Microbial community changes in biological phosphate-removal systems on altering sludge phosphorus content

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Biomarkers (respiratory quinones and cellular fatty acids) and denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA genes were used to characterize the microbial community structure of lab-scale enhanced biological phosphate-removal (EBPR) systems in response to altering sludge phosphorus (P) content. All the data suggest that the microbial community structures of sludge samples with a P content between 8 and 12.3% (sludge dry weight) (i.e. good EBPR activity) were very similar, but differed from those with 2% P content (i.e. no EBPR activity). For all samples analysed, ubiquinones Q-8 and Q-10, menaquinone MK-8(H₄), and fatty acids C_{16:0}, C_{16:1 ω9c} and C_{18:1 ω11c} were the major components. The dominance of Q-8, Q-10 and MK-8(H₄) suggested that large numbers of organisms belonging to the β and α subclasses of the *Proteobacteria* and the *Actinobacteria* from the high G+C Gram-positive bacteria, respectively, were present. DGGE analysis revealed at least 7–9 predominant DNA bands and numerous other fragments in each sample. Five major DGGE fragments from each of the 2% and 12% P-containing sludge samples, respectively, were successfully isolated and sequenced. Phylogenetic analysis of the sequences indicated that both 2% and 12% P-containing sludge samples shared three common phylotypes that were separately affiliated with a novel bacterial group from the γ subclass of the *Proteobacteria*, two MK-8(H₄)-containing actinobacteria previously isolated from the 2% P-containing sludge, and a *Caulobacter* spp. in the α subclass of the *Proteobacteria*. The phylogenetic analysis also revealed phylotypes unique to both sludge samples. Changes in sludge P content therefore had an effect on the composition and abundance of the predominant microbial populations, though specific phylotypes could not be unequivocally associated with EBPR.

Keywords: Activated sludge, biological phosphate removal, biomarker, DGGE, 16S rDNA

INTRODUCTION

Enhanced biological phosphate-removal (EBPR) processes couple a front-end anaerobic zone with a subsequent aerobic zone to selectively enrich for micro-organisms capable of biologically removing phosphate

Abbreviations: DGGE, denaturing gradient gel electrophoresis; EBPR, enhanced biological phosphate removal; PHA, polyhydroxyalkanoate.

The GenBank/EMBL/DBJ accession numbers for the sequences obtained in this report are AF109792 (strain Lpha5), AF109793 (strain Lpha7) and AF124650 to AF124659.

from waste water (Fuhs & Chen, 1975; Marais *et al.*, 1983). Empirical metabolic models (Comeau *et al.*, 1986; Mino *et al.*, 1987) suggested that EBPR is generally linked to the synthesis and consumption of intracellular storage polymers of phosphate (as polyphosphate) and carbon [as polyhydroxyalkanoates (PHA) or cellular carbohydrates]. In the anaerobic stage, the responsible micro-organisms may utilize energy derived from polyphosphate hydrolysis for carbon uptake and storage, and later under the aerobic stage, the previously stored carbon can be used for growth and for polyphosphate formation (Comeau *et al.*, 1986; Mino *et al.*, 1987). The

engineering mass-balance control strategy has been used to select microbial communities that carry out EBPR metabolism, but, unlike biological nitrification and denitrification processes, suitable operational conditions for EBPR process control have not been developed (Jenkins & Tandoi, 1991; Mino *et al.*, 1998). The failure to develop adequate EBPR process control has been related to the complexity of the microbial communities present that include both phosphate and non-phosphate accumulating populations with different preferences for carbon substrates and electron acceptors (Cech & Hartman, 1993; Kuba *et al.*, 1993; Liu *et al.*, 1996). This situation may be improved by first achieving a better understanding of the taxonomy of the phosphate-accumulating populations present, and then tailoring the process control strategy used to promote and maintain the growth of those populations under conditions most favourable to polyphosphate accumulation.

