論文内容の要旨

Abstract of Dissertation

Phylogenetic identification of estrone-degrading bacteria in activated sludge using microautoradiography-fluorescence *in situ* hybridization
（活性汚泥中に存在するエストロン分解細菌のマイクロオートラジオグラフィー蛍光 *in situ* ハイブリダイゼーション法を用いた系統学的同定）

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Endocrine disrupting chemicals (EDCs) are described as the exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)-populations (WHO/IPCS, 2002). In the last 20 years, a series of studies reported the reproductive changes in various species of fish and mollusks living downstream of sewage treatment plant (STP) outfalls (Howell *et al*., 1980, Bortone and Davis, 1994, Purdom *et al.* 1994, Tyler *et al.*, 1998, Irwin *et al.*, 2001 and Gagne *et al.*, 2002). The causative compounds were suggested to be the EDCs in sewage effluents such as 4-tertiary isomers of nonylphenol and octylphenol, natural and synthetic estrogen including 17β-estradiol (E2), estrone (E1), estriol (E3) and 17α-ethinylestradiol (EE2). Although a variety of EDCs have been observed in sewage effluents, the further toxicity identifications or evaluations of EDCs in sewage effluents suggested that natural estrogens and occasionally the synthetic estrogen were the main contributors to the estrogenic potency of sewage effluents (Desbrow *et al.*, 1998, Rodgers-Gray *et al.*, 2000 and Aerni *et al.*, 2004).

The natural estrogens after discharged by households are transported to STPs, where ideally, they are completely degraded before discharged to water environments. However, the removal efficiency of natural estrogens in real STPs varies greatly (Fujii *et al.*, 2002; Joss *et al.*, 2004; Johnson *et al.*, 2005 and Auriol *et al.*, 2006). So far, the reasons causing insufficient estrogen-removal are still unclear. It is likely that longer HRT and SRT contribute to better removal (Saino *et al.*, 2004 and Johnson *et al.*, 2005). Such findings indicated the importance of biological activity to estrogen removal.

To date, a number of bacterial strains have been isolated from activated sludge or soil, which can utilize E1, E2, E3 or even EE2 as carbon sources. Such isolates include *Novosphingobium tardaugens* sp. nov (Fujii *et al.,* 2002), *Rhodococcus zopfii* (Yoshimoto *et al.*, 2004), *Rhodococcus equi* (Yoshimoto *et al.*, 2004), *Alcaligenes xylosoxidans* (Weber *et al.*, 2005), *Ralstonia sp* (Weber *et al.*, 2005) and *Denitratisoma oestradiolicum* (Fahrbach *et al.*, 2006). In addition, *Nitrosomonas europaea*— well-known ammonia-oxidizing bacteria isolate was also proved capable of degrading E1 E2, E3 and EE2 to a certain degree by co-metabolic activity (Shi *et al.,* 2004). It is still unclear, however, who are the key members responsible for estrogen degradation in a complex microbial community such as activated sludge. This problem can be partially addressed using microautoradiography-fluorescence *in situ* hybridization (MAR-FISH) analysis.

MAR-FISH, as a powerful tool to directly link phylogenetic identity of a cell to its specific metabolic activity in a microbial community, has successfully analyzed the phylogeny and *in situ* physiology of microbial communities in many studies. So far, most MAR-FISH studies carried out are related to the nutrient uptake activities of microbial communities in different environments, and very limited work have been done to investigate the micropollutant-uptake activity by a microbial community in their habitat.

The aim of this study was to explore the bacteria responsible for the *in situ* E1 degradation in activated sludge, using the improved MAR-FISH.

In preliminary tests, slightly modified MAR-FISH technique with [^{3}H]E1 and [^{3}H]E1-3-sulfate conjugate ([^{3}H]E1-S) as radioisotope tracers was firstly applied to E1- and/or E1-S-degrading pure cultures *Rhodococcus* sp. ED7 and *Sphingomonas* sp. ED8 to demonstrate the feasibility of MAR-FISH for *in situ* analysis of E1- and E1-S-degrading bacteria. Before the validation, a slight modification of the washing step after MAR incubation in MAR-FISH analytical procedure was made in order to remove the adsorbed radioactive substrates from biomass more efficiently.

In the first stage of this study, the uptakes of [^{3}H]E1 and [^{3}H]E1-S by the major phylogenetic groups in activated sludge samples were investigated using MAR-FISH with broad group specific oligonucleotide probes. The activated sludge samples were taken from the anaerobic-anoxic-oxic process from two municipal wastewater treatment plants locating in Tokyo, Japan. Each sludge sample was incubated with 200 μg/L of tritium-labeled and unlabeled E1 mix. During E1 degradation process, the distribution patterns of tritium-assimilating cells among the major phylogenetic groups at different times were examined.

In the early phase of E1 degradation, about 1-2% of total bacterial community in both activated sludge samples were detected as [^{3}H]E1-assimilating cells. Around 60-80% of EUBmix-defined [^{3}H]E1-assimilating cells were hybridized with probe Bet42a for *β-proteobacteria* and 40-20% hybridized with probe GAM42a for *γ-proteobacteria*. Under the microscope, the betaproteobacterial MAR positive (+) cells were mostly rods in chain, but sometimes rods. While the gammaproteobacterial MAR (+) cells were in spherical or short rod shapes. Alphaproteobacterial tritium-assimilating cells were only detected in the late phase of E1 degradation. By the time of 90% of added E1 degraded, no tritium-assimilating cell associated with *Actinobacteria*, *Nitrospira*, *Planctomycetes* and *Cytophaga-flavobacter* cluster was detected. In the end of E1 degradation, a significant potion of tritium-assimilating filamentous cells related to *Chloroflexi* was detected in both sludge samples, which was likely caused by the cross-feeding of radioactive metabolites or microbial products derived from tritium-labeled bacteria.

