Extracellular polymeric substances responsible for bacterial adhesion onto solid surface

Satoshi Tsuneda * , Hirotoshi Aikawa, Hiroshi Hayashi, Atsushi Yuasa, Akira Hirata

Department of Chemical Engineering, Waseda University, Ohkubo 3-4-1, Shinjuku-ku, Tokyo 169-8555, Japan

Received 12 February 2003; received in revised form 15 April 2003; accepted 5 May 2003
First published online 5 June 2003

Abstract

The influence of extracellular polymeric substances (EPS) on bacterial cell adhesion onto solid surfaces was investigated using 27 heterotrophic bacterial strains isolated from a wastewater treatment reactor. Cell adhesion onto glass beads was carried out by the packed-bed method and the results were discussed in terms of the amount of each EPS component produced and cell surface characteristics such as zeta potential and hydrophobicity. Protein and polysaccharides accounted for 75–89% of the EPS composition, indicating that they are the major EPS components. Among the polysaccharides, the amounts of hexose, hexosamine and ketose were relatively high in EPS-rich strains. For EPS-poor strains, the efficiency of cell adhesion onto glass beads increased as the absolute values of zeta potential decreased, suggesting that electrostatic interaction suppresses cell adhesion efficiency. On the other hand, the amounts of hexose and pentose exhibited good correlations with cell adhesiveness for EPS-rich strains, indicating that polymeric interaction due to the EPS covering on the cell surface promoted cell adhesion. It was concluded that, if the EPS amount is relatively small, cell adhesion onto solid surfaces is inhibited by electrostatic interaction, and if it is relatively large, cell adhesion is enhanced by polymeric interaction.

Keywords: Extracellular polymeric substance; Bacterial adhesion; Zeta potential

1. Introduction

Extracellular polymeric substances (EPS), which are secreted by microorganisms during growth, consist of various organic substances such as polysaccharides, proteins, nucleic acids and lipids. However, the exact functions of EPS are not completely elucidated because of their extremely heterogeneous nature. From the physicochemical viewpoint, an EPS covering on a cell surface is regarded as polyelectrolytes adsorbed onto a colloidal particle. The molecular masses of EPS range from a few thousands to several millions and EPS components contain various functional groups including carboxyl, amino and phosphate groups [1]. It has been reported that EPS play significant roles in the formation and function of microbial aggregates, including adhesion phenomena, matrix structure formation and microbial physiological processes [2–6]. In particular, EPS significantly influence bacterial adhesion onto solid substratum surfaces, which is recognized as the initial stage in biofilm formation, because an EPS covering on a cell surface alters the physicochemical characteristics of the surface such as charge, hydrophobicity and the polymeric property [7–11].

There have been a number of studies with regard to the physicochemical aspects of bacterial cell adhesion [12,13]. Rijinaarts et al. [14] insisted that long-range forces, mainly electrostatic interactions due to the overlapping of diffuse layers and van der Waals forces, i.e. DLVO forces, are a very important factor of adhesion onto substratum surfaces at relatively lower ionic concentrations. Bos et al. [15] revealed that substratum hydrophobicity is a major determinant of bacterial cell retention although it hardly influences bacterial adhesion. Therefore, in order to elucidate the function of EPS affecting bacterial adhesion characteristics, the amount of each EPS component and its effect on cell surface properties such as electric potential and hydrophobicity and on cell adhesiveness onto solid surfaces should be precisely determined.
In this study, bacterial cell adhesion onto glass beads in connection with the amounts and compositions of the EPS produced was investigated using 27 heterotrophic bacterial strains isolated from a wastewater treatment reactor. Firstly, typical EPS components such as hexose, pentose, hexosamine, ketose, uronic acids, acetyl groups and proteins were quantitatively analyzed for each strain. Secondly, cell surface properties such as zeta potential and hydrophobicity were examined. Finally, bacterial cell adhesion onto glass beads was assayed by the packed-bed method and the relationship between cell surface properties and cell adhesiveness was investigated.

