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Global Gene Expression in *Bradyrhizobium japonicum* Cultured with Vanillin, Vanillate, 4-Hydroxybenzoate and Protocatechuate

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Pathways for aerobic degradation of naturally occurring aromatics were estimated from the entire genome sequence of *Bradyrhizobium japonicum* strain USDA110, a symbiotic nitrogen-fixing bacterium in soil. Many homologs for the genes encoding various oxygenases and enzymes for the β-ketoadipate pathway in the degradation of vanillin, vanillate, protocatechuate, and 4-hydroxybenzoate were scattered over nine loci of the genome. Using a macroarray developed for *B. japonicum* strain USDA110, we compared gene expression profiles in cells grown in each of these aromatic compounds as a sole carbon source with those of succinate-fed cells. One set of oxygenase genes homologous to *pcaGH*, *pobA*, and *vanAB* and structurally accompanied by transcriptional regulator homologs was markedly upregulated in their expression by one or more of the four aromatics, whereas no marked change was observed in the expression levels of *pcaBCDIJF* genes for the β-ketoadipate pathway. In addition, cells fed vanillin and vanillate showed high levels of expression of genes for a glutathione-dependent pathway of formaldehyde oxidation, suggesting that the formaldehyde generated from vanillate's demethylation is oxidized via C1 metabolism in *B. japonicum*. The expression of the above genes was confirmed by quantitative reverse transcription PCR. The implications of these results are discussed in terms of degradation pathways, gene regulation, and the soil environment.

Key words: *Bradyrhizobium japonicum*, aromatic degradation, C1 metabolism, transcriptome

Although advances in microbial genomics have been made in the last decade, the ecological functions and features of defined species of environmental microorganisms have not been fully evaluated[35]. It has now become technically feasible to study transcription on a global scale. Such studies will help to determine how microorganisms adapt to diverse environments[3,32].

Bradyrhizobium japonicum is a symbiotic nitrogen-fixing bacterium that is associated with soybeans[16]. The bacterium

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adapts to soil environments in upland and paddy fields, and is closely related phylogenetically to oligotrophs such as *Agromonas oligotrophica* and *Blastobacter denitrificans*[16,30,29].

Naturally occurring aromatic compounds are important sources of energy and carbon for soil-dwelling microorganisms and accumulate in the soil as a result of the degradation of plant-derived molecules, including lignin[15,19]. Indeed, *B. japonicum* is able to catabolize aerobically various aromatic compounds such as 4-hydrooxybenzoate[24], protocatechuate[14], and vanillin[8], probably via a β-ketoadipate pathway[10]. In particular, vanillin, a methoxylated phenolic aldehyde, is probably an intermediate in the degradation of humic substances and lignin[8]. Analysis of the entire genomic sequence of *B. japonicum* strain USDA110 has revealed the genomic basis of symbiotic nitrogen fixation[12,20,21]. The genome shows peculiarity and complexity, such as the existence of numerous gene copies for degradation, ABC transporters, the respiratory chain, C1 metabolism, and transcriptional regulation[7,12], which might permit the bacterium to adapt to diverse and varying environments in the soil.

The aim of this study was to illustrate global gene expression in *B. japonicum* strain USDA110 in the presence of naturally occurring aromatic compounds, and to find novel metabolic processes and responses during degradation of the compounds. To achieve this goal, we estimated the degradation pathways from the entire genome in advance, developed a macroarray for *B. japonicum* USDA110, and compared global gene expression in *B. japonicum* cells grown in the presence of each of four aromatic compounds or succinate.

Materials and Methods

Search for genes that degrade aromatic compounds

With the exception of *hcaB*, genes relevant to the degradation of vanillin, vanillate, 4-hydroxybenzoate, and protocatechuate, and genes for the enzymes involved in β-ketoadipate pathways, in *B. japonicum* USDA110 were searched for in the KEGG (Kyoto Encyclopedia of Genes and Genomics: http://www.genome.jp/kegg/) database. After the DNA sequences of the genes obtained had been searched using the BLASTP program[1] in the *Bradyrhizobium* section of Rhizobase (http://www.kazusa.or.jp/rhizobase/Bradyrhizobium/index.html), those genes that had E-values lower than 1×10^{-50} for amino acid similarity and formed clusters were regarded as paralog genes in the genome of *B. japonicum* strain USDA110. The *hcaB* gene encodes an enzyme involved in the transformation of vanillin to vanillate; we used the amino acid sequence of the protein product of this gene in *Acinetobacter* (accession number AAP78946.1) as a query sequence for a similarity search[25].

Strain and growth conditions

Bradyrhizobium japonicum strain USDA110[12] was used throughout the experiments. The strain was grown aerobically at 30°C in HM salt medium supplemented with 0.1% (w/v) arabinose[29,30] for preculture and in a defined mineral (DM) medium (a minimum medium for rhizobia[23]) for experiments with the feeding of aromatic compounds or succinate. DM medium contains the following components dissolved in 1 liter of water (pH 6.8): 220 mg of nitrilotriacetic acid; 580 mg of $MgSO_4$; 64 mg of $CaCl_2$; 0.2 mg of $(NH_4)_6Mo_7O_{24}\cdot7H_2O$; 2.0 mg of $FeSO_4\cdot7H_2O$; 1.0 ml of Hutner's "Metals 44" [2]; 1.0 g of $(NH_4)_2SO_4$; 12.5 mmol of KH_2PO_4; 12.5 mmol of Na_2HPO_4; and s 0.5 mg of biotin[23]. A filter-sterilized stock solution (100 mM) of vanillin, vanillate, 4-hydroxybenzoate, and protocatechuate was added to 200 mL of DM medium in a 500-mL Erlenmeyer flask at a final concentration of 1 mM. After cell inoculation, the concentration of the aromatic compounds in the culture supernatant was monitored with a spectrophotometer at intervals of 24 h or 12 h by measuring the ultraviolet absorbance at a wavelength of 251 to 280 nm. According to the concentrations, 1 ml of stock solution of the each aromatic compound was added to the 200-ml culture. This was done to maintain the concentration of each of the aromatic compounds (approximately 0.4 mM to 1.2 mM) in the culture during 4 days of cultivation. To examine cell growth and viability in the presence of each aromatic compounds, the cultures were serially diluted with a 0.85% (w/v) NaCl solution, the total cell number was determined directly by microscopy[28,34], and the cells were plated onto HM agar medium for evaluating CFUs.

