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Metagenomic Profile of Bacterial Community and Phage in Domestic Wastewater Treatment Plant

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Abstract. For a better understanding of the microbial community and associated phage abundance in domestic wastewater treatment system, this study did a metagenomics profiling of bacterial community and phage in activated sludge process and chlorination system. The Phylum *Proteobacteria* is the most abundant group in all streams but the most abundant subgroups of this is different between the raw wastewater and those in activated sludge. The former comprises *gamma* and *epsilon Proteobacteria* whereas activated sludge consist of *delta* and *alpha Proteobacteria*. The other less abundant Phylum in raw domestic wastewater are *Firmicutes*, *Bacteroidetes* and *Actinobacteria*. The group *Delta Proteobacteria* are most susceptible to chlorination. *Rhodocyclaceae (*of *beta Proteobacteria) and* the family *Oxalobacteraceae* and *Comamonadaceae* are also among the most affected by chlorination. *Rhodocylaceae* and *Procabacteriaceae* are resistant to chlorine treatment. *Rhodocyclaceae* are well known polyphosphate accumulating bacteria, which are also responsible for EBPR and their viability is high in activated sludge. On virome profiling, in the influent, the crAss phage**,** which is commonly found in fecal bacteria, predicted to the host Bacteroidetes, was dominant. The other phage such as *Pseudomonas*, *Escherichia coli*, *Aeromonas* and *Lactococcus* may also derived from the human gut. EBPR (enhanced biological phosphate removal)- associated phage were observed in all samples except influent. As their hosts, phosphate accumulating bacteria were present in activated sludge.

Keywords: activated sludge, aerobic, chlorination, microbiome, virome

1. Introduction

This study aimed to determine the metagenomics profile of bacterial community and phage in domestic wastewater treatment system, which includes an aerobic activated sludge process, settling and chlorination. To determine live cell DNA vs total cell, the bacterials cells were treated with ethidium monoazide (EMA) prior to DNA extraction. Most studies on microbial community profile in wastewater treatment system were based on DNA extracted on all cells, regardless of the viability of cells. EMA can penetrate through ruptured membrane of dead cells but not through intact membrane of live cells (Riedy et al., 1991). Once inside the dead cells and upon exposure to visible light, the azide group of EMA covalently cross-links with the nucleic acids resulting in fragmentation of the DNA of dead bacteria (Soejima et al., 2007). So far no studies have shown metagenomic analysis of both total and viable bacterial community and associated phage, in domestic wastewater treatment.

2. Methods

2.1. Wastewater Profile and Treatment System

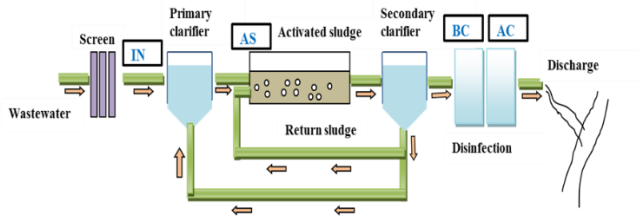

Figure 1. The domestic wastewater treatment system flow diagram and the sampling points for the influent raw wastewater (IN), activated sludge (AS), effluent before chlorination (BC or EBC), and effluent after chlorination (AC or EAC).

The wastewater and sludge samples were collected from a domestic wastewater treatment plant in Tokyo, which treats 112,900 m^3/d wastewater and includes a conventional activated sludge (AS) system (5h hydraulic retention time (HRT) mainly for organic matter removal and chlorination (ca. 1 mg/L of 12% NaClO solution, 20 minutes HRT) for disinfection (Fig. 1). The influent biological oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids, total N and total P were 220, 130, 200, 37.3 and 4.4 mg/L, respectively. The samples were processed and analyzed on the same day. Sludge samples were aerated while in transit.

