B−33 Pyro-sequencing-aided identification of microorganisms that were affected by activated sludge extract

○Purnika Ranasinghe*, Hiroyasu Satoh, Kenshiro Oshima, Masahira Hattori, Takashi Mino
Graduate School of Frontier Sciences, The University of Tokyo
(Kashiwanoha 5-1-5, Kashiwa, Chiba 277-8563, Japan)
* E-mail: *purr@k.u-tokyo.ac.jp*

1. INTRODUCTION

While knowledge on microbial population in wastewater treatment processes are being rapidly accumulated, there are researchers who argue that there are factors which affect microbial population but not yet well understood. For example, Satoh et al. (2009)[1] demonstrated that extract of activated sludge affected microbial population structure in activated sludge. In order to further clarify which microbial species were affected by the addition of activated sludge extract, some of the samples obtained in Satoh et al. (2009)[1] were further analyzed by the pyrosequencing method.

2. MATERIALS AND METHODS

(a) Activated sludge samples

Two of the activated sludge samples already reported in Satoh et al. (2009) were used in the present study. They were both from the first round experiment reported in the paper. In short, both the activated sludge had been incubated in microplate wells for 5 days with substrate and with or without ethanol extract from activated sludge. In the present study, the activated sludge incubated with and without the extract are referred to as "Sample X" and "Sample Y" respectively. that In Satoh et al.(2009)[1], they analyzed bacterial population structure in both of the samples by the polymerase chain reaction/terminal-restriction fragment polymorphism (PCR/T-RFLP) targeted at a partial sequence of 16S rRNA gene. As the primer set, 5'-FAM labelled 27f primer (AGAGTTTGATCMTGGCTCAG) and 519r primer (GWATTACCGCGGCKGCTG) were used for PCR, and *Hha*I was used for digestion. The T-RFLP profiles of the samples used in the present study are shown in Fig. 1.

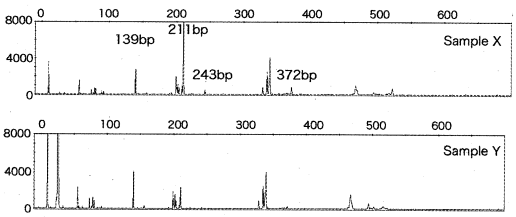

Fig. 1 T-RFLP profiles of X and Y Samples reported in Satoh et al. (2009)[1].

(b) Pyrosequencing

The Samples X and Y had been stored at -80oC. They were thawed, 50μL of the sample was diluted 20 times with Milli-Q water, sonicated by 250DA Advanced Digital Sonifier (Branson) with a special microtip at an amplitude of 40% (20W) for 20 seconds. The sonified liquid was directly used as the template for PCR. The primers used were 27f and 519r both added with barcode sequences. PCR reactions were performed with a Thermal cycler Dice (Takara, Japan) and thermal program of 95° C for 600s followed by 30 cycles of (94°C for 30s, 55.3°C for 30s and 72°C for 30s) followed by 72°c, 600s. The PCR products were purified using QIAQuick PCR purification KIT according to manufacture's instruction, and anlyzed by a GS FLX pyrosequencer (Roche).

(c) Data analysis

Fragments with lengths longer than 300bp were selected from the total number of reads. Reverse-read sequences were reverse-complemented and then used for further analyses.

Classification

Selected sequences were classified with RDP classifier tool in RDP 10 database[2), 3)], to identify the associated taxonomic groups.

Virtual Digestion Analysis

The sizes of terminal-labeled restriction fragments (T-RFs) were calculated by using FileMakerPro V10. The lengths of the calculated fragment sizes from 5' end and their abundances were compared with the real T-RFLP profiles shown in Fig. 1.

Phylogenetic Tree

The sequences were imported to ARB software environment (Version. 5.1)[4)], aligned by ClustalW fast DNA alignment, and then a phylogenetic tree was build by the neighbour joining method. Trees were further edited to illustrate the main taxonomic groups related to T-RFLP lengths and their abundances.

3.RESULTS AND DISCUSSION

By pyrosequencing, 4,495 reads longer than 300bp were obtained, where 2,358 reads were from Sample X and 2,137 reads were from Sample Y.

These sequence data were analyzed by the RDP Classifier and class level compositions were calculated for Samples X and Y, as shown in Fig. 2. More sequences were classified as class Gammaproteobacteria for Sample X which had been incubated with activated sludge extract.

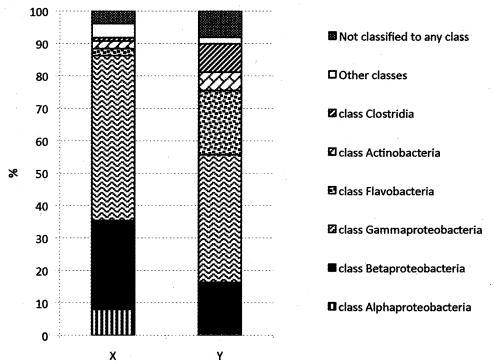

Fig. 2 Class level classification of pyrosequenced reads.

The expected T-RF lengths were calculated for each of the reads. And the reads were classified into 10 groups, Group A through Group J, based on the expected T-RF sizes. These groups were assigned only when number of reads affiliated to the group was bigger than 15. About half of the reads were affiliated to these 10 groups. By the addition of activated sludge extract, groups A, B and D moderately reduced, Groups C and J more severely reduced, and group H was significantly increased.

