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Horizontal transfer of antibiotic resistance genes in a membrane bioreactor

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ABSTRACT

Growing attention has been paid to the dissemination of antibiotic resistance genes (ARGs) in wastewater microbial communities. The application of membrane bioreactors (MBRs) in wastewater treatment is becoming increasingly widespread. We hypothesized that the transfer of ARGs among bacteria could occur in MBRs, which combine a high density of bacterial cells, biofilms, and antibiotic resistance bacteria or ARGs. In this study, the transfer discipline and dissemination of the RP4 plasmid in MBRs were investigated by the counting plate method, the MIDI microorganism identification system, and quantitative polymerase chain reaction (qPCR) techniques. The results showed that the average transfer frequency of the RP4 plasmid from the donor strain to cultivable bacteria in activated sludge was 2.76×10^{-5} per recipient, which was greater than the transfer frequency in wastewater and bacterial sludge reported previously. In addition, many bacterial species in the activated sludge had received RP4 by horizontal transfer, while the genera of *Shewanella* spp., *Photobacterium* spp., *Pseudomonas* spp., *Proteus* spp., and *Vibrio* spp. were more likely to acquire this plasmid. Interestingly, the abundance of the RP4 plasmid in total DNA remained at high levels and relatively stable at 10^4 copies/mg of biosolids, suggesting that ARGs were transferred from donor strains to activated sludge bacteria in our study. Thus, the presence of ARGs in sewage sludge poses a potential health threat.

1. Introduction

The constant emergence of antibiotic resistance is becoming a major global health issue due to health risks in humans (Levy, 2002; Munir et al., 2011). Various bacterial resistances are generally controlled by different types of antibiotic resistance genes (ARGs) (Zhang et al., 2009). Moreover, the ARGs can transfer among bacteria of different taxonomic and ecological groups by means of mobile genetic elements (Levy and Marshall, 2004), leading to the dissemination of resistance genes (Davies, 2007; Hawkey and Jones, 2009). Over the years, almost all kinds of ARGs, encoding resistances to aminoglycoside (Taviani et al., 2008), β-lactam (Antunes et al., 2006), trimethoprim (Ferreira da Silva et al., 2007), tetracyclines (Auerbach et al., 2007), and vancomycin (Caplin et al., 2008), have been detected in various water environments (Caplin et al., 2008), including wastewater, sewage, surface water, and groundwater. So, wastewater treatment plants (WWTPs) directly receive ARGs with the inflow of sewage water through the wastewater collection systems; thus, wastewater collection systems must be considered as an important pool of ARGs (Mohapatra et al., 2008; Schwartz et al., 2003).

It has been more than three decades since the horizontal transfer of plasmids in activated sludge and wastewaters has been first studied, and plasmid-mediated dissemination of ARGs has been widely recognized to take place in wastewater environmental compartments (Gealt et al., 1985). Multiple antibiotic-resistant bacteria can spread their ARGs to susceptible strains of the same species or other species or genera with different mechanisms, mainly by conjugative R plasmids (Mispagel and Gray, 2005). Moreover, in situ transfer of the plasmid RP4 can occur among released and indigenous bacteria in activated sludge (Geisenberger et al., 1999; Soda et al., 2008).

With people laying increasing emphasis on the water crisis, wastewater reclamation is considered to be a crucial way of reserving natural resources used for recreational purposes or even the drinking water supply. Furthermore, effluent standards have become increasingly stringent (Hussain et al., 2010). Compared with conventional wastewater treatment, membrane bioreactor (MBR) technology has been proven to be an efficient and

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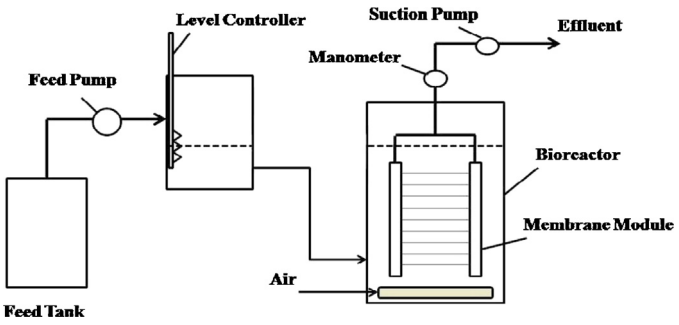

Fig. 1. Membrane bioreactor schematic diagram.

commercially viable process to reclaim water. Therefore, this process for municipal and industrial wastewater treatment is becoming increasingly widespread (Ben Aim and Semmens, 2003; Judd, 2008). The use of membrane filtration, which allows the complete physical retention of bacterial flocs and most of the suspended solids, leads to high mixed liquor suspended solid (MLSS) concentrations in a MBR with floating and biofilm formation (Rosenberger et al., 2002; Ueda and Hata, 1999; Wu and He, 2012; Wu et al., 2008). It has been reported that a significant level of antibiotic resistance transfer occurs in WWTPs and that a MBR is more likely to be considered one such hot spot that seems to promote transfer rates because it combines a high density of bacterial cells, biofilms, and antibiotic resistance bacteria or ARGs (Droge et al., 1998; Mach and Grimes, 1982; van Elsas and Bailey, 2002).

