Potential for Growth of *Candidatus* ‘Accumulibacter phosphatis’ in an Aerobic Shaking Culture

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ABSTRACT

Candidatus ‘Accumulibacter phosphatis’ (‘Accumulibacter’) is known as polyphosphate-accumulating organism found in the enhanced biological phosphorus removal (EBPR) activated sludge processes. ‘Accumulibacter’ is a yet-to-be isolated bacteria, and the growth of ‘Accumulibacter’ under aerobic conditions is still unknown. In this study, the potential for growth of ‘Accumulibacter’ in an aerobic shaking culture was evaluated. Activated sludge samples from a laboratory-scale EBPR reactor were incubated for 48 hours under different conditions (pH, temperature and carbon source), and the multiplication of the ‘Accumulibacter’ 16S rRNA gene was quantified by using quantitative real-time PCR (polymerase chain reaction). The gene showed statistically significant increase when the carbon source in the medium was glucose, the initial pH was 10.0 and the temperature was 20 °C ($p > 0.05$, $n=9$). Results in this study showed that ‘Accumulibacter’ most probably has the potential to grow in an aerobic shaking culture. The obtained results are desired to provide a hint for the successful isolation of ‘Accumulibacter’.

Keywords: aerobic shaking culture, *Candidatus* ‘Accumulibacter phosphatis’, enhanced biological phosphorus removal.

INTRODUCTION

Candidatus ‘Accumulibacter phosphatis’ (‘Accumulibacter’) is currently thought to be the most relevant polyphosphate-accumulating organism in the enhanced biological phosphorus removal (EBPR) process (Seviour *et al.*, 2003). ‘Accumulibacter’ has been identified by culture-independent approaches (Crocetti *et al.*, 2000; Hesselmann *et al.*, 1999), but has not yet been isolated despite tremendous research efforts.

‘Accumulibacter’ can assimilate organic substrates under both aerobic and anaerobic conditions (Kong *et al.*, 2004) but there have been no reports on the growth of ‘Accumulibacter’ under circumstances other than sequential anaerobic–aerobic conditions in the EBPR process. Although it is worthwhile to employ such conditions for the isolation of these organisms, the establishment of anaerobic–aerobic culture conditions is technically complicated and difficult. Thus, it would be helpful to investigate whether ‘Accumulibacter’ can grow under aerobic culture conditions. If so, this may lead to a simpler approach for its isolation.

In this study, we examined the growth potential of ‘Accumulibacter’ in an aerobic shaking culture. Activated sludge from laboratory-scale EBPR reactor was incubated for

48 hours under different conditions (pH, temperature and carbon source). The growth potential was evaluated by using the quantitative real-time PCR (polymerase chain reaction) method.

MATERIALS AND METHODS

Activated sludge

A laboratory-scale sequencing batch reactor (SBR) with a working volume of 10 L was operated under sequential anaerobic–aerobic conditions for EBPR. The SBR cycle was 6 h in total, comprising a 0.5-h feeding phase, a 1.5-h anaerobic phase, a 2.5-h aerobic phase, a 1-h settling phase, and a 0.5-h discharge phase. The sludge retention time (SRT) was about 8 days, and the hydraulic retention time (HRT) was about 10 h. The carbon concentration in the feed for each SBR cycle was 83.3 mg C/L, with acetate as the main carbon source. The phosphorus to carbon ratio (wt/wt) was 1:15.6. The concentrations of other nutrients in the feed were 7.3 mg/L $\text{CaCl}_2(2\text{H}_2\text{O})$, 75 mg/L $\text{MgCl}_2(6\text{H}_2\text{O})$, 35 mg/L KCl, 14.7 mg/L NH_4Cl , 18 mg/L $(\text{NH}_4)_2\text{SO}_4$, 15 mg/L K_2HPO_4 , 11.7 mg/L KH_2PO_4 , and 3.3 mg/L allylthiourea. The value of pH was maintained between 7.8 to 8.0 in the feeding, anaerobic and aerobic phases by using pH controllers (mk-250, Automatic System Research Co., Tokyo, Japan) that dosed 0.5 M of HCl or NaOH when the pH exceeded the setpoint. Seed sludge was obtained from a full-scale domestic wastewater treatment plant.

To monitor the reactor performance, phosphorus (P) contents of the sludge were measured as described previously (Fukushima *et al.*, 2007a).

