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Aerobic Vanillate Degradation and C_1 Compound Metabolism in *Bradyrhizobium japonicum*▽†

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***Bradyrhizobium japonicum*, a symbiotic nitrogen-fixing soil bacterium, has multiple gene copies for aromatic degradation on the genome and is able to use low concentrations of vanillate, a methoxylated lignin monomer, as an energy source. A transcriptome analysis indicated that one set of *vanA1B*, *pcaG1H1*, and genes for C_1 compound catabolism was upregulated in *B. japonicum* USDA110 cells grown in vanillate (N. Ito, M. Itakura, S. Eda, K. Saeki, H. Oomori, T. Yokoyama, T. Kaneko, S. Tabata, T. Ohwada, S. Tajima, T. Uchiumi, E. Masai, M. Tsuda, H. Mitsui, and K. Minamisawa, Microbes Environ. 21:240–250, 2006). To examine the functions of these genes in vanillate degradation, we tested cell growth and substrate consumption in *vanA1B*, *pcaG1H1*, and *mxaF* mutants of USDA110. The *vanA1B* and *pcaG1H1* mutants were unable to grow in minimal media containing 1 mM vanillate and protocatechuate, respectively, although wild-type USDA110 was able to grow in both media, indicating that the upregulated copies of *vanA1B* and *pcaG1H1* are exclusively responsible for vanillate degradation. Mutating *mxaF* eliminated expression of *gfa* and *flhA*, which contribute to glutathione-dependent C_1 metabolism. The *mxaF* mutant had markedly lower cell growth in medium containing vanillate than the wild-type strain. In the presence of protocatechuate, there was no difference in cell growth between the *mxaF* mutant and the wild-type strain. These results suggest that the C_1 pathway genes are required for efficient vanillate catabolism. In addition, wild-type USDA110 oxidized methanol, whereas the *mxaF* mutant did not, suggesting that the metabolic capability of the C_1 pathway in *B. japonicum* extends to methanol oxidation. The *mxaF* mutant showed normal nodulation and N_2 fixation phenotypes with soybeans, which was not similar to symbiotic phenotypes of methylotrophic rhizobia.**

Naturally occurring aromatics are important sources of energy and carbon for soil-dwelling microorganisms. Lignin represents an abundant carbon constituent of the vascular plant cell wall. Soluble phenolic lignin monomers, such as vanillate, are released by complex oxidative cleavage by some fungi (16). Indeed, vanillate and other lignin monomers have been found to be crucial components of dissolved organic matter in terrestrial (21) and marine (20) environments.

Bradyrhizobium japonicum, a symbiotic nitrogen-fixing soil bacterium, is able to aerobically catabolize low concentrations of aromatic compounds, such as vanillate and protocatechuate (3, 12, 24). Although many redundant copies of genes encoding proteins involved in aromatic degradation are scattered over nine loci of the *B. japonicum* genome, a previous transcriptome analysis (12) showed that vanillate and protocatechuate markedly upregulated the expression of only one set of oxygenase genes, *pcaG1H1* and *vanA1B* (Fig. 1).

This previous transcriptome analysis also indicated that genes for glutathione-dependent formaldehyde oxidation were highly expressed in *B. japonicum* cells grown in vanillate (12) (Fig. 1). The bacterial pathway for aerobic degradation of aromatic compounds has been investigated extensively (8). Although vanillate is demethylated to yield protocatechuate and formaldehyde (32) (Fig. 1), little is known about the fate of the methoxy group during degradation of methoxylated aromatics (10, 19, 32).

In *Burkholderia cepacia*, a bacterium that grows on several lignin monomers, the formaldehyde-fixing enzymes play important roles in the scavenging and assimilatory fixation of formaldehyde during vanillate degradation (18). A growth phase-specific activation of C_1 compound metabolism has also been observed in *Burkholderia xenovorans* LB400 during the degradation of polychlorinated biphenyl, although the involvement of C_1 metabolism in that degradation process remains unclear (4).

In this study, we examined the functions of the *vanA1B*, *pcaG1H1*, and *mxaF* genes during vanillate degradation by disrupting the *B. japonicum* USDA110 genes that were upregulated in the transcriptome analysis (12). The goal was to address whether the upregulated genes *vanA1B* and *pcaG1H1* are responsible for the degradation of vanillate and protocatechuate, a vanillate degradation product, in the bacterium. We also examined whether C_1 compound catabolism contributes to vanillate degradation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used are listed in Table 1. *Bradyrhizobium* cells were grown at 30°C in HM salt medium (2, 12) supplemented with 0.1% (wt/vol) arabinose and 0.025% (wt/vol) yeast extract (Difco, Detroit, MI) for preculture. For feeding of aromatic compounds or succinate, the cells were grown in a defined mineral medium that was a minimal medium for bradyrhizobia (12, 24). Cells in liquid media were cultured with reciprocal shaking at 300 rpm. Growth was monitored by measuring absorbance at a wavelength of 660 nm with a spectrophotometer (UV-1200; Shimazu, Kyoto, Japan). *Escherichia coli* cells were grown at 37°C in Luria-Bertani me-

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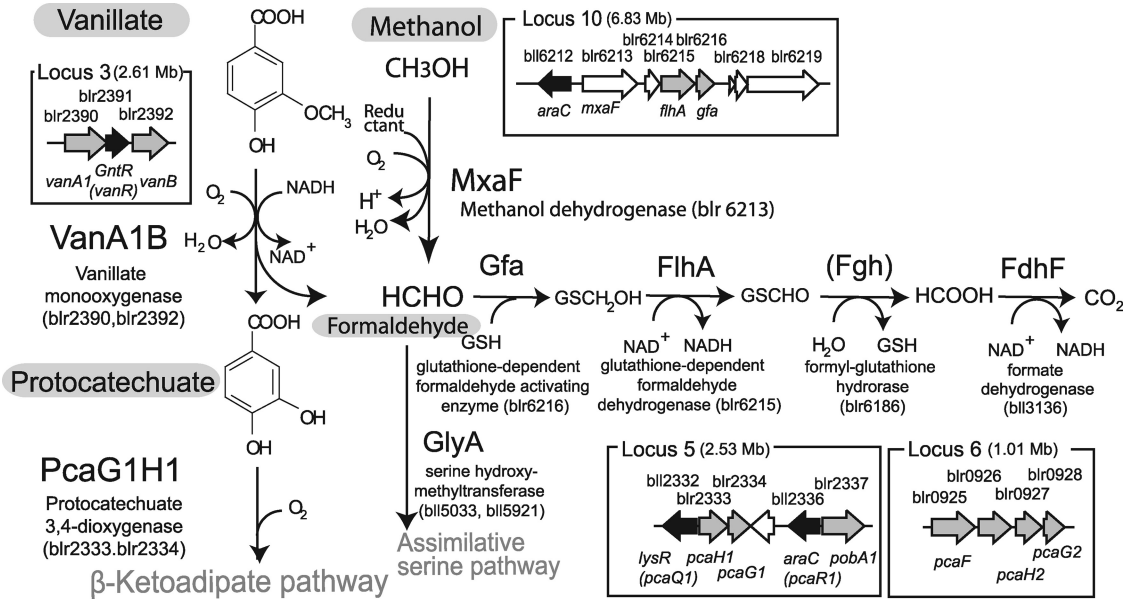

