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Identification of the 4-Hydroxycinnamate Decarboxylase (PAD) Gene of *Klebsiella oxytoca*

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A 7.1-kbp DNA fragment isolated from a wild strain of *Klebsiella oxytoca* was sequenced, leading to the identification of 10 open-reading frames (ORFs), including a 504-bp *Pad* gene. The *Pad* gene of the Gram-negative bacterium was subsequently expressed in *Escherichia coli* as a chimeric *Pad*. The deduced amino acid (AA) sequence of the *Pad* gene from wild-type *K. oxytoca* showed approximately 50% homology to those of other bacterial PADs from Gram-positive bacilli plus a coccus. These data and a genomic library search of some *γ*-proteobacteria, including *E. coli* and *Vibrio* sp., indicated that PAD of *K. oxytoca* is a member of the bacterial PAD family characteristic of Gram-negative bacteria. Using *Pad*-specific PCR primers designed from the Gram-negative bacterial *Pad* of *K. oxytoca*, *Pad* genes of two further strains of *K. oxytoca*, another wild isolate and JCM 1665 and two PAD-positive *Enterobacter* spp. were successfully amplified for specific *Pad* detection.

Key words: *Klebsiella oxytoca*; bacterial *Pad* gene; PCR detection of *Pad*; Gram-negative bacteria; 4-hydroxycinnamate decarboxylase gene

Some plant families, such as Convolvulaceae, Asteriaceae, and Umbelliferae, are representative chlorogenic acid-accumulating plant families that contain high concentrations of hydroxycinnamoyl quinic acids in their leaves and/or roots. Many of these plant polyphenols are now recognized as self-defensive substances, but the roles of these plant products in interactions between polyphenol-rich plants and host-associating microorganisms are not well understood.[1,2] In 1993, Hashidoko *et al.* investigated phylloplane bacteria from the chlorogenic acid-accumulating plant *Polymnia sonchifolia*, a member of the Asteriaceae family. A phylloplane bacterium was tentatively identified as *Klebsiella oxytoca* by morphological and biochemical characterization. This organism was found actively to decarboxylate 4-hydroxylated cinnamic acids by a substrate-inducible 4-hydroxycinnamate decarboxylase (4-HCD; also a synonym of phenylacrylic acid decarboxylase, PAD) to utilize the decarboxylative product, 4-hydroxylated styrene, as an antifungal agent.[3] In 1994, Hashidoko *et al.* successfully cloned the *Pad* gene from a genomic library prepared from wild-type *K. oxytoca*.[4] A 9.6-kbp genomic fragment carrying the *Pad* gene was obtained using *Cladosporium herbarum* AHU 9262 as a bioindicator which was highly sensitive to 4-hydroxystyrene produced from 4-hydroxycinnamic acid *via* PAD catalytic decarboxylation. Transformed *Escherichia coli* JM 109 harboring the cloned *Pad* gene region acquired the ability to decarboxylate (*E*)-4-hydroxycinnamate into 4-hydroxystyrene. Digestion with *Sac*I and *Eco*RI cleaved this 9.6-kbp DNA region into three fragments: *Hin*dIII-*Eco*RI (2.4 kbp, fragment C), *Eco*RI-*Sac*I (4.7 kbp, fragment B), and *Sac*I-*Hin*dIII (2.5 kbp, fragment A). These three fragments were independently cloned into the pUC19 vector. The expression of *Pad* in each fragment in transformed *E. coli* was unsuccessful. Only bacterial cells transformed with recombinant DNA containing fragment CB (7.1 kbp, *Hin*dIII-*Sac*I possessing an *Eco*RI site inside the DNA sequence) gained the ability to decarboxylate 4-hydroxycinnamic acid (unpublished data), but the DNA sequence of the *Pad* gene on fragment CB has not yet been characterized.

