Methanogenic degradation of lignin-derived monoaromatic compounds by microbial enrichments from rice paddy field soil

Souichiro Kato1,2,3, Kanako Chino4, Naofumi Kamimura4, Eiji Masai4, Isao Yumoto4 & Yoichi Kamagata1,2

Anaerobic degradation of lignin-derived aromatics is an important metabolism for carbon and nutrient cycles in soil environments. Although there are some studies on degradation of lignin-derived aromatics by nitrate- and sulfate-reducing bacteria, knowledge on their degradation under methanogenic conditions are quite limited. In this study, methanogenic microbial communities were enriched from rice paddy field soil with lignin-derived methoxylated monoaromatics (vanillate and syringate) and their degradation intermediates (protocatechuate, catechol, and gallate) as the sole carbon and energy sources. Archaeal community analysis disclosed that both aceticlastic (Methanosarcina sp.) and hydrogenotrophic (Methanoculleus sp. and Methanocella sp.) methanogens dominated in all of the enrichments. Bacterial community analysis revealed the dominance of acetogenic bacteria (Sporomusa sp.) only in the enrichments on the methoxylated aromatics, suggesting that Sporomusa spp. initially convert vanillate and syringate into protocatechuate and gallate, respectively, with acetogenesis via O-demethylation. As the putative ring-cleavage microbes, bacteria within the phylum Firmicutes were dominantly detected from all of the enrichments, while the dominant phylotypes were not identical between enrichments on vanillate/protocatechuate/catechol (family Peptococcaceae bacteria) and on syringate/gallate (family Ruminococcaceae bacteria). This study demonstrates the importance of cooperation among acetogens, ring-cleaving fermenters/syntrophs and aceticlastic/hydrogenotrophic methanogens for degradation of lignin-derived aromatics under methanogenic conditions.

Lignin is a major component of terrestrial plants and is a highly complex heteropolymer consisting of hydroxylated and methoxylated phenylpropanid units linked by various types of C–C and C–O–C bonds. As lignin represents a significant part of input of organic compounds, its degradation is integral for carbon and energy cycles in soil environments. Lignin is aerobically depolymerized by lignolytic fungi and some bacterial strains, followed by mineralization of the resulting low-molecular-weight products

1Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 2-17-2-1 Tsukisamu-Higashi, Toyohira-ku, Sapporo, Hokkaido 062-8517, Japan. 2Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Kita-9 Nishi-9, Kita-ku, Sapporo, Hokkaido 060-0810, Japan. 3Research Center for Advanced Science and Technology, The University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8904, Japan. 4Department of Bioengineering, Nagaoka University of Technology, 1603-1 Kamitomioka, Nagaoka, Niigata 940-2188, Japan. Correspondence and requests for materials should be addressed to S.K. (email s.katou@aist.go.jp)
by soil bacteria. Aerobic degradation of lignin-derived methoxylated monoaromatics, including vanillate (4-hydroxy-3-methoxybenzoate) and syringate (3,5-dimethoxy-4-hydroxybenzoate), has been thoroughly studied. For example, an aerobic vanillate- and syringate-degrading bacterium Sphingobium sp. SYK-6 firstly convert vanillate and syringate into protocatechuic acid (PCA, 3,4-dihydroxybenzoate) and 3-O-methylgallate (3-MGA), respectively, by O-demethylation reactions. PCA is oxidatively decomposed to pyruvate and oxaloacetate via the PCA 4,5-cleavage pathway. 3-MGA is degraded through multiple ring cleavage pathways and the resultant metabolites are further degraded via the PCA 4,5-cleavage pathway.

On the contrary, studies on anaerobic degradation of lignin and lignin-derived aromatics have been limited, despite some studies suggesting that their anaerobic biodegradation was evident in various natural environments. Acetogenic bacteria are the first anaerobes described to utilize methoxylated aromatics as the sole carbon and energy sources. Acetogenic bacteria utilize the O-methyl group as the carbon and energy sources, while they do not have the ability to degrade the aromatic ring structure. Similarly, some sulfate-reducing bacteria and fermentative bacteria utilize the O-methyl group as carbon and energy sources, and a part of them also have ability to decompose the aromatic rings. Anaerobic degradation of the O-demethylated derivatives of vanillate and syringate, namely PCA and gallate (3,4,5-trihydroxybenzoate), respectively, has been documented with nitrate-reducing, sulfate-reducing, and fermentative bacteria. A nitrate-reducer Thauera aromatica and a sulfate-reducer Desulfovibrio sp. were reported to anaerobically degrade PCA via the benzoyl-CoA pathway. Fermentative bacteria, such as Eubacterium oxidoreducens and Pelobacter acidigallici, were reported to anaerobically degrade gallate via the phloroglucinol pathway.

While methanogenesis is one of the most important microbial metabolisms in diverse anaerobic environments, including soil and freshwater/marine sediments, knowledge on methanogenic degradation of lignin-derived aromatics, especially on microorganisms responsible for the decomposition, has been quite limited. Kaiser and Hanselmann reported that microbial communities enriched from anaerobic sediments completely degraded lignin-derived aromatics, including vanillate and syringate, with concomitant generation of CH4. They demonstrated that the first step for methanogenic degradation is acetate production with O-demethylation of the methoxy-group. However, microorganisms participating in the O-demethylation and following ring-cleavage reactions under methanogenic environments were not identified.

In the present study, we enriched methanogenic microbial communities from rice paddy field soil with either lignin-derived aromatics (vanillate and syringate) or a model aromatic compound (benzoate) as the sole carbon and energy sources. The microbial communities were further enriched on the degradation intermediate compounds, namely PCA, catechol, and gallate. Microorganisms involved in methanogenic degradation of the lignin-derived aromatics were then identified with microbial community analysis based on their 16S rRNA gene sequences.

