

## **Chapter 5**

**Microbial community analysis in denitrifying process under high salinity conditions: a comparison between acetate and methanol as carbon source**

## Summary

*Acetate and methanol were compared in terms of their performance as carbon source for denitrification of wastewater various saline conditions with monitoring microbial community structure in respective reactors. Nitrogen removal performances were determined when salinity concentrations increase gradually in acetate- or methanol-fed denitrifying reactor. As a result, acetate-fed process attained stable and high nitrate removal at 0 - 10% NaCl, while methanol were proven beneficial electron donors at 0 - 3% NaCl. Changes of bacterial community structure with the increase of salinity monitored by molecular biological analysis (i.e. cloning, T-RFLP analysis). Time-course T-RFLP fingerprints of the acetate-fed sludge showed that a specialized microbial population (i.e. the genera Halomonas and Marinobacter) adapted to a high saline environment. On the other hand, there were no significant changes of bacterial populations in the methanol-fed reactor at 4% NaC, although the relative abundances of the genera Azoarcus and Methylophaga increased when salinity concentration was at 1 - 3% NaCl. Therefore, it suggested that methanol-utilizing populations in sewage sludge was unable to adapted to a high saline environments (> 4% NaCl). The results obtained in this study show that the selection of organic carbon source as an electron donor for denitrification was a matter of great important factor to trigger high nitrate removal activity in high saline environments.*

## 5.1 Introduction

With industrial diversification, the biological nitrogen removal technology is required to apply to the various industrial wastewater treatment systems. This study focused on the denitrification process for the industrial inorganic wastewater containing large amounts of nitrate and saline, such as metal refinery wastewater (Yoshie *et al.*, 2001). The biological nitrate removal from wastewaters is achieved by bacterial denitrification, which involves the reduction of nitrate, via nitrite and nitric oxide, to nitrous oxide or dinitrogen gas (Zumft, 1997). In the high saline wastewater treatment, there is a common view that a high saline wastewater is diluted to low salinity to prevent the inactivation of bacteria in the biological process (Hirata *et al.*, 2001; Diner and Kargi, 1999). However, there are some practical problems by diluting saline wastewater to low salinity. One is the augmentation of wastewater volume and operational costs by the use of tap water for dilution. Furthermore, it is difficult to ensure freshwater for dilution in the area that hardly gets freshwater, such as coastal area (Panswad and Anan, 1999). Therefore, it is crucial to develop a biological treatment system of a high saline wastewater without dilution by freshwater (e.g. tap water).

Our previous studies were shown some interesting findings about the biological nitrogen removal from the metal refinery wastewater. First, an improvement in denitrification efficiency was observed at a high salinity in comparison with that at low salinity in the sludge acclimated to a saline wastewater for a prolonged period. Second, the genera *Halomonas* and *Marinobacter* of the *Gammaproteobacteria*, most of which are known as halophilic bacteria, were identified as a major bacterial population in these denitrification systems by PCR-denaturing gradient gel electrophoresis (DGGE) (Yoshie *et al.*, 2001) and fluorescence in situ hybridization (FISH) (Yoshie *et al.*, 2004) and terminal restriction fragment length polymorphism (T-RFLP) (Yoshie *et al.*, 2006a) and culture-dependent method (Yoshie *et al.*, 2006b).

Some inorganic wastewater (e.g. metal refinery wastewater) lacks an organic carbon source as an electron donor for denitrification. Thus, it is necessary to add an external carbon source (e.g. acetate, ethanol, methanol) to achieve denitrification. Methanol has often been chosen because of its relatively low cost and the small amounts of sludge production compared with other organic carbon sources (Nyberg *et al.*, 1992). On the other hand, it has been demonstrated that only some bacterial population can utilize methanol as a carbon source under denitrifying conditions (Hallin *et al.*, 1996, 1998, 2006; Labbe *et al.*, 2003; Ginige *et al.*, 2004; Osaka *et al.*, 2006). Therefore, the external carbon source for denitrification needs to be selected depending on the characteristic of wastewater (Lee and Welander, 1996).

In this study, we characterized the microbial community structure in the denitrifying reactor using acetate or methanol as an external carbon source with the increase of salinity concentrations. Microbial community structure of each saline condition was monitored by T-RFLP and cloning analysis to identify the bacteria playing important role in high saline denitrifying process.

## **5.2. Materials and Methods**

### **5.2.1 Reactor operation and sampling**

The denitrification system used consisted of an anoxic reactor with stirred activated sludge (0.5 l) and sedimentation tank. The hydraulic retention time was 2 days. The characteristics of the synthetic wastewater were as follows: total organic carbon, 2,250 mg l<sup>-1</sup>; nitrate-nitrogen (NO<sub>3</sub>-N), 1500 mg l<sup>-1</sup>; and total phosphorus, 5.0 mg l<sup>-1</sup>. Acetate or methanol was used as an organic carbon source for denitrification, respectively. Initially, the concentration of mixed liquor volatile suspend solids was 5,000 mg l<sup>-1</sup>, and these sludge used in this study were acclimated with each organic carbon source under salinity-free and denitrifying conditions for 4 months. In this study, the saline concentration was steadily increased from 0% to 10% and 0% to 4% with NaCl in acetate-fed or methanol-fed denitrifying reactors, respectively. The water temperature was 25 ± 2°C. These experiments were carried out without controlling of pH because denitrification from high salinity wastewater favor high pH levels (Glass and Silverstein, 1999; Hwang *et al.*, 2006; Peyton *et al.*, 2001; Hoek *et al.*, 1987). For the characterization of bacterial communities, samples were collected from each saline condition and stored at -80°C. All the samples obtained from methanol and acetate reactors were filtered with a glass fiber filter (G/FC, Whatman, UK) and used in water quality measurement. Both NO<sub>2</sub>-N and NO<sub>3</sub>-N concentrations were measured by HPLC with a UV detector (column: IC-Anion-PW, Tosoh Corp., Tokyo, Japan). Total organic carbon (TOC) was measured using a TOC analyzer (TOC-5000A, Shimadzu Corp., Kyoto, Japan). MLVSS was measured according to the standard method (APHA *et al.*, 1992).