Bacterial *Pad* gene studies have been done in food chemistry, due to serious issues of off-flavor during the fermentation of foods and liquor. In 1995, bacterial *Pad* of Gram-positive *Bacillus pumilis* was first characterized by Zago *et al.*, and a full-length amino acid (AA) sequence of PAD was deduced.[5] Since this identification of *Pad*, additional *Pad* genes have been characterized in more than 20 different microorganisms, but almost all of these microorganisms are Gram-positive bacilli plus a coccus.[6–8] In contrast, among Gram-negative bacteria, only *Pseudomonas fluorescens*[9] and *K. oxytoca*[10] have been reported to possess PAD activity in crude protein from bacterial cells. Bacterial *Pad*-like ORFs have been found in genomic DNA from two Gram-negative proteobacteria, *Vibrio cholerae* and *Erwinia carotovora*,[11,12] but it is uncertain whether the

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encoded gene products possess PAD functionality *in vivo*. Hence both the enzymatic and the bacteriophysiological traits of PAD have yet to be examined in Gram-negative bacteria. In fact, the physiological and ecological roles of PAD-producing bacteria and PAD itself in the terrestrial ecosystem are unclear, because *K. oxytoca* immediately hydrolyzed chlorogenic acid to yield 4-hydroxylated styrenes in chlorogenic acid-containing potato-dextrose medium.[13] In this study, we aimed to determine the DNA sequence of *Pad* in fragment CB originating in epiphytic *K. oxytoca*, and to describe the PADs of other strains of *K. oxytoca* and two further wild isolates of *Enterobacter* spp.

Materials and Methods

General. The 7.1-kb DNA fragment (fragment CB) carrying the gene for PAD was identical to one previously cloned from chromosomal DNA of a wild-type *K. oxytoca* bacterial strain. This DNA fragment possessed *Hin*dIII and *Sac*I sites at the 5′ and 3′ ends respectively, and it was cloned into pUC19 vector plasmid. For PCR, HotStarTaq (Qiagen, Germantown, MD) was used at 97 °C for 15 min for preliminary heating. The reaction conditions are described in detail for each experiment below. DNA and amino acid (AA) sequence homology searches were performed on the website of NCBI (National Center for Biotechnology Information, USA) and DDBJ (DNA Data Bank of Japan, Mishima, Japan).

Primer design for direct sequencing. The primers used in this study are shown in Table 1. Based upon the sequence determination obtained from the initial round of sequencing, we designed customized forward/reverse internal primers. The DNA sequence of fragment CB was registered in the DDBJ DNA data bank under accession no. AB330293.

Table 1. Primers Used in Sequencing of Fragment CB

5′-GTTCCACGGTCTCTTCC-3′	(160–176)
5′-GATGTTGATGATCTGCC-3′	(395–411)
5′-GGATAATCTTCAGGTTCTC-3′	(837–856)
5′-GTTTCGCTTTCACCGT-3′	(1,707–1,722)
5′-ATGTCATCGCACAGCA-3′	(1,815–1,830)
5′-CATCCCGACGAACTGG-3′	(2,208–2,221)
5′-AGAGCGATGACGCTGCCGAA-3′	(2,336–2,355)
5′-GGATCTCAACCG-3′	(2,474–2,486)
5′-GTTGATCATCTTCGAGG-3′	(2,635–2,651)
5′-GCTTCGGTGAGCAT-3′	(2,888–2,901)
5′-GAGTCATCGCTGCT-3′	(2,909–2,922)
5′-AGCTCGCGCAGATAG-3′	(3,399–3,413)
5′-GGATAATTGCTTTGGTTC-3′	(3,832–3,849)
5′-CGTGAAACAGTTTATCG-3′	(4,843–4,859)
5′-GTCGGTGAGCTATAATCTC-3′	(5,412–5,430)
5′-ATGCGGTGCTGTATCAG-3′	(5,974–5,990)
5′-CGCCGAATGATATCAC-3′	(6,418–6,433)
5′-GCTCCGTCGCAGTTCAAAGA-3′	(6,840–6,859)
5′-ATGGGTACTGAAGG-3′	(6,905–6,918)

Characterization of ORF regions in fragment CB. The target DNA (fragment CB), possessing 7,102 bp between the *Hin*dIII and *Sac*I sites, did not contain any regions that exhibited high homology to *Pad* of *Bacillus* spp. Hence all of the ORF regions on this DNA fragment were searched by means of an ORF Finder tool provided by NCBI (http://www.ddbj.nig.ac.jp/search/blast-j.html). ORF regions were searched from both plus and minus strand directions with putative amino acid translations from three different frames (+1, +2, +3, −1, −2, and −3). A total of 20 ORF candidates of 250 bp or greater were identified on the target DNA. The deduced amino acid (AA) sequences of the putative proteins encoded on each ORF were further searched on the BLASTX database of DDBJ (http://www.ddbj.nig.ac.jp/search/blast-j.html).

