Title: 2,4-Dichlorophenoxyacetic acid (2,4-D)- and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T)-degrading gene cluster in the soybean root-nodulating bacterium *Bradyrhizobium elkanii* USDA94

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PII: S0944-5013(16)30187-2
DOI: http://dx.doi.org/doi:10.1016/j.micres.2016.04.014
Reference: MICRES 25888

To appear in:

Received date: 13-1-2016
Revised date: 29-4-2016
Accepted date: 29-4-2016

Please cite this article as: Hayashi Shohei, Sano Tomoki, Suyama Kousuke, Itoh Kazuhito.2,4-Dichlorophenoxyacetic acid (2,4-D)- and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T)-degrading gene cluster in the soybean root-nodulating bacterium Bradyrhizobium elkanii USDA94.*Microbiological Research* http://dx.doi.org/10.1016/j.micres.2016.04.014

2,4-Dichlorophenoxyacetic acid (2,4-D)- and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T)-degrading gene cluster in the soybean root-nodulating bacterium *Bradyrhizobium elkanii* USDA94

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ABSTRACT

Herbicides 2,4-dichlorophenoxyacetic acid (2,4-D)- and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T)-degrading *Bradyrhizobium* strains possess *tfdAα* and/or *cadABC* as degrading genes. It has been reported that root-nodulating bacteria belonging to *Bradyrhizobium elkanii* also have *tfdAα* and *cadA* like genes but lack the ability to degrade these herbicides and that the *cadA* genes in 2,4-D-degrading and non-degrading *Bradyrhizobium* are phylogenetically different. In this study, we identified *cadRABCK* in the genome of a type strain of soybean root-nodulating *B. elkanii* USDA94 and demonstrated that the strain could degrade the herbicides when *cadABCK* was forcibly expressed. *cadABCK*-cloned *Escherichia coli* also showed the degrading ability. Because co-spiked phenoxyacetic acid (PAA) could induce the degradation of 2,4-D in *B. elkanii* USDA94, the lack of degrading ability in this strain was supposed to be due to the low inducing potential of the herbicides for the degrading gene cluster. On the other hand, *tfdAα* from *B. elkanii* USDA94 showed little potential to degrade the herbicides, but it did for 4-chlorophenoxyacetic acid and PAA. The 2,4-D-degrading ability of the *cad* cluster and the inducing ability of PAA were confirmed by preparing *cadA* deletion mutant. This is the first study to demonstrate that the *cad* cluster in the typical root-nodulating bacterium indeed have the potential to degrade the herbicides, suggesting that degrading genes for anthropogenic compounds could be found in ordinary non-degrading bacteria.

Keywords: 2,4-Dichlorophenoxyacetic acid; 2,4,5-Trichlorophenoxyacetic acid; Herbicide-degrading gene; Non-degrading bacterium; Soybean root-nodulating bacterium; *Bradyrhizobium elkanii* USDA94

1. Introduction

2,4-Dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) have been used as chlorophenoxy herbicides to control broadleaf weeds since the 1940s. These herbicides were the main components of Agent Orange during the Vietnam War. 2,4-D is also a model compound for studying bacterial genes related to the catabolization of anthropogenic chemicals.

Diverse 2,4-D-degrading bacteria have been isolated from various environments, and they are categorized into three groups based on their physiological properties and degrading enzymes (Kamagata et al., 1997). Group I comprises copiotrophic and fast-growing bacteria, which belong to β- and γ-*Proteobacteria*. They have been isolated from agricultural and industrial soils and activated sludges (Fulthorpe et al., 1995; McGowan et al., 1998; Cavalca et al., 1999; Müller et al., 2001; Huong et al., 2007). These bacteria possess *tfdA*-encoding α-ketoglutarate-dependent dioxygenase, which degrades 2,4-D to 2,4-dichlorophenol (2,4-DCP). *tfdA* is often located on transmissible plasmids with *tfdBCDEF*, which are responsible for the further degradation steps (Don and Pemberton, 1981). Plasmid pJP4 in *Cupriavidus necator* JMP134 (formerly *Ralstonia eutropha* JMP134) contains the *tfd* cluster, and it has been extensively characterized. In the 2,4-D degradation pathway, TfdA converts 2,4-D to 2,4-DCP (Fukumori and Hausinger, 1993a,b). After the hydroxylation of 2,4-DCP to 3,5-dichlorocatechol by 2,4-DCP hydroxylase, which is encoded by *tfdB*, 3,5-dichlorocatechol is further degraded via a modified *ortho*-cleavage pathway encoded by *tfdCDEF* (Liu and Chapman, 1984; Perkins et al., 1990; Laemmli et al., 2000).

Group II consists of copiotrophic and fast-growing *Sphingomonas* spp. that belong to *α-Proteobacteria*. They have been isolated from environmental samples containing 2,4-D (Amy et al., 1985; Ka et al., 1994; Fulthorpe et al., 1995; Huong et al., 2007; Shimojo et al., 2009), and they possess *cadA* and *cadB* encoding 2,4-D monooxygenase large and small subunits, respectively, which convert 2,4-D to 2,4-DCP. CadA and CadB have no similarity with TfdA from *C. necator*

JMP134 but shares an approximately 50% identity with TftA and TftB, respectively, from 2,4,5-T-degrading *Burkholderia cepacia* AC1100. TftA and TftB of this strain, 2,4,5-T oxygenase large and small subunits, respectively, degrades 2,4,5-T to 2,4,5-trichlorophenol (2,4,5-TCP) (Danganan et al., 1994; Xun and Wagnon, 1995). It was demonstrated that *Escherichia coli* DH5α containing *cadAB* from *Sphingomonas agrestis* 58-1 exhibits 2,4-D-degrading activity (Shimojo et al., 2009).

Group III includes oligotrophic and slow-growing bacteria that belong to *Bradyrhizobium* spp. in *α-Proteobacteria.* They have been isolated from pristine soils in Hawaii, Canada, and Chile (Kamagata et al., 1997); arable soil without 2,4-D exposure history in Japan (Itoh et al., 2000); and 2,4-D-contaminated soil in south Vietnam (Huong et al., 2007). They are characterized by the presence of *cadAB* and *tfdAα.* CadA and CadB in group III share 54%–59% identity with TftA and TftB, respectively, from *B. cepacia* AC1100, and TfdAα shares 43%–46% identity with TfdA from *C. necator* JMP134. The *cadRABKC* from *Bradyrhizobium* sp. HW13 was transformed into *Sinorhizobium meliloti* Rm1021, and the transformant acquired the ability to degrade 2,4-D as well as related chlorophenoxy compounds such as 2,4,5-T and 4-chlorophenoxyacetic acid (4-CPAA). *cadRABKC* are assumed to encode an Ara/XylS-type positive transcriptional regulator, 2,4-D oxygenase large and small subunits, 2,4-D transporter, and ferredoxin component of *cadAB*, respectively. All *cadABC* genes were necessary for 2,4-D degradation in the *Sinorhizobium* transformant (Kitagawa et al., 2002). *tfdAα* in *Bradyrhizobium* sp. RD5-C2 was expressed in *E. coli* DH5α as a MalE-TfdA*α* fusion protein and it exhibited weak 2,4-D dioxygenase activity, which depended on α-ketoglutarate (Itoh et al., 2002).

2,4,5-T-degrading bacteria, such as *Burkholderia* spp. (Kellogg et al., 1981; Huong et al., 2007), *Nocardioides simplex* 3E (Golovleva et al., 1990), *Sphingomonas* spp. (Huong et al., 2007), and *Bradyrhizobium* spp. (Rice et al., 2005; Huong et al., 2007), have been less frequently reported than 2,4-D-degraders. *B. cepacia* AC1100 was the first example of a 2,4,5-T-degrading bacterium,

which was created by plasmid-assisted molecular breeding (Kellogg et al., 1981). After transformation of 2,4,5-T to 2,4,5-TCP, it is further oxidized to 2,5-dichloro-*p*-hydroquinone by TftC, chlorophenol 4-monooxygenase, and then to 5-chlorohydroxyquinol by TftD, $FADH_2$-utilizing monooxygenase of this strain (Gisi and Xun, 2003).

