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Original Article

Microbial Community Structure and Enumeration of *Bacillus* species in Activated Sludge

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ABSTRACT

Bacillus species is an important microbial species in the activated sludge process. Some researchers reported that the predominance of *Bacillus* spp. improves treatment performance and good sludge settleability. The viable count of *Bacillus* spp. is usually measured by the plate culture method. Recently developed massive sequence technology has been applied to activated sludge samples; this technique provides more detailed information on its microbial community. However, the relationship between the number of *Bacillus* spp. and microbial compositions is not yet well understood. In order to elucidate this relationship, microbial community analyses based on 16S rRNA gene sequence and cell count of *Bacillus* spp. were conducted. Activated sludge samples, including those from conventional activated sludge, sequencing batch, and oxidation ditch processes, were collected and subjected to analysis. The results of microbial community analysis revealed that Proteobacteria and Bacteroides were the predominant bacterial phyla and overall community compositions resembled each other at the phylum level. The detection ratio for *Bacillus* spp. was 0–0.33%, and the number of *Bacillus* spp. ranged from 10^5 to more than 10^8 colonies/g-MLSS. The results showed that the number of *Bacillus* spp. and detection ratio showed a similar trend, and thus, these analyses could be complementary to each other.

Keywords: sewage treatment, colony count, 16S rRNA gene, next generation sequencing

INTRODUCTION

Municipal sewage treatment using an activated sludge system is standardized and widely used; however, a large amount of excess sludge is produced that remains to be treated. According to the data from Ministry of Land, Infrastructure, Transport, and Tourism (MLIT) Japan, the recycling rate of sewage sludge was achieved to be 78% in 2010 on a dry solid basis, and over 70% of the excess sludge was incinerated and utilized as construction materials, such as cement or tiles [1,2]. The recycling of resource from a sewage treatment plant (STP), such as sewage sludge compost or reclaimed sewage, for agricultural use has become popular recently. However, beneficial utilization of excess sludge biomass as agricultural and plant fertilizers, including compost, accounted for 15% of the total generated sewage sludge in 2015 [2]. Thus, to increase the recycling rate of biomass generated from STP, the development of more value-added composting materials is required.

It was reported that certain night soil treatment facilities showed the presence of *Bacillus* spp. predominantly in the activated sludge, and those plants showed a highly efficient removal of organic compounds and nitrogen, and elimination of undesirable odors [3]. Thus, some of the treatment plants utilize the stimulation of *Bacillus* spp. in the activated sludge system [4]. *Bacillus* spp. are spore-forming aerobic or facultative anaerobic bacteria and belong to the phylum Firmicutes. Some strains of *Bacillus* spp. exhibit a suppressive

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effect on phytopathogenic microorganisms, and thus, *Bacillus* spp. are one of the well-studied biocontrol agents against *Fusarium* wilt [5–9]. These researchers added the specific effective *Bacillus* sp. to composting materials and used it as fertilizer or soil amendment. Therefore, it is considered that more value-added functional composting materials, which exhibit a suppressive effect on phytopathogenic microorganisms, could be produced from *Bacillus* spp. dominating excess sludge at a low cost.

To produce functional composting materials, increase in the number of *Bacillus* spp. in the activated sludge is important. Enumeration of *Bacillus* spp. in the activated sludge is commonly conducted by viable cell count using plate culture and identified by typical colony morphology [4]. This method requires skilled technique; hence, the results might be affected depending upon different analysts. In addition, it is well known that the cultivation-dependent method largely underestimates the diversity or number of microorganisms owing to the difficulty of cultivation of most of the microorganisms by conventional methods [10]. To overcome the difficulties of cultivation-dependent methods, culture-independent methods, especially for PCR based methods, have been developed and widely applied for microbiological studies including the studies of biological wastewater treatment. Recently, next-generation sequencing technology have employed intensively to reveal the microbial community structure or microbial dynamics of activated sludges [11,12]. However, to the best of our knowledge, application of these culture-independent technique for analysis of *Bacillus* spp. in the activated sludge have been limited on the one report [13]. In addition, there are no reports on the application of next-generation sequencing technology for analysis of *Bacillus* spp. in the activated sludge. Therefore, further research is required for a deeper understanding of the microbial community structure and the number of *Bacillus* spp. in the STP activated sludge.

