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Fine-scale differences between *Accumulibacter*-like bacteria in enhanced biological phosphorus removal activated sludge

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Abstract A lab-scale sequencing batch reactor (SBR) and six full-scale wastewater treatment plants (WWTPs) performing enhanced biological phosphorus removal (EBPR) were surveyed. The abundance of *Accumulibacter*-related organisms in the full-scale plants was investigated using fluorescent in situ hybridization. *Accumulibacter*-related organisms were present in all of the full-scale EBPR plants, at levels ranging from 9% to 24% of total cells. The high percentage of *Accumulibacter*-related organisms seemed to be associated with configurations which minimize the nitrate recycling to the anaerobic zone and low influent BOD:TP ratios. PCR-based clone libraries were constructed from the community 16S rRNA gene plus the internally transcribed spacer region amplified from the SBR and five of the full-scale WWTPs. Comparative sequence analysis was carried out using *Accumulibacter*-related clones, providing higher phylogenetic resolution and revealing finer-scale clustering of the sequences retrieved from the SBR and full-scale EBPR plants.

Keywords *Accumulibacter*; enhanced biological phosphorus removal; polyphosphate accumulating organisms

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Introduction

Phosphorus (P) is the critical factor leading to the eutrophication of surface waters. Enhanced biological P removal (EBPR) has been applied for several decades to achieve low P levels in treated wastewater effluent. EBPR employs the activities of polyphosphate accumulating organisms (PAOs) that release phosphate (P_i) anaerobically, then take up P_i aerobically and store it as intracellular polyphosphate (polyP), in excess of the amount released anaerobically. P is ultimately removed from the system through sludge wastage. In spite of its wide and successful application, our poor understanding of the underlying biological mechanisms responsible for EBPR requires that process design and operation be largely empirical. Therefore, some wastewater treatment plants (WWTPs) suffer from unreliable EBPR performance.

A significant amount of research effort has been expended in an attempt to identify the microorganisms responsible for EBPR. Early studies, based on culture-dependent methods, suggested that dominant PAOs were *Acinetobacter* spp.. However, later studies showed the insignificance of *Acinetobacter* in EBPR using cultivation independent molecular techniques (Wagner *et al.*, 1994; Mino *et al.*, 1998). Further investigation using such tools led to the identification of bacteria phylogenetically affiliated with the *Rhodocyclus* group of the beta-proteobacteria and those affiliated with gamma-proteobacteria as candidate PAOs (Bond *et al.*, 1995; Hesselmann *et al.*, 1999; Crocetti *et al.*, 2000; Liu *et al.*, 2001). The *Rhodocyclus*-like organisms are currently thought to be the most relevant PAO candidate and have been tentatively named *Accumulibacter phosphatis*

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(Hesselmann *et al.*, 1999). Several researchers (Zilles *et al.*, 2002; Saunders *et al.*, 2003) confirmed these organisms' involvement in EBPR in certain full-scale WWTPs.

Previous studies on the occurrence and phylogeny of *Accumulibacter*-related organisms in EBPR systems primarily relied on the use of 16S ribosomal RNA (rRNA)-based methods. However, the limited phylogenetic resolution of 16S rRNA makes it difficult to analyze very closely related bacteria (Fox *et al.*, 1992). Indeed, some of the *A. phosphatis*-related 16S rRNA sequences recovered from geographically and temporally distinct EBPR systems are nearly identical. Errors associated with PCR and sequencing procedures makes the difference between them statistically invalid (Acinas *et al.*, 2004). Do these sequences represent essentially identical "strains" of *A. phosphatis,* or can we expect significant genotypic and/or phenotypic differences between populations across EBPR systems? Clearly, investigation of fine-scale differences among *A. phosphatis* populations requires the use of more divergent genetic loci. The 16S–23S internally transcribed spacer (ITS) region in the *rrn* operon exhibits higher variation both in terms of length and sequence, and may provide enough resolution to observe such fine-scale differences in population structure. Despite the variance, the ITS region is still sufficiently conserved among closely related bacteria, making it possible to use this locus for stable classification (Iteman *et al.*, 2000).