Similarly, the investigation of E1-S degradation in activated sludge samples revealed that the betaproteobacterial MAR (+) cells with the same morphotypes accounted for the biggest portion in the total tritium-assimilating cell communities, while gammaproteobacterial MAR (+) cells ranked second.

In summary, both beta- and gamma-proteobacterial MAR (+) cells contributed the majority of [^{3}H]E1- or [^{3}H]E1-S-assimilating cells in the studied activated sludge samples. Betaproteobacterial MAR (+) cells contributed most to the *in situ* E1 and E1-S degradations.

In the second stage of this study, a set of hierarchic oligonucleotide probes targeting the subgroups of *β-proteobacteria* were applied in MAR-FISH, analyzing activated sludge M in order to target the most important *in situ* E1-degrading bacteria in more and more specific phylogenetic level. MAR-FISH analysis revealed that around 99% of betaproteobacterial MAR (+) cells were hybridized with probe BONE, which targets β1 subgroup of *Proteobacteria*. No MAR (+) cells was observed binding of probe BTWO which targets most members in order *Rhodocyclales*. The further screening for MAR (+) cells within β1 group revealed probe Cte, which mainly targets the members in family *Comamonadaceae* and *Incertae sedis*-group, targeted betaproteobacterial MAR (+) cells as efficiently as probe BONE. Subsequent screening for MAR (+) cells was carried out within family *Comamonadaceae* and *Incertae sedis*-group with more specific probes (or probe mix). The applied probes include probe ACI208 targeting genus *Acidovorax*; probe mix COM1424 and CteA targeting genus *Comamonas*; probe DEN220 targeting acetate-denitrifying bacteria affiliated with genera *Acidovorax* and *Comamonas* and some others in *Comamonadaceae* family; probe PSP-6 targeting genera *Aquabacterium*, *Leptothrix*, *Rubrivivax* and *Ideonella* in *Incertae sedis*-group, and probe PS-1 targeting four isolated *Leptothrix* spp. However, MAR-FISH analysis revealed that none of them could target the betaproteobacterial MAR (+) cells of interest.

In order to design oligonucleotide probes to target the betaproteobacterial MAR (+) cells of interest in activated sludge samples, DNA extracted from the same activated sludge sample was amplified by PCR with *β-proteobacteria*-specified primer set, followed by cloning and sequencing. A 690bp-16S rRNA clone library was constructed which contained 175 cloned sequences. One hundred and forty-seven cloned sequences out of 175 were different from each other. Of 175 cloned sequences, 129 of them affiliated with *β*-subclass of *Proteobacteria*.

The retrieved *β-proteobacteria*-associated sequences were diverse in phylogenetic identity. Within *β*-subclass of *Proteobacteria*, the highest number of cloned sequences affiliated with order *Burkholderiales* (71 clones) which was supposed to be targeted by probe BONE in hybridization. Order *Rhodocyclales* ranked second with 38 associated clones and followed by *Nitrosomonadales*, *Hydrogenophilales*, *Methyloversatilis* and *Neisseriales*. Most members in order *Rhodocyclales*, but not *Zoogloea* and *Dechlorosoma-Azospira* clusters were supposed to be targeted by probe BTWO. Within order *Burkholderiales*, 52 cloned sequences were grouped to family *Comamonadaceae*, and 12 cloned

sequences to *Incertae sedis*-group. Besides, three cloned sequences were affiliated with family *Alcaligenaceae* and two cloned sequences affiliated with family *Burkholderiaceae*.

Four oligonucleotide probes were designed according to the cloned sequences information, and used in fluorescence *in situ* hybridization to target most members in *Incertae sedis*-group (probe Inc1352); members in genera *Sphaerotilus*, *Leptothrix*, *Ideonella* and *Schlegelella* in *Incertae sedis*-group (probe Inc1197); and the cloned sequences affiliated with *Sphaerotilus* (probes Spha823 and Spha1037).

Finally, MAR-FISH results with newly designed oligonucleotide probes revealed that the key *in situ* E1-degrading bacteria in the studied activated sludge samples were affiliated with the sheathed *Sphaerotilus* in *Incertae sedis*-group. *Sphaerotilus* related E1-degrading bacteria contributed 60 to 80 % of [^{3}H]E1-assimilating cells during E1 degradations; and 78% of [^{3}H]E1-S-assimilating cells during E1-S degradation in activated sludge samples.

In summary, the *in situ* phylogenetic affiliation study of E1-degading bacteria in activated sludge revealed that betaproteobacterial E1-degrading bacteria contributed most to the *in situ* E1 and E-S degradations in the studied activated sludge samples and followed by gammaproteobacterial E1-degrading bacteria. MAR-FISH analysis combined with rRNA gene library survey successfully identified the most important E1-degrading bacteria as the sheathed *Sphaerotilus* with the newly designed probe Spha823. *Sphaerotilus*-related E1-degrading bacteria were phylogenetically different from all the previously reported E1-degrading bacterial isolates. They were proved as contributing most to the E1 and E1-S degradations in the studied activated sludge samples. The successful identification of the key *in situ* E1- and E1-S-degrading bacteria in activated sludge proved the feasibility of MAR-FISH technique as a discovery tool if coupled with 16S rRNA gene library survey to discover the bacteria with specific function.