Results and Discussion

Enrichment of methanogenic microbial communities on lignin-derived aromatics. Methanogenic microbial communities were enriched from rice paddy field soil using a freshwater basal medium supplemented with different aromatic compounds. Either lignin-derived methoxylated aromatics (5 mM of vanillate or syringate) or a control aromatic compound (5 mM of benzoate) were supplemented as the substrates. After several weeks of cultivation, CH4 was produced in all of the cultures supplemented with aromatics (Fig. S1). The amount of CH4 produced reached to 7 to 20 mmol l−1 within 2 month cultivation. On the contrary, only trace amount of CH4 (less than 0.1 mmol l−1) was produced in the cultures without supplementation of the aromatics (Fig. S1), indicating that most of CH4 produced in the enrichment cultures was derived from degradation of aromatic compounds. Production of CH4 from the aromatic compounds by respective enrichments (after five successive subcultures) are shown in Fig. 1A–C. It took more than one month to produce stoichiometrically expected CH4 in all cultures tested. The amounts of CH4 produced from 5 mM of benzoate, vanillate, and syringate were 17.1 ± 0.5, 18.4 ± 0.3, and 20.1 ± 0.3 mmol l−1, respectively, and were approximate to the theoretical values (18.75, 20, and 22.5 mmol l−1, respectively) calculated from the following equations:

\[
C_7H_5O_2^- \text{(benzoate)} + 7.75H_2O \rightarrow 3.75CH_4 + 3.25HCO_3^- + 2.25H_2^+,
\]

\[
C_8H_7O_4^- \text{(vanillate)} + 8H_2O \rightarrow 4CH_4 + 4HCO_3^- + 3H_2^+,
\]

\[
C_9H_8O_5^- \text{(syringate)} + 8.5H_2O \rightarrow 4.5CH_4 + 4.5HCO_3^- + 3.5H_2^+.
\]

These results suggest that the each enrichment culture completely degraded the aromatic compounds into CH4 and CO2.

Detection of degradation intermediates from the enrichment cultures. Among short chain alcohols and organic acids, only acetate was detected from the enrichment cultures throughout this
study by the high performance liquid chromatography (HPLC) analysis. Acetate accumulated in the enrichment cultures to 8 to 12 mM during methanogenesis, followed by the complete consumption of the accumulated acetate (Fig. 1A–C), indicating that acetate is one of the important intermediates of methanogenic degradation of the aromatic compounds.

In order to determine the degradation pathway of vanillate and syringate in the enrichment cultures, intermediate metabolites produced during cultivation were analyzed by HPLC with detection at 220 nm and 270 nm, respectively. The retention times of compound I, II, III, IV, and V were 1.43, 2.40, 3.34, 1.53, and 0.98 min, respectively. UV/VIS and mass spectra of compound I to V are shown in Figs S2 and S3.
3-hydroxybenzoate (3-HB), respectively (Figs S2 and S3). Generation of PCA at day 10 suggests that O-demethylation of vanillate is the first step of vanillate degradation. Catechol and 3-HB were appeared to be produced by the decarboxylation and dehydroxylation of PCA, respectively, probably as intermediates of methanogenic degradation pathway of PCA (discussed below). In the degradation of syringate, two metabolites, compound IV and V, were found and identified as 3-MGA and gallate, respectively (Fig. 2B, S2, and S3). These observations indicate that syringate is first converted to gallate with successive O-demethylation reactions via 3-MGA. In the benzoate enrichment cultures, no aromatic compound other than benzoate were detected (data not shown), suggesting that benzoate is firstly converted to benzy0l-CoA and then the ring structure is reductively cleaved via benzy0l-CoA pathway as reported25,26.

**Enrichment cultures on the degradation intermediates.** In order to further elucidate the mechanisms for degradation of the lignin-derived aromatics, the enrichment cultures on vanillate and syringate were subjected to further enrichments with respective degradation intermediates. Enrichment cultures on PCA and gallate were constructed with vanillate- and syringate-enrichments as the inoculum, respectively, and generated considerable amounts of CH4 within 40 days (Fig. 3A). Although enrichment cultures on catechol was also constructed using the vanillate-enrichments as the inoculum, the cultures supplemented with 5 mM catechol produced only little amounts of CH4 with a long lag time (>50 days, data not shown). Since cytotoxicity of relatively high concentration of catechol was considered as the reason for the low methanogenic activity, another set of enrichment cultures was constructed with

![Figure 3. Methanogenesis from PCA, gallate, and catechol by respective enrichment cultures.](image-url)
supplementation of 1 mM catechol. In this case, methanogenesis started with approximately a 20-day lag phase and plateaued at around 40 days of cultivation (Fig. 3B). After saturation of methanogenesis, another 1 mM of catechol was added to the culture, which resulted in resumption of methanogenesis with almost no lag time.

The amount of CH4 produced from PCA (5 mM), gallate (5 mM), and catechol (1 mM) were 15.5 ± 0.5, 14.4 ± 0.5, and 2.8 ± 0.3 mmol l−1, respectively, and were approximate to the theoretical values (16.25, 15, and 3.25 mmol l−1, respectively) calculated from the following equations:

\[
\text{C}_6\text{H}_5\text{O}_4^- \text{ (PCA)} + 7.25\text{H}_2\text{O} \rightarrow 3.25\text{CH}_4 + 3.75\text{HCO}_3^- + 2.75\text{H}^+;
\]

\[
\text{C}_7\text{H}_8\text{O}_6^- \text{ (gallate)} + 7\text{H}_2\text{O} \rightarrow 3\text{CH}_4 + 4\text{HCO}_3^- + 3\text{H}^+;
\]

\[
\text{C}_6\text{H}_4\text{O}_2\text{(catechol)} + 6.25\text{H}_2\text{O} \rightarrow 3.25\text{CH}_4 + 2.75\text{HCO}_3^- + 2.75\text{H}^+.
\]

These results suggest that methanogenic microbial communities that completely degrade the respective intermediates of vanillate and syringate were successfully enriched.