In our previous study, we found that *tfdAα* were present in all root-nodulating *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* strains that we examined, as well as *cadA* in some *B. elkanii* strains, but the strains exhibited no 2,4-D-degradaing activity (Itoh et al., 2004). Based on their GC contents, codon usage patterns, and phylogenetic properties, we suggested that *tfdAα* in *Bradyrhizobium* had been evolutionarily acquired without recent horizontal transfer and that *cadA* in 2,4-D-degrading *Bradyrhizobium* and non-degrading root-nodulating *Bradyrhizobium* have different origins. Our results indicated that *cadA* in 2,4-D-degrading *Bradyrhizobium* spp. was obtained via horizontal gene transfer to give them a novel degrading ability but that *tfdAα* and *cadA* in root-nodulating *Bradyrhizobium* strains were not related to this ability (Itoh et al., 2004). It was also reported that *tfdA* or its homologs were widespread among 2,4-D-non-degraders belonging to α- and γ-*Proteobacteria* and *Bacillus* (Hogan et al., 1997). These reports indicated that homologs of the 2,4-D-degrading genes were distributed in various bacteria regardless of their degrading ability. But it is unclear whether the homologous genes do not encode active enzymes or are not fully expressed.

In this study, we identified the *cad* cluster and *tfdAα* based on the available genome information for a type strain of the soybean root-nodulating bacterium *B. elkanii* USDA94 (Fig. 1), which has weak and no degrading abilities for 2,4-D and 2,4,5-T, respectively, and examined their 2,4-D- and 2,4,5-T-degrading potential by forcibly expressing them in *E. coli* JM109 and *B. elkanii* USDA94. We demonstrated that the lack of the degrading ability in the wild strain is due to poor inducing ability of 2,4-D and 2,4,5-T for the degrading gene. In addition, it was suggested that *B. elkanii* USDA94 possesses the other gene(s) that can degrade chlorophenoxyacetic acid based on the experiments using its *cadA* and *tfdAα* double deleted strain.

2. Materials and methods

2.1. Sequences of cad *cluster,* tfdAα*, and their surrounding regions in* B. elkanii *USDA94*

The sequences of the *cad* cluster, *tfdAα*, and their surrounding regions were identified in the genome of *B. elkanii* USDA94 (accession numbers. NZ_JAFC01000021 and NZ_JAFC01000038) using the partial sequences of *cadA* (AB119244) and *tfdAα* (AB119224) as the queries. The putative open reading frames (ORFs) were identified within the sequences via the Integrated Microbial Genomes system in the Joint Genome Institute (http://jgi.doe.gov/). The putative functions of the ORFs were assigned by comparing their deduced amino acid sequences with those in the NCBI databases using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2. Bacterial strains, plasmids and growth conditions

All bacterial strains and plasmids used in this study are listed in Tables 1 and S1, respectively. The *Bradyrhizobium* strains were grown at 25°C in HM medium (Minamisawa et al., 1998). *E. coli* JM109 and *E. coli* S17-1*λpir* were used as the cloning host and transconjugation donor, respectively. *E. coli* transformants were grown in Luria–Bertani (LB) medium (Sambrook and Russell, 2001) with appropriate antibiotics at 37°C with shaking.

2.3. Cloning of cadABCK *and* tfdAα *in* E. coli

DNA from the *E. coli* strains was manipulated as described previously (Sambrook and Russell, 2001). All primers used in this study are listed in Table S2. The *cadABCK* and *tfdAα* fragments (Fig. 1) were amplified from the genomic DNA of *B. elkanii* USDA94 using *Pfu* DNA polymerase (Bioneer, Seoul, Korea). The primers used were 94cadA-F-Bsp and 94cadK-R-Hind for *cadABCK*, and 94tfdAα-F-Bsp and 94tfdAα-R-Hind for *tfdAα*. The PCR cycle comprised a pre-run at 95°C for 5 min, 30 cycles of denaturation at 95°C for 30 sec, annealing at 71°C (*cadABCK*) or 60°C (*tfdAα*) for 30 sec, and extension at 72°C for 4 min (*cadABCK*) or 2 min (*tfdAα*), followed by a

post-run at 72°C for 10 min. The PCR mixture was prepared according to the manufacturer's instructions. The both PCR-generated fragments were digested with both *Bsp*HI and *Hin*dIII, and cloned into the *Nco*I and *Hin*dIII sites of pTV118N (TaKaRa, Tokyo, Japan) to generate pTV118N-94cadABCK and pTV118N-94tfdAα. The constructed plasmids were transformed into *E. coli* JM109, and the transformants were selected on LB agar medium with carbenicillin (50 mg l^{-1}). The fidelity of their plasmids was confirmed by sequencing using a BigDye Terminator Cycle Sequencing Ready Reaction Kit and an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, CA, USA). The primers used were RVN-new, 94cad-F1, 94cad-F2, 94cad-F3, 94cadC-R-Hind, and M4-new for pTV118N-94cadABCK and RVN-new and M4 for pTV118N-94tfdAα. The cycle sequencing was performed according to the manufacturer's instructions.

2.4. Cloning of cadABCK *and* tfdAα *in* B. elkanii

The fragments of *cadABCK* and *tfdAα* were amplified by PCR using KOD plus DNA polymerase (Toyobo, Tokyo, Japan) with 94cadA-F-Nde and 94cadK-R-Hind for *cadABCK* and 94tfdAα-F-Nde and 94tfdAα-R-Hind for *tfdAα*. The PCR cycle comprised 94°C for 2 min, 30 cycles at 95°C for 15 sec, 60°C for 15 sec and 68°C for 6.5 min. The PCR-generated fragments were digested with both *Nde*I and *Hin*dIII, and cloned into the *Nde*I and *Hin*dIII sites of pBBR1MCS2_START (Obranić et al., 2013) to generate pBBR2-94cadABCK and pBBR2-94tfdAα.

After the transformation of the each constructed plasmid into *E. coli* S17-1*λpir*, the transformant was incubated overnight in LB medium containing kanamycin (30 mg l^{-1}) at 37°C with shaking. The cells were harvested by centrifugation (2,430 ×g, 5 min, 4°C) and resuspended in 50 µl of HM medium. *B. elkanii* USDA94 was cultured in HM medium at 25°C for 5 days with shaking, collected by the centrifugation, and resuspended in 50 µl of HM medium. Both cell suspensions were mixed, and spotted onto HM agar medium. After incubation at 30°C for 3 days, the culture was scraped off, suspended in 500 µl of sterilized water, plated onto HM agar medium containing

kanamycin (150 mg l^{-1}) and tetracycline (10 mg l^{-1}), and incubated at 30°C for 1 week. The transconjugants were confirmed by colony PCR using the primers Km-check-F and Km-check-R for the kanamycin resistance gene.

2.5. Chlorophenoxyacetic acid-degrading ability of E. coli *and* B. elkanii *transformants*

The *E. coli* transformants were pre-cultured overnight in 10 ml of LB medium with carbenicillin (50 mg l^{-1}) at 37°C with shaking. After centrifugation (2,430 ×g, 5 min, 4°C) and washing with M9 medium (Sambrook and Russell, 2001), the cell suspension (OD_{660} = 1.1−1.3) was incubated at 37°C for 2 hours in 10 ml of M9 medium containing IPTG (100 μM) and carbenicillin with shaking. A 3-ml aliquot was placed in test tubes and spiked with 2,4-D, 2,4,5-T, 4-CPAA, and PAA at 100 μM, and continuously incubated at 25°C with shaking. For *B. elkanii* strains, their degrading abilities were examined in the same manner, except that the incubation was conducted using HM medium at 25 °C, and that the inoculation density was set at OD_{660} = 0.03−0.05. The experiment was performed in triplicate.

At appropriate intervals, an aliquot (200 μl) was centrifuged (21,880 ×g, 10 min) and the concentrations of the test compounds and their degradation products (the corresponding phenols) in the supernatants were determined using an ultra-fast liquid chromatography system (Prominence, Shimadzu, Kyoto, Japan) equipped with a photo-diode array (SPD-M20A, Shimadzu) and a Shim-pack XR-ODS column (2.2 μm, 100 mm length × 3.0 mm i.d., Shimadzu) under the following conditions: mobile phase; CH_3CN:0.5% HCOOH = 45:55 for 2,4-D, 2,4,5-T, 4-CPAA, 2,4-DCP, 2,4,5-TCP and 4-chlorophenol (4-CP), and 20:80 for PAA and phenol, column temperature; 40°C, flow rate; 0.4 ml min^{-1}, detection; 284 nm for 2,4-D, 2,4,5-T, 2,4-DCP, 2,4,5-TCP and 4-CP, 274 nm for 4-CPAA, and 269 nm for PAA and phenol.