### **5.2.2 DNA extraction and PCR amplification**

DNA extraction from sludge samples was performed using ISOPLANT (Nippon Gene Inc., Toyama, Japan) according to the manufacturer's instructions. DNA was

precipitated by adding ethanol and sodium acetate, resuspended in 50  $\mu$ l TE buffer (pH8.0) and stored at -20°C.

The following primer sets were used for PCR amplification: forward primer 8f (Amann *et al.*, 1995) and reverse primer 926r (Muyzer *et al.*, 1995). The PCR mixture contained 10 ng of extracted DNA, 0.2  $\mu$ M concentrations of each primer, 200  $\mu$ M concentrations of dNTP, 2 mM concentrations of MgCl<sub>2</sub>, 5 U of TaKaRa Ex Taq DNA polymerase (TaKaRa Bio Inc., Shiga, Japan), and 5  $\mu$ l of 10 $\times$  PCR buffer for TaKaRa Ex Taq. The PCR amplifications were performed in a total volume of 50  $\mu$ l in 0.2 ml reaction tubes by using a model 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) using the following programs: 3 min at 94°C, 25 cycles (30 sec at 94°C, 40 sec at 56°C, 60 sec at 72°C), and 3 min at 72°C. The presence of PCR products was confirmed by 2% agarose gel electrophoresis and the subsequent staining of the gels with ethidium bromide.

### **5.2.3 Cloning, sequencing, and phylogenetic analysis**

PCR products were purified by using a Wizard SV gel and a PCR clean-up system (Promega, Madison, WI, USA). The PCR amplicons were cloned using the pGEM-T Easy Vector System (Promega) according to the manufacturer's instructions. Then, colonies were randomly picked up with a needle and transferred to Insert Check Ready Solution (Toyobo, Osaka, Japan). Clones were sequenced by an ABI PRISM 3100-*Avant* DNA sequencing system (Applied Biosystems) using a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Freiburg, Germany) according to the manufacturer's instructions. The 16S rRNA gene sequences with more than 97% identity were considered to belong to the same operational taxonomic unit (OTU). A database search was conducted using BLAST from the DDBJ (DNA Data Bank of Japan). Sequences determined in this study and those retrieved from the database were aligned using Clustal W (Thompson *et al.*, 1994). Phylogenetic trees were constructed using a

neighbor-joining algorithm (Saitou and Nei, 1987). The 16S rRNA gene sequences of clones obtained in this study were analyzed *in silico* with respect to *HhaI* restriction site, and T-RFs of each clone were calculated to identify the predominant T-RFs detected in this study.

#### **5.2.4 T-RFLP analyses**

T-RFLP analysis of eubacterial 16S rRNA gene was carried out using the forward primer 8f labeled at the 5' end with the dye 6-carboxy-fluorescein and the reverse primer 926r (Liu *et al.*, 1997). After purification of PCR products with a Wizard SV gel and a PCR clean-up system (Promega), 4 µl of the PCR-products were digested with 10 U of the restriction enzyme *HhaI* [GCG'C] (TaKaRa) in the manufacturer's recommended reaction buffers for 4 h at 37°C. The enzyme was subsequently inactivated by incubation at 65°C for 20 min. Aliquots of the digested amplicons were desalted by ethanol precipitation. Desalted digests were suspended in 15 µl of Hi-Di formamide (Applied Biosystems) containing GeneScan-1000 size standard (Applied Biosystems), denatured (5 min at 94°C), cooled on ice, and resolved by an ABI PRISM 3100-*Avant* Genetic Analyzer automated sequence analyzer (Applied Biosystems) using the GeneScan software (Applied Biosystems). To avoid detecting primers and uncertainties in size determination, the length of T-RFs smaller than 30 bp was excluded. The percentage of detected T-RF was calculated from the height of the peak to the total peak height. The T-RFLP assays were performed in triplicate.

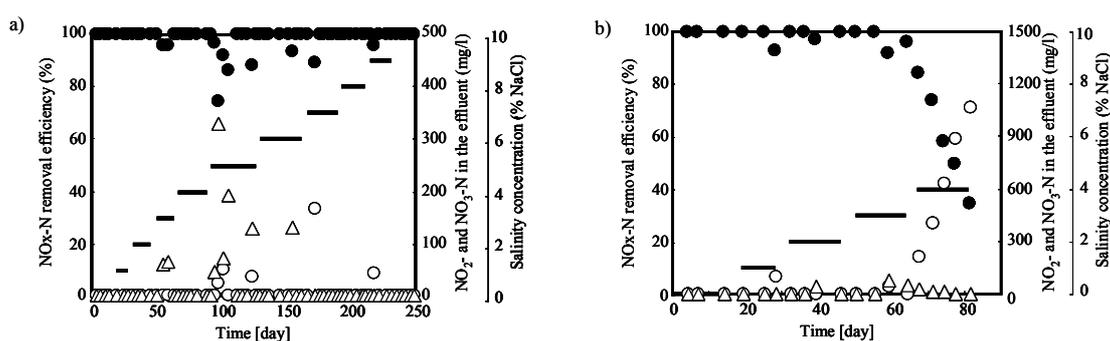
#### **5.2.5 Nucleotide sequence accession number**

The partial 16S rRNA gene sequences of clones obtained in this study were submitted to the DDBJ database under accession numbers AB297390 to AB297446.