Design of adapter primers for Pad *gene insertion into pGEX 4T-3.* In order to sub-clone target ORF regions into the pGEX vector, adapter primers were designed on the SIGMA Genosys website (http://www.genosys.jp/adt/SGJ_NN_3rd.html), and predictions for *Tm*, hairpin, and dimer structures were obtained. The designed adapter primers were: PAD-APF (forward, 5′-CGGAATTC-TATGAGCACATTCGACAAACA-3′, 29 mer, $Tm = 73\,^{\circ}C$), and PAD-APR (reverse, 5′-CGCTCGAGGAT-TACAGGTTGGCAGGAA-3′, 27 mer, $Tm = 77\,^{\circ}C$). These primers possessed *Eco*RI and *Xho*I restriction sites at the 5′ and 3′ ends respectively. The specificity of the primers was confirmed by PCR using fragment CB as the source of template DNA. PCR conditions were as follows for the amplification of the ORF-7 regions: 30 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min.

The resulting PCR product (523 bp) was double-digested with *Eco*RI and *Xho*I, and the enzymes were denatured by heating. The digested PCR product was then ligated into pGEX 4T-3 expression vector (Amersham Pharmacia Biotech, Uppsala, Sweden) at the *Eco*RI-*Xho*I cloning site for in-frame fusion to the internal GST gene in pGEX 4T-3. A set of adapter primers (−1, AP3, and AP4) was designed for *Pad* expression. The ligation reaction was set at a molar ratio of the vector plasmid:PCR product = 1:10. *E. coli* cells were transformed with the recombinant plasmid to facilitate the production of recombinant, PAD GST-fusion protein. Transformed cells were assayed for PAD activity with a decarboxylation test in the cultured medium using (*E*)-caffeic acid as the substrate.[10]

Confirmation of PAD activity in E. coli *transformed with recombinant pGEX 4T-3 plasmid. E. coli* JM109 competent cells (Nippon Gene, Toyama, Japan) were transformed with the recombinant pGEX 4T-3PAD plasmid carrying the *Pad* gene. Hundreds of colonies emerged on a selection plate containing 50 mg/liter of ampicillin. Among these, 96 colonies (B1-B96) were taken randomly from the selection plates and subjected

Table 2. ORFs on Fragment CB

no	Frame	Position	Size	Protein
1	−1	2083–98	1986	NAD-dependent epimerase/dehydratase, formyl transferase
2	−1	4315–3065	1251	UDP-4-amino-4-deoxy-L-arabinose oxoglutarate aminotransferase
3	+2	152–1264	1113	Not hit
4	−2	3117–2080	1038	Glycosyl transferase
5	+2	6248–7102	855	Bacterial extracellular solute-binding protein
6	+1	2530–3099	570	Carboxyl esterase (*Burkholderia* sp.)
7	−1	5107–4604	504	Bacterial phenolic acid decarboxylase (*Pad*)
8	+2	3221–3676	456	Not hit
9	+2	5645–6067	423	Transcriptional regulator LysR family
10	−2	1665–1251	405	Not hit
11	+2	2171–2569	399	Not hit
12	+3	5208–5588	381	Transcriptional regulator LysR family
13	−1	6658–6278	381	GTP binding protein (only for domain near N-terminal)
14	+2	4769–5131	363	Not hit
15	+2	1772–2101	330	Not hit
16	+3	1545–1835	291	Not hit
17	−1	7102–6815	288	Ni-binding periplasmic protein
18	+1	79–363	285	Not hit
19	+3	771–1055	285	Not hit
20	+3	390–656	267	Not hit

All of the ORFs greater than 250 bp are listed. ORF-7 encoded a putative protein that showed high sequence homology to bacterial PADs of Gram-positive bacteria.

to an insert check. Positive clones were then tested for decarboxylation activity. In this assay, (*E*)-caffeic acid was added as the substrate of PAD to 12-h-cultured medium to obtain a final concentration of 1 mM. After further incubation for 30 min, the medium was extracted with EtOAc, concentrated, and developed on TLC (Merck Kieselgel F_{254} Art 5715, 0.25 mm thick, Merck, Darmstadt, Germany) in $CHCl_3$-MeOH = 9:1 to check for the presence of 3,4-dihydroxystyrene.

