Detection of *Defluvicoccus*-related Glycogen-accumulating Organisms in Enhanced Biological Phosphorus Removal Processes

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To distinguish subgroups of *Defluvicoccus*-related glycogen-accumulating organisms (Defluvicoccus-related G-bacteria), new oligonucleotide probes were designed. Two of these probes, DF636 and DF827, were used successfully to detect each subgroup of Defluvicoccus-related G-bacteria. The morphological differences in clusters and cells between the DF636- and DF827-binding cells were confirmed. Interestingly, DF636-binding cells showed autofluorescence under UV light. Additionally, autofluorescence was observed in some alphaproteobacterial G-bacteria, which did not bind to any of the probes designed in this study. The results suggested that the Defluvicoccus-related G-bacteria consist of several physiologically different subspecies.

**Key words:** Alphaproteobacteria, Enhanced biological phosphorus removal (EBPR), Fluorescence in situ hybridization (FISH), G-bacteria, Genus *Defluvicoccus*

Some members of the *Alphaproteobacteria* have been reported as glycogen-accumulating organisms (GAOs), which are competitors to polyphosphate-accumulating organisms (PAOs) in enhanced biological phosphorus removal processes (EBPR). Alphaproteobacterial GAOs represent a group of the so-called “G-bacteria” or “tetrad-forming organisms (TFOs)” because of their characteristic morphotype as packaged in tetrad clusters. A member of the family *Sphingomonadaceae* and *Defluvicoccus*-related organisms (the DF1 G-bacteria group) had been identified as G-bacteria by culture-independent techniques. Fluorescence in situ hybridization (FISH) using probes targeting each G-bacteria group and post-chemical staining for poly-β-hydroxyalkanoate (PHA) have revealed that both the member of *Sphingomonadaceae* and the DF1 G-bacteria group actually exhibit the behavior of GAOs: accumulation of volatile fatty acids (VFAs) and transformation of VFAs into PHA under anaerobic conditions, and utilization of PHA as energy for growth under subsequent aerobic conditions. *Meyer et al.* also identified *Defluvicoccus*-related G-bacteria (the DF2 G-bacteria group), which are phylogenetically different from the DF1 G-bacteria group identified by Wong *et al.*. *Meyer et al.* designed two FISH probes (DF2MIX) specific for DF2 G-bacteria and two unlabelled helper probes which increased signal intensity. They also reported the phylogenetic and morphologic diversity among the DF2 G-bacteria group. In our previous study, the DF2 G-bacteria group and unclassified *Defluvicoccus*-related G-bacteria were detected in an acetate-fed sequencing batch reactor (SBR) with efficient phosphorus removal (Kondo *et al.*, presented at the IWA-ASPIRE Conference and Exhibition, Singapore, 10 to 25 July 2005) (Fig. 1). The sequences obtained in that study were highly diverse and thus were divided into subgroups (Fig. 1). In this study, to link the phylogenetic affiliations...
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Fig. 1. A phylogenetic tree of the members of the Alphaproteobacteria based on 16S rRNA gene sequences. The tree was constructed using the neighbor-joining method. Genetic distances were calculated using Kimura’s two-parameter method. The root of the tree was determined using the 16S rRNA gene of Methanosarcina mazei (AB065295) as an outgroup. The scale bar indicates the 10% estimated difference in nucleotide sequence position. Clones obtained in our previous study (Kondo et al., presented at the 1st IWA-ASPIRE Conference and Exhibition, Singapore, 10 to 25 July 2005) are in boldface. Solid lines indicate the sequences which have no mismatch to each probe. Broken lines indicate that there are no sequence data corresponding to the probe positions.

with their phenotypes, two FISH probes were designed to detect each subgroup in the DF2 G-bacteria group individually. Then the two probes were optimized for FISH using a fixed sludge sample collected from the SBR.