2.2. Preparation of Wastewater and Sludge Samples

The samples, IN (1.8L) and AS (80ml, with prior 10 min homogenization) samples were centrifuged at 8,000 rpm, 4˚C for 10 min. and the pellet was suspended in 10 ml of bacteria-free water (BFW). EBC and EAC samples (5 L each) were filtered (0.22 µm pore). The filter was washed with 2 ml BFW and kept at 4°C. All the samples were diluted with Milli-Q water to optical density OD_{600nm} value of 0.5 – 1. To distinguish between DNA of total cells and DNA of viable cells, one part of the suspended solids obtained from each wastewater or sludge samples

samples was subjected to EMA and UV light treatment before DNA extraction, while the other part was directly subjected to DNA extraction without prior EMA treatment. The applied EMA concentration was 100 µg/ml, as determined in a previous experiment, where this level resulted in a significant difference between total and viable cell counts.

2.3. DNA Extraction and Amplification

Genomic DNA from two samples each of IN, AS, EBC and EAC (with or without EMA treatment) were extracted by enzyme treatment (with 600 µl hexadecyl (=cetyl) trimethylammonium bromide buffer; Lysozyme solution, 10 µl of 10mg/ml; achromopeptidase solution, 40 µl of 0.2 mg/ml; Protease K solution, 50 µl of 1 mg/ml, in a series of 0.5 h incubations at 55˚C) and phenol-chloroform beads beating method. To the enzyme-treated samples, 100 µl of 25% SDS and 1 vol PCIA were added, sample was mixed, subjected to beads beating (2x 60 m/s, 30 s) and centrifuged. To the collected supernatant (ca. 450 µl), 1 vol chloroform: Isoamyl alcohol (CHISAM, 24:1 ratio) was added and the mixture was centrifuged again. To the supernatant (ca.300 µl), 0.1 vol 3M CH_3COONa, 0.6 vol iso-propanol and 1.5 µl of glycogen (Glycoblue™) were added, thoroughly mixed, allowed to stand (-20˚C, 2-3 h) to let nucleic acids precipitate. The sample was centrifuged and the pellet was washed with 1 ml 70% cold ethanol solution, centrifuged and the pellet was vacuum-dried for 15-20 minutes, re-dissolved in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and stored at -20˚C. Each centrifugation in the DNA extraction was at 20,000xg, 4˚C and 5 min.

Conventional PCR amplification for the extracted genomic DNA was carried out using the full length 16S rRNA universal primers 27F and 1492 R. The PCR mixture contained 0.75µl of primer 27F (5 µM), 0.75µl primer 1492R (5 µM), 2.5 µl dNTP (2.5 mM), 2.4 µl 10×Ex-Taq buffer, 0.125 µl Ex-Taq (5 U/µl), 1 µl sample DNA, and 17.4 bacteria-free water (BFW).

2.4. Roche/454 pyrosequencing and Analysis of Results

The 16S rRNA gene universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 519R (5'-GWATTACCGCGGCKGCTG-3') were used to amplify approximately 500 bp of the variable regions V1 to V3. Different tags were attached to the forward primer in order to distinguish different samples. The reaction mixture contained 1.0 µl primer 27F (5µM), 1.0µL primer 519R (5 µM), 2.4 µl dNTP (2.5mM), 3.0 µl 10×Ex Taq buffer, 0.15 µl Ex Taq (5U/µl) and 5.0 µl sample DNA, and 17.45 µl BFW. The PCR protocol includes denaturation at 95°C for 5 min, 30 cycles of amplification (denaturation at 95°C for 30 s, annealing at 50°C for 30 s, elongation at 72°C for 60 s), and a 5-min final extension at 72°C. PCR products were purified and multiplexed by combining 10 ng of purified DNA from each. Roche 454 GS-FLX Titanium pyrosequencing was performed by Hokkaido System Science Co., Ltd. All 16S rRNA gene pyrosequencing reads were analyzed using QIIME v 1.5.0. Quality criteria were 350 - 560 bp sequence length, at least 50 quality score, no ambiguous bases or mismatches in the primer sequence, 6 bp maximum homopolymer length. Sequences were clustered into operational taxonomic units (OTUs) at threshold of 97% similarity. Rarefaction curves and measures of alpha diversity were computed in QIIME's pipeline.