FIG. 1. Organization of genes and pathways in the genome of *B. japonicum* strain USDA110 for the degradation of vanillate (adapted from reference 12). *vanA1B* and *pcaG1H1* were exclusively expressed in vanillate-fed cells of *B. japonicum* USDA110 (12). The glutathione-dependent pathway from formaldehyde to CO_2 was derived from previous transcriptome data that included locus 10 (see the text). *mxaF* (blr6213) encodes a putative methanol dehydrogenase at locus 10 (12). The numbers in parentheses in the loci show genomic positions on *B. japonicum* USDA110 (14). GSH, glutathione.

dium (28). Antibiotics were added to the media at the following concentrations: for *B. japonicum*, 100 μg/ml of tetracycline, 100 μg/ml of spectinomycin, 100 μg/ml of streptomycin, 100 μg/ml of kanamycin, and 50 μg/ml of polymyxin B; for *E. coli*, 15 μg/ml of tetracycline, 50 μg/ml of spectinomycin, 50 μg/ml of streptomycin, 50 μg/ml of kanamycin, and 100 μg/ml of ampicillin.

DNA manipulations. Isolation of plasmids, DNA ligation, and transformation of *E. coli* were performed as described by Sambrook et al. (28). Genomic DNA was extracted from *B. japonicum* by using an AquaPure Genomic DNA kit (Bio-Rad Laboratories, Hercules, CA). Southern hybridization was carried out as described previously (30).

TABLE 1. Bacterial strains and plasmid used in this study

Strain or plasmid	Characteristics[a]	Source or reference
Strains		
B. japonicum		
USDA110	Wild-type strain	14
vanA1B mutant	USDA110 *vanA1B*::del/ins Ω cassette; Sm^r Sp^r	This study
pcaG1H1 mutant	USDA110 *pcaG1H1*::del/ins Ω cassette; Sm^r Sp^r	This study
mxaF mutant	USDA110 *mxaF*::del/ins Ω cassette; Sm^r Sp^r	This study
E. coli		
JM109	*recA* cloning strain	Toyobo Inc.[b]
Plasmids		
pRK2013	ColE1 replicon carrying RK2 transfer genes; Km^r	6
pHP45Ω	Plasmid carrying 2.1-kb Ω cassette; Sp^r Sm^r Ap^r	25
pK18mob	Cloning vector; pMB1 *oriT*; Km^r	31
pK19mob	Cloning vector; pMB1 *oriT*; Km^r	31
brp14282	pUC18 carrying *vanA1B* gene cluster; Ap^r	14
brp01186	pUC18 carrying *pcaG1H1* gene cluster; Ap^r	14
brp12520	pUC18 carrying *mxaF* gene cluster; Ap^r	14
pBI01	pK19mob carrying 4.5-kb *vanA1B* fragment; Km^r	This study
pBI07	pK19mob carrying 3.9-kb *vanA1B*::del/ins Ω cassette; Sm^r Sp^r Km^r	This study
pJN001	pK18mob carrying 3.7-kb *pcaG1H1* fragment; Km^r	This study
pJN002	pK18mob carrying 5.8-kb *pcaG1H1*::del/ins Ω cassette; Sm^r Sp^r Km^r	This study
pJN003	pK18mob carrying 4-kb *mxaF* fragment; Km^r	This study
pJN004	pK18mob carrying 6-kb *mxaF*::del/ins Ω cassette; Sm^r Sp^r Km^r	This study

[a] Ap^r, ampicillin resistant; Km^r, kanamycin resistant; Sm^r, streptomycin resistant; Sp^r spectinomycin resistant.
[b] Toyobo Inc., Tokyo, Japan.

Construction of *B. japonicum* USDA110 mutants. An NruI/HindIII fragment (4.5 kb) of plasmid brp14282 containing *vanA1B* was inserted into pK19mob to generate pB101 (Table 1). The Ω cassette (2.1 kb) from pHP45 was inserted into the XhoI/BstX1 sites of pB101, resulting in pB107. An XhoI fragment (3.7-kb) of brp001186 containing the *pcaG1H1*gene and a MunI/BglII fragment (4 kb) of brp12520 containing the *mxaF* gene were inserted into the SalI and EcoRI/BamHI sites of pK18mob to generate pJN001 and pJN003, respectively. The Ω cassette was inserted into the Eco0109I site of pJN001 and the NotI/SfiI sites of pJN003, resulting in pJN002 and pJN004, respectively. Triparental mating was conducted on HM agar plates using *E. coli* pRK2013 as a helper (29). The double-crossover events were verified by Southern hybridization.

Quantification of gene expression. Primers for real-time reverse transcription (RT)-PCR of blr6215 (*flhA*) and blr6216 (*gfa*) were designed using Beacon Designer software (Premier Biosoft International, Palo Alto, CA). The primer sequences were as follows: blr6215L, 5′-GCCAGAAGACCAATCTCTGC; blr6215R, 5′-AAGTTCGAGAAGGTCGAGCA; blr6216L, 5′-GACTATTGCA CTCCACCCATC; and blr6216R, 5′-GACCTGATGGTCCTTGCATT. The *sigA* gene (bll7349) was used as a control for quantification. PCR primers bll7349L (5′-GAGAACCAGATGTCGCTTGC) and bll7349R (5′-TGGATGT CCTGCTCCTGAAG) were used for RT-PCR of *sigA*. Total RNA was prepared as described previously (9). RT-PCR was carried out with the i-Cycler optical system (Bio-Rad Laboratories, Inc., Tokyo, Japan) as described previously (12, 29).

