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Short Communication

Bacterial diversity associated with empty oil palm fruit bunch compost as revealed by cultivation-independent analyses of PCR-amplified 16S rRNA genes

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Currently, Malaysia is producing about 15 million tons of crude palm oil annually. At the same time, this industry generated about 18 million tons of empty fruit bunches (EFB), one of the largest agricultural wastes in the country (Anon, 2008). EFB is the fibrous biomass left behind after the fruits are stripped for palm oil production. Composting EFB is a promising and sustainable alternative to recycle this enormous amount of waste into value-added and manageable products which later can be used as soil amendment or organic fertilizer. Besides, it is an environmentally acceptable solution to the growing amount of EFB. Although composting of EFB has a long history, there is no substantial information on its microbial diversity as compared to other aspects such as its nutritional requirement, kinetics and enumeration of specific microbial groups (Agamuthu et al., 2000; Thambirajah et al., 1995). It is therefore of much interest to identify the microbial community and particularly the microbial populations that dominate in the EFB compost, in this context, originating from the tropical environment of Malaysia.

Conventional culture-dependent methods are known to be able to reveal less than 1% of the bacterial communities from the environmental samples (Amann et al., 1995; Davis et al., 2005). The limitation of these methods has been in recent years replaced with various culture-independent approaches, some of which include direct analysis of phospholipid fatty acid (PLFA) patterns (Kato et al., 2005; Klamer and Baath, 1998), and DNA or RNA markers, such as 16S rDNA (Blanc et al., 1999; Hugenholtz et al., 1998; Ishii et al., 2000; Takaku et al., 2006). Analysis based on 16S rDNA is the most common, popular and convenient approach for bacterial community analysis. In this approach, 16S rRNA genes as the phylogenetic marker are PCR-amplified directly from community bulk DNA, then subjected to subsequent analysis, including amplified ribosomal DNA restriction analysis (ARDRA) (Dees and Ghiorse, 2001; Liew and Jong, 2008; Ntougias et al., 2004), denaturing gradient gel electrophoresis (DGGE) (Ishii et al., 2000; Jong et al., 2006; Narihiro et al., 2004; Pedro et al., 2001; Takaku et al., 2006), and terminal restriction fragment length polymorphism (T-RFLP) (Lazzaro et al., 2008; Liu et al., 1997; Thies,

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In this study, the authors examined the bacterial community structure using the culture-independent approach based on a 16S rDNA library. This study aims to provide new insight into the bacterial community that formed in direct association with decomposition of EFB fibrous residues, thereby generating useful information that might assist in future studies and further enhancement of EFB degradation. Furthermore, the results obtained may be useful in searching for novel microorganisms in the novel EFB compost environment.

The EFB composting experiment was conducted at a composting facility located in Serdang, Selangor, Malaysia. The compost heap was built at an outdoor open landsoil area using 1.5 tons of raw OPEFB from a local palm oil mill with the dimensions 1 m × 1 m × 1 m (length × width × height). Triplicate samples of mature compost, each 50 g, were aseptically collected into sterile sampling bags and were processed immediately. Bulk DNA was extracted from 1 g of each compost sample using the PowerSoil™ DNA isolation kit (MO BIO Laboratory, Carlsbad, USA) according to the manufacturer's instructions. The eluted DNAs were then diluted 250-fold for PCR amplification. PCR reactions were performed independently for the three bulk DNAs. The 16S rDNA fragments were PCR-amplified using bacterial-specific primer pair 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492r (5′-GGT TAC CTT GTT ACG ACT T-3′) (Lane, 1991). PCR was carried out in a 50-μl reaction mixture containing 25 ng of diluted genomic DNA, 1× reaction buffer, 1.25 U of *Taq* DNA polymerase (Yeastern Biotech, Shijr, Taiwan), 2 mM of $MgCl_2$, 0.2 mM of dNTP mix, and each primer at concentration of 0.25 mM. The PCR mixture was incubated in a Bio-Rad MyCycler thermal cycler (Bio-Rad, Hercules, USA), with an initial denaturation step at 95°C for 5 min, 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by a final extension step of 72°C for 15 min. The three PCR products of approximately 1.5 kb in size were combined and resolved on a 1% agarose gel, then recovered using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany).