Consequently, in this study aimed to elucidate the relationship between microbial community structure based on 16S rRNA gene sequence analysis and viable count of *Bacillus* spp. in the STP activated sludge. Recently developed massive sequence technology was applied for the analysis of microbial community structure in samples from several STP activated sludges and to conduct viable count of *Bacillus* spp. and quantitative PCR analysis for a comparison of the results.

MATERIALS AND METHODS

Sludge samples

Activated sludge samples used in this study were obtained from several municipal sewage treatment plants, agricultural sewage treatment facilities, and swine farm wastewater treatment facilities (**Table 1**). In addition, sewage-digested sludge (sample ND), which digested the excess sludge from NB and NG plants, was used for analysis to confirm the effect of anaerobic digestion.

Viable counts of *Bacillus* species

Twenty milliliters of activated sludge samples were homogenized and serially diluted ten-fold with sterile saline (0.85% [w/v] NaCl solution). The serially diluted samples were used for enumeration of bacterial cells after plating onto *Bacillus* medium. The medium composition was modified from Murakami et al. (1995) and was as follows: 8 g/L nutrient broth (Difco Detroit, MI), 8 g/L glucose, 10 g/L soluble starch, 6 g/L NaCl, and 17 g/L agar. Plates were incubated for 48 h at 32°C. The typical *Bacillus* colonies on the media were counted for a presumptive colony count on the basis of typical *Bacillus* morphology—large and round white-, gray-, or cream-colored colonies resulting from the generation of excessive slimy extracellular polymeric substances (EPS) [4]. The viable counts of *Bacillus* species were conducted by same person to avoid uncertainty regarding the difference of analytical person.

DNA extraction and sequencing

DNA was extracted from sludge samples using FastDNA SPIN Kit for Soil (MP Biomedicals Santa Ana, CA) according to the manufacturer's protocol. PCR amplification of 16S rRNA gene of each sample was performed using the universal primers for both bacteria and archaea with 515F and 806R primer set [14] with Premix Ex Taq Hot Start Version (Takara Bio, Kusatsu, Japan). The conditions for PCR amplification were as follows: 1 cycle of initial complete denaturation at 94°C (3 min); 25 cycles at 94°C (45 s), 50°C (60 s), 72°C (90 s); and final extension at 72°C (10 min). PCR products were purified using a MinElute PCR Purification Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol.

PCR products were sequenced using a MiSeq Reagent Kit v2 with MiSeq system (Illumina, San Diego, CA). Trimming and filtering of raw sequence reads, paired-end assembly, and operational taxonomic unit (OTU) picking at 97% identity were performed according to Kuroda *et al.* (2015a) [15].

Table 1 Summary of sludge samples and results of cell count and detection ratio of *Bacillus* spp.