Macroarray preparation

To prepare probes to load on the macroarray, we first selected 3739 clones out of the BRB library; each clone carried a 2.7-kb fragment (on average) of *B. japonicum* USDA110 genomic DNA in M13mp18 and had been sequenced by the whole-genome shotgun method[12]. Each insert of the selected clone was amplified by PCR with M13 universal/reverse primers[32]. For the gaps left on the genome, we designed 442 PCR primers (http://orca10.bio.sci.osaka-u.ac.jp/array02/) and amplified 221 DNA fragments of 1.2 kb (on average) using cosmid clones

(BRC library)[26] containing the corresponding regions. We used a spotting machine (MicroGrid II; Apogent Discoveries, Cheshire, UK) to spot a total of 3960 DNA fragments, which covered 98.4% of all the genes predicted in the genome, on nylon membranes (80×120 mm; Biodyne-A, Japan Pall Co. Ltd., Tokyo, Japan).

RNA preparation and macroarray analysis

When the cell turbidity had reached the mid-exponential phase, as shown by an optical density of 0.1 to 0.2 at a wavelength of 660 nm, the cells were harvested by centrifugation (25°C, 6,000×*g*, 10 min) and immediately suspended in RNAlater (Ambion, Austin, TX, USA). The cells were then washed and resuspended in 300 μL of TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) at 4°C. After the cell suspension had been sonicated three times at a power setting of 10 W with 50% active cycles for 30 s on ice (Branson Sonifier S-250, Danbury, CT, USA), total RNA was prepared using Trizol Max and TRIzol (Invitrogen, Carlsbad, CA, USA)[32]. After digestion with DNase I, the RNA was further purified[28,32,34]. cDNA labeling, hybridization, image acquisition, and data analysis were carried out as described previously[32]. At least three sets of biologically independent array analyses were carried out for each compound. The aromatics are aerobically catabolized and enter into the TCA cycle, whereas succinate is utilized directly as a substrate for the TCA cycle. Thus, we followed the significant upregulation caused by each of the four aromatic compounds as compared with succinate-fed cells. A *t*-test was performed to compare the expression level of each of the aromatic compounds with that of succinate.

Quantification of gene expression by real-time RT-PCR

The relative intensity of gene expression was estimated by real-time reverse transcription (RT)-PCR as described previously[28,32,34]. The primer sequences used in this work are available at http://orca10.bio.sci.osaka-u.ac.jp/array02/.

Results and Discussion

Prediction of degradation-related genes and pathways

It has been reported that *B. japonicum* degrades 4-hydroxybenzoate[23], protocatechuate[14], and vanillin[8]. Although Lorite *et al.*[14] found that a *pcaB* mutant was unable to catabolize protocatechuate and 4-hydroxybenzoate, the other genes relevant to aerobic degradation have not yet to be identified experimentally in *B. japonicum*.

When we searched and re-annotated the genes related to aerobic degradation, we found nine clusters that may be involved in the degradation of the four aromatics (Fig. 1). In most steps of the degradation process, two sets of gene clusters were found as follows: locus 1 (*hcaB1*) and locus 2 (*hcaB2*) for the conversion of vanillin to vanillate; locus 3 (*vanA1B1*) and locus 4 (*vanA2*) for the conversion of vanillate to protocatechuate; locus 5 (*pcaG1H1*) and locus 6 (*pcaG2H2*) for the conversion of protocatechuate to β-carboxy-*cis*, *cis*-muconate; locus 8 (*pcaI1J1*) and locus 9 (*pcaI2J2*) for the β-ketoadipate pathway, and locus 5 (*pobA1*) and locus 2 (*pobA2*) for the conversion of 4-hydroxybenzoate to protocatechuate (Fig. 1). On the other hand, single gene clusters containing *pcaBDC* and *pcaF* were found at locus 7 and locus 6, respectively. When these genes were again searched using the BLASTP program[1] on August 12, 2006 via DDBJ (DNA Data Bank of Japan, http://www.ddbj.nig.ac.jp/), they all showed E-values lower than 1×10^{-10} for amino acid sequence similarity with known genes. The scattered organization of the genes for degradation in *B. japonicum* is quite different from the compact *pob-pca* operon in *Agrobacterium*, *Pseudomonas*, *Sinorhizobium* and *Acinetobacter*[5,15,16].

