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Quantification of *Bacillus* Species in a Wastewater Treatment System by the Molecular Analyses

Koji Mori[1,2], Ryozo Iriye[3], Mutsunori Hirata[2], and Kazuhiro Takamizawa[2]*

[1] Biological Resource Center (NBRC), National Institute of Technology and Evaluation (NITE), Kisarazu, Chiba 292-0818, Japan
[2] Department of Bioprocessing, Faculty of Agriculture, Gifu University, Gifu 501-1193, Japan
[3] Departmentof Bioscience and Biotechnology, Faculty of Agriculture, Shinshu University, Kamiina, Nagano 399-4598, Japan

Abstract *Bacillus* species were observed and quantified by molecular approaches, using the 16S rDNA primers/probes, in a wastewater treatment plant designed for the purpose of stimulating the growth of *Bacillus* species. The plant has been operating as a test plant since 1997 in the city of Ina, Japan, with excellent treatment performance. Observations by *in situ* hybridization, using *Bacillus*-specific probes, indicated that *Bacillus* strains were inhabited in the plant and their numbers decreased during the treatment process. Similar results were obtained from a quantitative PCR analysis using a *Bacillus*-specific primer set, and the amount of DNA originating from various *Bacillus* species was maximally 1.91% of the total DNA in the wastewater treatment tank. Clone library analysis using the *Bacillus*-specific primers suggested that, while the population was noticeably increased, the phylogenetic diversity of the increasing *Bacillus* species was very low.

Keywords: *Bacillus*, wastewater treatment, fluorescence *in situ* hybridization (FISH), quantitative PCR

INTRODUCTION

Microbiological activity is typically used to treat wastewater containing soluble and particulate organic materials. Although there have been many improvements enhancing the treatment efficiency and speed, there is only limited understanding of the relationship among microbial community structures, treatment rates, and efficiency. This is largely due to the use of cultivation dependent analyses, such as isolation, identification and the most-probable number (MPN) method, with organisms identified by the cultivation methods typically representing less than 15% of the diversity of microorganisms associated with wastewater treatment processes [1].

Powerful culture independent analysis methods have recently been developed for the estimation of microbiological communities. In the study of wastewater treatment, several attempts have also been made to analyze the bacterial community structure. Quinone profiles [2,3], polyamine patterns [4], immunofluorescence [5,6], and 16S rDNA/RNA approaches have been used successfully. Particularly, 16S rDNA/RNA approaches, such as fluorescent *in situ* hybridization (FISH) [7-12], denaturing gradient gel electrophoresis (DGGE) [13-16], clone library [9,15-19] and dot blot hybridization [20], have yielded good information from the point of direct analysis of the total bacterial community structure.

One group [21] recently designed a wastewater treatment for the purpose of stimulating the growth of *Bacillus* species, called the ultra activated sludge (ULAS) process. The genus *Bacillus* belongs to the phylum Firmicutes, and members of this genus are spore-forming aerobic or facultatively anaerobic bacteria. As a group, members of this genus contain a variety of protein, starch and oil decomposing activities. Since such activities are well suited for wastewater treatment, methods have been developed to enhance their growth in the wastewater treatment systems [21-23]. In the city of Ina, Japan, the wastewater treatment system has been operating in a test plant since 1997. This has shown suitable removal rates for organic matter combined with high denitrification and dephosphorization activities and a decrease in the production of offensive odor. The results from our earlier studies using traditional culture-dependent methods suggested an increase in *Bacillus* species in this test plant compared with other plants [21-23], and some *Bacillus* species were isolated from the plant [24-26]. However, because of the limitation of the culture-dependent methods, the diversity of the *Bacillus* species inhabiting the plant could not be estimated.

In the present study, molecular approaches have been used to describe the *Bacillus* species present in the test plant at Ina. The FISH, clone library, and quantitative PCR analyses, using *Bacillus*-specific 16S rDNA probes/

***Corresponding author**
Tel: +81-58-293-2906 Fax: +81-58-293-2906
e-mail: tak2003@cc.gifu-u.ac.jp

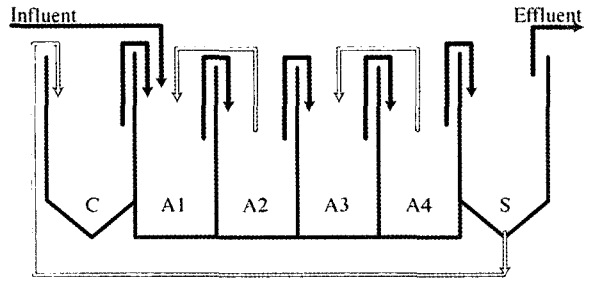

Fig. 1. The wastewater treatment process at Ina city, the prefecture of Nagano. Closed and open arrows show the flow and sludge return, respectively. C, concentrated sludge tank; A1~A4, aeration tanks; S, settling tank.

primers, permitted a more accurate estimation of the *Bacillus* population, and these results demonstrated the importance of *Bacillus* species in this sewage treatment system.

