Distribution of extracellular polysaccharides in anaerobic granular sludges

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Abstract Four fluorescent lectin probes specific for individual saccharides were applied to reveal the distribution of extracellular polysaccharides in methanogenic and hydrogen-producing granular sludges. Results indicated that most of extracellular polysaccharides were distributed in the outer layer of both granules. The main constituents of extracellular polysaccharide in the methanogenic granule were glucose/mannose and N-acetyl-galactosamine, whereas those in the hydrogen-producing granules were only glucose/mannose. There were little fucose and galactose in both granules.

Keywords Anaerobic; distribution; EPS; granule; polysaccharide; staining

Introduction
Extracellular polymeric substances (EPS) facilitate the aggregation of microbes, and thus play an important role in the development of biofilm, granular sludge and activated sludge flocs. Polysaccharides are the major component of EPS (Sutherland, 1997; Fang and Jia, 1996; Liu and Fang, 2002). The polysaccharide contents in EPS of activated sludge (Frolund et al., 1996), anaerobic granule (Fang and Jia, 1996) and biofilm (Jahn and Nielsen, 1998) are traditionally characterized from those fractions that can be extracted by physical and/or chemical means (Fang and Jia, 1996; Liu and Fang, 2002). However, polysaccharides may also be stained in situ for microscopic characterization. The staining agents generally used include calcofluor white M2R (Del Gallo et al., 1989), congo red (Allison and Sutherland, 1984) and alcian blue (Wetz et al., 1997). Lectins were developed recently for the staining of polysaccharide with better specificity than the traditional agents (Michael and Smith, 1995; Lawrence et al., 1998; Neu, 2000).

Lectins are a group of proteins, each of which has a specific binding characteristic for certain saccharides. Lectins labeled with fluorescent dyes can thus be used to map the distribution of specific saccharides in the microbial community. Although the distribution of polysaccharides in biofilm has been studied using lectin probes (Lawrence et al., 1998; Neu, 2000), little information is available so far on the distribution of polysaccharides in activated sludge and granular sludge. In this study, four fluorescent lectin probes specific for respective saccharides were applied to examine in situ the polysaccharides distribution in two types of anaerobic granular sludge.

Materials and Methods
Granular sludges
Two anaerobic granular sludges from previous studies were used. The methanogenic granule was sampled from a reactor treating brewery wastewater (Fang et al., 1995; Liu et al., 2002), whereas the hydrogen-producing granule was a reactor treating sucrose-rich wastewater (Fang et al., 2002).
Probes
Table 1 lists the probes used in the study, including two DNA-targeting probes SYTO9 and propodium iodide-PI (Molecular Probes, Eugene, OR), and four saccharides-targeting lectin probes labelled with either fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) (Sigma). The DNA-targeting SYTO9 and PI probes were used to stain bacteria cells, while the lectin probes were used to stain saccharides in the EPS. When excited by a laser at proper wavelengths, the SYTO9 and the FITC-labeled lectin probes emit green light, whereas the PI and the TRITC-labeled lectin probes emit red light. In order to reveal simultaneously the distributions of both EPS and bacterial cells for a given sample, SYTO9 was used jointly with the TRITC-labeled lectin probes, and PI with the FITC-labeled lectin probes. All the probe working solutions were prepared using a pH 7.2 phosphate-buffered saline solution (PBS; 0.13M NaCl, plus 10 mM Na2HPO4) and stored in -20°C before use.

Table 1 Cell and lectin probes and their key characteristics

<table>
<thead>
<tr>
<th>Probe description</th>
<th>Probe abbreviation</th>
<th>Concentration (µM)</th>
<th>Specific target</th>
<th>Ex/Em* (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell probes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SYTO9</td>
<td>SYTO9</td>
<td>5</td>
<td>nucleic acid</td>
<td>488/525</td>
</tr>
<tr>
<td>propodium iodide</td>
<td>PI</td>
<td>30</td>
<td>nucleic acid</td>
<td>536/617</td>
</tr>
<tr>
<td>Lectin probes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>from <em>Canavalia ensiformis</em></td>
<td>ConA-TRITC</td>
<td>1</td>
<td>glucose and mannose</td>
<td>540/566</td>
</tr>
<tr>
<td>labeled by TRITC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>from <em>Ulex europaeus</em></td>
<td>UEA I-TRITC</td>
<td>1</td>
<td>L-fucose</td>
<td>540/566</td>
</tr>
<tr>
<td>labeled by TRITC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>from <em>Erythrina cristagalli</em></td>
<td>ECV-FITC</td>
<td>1</td>
<td>N-acetyl-galactosamine and galactose</td>
<td>490/525</td>
</tr>
<tr>
<td>labeled by FITC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>from <em>Arachis hypogaea</em></td>
<td>AH-FITC</td>
<td>1</td>
<td>Galactose</td>
<td>490/525</td>
</tr>
</tbody>
</table>

* Ex/Em: maximum excitation and emission wavelengths.