**Dominant microorganisms in the enrichment cultures.** Microbial community structures of the enrichments were assessed based on 16S rRNA gene clone library analysis to elucidate the microorganisms participating in methanogenic degradation of the lignin-derived aromatics. The summarized features of the bacterial and archaeal community analyses are presented in Fig. 4. All phylotypes detected from each enrichment culture are listed in Tables S1 and S2.

Only 4 archaeal phylotypes were recovered from the enrichment cultures (Fig. 4A and Table S1). The phylotype ArA-01 (98.9% identity to *Methanosarcina vacuolata*), which dominated in all enrichment cultures, was the only phylotype classified as an aceticlastic methanogen. The phylotypes classified as hydrogenotrophic methanogens, namely the phylotype ArA-02 (97.9% identity to *Methanoculleus palmolet*) and ArA-03 (97.5% identity to *Methanocella arvoryzae*), were also detected from all enrichment cultures. These results suggest that both aceticlastic and hydrogenotrophic methanogenesis contribute to methanogenic degradation of the lignin-derived aromatics.

The predominant bacterial phylotype in the benzoate-enrichments was the phylotype ArB-25 (Fig. 4B and Table S2), which is classified into the class *Deltaproteobacteria* and has 99.0% identity to *Syntrophus aciditrophicus*. *S. aciditrophicus* has ability to degrade benzoate via benzoyl-CoA pathway in syntrophic association with methanogens. In the benzoate-enrichment cultures, *Syntrophus* sp. appears to...
decompose benzoate into acetate and $\text{H}_2/\text{CO}_2$, which is further converted into $\text{CH}_4$ by aceticlastic and hydrogenotrophic methanogens.

By contrast, almost no *Deltaproteobacteria* were recovered from the enrichment cultures with other aromatic compounds. Instead, the phylotypes classified into phylum *Firmicutes* dominated in the enrichments supplemented with the lignin-derived aromatics and their degradation intermediates (Fig. 4B). Among the *Firmicutes* phylotypes, the phylotype ArB-01 (99.6% identity to *Sporomusa sphaeroides*) and ArB-02 (89.8% identity to *Sporomusa paucivorans*) were abundantly detected from vanillate- and syringate-enrichments, but not from enrichments with their degradation intermediates. *Sporomusa* spp. were reported to have the ability to acetogenetically grow on diverse array of methoxylated aromatic compounds, such as vanillate, ferulate, and 3,4-dimethoxybenzoate, via respective $\text{O}$-demethylation reactions$^{29,30}$. These findings suggested that *Sporomusa* spp. contribute to the initial step of anaerobic degradation of the lignin-derived aromatics in the enrichments, namely the conversion of vanillate and syringate into PCA and gallate, respectively.

In the enrichments on vanillate and its degradation intermediates (PCA and catechol), the phylotypes classified into family *Peptococcaceae* were dominant, while the dominant phylotypes varied among the supplemented aromatic compounds (Fig. 4B and Table S2). The phylogenetic tree based on 16S rRNA gene sequences of representative *Peptococcaceae* isolates and the *Peptococcaceae* phylotypes detected in this study is shown in Fig. 5A. The dominant phylotypes ArB-04 (96.8% identity to *Pelotomaculum schinkii*) and ArB-06 (98.4% identity to *Cryptanaerobacter phenolicus*) were clustered into the *Desulfotomaculum* subcluster Ih$^{31}$. Microorganisms in the *Desulfotomaculum* subcluster Ih were characterized by their ability to anaerobically metabolize various organic substrates in syntrophic association with hydrogenotrophic methanogens$^{32}$. Furthermore, *Desulfotomaculum* subcluster Ih includes some strains that syntrophically degrade a range of aromatic compounds$^{33,34}$ and was frequently detected as the dominant
bacteria in microbial communities that anaerobically degrade diverse aromatic compounds\textsuperscript{35–38}. The other dominant phylotype ArB-05 (97.9% identity to Desulfosporosinus auripigmenti) was separately clustered from ArB-04 and -06 and affiliated with genus Desulfosporosinus (Fig. 5A). Desulfosporosinus spp. were generally characterized as sulfate-reducing bacteria and its syntrophic metabolism with methanogens has not been tested\textsuperscript{39}. However, all crucial domains for syntrophic metabolisms were found in the genome of Desulfosporosinus meridiei, suggesting their possible ability to grow in syntrophic association with methanogens\textsuperscript{40}. While there have been no reports on degradation of aromatic compounds by Desulfosporosinus spp., sequences related to Desulfosporosinus were frequently recovered from various aromatics-degrading microbial communities\textsuperscript{41–43}. Taken together, it is very likely that the Peptococcaceae phylotypes mainly contributed to the ring-cleavage of the vanillate and/or its degradation intermediates via syntrophic interaction with hydrogenotrophic methanogens.