Gas chromatography. The methanol concentration in culture was determined with a gas chromatograph (GC7A; Shimadzu, Kyoto, Japan) equipped with a Porapak Q column (80/100 mesh; diameter, 0.3 mm; length, 1 m) and a flame ionization detector (26). The temperature of injection was 150°C, and the temperature of the column was 185°C. The flow rate of the carrier gas (N_2) was 60 ml/min. After the culture was centrifuged at 18,000 × *g* for 5 min at 4°C, an aliquot (3 μl) of the sample was directly injected into the gas chromatograph.

HPLC analysis. Vanillate and protocatechuate in cultures were measured with a high-performance liquid chromatography (HPLC) apparatus (LC-10AD; Shimadzu, Kyoto, Japan) equipped with a reverse-phase column (ODS-80T; GL Sciences Inc., Tokyo, Japan) and UV detector (254 nm). The isotonic mobile phase consisted of methanol, water, and acetic acid (30:70:1 [vol/vol]). The flow rate was 0.9 ml min^{-1}. After the culture was centrifuged at 18,000 × *g* for 5 min at 25°C, an aliquot (5 μl) of sample was injected directly into the HPLC system.

Resting-cell experiment. Cells were grown in liquid minimum medium supplemented with 1 mM vanillate. The cells were harvested by centrifugation at 3,500 × *g* for 15 min at 4°C and washed twice with the medium. The washed cells were suspended in the medium at 10^{10} cells/ml and incubated at 30°C in the presence of 1 mM methanol. An aliquot of the cell suspension was analyzed with a gas chromatograph.

Plant test. Surface-sterilized soybean seeds (*Glycine max* cv. Enrei) were germinated and transplanted into Leonard jars that contained sterile vermiculite and nitrogen-free nutrient solution (23). *B. japonicum* inoculation and plant cultivation were carried out as described previously (23). Plant phenotypes were observed 4 weeks after inoculation.

RESULTS

Growth and metabolism of *vanA1B* and *pcaG1H1* mutants. To examine whether the *pcaG1H1*, *vanA1B*, and *mxaF* genes in *B. japonicum* are responsible for vanillate degradation, we constructed *pcaG1H1*, *vanA1B*, and *mxaF* deletion mutants of *B. japonicum* USDA110 (Table 1). *vanA1B* and *pcaG1H1* mutants (Fig. 2A and B) and wild-type USDA110 were grown in minimum medium supplemented with 1 mM of vanillate or protocatechuate, the concentration found to be optimal in this study (see Fig. S1 in the supplemental material). The *vanA1B* and *pcaG1H1* mutants did not grow in minimum medium containing vanillate and protocatechuate, respectively (Fig. 2C and D), although the wild-type strain of USDA110 was able to grow in these media (Fig. 2C and D). The mutants showed normal growth similar to that of the wild-type strain in a minimum medium supplemented with 1 mM succinate (data not shown).

The *vanA1B* and *pcaG1H1* mutants did not consume vanillate and protocatechuate, respectively (Fig. 2E and F), although wild-type USDA110 cells consumed these aromatic substrates during cell growth (Fig. 2E and F). This result clearly indicates that the *vanA1B* and *pcaG1H1* genes upregulated in the previous transcriptome analysis (12) are exclusively responsible for vanillate degradation in *B. japonicum*. In the presence of protocatechuate, the *vanA1B* mutant showed normal growth profiles (Fig. 2D and F) that were similar to wild-type USDA110, supporting the proposed pathway of vanillate degradation in *B. japonicum* USDA110 (Fig. 1).

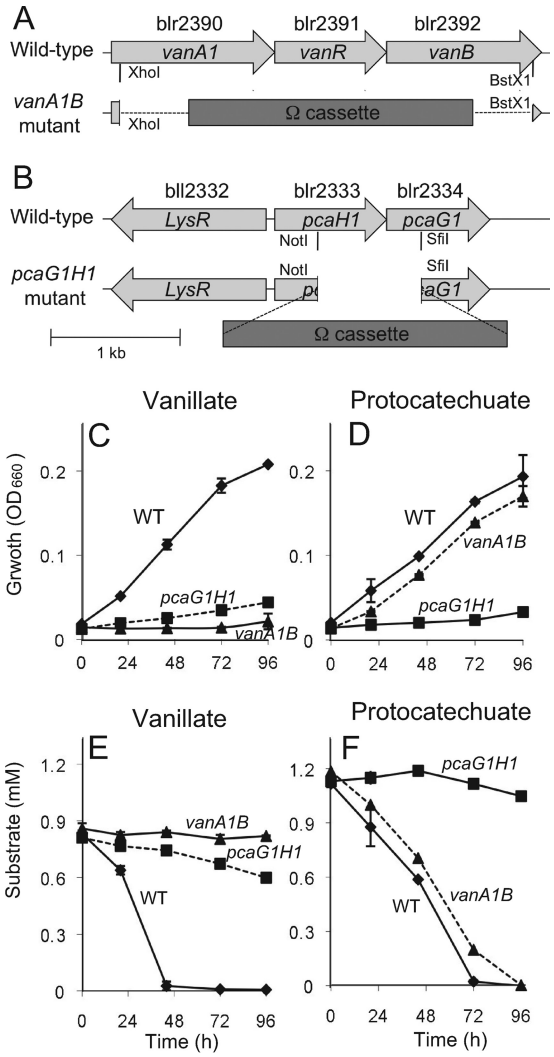

FIG. 2. Growth and substrate consumption in *vanA1B* and *pcaG1H1* mutants of *B. japonicum* USDA110. (A and B) Physical maps of *vanA1B* (A) and *pcaG1H1* (B) deletion mutants. Cell growth was monitored by measuring turbidity (optical density at 660 nm [OD_{660}]). (C and F) The *B. japonicum* cells were grown in minimal medium supplemented with vanillate (C and E) or with protocatechuate (D and F). The concentrations of vanillate (E) and protocatechuate (F) in culture were determined by HPLC. The error bars indicate standard deviations of triplicate determinations. WT, wild type. Dashed lines indicate cross-combinations of mutated genes and substrate (*pcaG1H1* mutant in vanillate-amended medium [E], *vanA1B* mutant in protocatechuate-amended medium [F]).

Expression of *flhA* and *gfa* genes in the *mxaF* mutant. The *flhA* and *gfa* genes, which likely function in the glutathione-

dependent C_1 pathway (Fig. 1), were also upregulated in the cells grown in vanillate (12). Although we tried to construct *flhA* and *gfa* mutants, the double-crossover steps needed for the mutant construction failed several times. However, on the basis of their DNA sequences, it is likely that *mxaF*, *flhA*, and *gfa* form a transcriptional unit from a single putative promoter (Fig. 3A). We therefore constructed an *mxaF* mutant with the omega cassette, an interposon with a strong polar effect (25), and expected that RNA and protein synthesis would be terminated beyond the site of insertion (Fig. 3). RT-PCR analysis indicated that the expression of *flhA* and *gfa* genes was drastically downregulated in the *maxF* mutant compared with the wild-type USDA110 (Fig. 3A). Thus, the *maxF* mutant can also be used instead of the *flhA* and *gfa* mutants.