MATERIALS AND METHODS

Process of Plant

Wastewater treatment has been operated at a test plant since 1997 in the city of Ina (Nagano prefecture, Japan). This process has been operating with the following performances: control of aeration, control of sludge return and the addition of magnesium ions and silicic acid [21-23]. The configuration of the process is shown in Fig. 1. The process consists of four aeration tanks (A1~4: 0.9 m^3, 0.55 × 1 × 2 m (H), 1.7 m depth), a settling tank (S: 1.5 m^3, 1 × 1 m surface area), and a concentration sludge tank (C: 1.75 m^3). Domestic wastewaters from Ina (Nagano prefecture) were used as influent, and the sludge in the settling tank was returned to the concentration tank through the aeration tanks after processing. The sludge was returned to the first aeration tank, with 2~4 h of residence time. The sludge returns were operated at regular intervals with an airlift. For the purpose of stimulating the growth of *Bacillus* species, the concentrations of dissolved oxygen in the aeration tanks, and magnesium ions, and silicate in the influent were maintained at 0.2~0.6, 1~2 and 1.2~2 mg/L, respectively [21-23]. In our earlier studies, the result found during the operations was that the colonies of *Bacillus* strains were obtained more from the plant than from conventional wastewater treatment systems.

The samples containing wastewater and sludge were collected on August 28, 2000. For comparative studies, a sample from a conventional sewage treatment plant in the city of Gifu (Gifu prefecture) was also collected and analyzed. Domestic wastewaters from Gifu prefecture were treated in the plant.

Analyses of Sludge

After collecting the samples, the following analyses were immediately performed. The pH of filtrated waters (final pore size, 0.6 μm) was measured with a B-212 pH meter (TOA Electronics Ltd., Tokyo, Japan). The values of the biochemical oxygen demand (BOD) and suspended solid (SS) were determined by standard methods [27]. Viable heterotrophic bacteria in the samples were measured as colony forming units, using a medium containing 10 g/L polypepton, 5 g/L meat extract, 5 g/L NaCl and 15 g/L agar. The numbers of cells from *Bacillus* species were also counted as colony forming units using a *Bacillus*-specific medium, containing 8 g/L nutrient broth, 8 g/L glucose, 0.5 g/L yeast extract, 5 g/L NaCl and 15 g/L agar. Although, in addition to *Bacillus* species, many microorganisms grew on the *Bacillus*-specific medium, with the rough and large colonies chosen and enumerated as *Bacillus* colonies. After enumeration, some representative colonies from the *Bacillus* species selective medium were picked and isolated.

Molecular Analyses of Sludge

For the molecular analyses, *Escherichia coli* JM109, *Clostridium bifermentans* DPH-1 [28] and various *Bacillus* strains isolated in this study (see results and discussion section) were used for the preparation of a standard curve and the determination of the hybridization conditions.

Genomic DNA from the samples, pure cultures and isolates were extracted and purified according to the methods of Mori *et al.* [29]. The samples containing wastewater and sludge were centrifuged (9,820 × g, 10 min), and then 1 g of the pellet samples with microorganisms was used for the extraction of genomic DNA. The genomic DNA concentration was quantified by the spectrophotometric measurement. The amplification and sequencing of the 16S rDNA from isolates were performed following the methods of Hattori *et al.* [30]. Quantitative PCR, 16S rDNA clone library and phylogenetic analyses, using *Bacillus*-specific primer set (Bs16S1 forward primer, *E. coli* position 67~89, 5′-ATGTTAGCGGCGGACGGG TGAG-3′; Bs16SR reverse primer, *E. coli* position 597~573, 5′-AAGTTCCCCAGTTTCCAATGACC-3′), were performed as described by Mori *et al.* [29]. A quantitative PCR method [29] was used for the enumeration of the *Bacillus* species in the samples, and a standard curve produced using genomic DNA extracted from *Bacillus* sp. A4-7-1-1 isolated from the wastewater treatment system (see results and discussion section):

$$y = (4.00 \times 10^6) \times 0.52^x$$

where x is the cycle number and y (ng) the initial amount of DNA (r^2 = 0.944). The amount of *Bacillus* sp. A4-7-1-1 DNA was supposed to be representative of the *Bacillus* species, and was calculated from the total DNA extracted from the samples.