Methods based on the analysis of various biomarkers and 16S rRNA genes (rDNA) have been used to characterize and monitor microbial communities in various ecological systems. The biomarkers, cellular fatty acids and respiratory quinones, have been used routinely in taxonomy to characterize, differentiate and identify genera, species, and strains of bacteria (Staley *et al.*, 1989). Some studies have further shown that the biomarker 'signature' of environmental samples can be statistically analysed and applied to differentiate community profiles (Haack *et al.*, 1994; Hiraishi *et al.*, 1989, 1998). 16S rDNA-based methods can provide more information on the phylogenetic structure of microbial communities than the biomarker method (Bond *et al.*, 1995; Muyzer *et al.*, 1993). For example, denaturing gradient gel electrophoresis (DGGE) can be used to resolve PCR-amplified 16S rDNA fragments by electrophoresis through an acrylamide gel that contains an increasing linear gradient of denaturants (Muyzer *et al.*, 1993). The number and intensity of resolved fragments gives an approximate estimate of the diversity of the predominant species, and further purification of fragments and sequence analysis provides an insight into the phylogenetic affiliation of individual populations (Muyzer *et al.*, 1995; Nielsen *et al.*, 1999). Combining the biomarkers and 16S rDNA-based approaches should greatly enhance the characterization of microbial communities found in various systems.

Previously, we varied the phosphorus:carbon (P/C) weight ratio of the feed used for lab-scale EBPR systems and successfully enriched communities that differed in their ability to accumulate phosphate (Liu *et al.*, 1997a). A gradual increase in the P/C ratio from 20:100 to 2:100 did not affect the carbon uptake and storage under anaerobic conditions, but caused a drop in the sludge P content from ~12% of sludge dry weight (or 33% polyphosphate) to a cellular constituent level (~2% P content or ~0% polyphosphate). As a result, the EBPR activity stopped, and apparent shifts in community structure were reflected by differences in the morphologies of the predominant populations. At a

12% P content the community was predominated by rod-shaped organisms that accumulated both polyphosphate and PHA, whereas at a 2% P content the community was dominated by PHA-accumulating cocci that neither accumulated polyphosphate nor aerobically took up phosphate when provided (Liu *et al.*, 1996, 1997a). It was suspected that the loss of polyphosphate-accumulating bacteria upon shifting the feed composition was due to loss of their energy pool (polyphosphate) which they used to transport and store carbon (e.g. PHA) under anaerobic conditions so that it could be subsequently used for growth under aerobic conditions. However, in that study we did not establish whether the apparent shifts in the morphology of the microbial community were indeed caused by changes in the relative abundance of different bacterial populations. Our initial assumption was that polyphosphate-accumulating bacteria would constitute a predominant fraction of the bacterial population in a 12% P-containing sludge, and on altering sludge P content to 2%, could be easily differentiated from non-polyphosphate accumulating bacteria using the biomarker and DGGE approaches. Furthermore, the phylogenetic affiliation of the predominant populations in 2% and 12%-P containing sludge could be easily identifiable from sequence analysis of the predominant 16S rDNA DGGE fragments. This study was aimed at testing the above assumption.

METHODS

Enriched activated sludge. Three sequential batch reactors (R1, R2 and R3) with a 1.8 l working volume were used with a mixture of acetate and peptone (~80:20, w/w) as carbon sources under alternating anaerobic and aerobic conditions as described previously (Liu *et al.*, 1994, 1997a). All the reactors were subjected to eight 3 h cycles per day that consisted of a 50 min anaerobic period followed by an 80 min aerobic phase and a 50 min sedimentation phase. At the beginning of the anaerobic phase, substrate was fed to maintain an organic loading of 0.4–0.9 kg C m⁻³. Sludge residence time was maintained between 7.5 and 8 d, and sludge pH was controlled between pH 7 and 8. The sludge P content in each reactor was regulated between approximately 2 and 12% using different total P to total organic carbon ratios (Liu *et al.*, 1997a). This was done by varying the influent KH₂PO₄ concentration at a constant influent total organic carbon level (~150 or 200 mg C l⁻¹). For R1 and R2, the P/C ratios were 2:100 and 10:100, respectively. For R3, the P/C ratio was initially maintained at 20:100, and decreased to 10:100 at d 70 and then to 2:100 at d 94. When the reactors were operated for more than one month, replicate samples from each P/C ratio were taken, immediately lyophilized and stored prior to the evaluation of microbial community structure.