Recovery of the Pad *gene fragment from the PAD positive clone.* The PAD assay-positive clones were cultured in LB-ampicillin medium for 24 h at 37 °C. The recombinant plasmid copied was recovered from the cultured *E. coli* cells by the lysozyme-alkaline method. The resulting plasmid was subjected to double-digestion with *Eco*RI/*Xho*I to obtain about 500 bp of inserted DNA. This fragment was subsequently sequenced (ABI PRISM™ 310 Genetic Analyzer, Applied Biosystems, Foster City, CA) in order to confirm the amplified DNA to be *Pad* from ORF-7.

Design of degenerate PCR primers for reliable Pad *gene detection by PCR.* The characterized *Pad* gene of *K. oxytoca* possessed some common motifs with those of *Bacillus* spp.,[5,7] *Lactobacillus* spp.,[6] and *Pediococcus pentosaceus*.[8] Bacterial *Pad* gene-detecting primers were designed from common motifs common to these organisms. The degenerate primers were then tested on Gram-negative bacteria isolated from the rhizosphere of wild plants collected throughout the Hokkaido University Campus.[14] Together with *K. oxytoca* JCM 1665, used as a reference bacterium,[13] these five newly isolated bacteria were tested for PCR detection of bacterial *Pad*s. These were tentatively identified as *K. oxytoca* 60E, two *Enterobacter* spp. (70S and 136A), and two *Burkholderia* spp. (67A and 67R). Among the tested Gram-negative bacteria, *K. oxytoca* JCM 1665, and *K. oxytoca* 60E possessed a substrate-dependent, inducible type of PADs. On the other hand, *Enterobacter* spp. 70S and 136A possessed a constitutive type of PAD, the latter of which was relatively weak in decarboxylation activity. *Burkholderia* spp. showed no PAD activity even in the presence of substrates, but they showed remarkable gallate decarboxylation activity equal to *Rhanella aquatilis*.[15] *K. oxytoca* 60E, *Enterobacter* sp. 70S, and *Enterobacter* sp. 136A were originally isolated from the roots of *Chenopodium album*, *Rudbeckia laciniata*, and *C. album* respectively. Two *Burkholderia* spp., 67A and 67R, were from a *Polygonum* sp.

Results

Identification of bacterial Pad *from* Klebsiella oxytoca

Full DNA sequencing of fragment CB (7.1 kb, *Hin*dIII-*Sac*I fragment from wild-type *K. oxytoca*) resulted in the identification 20 open reading frames (ORFs, ORF-1-20) that were greater than 250 bp in size. Among these, 11 ORFs encoded proteins/enzymes of known function (Table 2). Partial N-terminal amino acid (AA) sequences of ferulic acid decarboxylases purified from *L. plantarum* and *Pseudomonas fluorescens*[16,17] exhibited 50% homology to the AA sequence encoded in ORF-7 (5,107–4,604, on the reverse chain). The putative amino acid sequence encoded in ORF-7 was searched on InterProScan (http://www.ebi.ac.uk/InterProScan/).[18] The 504 bases of ORF-7 were characterized as the *Pad* gene in view of nearly 50% agreement of the coding