2. Materials and methods

2.1. Bacterial strains and cultivation

Twenty-seven heterotrophic bacterial strains were isolated from a biofilm formed on a cement-ball carrier (mean diameter: 0.23 mm) in a laboratory-scale aerobic three-phase fluidized-bed reactor (effective volume: 2.0 l) where synthetic domestic wastewater (dissolved organic carbon: 400 mg/l; total nitrogen: 250 mg/l) was continuously fed and sufficiently treated. Each strain was aerobically cultured for 30 h at 30°C by shaking at 115 rpm in a medium composed of the following: dextrin, 0.304 g; meat extract, 0.746 g; yeast extract, 0.654 g; bactopeptone, 0.186 g; KCl, 0.134 g; NH₄Cl, 0.817 g; distilled water, 1 l. Cells at their exponential growth phase were harvested by centrifugation (8000×g 10 min), and the pellets were used in the following experiments.

2.2. Extraction and analysis of EPS

The extraction of EPS from cell pellets was carried out by the formaldehyde/ultrasonication method according to Azeredo and Lazarova [16]. In cell fixation, pellets were suspended in a solution containing 0.22% formaldehyde and 8.5% NaCl, and then kept at 4°C for 2 h. The resulting cell suspensions were washed with distilled water and centrifuged, and then 1 g of each of the pellets obtained was redispersed in 50 ml of distilled water. The samples were sonicated using a homogenizer at 40 W for 3 min. The total quantity of extracted EPS was measured based on the weight of solids obtained after lyophilization. Subsequently, the amounts of typical EPS components such as hexose [17], pentose [18], hexosamine [19], ketose [20], uronic acids [21], acetyl groups [22] and proteins [23] were measured by color identification tests.

2.3. Cell adhesion by the packed-bed method

Bacterial cell adhesion was carried out by the packed-bed method [24]. A column made of glass with an inner diameter of 15 mm and a length of 350 mm (Shibata, Japan) was used. The solid substrata used were spherical glass beads with a mean diameter of 0.1 mm (Oshinriko, Japan). In this experiment, the glass bead was used because of its chemical stability and high reproducibility owing to the uniform surface structure. Twenty grams of glass beads was packed in the column up to a height of 80 mm and equilibrated by eluting 10 mM phosphate-buffered saline (PBS, pH 7.2) containing 0.68 mM KH₂PO₄, 0.21 mM K₂HPO₄, and 8.44 mM NaCl in deionized water. The bacterial suspension was fed into the column at a constant flow rate of 6.0 ml min⁻¹ for 20 min. The time course of OD₆₆₀ of the effluent was measured after it had flowed out for 1 min to discard the PBS solution that had existed in the column initially. The experiment was carefully performed under the condition that the cell adhesion has not reached the saturation point. The amount of cells adhering onto the glass beads was evaluated from the difference between cell concentrations of influent and effluent solutions. Cell adhesiveness, X, was defined as:

\[ X = \frac{\int_0^t \left( \frac{C_{in} - C_{out}}{C_{in}} \right) dt}{t} \]

where Cₐₕ and Cₐₜ are the OD₆₆₀ of influent and effluent solutions, respectively.

2.4. Zeta potential measurement of the cells

The electrophoretic mobilities of bacterial cells were measured using an electrophoretic light-scattering spectrophotometer (ELS-8000, Otsuka Electronics, Japan). Subsequently, the zeta potential was calculated from the measured electrophoretic mobilities using Smoluchowski’s equation. The cell suspension was dispersed in 10 mM PBS and sonicated for 3 min. All measurements were carried out in duplicate.