Analyses of chemotaxonomic biomarkers. Procedures for respiratory-quinone analysis were those described by Hiraishi *et al.* (1989). Briefly, respiratory quinones were extracted with a mixture of chloroform/methanol (2:1, v/v), purified by TLC and qualitatively analysed by reverse-phase HPLC using a Shimadzu model CTO6-A HPLC equipped with a photodiode array detector. Quinone standards were purchased from Wako Pure Chemicals or extracted from bacterial strains with known quinone compositions (Liu, 1995). Determination of

other quinones, including demethylmenaquinone and rhodoquinone, was not attempted since these quinones were not previously found in EBPR processes (Hiraishi *et al.*, 1989). Ubiquinones and menaquinones with *n* isoprene units are abbreviated Q-*n* and MK-*n*, respectively. MK-*n*(H_x) represents a partially hydrogenated menaquinone with *x* hydrogen atoms on the side chain containing *n* isoprene units. The total cellular fatty acids were analysed using a protocol described by Rajendran *et al.* (1992).

Cluster analysis (Statistica) was used to statistically discern patterns in the respiratory quinone and total cellular fatty acid data. Dendrograms were constructed by using the single linkage and Euclidean distance rules in the Statistica program. Analysis of variance (ANOVA) was further used to test the change of individual quinone components among sludge samples taken from reactors containing different sludge P contents.

Isolation and PCR amplification of DNA. DNA from activated sludge was obtained after cell lysis, phenol/chloroform extraction and ethanol precipitation using a previously described protocol (Liu *et al.*, 1997b). This DNA preparation was used as the template in PCR-reaction mixtures that contained 1 × PCR buffer (Gibco-BRL), 200 μM each dNTP, 1.5 mM MgCl₂, 0.1 μM each primer, 5% DMSO and 2.5 U *Taq* DNA polymerase (Gibco-BRL) in a final volume of 100 μl. For amplification of 16S rDNA for DGGE analysis, the 968FGC forward primer with a GC clamp (Heuer *et al.*, 1997) and the 1392R reverse primer (Ferris *et al.*, 1996) were used. The PCR was carried out in a Perkin Elmer 9600 thermocycler using a thermal program described previously (Nielsen *et al.*, 1999). Amplification of DNA was verified by electrophoresis of 2 μl of the PCR product through a 1% agarose gel in 1 × TAE buffer (20 mM Tris-acetate, pH 7.4, 10 mM sodium acetate, 0.5 mM EDTA).

DGGE. DGGE was performed using a D-Gene system (Bio-Rad) according to the manufacturer's instructions. PCR products were loaded onto a 6% acrylamide gel (37.5:1, acrylamide:N,N'-methylene-bis-acrylamide) in 1 × TAE buffer. The denaturing gradient in the gel was formed by mixing two stock solutions of 6% acrylamide that contained 40% denaturant (2.8 M urea and 18.7% (w/v) formamide; both from Sigma) and 60% denaturant (4.2 M urea, 24% formamide). Denaturants were deionized with AG501-X8 mixed bed resin (Bio-Rad) prior to being used. The DNA fragments were visualized by silver staining as described by Riesner *et al.* (1989).

Isolation, cloning and sequencing of DGGE fragments. The DNA sequences of specific fragments in the DGGE gels were determined. A fragment was excised from the gel using a razor blade and the DNA was eluted overnight in 100 μl TAE buffer. Individual DNA fragments were amplified by PCR with the DGGE primers described above, ligated into the pCRII vector and transformed into competent *Escherichia coli* cells according to the manufacturer's instructions (TA Cloning System; Invitrogen). Clones with the target DNA fragments were identified by amplifying ten randomly chosen clones using the DGGE primers and comparing the electrophoretic mobility of the amplicon with that of the fragments in the original sample. Selected DNA fragments were sequenced at the Michigan State University sequencing facility on an ABI DNA Sequencer model 373 (Applied Biosystems) using the 968FGC (without the GC clamp) and 1392R primers, and the *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems).

Phylogenetic analyses. Partial DNA sequences (~430 bp) obtained in this study were compared to available 16S rRNA sequences in GenBank using the NCBI BLAST program. The most closely related sequences from the NCBI BLAST searches, and important 16S rRNA sequences of environmental clones and bacterial isolates obtained from the EBPR process were retrieved and aligned to those sequenced DGGE bands using the CLUSTAL W program (Thompson *et al.*, 1994). A phylogenetic tree was constructed from the evolutionary distance matrix based on the Kimura two-parameter algorithm using the neighbour-joining method (Saitou & Nei, 1987). The analysis was performed with the MEGA program (Kumar *et al.*, 1993), and gap sites in the alignment were excluded in the pairwise comparison.