STP Name	Treatment system	MLSS (mg/L)	Number of *Bacillus* spp. (CFU/g-MLSS)	Detection ratio of the family Bacillaceae (%)	Detection ratio of genus *Bacillus* (%)
KT	SBR	3,040	5.3E+08	0.14	0.14
IG	SBR	3,084	2.1E+08	0.04	0.04
NK	SBR	5,192	1.2E+08	0.04	0.04
HO	SBR	3,832	4.3E+08	0.23	0.23
MB	IA-AS	2,840	2.4E+08	0.23	0.22
MO	OD	2,816	2.1E+08	0.13	0.10
OK	AS	1,795	1.8E+08	0.33	0.00
KO	AS	1,482	2.4E+08	0.09	0.09
KY	AS	1,564	2.5E+08	0.05	0.04
NO	AS	1,872	2.2E+08	0.07	0.07
IH	AS	991	3.1E+08	0.03	0.03
OT	AS	1,238	1.7E+08	0.09	0.08
KS	AS	637	2.5E+08	0.08	0.07
NC	AS	1,509	2.7E+08	0.28	0.27
N1	SBR	5,828	1.2E+06	0.20	0.20
N2	SBR	6,485	5.2E+05	0	0
S1	AS	9,875	1.0E+07	0	0
BE	Biofilm of BRC	(TS:5%)	8.1E+06	0.01	0.01
NN	OD	2,207	1.8E+08	0.07	0.07
TD	OD	3,010	4.2E+08	0.04	0.04
NW	SBR	2,250	5.3E+08	0.09	0.09
TC	AS	1,126	1.6E+08	0.08	0.07
OG	OD	1,564	2.2E+08	0.04	0.04
NB	AS	1,135	6.6E+08	0.09	0.09
NG	AS	595	2.4E+08	0.05	0.05
ND	Digested sludge	(TS:19.7%)	1.2E+08	0.32	0.32

SBR: Sequencing batch reactor; IA: Intermittent aeration; OD: Oxidation ditch; AS: Activated sludge; BRC: Biological rotating contactor

Sequence data analysis was conducted using QIIME software package v.1.9.1 [16], and basically according to Kuroda *et al.* [17]. Chimeric sequences were removed using ChimeraSlayer [18]. Each OTUs were assigned to taxonomy using blast on QIIME as a reference database of greengenes 13_8 [19]. Principal component analysis (PCA) was performed with STAMP software [20].

Quantitative PCR

Quantification of *Bacillus* spp. in extracted DNA was performed by real-time PCR using Thermal Cycler Dice Real Time System III (Takara Bio) and SYBR Premix Ex Taq II kit (Takara Bio). The reaction mixture for qPCR was prepared according to the manufacturer's instructions, using the following *Bacillus* spp.-specific 16S rRNA gene-targeted primer sets: B360f (5′-CAGTAGGGAATCTTCCGCAATG-3′) and B513r (5′-AGCCGTGGCTTTCTGGTTAG-3′) [21]. For preparation of the standard calibration curve, a dilution series of the 16S rRNA gene PCR products of *Bacillus subtilis* was used. The PCR product was used in each real-time PCR analysis to calculate the copy number of the 16S rRNA genes in the samples. To confirm the specificity of the real-time PCR assay, melting curve analysis was performed after the amplification and the sizes of the PCR products were confirmed by gel electrophoresis.

RESULTS AND DISCUSSION

Microbial community structure of activated sludge

Twenty-five aerobic activated sludge or biofilm samples of biological rotating contactor were collected from 5 different treatment processes; additionally, one excess digested