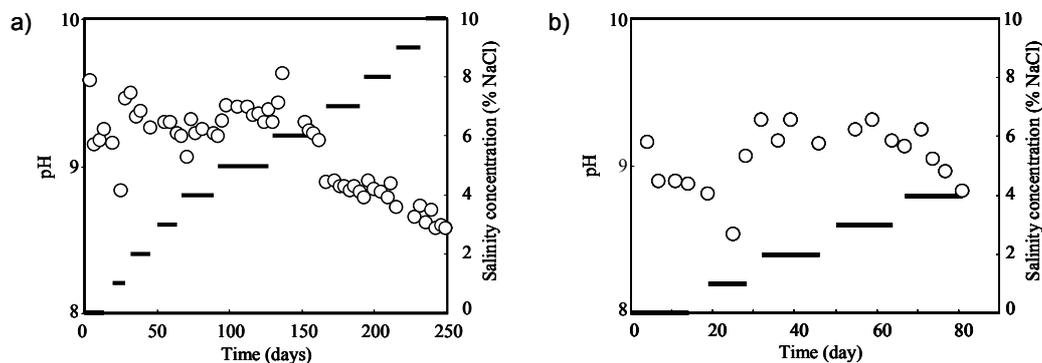
### 5.3. Results

#### 5.3.1 Monitors of denitrifying ability in acetate- or methanol-fed reactors with the increase of salinity concentrations

The time courses of NO<sub>x</sub>-N (NO<sub>2</sub>-N and NO<sub>3</sub>-N) removal efficiency, NO<sub>3</sub>-N and NO<sub>2</sub>-N concentrations in the effluent in the acetate- and methanol-fed reactors are shown in **Fig. 5.1**. In the acetate-fed reactor, accumulation of NO<sub>2</sub>-N was observed after increasing to 5% NaCl concentration, but NO<sub>2</sub>-N removal was recovered by day 112. After that, high NO<sub>x</sub>-N removal performance was observed even though salinity was steadily increased to 10% NaCl concentration. In contrast, in the methanol-fed reactor, NO<sub>x</sub>-N removal efficiency decreased at 4% NaCl although high NO<sub>x</sub>-N removal efficiency was stable until at 3% NaCl concentration. These results indicate that acetate is more useful than methanol as the external carbon source for denitrification under high salinity conditions. During the experiment periods, denitrification has been carried out at high pH levels (**Fig. 5.2**): acetate-fed reactor, 8.5-9.3; methanol-fed reactor, 8.5-9.6.



**Fig. 5.1** Time courses of NO<sub>x</sub>-N removal efficiency (closed circles), NO<sub>3</sub>-N (open circles) and NO<sub>2</sub>-N (open triangles) concentrations in the effluent of the (a) acetate- and (b) methanol-fed denitrifying reactors. The line indicates salinity concentrations.



**Fig. 5.2** Time courses of pH in acetate-fed (a) and methanol-fed (b) denitrifying reactors. The line indicates salinity concentrations.

### 5.3.2 16S rRNA gene clone libraries

Four 16S rRNA gene clone libraries were constructed in this study, and the phylogenetic positioning of OTUs in each clone library are shown in phylogenetic tree (Fig. 5.3). In acetate-fed experiments, 82 clones from the sludge acclimated under salinity-free condition (denoted the clone library Ac0P; 14 OTUs) and 75 clones from the sludge sampled at 10% NaCl concentration (denoted the clone library Ac10P; 5 OTUs) were obtained. The clone library Ac0P showed a clear predominance of sequences (71% of total clones) related to the family *Rhodocyclaceae* of the *Betaproteobacteria*. Sixty-seven percent of all clones were related to the genera *Azoarcus*. Furthermore, 7% of all clones were related to the genera *Halomonas*, and 6% of all clones were affiliated to the phylum *Bacteroidetes* (*in silico* T-RF size, 97 bp). In comparison with the clone library Ac0P, the diversity of sequences in the clone library Ac10P declined. Almost all of clones were related to the genera *Halomonas* (52% of all clones) and *Marinobacter* (45% of all clones) of the *Gammaproteobacteria*. The remaining two clone sequences (3%) were affiliated to the phylum *Firmicutes*.

In methanol-fed experiments, 85 clones from the sludge acclimated under salinity-free condition (denoted the clone library Me0P; 19 OTUs) and 75 clones from the sludge sampled at 4% NaCl concentration (denoted the clone library Me4P; 19

OTUs) were obtained. Both clone libraries Me0P and Me4P consisted exclusively of some *Alphaproteobacterial* clones: the genera *Hyphomicrobium* (Me0P, 35%; Me4P, 20%); the genera *Paracoccus* (Me0P, 7%; Me4P, 22%). A further 14% in Me0P and 29% in Me4P were affiliated to the phylum *Bacteroidetes*. Furthermore, the remaining relatively abundant population were related to the genera *Methylophaga* of *Gammaproteobacteria* (Me0P, 8%; Me4P, 11%) and the family *Rhodocyclaceae* of *Betaproteobacteria* (Me0P, 8%; Me4P, 13%), and were affiliated to the phylum *Chloroflexi* (Me0P, 7%) and the phylum *Firmicutes* (Me0P, 8%). The analysis of T-RF sizes based on sequenced data of clones obtained in this study allowed the assignments of T-RFs observed in T-RFLP fingerprints, and are summarized in **Table 5.1**.

**Table 5.1** Phylogenetic affiliations and numbers of 16S rRNA gene clones.<sup>a</sup>

Phylogenetic group	Ac0P <sup>b</sup>		Ac10P <sup>c</sup>		Me0P <sup>d</sup>		Me4P <sup>e</sup>	
	No. of clones	T-RFs (bp) <sup>f</sup>	No. of clones	T-RFs (bp) <sup>f</sup>	No. of clones	T-RFs (bp) <sup>f</sup>	No. of clones	T-RFs (bp) <sup>f</sup>
<i>Alphaproteobacteria</i>								
<i>Mesorhizobium</i>					1	61		
<i>Hyphomicrobium</i>					30	<b>513*</b>	15	<b>513*</b>
<i>Paracoccus</i>	1	341			6	<b>341, 512, 513*</b>	17	<b>341, 513*</b>
Uncultured <i>Phyllbacteriaceae</i>							1	513*
Uncultured bacteria					4	515		
<i>Betaproteobacteria</i>								
<i>Azoarcus</i>	55	<b>568, 570</b>			5	<b>212, 570</b>	5	<b>570</b>
<i>Thauera</i>	3	<b>208</b>						
Uncultured <i>Rhodocyclaceae</i>					2	<b>210</b>	5	<b>210</b>
<i>Gammaproteobacteria</i>								
<i>Halomonas</i>	6	<b>211, 365</b>	39	<b>211*</b>				
<i>Marinobacter</i>			34	<b>211*</b>				
<i>Methylophaga</i>					7	<b>564</b>	8	<b>368, 564</b>
<i>Oceanospirillum</i>	1	562						
<i>Pseudomonas</i>	1	155						
<i>Rheinheimera</i>	1	219						
Uncultured bacteria					1	371		
<i>Deltaproteobacteria</i>	1	95					1	95
<i>Bacteroidetes</i>	5	<b>97</b>			12	<b>92, 96</b>	22	<b>92, 93, 94*, 96*, 102</b>
<i>Chloroflexi</i>					6	208, <b>550</b>		
<i>Deinococcus-Thermus</i>					1	545		
<i>Firmicutes</i>	8	<b>228, 238, 389</b>	2	61, 377	7	<b>228, 388</b>		
<i>Planctomycetes</i>					3	<b>189</b>	1	863