Aerobic shaking culture

Activated sludge samples were taken at the end of the aerobic phase. The sludge samples were immediately diluted to 100 mg(dry) /L by the addition of a culture medium. The carbon concentration in diluted samples was 500 mgC/L. The concentrations of other nutrients were 17.6 mg/L $\text{CaCl}_2(2\text{H}_2\text{O})$, 181.6 mg/L $\text{MgCl}_2(6\text{H}_2\text{O})$, 84.0 mg/L KCl, 35.2 mg/L NH_4Cl , 42.2 mg/L $(\text{NH}_4)_2\text{SO}_4$, 36.0 mg/L K_2HPO_4 , and 28.0 mg/L KH_2PO_4 . The initial pH of the culture medium was adjusted to 6.0, 7.0, 8.0, 9.0, or 10.0 using 0.1M HCl or 0.1M NaOH. Ten milliliters of aliquots of each diluted sample were placed in sterile 50-mL tubes and incubated for 48 h with shaking at 150 rpm. The tubes were capped with silicone-sponge plugs to provide an oxygen supply. Incubations were performed in triplicate for each combination of cultural conditions. Incubations without a carbon source were performed and used as controls.

After incubation, pH and dissolved oxygen (DO) were monitored. The total volumes of the samples were then centrifuged at 2300 g, and both the supernatant and the sludge pellets were recovered. Volatile fatty acids (VFA) in the supernatant were detected by capillary electrophoresis (CIA, Waters, Milford, MA, USA). Dissolved organic carbon (DOC) was measured by a TOC analyzer (TOC-V, Shimadzu, Kyoto, Japan). These chemical analyses were performed with one representative tube for each incubation.

Culture conditions were investigated in the following order: (I) pH, (II) temperature and (III) carbon source. The conditions for experiments (II) and (III) were based on the

optimal conditions from the previous experiment. For the pH experiment (I), the activated sludge was supplied with glucose (500 mgC/L) and incubated at 20 °C, with initial pH values of 6.0, 7.0, 8.0, 9.0, or 10.0. For the temperature experiment (II), the activated sludge was supplied with glucose (500 mgC/L) and incubated at 10, 15, 20, 24, 30, or 37 °C, with an initial pH of 10.0. For the carbon source experiment (III), glucose, acetate, propionate, or lactate was supplied to the sludge at 500 mgC/L with an initial pH of 10.0 and temperature at 20 °C.

Quantification of 'Accumulibacter' 16S rRNA gene

DNA was extracted from the sludge samples using the MORA-EXTRACT kit (AMR Inc., Gifu, Japan). The DNA concentration was determined with a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

A LightCycler (Roche Diagnostics, Mannheim, Germany) was employed for the quantitative real-time PCR for the 16S rRNA genes of 'Accumulibacter'. The PCR conditions were the same as those described previously (Fukushima *et al.*, 2007b) except for the annealing step. To avoid unspecific amplification, annealing condition was changed to 66 °C for 15 s. The final reaction mixture of 20 µL contained: 1.0 U KOD-plus DNA polymerase (Toyobo, Tokyo, Japan), 1× PCR buffer, 0.3 µM (each) primer, 0.2 mM (each) dNTP, 1 mM MgSO₄, 25 µg/µL BSA (Promega, Madison, WI, USA), 25 nL/µL DMSO (Wako, Tokyo, Japan), 200 pL/µL SYBR Green I (Invitrogen, Carlsbad, CA, USA), and template DNA (or sterilized distilled water as a negative control). The 'Accumulibacter'-specific primers PAO651f (5'-CTG GAG TTT GGC AGA GGG-3') and PAO846r (5'-GTT AGC TAC GGC ACT AAA AGG-3') were used. The DNA extracted from the samples (5ng) or 1.0×10³ to 1.0×10⁷ copies of quantitative standard DNA were used as the template DNA. Cycling conditions included a pre-incubation step for 5 min at 94 °C, followed by 35 cycles of 94 °C for 15 s, 66 °C for 15 s, and 68 °C for 15 s. Specificity was evaluated from the analysis of the melting curve and the sequencing analysis of the PCR products.