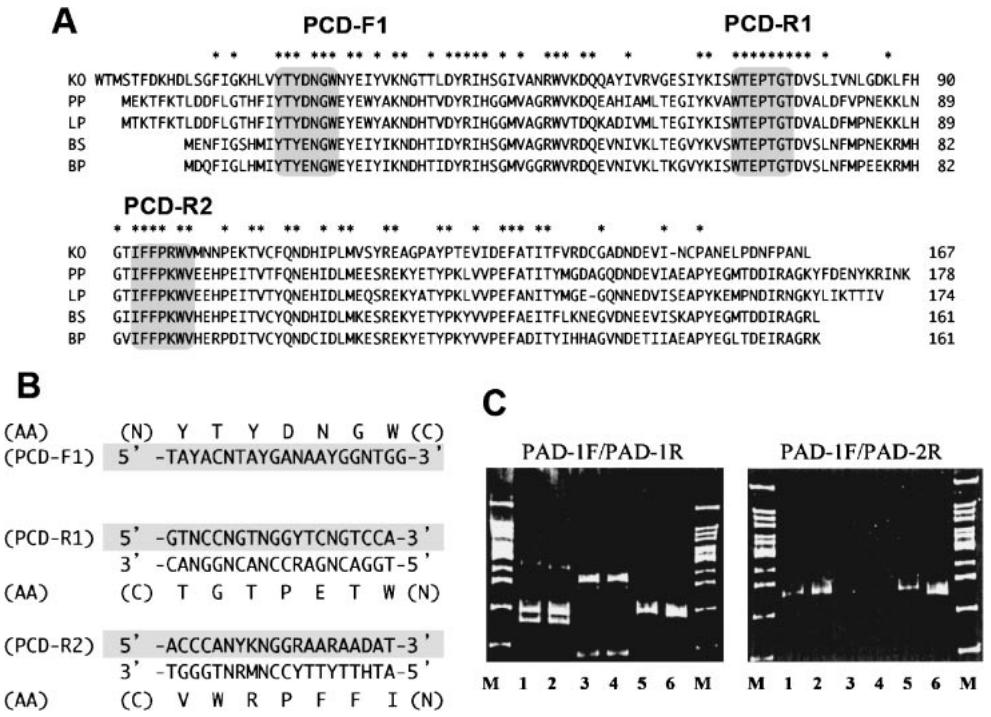

Fig. 1. Differences in PAD Characters between Gram-Positive Bacilli and Gram-Negative Rods.

A, The AA motifs indicated by colored backgrounds are preserved AA sequences among bacterial PADs. Using three motifs out of four, degenerate primers were designed for bacterial *Pad*-detecting PCR. KO, *Klebsiella oxytoca*; PP, *Pediococcus pentosaceus*; LP, *Lactobacillus plantarum*; BS, *Bacillus subtilis*; BP, *Bacillus pumilus*. ∗, identical amino acids. B, Oligonucleotide sequences of degenerate primers for bacterial PADs to cover Gram-negative bacteria. C, Bacterial PAD-detecting PCR assay for some test bacteria. Left, PCR products with PCD-F1/PCDR1; right, PCR products with PCD-F1/PCD-R2 primers. Electrophoresis was performed in a 4% stacking and 13% separating polyacrylamide gel. M, marker is a 100-bp ladder marker (100–1,000 and 1,500); 1, *Klebsiella oxytoca* 60E; 2, *K. oxytoca* JCM 1665; 3, *Burkholderia* sp. 67A; 4, *Burkholderia* sp. 67R; 5, *Enterobacter* sp. 70S; and 6, *Enterobacter* sp. 136A. Both PCR detections, with PAD-F1/PAD-R1 and PAD-F1/PAD-R2, were performed under the following PCR conditions: 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min. The volume of each primer solution (50 μM) added to the reaction solution (50 μl) was 1.5 μl. The resulting PCR products were run on a 13% polyacrylamide gel by slab gel electrophoresis along with a 100-bp ladder marker.

AA sequences with PADs reported from Gram-positive bacilli and a coccus. Furthermore, some conserved motifs common to PAD family proteins were identified in the PAD gene (Fig. 1A).[5–8)]

After full sequencing of fragment CB, it was evident that ORF-7 was located in reverse orientation to the *lac* promoter in the pUC19 plasmid. In addition, the sole *Eco*RI site was located at positions, 2,449–2,454, a region that was a long distance from ORF-7. Since neither fragment C nor B showed PAD activity on each recombinant plasmid when fragment CB was digested with *Eco*RI, we expected that the *Pad* gene would contain the *Eco*RI site, but this *Eco*RI site was not related to *Pad* gene expression. It is perplexing that the pUC plasmid carrying fragment C did not allow for the expression of PAD in transformed *E. coli*. On the other hand, it is reasonable in view of the reverse direction of the *Pad* gene against the *lac* promoter that IPTG failed to activate *Pad* expression in cells transformed with the recombinant pUC19-CB-carrying *Pad* gene.

Since we failed to observe induction in the pUC19 system, we inserted the ORF-7 region into the pGEX-4T-3 expression vector to obtain 10 independent colonies. The two clones (B35 and B43) tested were positive on caffeic acid decarboxylation assay (Fig. 2). The transformed *E. coli* JM109/pGEX-4T-3-PAD clone B43 was more active than pUC19-CB-transformed *E. coli* JM109. B35 and B43, selected as PAD-positive clones, were subsequently confirmed by PCR to carry the *Pad* gene in pGEX-4T-3-PAD plasmid. Expression of the *Pad* gene from Gram-positive bacteria in *E. coli* has been reported,[19)] but this is probably the first example of gene expression of a Gram-negative bacterial *Pad* in *E. coli*.

