Isolation and Characterization of Thermophilic Bacilli Degrading Cinnamic, 4-Coumaric, and Ferulic Acids

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Thirty-four thermophilic *Bacillus* sp. strains were isolated from decayed wood bark and a hot spring water sample based on their ability to degrade vanillic acid under thermophilic conditions. It was found that these bacteria were able to degrade a wide range of aromatic acids such as cinnamic, 4-coumaric, 3-phenylpropionic, 3-(*p*-hydroxyphenyl)propionic, ferulic, benzoic, and 4-hydroxybenzoic acids. The metabolic pathways for the degradation of these aromatic acids at 60°C were examined by using one of the isolates, strain B1. Benzoic and 4-hydroxybenzoic acids were detected as breakdown products from cinnamic and 4-coumaric acids, respectively. The β -oxidative mechanism was proposed to be responsible for these conversions. The degradation of benzoic and 4-hydroxybenzoic acids was determined to proceed through catechol and gentisic acid, respectively, for their ring fission. It is likely that a non- β -oxidative mechanism is the case in the ferulic acid catabolism, which involved 4-hydroxy-3-methoxyphenyl- β -hydroxypropionic acid, vanillin, and vanillic acid as the intermediates. Other strains examined, which are V0, D1, E1, G2, ZI3, and H4, were found to have the same pathways as those of strain B1, except that strains V0, D1, and H4 had the ability to transform 3-hydroxybenzoic acid to gentisic acid, which strain B1 could not do.

Lignin is the second most abundant carbon compound on the earth after cellulose, and it is composed of a polymer of phenyl propane units containing guaiacyl, syringyl, *p*-hydroxyphenyl, and biphenyl moieties. Cinnamic acid is a precursor for lignin biosynthesis, being transformed to 4-coumaric acid (4-hydroxycinnamic acid), ferulic acid (4-hydroxy-3-methoxycinnamic acid), and sinapic acid (4-hydroxy-3,5-dimethoxycinnamic acid) before its incorporation into a lignin polymer (64). These lignin-related phenylpropanoids are abundant in plant cells and are precursors not only of lignin but also of anthocyanins, phytoalexins, and flavonoids. Many phenylpropanoids are pharmacologically active and thus of pharmaceutical interest (17).

The biodegradation of phenylpropanoids is important for the global carbon cycle from an environmental point of view, since these compounds are released from plant wastes as breakdown products from lignin. In view of the potential use of phenylpropanoids as feedstock for the bioconversion into valuable molecules (49), we have attempted to isolate thermophilic bacteria capable of degrading phenylpropanoids such as cinnamic, 4-coumaric, and ferulic acids in this study.

The biodegradation of ferulic acid and its derivatives has been investigated in many bacteria, and the proposed degradative pathways have been classified into four categories according to the initial reactions: β -oxidation, non- β -oxidation, nonoxidative decarboxylation, and side chain reduction (47, 49). β -Oxidation has been proposed for the degradation of substituted cinnamic acids by *Pseudomonas putida* (63) and of ferulic acid in *Rhodotorula rubra* (30). This pathway, which is analogous to the β -oxidation of fatty acid, is thought to include thiolytic cleavage of 4-hydroxy-3-methoxyphenyl- β -ketopro-

pionyl-coenzyme A (CoA) to yield acetyl-CoA and vanillyl-CoA, which is catalyzed by a β -ketoacyl-CoA thiolase. However, almost no experimental evidence exists to support this proposal. In contrast, the non- β -oxidation pathway has been revealed in *Pseudomonas fluorescens* AN103 (23, 39, 41). This microbe transforms ferulic acid to feruloyl-CoA, which subsequently undergoes side chain cleavage by hydration to form vanillin and acetyl-CoA. The same pathway has been found in *Pseudomonas* sp. strain HR199 (45), *Sphingomonas paucimobilis* SYK-6 (37), *Delftia acidovorans* (46), and *Amycolatopsis* sp. strain HR167 (1). *para*-Hydroxylated aromatic acids, such as 4-coumaric and ferulic acids, have been incorporated as substrates in the catabolic route with *P. fluorescens* AN103 and *S. paucimobilis* SYK-6, but cinnamic acid has not been incorporated (37, 39). It is likely that hydroxy group at the *para* position is an essential structural requirement for this non- β -oxidation pathway. In *Pseudomonas acidovorans*, vanillin has been detected as an intermediate in the catabolism of ferulic acid, suggesting that a similar pathway for ferulic acid degradation exists also in this bacterium (58).

The nonoxidative decarboxylation of 4-coumaric and ferulic acids has been found in many bacteria (5, 8, 9, 30–33, 35, 48, 59, 62). This route involves one carbon being eliminated from the side chain of 4-coumaric and ferulic acids, respectively, resulting in the formation of 4-vinylphenol and 4-vinylguaiacol. This step is catalyzed by a nonoxidative decarboxylase. 4-Vinylguaiacol has been subsequently transformed to vanillin and then to vanillic acid by *R. rubra* (30), *Bacillus coagulans* (35), and *Pestalotia palmarum* (48). The fourth mechanism has been found under both anaerobic and aerobic conditions, by which ferulic acid has been reduced to 4-hydroxy-3-methoxyphenylpropionic acid (4, 10, 11, 20, 40, 44). In *Wolinella succinogenes*, 4-hydroxy-3-methoxyphenylpropionic acid has been further converted into vanillic acid through homovanillic acid (44). The degradation of ferulic acid has been proposed to proceed

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via 4-hydroxy-3-methoxyphenylpropionic acid, vanillic acid, and protocatechuic acid in *P. fluorescens* (4). *Acinetobacter calcoaceticus* has been found to degrade ferulic acid to vanillic acid, although the route for this conversion has not yet been elucidated (16, 50).