2.5. Contact angle measurement

Contact angle was measured according to the method of van Loosdrecht et al. [25]. The bacterial lawn for measuring contact angles was prepared by collecting bacterial cells on 0.45-μm-pore cellulose-acetate filters (HAWG-02500, Millipore, USA). Filters with a bacterial lawn were mounted on glass slides and dried in a desiccator for 0.5–3 h before measurement of the contact angle. It was confirmed that there was almost no change in contact angle data after 0.5 and 3 h of drying. The contact angle of 0.1 M NaCl aqueous solution was directly measured using a microscope with a goniometric eyepiece. Each contact angle was the average of at least six independent measurements.
3. Results and discussion

3.1. EPS composition

Fig. 1 shows the weight percentage of the EPS components for 1 g of dried biomass for four strains, P-1, P-2, R-1 and R-2. In this work, 27 bacterial strains were divided into two groups, namely, EPS-rich strains (R-strains) and EPS-poor ones (P-strains), and the borderline for the total amount of EPS produced by each strain was fixed at 100 mg g\(^{-1}\). The total percentages of proteins and polysaccharides including hexose, pentose, hexosamine, ketose, uronic acids and acetyl groups reached 75–90% of the total EPS amount, indicating that they are the major EPS components for these strains. This tendency was observed in all the 27 strains used in this study. In the R-strains such as R-1 and R-2, the amounts of hexose, hexosamine and ketose were relatively high; the amounts of pentose, uronic acids and acetyl groups were relatively low, less than 10%. In the P-strains such as P-1 and P-2, the percentage of protein relative to the total EPS amount was 34%, approximately twice as high as that in the R-strains such as R-1 and R-2. However, it is noteworthy that the absolute protein amounts were almost the same, 18–23 mg g\(^{-1}\), regardless of the strains.

3.2. Relationship between the cell surface property and cell adhesion onto glass beads

Fig. 2 shows the relationship between zeta potential and cell adhesiveness onto glass bead surfaces in 10 mM PBS. The zeta potentials of the strains varied from -50 to 0 mV, depending on the strain. Since the glass beads used in this study had a relatively high negative zeta potential, -29.0 mV, it was inferred that electrorepulsive forces are involved in the interaction between cells and glass beads due to the overlapping of electrical diffuse layers during cell adhesion. Therefore, the negative zeta potential of a cell surface has a negative effect on cell adhesion onto glass beads; a higher negative potential would lead to a lower degree of adhesion onto glass beads. In the experimental results, however, there seemed to be no correlation between zeta potential and cell adhesiveness onto the glass beads when all the strains were taken into account. Accordingly, we discuss the cell adhesiveness in terms of EPS production characteristics. In Fig. 2, open triangles represent the R-strains and closed circles represent the P-strains. It was found that the zeta potentials of the P-strains are relatively low compared with those of the R-strains. For the P-strains, a correlation between zeta potential and cell adhesiveness was observed; cell adhe-
siveness increased as the absolute value of zeta potential decreased. This tendency suggests that the suppression of electrorepulsive forces promotes cell adhesion onto glass beads for the P-strains. On the other hand, there seemed to be no correlation between zeta potential and cell adhesiveness for the R-strains, suggesting that there are other factors responsible for cell adhesion of these strains.

Fig. 3 shows the correlation between hydrophobicity and cell adhesiveness onto glass beads. Interestingly, the R-strains were more hydrophobic than the P-strains. However, there was no correlation between hydrophobicity and cell adhesiveness for both R-strains and P-strains. Since the glass surface was very hydrophilic, it is suggested that attractive forces due to the hydrophobic nature of a material are not involved in the interaction between cells and the solid during their approach. It is therefore con-

Table 1

<table>
<thead>
<tr>
<th>EPS component</th>
<th>Coefficient of correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>0.25</td>
</tr>
<tr>
<td>Hexose</td>
<td>0.88</td>
</tr>
<tr>
<td>Pentose</td>
<td>0.87</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>0.01</td>
</tr>
<tr>
<td>Uronic acids</td>
<td>0.48</td>
</tr>
<tr>
<td>Ketose</td>
<td>0.01</td>
</tr>
<tr>
<td>Acetyl groups</td>
<td>0.57</td>
</tr>
</tbody>
</table>
cluded that cell hydrophobicity does not influence the behavior of cell adhesion onto glass beads. It may do so to a hydrophobic surface.