RESULTS AND DISCUSSION

Community composition as revealed using chemotaxonomic biomarkers

Table 1 shows the respiratory-quinone profiles of activated sludge samples with different P contents. Both ubiquinones and menaquinones were detected in all sludge samples. Q-8 and Q-10 were the most dominant ubiquinones present in all samples, followed by Q-7 and Q-9. The high contents of Q-8 and Q-10 could reflect the predominance of members of the β and α subclasses, respectively, of the *Proteobacteria* (Hiraishi *et al.*, 1998). MK-8(H₄) was the dominant menaquinone detected (70%), and is found mainly in members of the actinobacteria of the high G+C Gram-positive bacteria (Hiraishi *et al.*, 1998). These findings closely matched previous quinone data obtained from laboratory-scale EBPR systems (Hiraishi *et al.*, 1989, 1998). Fatty-acid profiles revealed that two major monounsaturated fatty acids, C_{16:1 ω9c} and C_{18:1 ω11c}, and a saturated fatty acid, C_{16:0}, accounted for more than 60% of total fatty acids present in all sludge communities. In addition, lower amounts (6–7%) of C_{16:0i} were detected.

The profiles of both respiratory quinones and fatty acids for samples taken from different reactors at different P content were analysed statistically. The respiratory quinone profiles for samples taken from R3 (Fig. 1a) revealed that the microbial community in sludge samples with a 12.3% P content was more similar to that in samples with a 7.1% P content than to the community in samples with a 2% P content. When samples were taken from different reactors, the difference in community structure between samples with 12.3 and 6.7% P content, or 12.3 and 2% P content further increased. However, clear differences in the quinone profiles were also observed between samples with the same P content from different reactors (Fig. 1a), further suggesting that P content alone did not dictate the community structure in the reactors. ANOVA results of the respiratory-quinone profiles (Table 1) suggested that the marked difference in the structure of microbial communities between the 2 and 12.3% P-containing sludge samples from R3 were attributed to the differences (*P* < 0.05) in Q-10 and MK-8(H₄) content. Similar ANOVA results showed that a difference in the mean percentage of Q-8, Q-10 and MK-8(H₄) occurred between a 2% P sludge from R1 and a

Table 1. Respiratory-quinone profiles of sludge samples containing different P contents

Each number is expressed as a mean (%) with standard deviation shown in parentheses.

P content (% dry wt) ...	R1	R2	R3		
	2.0 (n=2)	6.7 (n=3)	2.0 (n=2)	7.1 (n=3)	12.3 (n=2)
P/C feed ratio (w/w) ...	2:100	10:100	2:100	10:100	20:100
Ubiquinones					
Q-7	0.1 (0.1)	1.9 (1.7)	0.4 (0)	0.6 (0.1)	9.2 (4.1)
Q-8	34.7 (5.3)	82.0 (7.4)	61.5 (1.5)	66.0 (13.4)	64.8 (3.2)
Q-9	2.8 (0.3)	2.5 (0.5)	1.6 (0)	2.3 (0.8)	4.0 (1.4)
Q-10	62.3 (5.5)	13.7 (9.1)	36.5 (1.6)	31.2 (12.6)	22.0 (5.9)
Menaquinones					
MK-6	0	1.0 (1.6)	0.6 (0.8)	0	5.6 (2.9)
MK-7	0	0	0	0	2.4 (3.4)
MK-8	1.1 (1.5)	1.5 (1.4)	0.5 (0.2)	0	5.6 (0.7)
MK-8(H ₄)	92.8 (1.9)	88.6 (3.0)	83.6 (4.7)	79.0 (2.2)	71.4 (1.8)
MK-9	1.7 (0.9)	2.1 (1.5)	2.6 (0.3)	0	0.4 (0.5)
MK-9(H ₄) or 10	1.6 (0.3)	3.4 (1.5)	7.8 (0.6)	0	0
other	2.8 (0.3)	3.5 (1.7)	4.9 (3.9)	21.0 (2.2)	14.7 (1.1)

12.3% P sludge from reactor R3. Since Q-8, Q-10 and MK-8(H₄) were major ubiquinone and menaquinone components, the difference observed on altering the sludge P content from 12.3 to 2% suggested population shifts occurring within the β and α subclasses of the *Proteobacteria*, and the *Actinobacteria*, respectively. Nevertheless, significant differences in Q-8, Q-10 and MK(H₄) in R1 with 2% P, and R3 with 2% P make it difficult to draw generalized conclusions regarding their role in phosphate removal. Like the respiratory-quinone-profiling result, the fatty acid data (Fig. 1b) also revealed marked differences in the structure of microbial communities in EBPR reactors containing different levels of P.