Studies on the catabolism of cinnamic acid are not as detailed as those on *para*-hydroxylated cinnamic acids such as ferulic acid. Most of the ferulic acid degraders just described fail to degrade cinnamic acid while several cinnamic acid degraders have the ability to degrade both cinnamic acid and its *para*-hydroxylated derivatives. It has been reported in *Pseudomonas* sp. strain CINNS (3), *Clostridium aerotolerans* DSM 5434 (10), and *Clostridium xylanolyticum* DSM 6555 (10) that the side chain of cinnamic and 4-coumaric acids is reduced to the propionic acid residue. However, the catabolic routes downstream from these phenylpropionic acids have not yet been elucidated. *Streptomyces setonii* (55) and *Rhodospseudomonas palustris* (29) have been shown to degrade cinnamic, 4-coumaric, and ferulic acids to their corresponding benzoic acid derivatives. The β -oxidation pathway has been proposed for the degradation of cinnamic acid by *Haloferax* sp. strain D1227 (21) and *R. palustris* (19). *Alcanivorax borkumensis* MBIC 4326 (18) and *Papillibacter cinnamivorans* (15) have transformed cinnamic acid to benzoic acid. Whether the β -oxidative pathway or non- β -oxidative pathway is used by these strains is unclear. The nonoxidative decarboxylation mechanism has also been reported for the conversion of cinnamic acid to styrene by *Pichia carsonii* (51) and *Cryptococcus elinovii* (38).

Ferulic acid is abundant in nature, and vanillic acid is one of the intermediates formed by the biodegradation of ferulic acid. Therefore, we have here screened bacteria capable of utilizing vanillic acid, and the biodegradation of other lignin-related compounds by the isolated bacteria has subsequently been examined.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased from Wako Pure Chemicals or Tokyo Kasei Kogyo Co.

Isolation of vanillic acid degraders. The W medium was composed of KH_2PO_4 (0.85 g/liter), $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (4.90 g/liter), $(\text{NH}_4)_2\text{SO}_4$ (0.50 g/liter), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.10 g/liter), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (9.50 mg/liter), MgO (10.75 mg/liter), CaCO_3 (2.00 mg/liter), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (1.44 mg/liter), $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (1.12 mg/liter), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.25 mg/liter), $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ (0.28 mg/liter), H_3BO_3 (0.06 mg/liter), and HCl (5.13×10^{-2} ml/liter). Decayed wood bark (0.1%) was added to 5 ml of W medium including either 10 mM vanillic acid and a 1/100-strength of Luria-Bertani medium (LB; 0.1 g of Bacto tryptone/liter, 0.05 g of yeast extract/liter, 0.05 g of NaCl/liter) or only 10 mM vanillic acid, and the mixture was incubated at 60°C to isolate the thermophilic degraders of vanillic acid. The microorganisms generally grew in 1 day, and a portion of the culture was plated on W medium agar plates including 10 mM vanillic acid plus 1/100 LB or only 10 mM vanillic acid to isolate single colonies.

Total DNA extraction and 16S rRNA gene sequencing. To isolate the total DNA, each bacterium was grown to the stationary phase in 10 ml of the LB medium at 60°C. The resulting cells were washed twice with a TES buffer (0.1 M NaCl, 10 mM Tris-HCl, and 1 mM EDTA at pH 8.0) and then suspended in 2.5 ml of the same buffer including 5 mg of lysozyme. After being kept at 37°C for 1 h, 256 μl of 10% (wt/vol) sodium dodecyl sulfate and 0.5 ml of the TES buffer containing 0.5 mg of protease K were added, and the mixture was incubated for 1 h at 65°C. Sodium dodecyl sulfate (10% [wt/vol], 1.8 ml) was then added, and the mixture was incubated for 1 h at 65°C. Total DNA was precipitated by adding 2 volumes of ethanol at room temperature. The precipitate obtained by centrifugation was washed with 70% (vol/vol) ethanol. The 16S rRNA gene-containing fragment of about 1.5 kb was amplified with the proR and 9Rev primers, re-

spectively, designed by Hanzawa et al. (28) and Takeuchi et al. (56). The proR, 1R, 2R, 2F, 4R, P7R, and 9Rev primers (28) were used to determine the DNA sequence of the 1.5-kb DNA fragment that had been purified from an agarose gel by using the Min Elute gel extraction kit (QIAGEN). The DNA sequence data were aligned by using Clustal W (57), and a neighbor-joining tree was then constructed with this software.

Growth of the isolates on vanillic acid. Each isolate was used to inoculate 5 ml of W medium including either 10 mM vanillic acid plus 1/100 LB or only 10 mM vanillic acid, and the mixture was incubated at 60°C with shaking (150 rpm). Aliquots were withdrawn at regular intervals, and the growth was monitored over a period of 1 day by measuring the turbidity at 600 nm.

Growth tests with various compounds. Each bacterium was grown overnight at 60°C in W medium including 0.2% (wt/vol) succinate. The resulting cells were washed twice with W medium and then suspended in 50 ml of the same medium to give a turbidity of 0.2 at 600 nm. Each of the test compounds was added to a final concentration of 5 mM, and the mixture was shaken (150 rpm) at 60°C. Aliquots were withdrawn at intervals of 2 h, and the growth was monitored as already described.

Degradation of various compounds by resting cells. Bacterial cells were grown to the stationary phase in LB medium at 60°C with shaking (150 rpm). The resulting cells were washed twice with W medium and then suspended in 5 ml of the same medium to give a turbidity of 1 at 600 nm. Each compound examined was added to a final concentration of 5 mM, and the mixture was incubated at 60°C with shaking (150 rpm). Aliquots were withdrawn at intervals of 1 day to assess the amount of residual initial compounds and their metabolic intermediates.