2.6. Construction of cadA *and* tfdAα *deletion mutants of* B. elkanii *USDA94*

To produce in-frame deletions of *cadA* and *tfdAα*, insertional inactivation *via* double crossover was performed. Each approximately 2-kb upstream and downstream region of the genes was PCR-amplified by KOD plus DNA polymerase using the following primers sets: DcadAup5-Kpn and DcadAup3-Xba, and DcadAdw5-Xba and DcadAdw3-Hind for the upstream and downstream regions of *cadA*, respectively, and DtfdAαup5-Kpn and DtfdAαup3-Xba, and DtfdAαdw5-Xba and DtfdAαdw3-Hind for the upstream and downstream regions of *tfdAα*, respectively. The PCR cycle was 94°C for 2 min, 30 cycles at 94°C for 15 sec, 60°C for 30 sec and 68°C for 2 min, and then 68°C for 7 min. After digesting the upstream and downstream fragments with *Kpn*I and *Xba*I, and *Xba*I and *Hin*dIII, respectively, they were ligated into the *Kpn*I and *Hin*dIII sites of pK18mob (Schäfer et al., 1994) to yield pK18mob-94cadAupdw and pK18mob-94tfdAαupdw. The plasmids were introduced into *B. elkanii* USDA94 by conjugative transformation *via E. coli* S17-1*λpir* transformants, as described above. Single crossover mutants were selected on HM medium with kanamycin (150 mg l^{-1}) and tetracycline (10 mg l^{-1}). After cultivating of the mutants in HM medium at 25°C for 7 days with shaking, the double crossover mutants were screened based on their kanamycin sensitivity on HM agar medium. The in-frame deletions of 1038 bp in *cadA* and 634 bp in *tfdAα* were confirmed by colony PCR using primers sets of 94cadA-F-Bsp and 94cadC-R-Hind, and 94tfdAα-F-Bsp and 94tfdAα-R-Hind, respectively, and sequencing of the new junction regions. For the in-frame double deletions of both *cadA* and *tfdAα*, pK18mobsacB-94cadAupdw was constructed by cloning an approximately 4-kb PCR-amplified fragment using the primers Dcad2Aup5-Bam and Dcad2Adw3-Hind from pK18mob-94cadAupdw into the *Bam*HI and *Hin*dIII sites of pK18mobsacB (Schäfer et al., 1994), and the deletion of *cadA* in *B. elkanii* USDA94ΔtfdAα was performed as described above. The double crossover mutant was screened on HM agar medium with 5% sucrose, which killed the cells that possessed *sacB* (levansucrase), and then by kanamycin sensitivity.

3. Results

3.1. cad *cluster and* tfdAα *in* B. elkanii *USDA94*

It has been reported that *B. elkanii* USDA94 possesses *cadA* (Itoh et al., 2004), and the surrounding DNA sequence is available in the NCBI database (NZ_JAFC01000021). Based on the deduced amino acid sequences of the putative ORFs around *cadA*, a *cad* cluster that comprised the transcription regulator (*cadR*), oxygenase large and small subunits (*cadA* and *cadB*, respectively), ferredoxin component (*cadC*) and transporter (*cadK*) was detected in the *B. elkanii* USDA94 genome as found in *Bradyrhizobium* sp. HW13 (Kitagawa et al., 2002) (Fig. 1 and Table 2).

CadR from *B. elkanii* USDA94 was predicted to be a LysR-type transcriptional regulator by BLAST search using the NCBI conserved domain database. The deduced amino acid sequence shared 44% and 24% similarity with BenM, a transcriptional activator for benzoate degradation from *Acinetobacter calcoaceticus* ADP1 (Collier et al., 1998), and CadR from *Bradyrhizobium* sp. HW13 (Kitagawa et al., 2002), respectively.

CadA and CadB was predicted to be large and small subunits, respectively, of a Rieske non-heme iron oxygenase. CadA from *B. elkanii* USDA94 possessed putative [2Fe-2S]-binding sites (Cys95, His97, Cys115, and His118) and putative mononuclear Fe-binding sites (Glu218, Asp222, His225, His230, and Asp378) in the N- and C-terminal regions, respectively (Fig. 2A). They are conserved in representative aromatic compound dioxygenases such as benzoate dioxygenase BenA from *A. calcoaceticus* ADP1, TftA from *B. cepacia* AC1100 (Neidle et al., 1991), toluene dioxygenase TodC1 from *Pseudomonas putida* F1 (Jiang et al., 1996), and naphthalene 1,2-dioxygenase NahAc from *Pseudomonas* sp. NCIB 9816-4 (Carredano et al., 2000). The deduced amino acid sequence of *cadA* shared 72% and 71% similarity with that of *cadA* from *S. agrestis* 58-1 (Shimojo et al., 2009) and *Bradyrhizobium* sp. HW13, respectively, and that of *cadB* did 65% and 62%, respectively (Fig. 1 and Table 2).

CadC was predicted to be a ferredoxin component of the Rieske non-heme iron oxygenase

family, and it possessed the Cys-X-His-X_{18}-Cys-X_2-His motif as [2Fe-2S]-binding sites, which are conserved in ferredoxin components of carbazole1,9a-dioxygenase from *Pseudomonas resinovorans* CA10 (Nam et al., 2005) and biphenyl dioxygenase from *Burkholderia* sp. LB400 (Colbert et al., 2000) (Fig. 2B). The deduced amino acid sequence shared 65% and 22% similarities with the ferredoxin component of the naphthalene 1,2-dioxygenase encoded by *nahAb* from *P. putida* NCIB 9816-4 (Carredano et al., 2000) and *cadC* from *Bradyrhizobium* sp. HW13, respectively (Fig. 1 and Table 2).

The deduced amino acid sequence of *cadK* shared 37% and 32% similarity with that of *tfdK*, a 2,4-D transporter gene, on plasmid pJP4 in *C. necator* JMP134 (Leveau et al., 1998) and *cadK* from *Bradyrhizobium* sp. HW13, respectively.

The deduced amino acid sequence of *tfdAα* (NZ_JAFC01000038) shared 99% and 61% similarity with that of *tfdAα* from *Bradyrhizobium* sp. HW13 and *tfdA* from *C. necator* JMP134, respectively. Most *tfdA* genes in the 2,4-D-degraders from group I are accompanied by the *tfdBCDEF* cluster (Don and Pemberton, 1981; Leveau et al., 1999), whereas there were no corresponding genes around *tfdAα* from *B. elkanii* USDA94.

3.2. Degradation of (chloro)phenoxyacetic acids by B. elkanii *USDA94*

The (chloro)phenoxyacetic acids-degrading ability of *B. elkanii* USDA94 was examined in this study (Fig. 3). The concentrations of 4-CPAA and PAA decreased in the culture of *B. elkanii* USDA94, and the corresponding molecular accounts of 4-CP accumulated, whereas phenol was not detected. 2,4-DCP was weakly detected in the culture after incubation for 3 days, but the decrease in 2,4-D was not clear. The concentration of 2,4,5-T did not decrease, and 2,4,5-TCP was not detected. Neither decrease in the substrates nor generation of corresponding degradation products was detected in the medium without *B. elkanii* USDA94 (data not shown).

3.3. Degradation of (chloro)phenoxyacetic acids by E. coli *JM109 harboring the* cadABCK *and* tfdAα

The *cadABCK* and *tfdAα* from *B. elkanii* USDA94 were expressed in *E. coli* JM109 to examine their (chloro)phenoxyacetic acids-degrading abilities (Fig. 4). *E. coli* JM109-118N94cadABCK harboring the *cadABCK* degraded approximately 30% of 2,4-D and 2,4,5-T, approximately 90% of 4-CPAA, and approximately 80% of PAA in 24 hours. The corresponding phenolic compounds were detected in the cultures. *E. coli* JM109-118N94tfdAα harboring *tfdAα* degraded neither 2,4-D nor 2,4,5-T. It weakly degraded PAA and the equivalent molecular amount of phenol was detected. 4-CP was slightly detected in the culture, although the degradation of 4-CPAA was not clear. *E. coli* JM109-118N did not degrade any test substances.

