CAR-SF required ferredoxin and ferredoxin reductase for biotransformation of carbazole. Although *carAc*, which encodes the ferredoxin component of carbazole 1,9a-dioxygenase, was not found immediately downstream of *carAaBaBbC*, the *carAc*-like gene may be located elsewhere based on Southern hybridization. This is the first report of genes involved in carbazole degradation isolated from a marine bacterium.

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Introduction

Carbazole, an environmental pollutant, is a heteroaromatic compound containing nitrogen found in coal–tar creosote. Because, bioremediation has proven to be an effective technique for cleaning sites polluted by persistent compounds such as carbazole, carbazole-degrading bacteria namely *Pseudomonas resinovorans* strain CA10 [20, 21], *Novosphingobium* sp. strain KA1 [6], *Janthinobacterium* sp. strain J3 [11], *Pseudomonas stutzeri* strain OM1 [17], *Sphingomonas* sp. strain CB3 [22], and *Nocardioides aromaticivorans* strain IC177 [10] have been isolated and studied. However, these are all terrestrial bacteria, and no genetic study has been performed on carbazole-degrading marine bacteria. Previously, our group has reported on the isolation and characterization of *car* gene cluster from the marine isolate strain OC7 [14]. Even though this strain was isolated from marine environments, it showed closest relation to bacteria in the *Lysobacter* genus and no evidence being a marine bacterium. The carbazole-degrading bacterium *Neptuniibacter* sp. strain CAR-SF was isolated from seawater [4]. In contrary to strain OC7, growth tests using artificial seawater mineral salt plates showed no growth of strain CAR-SF when NaCl was not present. Bacteria degrade carbazole by conversion to 2-hydroxypenta-2,4-dienoate and anthranilate via dioxygenation by carbazole 1,9a-dioxygenase (CarA), aromatic ring cleavage by the *meta*-cleavage enzyme (CarB), and hydrolytic cleavage by the *meta*-cleavage compound hydrolase (CarC). Next, 2-hydroxypenta-2,4-dienoate is converted to TCA cycle intermediates by 2-hydroxypenta-2,4-dienoate

hydratase (CarD), 4-hydroxy-2-oxovalerate aldolase (CarE), and acetaldehyde dehydrogenase (CarF). Anthranilate is also converted to TCA cycle intermediates via dioxygenation by anthranilate 1,2-dioxygenase (AntABC), spontaneous deamination, decarboxylation, and further degradation [16]. In this study, we isolated the first carbazole degradation genes from a marine bacterium strain CAR-SF. The genes were characterized by reverse transcription-polymerase chain reaction (RT–PCR) and biotransformant products were confirmed by GC/MS using resting cell reactions with *Escherichia coli*.

Materials and Methods

Gene Isolation

Total DNA of carbazole-utilizing bacterium CAR-SF was extracted from cells grown in artificial seawater mineral salts medium ONR7a [1] with carbazole as the sole carbon source at 30°C using standard protocols. Extracted total DNA was digested with *Eco*RI or *Eco*RV before ligation into plasmid vectors pUC119 or pBluescript II SK(−) linearized using similar restriction endonuclease and treated with calf intestinal alkaline phosphatase (Takara Bio Inc., Shiga, Japan). *E. coli* DH5alpha was transformed by recombinant plasmids constructed. Clones were screened for *meta*-cleavage enzyme activity by spraying with 2,3-dihydroxybiphenyl-acetone solution. Nucleotide sequences were determined as previously reported [13]. To analyze the obtained sequences, DNASIS Pro software (version 2.8; Hitachi Software Engineering Co. Ltd., Kanagawa, Japan) was employed. The BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) was used for homology searches and CLUSTAL W algorithm provided in DNASIS Pro was used to align sequences. The nucleotide sequences presented in this study have been deposited in DDBJ, EMBL, and GenBank nucleotide sequence databases under accession numbers AB365208 and AB365209.