2.4. Viral Purification: Phage Lysate Preparation

Phage were obtained from 5.4 L eachof IN and AS samples using polyethylene glycol (PEG) precipitation method, which includes a prior centrifugation (9000 rpm, 4˚C, 15 min). To the collected supernatant 10% w/v of PEG 6000 and 4% w/v of NaCl were added to precipitate the phage and teh mixture was stored at 4˚C, ca. 12 h. The mixture was centrifuged at 9000 rpm, 4˚C and 1 h. To the pellet obtained, 24 ml sodium-magnesium (SM) buffer solution was added to dissolve precipitated phage, and equal volume of chloroform and SM buffer was added to remove any bacteria in the sample. After mixing thoroughly, the resulting mixture was kept at 4˚C, 6 h, centrifuged (8000 rpm, 4˚C, 10 min), and the uppermost layer, which contains the phage lysate, was collected and stored at 4˚C until its use. EBC and EAC samples (3 L each) were centrifuged at 9000 rpm, 4˚C, 30 min. The supernatant was processed according to a modified procedure of Mendez et al. (2004). The phage lysate solutions were stored at 4˚C until it their use.

2.5. Phage DNA Extraction, MiSeq Illumina, Virome Analysis

To the phage lysate solution were added, DNase/RNase solution (composition per ml: 4 mg RNase A (bovine pancreas), 10 mg DNase I (bovine pancreas), 10 µl1M Tris-HCl (pH7.5), 5 µl1M MgCl2, 10 µl1M NaCl, and 500 µl glycerol) at 0.2% v/v, equal volume of 2.5 M NaCl-20% PEG solution. The resulting solution was kept in ice for 60 minutes and centrifuged at 17,4000x g, 1 min, 4˚C. The pellet was resuspended in 300 µL SM buffer. EDTA, Proteinase K and SDS were added in turnsto a final volume of ca. 500 µl. The mixture was incubated (1 h, 56˚C), cooled to room temperature and equal volume (500 µl) of Tris-EDTA saturated (TE-saturated) phenol was added. The mixture was gently mixed forming an emulsion, which was later centrifuged (3000xg, room temperature, 5 min). The aqueous phase was collected and equal volumes of TE-saturated phenol and CIAA mixture (PCIAA) were added. The mixture in the tube was inverted several times gently, centrifuged (3000xg, room temperature, 5 min). To the aqueous phase, equal volume of CIAA was added again and the mixture was centrifuged again similarly as the preceding one. The aqueous phase was separated and to it the following were added: 1 µl of Glygoen, 99% EtOH (2 volume) and 3M CH_3COONa (0.1 volume). The resulting mixture was thoroughly mixed and centrifuged at 17,400 x g for 30 minutes at 4˚C. After removing the supernatant, the pellet was washed gently with 70% ethanol (EtOH), centrifuged, and the EtOH was discarded. The pellet remaining in the tube was dried inside a desiccator and vacuum was applied for 15 minutes. TE buffer (30-100 µl) was added to resuspend the DNA. The phage DNA concentration was measured using Nanodrop 2000. The extracted phage DNA was also checked through gel electrophoresis (0.8 % agarose, 100V, 30 mins) with λ marker. The extracted DNA was sent to a company in Tokyo for MiSeq Illumina sequencing. Abundance of genes are presented in terms of number of reads and the relative abundance (number of reads for a particular gene divided by the total reads).

3. Results and Discussion

3.1. Diversity of bacterial community in influent domestic wastewater

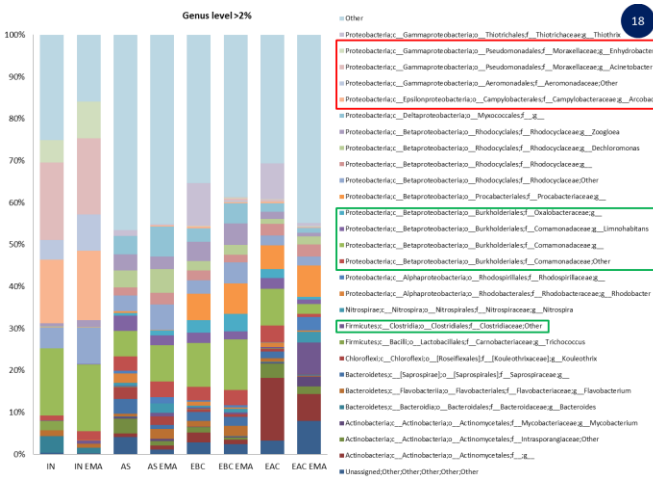

Fig. 2. Diversity of bacterial community in domestic wastewater.