Vanillate catabolism of the *mxaF* mutant. The *mxaF* mutant was subjected to growth experiments in vanillate-supplemented medium. The wild-type strain and the *vanA1B* mutant of *B. japonicum* USDA110 were used as positive and negative controls, respectively. The *mxaF* mutant had markedly lower growth in minimum medium containing vanillate than the wild-type strain (Fig. 3B), whereas the *mxaF* mutant was able to grow at the same rate as the wild-type strain in medium containing protocatechuate (Fig. 3C). The rate of vanillate catabolism by the *mxaF* mutant was also lower than catabolism by the wild-type strain (Fig. 3D), whereas the *mxaF* mutant and wild-type strains showed similar rates of protocatechuate catabolism (Fig. 3E). These results suggest that the *flhA* and *gfa* genes are required for efficient vanillate catabolism through the C_1 pathway (Fig. 3), probably because they alleviate the toxicity of the formaldehyde produced by vanillate monooxgenase, VanA1B (Fig. 1).

Methanol oxidation by the *mxaF* mutant. The *mxaF* gene encodes methanol dehydrogenase, which oxidizes methanol to formaldehyde in methylotrophs (17, 36). The *mxaF* gene is commonly used as a functional marker gene for environmental DNA analysis (27) and metagenomics (20) of the global C_1 cycle in the environment (1, 15). We therefore tested methanol oxidation in *mxaF* mutants and wild-type cells of *B. japonicum* USDA110. Since USDA110 showed very weak growth in minimum medium with methanol (data not shown), methanol oxidation was assayed using the condensed resting cells of *B. japonicum*.

USDA110 and *mxaF* mutant cells were grown in minimal medium with vanillate for 5 days. Then, the resting cells (10^{10} cells/ml) were incubated in the presence of 1 mM methanol. Methanol oxidation was observed in the wild-type strain, but not in the *mxaF* mutant (Fig. 3F). The rate of methanol oxidation from 2 h to 10 h was 18.8 ± 3.2 nmol methanol consumed h^{-1} $(10^9 \text{ CFU})^{-1}$ for the wild-type and 1.5 ± 2.2 for the *mxaF* mutant cells ($P < 0.05$; *t* test), indicating that the metabolic capability of the C_1 pathway in *B. japonicum* extends to methanol oxidation, as schematized in Fig. 1.

Symbiotic phenotype of the *mxaF* mutant. The *mxaF* gene has been shown to play an important role in symbiosis (13) and in competitive fitness for plant colonization (33). However, soybean plants inoculated with the wild-type strain and the *mxaF* mutant showed no apparent difference in shoot growth, nodule numbers, or nodule weights (see Fig. S2 in the supplemental material). This result suggests that the methanol oxidation and C_1 compound metabolism of *B. japonicum* are not

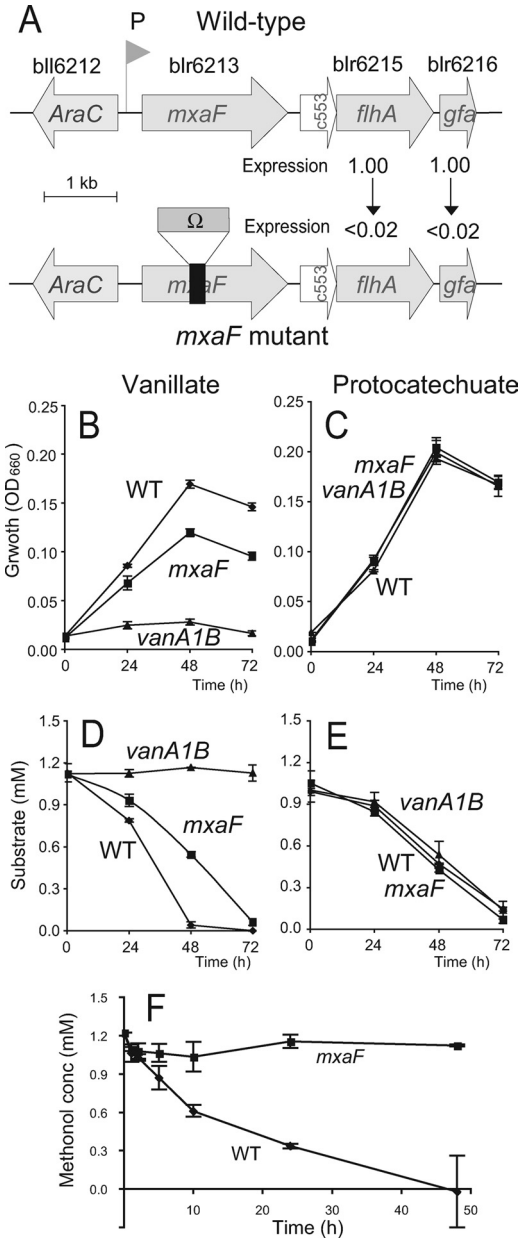

FIG. 3. Growth and methanol oxidation of the *mxaF* deletion mutant of *B. japonicum* USDA110. (A) *mxaF* gene cluster in wild-type strain USDA110 (top) and its *mxaF* deletion mutant (bottom). Ω indicates the omega cassette from pHP45Ω (Table 1). Expression, the relative expression levels, determined with RT-PCR, of the *flhA* and *gfa* genes in the *maxF* mutant compared with wild-type USDA110 based on *sigA* expression (see the text). c553 (blr6214) is a homolog of cytochrome *c*-553 of *Paracoccus denitrificans* and *Methylococcus capsulatus*. Other genes are shown in locus 10 in Fig. 1. (B to E) The wild-type strain (WT), *mxaF* mutant, and *vanA1B1* mutant of *B. japonicum* USDA110 cells were precultured in HM medium and then simultaneously transferred to minimal medium with vanillate (B and D) or with protocatechuate (C and E). Cell growth was monitored by turbidity (optical density at 660 nm [OD_{660}]) (B and C). The concentrations of vanillate (D) and protocatechuate (E) in culture were determined by HPLC. (F) Methanol-oxidizing activities of the wild-type strain and the *mxaF* mutant of *B. japonicum* USDA110. Resting cells of wild-type USDA110 and *mxaF* mutant cells prepared from vanillate-grown culture were incubated in a buffer containing 1 mM methanol. The error bars indicate standard deviations of triplicate determinations.

directly involved in soybean nodulation and symbiotic nitrogen fixation.