Community structure as revealed using DGGE

The 16S rDNA gene fragments amplified from sludge samples containing 12.3 (R3), 11 (R3), 8 (R2 and R3), or 2% (R3) P content were resolved using DGGE (Fig. 2). Based on the fragment intensity, about 7–9 predominant fragments and numerous other fragments were observed in each sample. The electrophoretic mobility and intensity of the major bands were similar in R3 sludge samples to sludges with P contents ranging from 8 to 12.3%, suggesting that the predominant populations of these microbial communities were not detectably different. Moreover, the patterns of 16S rDNA fragments from sludge samples with a P content of about 8% were very similar to each other (taken from R2 and R3; not operated in parallel), indicating that the enrichment of microbial populations was reproducible at this P content. Further reduction of the sludge P content from 8 to 2% caused a detectable shift in the DGGE fingerprints: at least three of the seven pre-

dominant bands in the 8% P sludge disappeared and six new predominant bands appeared in the 2% P sludge. Obviously, depletion of the energy pool (polyphosphate) for carbon uptake has excluded some of the predominant microbial populations that were possibly involved in EBPR activity, leading to the selection of other microbial populations in the process. Therefore, together with the biomarker results, it can be concluded that the change in P/C feed ratio from 20:100 to 2:100 caused not only the drop in sludge P content, but also the shift in the kinds and abundance of predominant microbial populations in the EBPR systems.

However, the observation of predominant common DGGE bands in both high- and low-percentage P sludge samples (e.g. 2% P band c and 12% P band d) has further suggested that the microbial ecology occurring in the EBPR system was more complicated than our initial assumption that polyphosphate-accumulating and non-polyphosphate-accumulating bacteria were represented by different DGGE bands. One can interpret a common band as 1) a bacterial population with two different metabolisms functioning in the 2 or 12% P reactor, 2) two functionally different species with an identical 16S rRNA sequence or 3) a non-polyphosphate-accumulating population that was not completely outgrown by phosphate accumulators in a 12% P-containing sludge. Identifying the 16S rRNA sequences of predominant DGGE bands allows such explanations to be postulated. However, it should be recognized that rRNA sequence information rarely allows physiological function (i.e. polyphosphate and PHA formation) to be assigned to individual populations. Furthermore, polyphosphate and PHA formation are not phylogenetically conserved traits (Dawes & Senior, 1973).

Fig. 1. Dendrogram of the cluster analysis on the profiles of respiratory quinones (a) and cellular fatty acids (b) for samples containing different sludge P contents. Samples in (a) are abbreviated as Ri_j (k%), where i is the reactor number, j is the sample number and k is the average P content of samples taken from the reactor at the same P/C ratio at different times. The dendrogram was constructed by selecting the single linkage and Euclidean distance rules in the Statistica program.

Phylogeny of predominant populations in EBPR processes

Seven and nine major fragments from sludge samples containing 12.3 and 2% P content, respectively, were excised from the gel and purified. The purified DNA was amplified using the same DGGE primers and electrophoresed in a DGGE gel along with the original sample. Possibly due to the quality of DNA retrieved, only five major fragments from each sample gave PCR products. Since reamplification of a single fragment often resulted in the formation of multiple amplicons, direct sequencing of the reamplified fragment was not possible (data not shown). Instead, the reamplified fragment was cloned and clones with the correct insert were identified by comparing the electrophoretic mobility of the cloned fragments with that of the target fragment found in the original sample. The comparative analysis of these partial 16S rRNA sequences (Fig. 3) revealed the phylogenetic affiliation of the ten sequences retrieved.

