APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1995, p. 1910–1916 Vol. 61, No. 5
0099-2240/95/$04.00+0

Bacterial Community Structures of Phosphate-Removing and Non-Phosphate-Removing Activated Sludges from Sequencing Batch Reactors

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Received 7 December 1994/Accepted 24 February 1995

The bacterial community structures of phosphate- and non-phosphate-removing activated sludges were compared. Sludge samples were obtained from two sequencing batch reactors (SBRs), and 16S rDNA clone libraries of the bacterial sludge populations were established. Community structures were determined by phylogenetic analyses of 97 and 92 partial clone sequences from SBR1 (phosphate-removing sludge) and SBR2 (non-phosphate-removing sludge), respectively. For both sludges, the predominant bacterial group with which clones were affiliated was the beta subclass of the proteobacteria. Other major groups represented were the alpha proteobacterial subclass, planctomycete group, and *Flexibacter-Cytophaga-Bacteroides* group. In addition, several clone groups unaffiliated with known bacterial assemblages were identified in the clone libraries. *Acinetobacter* spp., thought to be important in phosphate removal in activated sludge, were poorly represented by clone sequences in both libraries. Differences in community structure were observed between the phosphate- and non-phosphate-removing sludges; in particular, the *Rhodocyclus* group within the beta subclass was represented to a greater extent in the phosphate-removing community. Such differences may account for the differing phosphate-removing capabilities of the two activated sludge communities.

The activated sludge process used to treat wastewater involves the biological degradation of organic material. The removal of nutrients such as nitrogen (N) and phosphorus (P) from wastewater can be obtained in activated sludge under specific conditions. The present design for P removal, termed enhanced biological phosphate removal (EBPR), requires the wastewater to pass through an initial anaerobic treatment and then an aeration stage (52).

Empirical knowledge of the chemical transformations that occur throughout the different stages of the treatment has provided insight into the biological mechanism of EBPR. It was observed that EBPR was promoted if a sizable proportion of the influent organic carbon was in a form that was readily biodegradable (e.g., acetate) and present at the initial anaerobic stage of treatment (2, 12, 19, 37, 61).

There is interest to better understand the EBPR process, since it is not optimized and routinely fails. To achieve this, a more complete knowledge of microbial phosphate metabolism in activated sludge is required. Under favorable conditions, phosphate-removing sludge is observed to take several sludge ages to develop (41, 63). This suggests that the phosphate-removing community may need to be established and may not occur merely as a result of conditioning of the existing population.

Since Fuhs and Chen (18) first implicated *Acinetobacter* spp. as having an important role in EBPR, most subsequent studies have focused on this bacterial genus. The reasons for this attention have not been entirely unjustified. Culture-dependent methods consistently indicated that *Acinetobacter* spp. were the numerically dominant members of EBPR systems (7, 8, 29, 36, 60). Furthermore, some, but not all, *Acinetobacter* strains isolated from activated sludge accumulated excessive amounts of polyphosphate in pure culture, suggesting their importance in the EBPR process (3, 13, 18, 40, 56). However, researchers have had difficulties reconciling the carbon and phosphorus transformations in pure cultures of *Acinetobacter* strains with the biochemical model for EBPR (1, 5, 54). In recent years, serious doubts have been raised as to the significance of *Acinetobacter* spp. in EBPR processes. The most compelling evidence for this change of view has been the recent non-culture-dependent studies of phosphate-removing communities (11, 24, 59). In all cases, *Acinetobacter* spp. were found to represent only a small proportion of the total EBPR microbial population. Instead, other bacterial groups such as the gram-positive bacteria and the beta subclass of the proteobacteria were numerically dominant (59). While the use of these non-culture-dependent methods diminishes the significance of *Acinetobacter* spp. in EBPR processes, the resolution of the methods has not been sufficient to propose alternative EBPR candidate genera.

The non-culture-dependent molecular approach of cloning and sequencing 16S rDNA from environmental samples has previously been used to determine microbial community structure in soil (34), groundwater (16), and marine habitats (15, 20, 46). The aim of the present study was to apply this approach to determining the community structure of activated sludge samples obtained from two laboratory-scale sequencing batch reactors (SBRs) which differed in phosphate-removing capability. The bacterial community structures from two SBRs were compared in order to identify differences which may indicate groups or genera important in the EBPR process.