Analysis of the metabolites by GC-MS. Bacterial cells were grown to the stationary phase in 50 ml of W medium including each substrate at a concentration of 5 mM. The culture medium was acidified with HCl to pH 2, and the resulting metabolites were extracted with ethyl acetate. The organic phase was dried in vacuo and then treated with the trimethylsilylreagent. The resulting samples were analyzed by gas chromatography-mass spectrometry (GC-MS) (Shimadzu QP5050A instrument) with a DB-5 column (length, 30 m; diameter, 0.25 mm) (J&W Scientific). The column temperature was initially kept at 50°C for 5 min before being increased to 300°C at a rate of 2.5°C per min. The injector and detector temperatures were both 300°C.

Enzymatic activities. Strain B1 was grown in 1 liter of LB medium for 12 h at 60°C with shaking (150 rpm) and then washed twice with W medium. The resulting cells were resuspended in 300 ml of W medium including 5 mM concentrations of the individual substrates, and the mixture was incubated with shaking (150 rpm) at 60°C for 10 h. The cells were harvested and passed four times through a French press at a pressure of 1,500 MPa to give cell extracts.

The reaction mixture for assaying CoA ligase activity contained 100 μM substrate, 500 μM CoA, 500 μM ATP, 2 mM MgSO_4 , and 30 μl of the cell extracts (10 μg of total protein per μl) in 1 ml of 50 mM potassium phosphate buffer (pH 8.0). The ligase activity was determined at 60°C by spectrometrically measuring the rate of appearance of the CoA thioester product (37).

The reaction mixture for assaying catechol oxygenase activity contained 100 μM catechol, 500 μM MnSO_4 , and 100 μl of the cell extracts (10 μg of total protein per μl) in 1 ml of 50 mM potassium phosphate buffer (pH 8.0). The reaction mixture for assaying gentisic acid oxygenase activity contained 100 μl of the above cell extracts in 1 ml of 50 mM potassium phosphate buffer (pH 8.0). Both oxygenase activities were estimated at 60°C by the decrease in the amount of the individual substrates determined by GC-MS.

Nucleotide sequence accession number. The 16S ribosomal DNA (rDNA) sequences were deposited in the DDBJ, EMBL, and GenBank sequence databases under accession numbers no. AB089207 to AB089240.

RESULTS

Isolation and characterization of the vanillic acid degraders. Eleven strains (B1 to B7, D1, A3, and G62) were isolated from the wood bark samples after enrichment in W medium including vanillic acid as the sole carbon and energy source. Strain V0 was similarly obtained from the hot-spring water sample. Twenty-two strains (E1 to E3, F1, F2, G1 to G8, H1 to H6, and ZI3 to ZI5) were isolated from the wood bark samples after enrichment in W medium including vanillic acid in addition to 1/100-strength LB medium (1/100 LB).

Total DNA was extracted from each strain, and the 16S

FIG. 1. Phylogenetic tree of thermophilic isolates based on 16S rDNA sequences. Three 16S rDNA sequences of *Bacillus* sp. strains obtained from the GenBank database were aligned as the reference. *Bacillus vulcani* (accession no. AJ293805) was isolated from a shallow marine hydrothermal vent, and two *Bacillus* sp. (accession no. AB066336 and Z26929) were isolated from decomposed wood bark. The bootstrap value for each node (1,000 replications) is shown.

rRNA genes were amplified with the pro0R and 9Rev primers and then sequenced. A homology search of their 16S rDNA sequences in the GenBank database indicated that the rDNA sequences from the respective strains isolated in this study had high levels of identity with those of three strains belonging to the genus *Bacillus* (accession numbers AJ293805, Z26929, and AB066336). It has therefore been clarified that our strains belong to the genus *Bacillus*. The sequences were aligned with each other by using Clustal W along with the three *Bacillus* strains as references. The phylogenetic tree (Fig. 1) shows that most of the isolates were classified into two phylogenetic

groups (groups A and B), with the remaining one called group C as a matter of convenience. The overall identity of the isolates was 93%. Identity between the members within groups A and B were 98 and 99%, respectively, while group A shared 93% identity with group B.

The growth of the isolates on vanillic acid was investigated (Table 1). The strains in group A, including B1 to B7, G62, D1, A3, and V0, but excluding G6, were capable of growing on vanillic acid as the sole carbon and energy source. On the other hand, the strains belonging to groups B and C could not grow on vanillic acid without adding 1/100 LB, indicating that some

TABLE 1. Growth of isolates on vanillic acid and vanillic acid plus 1/100 LB and amount of residual vanillic acid culture medium