Degenerate PCR for detection of the Pad *gene using newly designed* Pad*-specific primers*

The amino acid sequence of the characterized PAD of wild-type *K. oxytoca* indicated that it is a decarboxylase of the bacterial PAD family. Sequence analysis also confirmed the presence of some motifs common to those of *Bacillus* spp. and *Lactobacillus plantarum*.[6)] For the characterization of PADs possessing motifs similar to those of Gram-positive bacilli, detection of *Pad* in wild-type *K. oxytoca* and *K. oxytoca* JCM 1665 by DNA homology searches failed. Furthermore, PCR detection with degenerate primers designed from Gram-positive bacilli were unsuccessful (data not shown) until the ORF

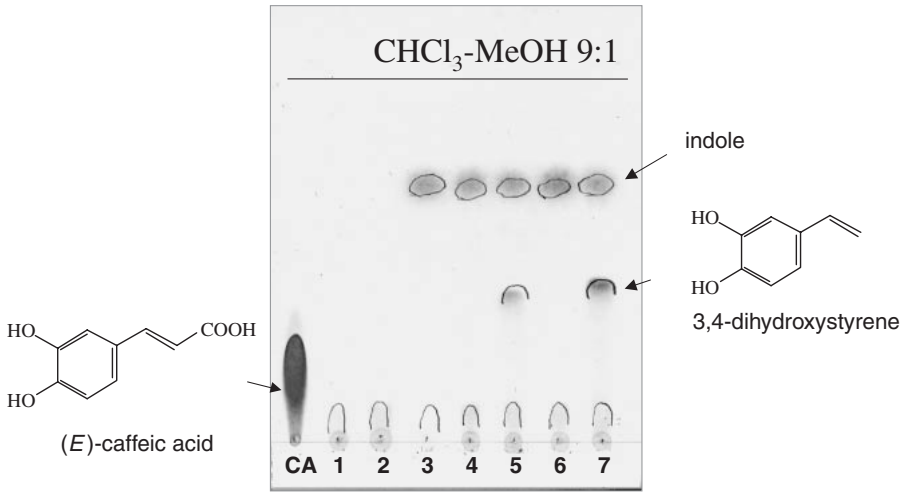

Fig. 2. PAD Activity of Transformed *E. coli* with the *Pad* Gene.

E. coli JM 109 that possessed ORF-7 inserted into pGEX-4T-3 expression vector plasmid (pGEX-4T-3-PAD). CA, caffeic acid only (5 mM in 10 ml of water); 1, LB broth medium only (10 ml); 2, LB medium + caffeic acid; 3, *E. coli* JM109/pUC19 + IPTG and caffeic acid; 4, *E. coli* JM109/pGEX-4T-3 + IPTG and caffeic acid; 5, *E. coli* JM109/pUC19-CB + IPTG and caffeic acid (positive control); 6, *E. coli* JM109/pGEX-4T-3-PAD B40 + IPTG and caffeic acid (negative control); 7, *E. coli* JM109/pGEX-4T-3-PAD B43 + IPTG and caffeic acid (positive). In all portions, IPTG was added to the cultures from the initiation of incubation. After overnight culture, 5 mM of caffeic acid was added as the decarboxylation substrate, and these transformed *E. coli* were further incubated for 30 min. After adjusting to pH 4.0 with 1 M HCl, 2 ml of EtOAc was added to the medium, and the test tube was vortexed for 1 min. The resulting EtOAc layer was then analyzed by TLC developed in $CHCl_3$-MeOH 9:1.

search in fragment CB. Hence, we designed new degenerate primers from common motifs of AA sequences that are encoded on bacterial *Pad* genes and *Pad*-like pseudo-genes.