Confocal laser scanning microscopy
A confocal laser scanning microscope (CLSM), (LSM 5 Pascal, Zeiss, Jena, Germany) was used to examine the granules after staining. It was equipped with two lasers (488 and 543 nm), a beam splitter NFT545, two filter sets (BP515-530 and LP560) and three lenses (10X/NA0.5, 40X/NA0.8 and 63X/NA1.4). To avoid interference between the emitted fluorescent lights, the two-track mode was applied in all image acquisition. For the first track, the sample was excited by the laser of 488 nm and the emitting light was collected by the filter set BP515-530; for the second track, the laser of 543 nm and the filter set LP560.

Cell and EPS staining
The hydrogen-producing granules were stained and were observed by CLSM at various depth levels. Due to poor light penetration, the methanogenic granules could not be observed. Instead, only the cross-sections of sliced methanogenic granules were stained and observed. The granules were first gently washed with PBS, embedded with the OCT compound (Miles, Elkhart, IN), and then sectioned
into 20 μm slices with a rotary cryo-microtome (CM 1510-Cryostat, Leica, Germany). The cross-sections of granules were collected on the wells of the glass slide coated by 0.01% poly-L-lysine solution. The slide was then immersed in PBS for 10 min to remove the OCT compound and air-dried.

The methanogenic granule sections and hydrogen-producing granules were placed into wells on the glass slides. Each sample in the well was covered with 20 μl of a staining solution, containing a cell probe and a lectin probe listed in Table 1. The slides containing methanogenic granule sections were then incubated in a dark moist chamber at room temperature for 20 min, whereas those containing hydrogen-producing granules were incubated for 60 min. After incubation, the sample wells were carefully rinsed with filtered PBS three times to remove the residual staining solutions.

Results and Discussion
Under proper conditions, microbes could aggregate forming granules in anaerobic (Lettinga et al., 1980; Fang et al., 1995) as well as aerobic (Beun et al., 1999) reactors. Although it has been extensively studied for over two decades, the granulation mechanism is still not fully understood (Fang, 2000). It is generally believed that EPS play an important role in the formation of methanogenic (Jia et al., 1996) as well as hydrogen-producing granules (Fang et al., 2002). However, little information is available on the distribution of EPS in these granules. In this study, lectin probes with fluorescent labels were used for the in situ analysis of polysaccharides and, thus, EPS in two types of anaerobic granules.

EPS in methanogenic granule
Figure 1 illustrates the images of a methanogenic granule stained by (a) the glucose/mannose-specific ConA-TRITC and (b) the DNA-specific SYTO9 probes. It clearly shows that the glucose/mannose-containing EPS were mostly distributed at the outer layer of the granule, whereas the distribution of bacteria was rather uniform without forming layers.

![Figure 1](image1.png)

Figure 1: Images of a methanogenic granule stained using (a) ConA-TRITC, and (b) SYTO9. (bar = 500 μm)

Figure 2 illustrates the images of another methanogenic granule stained by (a) the N-acetyl-galactosamine/galactose-specific EC-FITC and (b) the DNA-specific PI probes. It shows that the N-acetyl-galactosamine/galactose-containing EPS were also mostly distributed at the outer
layer of the granule, whereas the bacterial distribution was uniform without forming layers.

Images of methanogenic granules stained by L-fucose-specific UEA 1 and galactose-specific AH probes had a very weak fluorescent signal, indicating that there was little L-fucose and galactose in the EPS of methanogenic granules. Thus, the image in Figure 2(a) was most likely due to the presence of N-acetyl-galactosamine, not galactose, in the EPS.

![Figure 2](image)

**Figure 2** Images of a methanogenic granule stained using (a) EC-FITC, and (b) PI. (bar = 500 μm)

Results show that glucose, mannose and N-acetyl-galactosamine were the three major constituents of the extracellular polysaccharide of methanogenic granules. These constituents were previously reported in bacterial EPS by other researcher (Bryers and Drummond, 1998).

**EPS in hydrogen-producing granule**

Figure 3 illustrates the images of a hydrogen-producing granule stained by (a) the glucose/mannose-specific ConA-TRITC and (b) the DNA-specific SYTO9 probes. It also shows that the glucose/mannose-containing EPS were mainly distributed at the outer layer of the granule, whereas the bacteria cells were uniformly distributed.

![Figure 3](image)

**Figure 3** Cross-section images of a hydrogen-producing granule stained using (a) ConA-TRITC, and (b) SYTO9. (bar = 200 μm)
Images of hydrogen-producing granules staining by EC-FITC, UEA I-TRITC and AH-FITC probes produced very weak fluorescent signals, suggesting that there were little L-fucose and N-acetyl-galactosamine and galactose in the hydrogen-producing granules.

Conclusion
Results in this study show that extracellular polysaccharides of both methanogenic and hydrogen-producing granules were mostly distributed at the outer layer of the granules, whereas the bacterial distributions were rather uniform without a layered structure. EPS of the methanogenic granule were mostly composed of glucose/mannose and N-acetyl-galactosamine, whereas those of hydrogen-producing granule were mostly composed of glucose/mannose. There were little L-fucose and galactose in both granules.

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References