<sup>a</sup>Characteristic T-RFs for different clone groups are given.

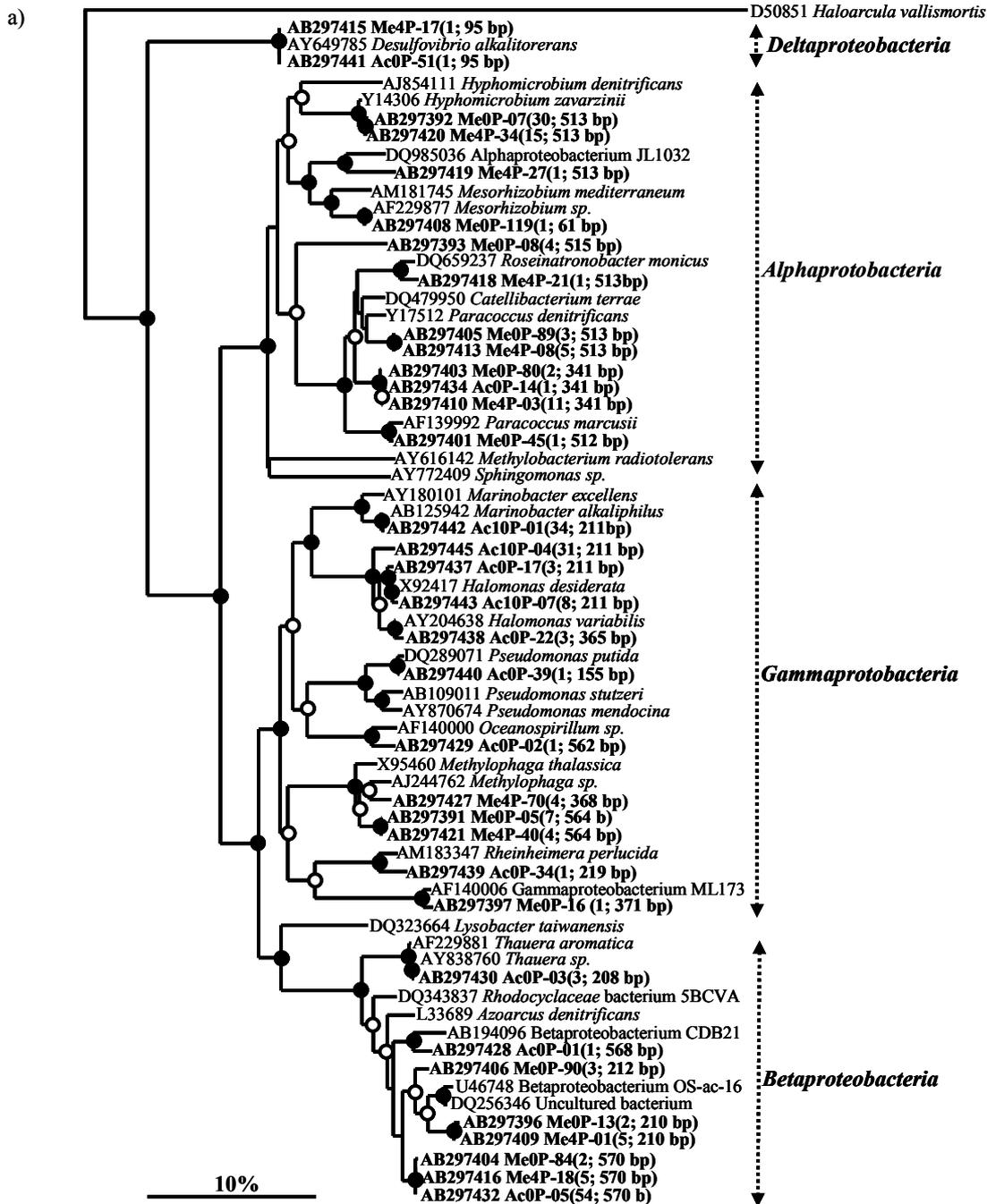
<sup>b</sup>Clone library "Ac0P" was constructed from DNA obtained from acetate-fed sludge under salinity-free conditions

<sup>c</sup>Clone library "Ac10P" was constructed from DNA obtained from acetate-fed sludge at 10% NaCl concentration.

<sup>d</sup>Clone library "Me0P" was constructed from DNA obtained from methanol-fed sludge under salinity-free conditions

<sup>e</sup>Clone library "Me4P" was constructed from DNA obtained from methanol-fed sludge at 4% NaCl concentration.

<sup>f</sup>Terminal restriction fragment length of each clone is shown in base pairs. T-RFs detected for more than one clone within one phylogenetic group are indicated in boldface. T-RFs detected in more than one phylogenetic group are marked with an asterisk (\*)



**Fig. 5.3** Phylogenetic affiliation of (a) the *Proteobacterial* clones (b) the remaining clones except *proteobacterial* clones from each clone library by neighbor-joining analysis. The prtial 16S rRNA gene sequences obtained from acetate-fed sludge at salinity-free or 10% NaCl are labeled as “Ac0P” or “Ac10P”, respectively. Those obtained from methanol-fed sludge at salinity-free or 4% NaCl are labeled as “Me0P” or “Me4P”, respectively. The number of clones assigned to each sequenced OTU with more than 97% identity and the theoretical length in silico analysis are shown in parentheses. *Haloarcula vallismortis* (accession no. D50851) is used as the outgroup. Bootstrap values >750 (●) and in the range of 500 to 750 (○) are indicated at branch points. Scal bar = 10% nucleotide substitution.