Construction of Expression Plasmids

To express the carbazole dioxygenase genes under control of the *lac* promoter in *E. coli*, the recombinant plasmids shown in Table 1 were constructed. The forward primers were designed to add an *Xba*I site and a Shine–Dalgarno sequence upstream of the initial codon. The reverse primers were designed to add a *Spe*I site downstream of the stop codon. Genes *carA*a and *orf6* were amplified from pBSF101 and pUSF01. Genes *carAc* and *carAd* from strain CA10, and *carAcI* from strain KA1 were amplified from pUCA1 [20] and pBKA102 [11]. Amplified products were cloned into pT7Blue(R) T-vector (Novagen, Madison, WI, USA) before subcloning into pBluescript II SK(−) to produce each designated plasmids.

RNA Extraction and RT–PCR

Total RNA samples were extracted on strains grown in ONR7a containing 0.1% (w/v) carbazole or 0.1% (w/v) anthranilate for 24 h. Samples from strain grown with 0.1% (w/v) succinate was used as negative control. For RT–PCR, primers were designed to amplify a 300-bp fragment of 16S rRNA, *carAa*, and *antA*. RT–PCR was run as follows: 50°C for 30 min; 94°C for 2 min; 30 cycles of 94°C for 2 min, 55°C for 15 s and 72°C for 30 s; and 72°C for 5 min. Total RNA extractions and RT–PCR preparations were conducted as reported previously [14]. The primer sets used were CarAa-L (5′-gctcaagctccttggtgaac-3′) and CARAa-R (5′-ggaggggtatctccatcacc-3′) specific for *carAa* gene, AntA-L (5′-ataagaccgacggcaaactg-3′) and AntA-R (5′-aaccatcaagaccgttttcg-3′) specific for *antA* gene, 16S-L (5′-tcgggaactctgagacaggt-3′) and 16S-R (5′-ttcatggagtcgagttgcag-3′) specific for 16S rDNA. Intensities of gene transcripts were measured and compared using Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Resting Cell Reaction

Resting cell reaction experiments were prepared as reported previously [14]. 0.01% (w/v) carbazole was used and reaction was continued for 24 h at 37°C. Reaction contents were extracted with the same volume of ethyl acetate, dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure. After trimethylsilylation with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (Nacalai Tesque, Kyoto, Japan) at 70°C for 20 min, each sample was injected into a GC/MS system (Thermo Electron, Kanagawa, Japan). Plasmids pUCARA [21] and pBluescript II SK(−) were used as positive and negative controls, respectively. The carbazole conversion ratio of each sample was determined by the formula given in Urata et al. [25]. All experiments were carried out in triplicate.

Results and Discussion

Isolation and Identification of *car* Gene Cluster

Three different plasmids were isolated and identified from clones showing *meta*-cleavage activity. A plasmid containing 4.2-kb fragment of *Sca*I-digested total DNA ligated into *Eco*RV-digested pBluescript II SK(−) was designated pBSF101. Two other plasmids containing a 6.8- and a 6.7-kb fragment of *Eco*RI-digested total DNA ligated into *Eco*RI-digested pUC119 were designated pUSF01 and

Table 1 Bacterial strains and plasmids used in this study

Plasmid	Relevant characteristics	Source or reference
Bacterial strains		
E. coli DH5α	F$^-$, 80d*lacZ*ΔM15, Δ(*lacZYA–argF*)U169, *deoR*, *recA1*, *endA1*,*hsdR17*(r_k^-, m_k^+), *phoA*, *supE44*, λ^-, *thi–1*, *gyrA96*, *relA1*	Takara Bio
E. coli JM109	*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*(r_k^-, m_k^+), *e14*$^-$(*mcrA*$^-$), *supE44*, *relA1*, Δ(*lac–proAB*)/*F*′[*traD36*, *proAB*$^+$, *lacI*^{q}, *lacZΔM15*]	Takara Bio
Neptuniibacter sp. strain CAR-SF	Carbazole degrading marine bacterium	[4]
Plasmids		
pBluescript SK(−)	Ap^r, *lacZ*	Stratagene
pT7Blue(R)	Ap^r, *lacZ*	Novagen
pUC119	Ap^r, *lacZ*	Takara Bio
pBSF101	Ap^r, pBluescript SK(−) with 4.2-kb *Sca*I fragment of CAR-SF DNA	This study
pUSF01	Ap^r, pUC119 with 6.8-kb *Eco*RI fragment of CAR-SF DNA	This study
pUSF09	Ap^r, pUC119 with 6.7-kb *Eco*RI fragment of CAR-SF DNA	This study
pBSF1	Ap^r, pBluescript SK(−) with *carAa* gene of CAR-SF	This study
pBSF2	Ap^r, pBluescript SK(−) with *carAa* and *orf6* genes of CAR-SF	This study
pBSF3	Ap^r, pBluescript SK(−) with *carAa* gene of CAR-SF and *carAc* gene of CA10	This study
pBSF4	Ap^r, pBluescript SK(−) with *carAa* gene of CAR-SF and *carAcI* gene of KA1	This study
pBSF5	Ap^r, pBluescript SK(−) with *carAa* gene of CAR-SF and *carAd* gene of CA10	This study
pBSF6	Ap^r, pBluescript SK(−) with *carAa* gene of CAR-SF and *carAcAd* genes of CA10	This study
pBKA102	Ap^r, pBluescript SK(−) with 8.7-kb *Hin*dIII fragment of *Novosphingobium* sp. KA1 DNA	[11]
pUCA1	Ap^r, pUC119 with 6.9-kb *Eco*RI fragment of *P. resinovorans* CA10 DNA	[20]
pUCARA	Ap^r, pUC119 with 5.6-kb *Eco*RI fragment of *P. resinovorans* CA10 DNA	[21]