Vanillate catabolism genes in *Bradyrhizobiaceae*. To examine whether *vanA1B*, *pcaG1H1*, and *mxaF* clusters are present on the genomes in members of the *Bradyrhizobiaceae* (19 strains, including *B. japonicum*, *Agromonas oligotrophica*, *Rhodopseudomonas palustris*, and *Bradyrhizobim* sp.), we extracted the previous array results (11) and summarized them in Table S1 in the supplemental material. Consequently, loci 3, 5, and 10, including *vanA1B*, *pcaG1H1*, *mxaF*, *flhA*, and *gfa*, were well conserved on the genomes of the 19 strains. Indeed, *Bradyrhizobium* sp. strains BTAi1 and ORS278 (7) carried *van* and *mxaF* gene clusters that were similar to those of USDA110 (see Fig. S3 in the supplemental material).

DISCUSSION

Recent efforts to find novel functional genes through global expression analyses have often failed in complicated biological systems; for example, the disruption mutants of genes upregulated in rhizobia during symbiosis generally show no symbiotic phenotype (9, 34). In the current study, the *vanA1B* and *pcaG1H1* genes that were upregulated in *B. japonicum* cells grown in vanillate (12) were shown to be exclusively responsible for the catabolism of vanillate and protocatechuate, respectively (Fig. 2). Because *vanA1B* (blr2390 and blr2392) at locus 3 represents the full set of *vanAB* gene homologs (vanillate monooxygenase) (Fig. 1), it is reasonable that the *vanA1B* mutant would completely lose the capability to catabolize vanillate (Fig. 2C and E). However, there are two full sets of *pcaGH* homologs (protocatechuate 3,4-dioxygenase): *pcaG1H1* (blr2334 and blr2333) at locus 5 and *pcaG2H2* (blr0928 and blr0927) at locus 6 (Fig. 1). The identities of the amino acids between *pcaG1H1* and *pcaG2H2* range from 48% to 54%. Thus, the results of the previous global expression study coincide with the functional information from postgenomic work for protocatechuate catabolism.

The growth and protocatechuate consumption of the *vanA1B* mutant were similar to those of the wild-type strain in protocatechuate-amended medium (Fig. 2D and F and 3C and E), but vanillate catabolism was drastically lower in the *pcaG1H1* mutant than in the wild-type strain (Fig. 2C and E). These facts are consistent with the pathway of vanillate catabolism (vanillate → protocatechuate → β-ketoadipate pathway) and the metabolic significance of C_1 compounds derived from the methoxy moiety of vanillate (Fig. 1). Interestingly, comparative genomic hybridization (11) suggested that these metabolic capabilities are conserved in members of the *Bradyrhizobiaceae* (see Table S1 in the supplemental material).

Bacterial cells that use vanillate as a carbon source ought to produce formaldehyde, which has a toxic effect on all organisms (5). In *B. japonicum*, the formaldehyde is likely to be converted into CO_2 in the C_1 pathway (Fig. 1), because the *gfa*, *flhA*, *fdhF*, and *fdhD* genes were coexpressed by vanillate addition (12). In this work, the *mxaF* mutant caused the downregulation of *flhA* and *gfa* in the glutathione-dependent C_1 pathway (Fig. 3A). The decreased growth (Fig. 3B) and vanillate consumption (Fig. 3D) of *mxaF* mutants supported the conversion of formaldehyde into CO_2 by the C_1 pathway. However, it is likely that an assimilatory serine pathway (Fig. 1) also functions for formaldehyde detoxification (1, 10, 18, 35), because the *mxaF* mutant retained its growth ability in the vanillate-amended medium (Fig. 3B and D).

In addition to our interest in the role of *mxaF* in the detoxification of formaldehyde via the C_1 pathway, we are interested in its ecological function in encoding methanol dehydrogenase (17, 36). The inability of the *mxaF* mutant to oxidize methanol in condensed resting cells (Fig. 3F) clearly shows that *mxaF* is responsible for methanol oxidation in *B. japonicum*.

It has been reported that the *mxaF* gene is required for symbiosis between *Methylobacterium nodulans* and *Crotalaria pedocarpa* (13) and for competitive colonization by *Methylobacterium extorquens* on plant surfaces (33). On the other hand, the methanol oxidation of *B. japonicum* USDA110 was not directly involved in nodulation and nitrogen fixation (see Fig. S2 in the supplemental material).

A sequence comparison of the *mxaF* gene in RhizoBase (http://genome.kazusa.or.jp/rhizobase/) found that only the stem-nodulating bradyrhizobia BTAi1 and ORS278 also carried a gene organization (*araC mxaF flhA gfa*) similar to that of *B. japonicum* USDA110 (see Fig. S3 in the supplemental material). Thus, rhizobial methanol oxidation by *mxaF* is likely to be restricted to *Bradyrhizobium* species. Because plants often produce methanol and formaldehyde (22), the ability to cope with these compounds might enhance the environmental fitness of plant-associated bradyrhizobia under competitive conditions.

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Aerobic vanillate degradation and C1 metabolism in *Bradyrhizobium japonicum*

Supplemental materials

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Running title: Vanillate catabolism and C1 metabolism in *B. japonicum*

Table S1. Comparative genomic comparison of gene regions for aromatic degradation in Beadyrhizobiaceae members