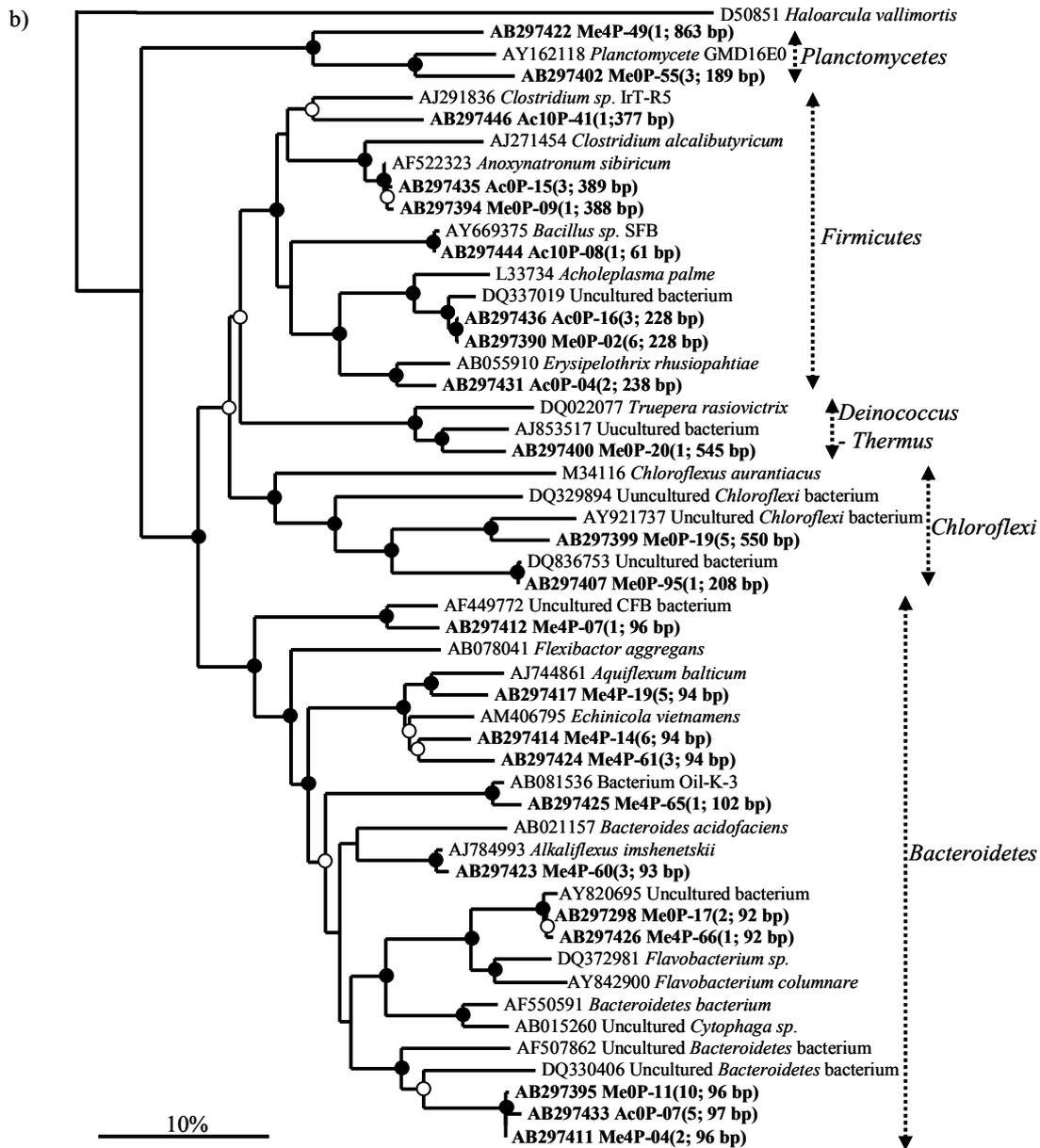
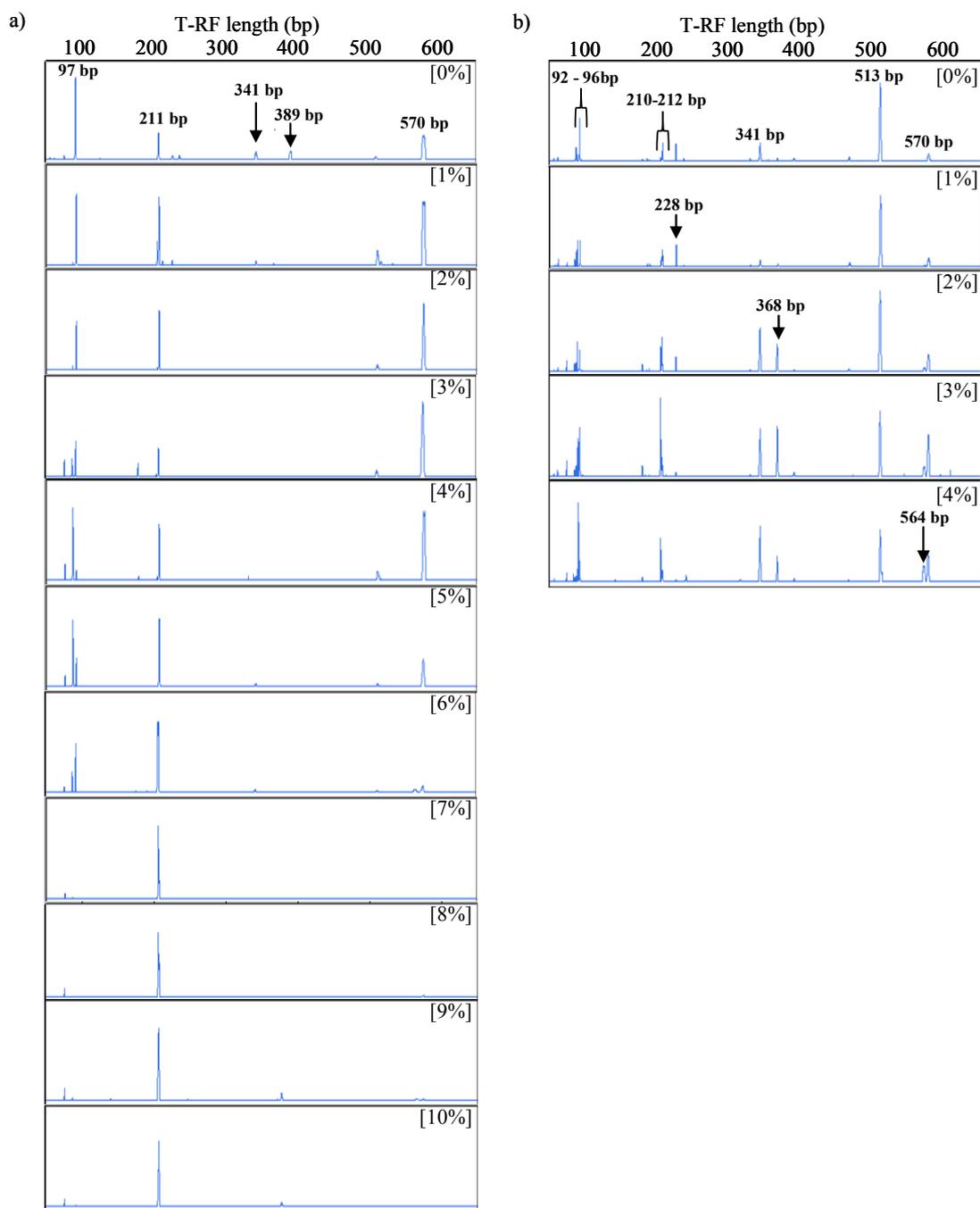