pUSF09. Plasmids pBSF101 and pUSF01 contained genes of the upper pathway and pUSF09 contained the genes responsible for the lower pathway degradation (Fig. 1). Nine complete open reading frames (ORFs) were identified from pBSF101 and pUSF01, among them five involved in carbazole degradation (Table 2). The deduced amino acid sequence of ORF2 had 77% identity to the terminal oxygenase component of carbazole 1,9a-dioxygenase (CarAa) from *Janthinobacterium* sp. strain J3. Strain J3 is known to possess carbazole degrading genes (*car* genes) with high similarity to *P. resinovorans* strain CA10 [11]. A Rieske-type [2Fe–2S] cluster and mononuclear nonheme iron-binding site [15, 18] motifs were also conserved. ORF3 and ORF4 were deduced as the small and large subunits of 2′-aminobiphenyl-2,3-diol 1,2-dioxygenase and showed 48 and 59% similarity with CarBa and CarBb of strain CA10. ORF5 gene product showed 67% identity with the *meta*-cleavage compound hydrolase (CarC) from strain J3. ORF6 showed a low similarity to Rieske [2Fe–2S] domain protein such as the ferredoxin component from *Burkholderia thailandensis* strain E264 (16% identity; [12]) and from strain J3 (12% identity). Notably, the 5′ terminal region of ORF6 showed 33% identity with $CarAc_{J3}$ and $CarAc_{CA10}$. However, no conserved motif of a Rieske [2Fe–2S] iron–sulfur protein was found. The ORF2 until ORF5 (*carAaBaBbC*) formed the *car* gene cluster which is similar to *car* gene clusters of strain CA10 and J3 (*carAaAaBaBbCAc*ORF7*Ad*). Even though the ferredoxin component was not included in the isolated fragments, a weak signal of a $CarAc_{CA10}$-like ferredoxin was detected from the total DNA extract using Southern hybridization (result not shown). This result suggested that a possible functional ferredoxin for $CarAa_{CAR\text{-}SF}$ existed but separated from the isolated *car* gene cluster. Plasmid pUSF09 contained six complete ORFs and one partial ORF (Fig. 1 and Table 3). The partial ORF10 was deduced to be a putative AraC/XylS-type transcriptional regulator and orientated similarly to the same gene found in *Pseudomonas fluorescens* strain MB214 [19]. The deduced amino acid sequences of ORF11 and ORF12 were similar to the large and small subunits of anthranilate 1,2-dioxygenase and showed 72 and 61% identity with AntA and AntB of strain CA10 [25]. A Rieske-type [2Fe–2S] cluster-binding site motif and mononuclear nonheme iron-binding site motif were also conserved. ORF13 gene product containing a plant-type [2Fe–2S] cluster-binding motif and FAD/NAD-binding domains showed 53% identity to anthranilate dioxygenase reductase (AntC) of *Acinetobacter* sp. strain ADP1 [2]. ORF15 gene product showed similarity to a *meta*-cleavage enzyme catechol 2,3-dioxygenase from various bacteria