		Genome of *B. japonicum* USDA110		Hybridization signal ratio based on USDA110 genomic DNA																
Locus	Array Clones	start position	end position	BJ USDA110	BJ NC6	BJ USDA122	BJ NC4	BJ NK2	BJ USDA124	BJ USDA6	BJ T7	BJ T9	BE USDA76	RP CGA009	Bsp ORS278	Bsp BTAi1	AO S58	Bsp G14130	Bsp HWK12	Bsp HW13	Gene
Locus 1																					
	brb05975	4432450	4434952	1.0	1.0	1.0	1.1	1.0	0.7	1.2	1.6	1.6	0.7	1.0	0.9	0.9	0.7	1.7	0.9	0.6	
	brb00339	4436345	4439010	1.1	1.0	1.2	1.1	0.9	0.4	0.6	0.7	1.0	0.7	0.4	0.5	0.8	0.9	0.9	1.1	1.1	*hcaB1*
	brb12607	4438658	4441422	0.9	1.1	1.5	1.0	1.7	0.4	0.6	0.6	0.5	0.5	1.0	1.2	1.0	0.8	0.5	1.0	0.2	
Locus 2 (trnK2)																					
	brb16353	7062073	7064459	0.9	0.8	0.1	0.2	0.1	0.1	0.2	0.2	0.1	0.2	0.4	0.4	0.3	0.4	0.2	0.2	0.2	
	brb20863	7063424	7065576	1.2	1.1	0.1	0.2	0.2	0.1	0.2	0.4	0.4	0.5	0.5	0.8	0.7	0.7	0.4	0.7	0.4	*hcaB2*
	brb13790	7064545	7067088	0.9	0.9	0.2	0.2	0.1	0.2	0.3	0.3	0.3	0.2	0.5	0.3	0.3	0.5	0.3	0.4	0.4	*hcaB2*
	brb09458	7067626	7070083	1.0	1.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.2	0.1	*pobA2*
	brb08949	7070514	7073257	1.0	0.9	0.2	0.4	0.1	0.2	0.1	0.5	0.8	0.6	0.8	0.9	0.8	0.9	0.6	0.6	0.6	
Locus 3																					
	brb02752	2600923	2604132	1.0	1.0	1.2	1.1	1.2	0.4	0.8	0.9	0.7	1.1	1.4	1.3	1.4	1.2	1.1	0.9	1.1	
	brb15955	2605202	2608400	1.0	1.1	1.0	1.3	1.3	0.6	1.1	1.8	1.5	1.3	1.6	1.4	1.3	1.2	1.2	1.5	1.8	*vanA1B1*
	brb21791	2608611	2611435	0.9	0.8	1.1	0.8	1.1	0.4	0.9	1.4	1.0	0.7	0.8	0.8	0.8	0.8	1.2	1.5	1.1	
Locus 5																					
	brb21553	2530539	2533254	0.8	0.8	1.1	1.1	1.2	0.2	0.4	0.4	0.5	0.6	0.9	1.8	1.4	1.8	0.5	0.7	0.5	
	brb01233	2533496	2535711	0.9	1.0	1.2	1.1	1.1	0.8	1.1	1.0	1.0	0.7	0.8	0.8	0.8	0.8	1.3	0.8	0.9	*pcaG1H1*
	brb07579	2534344	2537436	1.1	1.0	1.0	1.3	1.3	0.7	0.7	1.1	1.1	0.7	0.9	0.7	0.7	0.7	1.2	1.0	1.0	*pcaG1H1*
	brb02318	2537943	2540334	1.0	0.9	1.0	0.9	1.0	0.6	0.9	1.1	1.2	0.9	1.0	1.3	1.4	0.9	1.1	0.9	1.0	*pobA1*
	brb12908	2539700	2542142	1.0	1.1	0.8	0.8	0.8	0.8	0.9	1.2	1.3	1.0	1.0	1.1	1.2	1.2	1.4	1.1	1.1	
Locus 7																					
	brb17542	6219272	6221899	0.9	1.0	1.0	1.1	0.9	0.9	1.4	1.5	1.7	1.9	2.6	2.4	2.6	2.2	2.0	2.7	2.2	
	brb13961	6221335	6224608	1.0	1.1	1.3	1.5	1.7	0.7	1.4	1.4	1.6	1.6	2.8	2.4	2.1	2.1	1.8	2.1	2.1	
	brb02257	6225141	6227566	0.9	1.1	1.8	1.3	1.0	1.2	2.1	1.5	1.5	1.5	1.9	1.6	2.0	1.5	1.5	1.8	1.5	*pcaB*
	brb16014	6226822	6229653	1.2	1.1	1.0	1.3	1.2	0.7	1.5	1.6	1.3	1.0	1.1	1.4	1.3	1.6	1.7	2.6	3.1	*pcaBCD*
	brb22079	6230101	6232693	0.9	1.0	0.9	0.8	1.2	1.0	1.3	1.4	1.4	1.6	2.3	2.2	1.9	2.0	2.0	2.3	2.3	
Locus 10																					
	brb03580	6828411	6831030	1.0	1.3	1.0	1.0	1.5	0.7	1.1	1.0	1.3	0.7	0.8	0.7	0.7	0.8	1.1	1.4	1.2	
	brb23581	6829824	6832439	0.9	1.0	1.4	1.0	1.1	0.9	1.5	1.3	1.3	0.8	0.8	1.1	0.8	1.1	2.0	1.0	1.1	*mxaF*
	brb15565	6832515	6835422	0.9	0.9	1.2	1.0	1.1	0.8	1.4	1.1	1.2	1.0	1.1	1.5	1.8	1.7	1.3	1.3	1.7	*flhA, gfa*
	brb10395	6834420	6837301	0.9	0.9	1.3	1.1	1.0	0.8	1.0	1.1	1.1	1.1	1.3	1.4	1.5	1.5	1.3	1.5	1.9	*flhA, gfa*
	brb11784	6840320	6842085	1.0	1.0	1.2	1.2	1.0	0.9	1.4	1.5	1.5	1.1	0.8	1.6	1.6	2.0	1.6	1.5	1.9	
Locus 11																					
	brb07681	3459437	3462420	0.9	0.9	1.2	1.3	1.0	0.8	1.2	1.5	1.7	1.7	2.1	2.1	2.5	1.7	1.5	1.5	1.7	
	brb07054	3462635	3465249	1.2	1.1	0.9	1.1	1.2	0.7	1.3	1.6	1.7	1.2	2.2	1.9	1.4	1.6	1.5	2.0	2.1	*fdhDF*
	brb22696	3464701	3466982	1.0	0.9	1.0	0.9	0.9	0.9	1.2	1.5	1.1	1.6	2.5	1.9	1.7	1.5	2.0	2.0	2.4	*fdhF*
	brb08438	3466163	3469027	1.0	1.0	1.1	0.9	1.0	0.5	0.8	1.0	1.1	1.2	1.2	1.3	1.3	1.3	1.4	1.3	1.4	*fdhF*
	brb21813	3468222	3471184	0.9	1.1	1.2	1.0	0.9	0.8	1.3	1.3	1.6	1.1	1.9	1.8	1.8	1.8	1.7	2.0	1.8	
Locus 4																					
	brb06769	1182148	1185449	0.9	0.9	1.4	1.0	1.1	0.9	1.4	1.2	1.2	2.0	1.9	1.7	1.8	1.6	1.6	1.6	2.6	*vanA2*
	brb01244	1184443	1187013	0.9	0.9	1.0	0.9	0.9	0.4	0.5	0.7	0.5	1.1	1.0	1.3	1.0	1.1	0.8	1.0	1.2	
Locus 6																					
	brb12770	1007487	1009398	1.0	1.1	1.2	1.4	0.9	0.8	1.3	1.5	1.9	2.2	3.8	3.7	4.4	4.2	2.5	2.4	2.6	
	brb04424	1011439	1014733	1.0	1.1	1.2	0.8	1.0	0.8	1.4	1.6	1.8	1.2	1.5	1.2	1.4	1.5	1.8	2.2	2.4	*pcaFH2H3G2*
	brb21506	1012332	1015948	0.9	0.9	1.0	1.0	0.9	0.6	1.2	1.3	1.1	1.0	0.9	1.1	1.4	0.9	1.4	1.4	1.4	*pcaH2H3G2*
	BJ7094	1016920	1017851	1.2	1.0	1.4	1.2	1.0	0.8	1.2	0.8	1.2	0.6	0.7	0.5	0.6	1.0	1.1	0.6	0.8	
Locus 8																					
	brb19987	7805169	7807786	1.1	1.0	1.5	1.1	1.0	1.0	1.2	0.9	1.0	1.6	1.7	1.1	1.2	1.2	1.0	0.7	0.9	
	brb06538	7807655	7810187	1.0	1.0	1.0	1.0	1.0	0.8	1.4	1.5	1.4	1.7	2.1	1.6	1.7	1.5	1.9	2.2	2.0	*pcaJ1I1*
	brb09719	7810100	7811487	1.0	1.1	1.3	1.0	1.0	0.9	1.4	1.7	1.6	1.5	1.6	1.5	1.7	2.1	1.7	1.6	1.6	
Locus 9																					
	brb17676	3828018	3831068	1.0	1.0	1.2	1.1	1.0	0.3	0.7	0.9	0.8	0.6	0.7	0.8	0.8	1.3	0.4	0.6	1.0	
	brb07258	3829846	3833012	1.0	1.1	1.2	1.1	1.1	0.4	0.7	1.0	1.0	0.6	0.7	0.6	0.8	0.7	0.4	0.6	1.5	*pcaJ2I2*
	brb20152	3832986	3835526	1.1	1.1	0.8	0.9	0.9	0.1	0.1	0.2	0.3	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2	*pcaI2*
	brb24113	3834731	3837481	0.9	1.0	1.1	1.0	1.0	0.1	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.1	0.2	0.2	0.1	