The purified PCR fragments were ligated to the cloning vector, pTZ57R/T (Fermentas, Vilnius, Lithuania) and transformed into *Escherichia coli* DH5α competent cells. White colonies on LB-ampicillin agar containing 200 μM of IPTG and 80 μg/ml X-Gal were picked, and positive clones were determined by colony PCR using the M13/pUC primer set following the PCR cycling protocol as described earlier. From this study, 522 positive clones carrying the desired insert sizes of approximately 1.5 kb were collected for subsequent analysis. Cloned 16S rDNA fragments were double-digested with tetrameric restriction enzymes *Hae*III and *Hin*fI and analyzed as described previously (Liew and Jong, 2008). Restriction patterns obtained were sorted and grouped. Clones carrying discrete restriction patterns were sub-cultured in order to obtain single colonies. Amplicons were obtained by colony PCR amplification using the M13/pUC primer set, and purified using QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's instruction. Sequencing reactions of purified amplicons were carried out using DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare, Piscataway, USA) according to the manufacturer's instructions. Sequencing of the sample was carried out commercially.

The partial 16S rDNA sequence of first 550 bp in approximation for each clone was matched against the non-redundant sequence database at the National Center for Biotechnology Information (NCBI) using the nucleotide-nucleotide Basic Local Alignment Search Tool (BLASTn). The sequences were checked for possible chimeras by using the CHIMERA_CHECK program at the Ribosomal Database Project Web site (http://rdp8.cme.msu.edu). Multiple sequence alignment of the partial 16S rDNA sequences was carried out using ClustalW available in the MEGA 4.0 program (Tamura et al., 2007). Distance matrices were calculated using the Jukes-Cantor model (Jukes and Cantor, 1969) and a phylogenetic tree was constructed using neighbor-joining methods (Saitou and Nei, 1987). Bootstrap analysis of 1,000 data sets was performed to evaluate the tree topologies. The 16S rDNA sequences obtained in this study were deposited in the GenBank sequence database with accession numbers EF221882–EF221918, EF221920–EF222010, and EF222012–EF222022.

The 16S rDNA clone library of EFB compost, which consists of 522 clones, was clustered into 139 distinctive ARDRA patterns. Clones carrying the identical ARDRA pattern were presumptively the same bacteria. Sequencing analysis of these unique ARDRA patterns revealed a bacterial community of diverse phylogenetic groups. Among the library clones, 383 (73.37% of total library) were affiliated to cultivated counterparts

Table 1. Phylogenetic affiliation groups of EFB compost's clone library and their relative abundances.

Phylogenetic group	No. of clone(s)			% abundance of phylogenetic group
	Cultured[a]	Uncultured[b]	Total[c]	
Firmicutes	364		364	69.73
Proteobacteria				
Alpha-Proteobacteria		17	17	3.26
Beta-Proteobacteria	7	15	22	4.21
Gamma-Proteobacteria	10	14	24	4.60
Delta-Proteobacteria	1	16	17	3.26
Epsilon-Proteobacteria		2	2	0.38
Acidobacteria		37	37	7.09
Actinobacteria		10	10	1.92
Verrucomicrobia		3	3	0.57
Gemmatimonadetes		6	6	1.15
Cytophaga-Flexibacter-Bacteroides		12	12	2.30
Planctomycetes		3	3	0.57
Nitrospirae		1	1	0.19
Division OP11		3	3	0.57
Division OP10		1	1	0.19
Total clones	382	140	522	
% abundance	73.18	26.82		100.00

[a]Clone(s) with known closely related culturable counterpart(s).
[b]Clone(s) without closely related culturable counterpart(s).
[c]Total clone(s) representing the respective phylogenetic group.