3.4. Degradation of (chloro)phenoxyacetic acids by forcibly expressing the cadABCK *and* tfdAα *in* B. elkanii *USDA94*

Introduction of the *cadABCK* from *B. elkanii* USDA94 gave *E. coli* JM109 the ability to degrade 2,4-D and 2,4,5-T and *tfdAα* didn't as described above. The *tfdAα* from *Bradyrhizobium* sp. RD5-C2, which is highly similar to that from *B. elkanii* USDA94, exhibited a weak ability to degrade 2,4-D in a previous study (Itoh et al., 2002). However, *B. elkanii* USDA94 showed weak and no degrading abilities for 2,4-D and 2,4,5-T, respectively. Then, the genes were forcibly expressed in *B. elkanii* USDA94 to examine if they have the degrading ability in the original strain (Fig. 5). In *B. elkanii* USDA94-BBR2cadABCK, the degradation of 2,4-D was enhanced and that of 2,4,5-T was observed, and their corresponding degradation products were detected. In the culture of *B. elkanii* USDA94-BBR2tfdAα, on the other hand, there was little effect on the degradation of the substrates and the generation of the degradation products compared with *B. elkanii* USDA94-BBR2.

3.5. Degradation of (chloro)phenoxyacetic acids by cadA *and* tfdAα *deletion mutants*

To examine the contributions of the *cad* cluster and *tfdAα* to the degradation of (chloro)phenoxyacetic acids in *B. elkanii* USDA94, we quantified the degrading ability of the deletion mutants of the genes (Fig. 6). In the degradation of 2,4-D, little change was observed in the cultures of the deletion mutants compared with that of their wild type. In 4-CPAA and PAA, *B. elkanii* USDA94ΔcadA and *B. elkanii* USDA94ΔcadAΔtfdAα showed slower degradation than their wild type. In the case of *B. elkanii* USDA94ΔtfdAα, there was little change in the degradation of 4-CPAA and PAA. The degradation of 4-CPAA and PAA was still observed even in the culture of *B. elkanii* USDA94ΔcadAΔtfdAα.

The *cadABCK* showed the potential to degrade 2,4-D and 2,4,5-T, but *B. elkanii* USDA94 only weakly degraded 2,4-D and did not degrade 2,4,5-T. *B. elkanii* USDA94 degraded PAA and 4-CPAA and the *cadA* deletion mutants decreased the degrading ability. It was expected that PAA and 4-CPAA induce the *cadABCK* and that 2,4-D and 2,4,5-T don't. Thus, we examined the degradation of 2,4-D with co-spiked PAA or 4-CPAA (Fig. 7). When PAA was co-spiked, decreases in 2,4-D were observed in the cultures of *B. elkanii* USDA94 and *B. elkanii* USDA94ΔtfdAα and the degradation of PAA became slower, especially in the degradation by *B. elkanii* USDA94ΔcadA and *B. elkanii* USDA94ΔcadAΔtfdAα. In contrast to PAA, the simultaneous presence of 4-CPAA did not induce the degradation of 2,4-D in all strains. 4-CPAA was degraded by all strains, but the degradation rate by *B. elkanii* USDA94ΔcadA and *B. elkanii* USDA94ΔcadAΔtfdAα was slower than that by the wild type, as found with PAA.

4. Discussion

Based on analyses of the nucleotide sequences around *cadA* and the deduced amino acids sequences of the putative ORFs, we concluded that the *cad* cluster comprises five genes: *cadR* (positive transcriptional regulator), *cadA* (large subunit of 2,4-D oxygenase), *cadB* (small subunit of

2,4-D oxygenase), *cadC* (ferredoxin component of 2,4-D oxygenase), and *cadK* (2,4-D transporter). *cadA* and *cadB* shared higher similarity with their counterparts in *Bradyrhizobium* sp. HW13 than *cadR*, *cadC*, and *cadK*. The arrangement of *cadC* and *cadK* was reversed (Fig. 1). The GC contents of *cadRABC* in *B. elkanii* USDA94 (63%–67%) differed from those of *Bradyrhizobium* sp. HW13 (55%–58%), thereby suggesting that the origins of these genes in *B. elkanii* USDA94 and *Bradyrhizobium* sp. HW13 are different. This hypothesis was supported by the phylogeny and the codon usage pattern of *cadA* (Itoh et al., 2002). The GC contents of *cadK* were almost same in the strains, so they would share the same origin. It was interesting that the *cadRABC* had the same ability to degrade anthropogenic 2,4-D and 2,4,5-T if they have different origins.

It was reported that *B. elkanii* USDA94 could not degrade 2,4-D (Itoh et al., 2004). In the present study, but a small amount of 2,4-DCP was detected in the culture of *B. elkanii* USDA94 with 2,4-D (Fig. 3), implying that the strain had weak ability to degrade 2,4-D. An equivalent molecular amount of 4-CP accumulated in the degradation of 4-CPAA by *B. elkanii* USDA94, but phenol was not detected during the degradation of PAA, indicating that *B. elkanii* USDA94 can degrade phenol, but not 4-CP. It was confirmed by adding phenol and 4-CP in the culture (data not shown). The strain could not degrade 2,4,5-T and the degrading activity of 4-CPAA was higher than that of PAA, suggesting that the activity was affected by the number of chlorine atoms in the substrate.

E. coli JM109-118NcadABCK was able to degrade 2,4-D and 2,4,5-T (Fig. 4), indicating that the *cad* cluster from non-degrading *B. elkanii* USDA94 has the potential to degrade these chlorophenoxyacetic acids. This is the first study to show that ordinary root-nodulating bacteria possess the 2,4,5-T-degrading gene. *B. elkanii* USDA94 only weakly degraded 2,4-D and did not degrade 2,4,5-T, although the *cadABCK* from *B. elkanii* USDA94 degraded 2,4-D and 2,4,5-T in *E. coli*, thereby suggesting that the *cad* cluster was not expressed in *B. elkanii* USDA94. In fact, *B. elkanii* USDA94-BBR2cadABCK was able to degrade 2,4-D and 2,4,5-T when the *cadABCK* was forcibly expressed (Fig. 5). The degradation of 4-CPAA and PAA was faster than that of 2,4-D (Fig.

3). And the degradation of 2,4-D was enhanced in *B. elkanii* USDA94 when PAA was added to the culture as an inducer (Fig. 7). These results suggested that the *cad* cluster was induced by 4-CPAA and PAA, but neither by 2,4-D nor by 2,4,5-T. *E. coli* JM109-118NtfdAα was able to degrade PAA and 4-CPAA, but neither 2,4-D nor 2,4,5-T (Fig. 4). The degradation of 2,4-D and 2,4,5-T was not observed when *tfdAα* was expressed in *B. elkanii* USDA94 (Fig. 5). The results demonstrated that TfdAα from *B. elkanii* USDA94 has the ability to degrade PAA and 4-CPAA.

The *E. coli* JM109 transformants that harbored *cadABC* and *cadABCK* were able to degrade (chloro)phenoxyacetic acids at almost the same rate (data not shown). Previously, it was reported that the frameshift mutation of *cadK* from *Bradyrhizobium* sp. HW13 showed little effect on the degradation of 2,4-D in the *S. meliloti* Rm1021 transformant (Kitagawa et al., 2002) and that 2,4-D was transported into the *tfdK* mutant cells of *C. necator* JMP134 by diffusion (Leveau et al., 1998). The previous and our results suggest that the *E. coli* transformants took up the substrates by diffusion and that *cadK* was not essential for the degradation. *E. coli* transformant with only *cadAB* could not degrade 2,4-D (data not shown), so the ferredoxin component was essential for the degradation. However, in the case of *S. agrestis* 58-1, only *cadAB* was required for 2,4-D degradation in *E. coli* (Shimojo et al., 2009).