Transcription of predicted degradation genes by array

We compared the expression profiles of the cells grown in each of the four aromatic compounds as a sole source of carbon and energy with those of succinate-grown cells. Because the addition of a high concentration (10 mM) of each aromatic as a sole carbon source gave rise to growth inhibition or flocculation of *B. japonicum* cells, we supplied lower concentrations of each compound (around 1 mM) to the culture in DM minimum medium for 4 days. Under these conditions, normal cell growth was observed. The total number of cells increased 12 to 20 fold during culture for 3 days in the presence of each of the four aromatic compounds, and the cell viability (number of CFUs) increased by four or five fold; these results were similar to those in DM medium supplemented with 0.1% (w/v) arabinose as a sole carbon source.

Table 1 lists the array clones that showed both a more than 5-fold upregulation and statistically significant results with the *t*-test ($p<0.05$). Examples of array expression data are shown in Fig. 2.

In the presence of vanillin and vanillate, the expression of the array clone brb00339 containing *hcaB1* genes (Fig. 2A) and clone brb15955 containing *vanA1B1* genes (Fig. 2B) was markedly upregulated at locus 1 and locus 3 (Fig. 1), respectively. The level of expression of two clones (brb01233 and brb07579) containing *pcaG1H1* at locus 5 in cells fed each of the four aromatics was higher than that in

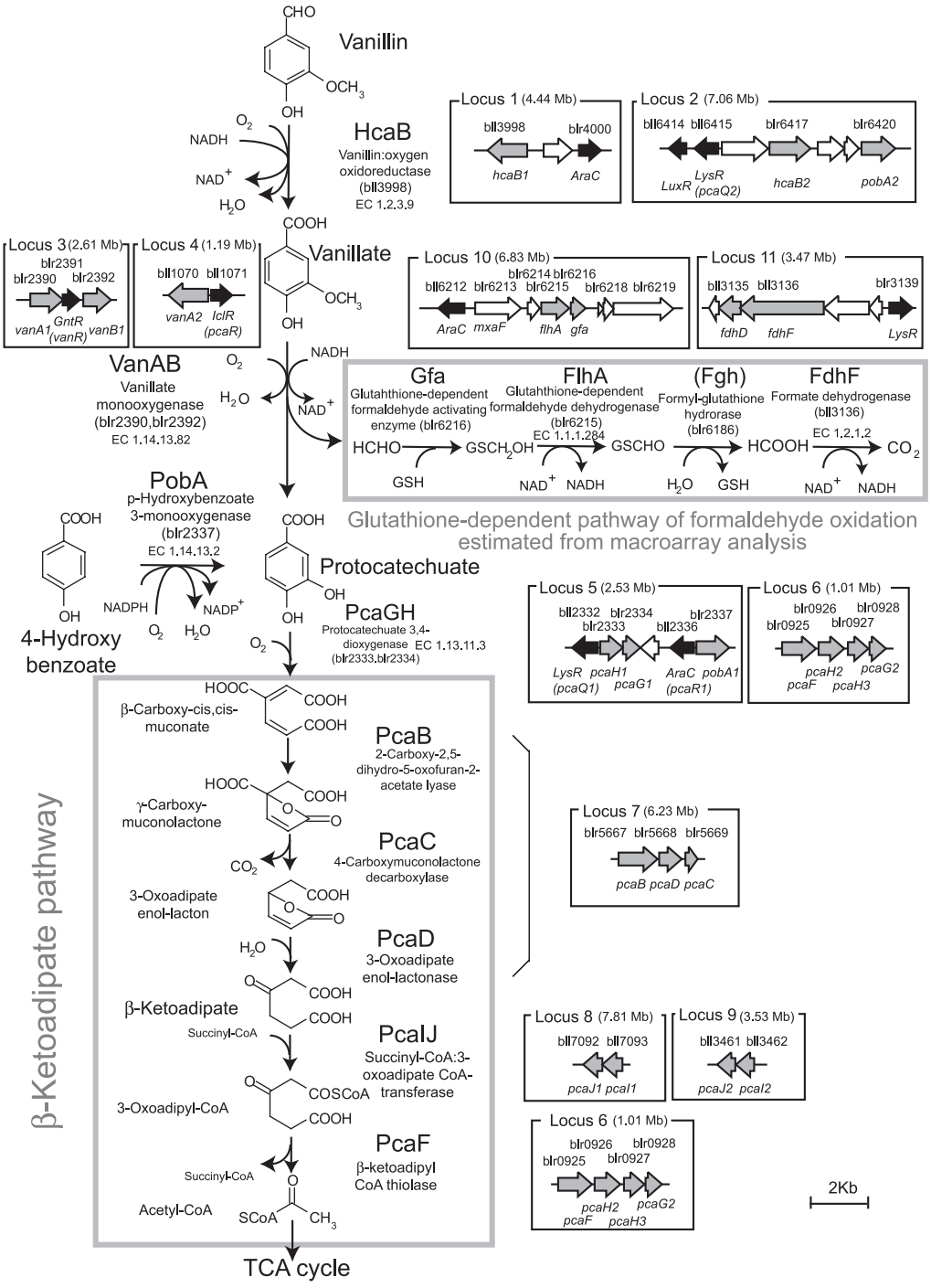

Fig. 1. Organization of genes and pathways in the genome of *Bradyrhizobium japonicum* strain USDA110 for the degradation of vanillin, vanillate, 4-hydroxybenzoate, and protocatechuate. Nine loci of gene clusters (loci 1–9) were found from information on the entire genome, along with a predicted main pathway of degradation from vanillin to acetyl-CoA (see text). Gray and black arrows are structural genes for degradation and putative regulator genes, respectively. Regulatory families[7] are shown for putative regulator genes (black arrow) with predicted gene names in parentheses. Rightward direction of the genes at all the loci indicates clockwise on the circular genome map of strain USDA110[12]. Figures in parentheses that follow the locus numbers indicate positions (Mb) on the 9.1-Mb genome of strain USDA110[12]. The enzyme names used were those in KEGG (Kyoto Encyclopedia of Genes and Genomics: http://www.genome.jp/kegg/). The glutathione-dependent C1 pathway from formaldehyde to CO_2 was estimated from array data, which involved loci 10 and 11 (see text). GSH indicates glutathione. *mxaF* (blr6213) encodes a putative methanol dehydrogenase at locus 10 (see text).