whereas 139 (26.63%) were affiliated to yet-uncultivated or uncultured counterparts in the GenBank databases (Table 1). It is interesting to observe that the majority of the EFB compost 16S rDNA clones (69.73%) were related to members of the genus *Bacillus* from *Firmicutes* (Fig. 1). Eight ARDRA patterns were closely associated with *Bacillus thermoamylovorans* BTa (GenBank accession no. AB121094), originally isolated from kitchen refuse in Japan (Haruta et al., 2002). The sequence types (based on sequence identity) recorded sequence similarity from 98–99%, accounting for approximately 46.43% (n=169) of the library *Firmicutes* (n=364) and 32.37% of the total library (n=522). *B. thermoamylovorans* is a moderately thermophilic bacterium (Combet-Blanc et al., 1995). The bacteria's abilities in degrading sewage sludge and food waste have been extensively studied by Ivanov et al. (2004). The high occurrence of thermophilic bacillus-like bacteria in the maturation phase was in agreement with Beffa et al. (1996). It is not clear if the clones of all these eight ARDRA patterns belong to the same strain, or the ARDRA method managed to separate isolates within this species. However, it could be due to sequence heterogeneities among the 16S rRNA genes. The observations of sequence heterogeneities are expected and are in agreement with previous studies (Liefting et al., 1996; Mylvaganam and Dennis, 1992; Yoon et al., 1999). The research groups found multiple (two or more) copies of ribosomal RNA genes with sequence heterogeneity of several nucleotides in a single microorganism. Fisher and Triplett (1999) pointed out that a single microorganism might be represented by multiple ARDRA patterns.

The second (n=154) and third (n=25) most abundant occurrences were *B. circulans* WSBC 20060 (GenBank accession no. Y13064) and *B. thermoamylovorans* R-19047 (GenBank accession no. AB034714) with 97–99% and 98–99% sequence similarities, respectively (Table 2). These sequence types account for 29.50% (8 ARDRA patterns, n=154) and 4.79% (8 ARDRA patterns, n=25) abundances, respectively, in the total library. Strain *B. circulans* WSBC 20060 was initially submitted to the GenBank databases in 1997, but no information on its habitat was provided. *B. circulans* was reported to be beneficial plant-growth-promoting rhizobacteria (Tilak and Reddy, 2006). The presence of *B. circulans* was also detected by Takaku et al. (2006) in their compost maturation phase.