Based on comparisons among AA sequences of some bacterial PADs, including that of phytoepiphytic *K. oxytoca* and ones obtained in a database search, YTYDNGW (19–25 AA, for forward primer), WTEPTGT (70–76 AA, for 1st reverse), and IFFPRWV (93–99 AA, for 2nd reverse) were selected as the target motifs in the wild-type *K. oxytoca* PAD to design *Pad*-specific primers (Fig. 1A). In this primer design, YTYDNQW and IYFPRWI of *Vibrio cholerae* (AE004296) PAD, located at 19–25 AA and 93–99 AA respectively,[12] and YTYANGW and IFFPQWI of *Mycobacterium avium* subsp. *paratuberculosis* (AE017229) at 28–34 AA and 102–108 AA respectively[20] were not included. These regions were excluded because these bacteria were assumed to be neither soil nor plant-associating bacteria.

From the preserved AA motifs of the target PAD, a degenerate forward (PAD-F1, 5′-TAYACNTAYGANAAYGGNTGG-3′) and two reverse (PAD-R1, 5′-GTNCCNGTNGGYTCNGTCCA-3′ and PAD-R2, 5′-ACCCANYKNGGRAARAADAT-3′) primers encoding the AA motifs were designed (Fig. 1B). Many of the PAD-positive bacteria isolated from the rhizoplanes of several plants in our systematic screening in the field showed decarboxylation activities almost equivalent to a reference strain, *K. oxytoca* JCM 1665. Five wild strains of Gram-negative eubacteria, isolated from the root surfaces of various host plants, were subjected to PCR assay using these degenerate primers for Gram-negative bacterial *Pad* gene detection. Using the fragment CB as a positive control, the combination of PAD-1F/PAD-1R and PAD-1F/PAD-2R produced amplified PCR products of 172 bp and 242 bp respectively.

Consequently, two *K. oxytoca* and two *Enterobacter* spp. tested by PCR with the combination PAD-1F/PAD-1R produced products of reasonable fragment sizes (> 200 bp), but the PCR products of two *Burkholderia* spp. were 300 bp in size (Fig. 1C, left). On the other hand, while four γ-proteobacteria of *K. oxytoca* (JCM 1665 and 60E) and *Enterobacter* spp. (70S and 136A) tested positive on PCR assay with PAD-1F/PAD-2R, two *Burkholderia* spp. were negative (Fig. 1C, right). The PCR products obtained by PAD-1F/PAD-2R primers were sequenced and compared for homology to their respective PADs on the amino acid level (Fig. 3). The PCR products of *K. oxytoca* JCM 1665 and 60E, both of which possessed an inducible type of active PADs, and *Enterobacter* sp. 70S with a constitutive PAD exhibited high homology to the partial AA sequence of bacterial PAD that was coded on the fragment CB from the phylloplane *K. oxytoca*. In contrast, *Enterobacter* sp. 136A, which possesses the constitutive type of PAD, exhibited low AA sequence homology to that of the inducible-type of Gram-negative bacteria.

Altogether, our degenerate primers, designed specifically for bacterial PAD gene detection, were applicable to γ-proteobacteria (genera *Klebsiella* and *Enterobacter*), but it is necessary to determine whether these primers are specific only to bacterial *Pad* of γ-proteobacteria (consisting mainly of inducible types of the PAD group), or whether they have broad specificity to

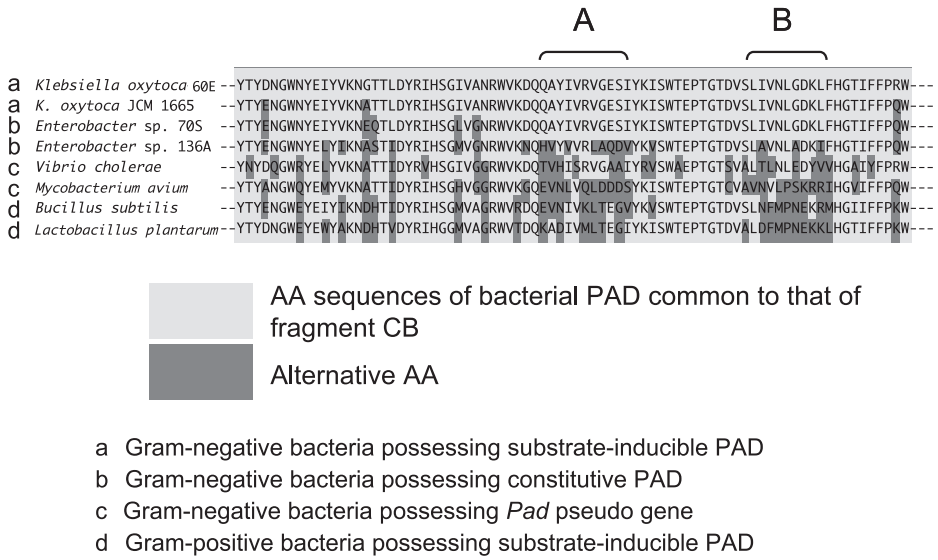

Fig. 3. Encoding Partial AA Sequences of the PCR Products from Bacterial *Pad*.