Two new FISH probes, DF636 and DF827, were designed to detect subgroup III and I of the DF2 G-bacteria, respectively. A probe specific for subgroup II was not designed in this study. These new probes were designed using GENETYX-MAC software (Genetyx, Tokyo Japan) and Probe match with Ribosomal Database Project II (http://rdp.cme.msu.edu/probematch/search.jsp)11 (Table 1). The specificity of each probe and the relative sequences of the Defluvicoccus-relative G-bacteria are shown in the phylogenetic tree (Fig. 1). The phylogenetic tree was constructed using Clastal W from the DDBJ (http://www.ddbj.nig.ac.jp/search/blast-e.html) and Tree View by the neighbor-joining method11,13. The sequences from our previous study (Kondo et al., presented at the 1st IWA-ASPIRE Conference and Exhibition, Singapore, 10 to 25 July 2005) and the published sequences from McMahon et al. (unpublished; direct submission to database), Meyer et al.10, Wong et al.14, and Wong and Liu (unpublished; direct submission to database)
are included in the phylogenetic tree (Fig. 1). The optimum formamide (FA) concentrations of the DF636 and DF827 probes were determined by changing FA concentrations (from 0 to 50%, 5% step) according to Meyer et al. The ALF1b probe and DF2MIX probes were used for detection of all alphaproteobacterial G-bacteria and the DF2 G-bacteria group, respectively. These probes were commercially synthesized and labeled at the 5' end with the fluorescein isothiocyanate (FITC) or indocarbocyanin 3 (Cy3) (TAKARA BIO, Shiga Japan). A sludge sample, which was collected from the SBR with efficient phosphorus removal operated in our previous study (Kondo et al., presented at the 1st IWA-ASPIRE Conference and Exhibition, Singapore, 10 to 25 July 2005), was immobilized on gelatin-coated glass slides and dehydrated by successive passages through 50, 80, and 98% ethanol. For direct counting of cells hybridized with each probe, the sludge sample was soaked in the same volume of sterile triplyphosphorus buffer solution (400 mg l\(^{-1}\)) and sonicated for 60 s with an ultrasonic disruptor (UR-20P, TOMY SEIKO, Tokyo Japan) to disperse microbial flocs before immobilization on slides. The hybridization was performed according to the standard hybridization protocol\(^{11}\). Then, the glass slides were mounted in VECTASHIELD Mounting Medium (Vector Laboratories, CA USA), and digital images (of the sludge samples) were taken under a confocal laser scanning microscope (TCS-SP5, Leica Microsystems Japan, Tokyo Japan). Images of each probe binding cell were collected and analyzed with the software Adobe Photoshop 6.0 (Adobe Systems, CA USA). At least 20 microscopic fields were examined to count the number of cells bound to each probe.

The newly designed FISH probes, DF636 and DF827, were tested. For both probes, the fluorescence intensity and the number of probe-binding cells were decreased markedly when the FA concentration was 25% or more. The optimum FA concentration was therefore determined as 20%. The cells bound to each probe formed tetrad clusters and were also detected by the DF2MIX probes, demonstrating that these new probes were successfully used to detect the DF2 G-bacteria group (Figs. 2A and B). No cell in the sludge sample collected from the acetate-fed SBR was double-stained with both DF636 and DF827, suggesting that the two probes detect different subgroups of DF2 G-bacteria, respectively. The morphological differences between the DF636- and DF827-bound cells were examined. The DF636-bound cells were packed in typical tetrad clusters and the microbial flocs of these clusters formed square tablets with spaces between clusters. On the other hand, the DF827-bound cells were also packed in typical tetrad clusters but the microbial flocs formed dense clusters (Figs. 2A and B). The morphological diversity among the DF2 G-bacteria group has been reported by Meyer et al.\(^{10}\) and our results, the morphological and phylogenetic differences between the DF636- and DF827-bound cells, additionally indicate the diversity of the DF2 G-bacterial physiology.

The diversity of the DF2 G-bacterial physiology was also supported by the microscopic analysis using UV excitation. Interestingly, UV excitation induced strong blue autofluorescence of the cell surface in some member of the DF2 G-bacteria group (Fig. 2A). Although we could not identify the material that emitted the autofluorescence, all of the DF636-bound cells, specific for subgroup III, exhibited this strong autofluorescence (referred to as Type A in Table 2). In contrast, not all the DF827-bound cells, specific for subgroup I, were induced by UV excitation (Type B). For the DF636 and DF827 negative DF2 G-bacteria group, some cells showed the autofluorescence (Type C) whereas the autofluorescence was not observed in the other cells (Type D) (Figs. 2C and D). Considering the specificity of the

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### Table 1. Oligonucleotide probes used in this study