The analysis of the pyrosequencing reads, as shown in fig. 2, show the Phylum Proteobacteria as the most abundant group in IN, AS, EBC, EAC. The next abundant Phylum are Firmicutes, Bacteroidetes and Actinobacteria. The lineage of Proteobacteria is totally different in the influent (IN) compared to other streams. The IN samples have γαβεσσ gamma γ- and ε-Proteobacteria, while AS has σ- and α-Proteobacteria. In IN, about 30% of reads are affiliated as γ-Proteobacteria, 15% are ε- and 20% are β-Proteobacteria. Many pathogens are γ-Proteobacteria. Most of the known ε-Proteobacteria species like Helicobacter and Campylobacter inhabit the digestive tract of animals and serve as symbionts or sometimes pathogen. They were significantly lost in AS suggesting high performance or sufficiently aerobic condition of the activated sludge system. Firmicutes and Bacteroidetes are the most abundant in both healthy human feces and human intestine or gut (Ley et al., 2006). Their viability decreases in the sewage treatment plant. The bacteria groups Firmicutes and Bacteroidetes have less viability in raw influent (IN-EMA sample). In AS, the most dominant class of Proteobacteria are delta, beta and alpha (7%, 25% and 8%, respectively).

Results for the viable community (shown in EMA treated samples), show that some bacterial groups shown in samples without EMA treatment are actually non-viable cells. Performing both, i.e., with and without EMA treatment, shows which bacterial groups are short-lived in the system. As an example, the sludge bulking bacteria group Thiothrix, a γ-Proteobacteria, constitute almost 10% in EBC and EAC reads but this group is no longer viable.

Delta-Proteobacteria are common in AS and EBC and its abundance is reduced to less than 1% after chlorination. Among β- Proteobacteria, the family Rhodocyclaceae was affected by disinfection (7% viability), whereas, the family Oxalobacteraceae and Comamonadaceae (4% viability) are the most effected by chlorination. Procabacteria group is less found in AS and increasing percentage in EBC and EAC. Less than 2% of the Phylum Firmicutes were in IN and AS, and about 10% in EAC. Bacteroidetes was no longer viable in AS and also in following treatment steps. Half percentage of Phylum Actinobacteria was killed by chlorination. The "unassigned, other" bacteria are resistant to chlorination. The viable bacterial groups found in AS are probably mostly essential for organic matter and nutrient removal. The viable microbial community population profile has similar patterns compared with that of the total population, which includes dead and viable bacterial cells. That β-Proteobacteria followed by γ-proteobacteria as the most abundant in AS (30-40%) as viable cells) is consistent with findings of Kwon et al. (2010) for total cells. One of the most dominant Betaproteobacteria classified into Rhodocyclaceae (ca. 20% viability in AS sample), is well known as polyphosphate accumulating bacteria. They are also responsible for enhancing biological phosphorus removal (EBPR). Further, the sludge bulking or flocking bacteria group Thiothrix was detected as non-viable in all effluent samples. They have likely become short-lived in the system due to unfavorable conditions.