Table 1. Significantly upregulated array clones in cells of *Bradyrhizobium japonicum* strain USDA110 fed vanillin, vanillate, 4-hydroxybenzoate, or protocatechuate, as compared with succinate-fed cells

Array clone[a]	Signal ratio[b]				Gene[c]					Annotated gene function[d]
	Vn	Va	4HB	P						
BJ7023	189		230		blr7741					
brb03583	31				blr2217	blr2218	blr2219			oxidoreductase with Fe-S subunit (blr2217), xanthine dehydrogenase (blr2218), dehydrogenase (blr2219)
brb15955	27	31			**blr2390**	blr2391	**blr2392**	blr2393		***vanA1* (blr2390),** GntR family (van) (blr2391), **putative dioxygenase subunit (*vanB*) (blr2392)**
brb01673	23	11			blr3044	blr3045				probable extracellular protease (EC 3.4.24.-) (blr3044)
brb07054	19	14			blr3133	bsl3134	bll3135	bll3136		Glu-NH3-ligase adenylyltransferase (blr3133), NAD-dependent formate dehydrogenase (bsl3134, bll3135, bll3136)
BJ7117	19				bll5689					TetR family (bll5689)
brb00218	18		19		blr8064	blr8065	blr8066			
brb14004	17				blr2439	blr2440	blr2441	blr2442		ABC transporter (blr2439, blr2440, blr2442)
brb21553	13				bll2327	bsl2328	bll2329	bll2330		nicotinate phosphoribosyltransferase (bll2327), putative monooxygenase (bll2329)
brb22696	11	9			bll3136	bll3137				formate dehydrogenase (bll3136), NADH dehydrogenase I chain F (bll3137)
brb00339	8				**bll3998**	blr3999	blr4000			***hcaB1* (bll3998),** 6-aminohexanoate-dimer hydrolase (blr3999), AraC family (blr4000)
brb07539	8				blr8000	bll8001	blr8002	bsr8003	blr8004	probable site-specific integrase/recombinase (blr8004)
brb19867	7				bll6988	bll6989	blr6990			
brb08438	7				bll3136	bll3137	bll3138	blr3139		formate dehydrogenase (bll3136), NADH dehydrogenase I (bll3137), NADH dehydrogenase I (bll3138)
brb04866	7	8			blr8167	bll8168	blr8169			Na+/H+ antiporter (bll8168)
brb18958	7		12	10	blr5489	bsl5490	blr5491	bll5492	blr5493	ATP-dependent DNA helicase (blr5493)
brb02404	6				bsr7564	bll7565	blr7566	blr7567		LysR family (bll7565), oxidoreductase (blr7566)
brb11912	6				blr7568	bll7569	bll7570	bll7571		flavoprotein (blr7568), probable *O*-methyltransferase (bll7569), *exoQ*-like protein (bll7571)
brb01233	6	20	7	13	blr2331	bll2332	**blr2333**	**blr2334**		MarR family (blr2331), LysR family (bll2332), ***pcaH1* (blr2333), *pcaG1* (blr2334)**
brb09579	6	6	7		bll3050	bll3051	bll3052	bsl3053	bll3054	methylmalonyl-CoA mutase (bll3054)
brb23118	5				blr0778	bll0779				catalase (blr0778), polyribonucleotide nucleotidyltransferase (bll0779)
BJ7193		77	71		blr4281					AraC family (blr4281)
brb02121		21	24		blr1746	blr1747	blr1748	bsr1749	bsr1750	nitrogenase Mo-cofactor synthesis protein (blr1746), Fe-Mo cofactor processing protein (blr1747), ferredoxin (bsr1750)
BJ7161		16			blr4673					
brb24054		11	8		bll6453	bll6454	bll6455			ABC transporter (bll6453, bll6454, bll6455)
brb22884		9			bll6902	bll6903	bll6904			outer membrane lipoprotein (bll6903), probable cation efflux system protein (bll6904)
brb13173		9	6		bll6455	blr6456				ABC transporter (bll6455), probable aliphatic sulfonates binding protein (blr6456)
brb07579		9		5	bll2332	**blr2333**	**blr2334**	bll2335	bll2336	LysR family (bll2332), ***pcaH1* (blr2333), *pcaG1* (blr2334),** AraC family (*pobR*) (bll2336)
brb14124		7			bll6450	bll6451	bll6452			substrate-binding protein (bll6450), alkanesulfonate monooxygenase) (bll6451), acyl-CoA dehydrogenase (bll6452)