The degradation of PAA and 4-CPAA by the *cadA* deletion mutants was slower than that by the wild type, but it was similar between *B. elkanii* USDA94ΔtfdAα and the wild type (Fig. 6), suggesting that the *cad* cluster rather than *tfdAα* was responsible for the degradation of PAA and 4-CPAA. This corresponded to the faster degradation of PAA and 4-CPAA by *E. coli* JM109-118NcadABCK than *E. coli* JM109-118NtfdAα. The enhancement of the degradation of 2,4-D by adding PAA was observed in *B. elkanii* USDA94 and *B. elkanii* USDA94ΔtfdAα, not in the *cadA* deletion mutants (Fig. 7), confirming that PAA induced the expression of the *cad* cluster to degrade 2,4-D. The induction of the degradation did not occur after addition of 20 μM phenol (data not shown), suggesting that PAA itself induced the expression of the *cad* cluster. In addition, our

results indicated that PAA and/or its degradation product(s) also induced chlorophenol-degrading gene(s) because 2,4-DCP was not detected during the degradation of 2,4-D. The simultaneous presence of 2,4-D inhibited the degradation of PAA by all strains although the mechanism was unclear. In contrast, the co-addition of 4-CPAA did not enhance the degradation of 2,4-D by *B. elkanii* USDA94. *B. elkanii* USDA94 degraded 4-CPAA without the addition of other substrates, so 4-CPAA and/or its degradation product(s) appeared to induce the *cad* cluster. We speculated that the induction of the *cad* cluster by 4-CPAA was too week to yield obvious 2,4-D-degradation but was sufficient for the degradation of 4-CPAA due to the high activity for the compound. The 4-CPAA- and PAA-degrading ability was still observed even in the *cadA* and *tfdAα* double-deleted strain, *B. elkanii* USDA94ΔcadAΔtfdAα, and the generation of 2,4-DCP by the strain was comparable to that of the wild type in the culture added 2,4-D (Fig. 6). These results suggested that *B. elkanii* USDA94 possesses other gene(s) that can degrade (chloro)phenoxyacetic acids.

2,4-D and 2,4,5-T are anthropogenic chemicals, which implies the existence of original substrate(s) for the *cad* cluster in nature, but the substrate(s) is still unknown. The deduced amino acid sequence of CadK shares similarity with 4-hydroxyphenylacetate permease from *E. coli* W (Prieto and Garcia, 1997). 4-Hydroxyphenylacetate and its structurally and metabolically related compounds were selected, and their abilities to induce the *cad* cluster were examined. When phenylalanine, tyrosine, 4-hydroxyphenylacetate, 4-hydroxyphenylpyruvate, and homogentisic acid were co-spiked with 2,4-D, *B. elkanii* USDA94 could not enhance the degradation of 2,4-D (data not shown). Thus, these compounds were not expected as the original substrate.

Previous reports presented that some 2,4-D-non-degrading root-nodulating *Bradyrhizobium* spp. possessed *cadA* homologs that were phylogenetically separated from *cadA* in 2,4-D-degrading *Bradyrhizobium* spp. (Itoh et al., 2004). Homologs of the *cad* cluster of *B. elkanii* USDA94 also present in *Bradyrhizobium* sp. DOA9 (NZ_BAWE01000006), *Bradyrhizobium* sp. OHSUIII (NZ_APJD01000006), *Bradyrhizobium* sp. Th.b2 (NZ_AUGA01000047), *B. elkanii* 587

(NZ_AJJK01002004), *B. elkanii* USDA76 (NZ_KB900701), *B. elkanii* USDA3254 (NZ_AXAH01000021), and *B. elkanii* USDA3259 (AXAW01000016). And the same arrangements of *cadA*, *cadB*, *cadC*, and *cadK* are conserved among the strains. The deduced amino acid sequences of these corresponding genes share high similarity (78%–100%) among them. Based on these results of *B. elkanii* USDA94 and the similarities, it was expected that these *cad* clusters in the *Bradyrhizobium* strains also have the potential to degrade 2,4-D and 2,4,5-T. Because the *cad* cluster is widely conserved in *Bradyrhizobium* strains, it must play important roles in the oxygenation of some natural compounds for survival of this strains in nature.

This study demonstrated that the *cad* cluster in the ordinary root-nodulating *B. elkanii* USDA94 had the ability to degrade 2,4-D and 2,4,5-T, although this strain could not degrade these herbicides. This phenotypic lack of degradation was not due to the difference in the amino acid residues of Cad enzymes but due to the inability of 2,4-D and 2,4,5-T to induce the *cad* cluster. It was considered that original substrate in nature should be presented and that 2,4-D and 2,4,5-T are incidentaly degraded due to similar chemical structure. In addition, the results of our study suggest that other degrading genes for anthropogenic compounds could be found in the non-degrading bacteria because genome information is rapidly accumulating in data bases.

Acknowledgements

The authors would like to express my gratitude to associate professor Gordana Maravić-Vlahoviček at University of Zagreb for the kind gift of pBBR1MCS2_START. The authors thank the faculty of Life and Environmental Science in Shimane University for help in financial supports for publishing this report.

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Figure captions

Fig. 1. Comparison of *cad* gene clusters and *tfdAα* in *B. elkanii* USDA94 and *Bradyrhizobium* sp. HW13. Each gene is represented by a large horizontal arrow within its CG content (mol%). Identity (upper) and similarity (lower) values (%) of the deduced amino acid sequences of the corresponding genes are represented with thin lines.

Fig. 2. (A) Multiple alignment of the partial deduced amino acid sequences of CadA from *B. elkanii* USDA94 (USDA94_CadA), CadA from *S. agrestis* 58-1 (58-1_CadA), CadA from *Bradyrhizobium* sp. HW13 (HW13_CadA), TftA from *B. cepacia* AC1100 (AC1100_TftA), BenA from *Acinetobacter* sp. ADP1 (ADP1_BenA), TodC1 from *P. putida* F1 (F1_TodC1), and NahAc from *P. putida* NCIB9816-4 (9816-4_NahAc). The putative [2Fe-2S]-binding sites and the putative mononuclear Fe-binding sites are marked by closed and open circles, respectively. Conserved residues are boxed.

(B) Multiple alignment of the deduced amino acid sequences from CadC of *B. elkanii* USDA94 (USDA94_CadC), NahAb from *P. putida* NCIB9816-4 (9816-4_NahAb), BphF from *Burkholderia* sp. LB400 (LB400_BphF), and CarAc from *P. resinovorans* CA10 (CA10_CarAc). The putative [2Fe-2S]-binding sites are marked by closed circles.

Fig. 3. Degradation of (chloro)phenoxyacetic acids by *B. elkanii* USDA94. Error bars indicate standard deviations based on triplicate cultures.

Fig. 4. Degradation of 2,4-D (A), 2,4,5-T (B), PAA (C), and 4-CPAA (D) by *E. coli* JM109 harboring the *cad* cluster or *tfdAα* forcibly expressed. Solid and dashed lines indicate substrates (2,4-D, 2,4,5-T, 4-CPAA and PAA) and the corresponding degradation products (2,4-DCP, 2,4,5-TCP, 4-CP, and phenol), respectively.

Fig. 5. Degradation of 2,4-D (A) and 2,4,5-T (B) by *B. elkanii* USDA94 harboring the *cad* cluster or *tfdAα* forcibly expressed. Solid and dashed lines indicate substrates and the corresponding degradation products, respectively. Error bars indicate standard deviations based on triplicate cultures.

Fig. 6. Degradation of 2,4-D (A), PAA (B) and 4-CPAA (C) by *cad*A and/or *tfdAα* deletion mutants of *B. elkanii* USDA94. Solid and dashed lines indicate substrates and the corresponding degradation products, respectively. Error bars indicate standard deviations based on triplicate cultures.

Fig. 7. Degradation of (chloro)phenoxyacetic acids in the simultaneous presence of 2,4-D and PAA (A and B), and 2,4-D and 4-CPAA (C and D) by *cad*A and/or *tfdAα* deletion mutants of *B. elkanii* USDA94. Solid and dashed lines indicate substrates and the corresponding degradation products, respectively. Error bars indicate standard deviations based on triplicate cultures.