The names of genus and species were abbreviated as follows: BJ, *Bradyrhizobium japonicum* ; BE, *Bradyrhizobium elkanii* ; RP, *Rhodopseudomonas palstris* ; Bsp, *Bradyrhizoboium* sp.; AO, *Agromonas oligotrophica* . See reference 11 for detailed characteristics of 17 strains and the methods. Figures mean relative intensity of macroarray hybridization signals of tested strain as compared with those of *B. japonicum* USDA110 (1) (http://orca10.bio.sci.osaka-u.ac.jp/array02/). Yellow and grey backgrounds indicate the presence and absence of homologous regions in *Bradyrhizobium japonicum* USDA110, respectively (1). Most loci for aromatic degradation were well conserved among the 19 members of Bradyrhizoibiaceae. Regions that are of interest in this work are shown as red on loci 3,5, and 10.

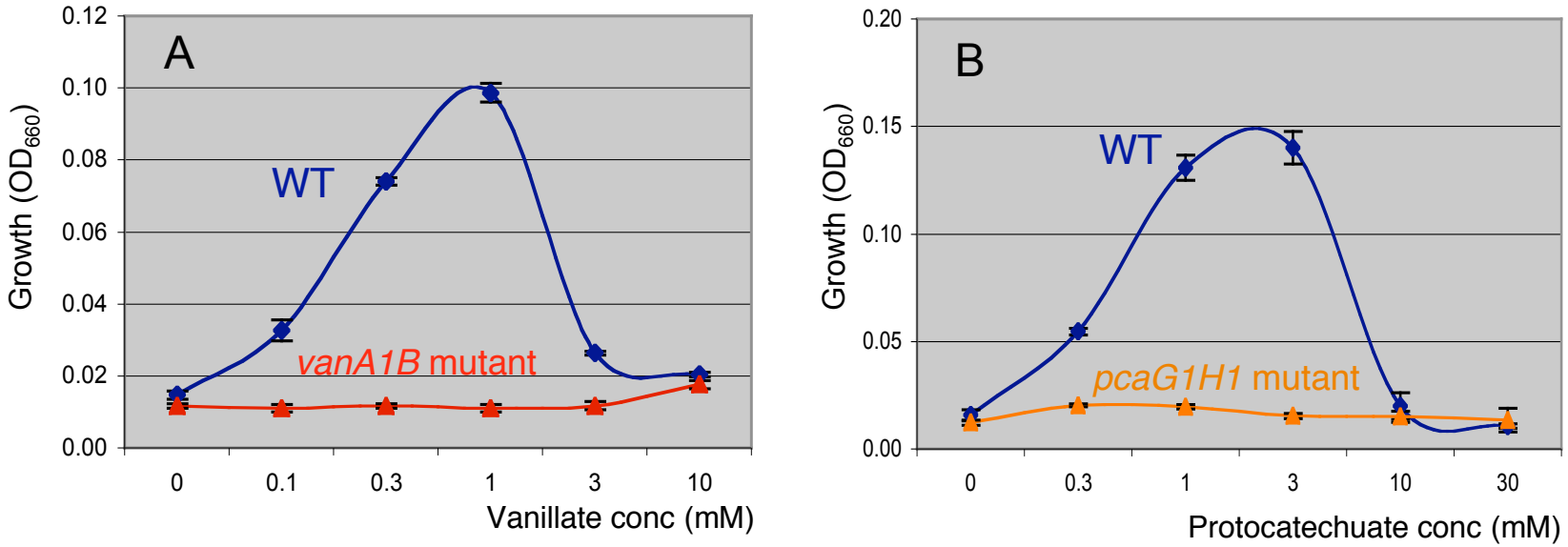
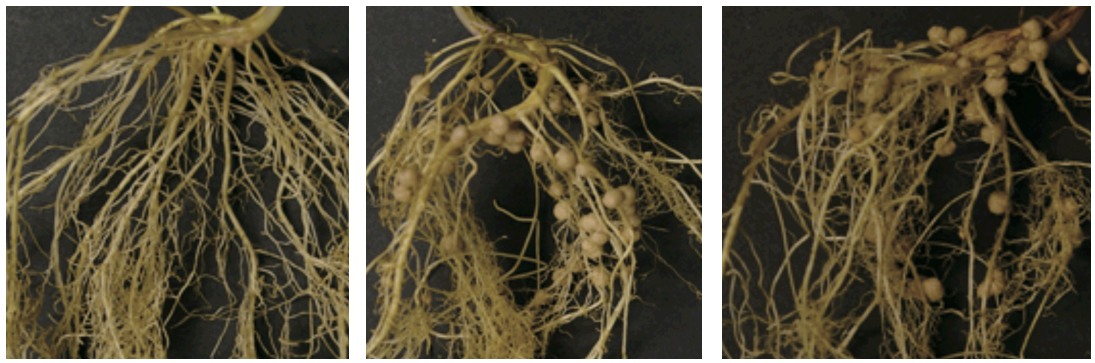