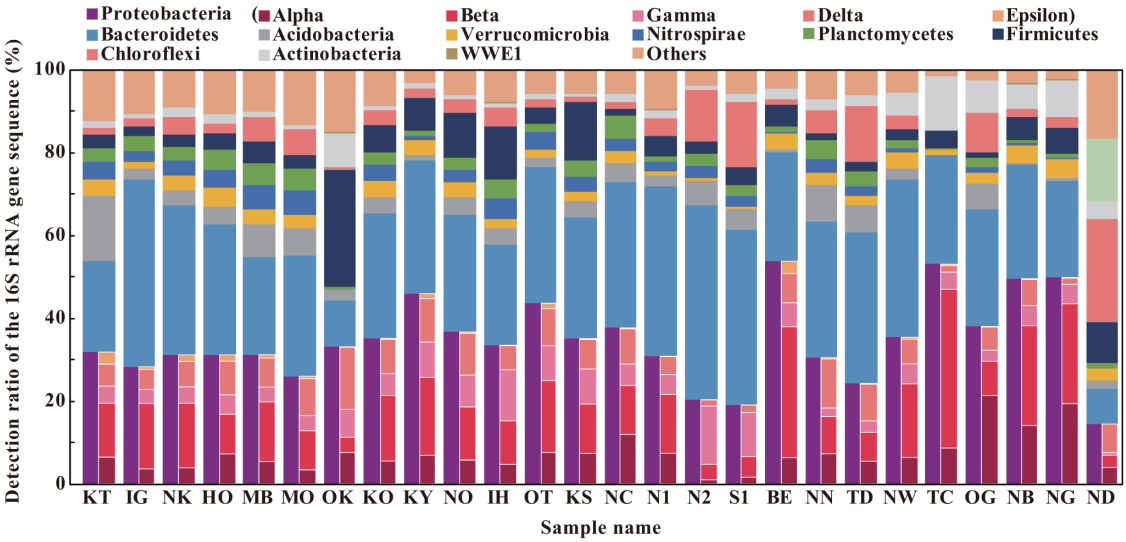

Fig. 1 Bacterial community structures in STP sludge samples at the phylum level.

activated sludge was collected (**Table 1**). Approximately, 7,000 − 24,000 sequence reads per sample were analyzed, and 284 − 2,665 OTUs per sample were found at 97% sequence similarity. The number of sequences was adequate for the analysis of microbial communities in sludge samples because the coverage value was over 0.92, which is sufficient to estimate the biodiversity in bioreactors [15].

Bacterial community structures in sludge samples were analyzed based on the phylum and sub-phylum level classification, as shown in **Fig. 1**. Principal bacterial groups in the activated sludge belonged to the phyla Proteobacteria and Bacteroidetes. Both the phyla accounted for at least 50% of the total microbes in each sample, except in the KO sample (**Fig. 1**). A similar tendency was noted for the other STP activated sludges from membrane bioreactor, oxidation ditch, and anaerobic/anoxic/oxic processes [22,23]. In addition, the analyzed activated sludge samples were obtained from 5 different reactors, but their dominant phylum-level bacterial group was similar. Actually, it was reported that bacterial compositions in most STP-activated sludges were highly consistent at higher taxonomical levels at different geographical locations and reactor configurations [24]. These results are consistent with the concept of the ecological coherence of high bacterial taxonomic ranks [24,25], and this concept is also applicable to STPs in Japan.

Phylum-level bacterial community structure in the activated sludge showed resemblance as mentioned above, but the genus-level community structure was not similar. PCA plots of genus-level bacterial communities in each sludge sample are shown in **Fig. 2**. The first two components of PCA explained 66% and PC1 account for more than half of variance. From the results of genus-level classification, genus belonging to Saprospiraceae have thought to large influence on PC1, because the genus is one of the dominant genes (average detection ratio is 11.9%) and the order of sample plot for x-axis is like the detection ratio of that genus. Sample ND was obtained from anaerobically digested excess sewage sludge; hence, the bacterial community structure was different from that in other aerobic sludge at phylum or genus levels (**Figs. 1 and 2**). Sample OK was almost similar to sample ND compared to the other samples, and this might be due to the dominance of phylum Firmicutes in the OK sludge. This was a characteristic feature of the STP from which sample OK was obtained; in addition, the type of dominant *Bacillus* sp. was different from that in the other STP sludge samples (**Fig. 3A**). Predominance of Firmicutes in the aerobic STP sludge is not common, but recent studies reported that Firmicutes was one of the dominant phyla in the sewage pipe system [26]. However, further studies are needed to elucidate the underlying causes.