Group	Isolate	Turbidity (600 nm) for isolates grown on:				Amt of residual VA (mM) for isolates grown on:		
		VA ^b for:		VA + 1/100 LB for:		VA for 3 days	VA + 1/100 LB for:	
		1 day	3 days	1 day	3 days		1 day	3 days
None	Control	0.02 ± 0.00	0.35 ± 0.08	0.02 ± 0.00	0.02 ± 0.00	9.12 ± 0.26	10.00 ± 0.27	10.00 ± 0.49
A	B1	0.35 ± 0.08	0.62 ± 0.04	0.25 ± 0.02	0.72 ± 0.02	0.00 ± 0.00	8.13 ± 0.23	0.00 ± 0.00
	G62	0.07 ± 0.04	0.71 ± 0.04	0.39 ± 0.02	0.70 ± 0.03	0.00 ± 0.00	7.44 ± 0.35	0.00 ± 0.00
	B2	0.14 ± 0.03	0.74 ± 0.02	0.28 ± 0.05	0.88 ± 0.14	0.00 ± 0.00	9.44 ± 0.24	0.00 ± 0.01
	B3	0.17 ± 0.03	0.65 ± 0.02	0.30 ± 0.01	0.74 ± 0.03	0.00 ± 0.00	9.81 ± 0.20	0.00 ± 0.00
	V0	0.21 ± 0.04	0.26 ± 0.03	0.28 ± 0.01	0.26 ± 0.01	0.00 ± 0.00	4.32 ± 0.34	0.00 ± 0.00
	E2	0.02 ± 0.02	0.07 ± 0.01	0.17 ± 0.01	0.57 ± 0.07	8.41 ± 1.64	10.02 ± 0.33	1.80 ± 0.62
	D1	0.27 ± 0.03	0.47 ± 0.10	0.31 ± 0.01	0.31 ± 0.02	0.00 ± 0.00	7.55 ± 0.48	0.00 ± 0.00
	A3	0.14 ± 0.04	0.66 ± 0.04	0.38 ± 0.04	0.61 ± 0.03	0.57 ± 0.42	8.40 ± 0.31	2.31 ± 0.98
	VA2	0.09 ± 0.03	0.66 ± 0.01	0.23 ± 0.01	0.71 ± 0.01	0.00 ± 0.00	9.62 ± 0.09	0.09 ± 0.09
B	G6	ND ^a	0.03 ± 0.00	0.62 ± 0.05	0.63 ± 0.02	9.32 ± 0.22	1.49 ± 1.26	ND
	E1	ND	0.02 ± 0.00	0.29 ± 0.01	0.35 ± 0.01	8.67 ± 1.29	0.00 ± 0.00	ND
	F1	ND	0.04 ± 0.03	0.44 ± 0.02	0.57 ± 0.03	9.21 ± 0.20	0.00 ± 0.00	ND
	F2	ND	0.04 ± 0.00	0.22 ± 0.03	0.26 ± 0.02	9.12 ± 0.24	0.01 ± 0.01	ND
	G1	ND	0.04 ± 0.00	0.27 ± 0.01	0.26 ± 0.01	7.96 ± 1.33	0.01 ± 0.00	ND
	G8	ND	0.05 ± 0.01	0.26 ± 0.02	0.28 ± 0.01	8.99 ± 0.48	0.00 ± 0.00	ND
	G2	ND	0.05 ± 0.00	0.26 ± 0.01	0.30 ± 0.02	8.88 ± 1.00	0.00 ± 0.00	ND
	ZI4	ND	0.09 ± 0.04	0.29 ± 0.03	0.27 ± 0.01	9.59 ± 0.26	2.84 ± 0.35	ND
	G3	ND	0.05 ± 0.02	0.23 ± 0.01	0.29 ± 0.02	9.74 ± 0.26	0.00 ± 0.00	ND
C	H5	ND	0.04 ± 0.00	0.22 ± 0.01	0.27 ± 0.02	9.90 ± 0.34	0.00 ± 0.00	ND
	ZI3	ND	0.08 ± 0.00	0.23 ± 0.02	0.27 ± 0.03	9.99 ± 0.25	0.01 ± 0.00	ND
	H4	ND	0.03 ± 0.00	0.24 ± 0.01	0.25 ± 0.01	9.47 ± 0.23	0.00 ± 0.00	ND
	G5	ND	0.10 ± 0.05	0.26 ± 0.29	0.29 ± 0.03	8.28 ± 0.69	0.00 ± 0.00	ND
	G4	ND	0.05 ± 0.03	0.20 ± 0.01	0.26 ± 0.00	9.28 ± 0.20	0.00 ± 0.00	ND
	H1	ND	0.03 ± 0.00	0.24 ± 0.02	0.27 ± 0.04	8.98 ± 0.44	0.01 ± 0.00	ND
	ZI5	ND	0.04 ± 0.00	0.25 ± 0.01	0.34 ± 0.03	9.06 ± 0.46	0.01 ± 0.00	ND

^a ND, not detected.^b VA, vanillic acid.

nutritional factors such as vitamins were needed for their growth. When the strains in group A were grown on vanillic acid along with 1/100 LB, the rates of degradation of vanillic acid were slower than those of the other two groups (Table 1). It seems that some factors in LB inhibited the degradation of vanillic acid in the strains belonging to group A.

Strains B1, V0, and D1 from group A, E1 and G2 from group B, and ZI3 and H4 from group C were selected as the representatives of these groups to investigate the ability to degrade other lignin-related aromatic acids, including cinnamic, 4-coumaric, and ferulic acids. All of the strains tested proved capable of degrading these compounds (the results are shown later). Strain B1 was found to grow fastest among the strains tested on these aromatic acids, so B1 was selected to study the pathways for the degradation of cinnamic acid and the related aromatic acids. Figure 2 shows the range of temperature in which strain B1 was able to grow on LB medium. This strain grew well at 50 to 70°C. A temperature of 60°C was used for further study.

Pathway for the degradation of cinnamic acid by strain B1.

Strain B1 was grown at 60°C on 5 mM cinnamic acid as the sole carbon and energy source, and aliquots withdrawn from the culture medium at regular intervals were subjected to GC-MS analysis. The concentrations of residual cinnamic acid and the products converted from cinnamic acid were determined from the standard curve for each. GC-MS data for the compounds

FIG. 2. Temperature range for strain B1 grown on LB medium.