Fig. 2. PCR-DGGE analysis of activated-sludge samples containing a phosphorus content of 12.3% (lane A), 11% (lane B), 8% (lane C), 8% (lane D) and 2% (lane E). The denaturant gradient used was 40–60% from top to bottom. Samples A and B were taken from R3 at a P/C ratio of 20:100. Samples C and D were taken from R3 and R2 (not operated in parallel) at a P/C ratio of 10:100. Sample E was taken from R3 at a P/C ratio of 2:100. Predominant bands that were excised from lanes A and E are marked with a bar adjacent to each band. Excised bands with successful reamplification of 16S rDNA were named a–e in each sample.

Of the ten sequences determined (five from the 12.3% P sludge and five from the 2% P sludge), two DGGE fragments found in both 2% and 12% P sludge samples were identified. The remaining six fragments were unique and found only in either 2% or 12% P sludge sample. One pair of common fragments (2% P content, band d, and 12% P content, band e; Fig. 2) was closely related to a novel but-not-yet-isolated bacterial group of the γ subclass of the *Proteobacteria* (i.e. Cle1 bands 4, 5 and 6; Fig. 3) found in an acetate-fed anaerobic–aerobic activated sludge that did not accumulate P (Nielsen *et al.*, 1999). This novel bacterial group could accumulate granular inclusions, possibly of PHA and consisted of two populations with an identical coccoid morphology (cells were $\sim 3\text{--}4\text{ }\mu\text{m}$ in diameter). Large cocci with similar morphological traits as the novel group were also observed as a predominant population earlier in our 2% P sludge sample (Liu *et al.*, 1996). Though the ability

Fig. 3. Phylogenetic distribution of numerically dominant populations in sludge samples containing either 2% or 12.3% P, based on the partial sequence of 16S rDNA genes resolved using DGGE. The distance-matrix consensus tree was calculated using the neighbour-joining method with bootstrapping. The 16S rDNA sequence of *Methanospirillum hungatei* (belonging to the domain *Archaea*) was used to root the tree. Bootstrap probabilities (Felsenstein, 1985) are indicated at the branch points (100 bootstrap data sets were used). The bar represents 4 nucleotide substitutions per 100 nucleotides in 16S rDNA sequences.

of this novel group to accumulate P remains unclear, it was also observed in a good EBPR process with PHA accumulation by the combined use of fluorescent *in situ* hybridization and PHB staining (data not shown). This was possibly because the novel group could perform the carbon metabolism in the EBPR process, and was not completely outgrown by phosphate accumulators. Thus, a low content of Q-9, a major ubiquinone component of

the γ subclass of the *Proteobacteria* (Hiraishi *et al.*, 1998), was observed in all sludge samples (Table 1).

Another DGGE fragment common to both 2 and 12.3% P sludges (2% P, band c and 12% P band d; Fig. 2) was closely affiliated with the *Actinobacteria*. The most closely related cultured bacteria were two isolates, strain Lpha5 and Lpha7, from the reactor containing 2% P

(Liu, 1995). These two isolates accumulated PHA but not polyphosphate as granular inclusions and contained the same major menaquinone component [MK-8(H₄)] (Liu, 1995) as observed in the sludge sample. It is likely that these two organisms were not specifically involved in P removal. However, it is also possible that organisms that can accumulate polyphosphate are phylogenetically related to organisms that cannot. A good example is between a phosphate-accumulating bacterium, *Micrococcus phosphovorans* (Nakamura *et al.*, 1995), and a non-phosphate-accumulating bacterium, *Micropruina glycogenica* (Shintani *et al.*, 2000), that are two phylogenetically related genera isolated from EBPR processes. Since different actinobacteria may contain different proportions of menaquinones (Collins & Jones, 1981), the change observed in MK-8(H₄) content on altering P content could reflect a population shift between non-phosphate-accumulating and phosphate-accumulating actinobacteria with different menaquinone contents. Nevertheless, these data confirmed that members of this novel bacterial population from the actinobacteria are widely distributed in EBPR systems, and as other reports have suggested (Bond *et al.*, 1999; Hiraishi *et al.*, 1989; Nakamura *et al.*, 1995; Wagner *et al.*, 1994), were responsible in part for carbon and possibly phosphate metabolism observed in both 2% and 12% P-containing reactors.