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brb15565	6			blr6213	blr6214	blr6215	blr6216		methanol dehydrogenase (blr6213), cytochrome c protein (blr6214), alcohol dehydrogenase class III, *flhA* (blr6215), glutathione-dependent formaldehyde activating enzyme (blr6216)
brb01751	6			bll2910	bll2911	blr2912	blr2913		class III aminotransferase (bll2910), ABC transporter (blr2912, blr2913)
brb01266	6			bll2908	bll2909	bll2910	bll2911		probable amino acid binding protein (bll2909), class III aminotransferase (bll2910)
brb15948	5	7	6	bll7181	bll7182	bll7183			hypothetical protein
brb01777	5	9		bll6309	bll6310				putative exopolysaccharide production protein (bll6310)
BJ7031		100		blr2581					putative D-fructose-1,6-bisphosphatase protein (blr2581)
brb00544		13		bll6302	bll6303	bll6304			putative lipopolysaccharide biosynthesis protein (bll6303)
brb02910		13		blr3695	blr3696				probable Flagellin (blr3695)
brb03629		8		bll0295	bsl0296				transcriptional regulator (bll0295)
brb00413		8		blr1619	blr1620	bsr1621	bsr1622	bll1623	conjugal transfer protein (blr1619, blr1620)
brb02410		7		bll6090	bll6091	bll6092			ABC transporter permease protein (bll6090), ABC transporter ATP-binding protein (bll6091), probable dihydroxy-acid dehydratase (EC 4.2.1.9) (bll6092)
brb00555		7		bll3668	blr3669				transcriptional regulatory protein AraC family (bll3668), putative 3-oxosteroid 1-dehydrogenase (EC 1.3.99.4) (bll3669)
brb12121		7		bll6778	bll6779	bsl6780			potassium-transporting ATPase B chain (bll6778), potassium-transporting ATPase A chain, (bll6779)
brb20737		7		blr3627	blr3628	blr3629	blr3630		aspartate aminotransferase A (blr3627), ABC transporter permease protein (blr3628, blr3629), ABC transporter substrate-binding protein (blr3630)
brb03146		7		bsl2014	bsl2015	bll2016			NolZ (bsl2015), NolY protein, (bll2016)
brb05095		7		blr1880	blr1881				transcriptional regulatory protein LuxR family (blr1880)
BJ7202		6		blr3324					flavoprotein oxidoreductase (blr3324)
brb03172		6		blr6052	blr6053				putative adenylate cyclase (blr6052), probable substrate-binding protein (blr6053)
brb05002		6		bsr1982	bsr1983	blr1984	bll1985		putative transposase (bsr1982, bsr1983, bsr1984), HipA protein (bll1985)
brb14381		6		blr3545	blr3546	blr3547			ABC transporter permease protein (blr3545. blr3546), ABC transporter substrate-binding protein
brb19013		6		blr2170	blr2171				probable ABC transporter substrate-binding protein (blr2170), probable ABC transporter permease protein (blr2171)
brb19034		6		bll3766	blr3767	bll3768			similar to flavohemoprotein (bll3766)
BJ7095		6		bll1608					putative transposase (bll1608)
brb16028		6		bll8274	bll8275	bll8276	bll8277		
brb10454		5		bll2066	bll2067				nodulate formation efficiency C protein (bll2067)
brb01869		5		bsr8213	bsr8214	blr8215	blr8216	blr8217	putative transposase (bsr8214, blr8215, blr8216, blr8217, blr8218, blr8219)
				blr8218	blr8219				
brb14961		5		blr3325	blr3326				transcriptional regulatory protein GntR family (blr3325), probable hydantoin utilization protein (blr3326)
brb03439		5		blr1934	blr1935				
brb01942		5		blr2131	blr2132				probable oxygenase (blr2131)
BJ7048		5		bll7880					putative oxidoreductase protein (bll7880)

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brb14662	5		blr0987	blr0988	bll0989	bll0990		3-methylcrotonyl-CoA carboxylase alpha subunit (EC 6.4.1.4) (bll0987), peptide ABC transporter ATP-binding protein (bll0989, bll0990)
brb02318	5		bll2336	blr2337	bll2338			transcriptional regulatory protein AraC family (bll2336), ***pobA* (bll2337)**
brb01972	5		bll3661	bll3662	bll3663			ABC transporter ATP-binding/permease protein (bll3661), ABC transporter permease protein (bll3662), ABC transporter substrate-binding protein (bll3663)
brb07073	5		bsl8058	bsl8059	blr8060	bsr8061	blr8062	two-component response regulator (blr8062)
			blr8063					
brb13250	5		blr3515	blr3516				probable Mn-dependent hydrolase (blr3515), probable sulfite oxidase molybdopterin subunit (blr3516)
brb23785	5		bll8258	bll8259	blr8260	blr8261		putative transposase (blr8260, blr8061)
BJ7107	18	23	blr8118	blr8119				ABC transporter (blr8118, blr8119)
brb11566		13	bll0888	bll0889				putative transport protein (bll0889)
brb04593	10	13	bll4814	bll4815	bll4816			
brb01203	10	9	bll6385	bll6386	bll6387	bll6388		3-dehydroquinate dehydratase (bll6386), ABC transporter (bll6387, bll6388)
brb05278		9	bll7072	bll7073	bll7074	bll7075	bll7076	biopolymer transport protein (bll7072, bll7073), Hemin receptor precursor (bll7076)
brb05924	11	8	bll1786	blr1787	blr1788	blr1789		
brb08383		7	bll7075	bll7076				Hemin receptor precursor (bll7076)
brb12316		7	bll0887	bll0888				ABC transporter (bll0887)
brb04182	7	6	bll6305	bll6306	bll6307			probable glycosyl transferase (bll6306), putative lipopolysaccharide biosynthesis protein (bll6307)
brb01746		6	blr3541	blr3542	blr3543			L-fuculose-1-phosphate aldolase subunit P (blr3542), shikimate 5-dehydrogenase (blr3543)
brb12879	5	6	bsr5197	bll5198	bll5199			
BJ7059		6	bll7071					TonB protein (bll7071)
BJ7129		6	bll3094					IclR family (bll3094)
brb19645		5	bll3874	bll3875	bll3876			amidase (bll3874), hypothetical metabolite transport protein (bll3875), aldehyde dehydrogenase (bll3876)
BJ7123		5	blr3214	blr3215				nitric oxide reductase subunit C (blr3214), subunit B (blr3215)

[a] Macroarray analyses were carried out in three biologically independent experiments. Listed are those array clones whose signal intensity in cells fed with aromatic compounds was increased more than 500% compared with that in succinate-fed cells by *t*-test ($p<0.05$).