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MATERIALS AND METHODS

Operation of SBRs. Two laboratory-scale SBRs were constructed. The influent to the SBRs was settled sewage collected from the Wacol Wastewater Treatment

Plant, Brisbane, Australia. The influent had a relatively weak organic content with an average chemical oxygen demand of approximately 370 mg/liter, while soluble phosphate concentrations averaged 18 mg/liter. The reactors were operated on a 6.0-h cycle consisting of three stages: (stage 1) a 2.5-h anaerobic fill period in which 4 liters of settled sewage was added to 8 liters of activated sludge; (stage 2) a 3.0-h react period during which the reactor was aerated to maintain dissolved oxygen levels between 1.8 and 2.5 mg/liter; and (stage 3) a 30-min settle and decant stage in which aeration was stopped, the biomass settled, and 4 liters of treated water was decanted. The sludge age of the SBRs was 15 days, and the working volume of the reactors was 12 liters. Further details of the operation of the SBRs are given by von Münch (57).

Soon after start-up of the reactors, the feed to SBR1 was supplemented with sodium acetate to increase the influent chemical oxygen demand by 100 mg/liter. This was done to develop the phosphate-removing microbial population relative to the unsupplemented reactor (SBR2) and thereby obtain two sludges with differing phosphate-removing capabilities. After 3 weeks of this treatment, samples for chemical analyses, direct cell counts, and molecular biological studies were obtained from each SBR during one complete SBR cycle.

Chemical analyses. Soluble phosphate content and chemical oxygen demand were measured in filtered samples according to von Münch (57).

Direct cell counts. For direct cell counts of the activated sludge, 1-ml samples were taken during the aerobic stage of the SBR cycle. Cells were fixed by adding 0.1 volume of glutaraldehyde, sonicated for 1 min to disperse flocs, and diluted 50-fold in filter-sterilized 0.03 mM tripolyphosphate solution. Dilute samples (200 μl) were added to 5 ml of 0.05% acridine orange solution for 2 min and then concentrated onto Irgalan black-stained membrane filters (21-mm diameter, 0.2-μm pore size) by using slight vacuum. Filters were then washed with 1 ml of tripolyphosphate solution and mounted on microscope slides. Cells were counted under blue light (450 to 490 nm), using an epifluorescent microscope (Leitz) at ×1,000 magnification. At least 12 fields of 243 μm^2 in size were counted to determine the number of cells per milliliter for each sample.

Extraction and purification of total genomic DNA. One sludge sample (60 ml) was collected from each reactor during the aerobic stage of the SBR cycle. The two samples were immediately frozen and stored at −20°C prior to DNA extraction. Samples were thawed and vortexed, and 6 ml was taken for each DNA extraction. The cells were concentrated by centrifugation at 15,000 rpm for 10 min and resuspended in 0.5 volume of 0.15 M NaCl–0.1 M EDTA. Lysozyme was added to a concentration of 2 mg/ml, and the suspension was incubated for 1 h at 37°C. The cells were subjected to three cycles of freeze-thaw by freezing in liquid N_2 and heating for 3 min at 65°C. Proteinase K and sodium dodecyl sulfate were added to final concentrations of 0.02 and 0.5% (wt/vol), respectively, and the mix was incubated at 60°C for 1 h.

The effectiveness of the cell lysis procedure was confirmed by microscopic examination of samples taken before and after lysis treatment. Preparations were smeared onto microscope slides, dried, and stained for 2 min with 0.05% acridine orange solution. The slides were observed by epifluorescence microscopy as described for direct cell counts.

Protein and polysaccharide complexes in the lysed cell suspensions were extracted into equal volumes of phenol, then phenol-chloroform (25:25), and finally, phenol-chloroform-isoamyl alcohol (25:24:1). Nucleic acids were precipitated by adding 0.25 volume of sodium acetate (3 M; pH 5.2) and 2 volumes of cold absolute ethanol and placed at −70°C for 1 h. The nucleic acids were recovered by centrifugation at 15,000 rpm for 20 min. The DNA pellets were then washed with 70% ethanol, dried, and suspended to a concentration of between 200 and 500 μg/ml in TE buffer (10 mM Tris-HCl [pH 7.8], 1 mM EDTA).

Purification of the DNA was by a cesium chloride density gradient (44) created in 13-ml polyallomer tubes centrifuged for 48 h at 45,000 rpm in a Beckman type 50Ti rotor. The band containing DNA was removed from the gradient with a syringe. From the DNA, ethidium bromide was extracted into 6 equal volumes of water-saturated 1-butanol, and the cesium chloride was removed by dialysis for 36 h against TE buffer.