TABLE 2. GC-MS data for growth substrates and metabolites

Substrate or metabolite ^a	Retention time (min)	<i>m/z</i> of major ion peaks (%) ^b
CA	17.68	220 (M ⁺) (28), 205 (100), 161 (68), 145 (28), 131 (89), 103 (67), 74 (50)
3PP	15.98	222 (M ⁺) (11), 207 (32), 104 (100), 91 (24), 75 (85)
BA	13.37	194 (M ⁺) (8), 179 (93), 135 (68), 105 (100), 77 (56)
3HB	17.98	282 (M ⁺) (31), 267 (100), 223 (52), 193 (45), 149 (11), 126 (21), 73 (53)
4CM	22.29	308 (M ⁺) (36), 293 (79), 249 (50), 219 (85), 179 (18), 139 (15), 73 (100)
3HPP	20.35	282 (M ⁺) (21), 267 (100), 223 (87), 193 (66), 149 (5), 126 (27), 73 (73)
4HB	18.76	308 (M ⁺) (36), 293 (79), 249 (50), 219 (85), 179 (18), 139 (15), 73 (100)
GTA	20.57	370 (M ⁺) (2), 355 (100), 267 (5), 223 (5), 149 (6), 73 (72)
FA	23.85	338 (M ⁺) (86), 323 (58), 308 (34), 293 (45), 279 (15), 249 (58), 219 (28), 191 (12), 146 (25), 73 (100)
Vanillin	17.58	224 (M ⁺) (26), 209 (44), 194 (100), 179 (5), 165 (6), 73 (22)
VA	20.38	312 (M ⁺) (57), 297 (100), 282 (54), 267 (96), 253 (69), 223 (72), 193 (25), 165 (17), 133 (29), 126 (80), 73 (93)
HMPHP	22.98	297 (100), 193 (10), 147 (13), 75 (37), 73 (37)

^a All compounds quoted in this table are trimethylsilyl derivatives. Identification was based on a library match and comparison with the data for relevant standards. Abbreviations: CA, cinnamic acid; 3PP, 3-phenylpropionic acid; BA, benzoic acid; 3HB, 3-hydroxybenzoic acid; 4CM, 4-coumaric acid; 3HPP, 3-(*p*-hydroxyphenyl)propionic acid; 4HB, 4-hydroxybenzoic acid; GTA, gentisic acid; FA, ferulic acid; VA, vanillic acid.

^b The ion abundance percentages are shown in parentheses.

analyzed in this study are listed in Table 2. The formation of three products derived from cinnamic acid was observed (Fig. 3A). These products that have retention times of 15.98, 13.37, and 17.98 min were identified as 3-phenylpropionic acid, benzoic acid, and 3-hydroxybenzoic acid, respectively, by comparing their retention times and mass spectra with those of the authentic standards. The former two compounds, 3-phenylpropionic and benzoic acids, could also support the growth of strain B1 as the sole carbon and energy source. When strain B1 was grown on 3-phenylpropionic acid, the formation of three products, cinnamic, benzoic, and 3-hydroxybenzoic acids, was observed (Fig. 3B). 3-Hydroxybenzoic acid was also detected as a degradation product from benzoic acid (Fig. 3C). 3-Hydroxybenzoic acid accumulated to a concentration ranging from 20 to 50 μ M, and it seemed to not be degraded by this strain. In fact, 3-hydroxybenzoic acid did not support the growth of strain B1 (Fig. 3D).

Based on these results, the pathway for the degradation of cinnamic acid by strain B1 is proposed as shown in Fig. 4. Cinnamic acid is first converted to benzoic acid. Although strain B1 was capable of transforming benzoic acid to 3-hydroxybenzoic acid, the latter compound seems to be a dead-end product. No substrate for ring fission was detected in this cinnamic acid-degradative pathway. Catechol is thought to be a promising candidate for the ring fission in this pathway. Our data also indicate that 3-phenylpropionic acid was degraded through cinnamic acid. The conversion step between 3-phenylpropionic acid and cinnamic acid was reversible because 3-phenylpropionic acid could be detected when cinnamic acid was used as the growth substrate. An anoxic condition at high temperatures may be responsible for the reduction of cinnamic acid to 3-phenylpropionic acid.

Pathway for the degradation of 4-coumaric acid by strain B1. When strain B1 was grown on 5 mM 4-coumaric acid as the sole carbon and energy source, the formation of 4-hydroxybenzoic and gentisic acids was observed in the culture medium (Fig. 5A). The identities of these two compounds were verified by comparing their retention times and mass spectra with those

of the authentic standards. Gentisic acid was also detected in the culture medium when 4-hydroxybenzoic acid was used as the growth substrate (Fig. 5B). Based on these results, the pathway for the degradation of 4-coumaric acid is proposed as shown in Fig. 4. 4-Coumaric acid is first converted to 4-hydroxybenzoic acid and then transformed to gentisic acid, which seems to be the target compound for ring fission.

The structure of 4-coumaric acid only differs from that of cinnamic acid by a *para*-substituted hydroxyl group. The reversible conversion between cinnamic and 3-phenylpropionic acids was reported in the previous section. However, 3-(*p*-hydroxyphenyl)propionic acid, a *para*-hydroxy derivative of 3-phenylpropionic acid, was not detected in the culture medium containing 4-coumaric acid as the growth substrate. Nevertheless, strain B1 could grow on 5 mM 3-(*p*-hydroxyphenyl)propionic acid, and the formation of 4-coumaric, 4-hydroxybenzoic, and gentisic acids was observed in the culture medium (Fig. 5C). Thus, 3-(*p*-hydroxyphenyl)propionic acid was found to be degraded through 4-coumaric acid. The reduction of 4-coumaric acid to 3-(*p*-hydroxyphenyl)propionic acid may be thermodynamically unfavorable due to the presence of the hydroxy group at the *para* position.