Fig. 5.3 Continued

### 5.3.3 T-RFLP analysis

T-RFLP fingerprints of eubacterial 16S rRNA gene demonstrated that the bacterial community changed with the increase of salinity concentration (Fig. 5.4). Additionally, T-RFLP patterns differed between the acetate- and methanol-fed experiments.

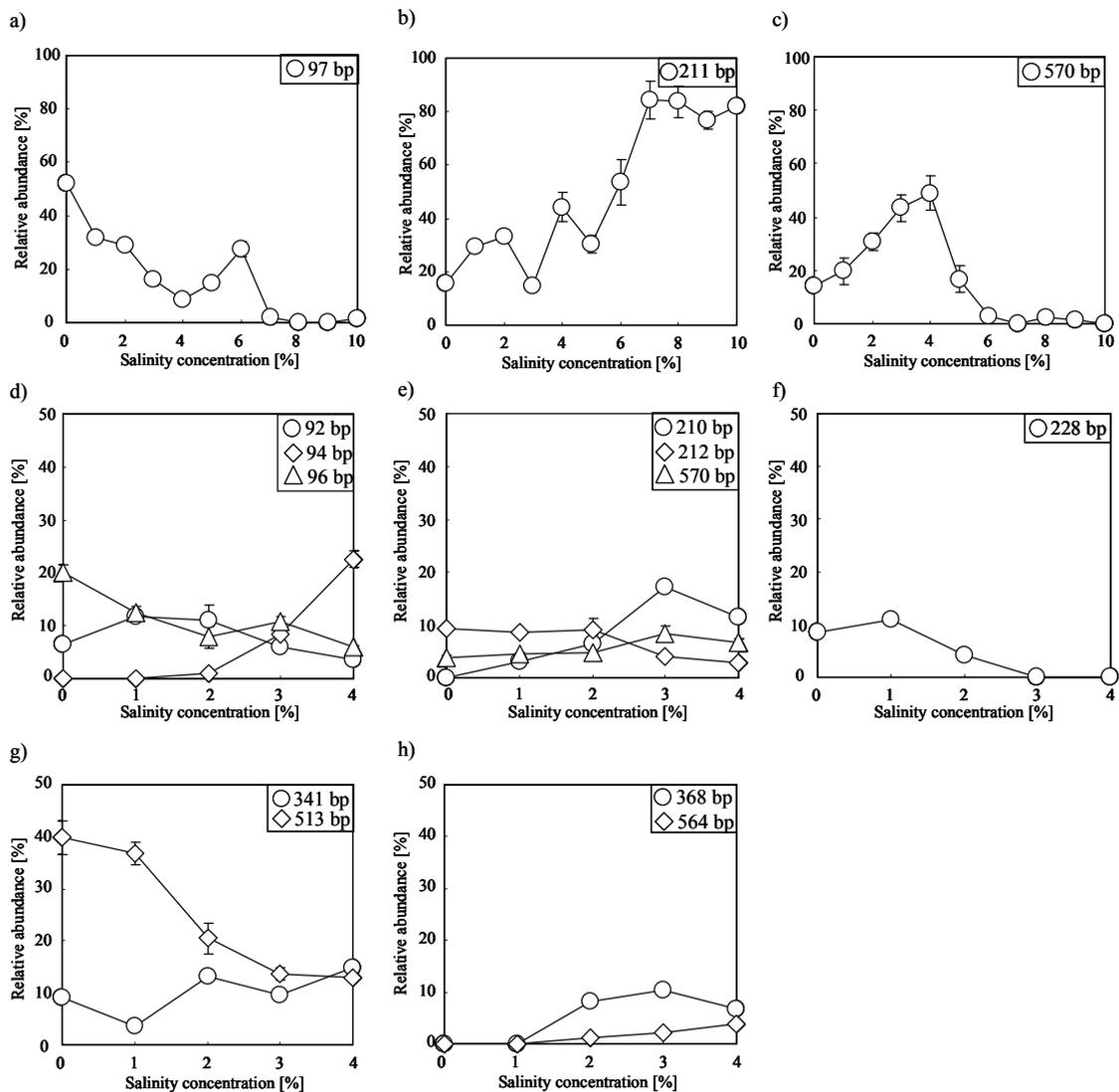


**Fig. 5.4** Time-course T-RFLP fingerprintings of 16S rRNA genes in the acetate-fed experiment (a) and the methanol-fed experiment (b). Amplicons with 8f-FAM/926r primers and digested with *HhaI*. Salinity concentration (% NaCl) are given in brackets. Sizes of important T-RFs (bp) are given.