AA sequences shown by a light-gray background were common AA with substrate-inducible PADs of *K. oxytoca*, a Gram-positive bacterium. AA sequences alternative to those of *K. oxytoca* are shown by a dark-gray background, in which AA sequence variations were confirmed among Gram-positive bacteria and constitutive PADs or pseudo-gene-like *Pad*-possessing Gram-negative bacteria.

several bacterial genera and subclasses. In the PCR assay and subsequent DNA sequence determination, a constitutive type of bacterial PAD of *Enterobacter* sp. 136A was found to possess a unique AA sequence region. In other words, substrate-inducible PADs of γ-proteobacteria, at least, possess common motifs (the A and B regions as shown in Fig. 3). Because many of the constitutive types of PAD-possessing Gram-negative bacteria have been isolated (data not shown), it is necessary to determine whether constitutive PAD is separated into sub-families of PAD among Gram-negative bacteria. In our bacterial collection, they showed a diverse and wide range of variations of enzymatic behaviors of PADs.[13,21] A different group of PADs has been reported in ferulate decarboxylases of yeast (named yeast PAD1). Since this group of *Pad* genes do not exhibit any AA sequence homology to the bacterial PADs,[22,23] it is speculated that the origins of the yeast and the bacterial *Pad* genes are completely different. In fact, a function-unknown UbiX-like decarboxylase (PAD1) of *Escherichia coli* O157:H7 possesses an AA sequence that is highly homologous to those of yeast PAD1,[24] but *E. coli* does not show any PAD-like activity that is either constitutive or inducible.

Discussion

PADs produced by Gram-negative bacteria are grouped into two types: inducible PADs and constitutive PADs.[21] In our investigations, bacteria possessing constitutive PADs tended to be weakly active, and conversively, bacteria that contain substrate-inducible PADs were highly active on decarboxylation assay (data not shown), but it is not clear why some bacteria are capable of inducing PADs under exposure to substrates, or why it is necessary to accumulate such decarboxylative products in their habitat. Probably, *de novo* synthesis in PAD production is a trade-off between detoxification and energy consumption.

In our current research, *Pad* characterization from *K. oxytoca*, a Gram-negative phytoepiphyte (phylloplane bacterium) of γ-proteobacteria was done first, along with sequence determination of the upstream and downstream regions of the *Pad* gene. This is significant, because it will be possible to focus on important DNA regions associated with the regulation mechanism of substrate-dependent, inducible *Pad* gene expression in *K. oxytoca*. Since substrate-specificity and substrate-dependent induction of PAD has been well studied in *K. oxytoca* JCM 1665, genetic information from fragment BC is probably applicable to JCM 1665. In fact, it has been found that an indigested substrate analog, 6-hydroxynaphthoic acid, acts in *K. oxytoca* JCM 1665 cells as a powerful PAD inducer, maintaining its induction activity longer than (*E*)-caffeic acid or other natural substrates. Thus, both chemical and genetic tools for further biochemical study became available. Barthelmebs *et al.* have reported as a pioneer work that *PadA* gene expression in *Pediococcus pentosaceus* is regulated by bicistronic transcription of a *PadR* gene located downstream of *PadA*. In this bacterium, the PadR protein acts as a transcriptional repressor of the PadA operon upstream of the *PadA* gene. It is important to note that similar repressor regions were not found upstream or downstream of ORF-7 in fragment BC.[8]

The *Pad* gene-specific primers that were developed in this study should serve as a useful *Pad*-detection tool also for study of the environmental roles of the PAD-

producing bacteria. *Pad* gene distribution and expression in phenolic compound-accumulated habitats of phytoepiphytic bacteria indicate the importance of the PAD reaction in the terrestrial ecosystem.

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