The bacteria concentration dropped by two orders of magnitude after chlorination as indicated by quantification of 16S rRNA gene (data not shown). Procabacteriaceae (a β-proteobacteria) were found in all effluent samples, EBC and EAC, with or without EMA treatment but not in the activated sludge, indicating their viable presence and resistance to chlorination. Clostridiaceae belonging to the phylum Firmicutes is shown in this study to be probably chlorine-resistant as chlorination disinfection treatment did not kill nor destroy their cells. Its viable percentage in EAC (EMA-treated sample) was almost 10%. The spore of anaerobic bacteria Clostridium perfringens are resistant to primary and secondary treatment and chlorine disinfection of wastewater and they are used as indicators of presence of protozoa cysts in water (Sobsey et al., 1998). The pyrosequencing result in this study revealed family level as the lowest classification for this Phylum and it cannot show whether all Clostridiaceae in EAC belong to the species perfringens. Clostridia include species that are capable of producing heat-resistant endospores and they are related to some important human pathogens that are responsible for deadly diseases such as anthrax, botulism, gas gangrene and tetanus (Galperin et al., 2012). The behavior of Actinobacteria, which is classified as genus Mycobacterium, towards chlorination is similar to that of Clostridiaceae to chlorination. Their tolerance to chlorine is probably attributed to their having hard cell wall, which contains mycolic acid, peptidoglycan, polysaccharides and arabigalactan. Alterations in mycolic acids would be the reason to change the membrane permeability to chlorine (Steed and Falkinham, 2006), because the mycolic acid structure is a determinant of membrane fluidity in Mycobacteria (Liu et al., 1996). Unassigned "other" bacteria are also resistant to chlorination. Among β-proteobacteria, Comamonadaceae are sensitive to chlorination. They have the lowest relative abundance in EMA-treated EAC. Rhodocyclaceae and Procabacteriaceae are potentially resistant to chlorine treatment. Clostridium and Mycobacterium are of great concern. Their presence in effluents is consistent with

other studies and their viability is shown strong in this study.

3.2. Phage in domestic wastewater treatment system

The phage concentration (table 1) is one order higher than the bacterial concentrations. The virome and bacterial community analysis of the wastewater and sludge samples did not show a clear relationship between them. But, several features of the virome profile may give insights on the bacterial groups present in the wastewater or sludge samples. The information on the viruses present in the samples may be viewed with the premise that viruses coexist with their target bacteria. In the influent sample (IN), crAssphage is highly dominant (table 1). These crAss phages are highly abundant bacteriophage that is recently discovered in the unknown sequences of human fecal metagenomes using a new occurrence profiling approach. They are predicted to infect bacteria of the genus Bacteroides, the abundant group in the wastewater. Its low viability is attributable to the high abundance of crAss phage in the influent wastewater.

In AS, EBC and EAC samples (table 1-b,c,d), there are phages for bacteria associated with enhanced biological phosphate removal (EBPR). The abundance of the combination of all EBPR-associated bacterial phage in terms of sequence reads is consistent in all samples. In bacterial consortia, the EBPR bacterial groups Rhodocyclales are found to be resistant to chlorination disinfection. The abundance of those EBPR-associated bacteria is decreasing from AS to EBC and EAC. This resistance of viruses is consistent with the results of Wu and Liu (2009) that indicated that viruses are resistant to chlorination while some bacterial groups are no longer found on wastewater after chlorination.

Comparison between the abundance of bacteria and their respective phage shows that the decrease or increase in their abundance (i.e., in wastewater to and from aerobic treatment, i.e., activated sludge process and from aerobic process to chlorination, and comparing influent and sludge in AS process) are consistent. That is, both bacteria and their respective phages increase or both decrease as they go though the treatment process. The quantity of associated phage for a bacterial species is generally higher than that of the bacteria.

4. Conclusion

This study showed the diversity of bacterial community in domestic wastewater treatment system. With EMA, the viable community was distinguished from the total bacterial metagenomics profile, showing the short-lived groups and the persistent ones. Generally, phage increases with increasing bacterial cells. The raw domestic wastewater bacterial community is influenced by human fecal or gut microbiome. The phylum Proteobacteria is the most abundant group in all streams but the subgroup abundance varies between the raw wastewater and those in activated sludge. The group σ-Proteobacteria are most susceptible to chlorination.

5. Acknowledgement

Funding support by AUN/SEED-Net is acknowledged.