Amplification, cloning, and sequencing of 16S rRNA genes. Amplification of 16S rRNA genes from purified genomic DNA of both the SBR1 and SBR2 sludge samples was carried out in 100-μl reactions. Each reaction tube contained Tth *plus* reaction buffer (Biotech International, Perth, Western Australia), 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleotide triphosphate, 2 U of Tth *plus* DNA polymerase (Biotech International), 0.2 μg of each primer, and 150 ng of purified template DNA. The primers used in the amplification were 27f and 1492r (31). The reactions were covered with mineral oil and placed in a thermocycler. After an initial denaturation at 96°C for 2 min, 25 cycles were carried out: 48°C for 1 min, 72°C for 2 min, and 94°C for 1 min.

To prepare the rRNA gene libraries for each SBR, the products of three PCR amplifications were pooled and purified on Magic DNA Clean Up columns (Promega, Madison, Wis.). The purified product was then ligated into the TA Cloning System according to the manufacturer's instructions (Invitrogen, San Diego, Calif.). The ligation product was transformed into competent cells supplied in the TA cloning kit or into XL1-Blue MRF′ competent cells (Stratagene), using IPTG (isopropyl-β-D-thiogalactopyranoside). Plasmids were extracted by an alkaline lysis-polyethylene glycol precipitation method (Applied Biosystems, Foster City, Calif.) and inserts were detected by agarose gel electrophoresis. Plasmid inserts were sequenced with the PRISM Ready Reaction Dyedeoxy terminator sequencing kit (Applied Biosystems) with 500 to 1,000 ng of template DNA and 27f as the sequencing primer. Extension products were purified by phenol-chloroform, and an ABI model 373A automated sequencer was used to separate amplified fragments and to read the sequences.

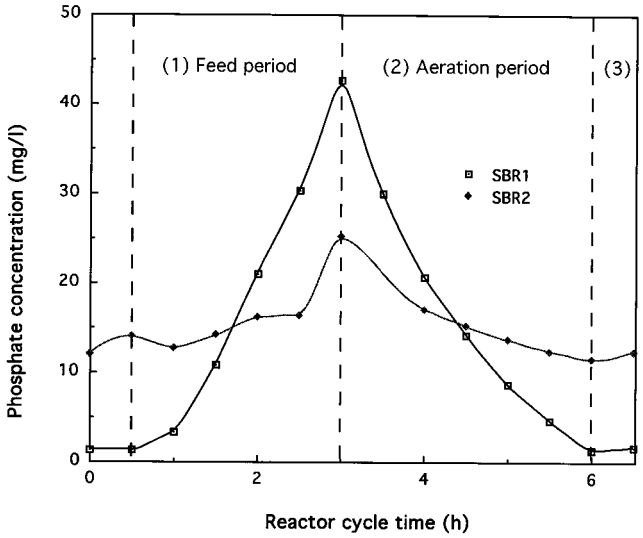

FIG. 1. Soluble phosphate concentrations in SBR1 and SBR2 during the period of a complete cycle in the reactors. Numbers in parentheses correspond to the reactor stages described in Materials and Methods.

Phylogenetic analysis. Partial sequences were manually aligned against reference sequences representative of the domain bacteria, using the editing program SeqEd (Applied Biosystems). All reference sequences were obtained from the ribosomal database project (32). Aligned sequence data were transferred to the ae2 editor (32) for construction of similarity matrices and preparation of masked data sets. A single representative of clones with ≥98% sequence similarity was selected to reduce the total number of sequences used in the subsequent analyses. Variable regions from positions 69 to 100 and 181 to 219 were excluded from data sets per the bacterial mask of Lane (31). Evolutionary distance trees with bootstrap resampling were constructed as previously described (4).

Nucleotide sequence accession numbers. Partial 16S rDNA sequences of the 189 SBR clones have the accession numbers X84449 to X84637.

RESULTS

SBR performance. Two SBRs were operated with the objective of obtaining sludges with differing phosphate removal capabilities. Sodium acetate was added to the feed of SBR1 to promote phosphate removal. After operation for 3 weeks, superior phosphate removal was observed in SBR1 relative to SBR2 (Fig. 1). These results are typical of good and poor phosphate removal in activated sludge as evidenced by excessive anaerobic phosphate release and aerobic phosphate uptake (39, 52). The influent phosphate concentration for both SBRs was 18.0 mg/liter. Anaerobic phosphate release by the SBR1 biomass was 35.4 mg/liter and that by the SBR2 biomass was 5.2 mg/liter. Phosphate levels in the effluents of SBR1 and SBR2 were 1.5 and 12.0 mg/liter, respectively. These data indicated that there were significant differences in the microbial communities of SBR1 and SBR2 with respect to the biochemical transformation of phosphate. Direct cell counts for the studied SBR cycle were not significantly different in SBR1 and SBR2 (9.1×10^9 and 7.6×10^9 cells per ml, respectively).