The phylotypes classified into family Ruminococcaceae were dominant in the enrichments with syringate and gallate (Fig. 4B and Table S2). The phylogenetic tree for family Ruminococcaceae is shown in Fig. 5B. One of the phylotypes, ArB-08, was not clustered with known Ruminococcaceae isolates and has only quite low 16S rRNA gene sequence identity to isolated microorganisms (92.0% identity to Acetivibrio sp. 6–13). The other dominant phylotype ArB-09 (97.1% identity to Intestinimonas butyriciproducens) was placed within the “Clostridium cluster IV”\textsuperscript{44,45}. Although the closest relative of ArB-09 (I. butyriciproducens) has not been reported to degrade aromatic compounds\textsuperscript{46}, some strains in the Clostridium cluster IV were reported to fermentatively decompose diverse aromatic compounds. For example, Flavonifractor plautii (formerly Clostridium orbiscindens) has the ability to degrade flavonoids via phloroglucinol (1,3,5-trihydroxybenzene) as the intermediate\textsuperscript{47}. Most notably, Sporobacter termitis grows exclusively on a limited range of methoxylated aromatic compounds, including syringate and vanillate, through their O-demethylation and ring cleavage\textsuperscript{47}. These reports support the assumption that the Ruminococcaceae phylotypes dominant in the syringate- and gallate-enrichments mainly contributed to their ring cleavage.

**Proposed model for methanogenic degradation of vanillate and syringate.** The proposed models for methanogenic degradation of the lignin-derived aromatics are shown in Fig. 6. Analysis on degradation intermediates and microbial communities in the enrichments suggested that vanillate is firstly converted into PCA by Sporomusa spp. with acetate generation via O-demethylation reactions (Fig. 6A). Peptococcaceae bacteria (Desulfotomaculum subcluster Ih and/or Desulfosporosinus spp.) are the plausible candidates for decomposing PCA into acetate and H$_2$/CO$_2$, which are the substrates for methanogenesis by acetoclastic methanogens (Methanosarcina spp.) and hydrogenotrophic methanogens (Methanoculleus and Methanocella spp.), respectively. Anaerobic degradation of PCA has been investigated for some
nitrate- and sulfate-reducing bacteria\textsuperscript{20,21}, by which PCA is firstly activated to protocatechuyl-CoA and reductively dehydroxylated to 3-HB-CoA, followed by ring cleavage via the (3-hydroxy)benzoyl-CoA pathway. However, we could not confirm the existence of this degradation pathway in the enrichment cultures, because CoA-derivatives of aromatic compounds could not be detected by our analytical protocol. Instead, catechol and 3-HB were detected as the possible intermediate compounds through degradation of PCA (Fig. 2A). Although there have been no reports on microbial enzymes that dehydroxylate PCA into 3-HB under anoxic conditions, it might be possible that certain bacteria utilize such reaction followed by CoA activation and ring cleavage via the (3-hydroxy)benzoyl-CoA pathway. While enzymatic activities to generate catechol by decarboxylation of PCA were reported for some anaerobic microorganisms\textsuperscript{48,49}, further catechol degradation routes have not been identified. Some nitrate- and sulfate-reducing bacteria were reported to decompose catechol\textsuperscript{20,21}. These bacteria initially convert catechol into PCA by carboxylation, followed by activation with CoA-ligation, dehydroxylation and reductive ring cleavage via the (3-hydroxy)benzoyl-CoA pathway. Although there are some reports on anaerobic degradation of catechol under methanogenic conditions\textsuperscript{51–53}, the responsible microorganisms and the degradation pathway were not identified.

As in the case of vanillate, the first step of syringate degradation appears to be acetogenic O-demethylation catalyzed by Sporomusa spp. (Fig. 6B). The demethoxylated product, gallate, seems to be further decomposed into acetate and H\textsubscript{2}/CO\textsubscript{2} mainly by Ruminococcaceae bacteria. Anaerobic degradation of gallate were reported for some fermenting bacteria, including E. oxidoreducens, which is classified into Firmicutes and distantly related to the Ruminococcaceae phylotypes detected in this study\textsuperscript{22,23}. E. oxidoreducens initially converts gallate into trihydroxybenzene by decarboxylation reaction, followed by ring cleavage via the phloroglucinol pathway\textsuperscript{22}. Although the decarboxylated products of gallate were not detected in this study, it is highly possible that the trihydroxybenzene degradation is much faster than its generation.

**Concluding remarks.** In this study, we successfully enriched methanogenic microbial communities that decompose the lignin-derived monoaromatics, namely syringate and vanillate. This study is the first to demonstrate that cooperation of microorganisms with a diverse range of trophic groups are required for methanogenic degradation of the lignin-derived aromatics. The initial step appears to be catalyzed by Sporomusa spp. that generate acetate via O-demethylation of the methoxylated aromatics. The resultant demethoxylated aromatics were decomposed into acetate and H\textsubscript{2}/CO\textsubscript{2} by Firmicutes bacteria, while the bacterial groups responsible for PCA (family Peptococcaceae) and gallate (family Ruminococcaceae) were not identical. Finally, both aceticlastic and hydrogenotrophic methanogens generate methane from acetate and H\textsubscript{2}/CO\textsubscript{2}, respectively. Although the details in the pathway of aromatic ring cleavage have not been characterized, isolation of aromatics-degrading Peptococcaceae and Ruminococcaceae strains identified in this study followed by genomic and enzymatic studies will shed light on novel aspects of methanogenic degradation of lignin-derived aromatics in anaerobic environments.

**Methods**

**Enrichment cultures of methanogenic microbial communities.** Methanogenic microbial communities were enriched in serum bottles (68 ml in capacity) filled with 20 ml of a bicarbonate- and HEPES-buffered freshwater basal medium (pH 7.0)\textsuperscript{54} supplemented with aromatic compounds (benzoate, vanillate, or syringate) as a substrate. The cultures without supplementation of organic substrates were also prepared as a no-amendment control. Approximately 50 mg (wet weight) of rice paddy field soil was inoculated as a microbial source. The cultures were incubated at 30°C under an atmosphere of N\textsubscript{2}/CO\textsubscript{2} [80:20 (v/v)] without shaking. When methanogenesis reached a plateau, 0.5 ml of culture solution was transferred to the fresh media. Enrichment cultures on PCA/catechol and gallate were constructed in the same medium with the vanillate- and syringate-enrichments as the inoculum, respectively. After more than three passages, the enrichment cultures were subjected to chemical and phylogenetic analyses. All culture experiments were conducted in triplicate.