In the current study, we investigated the abundance of *Accumulibacter*-related organisms, and the fine-scale population structure of these candidate PAOs. To do this, we sampled activated sludge from one lab-scale sequencing batch reactor (SBR) and six full-scale EBPR facilities in the USA. Several of these WWTPs were recently included in a survey of EBPR system performance (Stephens *et al.*, 2004; Gu *et al.* in preparation).

Methods

The lab-scale SBR was inoculated with sludge from the Madison, WI, USA, Nine Springs WWTP (operated as a UCT (University of Cape Town) process), performing good EBPR. The reactor, with a working volume of 2 L, was operated on a 6 h cycle, including 130 min anaerobic phase, 190 min aerobic phase, 30 min settling and 10 min effluent withdrawing. The hydraulic residence time was 12 h, and the solids retention time (SRT) was 4 d. The pH was maintained at 7.0–7.3. The SBR was fed with acetate, casamino acids, and a mineral salts medium with P_i to achieve a COD:P of 14 (mg COD:mg P) (McMahon *et al.*, 2002b; Schuler and Jenkins, 2003).

Activated sludge samples were collected at the end of the aerobic stage in six full-scale EBPR WWTPs. Important physical and chemical characteristics of these plants are summarized in Table 1. The samples were transported overnight on ice for analysis within 24 h of sampling.

PAOs were visualized by staining intracellular polyP with 4′,6-Diamidino-2-phenylindole (DAPI). *Accumulibacter*-related organisms were visualized by 16S rRNA-targeted fluorescent in situ hybridization (FISH). The sludge flocs were mechanically disrupted by repetitively pushing sludge sample through a 26-gauge needle for 20 min, fixed with 3% paraformaldehyde and then transferred to slides. A probe mixture (RHC439, PAO462b, PAO651 and PAO846b), targeting the *Accumulibacter*-related organisms was applied with the same hybridization conditions used by Zilles *et al.* (2002). PolyP staining and FISH results were expressed as the percentage of the total cells determined by DAPI stain.

Genomic DNA was extracted by a modified enzyme digestion method followed by phenol:chloroform extraction and isopropanol precipitation (Purkhold *et al.*, 2000; McMahon *et al.*, In submitted). The 16S + ITS region of the *rrn* operon was amplified from the community DNA using bacterial-specific 8f and 23Sr primers, and KOD Hot Start high fidelity DNA polymerase (Novagen, WI). Clone libraries were constructed

Table 1 Summary of characteristics of the SBR and full-scale EBPR plants

	SBR	Nansemond	VIP	Durham	Las Vegas 1[a]	Las Vegas 2[a]	Nine Springs
State	WI	VA	VA	OR	NV	NV	WI
Process	SBR	VIP	VIP	A^2O	MUCT[b]	MUCT	UCT
Chemical addition to precipitate P	None	Ferric/SC[b] on demand	None	Lime/PE[b] Alum/SC[b],T [b] Ferric/RS[b]	Ferric[c]/PC[b]	Ferric[c]/PC[b]	None
SRT (d)	4.0	8.5	9.5	10.9	8.4[d]	8.4[e]	8.5 ~ 9.0
SI[b] total P(mg-P/L)	7.0	14.2	5.3	6.1	6.5	6.5	7.0
SI[b] PO_4-P (mgP/L)	7.0	11.3	4.1	3.4	4.2	4.2	ND[b]
SE[b] total P(mg-P/L)	0.10	1.2	0.64	0.51	0.14	0.12	0.40
BOD:P (mg/mg-P)[f]	14	14 ± 3	29 ± 4	20 ± 4	42	42	26