The copy number of the 'Accumulibacter' 16S rRNA gene in the tube was calculated by using equation (1). The multiplication of 'Accumulibacter' 16S rRNA gene in incubation was calculated by dividing the copy number in the tube after incubation by the initial copy number.

$$\text{copy number of the 'Accumulibacter' 16S rRNA gene in the tube} = \frac{(\text{DNA amount in the tube}) \times (\text{copy number in the template DNA})}{5} \quad (1)$$

Reproducibility test

Reproducibility of the increase in the 'Accumulibacter' 16S rRNA gene was evaluated using a *t*-test (Excel 2002, Microsoft, Redmond, WA, USA), when the activated sludge was supplied with glucose and incubated at 20 °C at an initial pH of 10.0. To confirm the reproducibility, nine replicates of the quantification results were obtained from the three incubation experiments (i.e., incubations in each experiment were performed in triplicate) by using different activated sludge.

Reproducibility of DNA extraction and quantitative real-time PCR was also tested with six replicates. Variance was shown as the coefficient of variation based on the

calculated copy number.

RESULTS AND DISCUSSION

Aerobic shaking culture under different conditions

P contents in the sludge samples used for aerobic shaking culture, ranged from 3.7 to 10.4%.

Table 1 presents the pH results. Except for the control, the value of pH decreased noticeably during incubation. Incubation at an initial pH of 10.0 produced the largest increase in the 'Accumulibacter' 16S rRNA gene, thus subsequent experiments were conducted with an initial pH of 10.0.

Table 2 shows the temperature results. Incubation at 20 °C produced the largest increase in the 'Accumulibacter' 16S rRNA gene.

Carbon source results are presented in Table 3. Increase of the 'Accumulibacter' 16S rRNA gene was observed only when glucose was fed. Acetate and propionate, which are generally thought to be utilized by 'Accumulibacter' (Seviour *et al.*, 2008), were ineffective under our study conditions.

When we supplied activated sludge with glucose and incubated it at 20 °C with an initial pH of 10.0, we detected 10 to 20 mgC/L of acetate and propionate after incubation, even though the DO concentrations in each incubation ranged between 2 and 4 mg/L. The pH in the samples decreased to 6.

Table 1 - Different initial pH conditions at 20 °C and fed with glucose.

initial pH	final pH	Carbon concentration after incubation	Copies of 'Accumulibacter' 16S rRNA gene (10 ⁸ copy/tube)		48-hr incubation/ inoculum ratio ± SD ²
			before incubation ¹	after incubation ±SD	
6.0	3.1	113		2.80±0.07	0.71±0.02
7.0	3.2	97		4.33±0.90	1.10±0.23
8.0	3.4	93		3.77±0.83	0.95±0.21
9.0	3.9	194	3.95	5.59±1.21	1.41±0.31
10.0	6.0	219		6.76±2.73	1.71±0.69
control ³	7.0	-		3.41±0.90	0.86±0.23

¹ Activated sludge sample was taken before inoculation into tubes.

² Copies of 'Accumulibacter' 16S rRNA gene in activated sludge sample after 48-hr culture divided by those in activated sludge before culture.

³ Carbon sources were not fed and incubated with an initial of pH 8.0.

Table 2 - Different temperatures (A: 10, 15 and 20 °C; B: 24, 30 and 37 °C) with an initial pH of 10.0 and fed with glucose.

A

Temperature (°C)	Carbon concentration after incubation	Copies of 'Accumulibacter' 16S rRNA gene (10 ⁸ copy/tube)		48-hr incubation/ inoculum ratio ± SD ²
		before	after incubation ±SD	
10	459		1.59±1.18	0.84±0.62
15	440	1.90	2.68±1.90	1.41±1.00
20	401		2.89±1.60	1.52±0.84
control ³	-		2.27±0.70	1.19±0.37

¹ Activated sludge sample was taken before inoculation into tubes.² Copies of 'Accumulibacter' 16S rRNA gene in activated sludge sample after 48-hr culture divided by those in activated sludge before culture.³ Carbon sources were not fed and incubated at 20 °C.

B

Temperature (°C)	Carbon concentration after incubation	Copies of 'Accumulibacter' 16S rRNA gene (10 ⁸ copy/tube)		48-hr incubation/ inoculum ratio ± SD ²
		before incubation ¹	after incubation ±SD	
24	192		6.56±0.35	0.74±0.04
30	101	8.85	3.24±0.43	0.37±0.05
37	54		1.69±0.29	0.19±0.03
control ³	-		7.86±0.46	0.89±0.05

¹ Activated sludge sample was taken before inoculation into tubes.² Copies of 'Accumulibacter' 16S rRNA gene in activated sludge sample after 48-hr culture divided by those in activated sludge before culture.³ Carbon sources were not fed and incubated at 24 °C.

