Cation-dependent Flocculation in a *Flavobacterium* Species Predominant in Activated Sludge

YASUHIKO TEZUKA

*Faculty of Science, Tokyo Metropolitan University, Setagaya-ku, Tokyo, Japan*

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The predominant bacterium of activated sludge classified as belonging to the genus *Flavobacterium* showed good flocculent growth in the presence of both calcium and magnesium ions, although capsular material or gelatinous matrix was not detectable in the flocs. The bacterium accumulated a large amount of poly-β-hydroxybutyrate when grown on glucose, but not on peptone, in spite of good flocculent growth on both substrates. When the flocs formed during growth phase were suspended in deionized water and shaken for a few minutes, they disintegrated perfectly, and a uniformly dispersed cell suspension was obtained. Furthermore, when the dispersed cell suspension thus obtained was added with each of various mineral salts and shaken for a few minutes, the cells flocculated again, the resultant supernatant solution being almost clear. Even if the dispersed cells were killed by heat or treated by trypsin, they did not lose the ability to form flocs when added with mineral salts. Since the electric charge of the cell surface was negative, a possible mechanism of flocc formation was suggested as follows: negatively charged surfaces of adjacent cells are bridged by ionic bonds intermediated by cations. The bacterium utilized a relatively wide variety of organic compounds and showed high metabolic activity comparable to that of naturally activated sludge. These properties, along with flocc-forming ability, were considered to be factors for making the bacterium predominant in activated sludge.

Activated sludge process is one of the most effective means by which organic wastes are stabilized microbiologically. Although bacteria have been considered to be the major microorganisms responsible for the purification process, and hence the predominant bacteria or flocc-forming bacteria in activated sludge have been investigated for many years, there have been some discrepancies among the results obtained by various investigators (1-3, 6, 7, 9, 13, 14). These discrepancies may be partly attributable to the different methods employed. But the differences of the type of activated sludge examined seem to be more decisive, since the microflora of activated sludge is considered to be mainly dependent on the composition of the waste water to be stabilized. Therefore, comprehensive studies on each type of activated sludge are needed before any microbiological principle is established for activated sludge processing.

In a previous paper (Y. Tezuka, J. Japan. Biol. Soc. Water Waste, in press), the number of heterotrophic bacteria, the kinds of predominant bacteria, and those with flocc-forming ability were reported in an activated sludge obtained from Shibaura sewage treatment facility in Tokyo, where sewage to be purified was mainly of municipal origin. Agar plates containing half strength of the standard mineral base (11), with 0.1% yeast extract and 0.2% polypeptone, gave the highest viable count of 2.8 × 10^9 per ml of settled sludge among various media examined. Of the isolates obtained from these plates, about 60% were yellow-pigmented and appeared to belong to the genus *Flavobacterium*. The remaining 40% seemed to be composed of various bacterial species such as *Zoogloea, Pseudomonas*, and so on. Furthermore, many isolates which appeared to belong to the genus *Flavobacterium* showed good flocculent growth in media containing both polypeptide and glucose.

To understand the role of bacteria in activated sludge processing, it seemed important to investigate the taxonomical properties, flocculation process, and the metabolic activity of the isolates presumably belonging to *Flavobacterium*.

**MATERIALS AND METHODS**

**Materials.** Fifteen isolates which seemed to belong to the same species of *Flavobacterium* were used for the examination of taxonomical properties and the
ability to form flocs. Of these isolates, strain 18 was used mainly for detailed studies on flocculation process, substrate utilization, and metabolic activity.

Stock cultures were maintained on agar slants containing the standard mineral base (11) with 0.1% yeast extract (Difco) and 0.2% polypeptone.

Methods. Taxonomical properties of the isolates were determined mainly by the methods described in the Manual of Microbiological Methods (10). Several tests were carried out by modifying the methods described in the manual. Oxidative acid formation was determined by using a medium containing bromothymol blue and glucose with shaking. The oxidase test was carried out by the method of Stanier et al. (11). Poly-β-hydroxybutyrate (PHB) was determined by the method of Crabtree et al. (2).

Nutritional requirements of the isolates were determined by growing them in each of the following media: medium 1, the standard mineral base + 0.5% glucose; medium 2, medium 1 + 0.1% Vitamin Free Casamino Acids (Difco); medium 3, medium 2 + vitamin mixtures; medium 4, medium 1 + 0.1% yeast extract (Difco). Vitamin mixtures contained (per liter of medium) the following: 0.5 mg each of thiamine hydrochloride, riboflavine, pyridoxine hydrochloride, nicotinic acid, p-aminobenzoic acid, calcium panthetheate, and folic acid; 2 µg each of biotin and cyanocobalamin.

Substrate utilization by strain 18 was determined by growing the strain in a medium containing the standard mineral base, with 0.1% yeast extract and 0.5% of each of the substrates.