[b] Ratios of signals of cells grown in vanillin (Vn), vanillate (Va), 4-hydroxybenzoate (4HB), or protocatechuate (P) to those of succinate-fed cells.

[c] Gene numbers are based on Rhizobase (http://www.kazusa.or.jp/rhizobase/index.html)[12], in which putative functions have been assigned to the underlined genes.

[d] Gene function annotated in Rhizobase. Bold indicates predicted degradation-related genes (Fig. 1). Double-underlines indicate putative genes relevant to formaldehyde oxidation via C1 metabolism in the presence of vanillin and vanilate. Single-underlines indicate putative genes relevant to iron metabolism.

succinate-fed cells (Fig. 2B). In the presence of 4-hydroxybenzoate, the expression of brb02318, containing *pobA1*, was upregulated at locus 5 as well (Fig. 2B). No marked change was observed in array clones containing another set of oxygenase genes, *hcaB2*, *pobA2*, *vanA2*, and *pcaG2H2* (Fig. 2A for *hcaB2* and *pobA2*, data not shown for *vanA2* and *pcaG2H2H3*). The expression of two clones (brb02257 and brb16014) containing *pcaBCD* did not differ between cells fed any of the four aromatics and succinate (Fig. 2B). The *pcaIJF* gene expression was not upregulated at loci 7, 8, or 9 (data not shown). The data are available at a Web site for the MacroArray Analysis Page of *Bradyrhizobium japonicum* (http://orca10.bio.sci.osaka-u.ac.jp/array02/).

In summary, the expression of four oxygenase genes in *B. japonicum* USDA110—*hcaB1*, *vanA1B1*, *pobA1*, and *pcaG1H1*—was upregulated upstream of the β-ketoadipate pathway in response to one or more of the aromatic compounds, which was reasonable given that the respective aromatic compounds act as an inducing substrate (Fig. 1). On the other hand, the expression levels of another set of oxy-