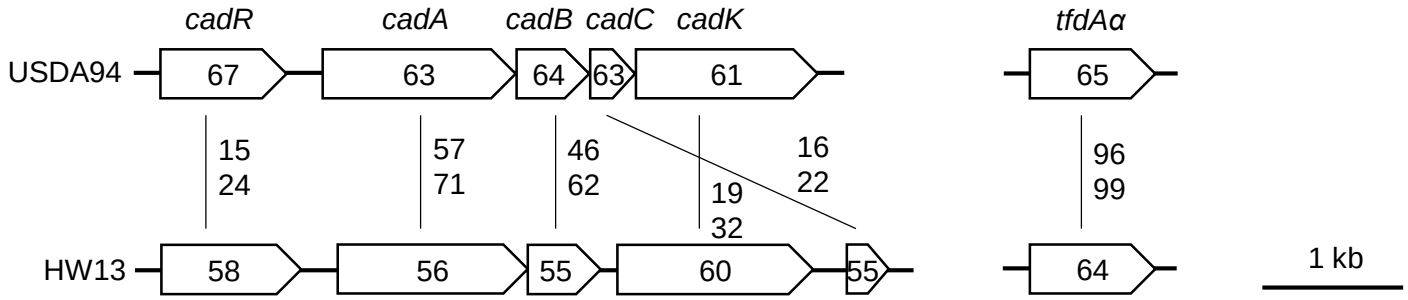


Figure 1 S. Hayashi et al. 2016

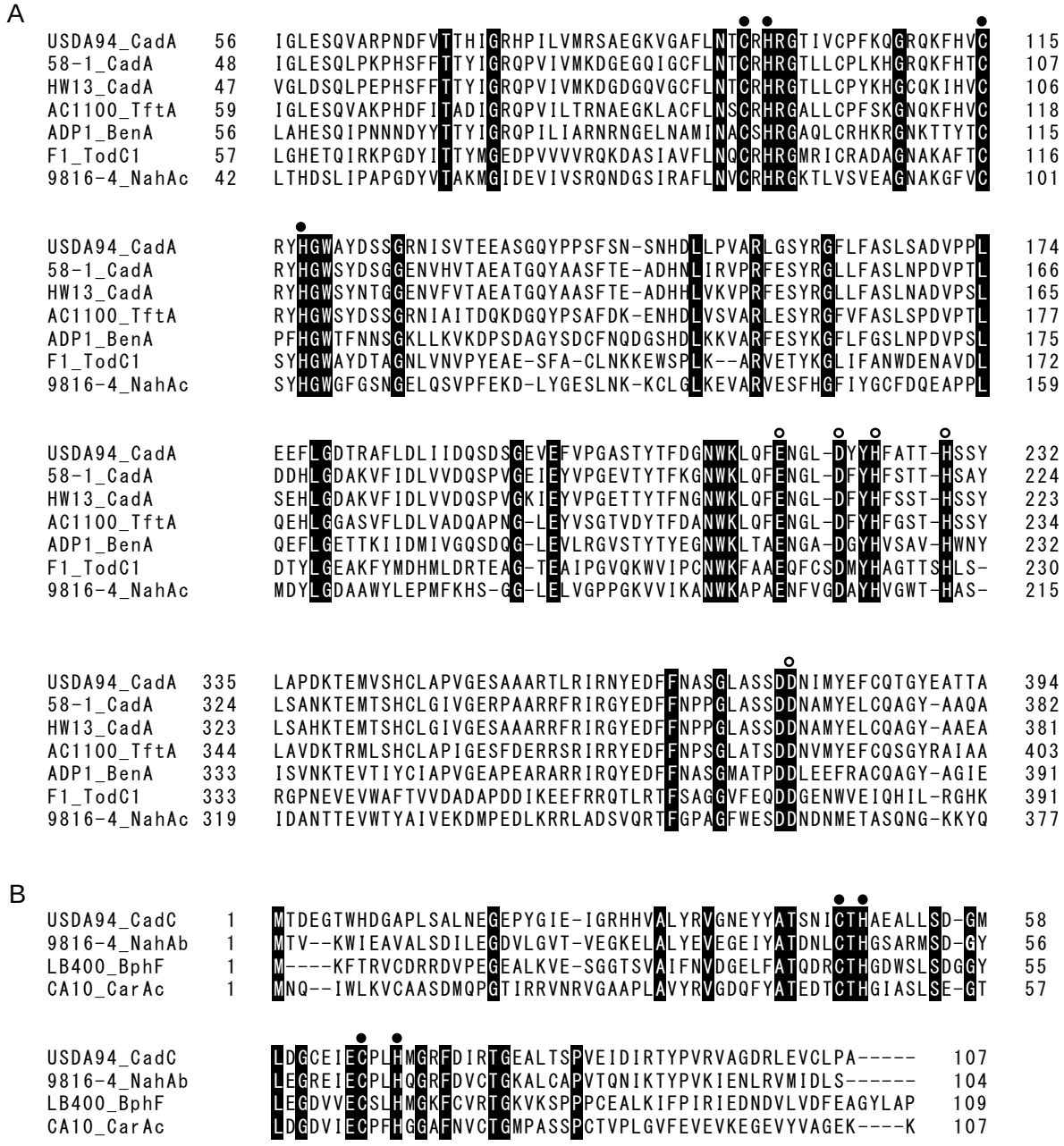


Figure 2 S. Hayashi et al. 2016

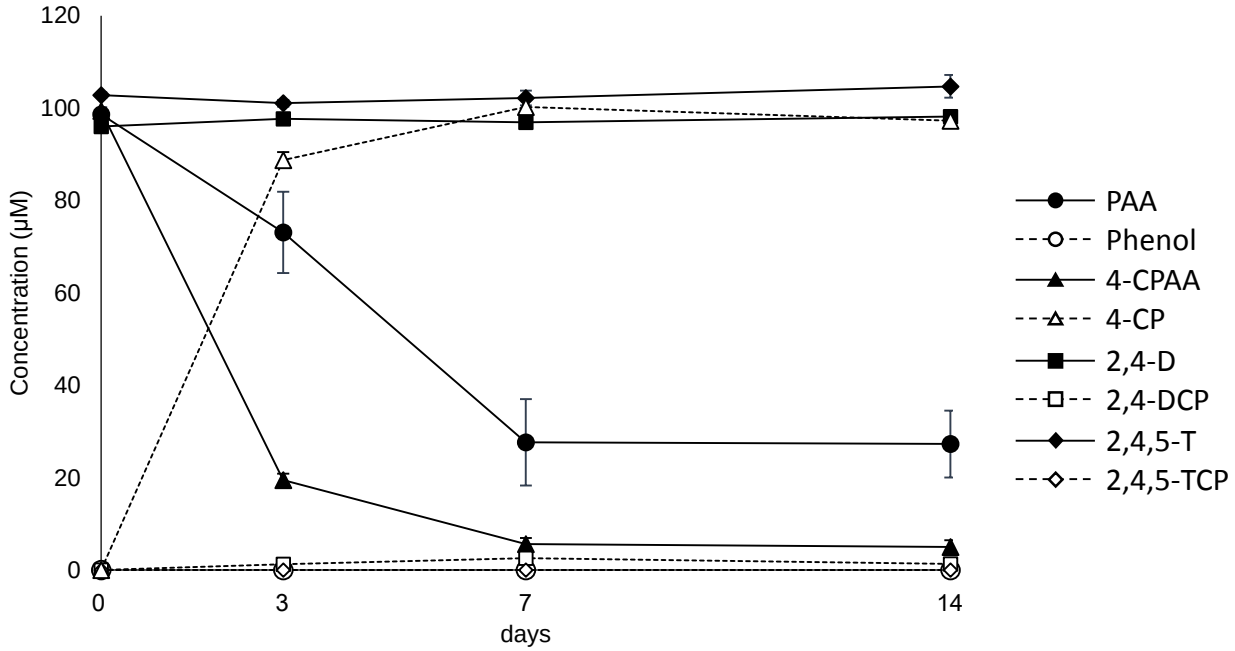
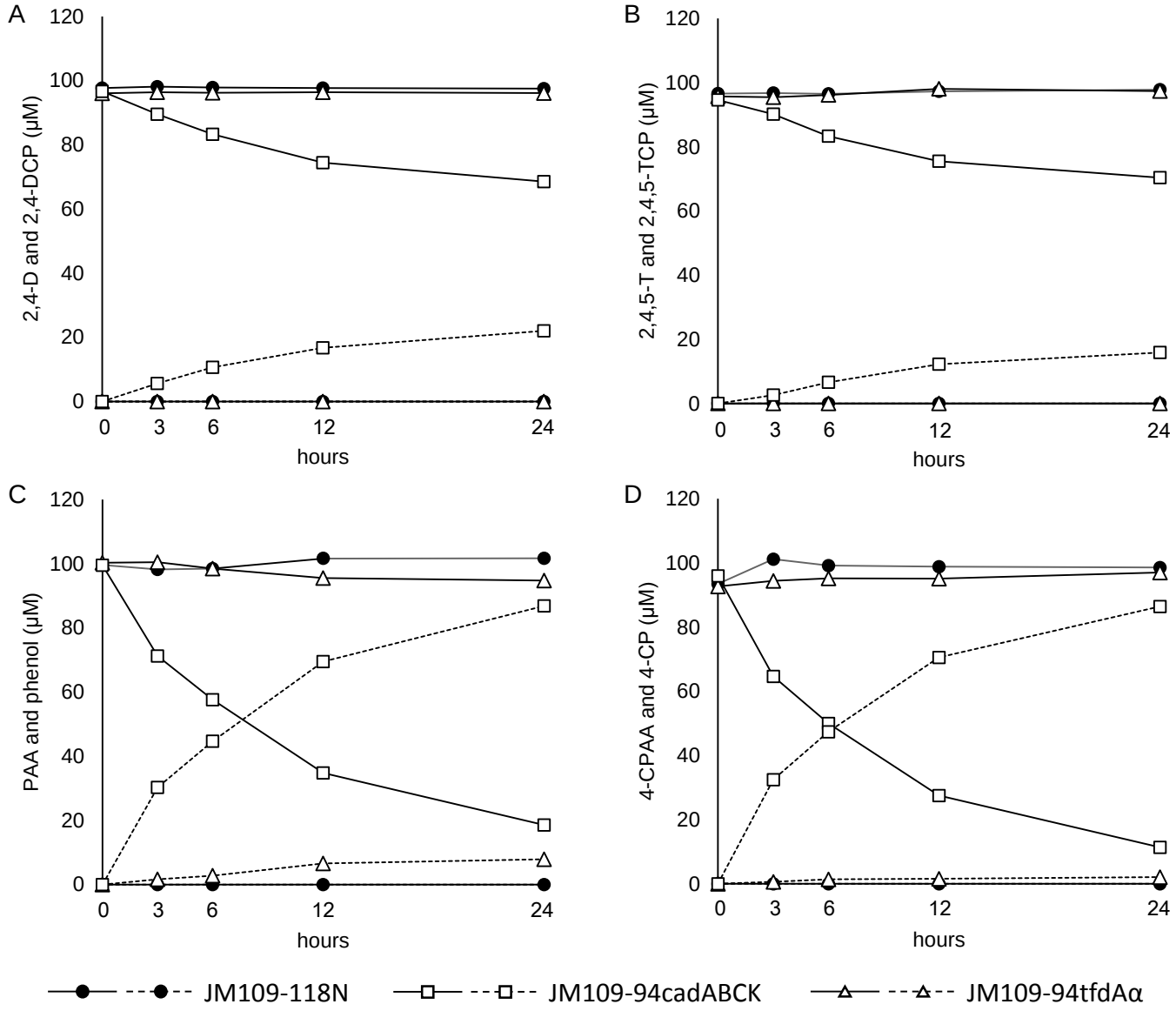