**Chemical analyses.** The partial pressure of CH\textsubscript{4} was determined using a gas chromatograph (GC-2014, Shimadzu) as described previously\textsuperscript{55}. The concentrations of organic acids were determined using a HPLC (D-2000 LaChrom Elite HPLC system, HITACHI) as described previously\textsuperscript{56}. To identify metabolic intermediates, the authentic compounds and supernatant of enrichment cultures were analyzed by HPLC (ACQUITY UPLC system, Waters) coupled with ESI-MS (ACQUITY TQ detector, Waters) using a TSKgel ODS-140HTP column (2.1 by 100 mm, Tosoh) as described previously\textsuperscript{57}. In the HPLC analysis, the mobile phase was a 90:10 (v/v) water:acetonitrile at a flow rate of 0.3 ml/min. Formic acid (0.1%) was added to the mobile phase solvent as a means of increasing ionization efficiency for ESI-MS. Degradation of PCA into 3-HB under anoxic conditions, it might be possible that certain bacteria utilize such reaction followed by CoA activation and enzyme cleavage via the (3-hydroxy)benzoyl-CoA pathway. While enzymatic activities to generate catechol by decarboxylation of PCA were reported for some anaerobic microorganisms\textsuperscript{48,49}, further catechol degradation routes have not been identified. Some nitrate- and sulfate-reducing bacteria were reported to decompose catechol\textsuperscript{20,21}. These bacteria initially convert catechol into PCA by carboxylation, followed by activation with CoA-ligation, dehydroxylation and reductive ring cleavage via the (3-hydroxy)benzoyl-CoA pathway. Although there are some reports on anaerobic degradation of catechol under methanogenic conditions\textsuperscript{51–53}, the responsible microorganisms and the degradation pathway were not identified.

As in the case of vanillate, the first step of syringate degradation appears to be acetogenic O-demethylation catalyzed by Sporomusa spp. (Fig. 6B). The demethoxylated product, gallate, seems to be further decomposed into acetate and H\textsubscript{2}/CO\textsubscript{2} mainly by Ruminococcaceae bacteria. Anaerobic degradation of gallate were reported for some fermenting bacteria, including E. oxidoreducens, which is classified into Firmicutes and distantly related to the Ruminococcaceae phylotypes detected in this study\textsuperscript{22,23}. E. oxidoreducens initially converts gallate into trihydroxybenzene by decarboxylation reaction, followed by ring cleavage via the phloroglucinol pathway\textsuperscript{22}. Although the decarboxylated products of gallate were not detected in this study, it is highly possible that the trihydroxybenzene degradation is much faster than its generation.

**Concluding remarks.** In this study, we successfully enriched methanogenic microbial communities that decompose the lignin-derived monoaromatics, namely syringate and vanillate. This study is the first to demonstrate that cooperation of microorganisms with a diverse range of trophic groups are required for methanogenic degradation of the lignin-derived aromatics. The initial step appears to be catalyzed by Sporomusa spp. that generate acetate via O-demethylation of the methoxylated aromatics. The resultant demethoxylated aromatics were decomposed into acetate and H\textsubscript{2}/CO\textsubscript{2} by Firmicutes bacteria, while the bacterial groups responsible for PCA (family Peptococcaceae) and gallate (family Ruminococcaceae) were not identical. Finally, both aceticlastic and hydrogenotrophic methanogens generate methane from acetate and H\textsubscript{2}/CO\textsubscript{2}, respectively. Although the details in the pathway of aromatic ring cleavage have not been characterized, isolation of aromatics-degrading Peptococcaceae and Ruminococcaceae strains identified in this study followed by genomic and enzymatic studies will shed light on novel aspects of methanogenic degradation of lignin-derived aromatics in anaerobic environments.

**Methods**

**Enrichment cultures of methanogenic microbial communities.** Methanogenic microbial communities were enriched in serum bottles (68 ml in capacity) filled with 20 ml of a bicarbonate- and HEPES-buffered freshwater basal medium (pH 7.0)\textsuperscript{54} supplemented with aromatic compounds (benzoate, vanillate, or syringate) as a substrate. The cultures without supplementation of organic substrates were also prepared as a no-amendment control. Approximately 50 mg (wet weight) of rice paddy field soil was inoculated as a microbial source. The cultures were incubated at 30°C under an atmosphere of N\textsubscript{2}/CO\textsubscript{2} [80:20 (v/v)] without shaking. When methanogenesis reached a plateau, 0.5 ml of culture solution was transferred to the fresh media. Enrichment cultures on PCA/catechol and gallate were constructed in the same medium with the vanillate- and syringate-enrichments as the inoculum, respectively. After more than three passages, the enrichment cultures were subjected to chemical and phylogenetic analyses. All culture experiments were conducted in triplicate.