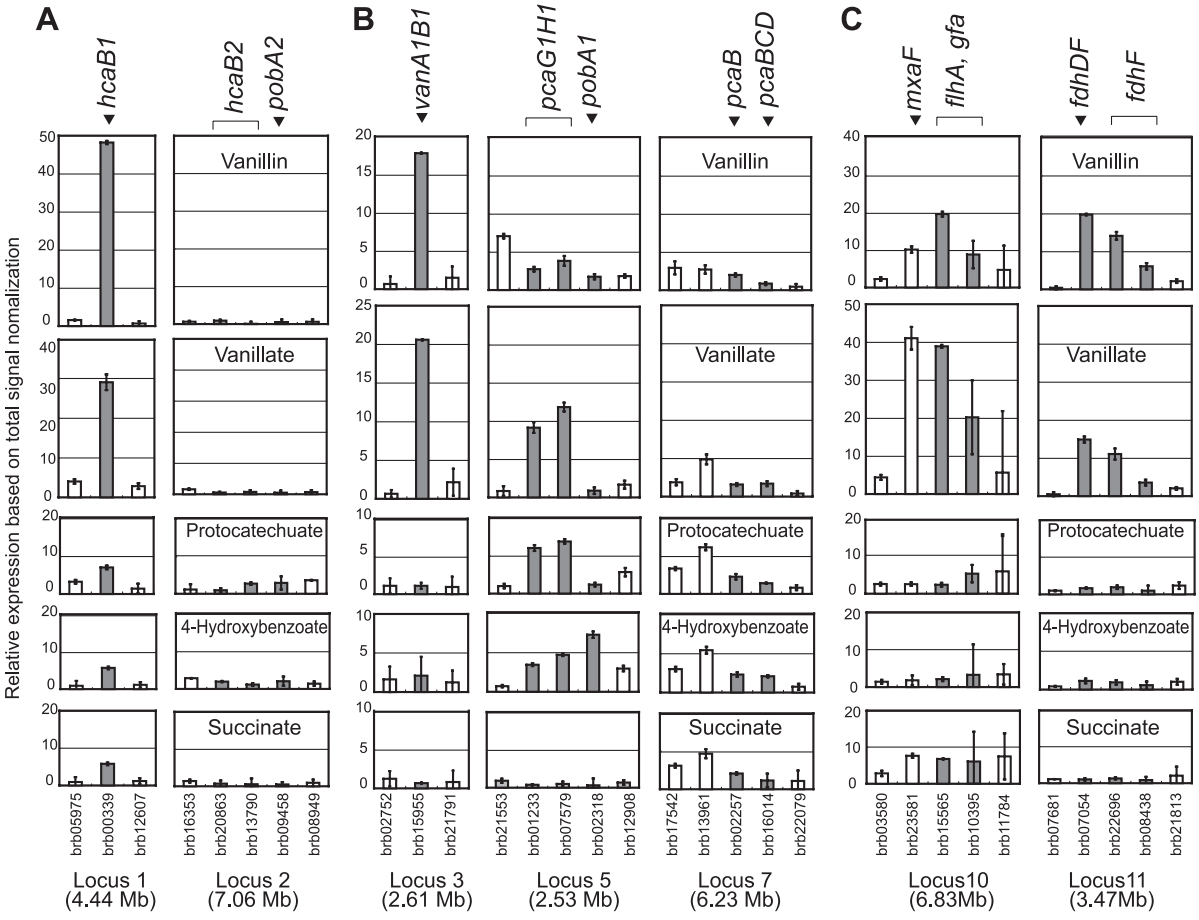

Fig. 2. Expression profiles of cells fed each of four aromatics or succinate. Panel A shows locus 1 (*hcaB1*) and locus 2 (*hcaB2* and *pobA2*). Panel B shows locus 3 (*vanA1B1*), locus 5 (*pcaG1H1* and *pobA1*), and locus 7 (*pcaBCD*). Panel C shows locus 10 (*flhA* and *gfa*) and locus 11 (*fdhDF*). Numbers with "brb" prefixes are clone numbers on the array membrane. The array clones normally contained two to five genes (Table 1). The loci and genome positions are identical to those in Figure 1. Expression levels were normalized based on total signal intensity[32] and show standard deviations (as bars) from three biologically independent experiments. Gray bar indicates array clone containing degradation genes as shown in Fig. 1, while open bar shows clone surrounding the degradation genes.

genase genes (*hcaB2*, *vanA2*, *pobA2*, and *pcaG2H2H3*) and the *pcaBCDIJF* genes involved in the β-ketoadipate pathway were not markedly altered.

Quantitative RT-PCR analysis

The above results were confirmed by conducting a quantitative RT-PCR analysis (Table 2). The expression of *hcaB1* was significantly upregulated by vanilline and vanilate. That of *vanA1* and *vanB* was significantly upregulated by vanillin and vanillate. The *pobA1* gene was significantly upregulated in its expression by 4-hydroxybenzoate. The expression of *vanG1* and *vanH1* genes was significantly upregulated by each of the four aromatic compounds.

The expression of *vanA1B1*, *pobA1*, and *pcaG1H1* was highly upregulated by one or more aromatics as compared with succinate-fed cells, although the increase in the expression of *hcaB1* caused by vanillin and vanillate was relatively small (Table 2).

Characteristics of gene expression for β-ketoadipate pathway

Parke and Ornston[24] compared enzymatic activities in quinate-treated and untreated cells of *Bradyrhizobium* spp. Protocatechuate-3,4-dioxygenase activity increased by five to 15 fold in the treated cells. However, the activities of four enzymes (PcaB, β-carboxy-*cis*, *cis*-muconate lactonizing enzyme; PcaC, 4-carboxymuconolacton decarboxylase; PcaD, β-ketoadipate enol-lactone hydrolase; PcaI, β-ketoadipate succinyl-CoA transferase) involved in the β-ketoadipate

Table 2. Validation by real-time RT-PCR of putative genes of *Bradyrhizobium japonicum* strain USDA110 upregulated by aromatics

Gene anotation	Gene number	Relative expression[a]			
		Vn	Va	4HB	P
hcaB1	bll3998	**10***	5*	1	4
hcaB2	blr6417	3	5	1	0
vanA1	blr2390	**23****	**61****	1	0
VanB1	blr2392	**20***	**49****	6	7
vanA2	bll1070	1	2	1	1
pobA1	blr2337	3	8	**111***	5
pobA2	blr6420	2	7	1	1
pcaG1	blr2334	**10***	**80****	**13****	**19****
pcaH1	blr2333	**28****	**415****	**25****	**98****
pcaG2	blr0928	3	8	10	4
pcaH2	blr0926	4	8	3	4
pcaH3	blr0927	2	3	1	2
pcaB	blr5667	2	8	6	1
pcaI1	bll7093	2	6	3	3
pcaI2	bll3462	2	6	2	2
pcaF	blr0925	2	7	3	2
mxaF	blr6213	**12***	**23***	1	1
gfa	blr6216	**24****	17	0	1
flhA	blr6215	**12****	**55***	1	2
fdhF	bll3136	**26***	**67****	2	1
fdhD	bll3135	**44***	**45****	2	1

[a] Relative expression: Ratios of signals of cells grown in vanillin (Vn), vanillate (Va), 4-hydroxybenzoate (4HB), or protocatechuate (P) to those of succinate-fed cells. Values are means of three independent experiments by real-time RT-PCR. Star marks indicate a significant difference between aromatics and succinate by t-test (* $p<0.05$, ** $p<0.01$).

pathway (Fig. 1) were not induced, and these enzymes were therefore constitutive in *Bradyrhizobium* spp., unlike in other bacteria[21,22]. Our results support the enzymatic properties of the β-ketoadipate pathway in bradyrhizobia in that the *pcaBCDIJF* gene was constitutively expressed.

In this regard, none of the transcriptional regulators preceded the *pcaBCDIJF* genes at loci 6, 7, 8, and 9 (Fig. 1). In contrast, the appropriate transcriptional regulators *vanR* (GntR family), *pcaR1* (AraC family), and *pcaQ1* (LysR family) at loci 3 and 5 were adjacent to the *vanA1B1*, *pobA1*, and *pcaH1G1* genes, the expression of which was markedly upregulated (Fig. 1, Table 2).

B. japonicum is able to survive and even grow in distilled water as an oligotrophic bacterium[4]. Park and Ornston[24] discussed that a severe limitation of growth substrate might result in an unregulated catabolic enzymes. If so, *B. japonicum* may reduce the physiological expense of transcriptional control for the β-ketoadipate pathway, a central pathway of aerobic aromatic degradation, to adapt to soil environments through elimination of the transcriptional regulator as a oligotroph.