3.3. Cell adhesion behavior for EPS-rich strains

It is considered that cell adhesion properties depend on EPS properties for the R-strains. In order to determine the influence of each EPS component on cell adhesion characteristics, eight strains having almost equal zeta potentials were selected (Fig. 2), and then the correlations between the amount of each EPS component produced by each strain and cell adhesiveness were evaluated. The results are shown in Fig. 4a–g. There seem to be positive correlations between the amount of each EPS component and cell adhesiveness, although the tendency differed among the components. Table 1 summarizes the coefficient of correlation between the amount of each EPS component and cell adhesiveness. Relatively high correlations were obtained for hexose (0.88) and pentose (0.87), both of which are important EPS constituents for the R-strains, indicating that large amounts of hexose and pentose facilitate cell adhesion onto glass beads. In contrast, the amounts of hexosamine, uronic acids, ketose and acetyl groups exhibited relatively low coefficients of correlation with cell adhesiveness. Although the specific interaction concerning functional groups could not be determined, it was suggested that polymeric interaction due to the EPS covering on the cell surface promotes cell adhesion onto glass beads because high-molecular-mass polysaccharides could serve as polyelectrolytes adsorbed onto the cell surface. EPS has been often reported to enhance bacterial adhesion onto solid surfaces [26, 27]. On the contrary, Gomez-Suarez et al. [28] reported that the adhesiveness onto solid surfaces of non-EPS-producing Pseudomonas aeruginosa SG81R1 was higher than that of EPS-producing P. aeruginosa SG81. Although those are very interesting findings, it is necessary to examine them using a number of isolated bacteria and construct a systematic theory with regard to the role of EPS in bacterial adhesion onto solid surfaces.

Electrical force from overlapping of electrical double layers, van der Waals force (these two are DLVO forces) and polymeric interaction are all responsible for the cell adhesion process [26]. Electrical force is repulsive one because both cell and glass have negative surface potential, whereas van der Waals force and polymeric interaction are attractive ones. Taking the above findings into consideration, electrical force is the decisive factor for preventing P-strains cells from adhesion onto glass beads, because polymeric interaction is weak and van der Waals force is almost independent of bacterial species. In contrast, in the case of the R-strains, the EPS covering on the cell substantially facilitates cell adhesion onto glass beads. In other words, cell adhesion onto solid surfaces is inhibited by electrostatic interaction if the EPS amount is small and enhanced by polymeric interaction if the EPS amount is large.

4. Conclusion

In this study, cell adhesion onto a glass bead surface in correlation with the amount of EPS components and cell surface characteristics such as zeta potential and hydrophobicity was investigated using 27 heterotrophic bacterial strains isolated from a wastewater treatment reactor. The findings are summarized as follows.

1. Protein, hexose, pentose, hexosamine and ketose are the major EPS components of EPS-rich strains.
2. There was a weak correlation between zeta potential and cell adhesiveness for EPS-poor strains, suggesting that the suppression of electrorepulsive forces promotes cell adhesion onto glass beads.
3. The amounts of hexose and pentose exhibited good correlations with cell adhesiveness for EPS-rich strains, indicating that hexose and pentose facilitate cell adhesion onto glass beads.
4. There was no correlation between hydrophobicity and cell adhesiveness for both EPS-rich and EPS-poor strains.

Therefore, it could be concluded that, if the EPS amount is small, cell adhesion onto solid surfaces is inhibited by electrostatic interaction, and if it is large, cell adhesion is enhanced by polymeric interaction.

Acknowledgements

We thank for Prof. H. Sasaki (Waseda University) for the technical advice with regard to zeta potential measurements.

References