Figure 3 S. Hayashi et al. 2016



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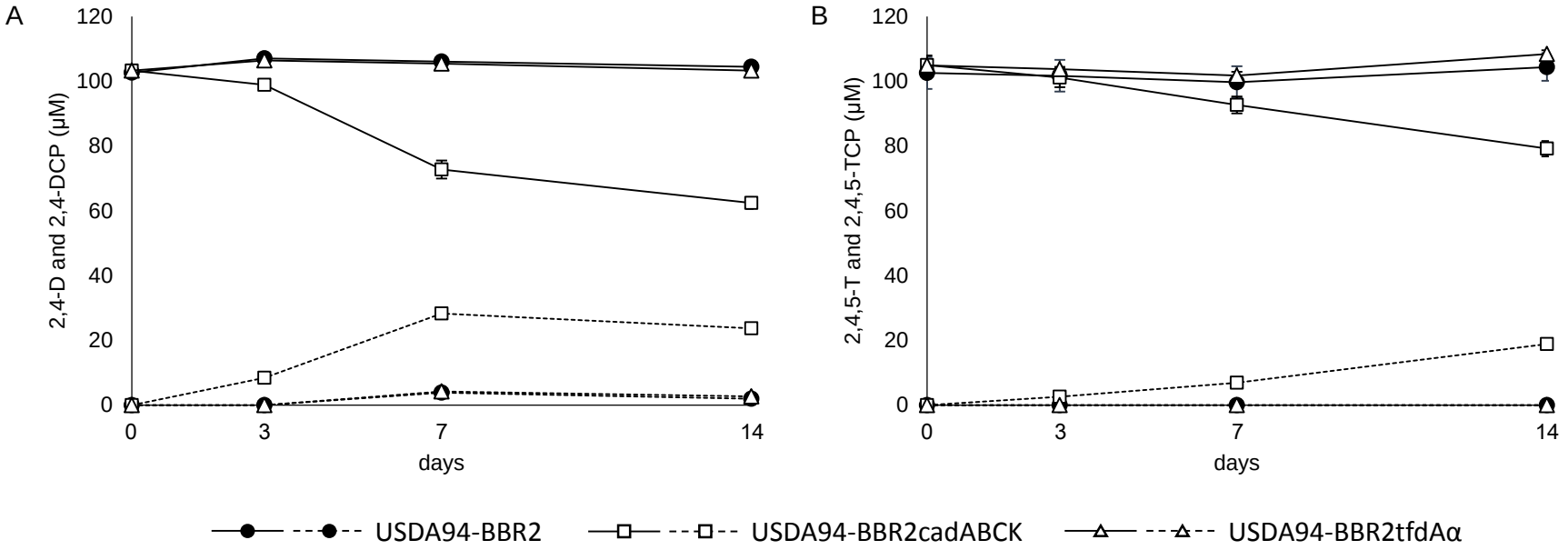
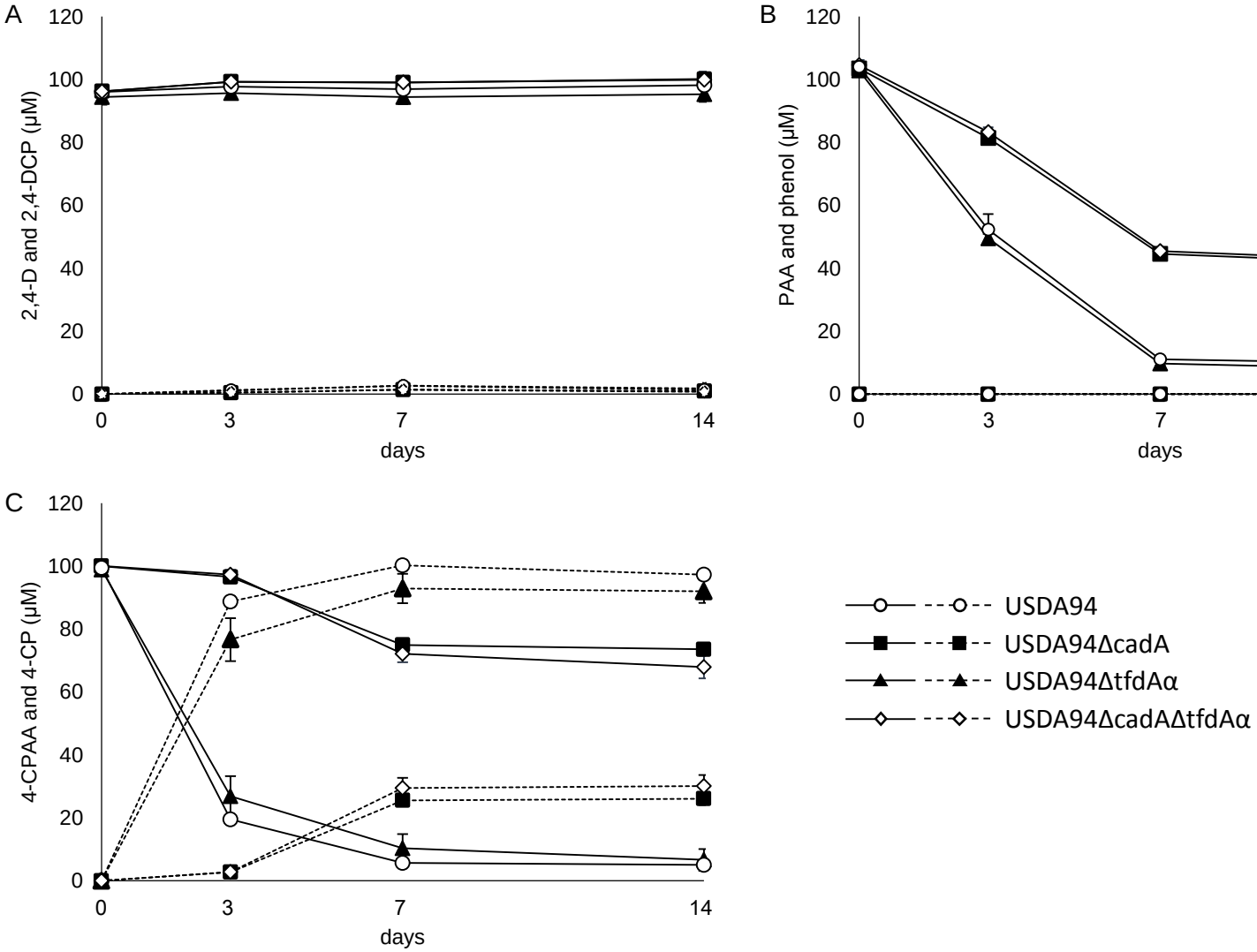