DNA extraction and PCR cloning. Approximately 22 μg of purified genomic DNA was obtained from the two SBR sludge samples. Following 16S rDNA amplification and ligation into the vector, the efficiencies of transformation of the clone libraries into cells supplied in the TA Cloning System were very low. Therefore, a second transformation into XL1-Blue MRF′ competent cells was carried out. Much higher efficiencies were

obtained for both clone libraries, in the order of 10^7 transformants per μg of plasmid. Clone libraries were established with approximately even numbers of β-galactosidase-negative recombinants selected from each transformation event. Transformation efficiency did not affect microbial community structure, as determined by phylogenetic analyses. Of the recombinants chosen, approximately 80% contained a 16S rRNA gene insert and provided readable sequences of usually greater than 400 bases.

Phylogenetic analysis. Partial sequences of the 16S rRNA gene, ranging in length from 376 to 569 nucleotides, were determined for 189 SBR clones (97 SBR1 and 92 SBR2 clones). A sequence similarity matrix is not presented due to the large size of the data set. The number of SBR clone sequences used in the initial analysis was reduced from 189 to 137 by including a single representative of clones with ≥98% sequence similarity. On the basis of a comparative analysis of 318 nucleotide positions, an evolutionary distance tree was constructed with SBR clones and representatives of the domain bacteria. To confirm the groupings established from this initial tree, subsets of the data were reanalyzed with bootstrap resampling (100). Typically, these subsets consisted of reference sequences and the sequences of the clone group of interest. Approximately 30 bootstrapped trees were constructed in this manner. The data from these analyses are summarized in Table 1. The criteria used to position clones in the bacterial domain were based in most instances on significant bootstrap values (>75%) (64). However, when bootstrap values did not support groupings, tree topology alone was used to infer relationships.

Most SBR clones (133 of 189) were affiliated with recognized "phyla" of the domain *Bacteria* (32), predominantly the proteobacteria (89 of 189) and, in particular, the beta subclass of the proteobacteria (54 of 189). The remainder of the SBR clones were either outliers to recognized phyla (27 of 189) or unaffiliated (29 of 189). The bacterial groups containing the majority of clones (beta and alpha subclasses and planctomycetes) were approximately evenly represented in both SBRs with the exception of the *Flexibacter-Cytophaga-Bacteroides* group, which was represented in SBR2 to a greater extent (Table 1). Individually, all other clone groups represented only a small percentage of one or both clone libraries and were considered minor constituents of the community.

To further observe differences between the community structures of the reactors, comparisons were made between the subgroups of the predominant phyla represented in each clone library. The gamma subclass was also considered because it includes the genus *Acinetobacter* (Fig. 2). The subgroups were defined, in most cases, according to the RDP release 4.0 phylogenetic list (32) and on the basis of tree topology since the bootstrap values within the major phyla were often low. Within the beta subclass, the *Rubrivivax* group was represented approximately evenly in the SBR1 (14.4% of sequenced SBR1 clones) and SBR2 (17.4% of sequenced SBR2 clones) clone libraries. However, representatives of the *Rhodocyclus* group were more prominent in SBR1 (12.4%) than in SBR2 (3.3%). In the alpha subclass, most of the clones were divided evenly into the alpha-2 and alpha-4 groups (62). Within the planctomycetes subdivision, the *Planctomyces* and *Gemmata* groups (33) were both represented to a greater extent by SBR1 clones. Within the *Flexibacter-Cytophaga-Bacteroides* group, both subdivisions were represented to a greater extent in the SBR2 clone library. The gamma subclass made up a relatively small percentage of the clone libraries (ca. 5% in both). Within this subclass, the *Acinetobacter* group was equally represented in the SBR1 and SBR2 clone libraries (ca. 2% in each).