[a] Las Vegas 1 and 2 are parallel trains in the same WWTP
[b] SC-secondary clarifier, PE-primary effluent, T-tertiary treatment, RS-recycled stream, PC-primary clarifier, SI-secondary influent, SE-secondary effluent, MUCT-modified UCT, ND-not determined
[c] Ferric was intermittently added. There was no ferric addition during the month in which the samples for the current study were collected
[d] SRT was 7.5 d previously, and switched to 9.5 d for 18 d then to 8.4 d. Sample was taken 5 d after switching to 8.4 d
[e] SRT was 9.5 d and then switched to 8.4 d. Sample was taken 5 d after switching to 8.4 d
[f] Primary effluent BOD:total P ratio ± standard deviation (when available)

using the TOPO TA cloning kit (Invitrogen, CA) according to the manufacturer's instructions. Ninety-six clones from each library were picked randomly. For full-scale EBPR samples, *Accumulibacter*-related clones were screened by real-time PCR (Warnecke *et al.*, 2004), using a mixture of forward primers (RHC439f, PAO651f and PAO846bf) and 1492r, at the annealing temperature of 60 °C. For the SBR, unique clones were determined by restriction fragment length polymorphism (RFLP) (McMahon *et al.*, 2002a). The entire cloned 16S + ITS region of *Accumulibacter*-positive clones from full-scale WWTPs and unique clones from the SBR were sequenced. Sequences from each full-scale WWTPs were grouped into operational taxonomic units (OTUs) based on 99% identity shared between 16S + ITS sequences. Unique sequences associated with *Accumulibacter* spp., as determined using the BLAST network service (Altschul *et al.*, 1990) were selected for phylogenetic analysis. The 16S + ITS sequences were aligned with the GCG software package (Accelrys, CA). MrBayes version 3.0 was used for Bayesian analyses (Ronquist and Huelsenbeck, 2003), running for 1,000,000 generations with sampling every 10 generations to create 100,000 trees. The consensus tree was visualized and printed in PAUP * 4.0 (Sinauer Associates, MA).

Results and discussion

SBR performance

At steady state, the SBR exhibited characteristic EBPR carbon and P transformations. P content of the sludge biomass was 18% (mg P/mg VSS).

The abundance of *Accumulibacter*-related organisms

The abundance of *Accumulibacter*-related organisms was quantified using FISH. In the SBR, the microbial community was dominated (>~80%) by *Accumulibacter*-related organisms (data not shown). For the six WWTPs studied, *Accumulibacter*-related organisms were present, at levels ranging from 9% to 24% of total cells, confirming that they play a significant role in EBPR processes. Nansemond and Viginia Initiative Process (VIP)

(operated as VIP processes), and Nine Springs (operated as a UCT process) had higher percentages of *Accumulibacter*-related organisms than Durham (operated as an A^2O process) (Figure 1). The major difference between the VIP, UCT and anaerobic – anoxic – oxic (A^2O) processes is that the VIP and UCT configurations minimize the amount of nitrate return to the anaerobic zone. Lindrea *et al.* (1998) showed that the amount of nitrate returned to the anaerobic zone negatively affected the P removal stability, and that this was associated with the distribution of P between different cellular polyP fractions (short-chain polyP vs. long-chain polyP) in EBPR sludge. The lower level of *Accumulibacter* in A^2O processes may be due to their higher sensitivity to nitrate and/or their preferred polyP formation mechanism. VIP processes and modified UCT (MUCT) processes are very similar. However, Nansemond and VIP had markedly higher percentages of *Accumulibacter*-related organisms than the two Las Vegas plants, which were operated as MUCT processes. The low abundance of *Accumulibacter* in the Las Vegas plants might arise from the higher influent BOD:total P ratios (Table 1). Previous studies showed that low BOD:P ratios favored *Accumulibacter*-related organisms (McMahon *et al.*, 2002b; Schuler and Jenkins, 2003), while high BOD:P ratios favored glycogen-accumulating organisms (Schuler and Jenkins, 2003). Also, from Figure 1 it is noticeable that the percentage of *Accumulibacter*-related organisms was less than the percentage of PAOs, suggesting a possibility of non-*Accumulibacter* PAOs in the full-scale EBPR plants, especially in Durham and the two Las Vegas plants.