Unless otherwise stated, liquid cultures were grown in L-shaped tubes, each containing 30 ml of medium, at 30 C with Monod-type shaking.

The rates of glucose and glutamate uptake by the flocs of strain 18 were determined as follows. In the glucose experiment, the bacterium was precultivated in a medium containing the standard mineral base with 0.1% yeast extract and 0.5% glucose. After 88 hr, the flocs formed were harvested and washed once with a mineral solution containing CaCl₂ and MgSO₄. The flocs were then suspended in a medium containing glucose (about 1,000 mg/liter), yeast extract (200 mg/liter), CaCl₂ (40 mg/liter as Ca), and MgSO₄ (20 mg/liter as Mg), and the suspension was incubated for 6 hr at 30 C with Monod-type shaking. At 2 hr intervals, one portion of the suspension was withdrawn, added with a few drops of 50% H₂SO₄, and centrifuged. The concentration of glucose in the supernatant solution was determined by the anthrone method. In the glutamate experiment, the bacterium was precultivated in a medium containing yeast extract (0.1%), polypeptone (0.5%), CaCl₂ (40 mg/liter as Ca), and MgSO₄ (20 mg/liter as Mg). After 46 hr, the flocs were harvested and washed twice with the same mineral solution as that used in the glucose experiment. The flocs were then suspended in a medium containing sodium glutamate (about 1,500 mg/liter), yeast extract (200 mg/liter), CaCl₂ (40 mg/liter as Ca), and MgSO₄ (20 mg/liter as Mg). The suspension was incubated under the same conditions as those used in the glucose experiment. After 1, 2, 4, and 6 hr, one portion of the suspension was withdrawn and filtrated with an HA filter (Millipore Corp., Bedford, Mass.). The total organic carbon in the filtrate was determined by a wet combustion method (12). Although organic carbon derived from the yeast extract and from the metabolites of the cells was probably present in the filtrate, the carbon content of the filtrate was converted directly to the content of glutamic acid by calculation. In both experiments, the initial cell concentrations in the reaction mixtures were determined.

RESULTS

Characteristics of the isolates. Some morphological, cultural, and biochemical properties of the isolates are shown in Table 1. The isolates were all small rods without flagella and formed translucent, pale-yellow colonies. Nutritional requirements were complex; all of the isolates required both amino acid(s) and vitamin(s). Although the isolates grew in medium 3, the growth was slower than in medium 4, suggesting that some other growth factor(s) is necessary or stimulatory. When the isolates were grown in a medium containing the standard mineral base plus 0.1% yeast extract and 0.5% glucose, they accumulated a considerable amount of PHB within the cells. For example, the cells of strain 18 grown in the above medium for 92 hr contained 44% of PHB on a dry-weight basis. PHB was not accumulated when grown on polypeptone. The above results, along with the data on substrate utilization described later, may be

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Table 1. Some characteristics of the isolates classified as belonging to the genus Flavobacterium

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Description</th>
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<tbody>
<tr>
<td>Morphology</td>
<td>Rod, 0.7 X 1-2 µm</td>
</tr>
<tr>
<td>Gram reaction</td>
<td>-</td>
</tr>
<tr>
<td>Spore formation</td>
<td>-</td>
</tr>
<tr>
<td>Relation to O₂</td>
<td>Strict aerobic</td>
</tr>
<tr>
<td>Motility</td>
<td>-</td>
</tr>
<tr>
<td>Chromogenesis</td>
<td>Pale yellow</td>
</tr>
<tr>
<td>Capsule or slime layer</td>
<td>-</td>
</tr>
<tr>
<td>Gelatin liquefaction</td>
<td>+</td>
</tr>
<tr>
<td>Denitrification</td>
<td>-</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
</tr>
<tr>
<td>Oxidative acid production</td>
<td>Variable</td>
</tr>
<tr>
<td>Litmus milk</td>
<td>Variable</td>
</tr>
<tr>
<td>Growth factor requirement</td>
<td>Amino acid(s) and vitamin(s)</td>
</tr>
<tr>
<td>Growth on nutrient agar</td>
<td>Poor</td>
</tr>
</tbody>
</table>

Accumulation of PHB by the cells grown on:
- Glucose +
- Polypeptone -
enough to classify the isolates as belonging to one species of the genus *Flavobacterium*, although further identification of these isolates into species is difficult (5; Bergey's Manual of Determinative Bacteriology, 7th ed.).

Microscopic observation of the stained cells showed the absence of capsular material. To obtain further evidence to support this, anthrone-reactive substances of the cells were determined with the cells grown on polypeptone. Carbohydrate content expressed as glucose was only 1.4% of dry weight, suggesting the absence of capsular material at least containing polysaccharides.