**Chemical analyses.** The partial pressure of CH\textsubscript{4} was determined using a gas chromatograph (GC-2014, Shimadzu) as described previously\textsuperscript{55}. The concentrations of organic acids were determined using a HPLC (D-2000 LaChrom Elite HPLC system, HITACHI) as described previously\textsuperscript{56}. To identify metabolic intermediates, the authentic compounds and supernatant of enrichment cultures were analyzed by HPLC (ACQUITY UPLC system, Waters) coupled with ESI-MS (ACQUITY TQ detector, Waters) using a TSKgel ODS-140HTP column (2.1 by 100 mm, Tosoh) as described previously\textsuperscript{57}. In the HPLC analysis, the mobile phase was a 90:10 (v/v) water:acetonitrile at a flow rate of 0.3 ml/min. Formic acid (0.1%) was added to the mobile phase solvent as a means of increasing ionization efficiency for ESI-MS. Degradation products of vanillate and syringate were detected at the wavelength of 220 nm and 270 nm, respectively. Wavelength for the detection and the retention times of authentic compounds were described in Figs S2 and S3. In the ESI-MS analysis, mass spectra were obtained by negative and positive modes with the following settings: capillary voltage, 3.0 kv; cone voltage, 10 to 40 V; source temperature, 120°C; desolvation temperature, 350°C; desolvation gas flow rate, 650 liter h\textsuperscript{-1}; and cone gas flow rate, 50 liter h\textsuperscript{-1}.
Phylogenetic analyses. Microbial DNA was extracted with the FAST DNA Spin Kit for soil (MP Biomedicals) according to the manufacturer’s instructions. PCR amplification of 16S rRNA gene fragments for clone library analyses was conducted as described previously60, with primer sets of U515f and U1492r for bacteria and A25f and A958r for archaea60. The sequences obtained were assigned to each phylotype using BLASTClust program61 with a cut-off value of 97% sequence identity. Classification of phylotypes was performed using the Classifier program in the Ribosomal Database Project database62. The sequence of each phylotype was compared to those stored in the GenBank nucleotide sequence database using the BLAST program63 to infer the closest relatives. Phylogenetic trees were constructed by the neighbor-joining method64 using program MEGA65. The bootstrap resampling method66 was used with 1000 replicates to evaluate the robustness of the phylogenetic trees.

Nucleotide sequence accession numbers. The nucleotide sequence data reported here have been submitted to GenBank under Accession No. LC036665–LC036702.

References
1. Zimmermann, W. Degradation of lignin by bacteria. J. Bacteriol. 13, 119–130 (1990).
2. Martínez, A. T. et al. Biodegradation of lignocellulosic: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. Int. Microbiol. 8, 195–204 (2005).
3. Bugg, T. D., Ahmad, M., Hardiman, E. M. & Rahmanpour, R. Pathways for degradation of lignin in bacteria and fungi. Nat. Prod. Rep. 28, 1883–1896 (2011).
4. Masai, E., Katayama, Y. & Fukuda, M. Genetic and biochemical investigations on bacterial catabolic pathways for lignin-derived aromatic compounds. Biosci. Biotechnol. Biochem. 71, 1–15 (2007).
5. Abe, T., Masai, E., Miyauchi, K., Katayama, Y. & Fukuda, M. A tetrahydrofolate-dependent O-demethylase, LigM, is crucial for catalysis of vanillate and syringate in Sphingomonas paucimobilis SYK-6. J. Bacteriol. 187, 2030–2037 (2005).
6. Kasai, D., Masai, E., Katayama, Y. & Fukuda, M. Degradation of 3-O-methylgalate in Sphingomonas paucimobilis SYK-6 by pathways involving protocatechuate 4,5-dioxygenase. FEMS Microbiol. Lett. 274, 323–328 (2007).
7. Kamimura, N. & Masai, E. The protocatechuate 4,5-cleavage pathway: Overview and new findings. In Biodegradative Bacteria. Nojiri, H., Tsuda, M., Fukuda, M. & Kamagata, Y. (eds). Springer Japan, pp. 207–226 (2014).
8. Hackett, W. F., Connors, W. J., Kirk, T. K. & Zeikus, J. G. Microbial decomposition of synthetic 14C-labeled lignins in nature: lignin biodegradation in a variety of natural materials. Appl. Environ. Microbiol. 33, 43–51 (1977).
9. Benner, R., Maccubbin, A. E. & Hodson, R. E. Anaerobic biodegradation of the lignin and polysaccharide components of lignocellulosic and synthetic lignin by sediment microflora. Appl. Environ. Microbiol. 47, 998–1004 (1984).
10. Young, L. Y. & Frazer, A. C. The fate of lignin and lignin-derived compounds in anaerobic environments. Geomicrobiol. J. 5, 261–293 (1987).
11. Cookson, L. J. The site and mechanism of 14C-lignin degradation by Nasatiternnes exquisitus. J. Insect. Physiol. 34, 409–414 (1988).
12. Dittmar, T. & Lara, R. Molecular evidence for lignin degradation in sulfate-reducing mangrove sediments (Amazonia, Brazil). Geochim. Cosmochim. Acta 65, 1417–1428 (2001).
13. Ko, J. I. et al. Biodegradation of high molecular weight lignin under sulfate reducing conditions: lignin degradability and degradation by-products. Bioresource Technol. 100, 1622–1627 (2009).
14. Bache, R. & Pfennig, N. Selective isolation of Acetobacterium woodii on methoxylated aromatic acids and determination of growth yields. Arch. Microbiol. 130, 255–261 (1981).
15. Daniel, S. L., Keith, E. S., Yang, H., Lin, Y. S. & Drake, H. L. Utilization of methoxylated aromatic compounds by the aceto- gen Clostridium thermoaceticum: expression and specificity of the co-dependent O-demethylating activity. Biochem. Biophys. Res. Commun. 180, 416–422 (1991).
16. Tasaki, M., Kamagata, Y., Nakamura, K. & Mikami, E. Utilization of methoxylated benzoates and formation of intermediates by Desulfotomaculum thermoceticum in the presence or absence of sulfate. Arch. Microbiol. 157, 209–212 (1992).
17. Grech-Mora, I. et al. Isolation and characterization of Sporobacter termiditis gen. nov., sp. nov., from the digestive tract of the wood-feeding termite Nasatiternnes lajue. Int. J. Syst. Bacteriol. 46, 512–518 (1996).
18. Mechichi, T., Labat, M., Garcia, J., Thomas, P. & Pate, B. K. C. Sporobacter oleaearum gen. nov., a new methanethiol-producing bacterium that degrades aromatic compounds, isolated from an olive mill wastewater treatment digester. Int. J. Syst. Bacteriol. 49, 1741–1748 (1999).
19. Neumann, A. et al. Phenyl methyl ethers: novel electron donors for respiratory growth of Desulfibacterium hafniense and Desulfobacterium sp. strain PCE-S. Arch. Microbiol. 181, 245–249 (2004).
20. Gorny, N. & Schink, B. Anaerobic degradation of catechol by Desulfo bacterium sp. strain Cat2 proceeds via carboxylation to protocatechuate. Appl. Environ. Microbiol. 60, 3396–3400 (1994).
21. Philipp, B. et al. Anaerobic degradation of protocatechuate (3,4-dihydroxybenzoate) by Thauera aromatica strain AR-1. FEMS Microbiol. Lett. 212, 139–143 (2002).
22. Krumholz, L. R. & Bryant, M. P. Characterization of the pyrogallol-phenolglucin isolomer of Eubacterium oxidireducens. J. Bacteriol. 170, 2472–2479 (1988).
23. Brune, A. & Schink, B. Phenolglucin pathway in the strictly anaerobic Pelobacter acidigallici: fermentation of trihydroxybenzenes to acetate via tricarboxylic acid. Arch. Microbiol. 157, 417–424 (1992).
24. Kaiser, J. & Hanselmann, K. Fermentative metabolism of substituted monooaromatic compounds by a bacterial community from anaerobic sediments. Arch. Microbiol. 133, 185–194 (1982).
25. Peters, F., Shimoda, Y., McInerney, M. J. & Boll, M. Cyclolacta-1,5-diene-1-carbonyl-coenzyme A (CoA) hydratases of Geobacter metallireducens and Syntrophus aciditrophicus: Evidence for a common benzoyl-CoA degradation pathway in facultative and strict anaerobes. J. Bacteriol. 189, 1055–1060 (2007).
26. Fuchs, G., Boll, M. & Heider, J. Microbial degradation of aromatic compounds - from one strategy to four. Nat. Rev. Microbiol. 9, 803–816 (2011).
27. Jackson, B. & Bhupathiraju, V. Syntrophus aciditrophicus sp. nov., a new anaerobic bacterium that degrades fatty acids and benzoate in syntrophic association with hydrogen-using microorganisms. Arch. Microbiol. 171, 107–114 (1999).
28. McInerney, M. J. et al. The genome of Syntrophus aciditrophicus: life at the thermodynamic limit of microbial growth. Proc. Natl. Acad. Sci. USA 104, 7600–7605 (2007).
29. Stupperich, E. & Konle, R. Corrinoid-dependent methyl transfer reactions are involved in methylol-3,4-dimethoxybenzoate metabolism by Sporomusa ovata. Appl. Environ. Microbiol. 59, 3110–3116 (1993).
30. Kuhner, C. H. et al. Sporomusa silvicetica sp. nov., an acetoogenic bacterium isolated from aggregated forest soil. Int. J. Syst. Bacteriol. 47, 352–358 (1997).
Acknowledgements