Fluorescent *in situ* identification of microorganisms, by whole cell hybridization with rRNA-targeted nucleic acid probes, was carried out in this study using universal (Uni1392, *E. coli* position 1,392~1,406, 5′-GACGGGC

Table 1. The characteristics of the samples used in this study

Sample	pH*	BOD* (mg/L)	SS (mg/L)	Total heterotrophic bacteria (cfu/mL)	*Bacillus* species (cfu/mL)
Sludge concentration tank	5.4	7.1	8,200	1.08×10^7	6.80×10^5
Aeration tank 1	6.7	10.2	6,510	n.d.	n.d.
Aeration tank 2	5.9	11.5	6,650	n.d.	n.d.
Aeration tank 3	5.5	5.8	6,770	n.d.	n.d.
Aeration tank 4	5.3	5.9	6,710	9.56×10^6	5.20×10^5
Conventional plant in Gifu city	7.2	8.6	3,983	1.36×10^6	less than 1

n.d.; not determined.
*; supernatant was analysed.

GGTGTGTRC-3′) [31] and *Bacillus*-specific probes (Bt16S2P, *E. coli* position 597~619, 5′-GGGTCATTGG AAACTGGGGAAC-3′). For double staining of the sections, Uni1392 and Bt16S2P probes were labeled with rhodamine and FITC, respectively. The optimal hybridization conditions were determined using *E. coli* JM109, *C. bifermentans* DPH-1, and the isolated strains of *Bacillus*. All samples containing activated sludge and wastewater were centrifuged (9,820 × g, 10 min), and 1 g of the resulting pellets re-suspended. Cell fixation using paraformaldehyde and whole cell hybridization was performed following the method of Amann [32]. Hybridization was performed at 45°C for 2 h in hybridization buffer, with its stringency adjusted by the addition of formamide to the hybridization buffer (5 and 10% for Uni1392 and Bt16S2P probes, respectively). The sections hybridized with the probes were observed with a confocal laser scanning microscope (LSM 510 model; Carl Zeiss Co., Ltd., Germany).

RESULTS AND DISCUSSION

Conditions in the Wastewater Treatment Systems

The characteristics of the samples containing the activated sludge are shown in Table 1. The measured pH, BOD and SS values were normal in the wastewater treatment system, indicating that the wastewater treatment was operating properly. Because the sludge returns were performing at every treatment tanks (Fig. 1), the SS values tended to be higher than those of the conventional plant at Gifu. The colony forming units of heterotrophic bacteria and *Bacillus* strains are also shown in Table 1. The numbers of cells observed on the *Bacillus* selective media were $5.20 \sim 6.80 \times 10^5$ cfu/mL, corresponding to 5.4~6.2% of the total number of heterotrophic bacteria. Conversely, the growth of *Bacillus*-like strains was not observed on the *Bacillus* selective medium when the sample from the conventional plant was used as the inoculum.

Isolation of *Bacillus* strains from the Wastewater Treatment System

Four different strains were isolated on the *Bacillus* selective medium from the samples in the wastewater treatment tank (strains A4-7-1-1, A4-7-1-2, A4-20-12, and ICSC-1). Almost complete sequences of the 16S rDNA from these isolates were obtained (DDBJ accession numbers: AB159767, AB159769, AB159768, and AB159770, respectively). Phylogenetic analysis, based on the neighbor-joining method [33,34], demonstrated that the isolates belonged to the genus *Bacillus* (Fig. 2).

Fluorescent *in situ* Hybridization of the Wastewater Treatment Systems

Phase-contrast and simultaneous hybridized images of the samples containing the activated sludge from the wastewater treatment tank are shown in Fig. 3A~O. Using *in situ* hybridization with the Uni1392 probe revealed that the cells were rich in 16S rRNA, indicating that the microorganisms were very active in the sludge from the concentration sludge tank (Fig. 3B) and aeration tank 1 (Fig. 3E). Because some cells from these tanks were hybridized with Bt16S2P probe (Fig. 3C and F), these microbial communities included some *Bacillus* species. The detection of microorganisms, including *Bacillus* species, by *in situ* hybridization were reduced to lower levels as the wastewater treatment was passed its way through the various aeration tanks (Fig. 3G~O). The reason for the lower number of microorganisms in the latter stages of treatment is unclear, but the many hours of aeration and a decrease in the organic compounds may have caused this observed decline.