<table>
<thead>
<tr>
<th>Probe</th>
<th>Probe Sequence (5'–3')</th>
<th>Target Site(^a)</th>
<th>FA (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DF636</td>
<td>AAGCCCAAGCAGTCTGAAG</td>
<td>636–653</td>
<td>20</td>
<td>This study</td>
</tr>
<tr>
<td>DF827</td>
<td>AAGCTTCCAACATCAG</td>
<td>827–843</td>
<td>20</td>
<td>This study</td>
</tr>
<tr>
<td>DF988(^b)</td>
<td>GATACGACGCCCAGGGAG</td>
<td>988–1009</td>
<td>35</td>
<td>Meyer et al.(^{10})</td>
</tr>
<tr>
<td>DF1020(^b)</td>
<td>CCGGCCGAACCGACTCCC</td>
<td>1020–1037</td>
<td>35</td>
<td>Meyer et al.(^{10})</td>
</tr>
<tr>
<td>H966(^b)</td>
<td>CTTGGAAGATTCTGGCTGTTGC</td>
<td>966–987</td>
<td>—</td>
<td>Meyer et al.(^{10})</td>
</tr>
<tr>
<td>H1038(^b)</td>
<td>AGCAGCCATGCAGCACCTGTGCGT</td>
<td>1038–1064</td>
<td>—</td>
<td>Meyer et al.(^{10})</td>
</tr>
<tr>
<td>ALF1b</td>
<td>CGCTCG(C/T)TCGGCGCCAG</td>
<td>19–35</td>
<td>20</td>
<td>Manz et al.(^{9})</td>
</tr>
</tbody>
</table>

\(^a\) E. coli rRNA numbering.

\(^b\) A mixture of the DF988, DF1020, H966, and H1038 probes was used as the DF2MIX probe according to Meyer et al.\(^{10}\). The unlabeled H966 and H1038 probes were used as helper probes\(^{10}\).
Fig. 2. Images of each type of the alphaproteobacterial G-bacteria. The arrow in each figure shows each type listed in Table 2: (A) Type A, (B) Type B, (C) Type C, (D) Type D, (E) Type E, and (F) Type F. Scale bar, 10 μm.
probes and the phylogenetic analysis in our previous study, Types C and D might be affiliated with subgroup II. The intensity of the autofluorescence of Type C was lower than that of Type A. The results of the direct count-FISH analysis and UV excitation are summarized in Table 2. Figure 2E and F show the existence of phylogenetically different alphaproteobacterial G-bacteria, which was not detected by the DF2MIX probes but was detected by the ALF1b probe. Some of them exhibited strong autofluorescence the same as Type A (Type E) but the others exhibited no autofluorescence (Type F) (Figs. 2E and F). According to the sequences obtained in our previous study (Fig. 1) and the results of FISH analysis (Figs. 2E and F), Types E and F might be affiliated with the sequences which are not closely related to the DF2 G-bacteria group (AB231376, AB231385 and AB231392 shown in Fig. 1). It was difficult to link the results of FISH and the autofluorescence induced by UV excitation for all types; however, at least for Types A and B, the differences in phenotype seemed to be derived from the difference in phylogenetic affiliation.

The direct count-FISH analysis revealed that only 13.8% and 14.7% of the alphaproteobacterial G-bacteria were detected by the DF636 and DF827 probes, respectively. Types C and D, which were the DF636 and DF827 negative DF2 G-bacteria group, were prominent in the alphaproteobacterial G-bacteria whereas the unidentified alphaproteobacterial G-bacteria (Types E and F) exhibited low abundance in the acetate-fed SBR with efficient phosphorus removal (Table 2).

In conclusion, two probes, DF636 and DF827, were successfully able to detect each subgroup of the DF2 G-bacteria group individually. The DF636 probe could detect some of the cells with UV-induced autofluorescence and the DF827-bound cells were not induced by UV excitation. The morphology of clusters and cells also differed between the DF636- and DF827-bound cells. The difference in phenotypes suggested that DF2 G-bacteria consist of several subspecies. Meyer et al. suggested that the DF2 G-bacteria group contribute to the deterioration of the phosphorus removal process while our SBR maintained an efficient rate of phosphorus removal even with the prominence of the DF2 G-bacteria group. Clarification of the relation between the DF2 G-bacterial subspecies and the roles in EBPR processes is a challenge for the future.

**References**


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