Clone library screening

Clone libraries were constructed for the lab-scale SBR and the full-scale WWTPs, except for Nine Springs, since 16S rRNA-based phylogenetic analysis had previously been performed on *Accumulibacter*-related organisms in that plant (Zilles *et al.*, 2002). A total of 96 clones from each full-scale WWTP's library were screened by real-time PCR to identify potential *Accumulibacter*-related 16S + ITS clones for sequencing. The numbers of positive clones identified with this screen are listed in Table 2. However, comparative sequence analysis led to the observation that not all of the positive clones were related to *Accumulibacter* (Table 2). This could be attributed to non-specific amplification of some 16S rRNA outside the *Accumulibacter* group. However, this is not surprising since the multiplex PCR was carried out under less stringent conditions to assure retrieval of all

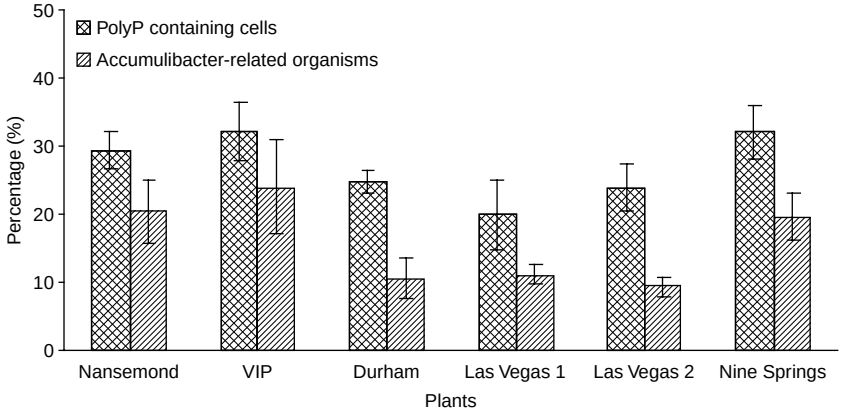

Figure 1 Percentages of *Accumulibacter*-related organisms and polyP containing cells. Error bars represent standard deviations for replicates

Table 2 Results of 16S + ITS rRNA clone library screening

	SBR	Nansemond	VIP	Durham	Las Vegas 1	Las Vegas 2
Number of positive clones by real-time PCR	13[a]	15	27	7	7	6
Number of *Accumulibacter*-related clones	2[b]	11	21	2	0[c]	0[c]

[a] The number of unique operational taxonomic units (OTUs) determined by RFLP
[b] The number of *Accumulibacter*-related sequence types out of the 13 unique OTUs
[c] The number of *Accumulibacter*-related clones was 7 for Las Vegas 1 and 8 for Las Vegas 2 in additionally constructed 16S + ITS clone libraries

Accumulibacter-related sequences. Thus, this screening strategy successfully reduced the number of clones to be sequenced, but did result in some false positives.

A larger number of *Accumulibacter*-related sequences were recovered from the Nansemond and VIP plants. These results confirm the abundance of *Accumulibacter* group members as determined by FISH. The lower recovery of *Accumulibacter*-related sequences from Durham and two Las Vegas sludges may partly due to PCR bias (Wintzingerode *et al.*, 1997). However, 16S + ITS amplicons were easily obtained from these sludges using universal primers (data not shown), therefore it is reasonable to rule out general PCR inhibition. Since *Accumulibacter*-related organisms were present in lower abundances in these three plants, as indicated by our FISH results, it is probable that *Accumulibacter*-related clones were simply missed by our rather small clone libraries. Additional 16S + ITS clone libraries were constructed for the two Las Vegas sludges. A total of 7 and 8 *Accumulibacter*-related clones were recovered from Las Vegas 1 and 2 respectively from additionally constructed clone libraries.

Finer scale phylogenetic analysis of *Accumulibacter*-related sequences retrieved

The ITS sequences retrieved from the current study consisted of both conserved regions (corresponding to coding regions for tRNA-Alanine and tRNA-Isoleucine) and variable regions. The variable regions may lead to the design of specific rRNA-targeted probes or primers with higher resolution, distinguishing closely related *Accumulibacter* organisms. The conserved regions made it possible to align the sequences with different ITS lengths and build the 16S + ITS phylogenetic tree, as shown in Figure 2.

By comparative sequence analysis, it is noticeable that the OTUs in Clade I form a well-supported cluster (0.99 posterior probability). PCR bias notwithstanding, this result suggests that the *Accumulibacter*-like organisms found in the lab-scale SBR were present in full-scale EBPR plants operated using the VIP process. Taken together with the FISH results, it is likely that these *Accumulibacter*-like organisms are important PAOs in plants with the VIP configuration.