A sample containing the activated sludge from the conventional tank at the city of Gifu is also shown in Fig. 3P and Q. Although active microorganisms inhabited this sludge, the cells did not hybridize with the Bt16S2P probe, indicating that the population of *Bacillus* species was very low.

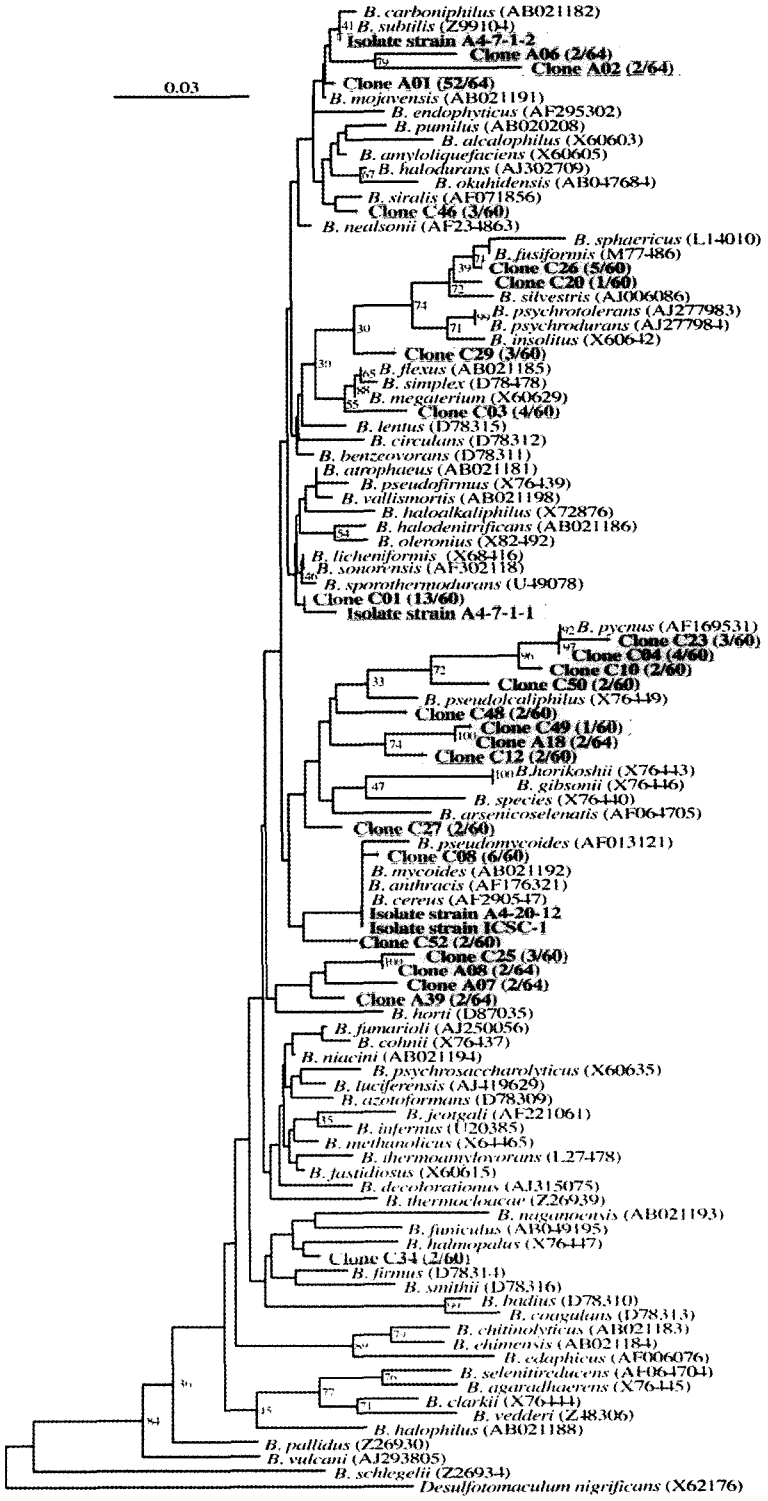

Fig. 2. A neighbor-joining tree of the 16S rDNA of *Bacillus* strains, and their clones, from the wastewater treatment systems. The significance of each branch is indicated by bootstrap values. The isolates and clones in this study are represented in bold. The accession number is shown in parentheses. Clones A01~39 and C01~52 originated in the samples from the concentrated and conventional sludge tanks, respectively. The parentheses next