In Figure 1, it is seen that T-RFs with sizes 211bp, 243bp, and 372bp were increased, and that with a size of 139bp was decreased by the addition of activated sludge extract. Especially, the increase of the T-RF 211bp was very significant. This observation on T-RF 211bp is in consistent with the increase of the number of reads in Group H, though expected T-RF size for Group H is a little bit bigger than 211. On the other hand, none of the groups in Table 1 corresponded to the T-RFs 243bp and 372bp. Either Group C or D is thought to correspond to T-RF 139bp. In T-RFLP profile, the reduction of T-RF 139bp by the addition of activated sludge extract was small, but more differences were found in the number of reads of Group C and D.

Table 1. Grouping of reads based on the expected T-RF sizes.

Group	Expected T-RF size (bp)	No. of Reads (% in reads) X	Y	Enhancement X/Y
A	60-61	14 (0.6)	36 (1.7)	0.35
B	85-86	15 (0.6)	30 (1.4)	0.45
C	143-145	29 (1.2)	104 (4.9)	0.25
D	148-150	49 (2.1)	103 (4.8)	0.43
E	205-206	158 (6.7)	179 (8.4)	0.80
F	207-210	45 (1.9)	68 (3.2)	0.60
G	211-212	20 (0.8)	23 (1.1)	0.79
H	213-216	558 (23.7)	236 (11.0)	2.14
I	225-226	15 (0.6)	- (0)	-
J	339-342	24 (1.0)	128 (6.0)	0.17

The pyrosequenced reads which were assigned to Groups A through J were used to construct a phylogenetic tree as shown in Fig. 3. In Fig. 3, cluster indicators starting with "X", "Y" or "XY" are for clusters that contain reads from Sample X only, Sample Y only, or both Samples X and Y, respectively. Phylogenetic identity information is added for clusters with more than 50 reads.

Bacterial species that corresponds to clusters that are indicated with "XY-" were not affected or affected to a smaller degree by the addition of activated sludge extract.

Clusters that are corresponed to family Rhodocyclaceae in class Betaproteobacteria were found to contain reads from Sample Y only. Rhodocyclaceae family bacteria were adversely affected by the addition of activated sludge extract.

Based on data shown in Table 1, clusters that are related to Group H are of interest. Most of clusters related to Group H are found at the bottom of the tree. As can be seen, one of them contains reads only from Sample X, and another from Sample Y. The rest of the clusters contain reads from Samples X and Y, but mostly from Sample X.

4. CONCLUSIONS

The effect of the addition of activated sludge extract on activated sludge bacterial communities was investigated by the pyrosequencing method. The outcomes were in good correspondence with those obtained by the T-RFLP method, but more detailed insight was achieved. Rhodocyclaceae family was negatively affected, while some part but not all of Xanthomonas family was positively affected by the addition of activated sludge extract.

ACKNOWLEDGEMENT

The project was supported by the GCOE program Deciphering Biosphere from Genome Big Bang, and Grant-in-Aid for Scientific Research (A) (22246069).

REFERENCE 1) Satoh, H. et al. (2009)Env. Eng. Research, 46, 503 – 510. 2) Cole, J.R. et al.(2007) Nucleic Acids Res. 35: D169-D172. 3) Wang, Q,G.M. et al. (2007) Appl. Environ. Microbiol. 73(16):5261-7. 4) Ludwig,W. et al. (2004) Nucleic Acids Research. 2004. 32(4):1363-1371.

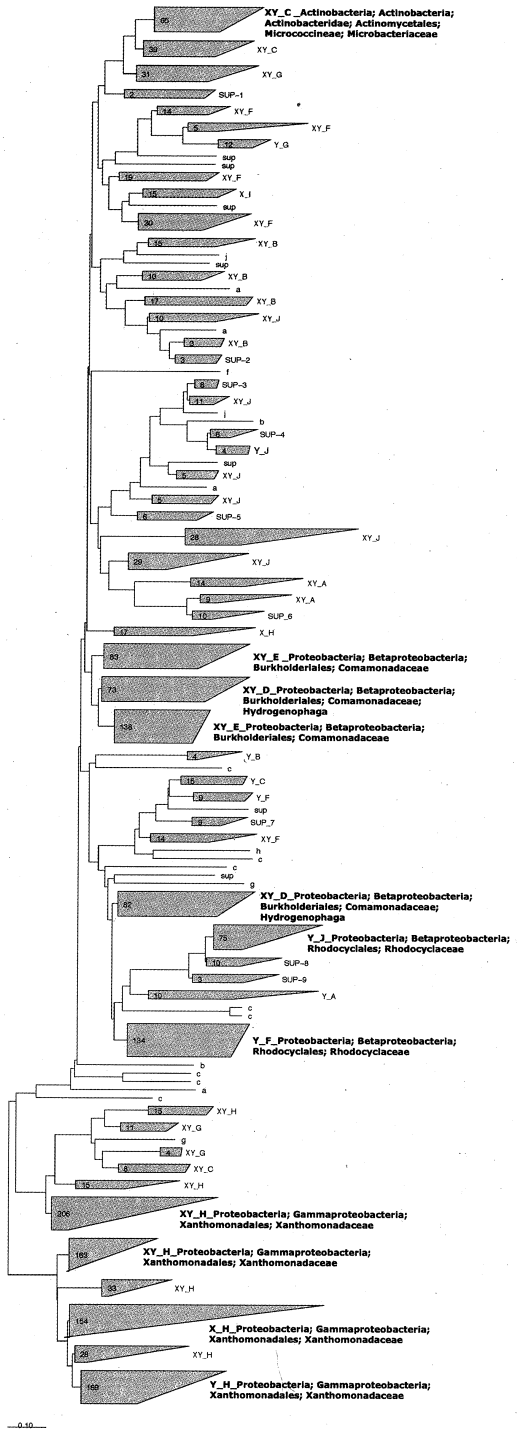

Fig. 3 Phylogenetic Tree of the Reads