Pathway for the degradation of ferulic acid by strain B1.

When strain B1 was grown on 5 mM ferulic acid as the sole carbon and energy source, three converted products were detected (Fig. 6A). Two of them were identified as vanillin and vanillic acid by comparing their retention times and mass spectra with those of authentic standards. The other one with a retention time of 22.98 min had major fragment ions at *m/z* of 297, 193, 147, 75, and 73. A library search (National Institute of Standards and Technology library of mass spectra) listed three compounds as best matches. The first one was 4-hydroxy-3-methoxymandelic acid, which we found to have a retention time of 21.76 min. The second one was 4-hydroxy-3-methoxyphenethylene glycol, with a retention time of 21.24 min. The third one was 4-hydroxy-3-methoxyphenyl- β -hydroxypropionic acid (HMPHP), whose authentic sample was not available. The retention times of these first two compounds did not agree with

FIG. 3. Growth of strain B1 on cinnamic acid (CA) (A), 3-phenylpropionic acid (3PP) (B), benzoic acid (BA) (C), and 3-hydroxybenzoic acid (3HB) (D). The kinetics for the product formation from each compound are also presented in each graph. For the results shown in panel A, each unit of concentration corresponds to 1 mM for CA, 5 μ M for 3PP, 500 μ M for BA, and 10 μ M for 3HB. For the results shown in panel B, each unit of concentration corresponds to 1 mM for 3PP, 5 μ M for CA, and 10 μ M for BA and 3HB. For the results shown in panel C, each unit of concentration corresponds to 1 mM for BA and 10 μ M for 3HB. For the results shown in panel D, each unit of concentration corresponds to 1 mM for 3HB. Tur., turbidity.

the retention time (22.98 min) of the product converted from ferulic acid. It was therefore thought that the product with a retention time of 22.98 min was HMPHP. No degradation product was detected in the culture medium when vanillic acid was used as the growth substrate (Fig. 6B).

Based on these results, the pathway for the degradation of ferulic acid is proposed as shown in Fig. 4. Ferulic acid is converted to vanillin via HMPHP; vanillin is then oxidized to give vanillic acid. The steps following the formation of vanillic acid are unclear, but the most feasible route is subject to ring fission via protocatechuic acid.

Catabolic pathways for degradation of cinnamic acid derivatives by other bacilli. We also investigated the degradation of cinnamic, 4-coumaric, ferulic, benzoic, 3-hydroxybenzoic, and 4-hydroxybenzoic acids by using several of the other isolated strains. Strains V0 and D1 from group A, E1 and G2 from group B, and ZI3 and H4 from group C were selected for this purpose. A few of these strains showed a very slow growth rate on the aromatic acids, so the resting cells grown on the LB medium were used for the experiments. The resting cells of six strains were incubated with W medium including 5 mM cinnamic, 4-coumaric, ferulic, benzoic, 3-hydroxybenzoic, or 4-hydroxybenzoic acid at 60°C. Aliquots were withdrawn after incubation for 1 and 2 days for GC-MS analysis (Table 3). The

degradation of cinnamic acid was observed in all of the six strains and benzoic acid was detected as an intermediate, except for strain ZI3, in which only 3-hydroxybenzoic acid was detected. The ability of the respective strains to degrade benzoic and 3-hydroxybenzoic acids was not identical. Strains V0 and E1 did not significantly degrade benzoic acid, whereas the other strains (D1, G2, ZI3, and H4) did. The degradation of 3-hydroxybenzoic acid was not observed in strains E1, G2, and ZI3, whereas, significantly, strains V0, D1, and H4 were capable of degrading this compound. The formation of gentisic acid from 3-hydroxybenzoic acid was observed in strains D1 and H4. Although gentisic acid formation was not experimentally confirmed in strain V0, 3-hydroxybenzoic acid is likely to be metabolized via gentisic acid also in this strain.

All of the six strains degraded 4-coumaric and 4-hydroxybenzoic acids faster than they degraded cinnamic and benzoic acids. Gentisic acid was detected as an intermediate when using both 4-coumaric and 4-hydroxybenzoic acids as initial substrates. The transformation of ferulic acid into HMPHP, vanillin, and vanillic acid was observed in all six of the strains.

These results indicate that the pathways for the degradation of cinnamic acid and the related aromatic acids were the same as those of strain B1, except that strains V0 and E1 were not

FIG. 4. Pathways for the thermophilic biodegradation of cinnamic acid and benzoic acid and their substituted aromatic acids by the *Bacillus* isolates. The open arrows represent the unidentified steps.

able to degrade benzoic acid and strains V0, D1, and H4 were able to degrade 3-hydroxybenzoic acid.

Cinnamic acid-CoA ligase activity in strain B1. The degradation of the cinnamic acid and its substituents may start with the conversion of these initial substrates into their acyl-CoA derivatives. Accordingly, crude extracts were prepared from cells of strain B1 grown on 4-coumaric acid. The activities of cinnamate-CoA ligase, 4-coumarate-CoA ligase, and ferulate-CoA ligase were detected in the presence of cofactors CoA, ATP, and Mg^{2+} with the respective specific activities of 31.3 ± 2.5 , 32.7 ± 3.8 , and 35.5 ± 1.3 nmol min⁻¹ mg⁻¹.