As mentioned above, we considered that ARG transfer would occur as bacteria entered into a MBR with sewage, resulting in dissemination of antibiotic resistance in sludge communities and development of new resistant bacteria. Additionally, most ARGs cannot be effectively removed through treatment processing (Auerbach et al., 2007; Volkmann et al., 2004). Therefore, ARGs may accumulate and be released into the environment with effluents and discharged biosolids, posing ecosystem risk and health hazards. This study is the first to investigate the transfer discipline of ARGs in microbial communities in a MBR.

In this study, we have evaluated the profile of conjugative transfer mediated by plasmid RP4 in the indigenous bacterial population of activated sludge in a MBR, including cultivable and uncultivable bacteria. The results will help (I) to survey the transfer of ARGs in microbial communities when ARGs enter into a MBR with sewage; (II) to quantify the abundance of ARGs in biosolids; (III) to further shed light on ARG dissemination in a MBR; and (IV) to evaluate the operation performance of the MBR after being inoculated with donor bacteria.

2. Materials and methods

2.1. Construction of a lab-scale MBR

A conjugation experiment was conducted in a lab-scale submerged MBR, which was a cube-type reactor (23 cm × 13 cm × 60 cm) with a working volume of 10 L. Fig. 1 shows the schematic diagram of the MBR. The hollow-fiber membrane module was made of polyvinylidene fluoride (PVDF, Tianjin Motian Membrane Eng. Co. Ltd., China) with a frame dimension (L × W) of 17 cm × 15 cm, a pore size of 0.22 μm, and a total surface area of 0.2 m^2. A level controller was used to regulate the feeding pump, and the effluent was drawn directly from the MBR through the membrane by a suction pump. The effluent was removed by a pump that operated periodically for 8 min, separated by 2-min intervals. A manometer was mounted between the membrane module and the suction pump to monitor the trans-membrane pressure (TMP) (Li and Chu, 2003). Air was applied through a dispenser installed at the bottom of the reactor. The initial MLSS concentration of the activated sludge biomass was about 2.7 g/L. Air was applied through a dispenser installed at the bottom of the reactor at a flow rate of 0.2 L/min. The temperature was maintained at approximately 20–30 °C, and the pH was controlled at 7.0–8.0. The chemical oxygen demand (COD_{cr}) concentration in the synthetic wastewater was in the range of 194.3–315.9 mg/L, and the composition of the synthetic wastewater is detailed elsewhere (Moy et al., 2002). MLSS, COD_{cr}, and ammonia nitrogen concentration (NH_4^+-N, mg/L) were measured according to standard methods (APHA, 1992).

2.2. Conjugation experiment in the MBR

The activated sludge seeded in the MBR was collected from the secondary settling tank of the Ji Zhuangzi Wastewater Treatment Plant in Tianjin, China. The donor strain used in this study was rifampicin (Rif)-resistant *Escherichia coli* K12 (ATCC 47076), harboring the plasmid RP4 (accession number: L27758). The donor strain was grown at 37 °C in Luria-Bertani (LB) broth with appropriate antibiotics: 60 mg/L kanamycin (Km, Sigma–Aldrich, USA) and 40 mg/L Rif (Sangon Biotech, Shanghai, China). To reduce the impact of the environmental matrix and microbial communities, a self-control experiment was applied in this study. In other words, the donor strain was inoculated into the MBR after the reactor had reached steady state for 20 days, and the reactor operation parameters remained the same as previously described. Before donor strain inoculation, background counts of the donor strain and the bacteria carrying RP4 were determined, and the results showed that they were undetectable. The initial concentration of potential cultivable recipient bacteria was 7.0×10^6 CFU/mL. The donor strain added to the reactor was at a concentration of 5.5×10^5 CFU/mL in the system.

Following donor strain inoculation, 20 mL of sludge was sampled from the bioreactor every 24 h; and these samples were used for plate counting and DNA extraction. Detecting the number of transconjugants with RP4 transfer was performed as follows (Soda et al., 2008; Sorensen, 1993). The number of indigenous potential recipients (*Nr*) was determined with culturing on LB agar plates. The number of donor strains (*Nd*) was enumerated using LB agar plates containing 60 mg/L Km, 60 mg/L Ampicillin (Amp, Sigma–Aldrich, USA), 40 mg/L tetracycline (Tc, Sangon Biotech, Shanghai, China), and 40 mg/L Rif. The number of bacteria carrying plasmid RP4 (*Np*) was determined with culturing in LB agar plates containing Amp, Km, and Tc. Cycloheximide (Sangon Biotech, Shanghai, China, 50 mg/L) was added to all solid media to prevent fungal growth. The transfer frequency (*f*) of plasmid RP4 was obtained on the basis of the parameters mentioned above using the formula $f = (Np - Nd)/Nr$.

2.3. Identification of the cultivable transconjugants

Transconjugant strains were grown in LB broth containing 60 mg/L Amp, 60 mg/L Km, and 40 mg/L Tc overnight in a reciprocal shaker (120 rpm) at 37 °C. Then, they were confirmed to possess the RP4 plasmid using PCR-based detection technology. Plasmid DNA extraction was performed using an EZ Spin Column Plasmid Isolation Kit according to the manufacturer's instructions (Biomega Inc., USA, product number 121101100914). In this study, oligonucleotide primers P1 (5′-AAAGCGGACAGCATCAGTAACGAA-3′) and P2 (5′-GAGCTTGGTGGCCGCATAGTGTAG-3′) were designed for detection of RP4 (aphA gene). PCR conditions were 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s. Aliquots (5.0 μL) of the PCR products (140 bp)