The T-RFLP fingerprint of the acetate-fed sludge at salinity-free condition (0% NaCl) showed three dominant T-RFs (i.e. 97, 211, and 570 bp), indicating the acclimation and enrichment of bacterial population due to long-term enrichment (**Fig. 5.4a**). Distinct patterns of T-RFLP fingerprints observed after the increase of salinity concentrations. Changes of the relative abundances of each characteristic T-RF with the increase of salinity concentrations are shown in **Fig. 5.5**. The T-RF of 97 bp (i.e. the phylum *Bacteroidetes*) was a clear dominant population (relative abundance, 52%). However, the relative abundances of this T-RF gradually decreased with the increase of salinity concentration, and were less than 2% above at 6% NaCl concentration (**Fig. 5.5a**). On the other hand, the abundance of the T-RF of 211 bp (i.e. the genus *Halomonas* and *Marinobacter* of *Gammaproteobacteria*) increased from 16% to 82% with increasing salinity concentrations, and this T-RF was a dominant peak in the T-RFLP profile at 10% NaCl (**Fig. 5.5b**). Although the abundance of the T-RF of 570 bp (i.e. the *Azoarcus*-like populations) increased to 49% at 4% NaCl, this T-RF decreased drastically after further increase of salinity concentration (**Fig. 5.5c**).

In the methanol-fed sludge at salinity-free condition, some characteristic T-RFs (e.g. 96, 212, 228, 341, 513, and 570 bp) were detected from the sludge acclimated with methanol as an organic carbon source for denitrification under salinity-free condition (0% NaCl). The T-RFLP fingerprint at 0% NaCl showed a clear dominance of the 513 bp T-RF derived from the *Alphaproteobacteria* (i.e. the genus *Hyphomicrobium* and *Paracoccus*). Above at 2% NaCl concentration, the relative abundance of some T-RFs (i.e. T-RFs of 94, 210, 368, 564 bp) increased, whereas the relative abundance of the 513 bp T-RF decreased to 13% (**Fig. 5.5**). The 94 bp T-RF (i.e. the phylum *Bacteroidetes*) increased to 23% at 4% NaCl. Meanwhile, other *Bacteroidetes*-derived T-RFs (e.g. T-RFs of 92 and 96 bp) remained stable or decreased with the increase of salinity concentrations (**Fig. 5.5d**). The T-RF of 210 bp (i.e. the *Azoarcus*-like populations) increased gradually from 0% to 11% with the increased of salinity

concentrations (**Fig. 5.5e**). The abundance of the *Methylophaga*-derived T-RFs (i.e. 368 bp and 564 bp) increased to about 10% of the total community above at 2% NaCl (**Fig. 5.5h**). Additionally, the relative abundance of the remaining characteristic T-RFs of 212 bp and 570 bp (i.e. the *Azoarcus*-like populations) and 228 bp (i.e. the phylum *Firmicutes*) are also shown in **Fig. 5.5**.



**Fig. 5.5** Effect of the increase of salinity concentrations on the composition of the bacterial community in acetate-fed sludge or methanol-fed sludge given by the relative abundance of characteristic T-RF: (a) to (c), acetate-fed sludge sample; (d) to (g), methanol-fed sludge sample. (a) Bacteroidetes, (b) *Halomonas* and *Marinobacter*, (c) *Azoarcus*-like populations, (d) Bacteroidetes, (e) *Azoarcus*-like populations, (f) Firmicutes, (g) *Hyphomicrobium/Paracoccus* populations, (h) *Methylophaga*. Some of the error bars, which indicate standard deviations ( $n=3$ ), are smaller than the symbols.

#### 5.4. Discussion

Previous studies have shown that a dose of acetate or methanol to denitrifying reactors leads to a selection of specific bacterial populations (Ginige *et al.*, 2004, 2005; Osaka *et al.*, 2006). In those studies, acetate has been mainly assimilated by denitrifying bacteria belonging to the families *Rhodocyclaceae* (e.g. *Azoarcus*, *Thauera*) and *Comamonadaceae* (e.g. *Acidovorax*, *Comamonas*) (Ginige *et al.*, 2005; Osaka *et al.*, 2006), while methanol has been mainly assimilated by methylotrophs belonging to the families *Hyphomicrobiaceae* and *Methylophilaceae* (Ginige *et al.*, 2004; Osaka *et al.*, 2006). However, we found some discrepancies between this study and those previous studies. For example, our results showed that bacterial diversity in sludge acclimated to acetate before NaCl loading was lower than foreseen, e.g. the selection of the *Azoarcus*-like populations. Hwang *et al.* (2006) also showed a low diversity and predominance of the *Azoarcus*-like populations at high pH environments. Likewise, our denitrifying reactors have been operated without controlling of pH because denitrification from high salinity wastewater favor high pH levels (Glass and Silverstein, 1999; Peyton *et al.*, 2001; Van der Hoek *et al.*, 1987). Therefore, it has been suggested that the bacterial populations is naturally adapted to a high pH environments in addition to the selection of bacteria by the supplement of acetate.