Figure 5 S. Hayashi et al. 2016



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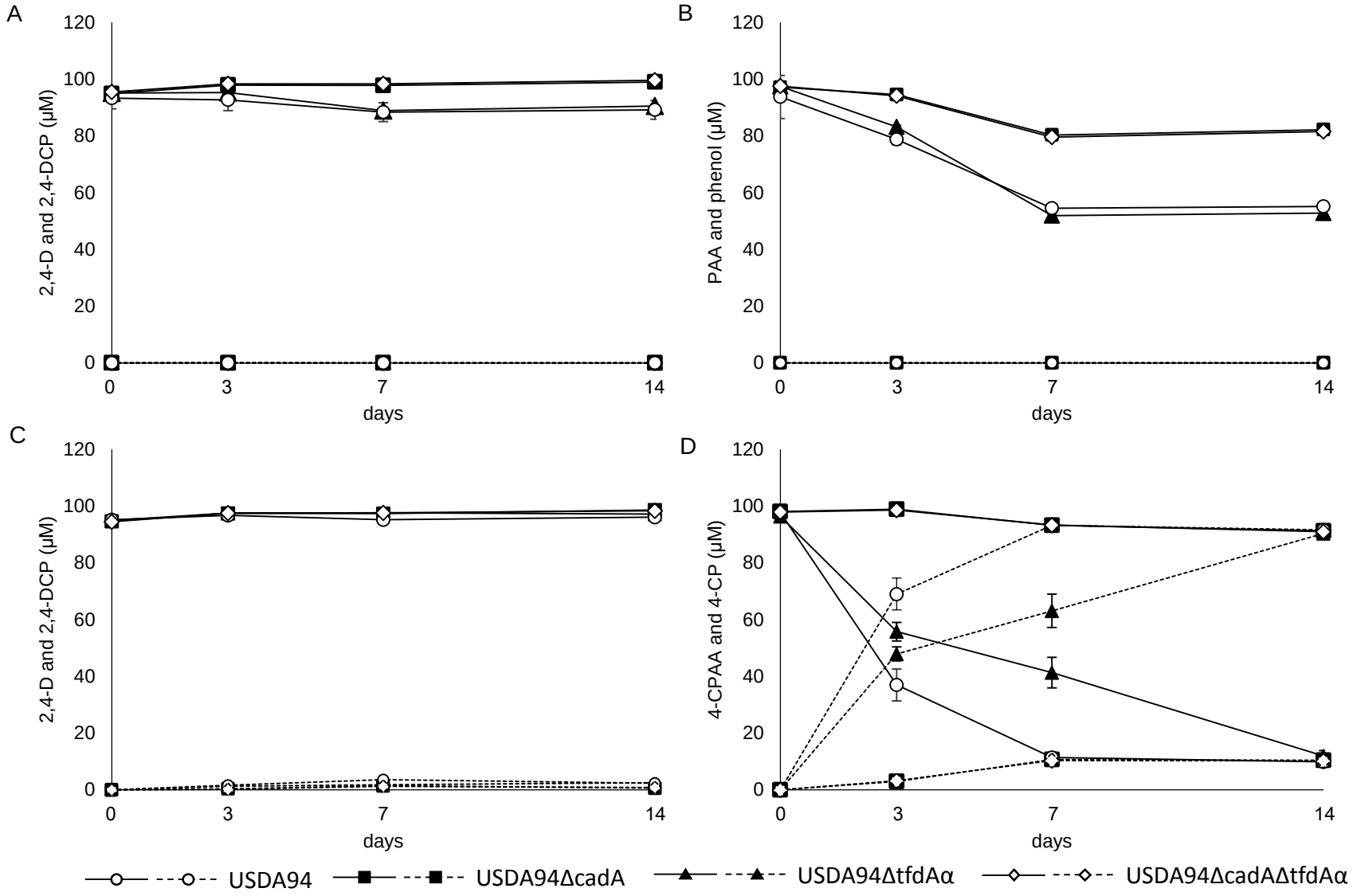

Figure 7 S. Hayashi et al. 2016

Table 1 Strains used in this study.

Strain	Characteristic	Source or reference
Escherichia coli		
JM109	Cloning strain (*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17(r_K^- m_K^+), *e*14$^-$ (*mcrA*$^-$), *supE*44, *relA*1, Δ (*lac-proAB*)/F′[*traD*36, *proAB*$^+$, *lac I*^{q}, *lacZ*ΔM15]) Tc^S, Km^S	TaKaRa
JM109-118N94cadABCK	*E. coli* JM109 harboring pTV118N-94cadABCK	This study
JM109-118N94tfdAα	*E. coli* JM109 harboring pTV118N-94tfdAα	This study
JM109-118N	*E. coli* JM109 harboring pTV118N (empty vector)	This study
S17-1λpir	Donor strain in conjugation with *Bradyrhizobium* (hsdR, [RP4-2 Tc::Mu Km::Tn7 (Tp Sm)], λpir) Tc^S, Km^S	Mazodier et al. (1989)
Bradyrhizobium elkanii		
USDA94	Wild-type strain, Tc^R, Km^S	Minamisawa et al. (2002)
USDA94ΔcadA	In-frame disruption mutant of *cadA* of USDA94	This study
USDA94ΔtfdAα	In-frame disruption mutant of *tfdAα* of USDA94	This study
USDA94ΔcadAΔtfdAα	In-frame disruption mutant of *cadA* and *tfdAα* of USDA94	This study
USDA94-BBR2cadABCK	USDA94 harboring pBBR2-94cadABCK	This study
USDA94-BBR2tfdAα	USDA94 harboring pBBR2-94tfdAα	This study
USDA94-BBR2	USDA94 harboring pBBR1MCS2_START (empty vector)	This study

Table 2 Proteins homologous to CadRABCK and TfdAα (deduced amino acid sequences)

Protein (size aa)	Homolog (% similarity)	Origen	Function (size aa)	Accession No.
CadR (298)	BenM (44)	*Acinetobacter calcoaceticus* ADP1	LysR-type transcriptional activator (304)	AAC46441
	CadR (27)	*Bradyrhizobium* sp. HW13	*cad* gene transcriptional regulator (336)	AB062679
CadA (457)	CadA (72)	*Sphingomonas agrestis* 58-1	2,4-D oxygenase large subunit (448)	AB353895
	CadA (71)	*Bradyrhizobium* sp. HW13	2,4-D oxygenase large subunit (448)	AB062679
CadB (171)	CadB (65)	*S. agrestis* 58-1	2,4-D oxygenase small subunit (177)	AB353895
	CadB (62)	*Bradyrhizobium* sp. HW13	2,4-D oxygenase small subunit (177)	AB062679
CadC (107)	NahAb (65)	*Pseudomonas* sp. NCIB 9816-4	ferredoxin component of naphthalene 1,2-dioxygenase (104)	NP_863070
	CadC (23)	*Bradyrhizobium* sp. HW13	ferredoxin component of 2,4-D oxygenase (105)	AB062679
CadK (437)	TfdK (37)	*Ralstonia eutropha* JMP134	2,4-D transporter (460)	YP_009074945
	CadK (32)	*Bradyrhizobium* sp. HW13	2,4-D transporter (467)	AB062679
TfdAα (295)	TfdAα (99)	*Bradyrhizobium* sp. HW13	α-ketoglutarate-dependent 2,4-D dioxygenase (295)	AB074492
	TfdA (61)	*R. eutropha* JMP134	α-ketoglutarate-dependent dioxygenase (287)	EU827467