TABLE 1. Phylogenetic position and frequency of SBR clones (from that reactor) in the bacterial domain[a]

RDP group no.	Bacterial phylum	% Clones from:		Bootstrap value
		SBR1	SBR2	
2.1	Thermophilic oxygen reducers			
2.2	Thermotogales			
2.4.1	*Chloroflexus* group		1.1	100
	Chloroflexus group outlier P	1.0	1.1	63
2.4.2	Deinococci			
2.5	*Leptospirillum*			
	Leptospirillum outliers			
	Q	3.1	2.2	100
	R	2.1	1.1	100
2.6	*Flexibacter-Cytophaga-Bacteroides*	5.2	13.0	56
2.7	*Chlorobiaceae*			
2.8	*Flexistipes*		1.1	52
	Flexistipes outlier T	1.0		
2.9.1	*Planctomyces*	12.4	8.7	90
	Planctomyces outlier I	1.0		
2.9.2	Chlamydia		1.1	62
2.9.3	*Verrucomicrobium*	1.0	1.1	87
	Planctomyces (and relatives) outliers			
	D	1.0		
	N	1.0		
2.10	Cyanobacteria			
2.12	Fibrobacteria			
2.13	Spirochaetes			
2.14.1	Alpha-subclass proteobacteria	11.3	15.2	67
2.14.2	Beta-subclass proteobacteria	32.0	25.0	89
2.14.3	Gamma-subclass proteobacteria	5.2	5.4	27
2.14.4	Delta-subclass proteobacteria			
2.14.5	Epsilon-subclass proteobacteria			
	Proteobacterial outliers			
	B		1.1	
	C	3.1	4.3	73
	G	1.0		
	U	1.0		
	V		1.1	
	X	1.0		
2.15	Fusobacteria			
2.16.1	High G+C, gram positive	1.0		96
2.16.2	Low G+C, gram positive	1.0		90
	Low G+C, gram-positive outlier S	1.0		
	Unaffiliated groups			
	A	1.0		
	E	1.0	7.6	67
	H		2.2	89
	J	3.1	2.2	72
	K	1.0		
	L		1.1	
	M	3.1		100
	O	4.1	4.4	55

[a] The nomenclature used is that of the RDP release 4.0 phylogenetic list (32) (not including group 2.3 as comparable sequence data were not available). Clones which could not be conclusively incorporated into existing bacterial phyla were categorized into groups A to X. Bootstrap values (100 bootstrap resamplings) supporting inclusion of both SBR1 and SBR2 clones in a defined group are presented.

The bacterial "species" composition of the major groups (according to Fig. 2) in the SBR1 and SBR2 clone libraries is presented in Table 2. A species was defined as a group of clones with greater than 97% sequence similarity. Clones which did not have >97% similarity with any other clones were considered sole representatives of a species. The 116 clones which represented the major phylogenetic groups were distributed among 69 species. Of the 69 species, 20 were common to

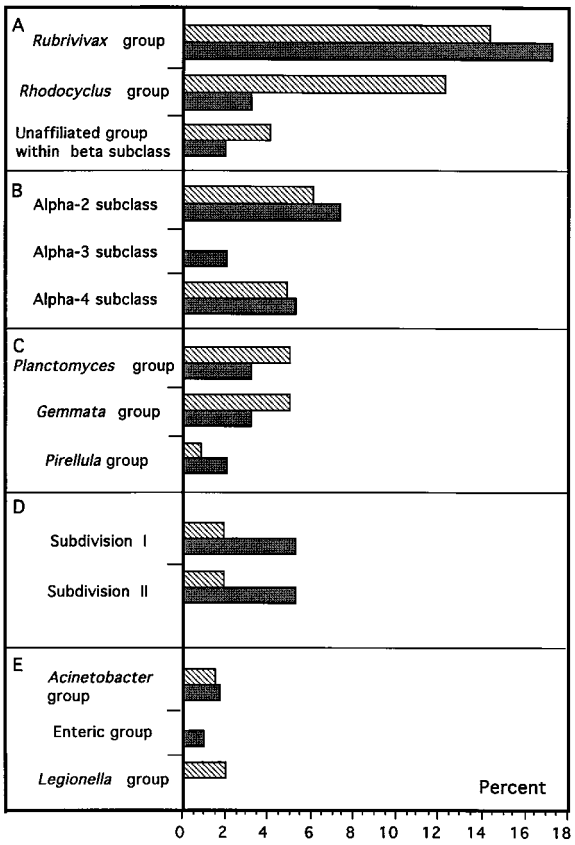

FIG. 2. Comparison of bacterial community structures of the major groups identified in the SBR1 library (▨) and the SBR2 library (■), expressed as a percentage of clones in each library: (A) beta subclass proteobacteria; (B) alpha subclass proteobacteria; (C) planctomycete group; (D) *Flexibacter-Cytophaga-Bacteroides* group; (E) gamma subclass proteobacteria.

both clone libraries and accounted for 45% of the 116 clone sequences. The diversity at the species level was quite different for the *Rubrivivax* and *Rhodocyclus* groups (Table 2). In the *Rubrivivax* group, four of the nine species had clone sequences in both the SBR1 and SBR2 libraries, making up 73% of the total clones in this group. The 15 clone sequences in the *Rhodocyclus* group were distributed among 12 species, and only 1 of these species, consisting of two clones, was observed in both libraries.