The selection of external carbon source for denitrification affects management cost of wastewater treatment plants. Methanol is particularly used because of its relatively low cost and low sludge production among organic compounds at wastewater treatment plants. However, it should be carefully evaluated because the selection of source has strong effects on the denitrification performance in wastewater treatment plants. In this study, we found that the type of organic carbon source (i.e. acetate, methanol) had a strong effect on the activity of nitrate removal and bacterial populations in a saline environment. Acetate-fed process kept a high performance of nitrate removal even at 10% NaCl, while methanol-fed process resulted in the drastic reduction of nitrate

removal at 4%. The community analysis by cloning targeting on 16S rRNA gene revealed that the selection of bacteria belonging to the genera *Halomonas* and *Marinobacter* occurred under high saline conditions in the acetate-fed reactor. Members of the genera *Halomonas* and *Marinobacter* grow in a wide range of NaCl concentrations (0-20%), and the optimum NaCl concentrations for these bacterial growth are less than 10% NaCl (Mata *et al.* 2002; Takai *et al.* 2005). Additionally, the *Halomonas* and *Marinobacter* populations were also detected as most abundant population in acetate-fed denitrifying reactor treated with the metal refinery wastewater containing high nitrate and salinity (Yoshie *et al.* 2001, 2004, 2006a, 2006b). On the other hand, the microbial community analysis of methanol-fed sludge indicated the predominance of *Bacteroidetes*, *Hyphomicrobium*, *Methylophaga*, and *Paracoccus*. The genus *Hyphomicrobium* has been identified as the active methanol-utilizing denitrifiers in a sewage sludge by stable-isotope probing of DNA (Osaka *et al.*, 2006), and the appearances of the genera *Methylophaga* and *Paracoccus* have been reported in a methanol-fed denitrifying reactor (Labbe *et al.*, 2003; Neef *et al.*, 1996). Therefore, it suggested that these bacteria play an important role in the methanol-fed denitrifying reactor in the presence of saline. However, there was no significant change of predominant bacterial populations between at 0% and 4% NaCl. This observation is in agreement with previous studies that the optimal salinity concentrations of the genera *Hyphomicrobium*, *Methylophaga*, and *Paracoccus* were less than 5% NaCl (Doronina *et al.*, 2003; Gliesche *et al.*, 2005; Janvier *et al.*, 1985; Van Spanning *et al.* 2005). Thus, the suppression of these bacterial growths could be resulted in the deterioration of nitrate removal at 4% NaCl. However, PCR bias, small sampling size or cloning bias clearly limits the significance of clone libraries for understanding microbial community compositions in the cloning analysis. Therefore, we can not rule out the possibility of overestimation or underestimation of the population size by the cloning analysis.

The combination of T-RFLP fingerprinting and clone library construction of 16S

rRNA genes has documented the community transitions with the increase of salinity concentration. In acetate-fed reactor, time-course T-RFLP analysis clearly revealed the relationship between the population dynamics of the *Azoarcus*-like populations and salinity concentrations (**Fig. 5.5c**), which is in agreement with a previous report showing that the *Azoarcus* isolates can not grow at a NaCl concentration of 5% or more (Zhou *et al.*, 1995). Furthermore, time-course T-RFLP analysis also showed a transient change of bacterial community structure from non-halophilic denitrifying population (i.e. *Azoarcus*-like populations) to halophilic denitrifying population (i.e. *Halomonas*, *Marinobacter*) at 5% NaCl (**Figs. 5.4, 5.5b, and 5.5c**), at when nitrite was transiently detected in effluent. Moreover, in methanol-fed sludge, we observed a NaCl-dependent increase of the relative abundance of the genus *Methylophaga* (i.e. T-RFs of 368 bp and 564) and the *Azoarcus*-like populations (i.e. T-RF of 210 bp). The genus *Methylophaga* is known to a haloalkaliphilic restricted facultative methylotroph (Doronina, *et al.*, 2003). The genus *Azoarcus* can not utilize only acetate but also methanol under denitrifying conditions (Hagman *et al.*, 2007). Therefore, it suggested that these bacteria played an important role in denitrification at the salinity condition. However, we keep in mind that quantitative T-RFLP can not necessarily reflected on the real microbial community compositions, though it is a simple and quick tool for characterizing of microbial community. Some explanations for these findings were discussed as follow. First, T-RFLP analysis is subject to a potential PCR bias. Second, the copy number of ribosomal RNA operon differs among the bacterial lineage (Acinas *et al.* 2004). Third, the phylogenetically distant bacteria could generate identically sized T-RFs (e.g. some of *Hyphomicrobium* and *Paracoccus* generate the T-RF with size of 513 bp), or, conversely, phylogenetically close relationship could generate different sized T-RFs (e.g. the *Methylophaga* generates the T-RFs with size of 368 bp and 564 bp). Furthermore, T-RFLP analysis has a specific problem related to the restriction digestion: incomplete digestion due to limiting enzyme concentration or suboptimal reaction

conditions (Osborn *et al.*, 2000), the formation of pseudo-T-RFs (Egert and Friedrich, 2003), and 16S rRNA gene sequence heterogeneity (Clement *et al.*, 1998). Therefore, the relative abundance of a certain T-RF does not necessarily correspond to the actual population size. In order to resolve these points, other quantitative approach (e.g. FISH, the real-time PCR) is required.

We can not necessarily identify the active denitrifiers under each condition from the results of the 16S rRNA gene analysis, although time-course T-RFLP fingerprints of 16S rRNA genes is easy-to-use methods for characterizing the population dynamics. It is anticipated that molecular biological investigation targeted mRNA of functional genes involved in denitrification and functional characterization combined with microautoradiography (MAR)-FISH (Lee *et al.*, 1999) or stable-isotope probing (SIP) (Radajewski *et al.*, 2000) will clarify the true denitrifying bacteria in nitrogen removal system for saline wastewater.

## 5.5 Conclusions

This study show that the selection of organic carbon source as an electron donor for denitrification is a matter of great importance to trigger high nitrate removal activity in high saline environment: acetate-fed process attained stable and high nitrate removal at 0 - 10% NaCl, whereas methanol was proven beneficial electron donors at less than 3% NaCl. By combination of T-RFLP and cloning analysis, it was shown that the succession of microbial community structure has a correlation with the increase of salinity concentration. Furthermore, it was shown that the genera *Azoarcus*, *Marinobacter* and *Halomonas* play an important role in the acetate-fed reactor, while the genera *Azoarcus*, *Hyphomicrobium*, *Methylophaga*, and *Paracoccus* play an important role in the methanol-fed reactor.

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