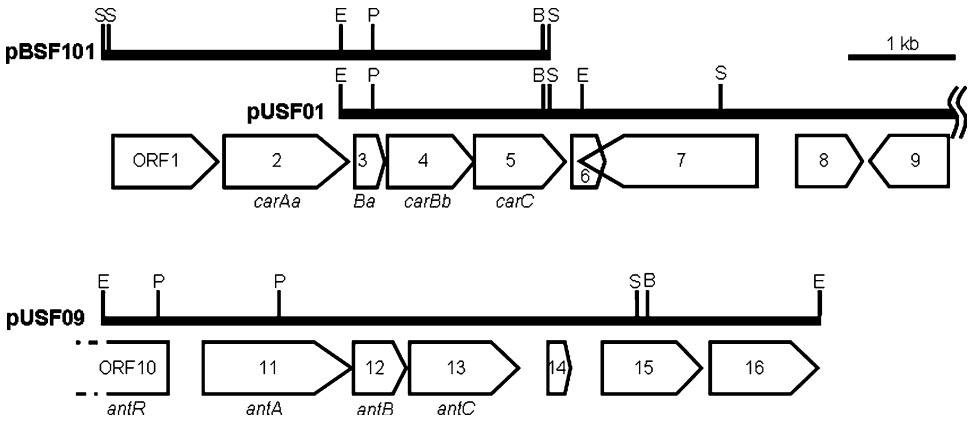

Fig. 1 Organization of the genes responsible for the upper and lower carbazole degradation pathways of the strain CAR-SF. Restriction sites shown: *B*, *Bam*HI; *E*, *Eco*RI; *P*, *Pst*I; *S*, *Sca*I. Pentagons indicate the direction and region of gene transcription

such as the *Acinetobacter* sp. strain YAA (78% identity; [3]), *P. stutzeri* strain OM1 (77% identity; [17]), and *P. putida* strain G7 (75% identity; [5]). This enzyme showed strong cleaving activity toward 2,3-dihydroxybiphenyl which led to the isolation of this gene cluster. Such strong activity indicated the possibility of this enzyme being able to assist the ring cleavage in the upper pathway of carbazole degradation. The genes of this cluster suggested that strain CAR-SF converts anthranilate to catechol by anthranilate 1,2-dioxygenase (AntABC), similar to strain CA10. However, strain CA10 continues the downstream conversion using an *ortho*-cleavage enzyme, whereas strain CAR-SF presumably utilizes a *meta*-cleavage enzyme. Catechol catabolic genes such as the *xyl* genes of the TOL plasmid pWW0 and the *nah* genes of plasmid NAH7 [7, 8] also convert catechol to a TCA cycle intermediate through *meta*-cleavage. Similarly, the carbazole-degrading *P. stutzeri* strain OM1 metabolizes catechol using the same pathway [17]. Nevertheless, detection of metabolism products is needed to confirm the degradation pathway for strain CAR-SF.

Transcriptional Analyses of *car* and *ant* Genes by RT–PCR

The transcripts of *carA* were detected while *antA* transcripts were not detected when grown with succinate. The *antA* transcripts detected from cells grown with carbazole were approximately 15% more when compared to the same transcripts from anthranilate-grown cells. When cells grown with carbazole or anthranilate, the intensities of *carA* transcripts were 380 and 460%, respectively, in comparison to the intensity of *carA* transcripts from cells grown with succinate (Fig. 2). These results proved the involvement of the isolated genes in carbazole degradation and the expression of both genes were induced when carbazole or anthranilate was supplied. Previously, *car* operon of strain CA10 was reported to be induced by the presence of carbazole or anthranilate [24]. However, *car* operon of strain IC177 was reported to be induced by carbazole but not anthranilate [9]. Thus, the transcriptional mechanism of *car* operon of strain CAR-SF may be similar to the strain CA10. The promoter region of *car* gene cluster was not included in the isolated gene fragments from strain CAR-SF. Thus, it is unknown whether *car* gene cluster of strain CAR-SF and strain CA10 share the same promoter region.