C1 metabolism suggested by array analysis

Apart from the degradation-related genes, several other array clones were unregulated (Table 1). Clones brb07054, brb22696, and brb08438, all containing the *fdhF* gene (NAD-dependent formate dehydrogenase) (double-underlined in Table 1, Fig. 2C) at locus 11 (Fig. 1), were highly expressed in the presence of the methoxy phenolics vanillin and vanillate. VanAB, vanillate monooxygenase, produces formaldehyde as a byproduct of the demethylation of vanillate[26] (Fig. 1). Thus, the *fdhF* gene encoding formate dehydrogenase participates downstream of formaldehyde oxidation (Fig. 1). Interestingly, clone brb15565 was also upregulated in its expression by the methoxy phenolics (double-underlined in Table 1, Fig. 2C); it contained *flhA* encoding glutathione-dependent formaldehyde dehydrogase and *gfa* for glutathione-dependent formaldehyde-activating enzyme at locus 10 (Table 1, Fig. 2C). On the other hand, the *fgh* gene homolog encoding formyl-glutathione hydrolase (Fig. 1) exists as blr6186 which is located with two component regulatory protein genes for methanol utilization control at a position of 6.81 Mb in the strain USDA110 genome, although it was not markedly upregulated in the array analysis (data not shown).

It has recently been shown that there are several cofactor-dependent pathways of formaldehyde oxidation in methylotrophic bacteria[33]. Taken together, the above results suggest a pathway that is very similar to the glutathione-dependent formaldehyde oxidation pathway found in *Paracoccus denitrificans* and *Rhodobacter sphaeroides*[35] (Fig. 1). This idea was supported by the upregulated expression of the *gfa*, *flhA* and/or *fdhF* genes in the presence of vanillin or vanillate in the quantitative RT-PCR analysis (Table 2). The significant increase in the expression of *gfa* by vanillate was not shown by the quantitative RT-PCR analysis (Table 2). However, the expression of *gfa* is probably upregulated under the conditions because the expression of *mxaF* and *flhA* located upstream of *gfa* (Fig. 1) was significantly upregulated (Table 2). A growth phase-specific activation of C1 metabolism was also observed in *Burkholderia xenovorans* strain LB400 during the degradation of polychlorinated biphenyl (PCB), although the involvement of C1 metabolism in the degradation process remains unclear[5,6].

A two-component system, FlhRS regulates formaldehyde oxidation and plays a key role in controlling the concentration of the toxic formaldehyde in *Paracoccus denitrificans* although it has not yet been verified whether FlhRS directly senses formaldehyde in the cell[9]. The homologs of *Paracoccus denitrificans* FlhRS were found as blr1194 and bll1199 in strain USDA110 genome. The glutathione pathway in *B. japonicum* could detoxify the large amount of formaldehyde produced by the demethylation of vanillate. Mitsui *et al.*[17] reported that the non-methylotrophic *Burkholderia cepacia* assimilatively fixed formaldehyde derived from the methoxy moiety of vanillate into the ribulose monophosphate (RuMP) pathway. The dissimilatory pathway of glutathione-dependent formaldehyde oxidation to CO_2 thus appears intriguing and novel in the non-methylotrophic *B. japonicum*. In this regard, it is interesting that *mxaF* encoding methanol dehydrogenase was upregulated in its expression probably as a transcriptional unit for C1 metabolism with *flhA* and *gfa* (Fig. 1 and Fig. 2C).

Other upregulated genes

Several clones containing genes of unknown or unexpected function were highly upregulated in their expression in the presence of the four aromatic compounds (Table 1). However, we have not yet fully explained the involvement of most of theses genes in cell functions so far. For example, although the expression of clone BJ7023 was markedly upregulated in the presence of vanillin and 4-hyroxybenzoate (Table 1), the putative blr7741 gene contained in the clone is still a hypothetical protein that only showed a low degree of similarity to the polyneuriden-aldehyde esterase precursor in *Rauvofia seropentina* by BLASTP search.

In the cells fed protocatechuate, the expression of clones brb05278 and brb08383, containing the gene encoding the hemin receptor precursor, and clone BJ7059, containing the gene encoding *tonB*, was significantly upregulated by protocatechuate (Table 1). Because these genes are involved in the uptake of iron-loaded siderophores[27], *B. japonicum* might respond to an iron-deficient environment in DM medium containing protocatechuate.

Implications for environmental microbiology

Recently, genomic and molecular approaches have been introduced into environmental microbiology[5,11,13,18,31]. Array-based technologies have produced vast data sets, and sometimes the question of how to deal with the data bothers scientists. In this work, we focused on the metabolism of four aromatic compounds and simplified the substrate supply. It was therefore easy to analyze the array data, and we were able to predict a novel glutathione-dependent C1 pathway that occurs during the degradation of lignin-derived compounds in *B. japonicum*.

Dissolved soil organic matter consists of humic substances and low-molecular-weight organic compounds such as aromatics. Nadri *et al.*[19] detected 0.1 to 0.4 mM of vanillate, 1.5 to 2.7 mM of 4-hydroxybenzoate, and 0.1 to 0.5 mM of protocatechuate in the top layers of a forest soil. Thus, our results provide insight into how free-living bradyrhizobia respond to, and cope with, these aromatic compounds in soil environments. In addition, these array data sets will contribute to expression database for metabolism, cell function, and environmental adaptation of bradyrhizobia.

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