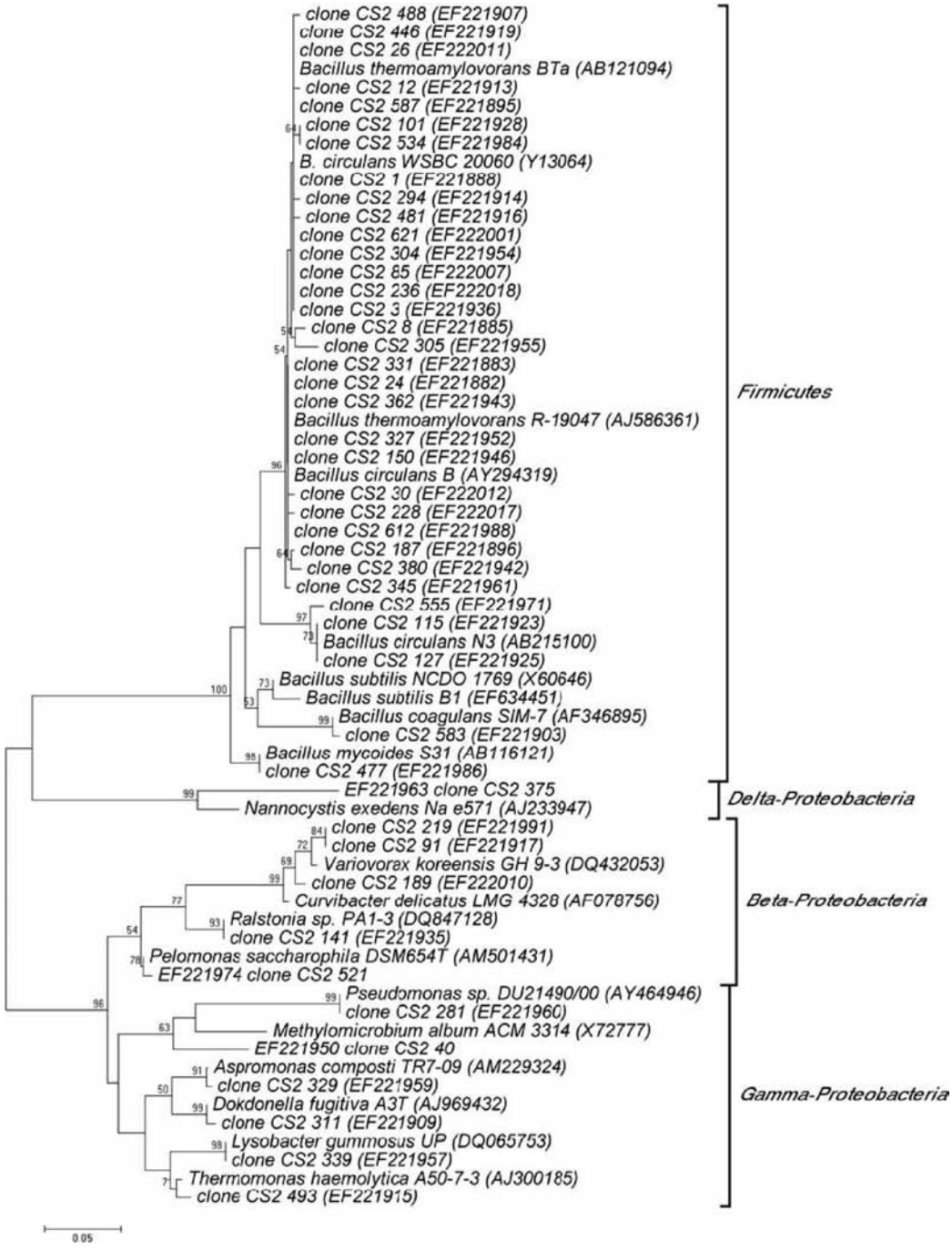

Fig. 1. The 16S rRNA gene-based neighbor-joining phylogenetic tree for the clones affiliated to the cultivated bacterial members in the GenBank databases.

The accession numbers for 16S rRNA gene sequences are shown in parentheses behind the clone name. The evolutionary distance was estimated based on the Kimura 2-parameter nucleotide substitution model. The scale bar indicates 0.05 substitutions per site.

B. thermoamylovorans R-19047 was originally isolated from gelatine extract samples. However, the presence of clones associated with this strain in the EFB compost was unknown. Similar to *B. thermoamylovorans* BTa, it could be a moderately thermophilic bacterium. Other affiliates of *Firmicutes* include *B. circulans* strain WSBC 20061 (Y13065), *B. circulans* isolate B (AY294319), *B. mycoides* S31 (AB116121), *B. coagulans* SIM-7 (AF346895), *B. circulans* N3 (AB 215100), and *Bacillus* sp. SMB8 (DQ868674) as shown

Table 2. Cultivated counterparts of OPEFB clone library based on partial 16S rRNA gene sequences (~ first 550 base pairs) with reference to distinctive ARDRA patterns.