Our survey was designed to begin to search for evidence of biogeographical isolation of *Accumulibacter*-related PAOs. Although in previous studies, some of the *Accumulibacter*-related 16S rRNA sequences recovered from geographically distinct EBPR systems were nearly identical, implying a lack of biogeography, it is hard to draw such a conclusion due to the limited phylogenetic resolution provided by 16S rRNA. However, phylogenetic analysis based on 16S + ITS rRNA could provide additional resolution. From our 16S + ITS rRNA tree, there are no obvious coherent patterns of the sequences retrieved from geographically isolated systems. Interestingly, clone Dur D8, from the state of Oregon, affiliated with clones Vir D2 and Nan E6, both from the state of Virginia. Does this suggest that geographical factors do not influence the global population structure of *Accumulibacter*-related organisms? Due to the small sample size and limited sequences recovered in the current study, it is not possible to answer this question yet.

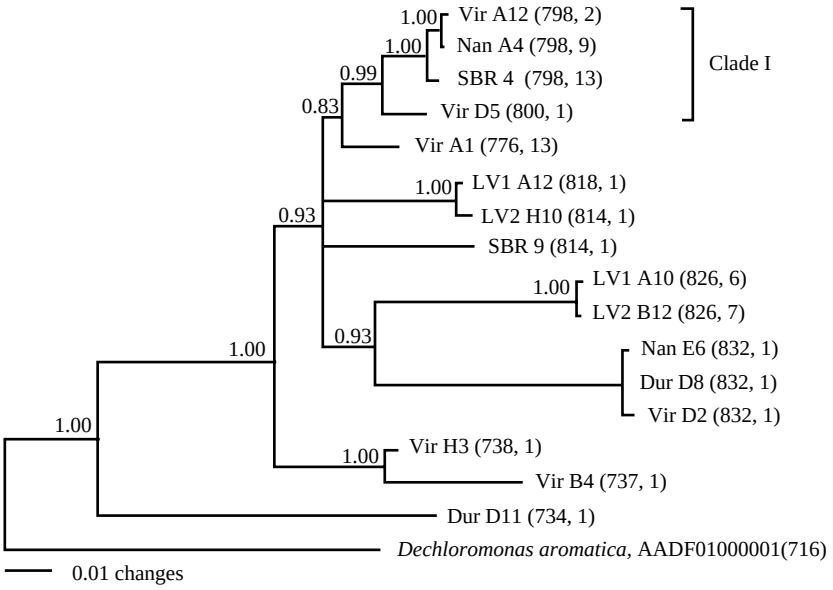

Figure 2 Unrooted phylogenetic tree for *Accumulibacter*-related 16S + ITS rRNA sequences retrieved from SBR and full-scale WWTPs (Nan from Nansemond, Vir from VIP, Dur from Durham, LV1 from Las Vegas 1 and LV2 from Las Vegas 2). The outgroup is the 16S + ITS rRNA sequence from *Dechloromonas aromatica*. The posterior probabilities are shown next to the nodes. The first number in the parentheses indicates the automated ribosomal intergenic spacer analysis (ARISA) fragment length, which is the number of bases between the annealing sites of primers 1406f and 23Sr (primer sequences included) and the second number indicates the number of the clones belong to this OTU. For all *Accumulibacter*-related sequences retrieved in this study, ITS lengths were 282 bp shorter than ARISA fragment lengths, except Vir A1, Vir H3 and Dur D11, whose ITS lengths were 283 bp shorter than ARISA fragment lengths

Conclusions

Accumulibacter-related organisms were detected using FISH in all of the full-scale EBPR plants surveyed. The high percentage of *Accumulibacter*-related organisms seemed to be associated with configurations which minimize the nitrate recycling to the anaerobic zone and low influent BOD:TP ratios.

The percentage of *Accumulibacter*-related organisms was less than the percentage of PAO populations, suggesting a possibility of non-*Accumulibacter* PAOs in the full-scale EBPR plants, especially in the Durham and two Las Vegas plants.

A 16S + ITS rRNA phylogeny was reconstructed and a finer-scale classification of *Accumulibacter*-related organisms retrieved from the current study was revealed.

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