We thank Dr. Mía Terashima for critical reading of the manuscript and Hiromi Ikebuchi for technical assistance. This work was supported by grants from the Japan Society for the Promotion of Science (JSPS).

31. Stackebrandt, E. et al. Phylogenetic analysis of the genus Desulfofaculomaculum: evidence for the misclassification of Desulfofaculomaculum guttoides and description of Desulfofaculomaculum orientis as Desulfofaculomaculum orientis gen. nov., comb. nov. Int. J. Syst. Bacteriol. 47, 1134–1139 (1997).

32. Imachi, H., Sekiguchi, Y. & Kamagata, Y. Non-sulfate-reducing, syntrophic bacteria affiliated with Desulfofaculomaculum cluster I are widely distributed in methanogenic environments. Appl. Environ. Microbiol. 72, 2080–2091 (2006).

33. Juteau, P. et al. Cryptanaerobacter phenolicus gen. nov., sp. nov., an anaerobe that transforms phenol into benzoate via 4-hydroxybenzoate. Int. J. Syst. Evol. Microbiol. 55, 245–250 (2005).

34. Qi, Y.L. et al. Pelotomaculum terophilicum sp. nov. and Pelotomaculum isophilicum sp. nov.: two anaerobic bacteria that degrade phthalate isomers in syntrophic association with hydrogenotrophic methanogens. Arch. Microbiol. 185, 172–182 (2006).

35. Chen, C. L., Wu, J. H. & Liu, W. T. Identification of important microbial populations in the mesophilic and thermophilic phenol-degrading methanogenic consortia. Water Res. 42, 1963–1976 (2008).

36. Kleinsteuber, S. et al. Characterization of bacterial communities mineralizing benzene under sulfate-reducing conditions. FEMS Microbiol. Ecol. 66, 143–157 (2008).

37. Winderl, C., Penning, H., Netzer, F., Meckenstock, R. U. & Lueders, T. DNA-SIP identifies sulfate-reducing Clostridia as important tolucne degraders in tar-oil-contaminated aquifer sediment. ISME J. 4, 1314–1325 (2010).

38. Perkins, S. D., Scalfone, N. B. & Angenent, L. T. Comparative 16S rRNA gene surveys of granular sludge from three upflow anaerobic bioreactors treating purified teraphthalic acid (PTA) wastewater. Water Sci. Technol. 64, 1406–1412 (2011).