Closest relative	ARDRA pattern	# of clones	Accession no.	% similarity	Source, Country
Firmicutes					
Bacillus circulans WSBC 20060	1,3,9,25,26,52, 90,108	154	Y13064	97–99	Unknown, Germany
Bacillus thermoamylovorans BTa	4,10,29,31,51, 115,133,139	169	AB121094	98–99	Kitchen refuse, Japan
Bacillus thermoamylovorans R-19047	7,12,49,57,59, 62,69,129	25	AJ586361	98–99	Gelatine extracts, Belgium
Bacillus circulans WSBC 20061	21	1	Y13065	99	Unknown, Germany
Bacillus coagulans SIM-7	34	2	AF346895	99	Overheated and microbiologically degraded wheat, Estonia
Bacillus circulans N3	37,39	4	AB215100	99–100	Commercial Natto products, Japan
Bacillus circulans isolate B	42,46,65,89	7	AY294319	99	Milk powder, Poland
Bacillus mycoides S31	107	1	AB116121	99	Leaf mold, Japan
Bacillus sp. SMB8	120	1	DQ868674	99	Marine biofilm, Singapore
Proteobacteria					
Beta-Proteobacteria					
Variovorax koreensis GH 9-3	30,87	4	DQ432053	98	Greenhouse soil, Korea
Ralstonia sp. PA1-3	41	1	DQ847128	100	*p*-Aminobenzoic acid degradation, China
Curvibacter delicatus LMG4328	47	1	AF078756	97	Unknown, Australia
Pelomonas saccharophila DSM654T	112	1	AM501431	98	Industrial and hemodialysis water, Spain
Gamma-Proteobacteria					
Methylomicrobium album ACM 3314	20	1	X72777	87	Soil, Australia
Dokdonella fugitiva A3T	55	3	AJ969432	99	Potting soil, Portugal
Aspromonas composti TR7-09	58	1	AM229324	98	Unknown, South Korea
Lysobacter gummosus UP	61	1	DQ065753	99	Unknown, Canada
Pseudomonas sp. DU21490/00	75	1	AY464946	99	Unknown, Singapore
Thermomonas haemolytica A50-7-3	88	3	AJ300185	98	Kaolin slurry, Finland
Delta-Proteobacteria					
Nannocystis exedens Na e571	67	1	AJ233947	90	Unknown, Germany

in Table 2. The predomination by bacteria from the phylum *Firmicutes*, especially *Bacillus*, in the compost concurred with many previously reported compost environments (Apun et al., 2000; Beffa et al., 1996; Haruta et al., 2002; Ryckeboer et al., 2003). The abundance of *Bacillus* in the EFB's high ligno-hemicellulosic environment may be explained by their many well-known abilities in relation to recalcitrant xylan and cellulose as reviewed by Priest (1977).

Phylum *Proteobacteria* is the second most abundant bacterial group observed in the total EFB compost library with 15.71% (n=82) occurrences (Table 1). The clones related to the cultivated *Proteobacteria* were of the subdivisions of *beta, gamma* and *delta*. The cultivated affiliates belonging to the subdivision *beta-Proteobacteria* include *Variovorax koreensis* GH 9-3 (DQ432053), *Curvibacter delicatus* LMG 4328 (AF 078756), *Ralstonia* sp. PA1-3 (DQ847128), and *Pelomonas saccharophila* DSM654T (AM501431) as shown in Table 2 and Fig. 1. Other clones related to the gamma subdivision are affiliated to *Dokdonella fugitiva* A3T (AJ969432), *Aspromonas composti* TR7-09 (AM229324), *Lysobacter gummosus* UP (DQ065753), *Pseudomonas* sp. DU21490/00 (AY464946), *Thermomonas haemolytica* A50-7-3 (AJ300185) and *Methylomicrobium album* ACM 3314 (X72777). Meanwhile,

clone CS2 375 (EF221963) was related to *Nannocystic exedens* Na e571 (AJ233947) of the delta subdivision. A few of these bacteria originate from greenhouse soil, potting soil, compost and kaolin slurry (paper-making chemicals). Members of *Variovorax*, *Pseudomonas*, and *Lysobacter* were previously reported to carry plant-growth-promoting properties (Saleem et al., 2007; Schmalenberger et al., 2008).