Table 1 a, b, c, d Phage profile in IN, AS, EBC and EAC (top to bottom)

Acc. #	Reads	virus/phage
NC024711	83885	crAssphage
HF679133	2862	Choristoneura rosaceana entomopox virus 'L'
GU911519	2384	Acinetobacter phage Acj61
EU081845	2094	Lactococcus phage 1706
KM672662	2038	Acinetobacter phage YMC13/03/R2096
AY625898	1660	Pseudomonas aeruginosa phage F116
KC348599	1619	Streptococcus phage phi30c
KC262634	1324	Pseudomonas phage H66
KJ473422	1235	Acinetobacter phage vB_AbaM_Acibel004
GU196277	1192	Escherichia phage K1G
KM389247	1132	Pseudomonas phage JBD90
KM058087	1081	Cronobacter phage vB_CsaP_Ss1
JX100810	1042	Caulobacter phage CcrColossus
KJ003983	1008	IAS virus
GU196278	983	Escherichia phage K1H
KC959568	951	Flavobacterium phage 6H
JQ177063	950	Aeromonas vB_AsaM-56
GU196279	944	Escherichia phage K1ind1
KC348660	939	Streptococcus phage phi5218
KP861230	923	Acinetobacter phage YMC11/11/R3177
AM491472	894	Salmonella phage E1

Acc. #	Reads	virus/phage
KC977571	9635	Pandoravirus salinus
KC977570	7961	Pandoravirus dulcis
AY605181	2569	Burkholderia cepacia complex phage BcepC6B
JF412295	2151	EBPR podovirus 2
JF412294	1152	EBPR podovirus 1
AF281817	1088	Tupaia herpesvirus strain 2
AY349011	910	Burkholderia cepacia complex phage Bcep22
KC821618	599	Cellulophaga phage phi10:1
JN662425	585	Burkholderia phage DC1
KC821620	584	Cellulophage phage phi18
AB720064	511	Xanthomonas citri phage CP2
HQ634153	503	Salicola phage CGphi29
AY625898	461	Pseudomonas aeruginosa phage F116
NC024711	447	crAssphage

Acc. #	Reads	virus/phage
AF191073	5011	Stealth virus 1 clone 3B43
JF412295	2167	EBPR podovirus 2
NC024711	1119	crAssphage
JF412294	991	EBPR podovirus 1
AY349011	777	Burkholderia cepacia complex phage Bcep22
KC821624	754	Cellulophage phage phi14:2
AY625898	718	Pseudomonas aeruginosa phage F116
AF065755	661	Stealth virus 1 clone 3B43 T3
JN662425	608	Burkholderia phage DC1
KC821618	590	Cellulophaga phage phi10:1
JX507079	585	Acidithiobacillus phage AcaML1
AB720064	551	Xanthomonas citri phage CP2
KF301602	548	Caulobacter phage Cr30
KM389247	526	Pseudomonas phage JBD90
JQ067093	524	Pseudomonas phage PaMx74

Acc. #	Reads	virus/phage
AF191073	5720	Stealth virus 1 clone 3B43
AF063866	4496	Melanoplus sanguinipes entomopoxvirus
AF250284	4449	Amsacta moorei entomopoxvirus
HF679132	3536	Choristoneura biennis entomopoxvirus 'L'
HF679131	3478	Adoxophyes honmai entomopoxvirus 'L'
HF679133	3337	Choristoneura rosaceana entomopoxvirus 'L'
JF412296	2111	EBPR podovirus 3
AP008983	1777	Clostridium phage c-st
JF412295	1438	EBPR podovirus 2
AF281817	1410	Tupaia herpesvirus strain 2
NC024711	1386	crAssphage
JX507079	1334	Acidithiobacillus phage AcaML1
EU826466	1303	Mycobacterium phage Myrna
JF412294	1221	EBPR podovirus 1
HF586477	812	Cotesia congregata bracovirus proviral locus 6 (PL6)
AY605181	660	Burkholderia cepacia complex phage BcepC6B
AB720064	659	Xanthomonas citri phage CP2
AY349011	654	Burkholderia cepacia complex phage Bcep22

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