Samples N2 and S1 had formed bacterial communities distinct from those of the other samples (**Fig. 2**). These two samples had the lowest Proteobacteria ratio (**Fig. 1**), and the family Bacillaceae was not detected in the 16S rRNA gene-based sequence analysis (**Fig. 3A**). Although samples N1 and N2 were obtained from parallelly operated sequencing batch reactors (SBRs) receiving wastewater from the same swine farm, their communities were found to be different. One of the SBRs, from which the N1 sample was obtained, had received mineral addition to increase the treatment efficien-

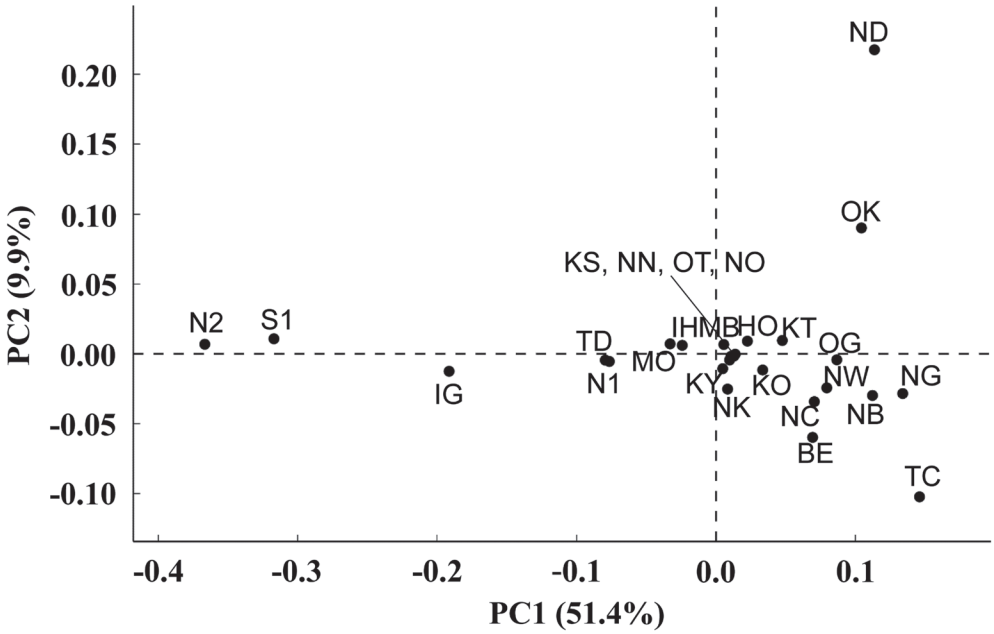

Fig. 2 Principal component analysis plots of each STP sludge microbial community at the genus level.