Besides the well-known cultivated bacteria, the analysis has revealed a rather large community portion of uncultured bacteria in the EFB compost. It was observed that more than a quarter (26.63%) of the sequence library is constituted by still-unknown or yet-uncultivated bacterial members (Table 1). Sequence comparison relates these uncultured members to *alpha-, beta-, gamma-, delta-* and *epsilon-Proteobacteria, Acidobacteria, Cytophaga-Flexibacter-Bacteroidetes* (CFB), *Actinobacteria, Gemmatimonadetes, Nitrospirae, Planctomycetes, Verrucomicrobia*, Division OP11 and Division OP10 (Table 1). These uncultured library sequences demonstrated sequence similarities ranging from 88 to 100% to their close affiliates (data not shown). Most of these clones are mainly associated with uncultured bacterial clones from environment samples of soil, sediment, and rhizosphere. The uncultured community was predominated by clones that closely affiliated to *Proteobacteria* ($n=64$), including the *alpha-, beta-, gamma-, delta-* and *epsilon*-subdivisions, and seconded by those of *Acidobacteria* constituting 25.53% ($n=37$) of the total uncultured community. The CFB group ($n=12$) was the third major uncultured members in the EFB compost. Predomination of the CFB group has also been reported from garbage-, sawdust- and spent mushroom-composts (Green et al., 2004; Ntougias et al., 2004; Takaku et al., 2006). Besides, widely varied members of this group have the ability to degrade macromolecules such as cellulose and xylan, which are the main components in EFB.

The fourth major group was associated with *Actinobacteria* ($n=10$) suggesting the maturity of the EFB compost. *Actinobacteria* were reported to play important roles, especially in the degradation of macromolecules in waste material, such as hemicellulose, lignin and cellulose, at the later stages of composting (Ryckeboer et al., 2003; Steger et al., 2007). The process reportedly releases inorganic nutrients and also plays an important role in humus formation (Epstein, 1997). It is interesting that clones related to the uncultured divisions of OP10 and OP11 were obtained. However, the function of their presence was not clear. The observation of highly diverse sequences in addition to the high proportion (>25%) of unknown sequences in the library indicates the novelty of the microbial community in the EFB compost environment. Thus, the relationships between the microbial diversity with the community structure and function need to be further examined and better understood. Although biases were reported in the 16S rRNA gene's sequence analysis (Acinas et al., 2004; Gevers et al., 2005; Pontes et al., 2007), the information obtained is definitely more comprehensive than that obtained using the culture-dependent approaches. The presence of this huge percentage (>25%) of not-yet-cultured sequences in the EFB community library should be carefully examined.

To the best of our knowledge, this is the first report on a study of an EFB microbial community structure via culture-independent methods. Generally, although microbiological evaluation has been performed at various stages of composting, it is the mature compost that poses the greatest concern to the agriculture industry, where the microbiological content may act as one of the quality indicators of a finished compost product. The culture-independent approach has been most useful in discovering the greater microbiological richness of the EFB compost. The high microbial anonymity, consisting of 11 major bacterial groups including *Firmicutes, Proteobacteria, Acidobacteria, Actinobacteria, Verrucomicrobia, Gemmatimonadetes, Cytophaga-Flexibacter-Bacteroidetes, Planctomycetes, Nitrospirae*, uncultured divisions OP11 and OP10, with 26.82% of still unknown bacterial members, makes the EFB compost worthy of further explorations. In addition to the still limited resources on the lignocellulosic compost microorganisms, information on active composting microorganisms is still very crucial. In this paper, a large microbial diversity was uncovered in EFB compost, which was mainly of environmental origins. The data obtained may be useful to answer some of the intriguing questions on the effect of EFB on the microbial community in a composting system. The revelation of a significant portion of uncultured populations among the compost community increases the research challenge to uncover the microbiological and functional aspects of the compost microbial community. In total, this study elucidates useful information and in-depth microbiological insight of an EFB-associ-

ated bacterial community structure.

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