TABLE 2. Species and number of clones in each species for the major phyla (as grouped in Fig. 2) in the SBR1 and SBR2 clone libraries[a]

Phylum	Group	Species no.	No. of clones		Clones present in species common to both clone libraries (%)
			SBR1	SBR2	
Beta subclass proteobacteria	*Rubrivivax*	1	2	3	
		2	2	1	
		3	1	2	
		4	6	5	
		5	3	0	
		6	0	2	
		7–9*	0	3	
	Total		14	16	73
	Rhodocyclus	1	2	0	
		2	2	0	
		3	1	1	
		4–12*	7	2	
	Total		12	3	13
	Unaffiliated group	1	2	1	
		2–4*	2	1	50
Alpha subclass proteobacteria	Alpha-2 subclass	1	3	1	
		2–10*	3	6	31
	Alpha-3 subclass	1–2*	0	2	0
	Alpha-4 subclass	1	4	2	
		2–5*	1	3	60
Planctomycete group	*Planctomyces*	1	2	1	
		2–6*	3	2	37
	Gemmata	1	2	1	
		2	2	1	
		3–4*	1	1	75
	Pirellula	1–3*	1	2	0
Flexibacter-Cytophaga-Bacteroides	Subdivision I	1	1	1	
		2	1	3	
		3*	0	1	86
	Subdivision II	1	0	2	
		2–6*	2	3	0
Gamma subclass proteobacteria	*Acinetobacter*	1	2	0	
		2–3*	0	2	0
	Enteric group	1*	0	1	0
	Legionella	1	2	0	0

[a] A species was defined as a group of clones having >97% sequence similarity. Clones which did not have >97% similarity with any other clones were considered sole representatives of a species; these are indicated by asterisks.

DISCUSSION

The addition of acetate to promote phosphate removal in activated sludge wastewater treatment systems is well established (2, 37, 61). The acetate addition to SBR1 resulted in significantly better phosphate removal in this reactor than in SBR2. No significant difference in cell counts between the reactors was observed, suggesting that phosphate removal was a result of variations in community structure and/or metabolism. The present study was aimed at examining variations in the bacterial community structure by using a molecular approach. The methodology and results of this approach are discussed below.

Methodology. A 16S rDNA PCR cloning approach was used to determine the bacterial community structure of sludges from SBR1 and SBR2. Problems associated with culturing methods to study microbial populations are well known (6, 38, 58), and the use of non-culture-dependent methods such as PCR cloning is not without its limitations. In the PCR cloning approach several factors may bias the inferred community-structure, such as total DNA extraction from samples, PCR amplification bias, gene copy number bias, and cloning and sequencing artifacts (15, 16, 35, 42, 46). To reduce bias introduced from incomplete extraction and purification of total

DNA from sludge samples, rigorous cell lysis and DNA purification protocols were used. Few cells were observed in lysed samples, suggesting minimal selective loss of rRNA genes. Additionally, the use of a cesium chloride gradient in the purification procedure ensured removal of organic substances and metals known to be present in activated sludge samples and which inhibit PCR amplification (50).

An initial attempt to entirely remove the PCR amplification bias from the cloning procedure was made by using a bacteriophage shotgun cloning approach. This method was used successfully to study a marine picoplankton community (46). Sludge DNA was partially digested with the restriction enzyme *Sau*3A and cloned directly into a bacteriophage λ vector. Phage plaques were then probed for bacterial 16S rDNA sequences, using a digoxigenin labelling and detection system (data not shown). However, we were unable to routinely detect putative positive recombinants against the positive signal from the bacterial host of the phage. A possible improvement to this approach, without resorting to radiolabel detection of recombinants, would be the use of an archaeal or eucaryal phage cloning vector to avoid the interfering host signal.