to the clone numbers also indicate the frequency of appearance of similar sequences (<1% dissimilarity) in all the clones. Scale bar estimated substitutions per nucleotide position. Sequences of isolates and clones have been submitted to the DDBJ, with accession numbers AB159767 for strain A4-4-7-1-1, AB159768 for strain A4-20-12, AB159769 for strain A4-7-1-2 and AB159770 for strain ICSC-1. For clones, AB159742 to AB159766 were respectively registered.

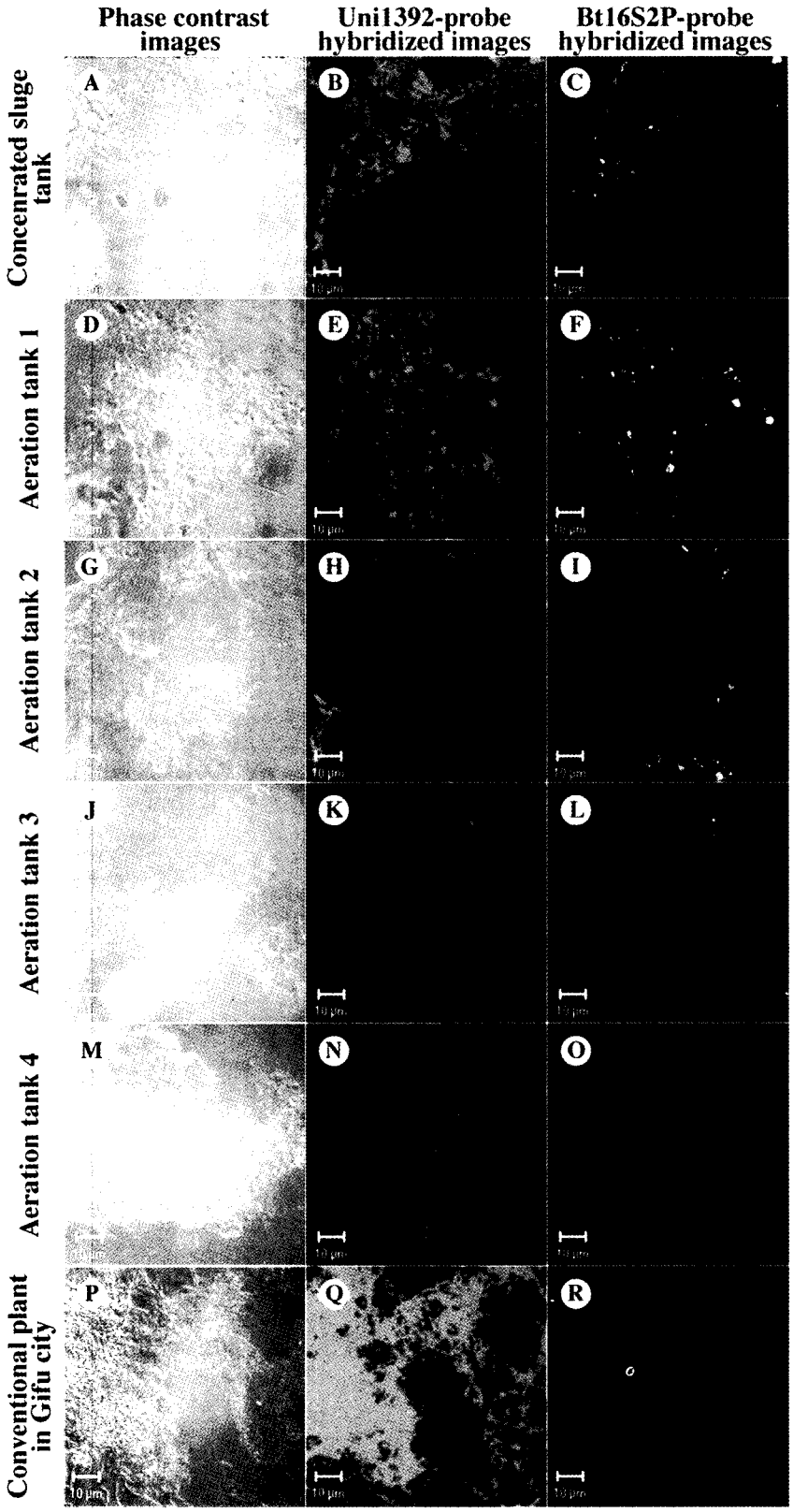

Fig. 3. Simultaneous phase-contrast and fluorescent *in situ* hybridized images of the samples contained within the activated sludge. Sections hybridized with carboxyl rhodamine-labeled universal probe (Uni1392 probe) and FITC-labeled *Bacillus*-specific probe (Bt16S2P probe).