Oxygenase activities in strain B1. To confirm whether catechol, gentisic acid, and protocatechol are the substrates for the ring fission in each pathway, the activities of the respective oxygenases were examined. Catechol oxygenase activity was detected in the presence of catechol, Mn^{2+} , and cell extracts prepared from the cells grown on cinnamic acid with specific activity of $7.8 \pm 0.5 \text{ nmol min}^{-1} \text{ mg}^{-1}$. No activity was detected without adding Mn^{2+} to the reaction mixture, indicating that catechol oxygenase is dependent on Mn^{2+} . Gentisic acid oxygenase activity was also detected in the presence of gentisic acid and cell extracts prepared from the cells grown on 4-coumaric acid with a specific activity of $11.3 \pm 1.2 \text{ nmol min}^{-1}$.

mg⁻¹. A cofactor requirement is not observed in this reaction. On the other hand, the protocatechuic acid oxygenase activity was not detected when protocatechuic acid, Fe²⁺, or Mn²⁺, and cell extracts prepared from the cells grown on ferulic acid were added to the reaction mixture.

The above data strongly support our proposed pathways and that cinnamic acid and 4-coumaric acid were degraded through catechol and gentisic acid for the ring fission, respectively. The failure to detect the protocatechuic acid oxygenase activity may be due to the absence of any cofactor needed in the reaction, which we could not find.

DISCUSSION

Bacilli are widely distributed in soil and water environments: thermophilic *Bacillus* species, with growth temperature optima between 45 and 70°C, have been isolated from a wide range of environments (53). Research interest in these organisms has increased due to their biotechnological potential, especially as sources of such thermostable enzymes as amylases and neutral proteases (53). Some *Bacillus* species can degrade aromatic compounds (13). However, no study has previously been re-

FIG. 5. Growth of strain B1 on 4-coumaric acid (4CM) (A), 4-hydroxybenzoic acid (4HB) (B), and 3-(*p*-hydroxyphenyl)propionic acid (3HPP) (C). The kinetics for the product formation from each compound are also presented in each graph. For the results shown in panel A, each unit of concentration corresponds to 1 mM for 4CM, 10 μM for 4HB, and 5 μM for gentisic acid (GTA). For the results shown in panel B, each unit of concentration corresponds to 1 mM for 4HB and GTA. For the results shown in panel C, each unit of concentration corresponds to 1 mM for 3HPP and 10 μM for 4CM, 4HB, and GTA. Tur., turbidity.

ported on the pathways for degradation of lignin-related compounds such as cinnamic acids under thermophilic conditions.

We isolated in this study 33 strains capable of degrading a variety of phenylpropanoids from decayed wood bark in thermophilic conditions. The optimal temperature for their growth seems to range from 50 to 70°C, as shown for strain B1. The 16S rDNA sequences of our isolates showed high levels of identity with those of three bacilli which were previously isolated from the thermophilic environment in decomposed wood bark and a shallow marine hydrothermal vent (accession numbers AJ293805, Z26929, and AB066336), indicating that the 33 isolates belong to the genus *Bacillus*. These bacilli are likely to play a major role in the degradation of lignin-related compounds in decayed wood bark under thermophilic conditions. The metabolic pathways shown in Fig. 4 were also applicable to those of strain V0 isolated from a hot spring, indicating that those pathways may be widely distributed in the thermophilic environment.

The pathway for the conversion of cinnamic acid to benzoyl-CoA, which is then converted to gentisic acid via 3-hydroxybenzoyl-CoA, with subsequent ring fission leading to the gentisic acid pathway has been proposed for *Haloferax* sp. D1227 (21). This conversion requires several cofactors, including ATP, CoA, and NAD⁺. CoA ligase activity toward cinnamic acid was detected in strain B1. Furthermore, benzaldehyde and styrene, respective intermediates typical for the non-β-oxidation and nonoxidative decarboxylation pathways, were not detected as intermediates in strain B1. Consequently, the β-oxidative path-

way is likely to be involved in the degradation of cinnamic acid by strain B1, as has been reported for *Haloferax* sp. D1227. However, more studies are required to validate this notion.

The catabolic pathways for the degradation of (substituted) benzoic acid in various organisms have been reviewed by Hammann and Kutzner (27). Monooxygenases or dioxygenases catalyze the formation of the 2-, 3-, and 4-hydroxy derivatives of (substituted) benzoic acid and/or (substituted) catechols. A CoA-dependent mechanism for the conversion of benzoic acid to 3-hydroxybenzoic acid via benzoyl-CoA has recently been reported for *Pseudomonas* sp. (2, 42). Strain B1 also had the ability to transform benzoic acid to 3-hydroxybenzoic acid, but it was a dead-end product. The presence of catechol oxygenase activity in the cell extracts prepared from the B1 cells grown on cinnamic acid indicated that benzoic acid was converted to catechol. On the other hand, the degradation of cinnamic acid by strains V0, D1, and H4 provided 3-hydroxybenzoic acid, which was then converted to gentisic acid. The transformation of 3-hydroxybenzoic acid to gentisic acid catalyzed by a 3-hydroxybenzoic acid 6-hydroxylase has been proposed for several bacteria (13, 27, 54). Strains D1, V0, and H4 may also have such an enzyme.

The catalytic reactions involved in the conversion of 4-coumaric acid to 4-hydroxybenzoic acid by strain B1 and other members may be equivalent to those found in the catabolic pathway for cinnamic acid. The reaction for converting 4-hydroxybenzoic acid to gentisic acid was reported about 20 years ago for only three *Bacillus* strains (7, 12, 14), no more-recent report concerning the enzyme and gene for this reaction having been published. A mechanism analogous to the NIH shift (26, 34) can yield gentisic acid from 4-hydroxybenzoic acid: hydroxylation at C1 of the 4-hydroxybenzoic acid ring, with concomitant migration of the carboxyl group to an *ortho* position on the ring, yields gentisic acid. However, no experimental data

FIG. 6. Growth of strain B1 on ferulic acid (FA) (A) and vanillic acid (VA) (B). The kinetics for the product formation from each compound are also presented in each graph. The concentration of HMPHP was calculated by using a standard curve for ferulic acid. For the results shown in panel A, each unit of concentration corresponds to 1 mM for FA, 5 μ M for HMPHP and vanillin (VN), and 50 μ M for VA. For the results shown in panel B, each unit of concentration corresponds to 1 mM for VA. Tur., turbidity.

are available to support this proposal. The catabolic pathways for 4-coumaric acid so far reported yielded protocatechuic acid (37, 52), and this is the first report on the catabolic pathway for 4-coumaric acid yielding gentisic acid.