Biotransformation of Carbazole with *E. coli* Cells

Based on GC/MS analyses, no product was detected other than 2′-aminobiphenyl-2,3-diol (the conversion product of carbazole through angular dioxygenation by carbazole 1,9a-dioxygenase and a spontaneous reaction). The mass spectrum of 2′-aminobiphenyl-2,3-diol detected was trimethylsililated [23] for each sample. The carbazole conversion ratios are shown in Table 4. The products of *carAa*, *carAc*, *carAd*, and *orf6* genes from *E. coli* cells harboring plasmids encoding respective genes were detected using 13% SDS polyacrylamide gel (Fig. 3). Peptides with molecular masses of approximately 44, 36, 12, and 11 kDa correspond to the predicted molecular masses of *carAa*, *carAd*, *orf6*, and *carAc* gene products, respectively. *E. coli* JM109 harboring pBSF1 or pBSF2 converted carbazole at the rate of about 1.0%, suggesting that $CarAa_{SF}$ was able to weakly utilize the ferredoxin and ferredoxin reductase available in *E. coli*. Although a part of ORF6 showed low identity to a Rieske-type ferredoxin, it did not function as a ferredoxin. *E. coli* JM109 carrying pBSF3 exhibited a high conversion rate (approximately 86%), implying that $CarAa_{SF}$ is capable of utilizing $CarAc_{CA10}$ efficiently. In addition, *E. coli* JM109 harboring pBSF6 converted carbazole at a rate of over 99%. This result indicates that the carbazole 1,9a-dioxygenase from strain CAR-SF is a multicomponent dioxygenase system consisting of three components, as in other well-known carbazole degrading bacteria [9, 21, 25] even though

Table 2 Homology of deduced ORFs in the 4.2-kb *Sca*I and 6.8-kb *Eco*RI fragments of the strain CAR-SF

ORF[a]	Amino acid residues (% identity)	Deduced function	Similar protein	Accession no.
ORF1	326 (59)	Unknown	Hypothetical protein, *Janthinobacterium* sp. strain J3	BAC56741
ORF2 (*carAa*)	385 (77)	Terminal oxygenase component of carbazole 1,9a-dioxygenase	CarAa, *Janthinobacterium* sp. strain J3	BAC56742
ORF3 (*carBa*)	90 (48)	Small subunit of *meta*-cleavage enzyme	CarBa, *P. resinovorans* strain CA10	BAC41546
ORF4 (*carBb*)	270 (59)	Large subunit of *meta*-cleavage enzyme	CarBb, *P. resinovorans* strain CA10	BAC41547
ORF 5 (*carC*)	282 (67)	*meta*-Cleavage compound hydrolase	CarC, *Janthinobacterium* sp. strain J3	BAC56745
ORF6	101 (16)	Unknown	Naphthalene 1,2-dioxygenase System ferredoxin, *B. thailandensis* strain E264	YP_439699
ORF7	551 (87)	Elongation factor G	Elongation factor G, *Vibrio shilonii* strain AK1	EDL53362
ORF8	203 (71)	tRNA-hydroxylase	tRNA-hydro*xy*1ase, *Marinobacter aquaeolei* strain VT8	YP_959127
ORF9	244 (66)	UDP-2,3-diacy1glucosamine hydrolase	UDP-2,3-diacylglucosamine hydrolase, *Oceanospirillum* sp. strain MED92	ZP_0l168199

[a] ORFs encoding proteins similar to those from aromatic-degrading bacteria are shown

Table 3 Homology of deduced ORFs in the 6.7-kb *Eco*RI fragment of strain CAR-SF

ORF[a]	Amino acid residues (% identity)	Deduced function	Similar protein	Accession no.
ORF10 (*antR*)	205 (45) (partial)	Putative transcrptional regulator	Probable transcriptional regulator, *Pseudomonas aeruginosa* strain PA7	YP_001348087
ORF11 (*antA*)	463 (72)	Anthranilate dioxygenase large subunit	AntA, *P. resinovorans* strain CA10	NP_758548
ORF12 (*antB*)	162 (64)	Anthranilate dioxygenase small subunit	AntB, *P. fluorescens* strain MB214	ABA06560
ORF13 (*antC*)	339 (54)	Anthranilate dioxygenase reductase	AntC, *Acinetobacter* sp. strain ADP1	YP_047248
ORF14	63 (60)	4-Oxalocrotonate tautomerase	4-Oxalocrotonate tautomerase, *Marinobacter algicola* strain DG893	ZP_01892834
ORF15	307 (78)	Catechol 2,3-dioxygenase	Catechol 2,3-dioxygenase, *Acinetobacter* sp. strain YAA	BAA23555