The use of the PCR cloning and sequencing protocol proved to be fruitful in our attempt to investigate bacterial community structure of activated sludge. A high percentage of the chosen clones had 16S rRNA gene inserts that were readily sequenced. In conjunction with automated sequencing, this resulted in a rapid turnaround of high-quality sequence data. The first 500 nucleotide positions of the 16S rDNA were selected for sequencing because phylogenetic analyses based on this region have been shown to be consistent with analyses based on the entire gene sequence (46). We also verified this observation. On the basis of a distance analysis of our near-complete data set (1,201 nucleotides) of representative bacterial sequences, most of the stable clades described by Embley et al. (17) were supported by bootstrap resampling (>75%) (64; data not shown). Exceptions were the branching points for the proteobacterial group and the gamma subclass. The low bootstrap value for the gamma subclass is shown in Table 1. We carried out several distance analyses with partial data sets of the reference sequences to compare the resultant trees with that from the near-complete data set. Sequence zones from positions 1 to 500, 501 to 1000, and 1000 to 1500 of the 16S rDNA reference sequences were analyzed. The analysis of the region from positions 1 to 500 of our reference data set showed that two stable clades from our near-complete data set analysis were no longer supported by bootstrapping: the *Leptospirillum* group and Thermotogales. Our clone data were then included in distance analyses. As the number of clones for a given group increased, the associated bootstrap value for that group fell. This was particularly the case for the *Flexibacter-Cytophaga-Bacteroides* group.

Bacterial community structure. Historically, it has been thought that bacteria of the gamma subclass of the proteobacteria, such as *Acinetobacter* species, dominate phosphate-removing activated sludge populations. In a sludge that was conditioned to remove phosphate by the addition of acetate, bacteria from the genus *Acinetobacter* were reported to make up 90% of the cultivated heterotrophs (60). Other members of the gamma subclass such as aeromonads, vibrios, and coliforms were reported to dominate an activated sludge community as determined by plate culturing onto various media (28). By contrast, non-culture-dependent methods such as quinone profiles (24, 25) and fluorescent in situ hybridization probes (59) indicated that *Acinetobacter* spp. were present in small proportions in activated sludge (ca. 3 to 6%). As well, Wagner et al. (58, 59) found that members of the gamma proteobacterial subclass were selected for by cultivation with nutrient media, explaining the results of Kavanaugh and Randall (28). Results from our study also indicated that *Acinetobacter* spp. made up only a small proportion (ca. 2%) of the SBR communities. These data suggest that culturing techniques have provided a misleading picture of bacterial community structure in activated sludge and that, in general, the role of *Acinetobacter* spp. in activated sludge processes has been overrated.

The largest percentages of clones from both SBR1 and SBR2 were members of the proteobacterial beta subclass (ca. 28%). Bacteria from this subclass have recently been observed by fluorescent in situ hybridization to be dominant in activated sludge communities (38, 58, 59). Given their numerical dominance, it is likely that representatives of the beta subclass play roles in aspects of activated sludge such as degradation of organic material, removal of nutrients, and formation of floc structure.

The differences observed between the two SBR communities within the beta subclass (Fig. 2) suggest that the *Rhodocyclus* group may have a specific role in phosphate removal. Consistent with this observation is the fact that a high percentage of species within the *Rubrivivax* group are common to both SBRs (73%; Table 2) as opposed to *Rhodocyclus* species, with only 13% of clones being common to both SBRs. However, the high species diversity apparent in the SBRs (Table 2) and the limited number of clones analyzed make it difficult to identify one or more bacterial species which may be important in phosphate removal. Further analysis of SBR clones until all species are represented by at least two clones would increase the resolution of the study and confirm the possible importance of a given species or group in the phosphate removal process.

The *Rhodocyclus* group contains members of the phototrophic genus *Rhodocyclus*, as well as members of *Azoarcus* and *Zoogloea* (RDP phylogenetic list version 4.0 [32]). *Rhodocyclus* and *Zoogloea* spp. have been previously isolated from activated sludge (23, 48), and the latter are considered typical floc-forming organisms (55). However, caution should be taken in concluding that *Zoogloea* spp. are important in phosphate removal because *Zoogloea* spp. are phylogenetically heterogeneous. Species of this genus are also present in the alpha subclass and *Burkholderia* group of the beta subclass (48). Further sequencing of the *Rhodocyclus* clones from SBR1 and SBR2 is required to definitively place them within the group.

Following the beta subclass, the alpha proteobacterial subclass was the next most predominant group represented by SBR clones (ca. 13% of total clones). Bacteria from the alpha subclass were observed to be dominant in an activated sludge community also determined by a PCR cloning approach (47). This suggests that the alpha subclass may be important in activated sludge processes. However, at the group level no significant differences between the phosphate- and non-phosphate-removing communities were noted (Fig. 2), although certain species were observed in greater numbers in the SBR1 clones than in the SBR2 clones (Table 2).