39. Robertson, W. J., Bowman, J. P., Franzmann, P. D. & Mee, B. J. Desulfosporosorus meridiei sp. nov., a spore-forming sulfate-reducing bacterium isolated from gasoline-contaminated groundwater. Int. J. Syst. Evol. Microbiol. 51, 133–140 (2001).

40. Worm, P. et al. A genomic view on syntrophic versus non-syntrophic lifestyle in anaerobic fatty acid degrading communities. Biochim. Biophys. Acta 1837, 2004–2016 (2014).

41. Sun, W. & Cupples, A. M. Diversity of five anaerobic tolucne-degrading microbial communities investigated using stable isotope probing. Appl. Environ. Microbiol. 78, 972–980 (2012).

42. Kuppardt, A. et al. Phylogenetic and functional diversity within tolucne-degrading, sulphate-reducing consortia enriched from a contaminated aquifer. Microb. Ecol. 68, 222–234 (2014).

43. Fowler, S. J., Gutierrez-Zamora, M. L., Manefield, M. & Gieg, L. M. Identification of toluene degraders in a methanogenic environment. FEMS Microbiol. Ecol. 89, 625–636 (2014).

44. Collins, M. D. et al. The phylogeny of the genus Clostridium: proposal of five new genera and eleven new species combinations. Int. J. Syst. Bacteriol. 44, 812–826 (1994).

45. Carlier, J. P., Bedora-Faure, M., K'ouas, G., Alauzet, C. & Mory, F. Proposal to unify Clostridium and reassignment of Bacteroides capillosus to Pseudoflavinfractor capillosus gen. nov., comb. nov. Int. J. Syst. Evol. Microbiol. 60, 585–590 (2010).

46. Klärning, K. et al. Intestinimonas butyriciproducens gen. nov., sp. nov., a butyrate-producing bacterium from the mouse intestine. Int. J. Syst. Evol. Microbiol. 63, 4606–4612 (2013).

47. Schoefer, I., Mohan, R., Schwieritz, L., Braune, A. & Blaut, M. Anaerobic degradation of flavonoids by Clostridium orbiscindens. Appl. Environ. Microbiol. 69, 5849–5854 (2003).

48. He, Z. & Wiegel, J. Purification and characterization of an oxygen-sensitive, reversible 3,4-dihydroxybenzoate decarboxylase from Clostridium hydroybenzoicum. J. Bacteriol. 178, 3539–3543 (1996).

49. Ostermann, A., Gallus, C. & Schink, B. Decarboxylation of 2,3-dihydroxybenzoate to catechol supports growth of fermenting bacteria. Syst. Appl. Microbiol. 35, 270–273 (1997).

50. Dvornik, G., Schmeling, S. & Fuchs, G. Anaerobic metabolism of catechol by the denitrifying bacterium Thauera aromatica - a result of promiscuous enzymes and regulators? J. Bacteriol. 190, 1620–1630 (2008).

51. Szewzyk, U., Szewzyk, R. & Schink, B. Methanogenic degradation of hydroquinone and catechol via reductive dehydroxylation to phenol. FEMS Microbiol. Ecol. 31, 79–87 (1985).

52. Subramanyam, R. & Mishra, I. M. Biodegradation of catechol (2-hydroxy phenol) bearing wastewater in an UASB reactor. Chemosphere 69, 816–824 (2007).

53. Kuschik, P. et al. Batch methanogenic fermentation experiments of wastewater from a brown coal low-temperature coke plant. J. Environ. Sci. 22, 192–197 (2010).

54. Kato, S., Yamamoto, I. & Kamagata, Y. Isolation of aceticogenic bacteria that induce biocorrosion by utilizing metallic iron as the sole electron donor. Appl. Environ. Microbiol. 81, 67–73 (2015).

55. Kato, S., Sasaki, K., Watanabe, K., Yumoto, I. & Kamagata, Y. Physiological and transcriptomic analyses of a thermophilic, acetoclastic methanogen Methanoseta thermophila responding to ammonia stress. Microbes Environ. 29, 162–167 (2014).

56. Kato, S. et al. The effects of elevated CO2 concentration on competitive interaction between acetoclastic and syntrophic methanogenesis in a model microbial consortium. Front. Microbiol. 5, 575 (2014).

57. Kamimura, N. et al. Characterization of the protocatechuate 4,5-cleavage pathway operon in Comamonas sp. strain E6 and discovery of a novel pathway gene. Appl. Environ. Microbiol. 76, 8093–8101 (2010).

58. Kato, S., Kai, F., Nakamura, R., Watanabe, K. & Hashimoto, K. Respiratory interactions of soil bacteria with (semi-)conductive iron-oxide minerals. Environ. Microbiol. 12: 3114–3123 (2010).

59. Dojka, M. A., Hugenholtz, P., Haack, S. K. & Pace, N. R. Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. Appl. Environ. Microbiol. 64, 3869–3877 (1998).

60. Altschul, S. F. et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402 (1997).

61. Wang, Q., Garrity, G. M., Tiedje, J. M. & Cole, J. R. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. 73, 5261–5267 (2007).

62. Saitou, N. & Nei, M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425 (1987).

63. Tamura, K., Dudley, J., Nei, M. & Kumar, S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599 (2007).

64. Felsenstein, J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791 (1985).
Author Contributions
S.K. designed the experiments, carried out the culture experiments and microbial community analysis, analyzed the data, and wrote the paper. K.C. carried out the culture experiments and microbial community analysis. N.K. and E.M. carried out the ESI-MS experiments and analyzed the data. I.Y. and Y.K. were involved in the design of the experiments and helped interpret the data. All authors reviewed the paper.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Kato, S. et al. Methanogenic degradation of lignin-derived monoaromatic compounds by microbial enrichments from rice paddy field soil. Sci. Rep. 5, 14295; doi: 10.1038/srep14295 (2015).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/