Fig. S1. Cell growth of wild-type (WT), *vanA1B* and *pacG1H1* mutants of *Bradyrhizobium japonicum* USDA110 in minimal medium containing various concentrations of vanillate (A) or protocatechuate (B) at 3 days after inoculation

Growth was expressed as cell turbidity (OD_{660}). Bar indicate SD by tripricate determinations. Maximum growth of wild-type USDA110 was observed in minimal media supplemented with 1 mM of vanillate and 3 mM protocatechuate, respectively. In higher concentrations of vanillate or protocatechuate, the cell growth of wild-type USDA110 was reduced. On the other hand, growth of *vanA1B* and *pcaG1H1* mutants were not supported by vanillate and protocatechuate addition, respectively. These results indicated that the bacterium adapts low concentrations (1-3 mM) of vanillate and protocatechuate.

Nodulated root system



	WT	*mxaF* mutant
Number of nodules (soybean plant^{-1})	31 ± 6	32 ± 3
Nodule fresh weight (g)	0.52 ± 0.16	0.64 ± 0.13

Fig. S2. Inoculation of wild-type (WT) and *mxaF* mutant of *Bradyrhizobium japonicum* to soybeans. Soybean plants inoculated with wild-type USDA110 and *mxaF* mutant (10^7 cells /plant) were grown for 3 weeks at 25° C using nitrogen-free nutrient solution (2). Plant phenotypes were photographed. Nodule numbers and nodule weight were determined with triplicates. There is no difference in plant phenotypes including nodule numbers and weight between the wild-type and *mxaF* mutant of *B. japonicum.*

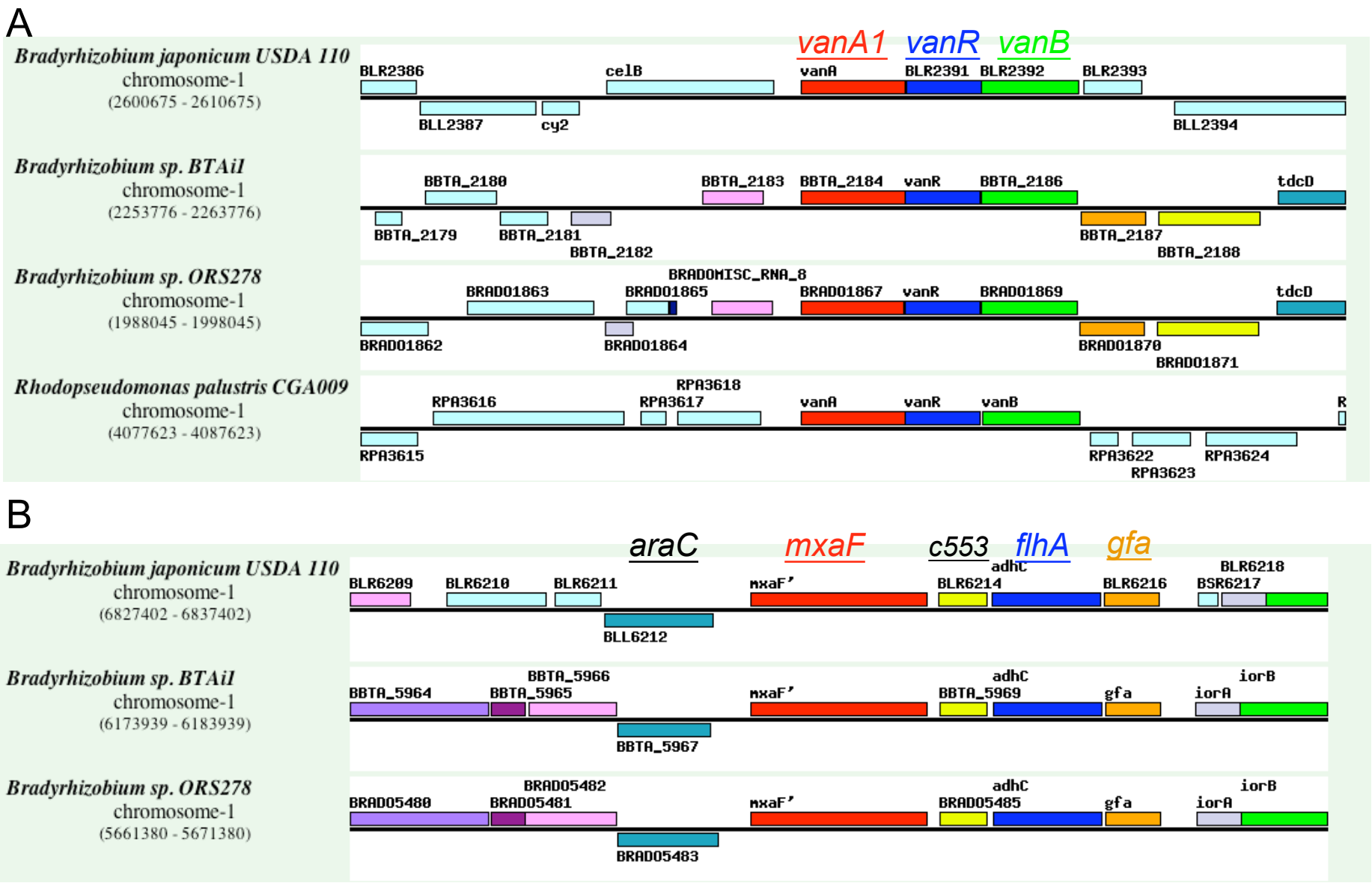


Fig. S3. Multiple genome map comparison of *van* (A) and *mxaF* (B) gene clusters in Bradyrhizobiaceae members. Underlined gene names were corresponded to those in Fig. 1, Fig. 2AB and Fig. 3A. The comparison was conducted by Microbial Genome Database for Comparative Analysis (MBGD; http://mbgd.nibb.ac.jp/) with default parameters.

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