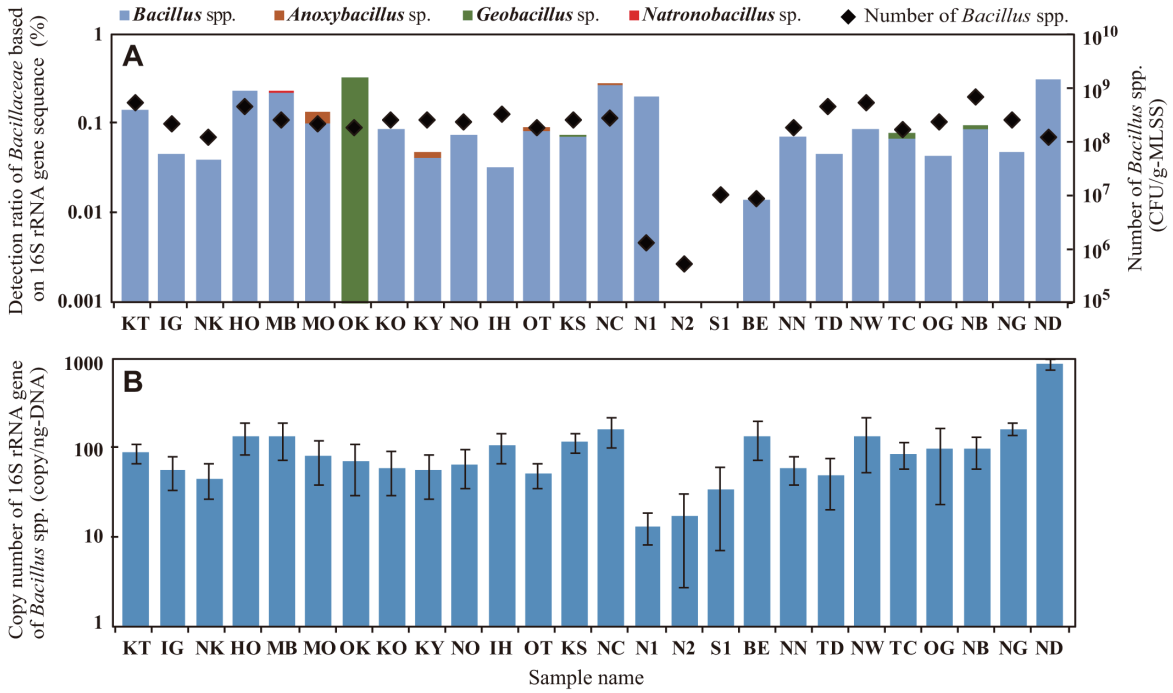

Fig. 3 Detection ratio of the family Bacillaceae and (A) number of *Bacillus* spp. and (B) copy number of 16S rRNA gene of *Bacillus* spp. in each STP sludge sample.

cies. This might have affected the bacterial community composition, but additional research is required to clarify this. In addition, there was no obvious tendency found for microbial community structure and treatment system configurations.

Numeration of *Bacillus* species and its predominance

The total number of viable cells of *Bacillus* spp. is shown in **Fig. 3A**. Except for four samples—N1, N2, S1, BE—the remaining 21 samples showed more than 10^8 CFU/g mixed liquor suspended solids (MLSS) of *Bacillus* spp.; thus, these samples were considered to predominantly contain *Bacillus* spp. Real-time PCR assay for *Bacillus* spp. was also performed (**Fig. 3B**). 16S rRNA gene copy number in the DNA of *Bacillus* spp. showed trends similar to those of the viable cell count or detection ratio of species belonging to the family Bacillaceae (**Fig. 3**). Interestingly, sample ND obtained from anaerobically digested sewage sludge also had a high number of viable *Bacillus* spp. and 16S rRNA gene copy number (**Fig. 3**). The anaerobic digester, from which the ND sample was obtained, had received excess NB and NG sludge; hence, *Bacillus* spp. might have survived during anaerobic digestion and could have been detected in the viable cell counts. *Bacillus* spp. belonging to the family Bacillaceae were predominantly detected during 16S rRNA gene sequence analysis. However, in case of sample OK, only *Geobacillus* sp. was detected, which means that the viable cell count measured not only the *Bacillus* spp., but also the *Geobacillus* sp., in sample OK. This could also be the case in qPCR assays.

Based on the 16S rRNA gene sequence analysis, the detection ratio of the species belonging to the family Bacillaceae in the four samples, N1, N2, S1, and BE, which showed lower viable cell number, was 0 − 0.014%. On the contrary, *Bacillus* spp.-dominated samples showed a detection ratio of 0.032 − 0.33%. The tendency of the viable count of *Bacillus* spp. and that of the detection rate of 16S rRNA gene sequence analysis was similar. From the qPCR assay, samples with lower viable cell number, N1, N2, and S1, also had lower copy number of 16S rRNA genes of *Bacillus* spp. with 12 − 33 copies/ng DNA, whereas *Bacillus* spp.-dominated samples showed approximately 100 copies/ng-DNA, and the highest number was detected from sample ND with 838 copies/ng DNA (**Fig. 3B**).