Few bacteria can attack both cinnamic and *para*-hydroxylated cinnamic acids (4-coumaric and ferulic acids). The degradation rates of 4-coumaric and 4-hydroxybenzoic acids by our strains were faster than those of cinnamic and benzoic acids. The enzymes in the pathways may prefer 4-coumaric acid and its metabolites to cinnamic acid and its metabolites.

The metabolism of ferulic acid by strain B1 seems to be different from that of cinnamic and 4-coumaric acids. In addition to vanillin and vanillic acid detected, HMPHP seems to exist as an intermediate of the ferulic acid biodegradation. It is

likely that the non- β -oxidation pathway is used by strain B1 for the catabolism of ferulic acid, which can give the valuable flavor compound vanillin. The enzymes and genes involved in the ferulic acid pathway have been studied in detail for this reason in many bacteria (23, 37, 39, 41, 45). The formation of feruloyl-CoA was catalyzed by a ferulic acid-CoA ligase in this pathway, and feruloyl-CoA was then converted to vanillin and acetyl-CoA by the single enzyme enoyl-CoA hydratase/lyase. This enzyme demonstrated two partial activities: enoyl-CoA hydratase activity that can hydrate feruloyl-CoA to HMPHP-CoA and lyase activity that can cleave HMPHP-CoA to vanillin and acetyl-CoA. However, the formation of HMPHP-CoA from feruloyl-CoA was not detected, suggesting that it was tightly bound to the enzyme during catalysis (39). Why free

TABLE 3. Degradation of various aromatic acids by *Bacillus* strains belonging to different taxonomic groups

Initial compound ^a	Product	Amt of product formed from each initial compound (μ M) on strain:											
		VO		D1		E1		G2		ZI3		H4	
		1 day	2 days	1 day	2 days	1 day	2 days	1 day	2 days	1 day	2 days	1 day	2 days
CA	CA	4,986	4,571	4,946	0	4,153	0	2,336	0	0	0	170	0
	3PP	0	0	0	0	Trace	0	Trace	0	0	0	Trace	0
	BA	6.8	15.1	4.9	0	45.5	0	428.7	0	0	0	890.7	0
	3HB	0	0	0	0.3	0	0	20.0	0	4.4	0	20.4	0
BA	BA	5,135	4,967	4,977	0	4,816	4,996	3,165	0	407	0	1,349	0
	3HB	0	0	0	0.3	0	0	3.0	0	0.8	0	2.5	0
3HB	3HB	4,968	0	4,734	0	4,779	4,898	4,941	4,981	4,687	5,051	1,270	0
	GTA	0	0	132	0	0	0	0	0	0	0	19.1	0
4CM	4CM	0	0	0	0	Trace	0	0	0	0	0	0	0
	3HPP	0	0	0	0	0	0	0	0	0	0	0	0
	4HB	0	0	0	0	0	0	0	0	0	0	0	0
	GTA	0	0	1.9	0	2.2	0	1.9	0	0.4	0	1.4	0
4HB	4HB	3,223	0	2,277	0	Trace	0	0	0	Trace	0	0.1	0
	GTA	0.4	0	13.7	0	0	0	0.4	0	0	0	0	0
FA	FA	4,109	2,271	2,973	0	3,987	0	4,223	0	4,293	0	4,831	0
	HMPHP	Trace	0	Trace	0	Trace	0	Trace	0	Trace	0	Trace	0
	VN	2.5	7.8	11.7	0	11.5	0	9.1	0	13.9	0	6.8	0
	VA	3.1	15.8	1.1	0	2.9	0	3.8	0	3.6	0	1.5	0

^a The initial concentration of each growth substrate was 5,000 μ M. Abbreviations: CA, cinnamic acid; 3PP, 3-phenylpropionic acid; BA, benzoic acid; 3HB, 3-hydroxybenzoic acid; 4CM, 4-coumaric acid; 3HPP, 3-(*p*-hydroxyphenyl)propionic acid; 4HB, 4-hydroxybenzoic acid; GTA, gentisic acid; FA, ferulic acid; VA, vanillic acid; VN, vanillin.

HMPHP was formed by strain B1 is unclear. No substrate for ring fission, such as protocatechuic acid, gentisic acid, or catechol, could be detected in the culture medium when using either ferulic or vanillic acid as the growth substrate. Structural considerations indicate the likelihood that vanillic acid might be metabolized via protocatechuic acid for its ring fission rather than via gentisic acid (6, 43).

Cinnamic, benzoic, and gentisic acids are intermediates formed by the biodegradation of environmental contaminants such as biphenyl (36, 61), naphthalene (22, 24, 25, 65), dibenzo-*p*-dioxin (60), and *n*-alkylbenzenes (18). The *Bacillus* species isolated in this study may provide useful information about the evolution and functions of catabolic enzymes for these aromatic compounds.

We have described in this study the thermophilic degradation of cinnamic acid and its related compounds by *Bacillus* sp. It is expected that the thermostable enzymes of these strains will have industrial applications.

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