Table 2. Amount of *Bacillus* DNA in the samples using the quantitative PCR method

Sample	Extracted DNA (μg/L)	*Bacillus* species (% of extracted DNA)
Sludge concentration tank	7.45	0.72
Aeration tank 1	4.25	1.91
Aeration tank 2	2.41	0.72
Aeration tank 3	1.98	0.13
Aeration tank 4	3.39	0.12
Conventional plant in Gifu city	0.92	0.18

Quantitative PCR and Clone Library Analyses

In order to remove the inherent bias associated with enumeration using cultivation dependent methods, the enumeration was performed using molecular techniques. The results of the quantitative PCR analyses are shown in Table 2. DNA samples for the PCR reactions were collected, with the concentration range 0.92~7.45 μg/L. DNA originating from the *Bacillus* species in the total extracted DNA ranged form 0.12 to 1.91%, which is consistent with the observations obtained from the *in situ* hybridization techniques. There were large amounts of DNA originating from *Bacillus* species in the concentration sludge tank and aeration tanks 1 and 2, but these were decreased to levels similar to those observed in the conventional plant in tanks 3 and 4.

For the purpose of specifying the PCR products, cloning and sequencing were performed. For these procedures, the PCR products from the samples obtained from the concentrated sludge tank and conventional tank at the city of Gifu were selected. Approximately 500 base sequences of 64 and 60 clones were retrieved from the samples taken from the concentration sludge tank and the conventional tank, respectively. The results of the phylogenetic analysis are shown in Fig. 2. In the clone library of the concentration sludge tank, the clones (clone A01~64) were classified into 7 types. The most common sequence, clone A01 (52 clones/64 total clones) was close to that of *Bacillus majavensis* (sequence similarity 99.6%). Clone A01 was also related to *Bacillus* sp. A4-7-1-2 isolated from the sludge, with 99.3% sequence similarity. Conversely, in the clone library of the conventional tank, the clones (clone C01~60) were classified into 18 types, and the diversity was higher than that of the concentration sludge tank.

CONCLUSION

Bacillus species were thought to be suitable for wastewater treatment due to their decomposition activities, and wastewater treatment using *Bacillus* species has been reported [35]. The plant at Ina maintained good treatment, as determined by measurement of various parameters. Compared with conventional sewage treatments, the plant has shown a suitable removal rate for organic matter and decreasing in the production of offensive odor. However, the detection of *Bacillus* species was proven only by cultivation methods [21-23]. As observed in the previous reports, the colonies on the *Bacillus* selective medium were mainly observed in samples taken from the sludge concentration tank. Although the plant has been operated for the purpose of stimulating the growth of the *Bacillus* species, we could not tell exactly whether the activity of *Bacillus* species caused the good performance.

In this study, the observation from the *in situ* hybridization and quantitative PCR analysis confirmed the existence of *Bacillus* species in the wastewater treatment tank. The amount of DNA originating from *Bacillus* species was maximally 1.91% of the total DNA, which was significantly higher than that of the conventional tank at the city of Gifu. The clone library analysis using the *Bacillus* specific primers suggested that the specific species of the genus *Bacillus* had increased in the plant. The molecular approaches typically indicated that *Proteobacteria* are generally dominant in wastewater treatment plants [9,11, 12,15], with a few reports suggesting that the retrieval of Gram-positive bacteria, with a low DNA G+C content, from wastewater treatment plants [9,15]. In our study, although they were not dominant, representatives of the genus *Bacillus* were indeed stimulated by the various operations at the Ina wastewater treatment plant. The study using the molecular approaches suggested that the specific species of genus *Bacillus*, indicated by the clone library, played a part in the good performance of the plant. This is the first report using molecular methods to prove a wastewater treatment process is capable of stimulating the proliferation of *Bacillus* species.

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