The relationship between the detection ratio of the species belonging to the family Bacillaceae and viable count of *Bacillus* spp. was established (**Fig. 4**). From **Fig. 4A**, the detection ratio of the species belonging to the family Bacillaceae was over 0.032% and the viable cell count was above 10^8 CFU/g-MLSS of *Bacillus* spp. Additionally, from **Fig. 4B**, approximately more than 50 copies of 16S rRNA genes/ng-DNA of *Bacillus* spp. accounted for over 10^8 CFU/g-MLSS of *Bacillus* spp. Thus, we could roughly estimate the dominance of viable cell number of *Bacillus* spp. by 16S rRNA gene-based bacterial community analysis or qPCR assay. To exert suppressive or antagonistic effects on wilt pathogens, such as *Fusarium* or *Rhizoctonia*, 10^8 − 10^9 CFU/g-dry material of *Bacillus* spp. is required [7,27,28]. During the production of mature compost material, the number of antagonists, such as *Bacillus* spp., could be increased [7,28]; thus, *Bacillus* spp.-dominated excess sludge should be considered a potential seeding material to produce more value-added composting materials. This study revealed that most of activated sludges plus anaerobically digested sludge have enough number of *Bacillus* spp. (**Table 1**). This means most of excess sludge from STP could be used for production of more value-added composting materials, which is connected to increase the recycling rate of biomass generated from STP in future.

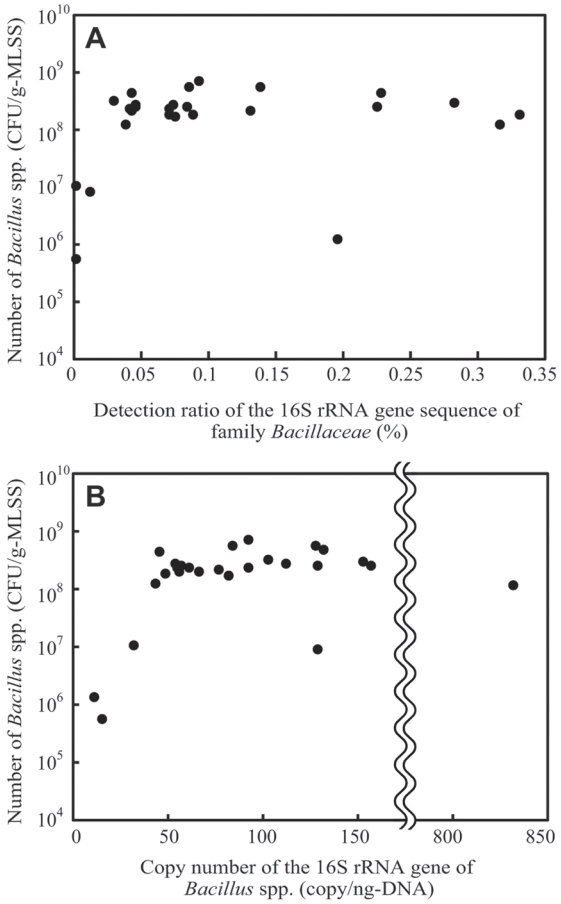

Fig. 4 Relationship between the viable cell number of *Bacillus* spp. and (A) detection ratio of the species belonging to the phylum Bacillaceae, and (B) copy number of 16S rRNA gene of *Bacillus* spp.

CONCLUSION

Twenty-six aerobic activated sludge, biofilm, and anaerobically digested sludge samples were analyzed. The relationship between the detection ratio or 16S rRNA gene copy number and viable count of *Bacillus* spp., a detection ratio of over 0.032% of the species belonging to the family Bacillaceae or over 50 copies/ng DNA of 16S rRNA gene copy number of *Bacillus* spp. accounted for more than 10^8 CFU/g MLSS of *Bacillus* spp. Surprisingly, anaerobically digested sewage sludge samples also showed a high number of viable *Bacillus* spp.; therefore, digested sewage sludge could also be a promising candidate as raw material for the production of functional compost. But further work is needed to confirm its validity.

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