Table 3 - Different carbon sources with an initial pH of 10.0 and temperature at 20 °C.

Carbon Source	Carbon concentration after incubation	Copies of 'Accumulibacter' 16S rRNA gene (10 ⁸ copy/tube)		48-hr incubation/ inoculum ratio ± SD ²
		before incubation ¹	after incubation ±SD	
Glucose	365		7.10±0.72	1.16±0.12
Acetate	228		5.43±1.76	0.89±0.29
Propionate	334	6.10	2.23±0.28	0.37±0.05
Lactate	258		3.54±1.24	0.58±0.20
control ³	-		5.60±0.75	0.92±0.12

¹ Activated sludge sample was taken before inoculation into tubes.² Copies of 'Accumulibacter' 16S rRNA gene in activated sludge sample after 48-hr culture divided by those in activated sludge before culture.³ Carbon sources were not fed.

Reproducibility test

We confirmed the reproducibility of the increase in the 'Accumulibacter' 16S rRNA gene, when we supplied activated sludge with glucose and incubated it at 20 °C with an initial pH of 10.0. The copy numbers of the 'Accumulibacter' 16S rRNA gene showed a statistically significant increase ($p < 0.05$, $n = 9$), with a mean multiple of 1.47 (min 0.98, max 2.50).

Reproducibility of the sample preparation procedure comprising of DNA extraction and quantitative real-time PCR, was determined to confirm the significance of experimental error. Coefficient of variation was 15 % for the calculated copy numbers ($n = 6$). Melting curve analysis and sequencing analysis were performed using DNA extract from sludge sample which was incubated with glucose at 20 °C with an initial pH of 10.0. We confirmed the absence of unspecific peaks in melting curve. Sequence of PCR product was related to 'Accumulibacter'. We did not observe any contaminant peaks in the results of the sequence. These results also supported statistically significant increase of 'Accumulibacter' gene due to their growth.

Growth potential of 'Accumulibacter' in an aerobic shaking culture

The copy numbers of the 'Accumulibacter' 16S rRNA gene showed a statistically significant increase ($p < 0.05$, $n = 9$) when the activated sludge samples were incubated with glucose at an initial pH of 10.0 and a temperature of 20 °C. Although the increased copy numbers of the 16S rRNA gene do not necessarily indicate an increase in the rate of cell division, they do show that DNA synthesis by 'Accumulibacter' has increased.

The carbon utilization pattern observed in this study does not agree with that reported by Kong *et al.* (2004). In the latter study, microautoradiography combined with fluorescence *in situ* hybridization revealed that 'Accumulibacter' assimilated acetate and propionate, but not glucose, under aerobic conditions. This is the opposite of our results. However, in the study by Kong *et al.* (2004), substrate assimilation was investigated by using 36 to 72 mgC/L of acetate, 18 to 54 mgC/L of propionate, or a combination of the two. In our incubations fed with 500 mgC/L of glucose, 10 to 20 mgC/L of acetate and propionate were detected after 48 h of incubation. Thus, our observations and those of Kong *et al.* (2004) may be suggesting that 'Accumulibacter' can grow by assimilating low concentrations of acetate and propionate aerobically, but cannot grow when they are supplied with 500 mgC/L of acetate or propionate because the carbon concentration is too high.

Therefore, our batch experiments might indirectly evaluate the growth of 'Accumulibacter', because its growth depends on the metabolites of other bacteria. Although our results could show significant increase of 'Accumulibacter' 16S rRNA gene under the aerobic shaking cultivation, further experiments are required to determine the growth characteristics of 'Accumulibacter' under aerobic condition.

CONCLUSIONS

In this study, we observed the increase of 16S rRNA gene concentration of 'Accumulibacter' in aerobic shaking cultures with glucose as the carbon source with an initial pH of 10.0 and temperature set at 20 °C. The increase of 16S rRNA gene was

statistically significant. Our results showed that ‘Accumulibacter’ most probably has the potential to grow in an aerobic shaking culture. We hope that these results will provide a hint for the successful isolation of ‘Accumulibacter’.

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