DGGE fragments (2%, band a, and 12%, band b; Fig. 2) that were phylogenetically related to species of *Caulobacter* from the α subclass of the *Proteobacteria* were observed both in 2% and 12% P-containing systems, even though these fragments did not migrate to the same position on the DGGE gel. It is therefore apparent, as demonstrated here and by other studies (Felske *et al.*, 1998; Nielsen *et al.*, 1999), that 16S rDNA fragments that are phylogenetically closely related can have different migration positions in a DGGE gel. *Caulobacter* spp. are often found in environments with low organic carbon contents (Stahl *et al.*, 1992), but have been detected in sewage-treatment systems (MacRae & Smit, 1991) and a full-scale EBPR process (Schuppler *et al.*, 1995). The presence of *Caulobacter* may be one reason for the significant amount of Q-10 detected in all sludge samples (Table 1).

In addition to the common groups, the phylogenetic analysis (Fig. 3) revealed fragments or bacterial populations specific to both 2% and 12% P-containing sludge. Due to the short 16S rRNA sequence obtained and the lack of closely related sequences in the 16S rRNA database, the exact phylogenetic placement of these populations was difficult. Band e (2% P) and band a (12% P) were both associated with the α subclass of the *Proteobacteria*. Band c (12% P) and band b (2% P) were associated with the high G+C group and unidentified green sulfur bacteria, respectively. These findings were consistent with the result that significant amounts of Q-10 and MK-8(H₄) were present in all sludge samples, and a shift in their composition on altering P content was detected. Although those populations unique to the 12% P sludges were possibly the

predominant populations performing the EBPR metabolism, their abilities with respect to polyphosphate and PHA metabolism is unknown and warrants further study. Further, in contrast to the respiratory-quinone result, none of the predominant DGGE fragments retrieved and sequenced were associated with the β subclass of the *Proteobacteria*. This could be due to our inability to retrieve sequences of some predominant DGGE bands (Fig. 2) that may have belonged to the β subclass. The biases associated with community DNA extraction and PCR amplification in some cases (Picard *et al.*, 1992; Tebbe & Vahjen, 1993; Wilson, 1997) could also lead to the misrepresentation of the true community fingerprint. It is also possible that bacteria that produce large quantities of Q-8 but do not belong to the β -*Proteobacteria* exist and may be present in activated sludge systems.

In summary, our current understanding of the diversity of microbial populations in EBPR processes has primarily come from studies using fluorescent *in situ* hybridization (Bond *et al.*, 1999; Kämpfer *et al.*, 1996; Schuppler *et al.*, 1998; Wagner *et al.*, 1994) and analysis of environmental 16S rDNA clone libraries (Bond *et al.*, 1995; Schuppler *et al.*, 1995). All studies suggest a high degree of phylogenetic diversity, including at least 30 different phylotypes from major phyla of the domain *Bacteria*. Nevertheless, no study to date has firmly identified a specific phylotype directly associated with the accumulation of polyphosphate, PHA or both. We initially suspected that the inconclusive findings were attributed to the use of sludge samples from full-scale EBPR processes, in which the population diversity is influenced by a variety of environmental factors (e.g. multiple substrates, electron acceptors and constant changes in the P/C feeding ratio) (Cech & Hartman, 1993; Kuba *et al.*, 1993; Liu *et al.*, 1997a), and phosphate-accumulating bacteria were not necessarily the major population. Our study indicated that even under well-controlled and enriched EBPR systems, the detectable microbial populations were phylogenetically diverse. While a shift of microbial community structure on altering sludge P content was observed from the biomarker and DGGE fingerprints, our results further found that microbial communities in 2% and 12% P-containing sludge not only included predominant bacterial populations specific to each sludge, but also shared phylogenetically closely related populations. It was further suspected that specific phylogenetic groups might include both non-phosphate-accumulating and phosphate-accumulating populations. Thus, the combined use of biomarkers and DGGE methods was insufficient to identify organisms that accumulate phosphate. A promising approach will be to combine microautoradiography with fluorescent *in situ* hybridization to link functional traits to a phylogenetic population in activated sludge processes (Lee *et al.*, 1999). This approach with a refined hierarchical set of probes (e.g. Mobarry *et al.*, 1996; Raskin *et al.*, 1994) should provide a better understanding of the organisms responsible for EBPR.

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