[a] ORFs encoding proteins similar to those from aromatic-degrading bacteria are shown

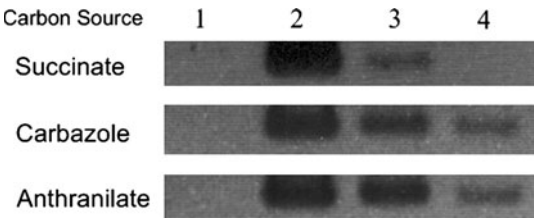

Fig. 2 RT-PCR amplications of 16S rRNA, *carAa*, and *antA* from total RNA extracted from cells when grown with succinate, carbazole, or anthranilate as sole carbon source. *Lane 1*, amplication of 16S rRNA using *Taq* polymerase (negative control); *Lane 2*, amplication of 16S rRNA using RT polymerase (positive control); *Lane 3*, amplication using *carAa* specific primer set; *Lane 4*, amplication using *antA* specific primer ser

Table 4 Carbazole conversion ratios obtained using *E. coli* cells carrying different biotransformation plasmids

Plasmid	Components of carbazole 1,9a-dioxygenase	Carbazole conversion ratio (%)[a]
pBSF1	$CarAa_{SF}$	1.0 (±0.19)
pBSF2	$CarAa_{SF}ORF6_{SF}$	1.1 (±0.23)
pBSF3	$CarAaSFAc_{CA10}$	85 (±2.9)
pBSF4	$CarAa_{SF}AcI_{KA1}$	1.2 (±0.071)
pBSF5	$CarAaSFAd_{CA10}$	1.1 (±0.083)
pBSF6	$CarAaSFAc_{CA10}Ad_{CA10}$	99 (±0.067)

[a] The carbazole conversion ratio (%) is 100 × [total ion current (TIC) peak area of 2′-aminobiphenyl-2,3-diol]/(TIC peak area of 2′-aminobiphenyl-2,3-diol + TIC peak area of carbazole)

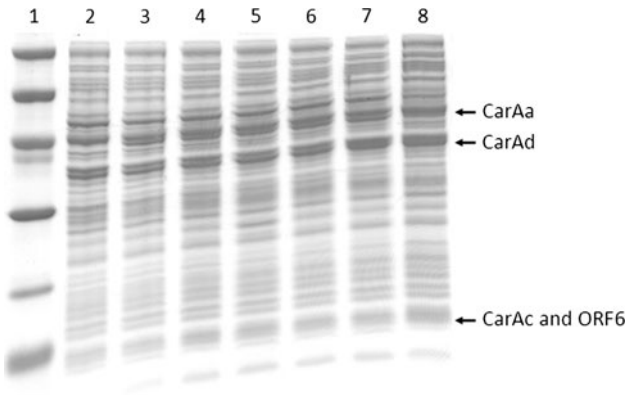

Fig. 3 Detection of the products of *carAa*, *carAc*, *carAd*, and *orf6*. Total cellular proteins of *E. coli* strains were analyzed by SDS–PAGE. *Lane 1*, molecular mass standards of 94, 67, 43, 30, 20.1, and 14.4 kDa (top to bottom); *Lane 2*, JM109(pBluescript SK(−)); *Lane 3*, JM109(pBSF1); *Lane 4*, JM109(pBSF2); *Lane 5*, JM109(pBSF3); *Lane 6*, JM109(pBSF4); *Lane 7*, JM109(pBSF5); *Lane 8*, JM109(pBSF6)

its ferredoxin and ferredoxin reductase component were not found in the isolated cluster.

In conclusion, these genes are clustered in similar fashion with their terrestrial counterparts especially genes isolated from strain CA10 and strain J3. However, ferredoxin and ferredoxin reductase which are necessary for initial dioxygenation of carbazole were not found in the cluster and is believed to be located elsewhere. The ORF6 may be regarded as a trace of genetic movements during the formation of this cluster. This result might be a unique finding that may help reveal the evolutionary relationships among other reported *car* gene clusters.

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