Our data suggesting a role for the *Rhodocyclus* group in EBPR are interesting since phototrophic proteobacteria belonging to the alpha and beta subclasses have been observed in conventional activated sludge samples (23, 49) and in photosynthetic activated sludge (26). The contribution of these phototrophic bacteria in conventional processes was thought to be negligible because they were detected in low numbers by culture techniques (23, 49). The number of these bacteria was observed to increase in activated sludge when the levels of volatile fatty acids were increased (23). This is similar to the relationship observed between EBPR and volatile fatty acid levels (52). Polyphosphate accumulation by strains of the pho-

totrophic proteobacteria in the alpha and beta subclasses has been observed (22, 27). It would be interesting to compare the physiological aspects of phosphate metabolism used by the phototrophic proteobacteria with the biochemical model for EBPR.

A surprising proportion of both clone libraries (ca. 10%) was observed in the planctomycetes group. Bacteria from this group have not previously been observed in activated sludge. However, *Planctomyces bekefii* has been documented in eutrophic aquatic habitats contaminated with domestic sewage (30), and recently *Pirellula*-like and unidentified planctomycetes were isolated from sewage sludge (45). Of the clones within the *Planctomyces* and *Gemmata* groups, potentially significant differences were observed between the SBR communities (Fig. 2), suggesting their possible involvement in the EBPR process. However, no single species within the planctomycete groups appeared to dominate significantly in the SBR1 clones relative to SBR2 (Table 2), indicating the need for further screening of the clone libraries to substantiate the possible importance of this group to the process.

A sizable proportion of SBR clones were affiliated with the *Flexibacter-Cytophaga-Bacteroides* group, particularly in the non-phosphate-removing (SBR2) library (13%). Bacteria from this group have frequently been observed in activated sludge samples (21, 38, 51, 59). The larger percentage of *Flexibacter-Cytophaga-Bacteroides* group clones in SBR2 relative to SBR1 may reflect opportunistic occupation of a niche vacated by phosphate-removing bacteria in the sludge habitat of SBR2. Alternatively, they may actively inhibit phosphate removal, as has been postulated for some bacteria (10).

Noticeably absent from the clone libraries were representatives of the gram-positive bacteria. This is consistent with most previous reports of activated sludge bacterial flocs being dominated by gram-negative genera (7, 8, 21). However, at least one survey found that the culturable fraction of a number of activated sludge treatment plants was dominated by *Bacillus* species (9). Gram-positive bacteria with high G+C contents have also been observed as dominant members of a phosphate-removing activated sludge community as determined by a non-culture-dependent method (fluorescent in situ hybridization), and it was suggested that bacteria belonging to this group may have been responsible for the observed phosphate removal (59). The lack of gram-positive clones in our study compared with that of Wagner et al. (59) may be a result of the different bacterial communities present or because of the different approaches used.

A relatively large proportion of the SBR clones were unaffiliated with, or were outliers of, existing bacterial groups (ca. 30%). This is typical of recent cloning studies of environmental samples (14, 34, 46) and reflects the fact that most bacteria in nature have not been cultured and described (6, 43, 53). Further sequencing of our potentially novel clones is required to definitively position them within the bacterial domain and will provide the focus for future studies.

While the results observed in the analyses of the SBR clone libraries offer exciting insights into the bacterial ecology of phosphate removal in activated sludge, the conclusions drawn from this study require further support from other sludge community structure studies. The immediate use of the sequence data obtained from the clone libraries will be to construct RNA-directed probes for screening clone libraries and probing activated sludge. Results of these types of approaches will identify the bacteria responsible for phosphate removal. The next goal is to investigate phosphate transformations in pure cultures of bacteria identified as important members of EBPR processes. For the moment, bacterial phosphate removal in activated sludge remains an intriguing microbiological puzzle.

ACKNOWLEDGMENTS

This work was funded by the CRC for Waste Management and Pollution Control Ltd., a center established and supported under the Australian Government's Cooperative Research Centres Program.

We appreciate the expertise of Jens Gösswein and Elisabeth von Münch for the construction and operation of the SBRs and access to their results. We thank Jenny Cassady and Emma Puttick (DNA Sequencing Facility, The University of Queensland) for assisting with DNA sequencing and John Fuerst for useful discussions on planctomycete ecology.

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