**Cation-dependent, flocculent growth.** When the bacterium (strain 18) was grown in a medium containing the standard mineral base plus 0.1% yeast extract and 0.5% polypeptone or in a medium containing 0.5% glucose in place of polypeptone, good flocculent growth was observed within 48 to 72 hr in both media; the resultant supernatant solutions after settling were almost clear. The amount (volume) of settled flocs was about 10% of the medium, and the dry weight of flocs was almost clear. The amount (volume) of settled flocs was about 10% of the medium, and the dry weight of flocs was 2,000 mg/liter. However, the size of flocs formed was larger in those formed on polypeptone (1 to 2 mm) than on glucose (100 μm). Microscopic observation of the flocs showed neither the presence of slime substances nor gelatinous matrix. The cells seemed to be closely attached to each other.

When the concentration of the standard mineral base was decreased to one fifth or less of normal medium, the bacterium did not show any flocculent growth. This result suggests that some mineral component(s) in the medium is necessary for flocculation by this bacterium.

To determine what kind of mineral components affect flocculation, the bacterium was grown for 72 hr in each of the three media described in Table 2. The bacterium grew well in all three media, but good flocculation was observed only in medium C. The bacterium showed no flocculent growth in medium B and poor flocculent growth in medium A. Mineral salts other than calcium and magnesium did not induce flocculent growth. Thus, both calcium and magnesium ions are indispensable for flocculation by the growing cells, although the role of calcium ion is more important than that of magnesium ion. In another experiment, calcium and magnesium ions, at about 5 mg/liter each, brought about good flocculent growth. All of the 15 isolates showed good flocculent growth in medium C, although the size of flocs varied from strain to strain.

**Disintegration of the flocs and reflocculation of the dispersed cells.** When the flocs formed in medium C were suspended in deionized water, and the suspension was shaken gently for a few minutes, the flocs disintegrated perfectly, and a uniformly dispersed cell suspension was obtained.

Moreover, when the suspension of the dispersed cells thus obtained was added with 0.05 m CaCl₂ and shaken for a few minutes, the cells flocculated again, and the resultant supernatant solution was clear. For instance, optical densities of the dispersed cell suspension and the supernatant solution after reflocculation were 0.675 and 0.02, respectively. When the dispersed cells grown in a calcium-deficient medium were added with 0.05 m CaCl₂ and shaken, flocculation occurred as well.

To determine whether ions or compounds other than calcium ion can induce flocculation of the dispersed cells, the following mineral salts or organic compounds were added to the dispersed cell suspension at a concentration of 0.05 m: KCl, NaCl, (NH₄)₂SO₄, MgSO₄, BaCl₂, ZnCl₂, CuSO₄, MnCl₂, FeSO₄, phosphate buffer (KH₂PO₄ + Na₂HPO₄), glucose, and glycine. All of these compounds except glucose and glycine induced good flocculation. The effective concentration of potassium, calcium, and magnesium ions was above 5 mm. Reflocculation of the dispersed cells by mineral salts was not affected by a pH in the range of 4.5 to 8.3.

When the flocs formed by the addition of mineral salts were resuspended in deionized water and shaken, they again disintegrated perfectly. Thus, the process of flocculation by mineral salts and disintegration of the flocs in deionized water is reversible, suggesting that the mechanism of the process is physicochemical rather than physiological.

**Negative charge of the cell surface.** To determine whether the electric charge of the cell surface is positive or negative, the following test was performed. A 3-ml amount of cation exchange resin (Amberlite IR-120, H-form) or anion exchange resin (Amberlite IR-400, OH-form) was added to 5 ml of the dispersed cell suspension. The mixture was shaken for a few minutes and allowed to settle; optical densities of the supernatant solu-

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**Table 2. Effect of calcium and magnesium ions on floc formation by growing cultures of strain 18**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Growth</th>
<th>Flocculation</th>
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<tbody>
<tr>
<td>(A) BM + CaCl₂ (10 mm)</td>
<td>+++++</td>
<td>+</td>
</tr>
<tr>
<td>(B) BM + MgCl₂ (10 mm)</td>
<td>+++++</td>
<td>-</td>
</tr>
<tr>
<td>(C) BM + CaCl₂ (5 mm) + MgCl₂ (5 mm)</td>
<td>+++++</td>
<td>+++</td>
</tr>
</tbody>
</table>

* BM = basal medium containing 0.1% yeast extract and 0.5% polypeptone.
tions were determined. Optical densities were 0.53 (IR-400) and 1.20 (IR-120); that of the untreated cell suspension was 1.30. This result indicates clearly that the electric charge of the cell surface is negative, as in the case of many bacteria.

**Effect of heat or trypsin treatment of the dispersed cells on reflocculation.** To show that the reflocculation process is physicochemical, the following experiments were performed. Dispersed cells were killed by heating the cell suspension at 70°C for 15 min. Then the suspension was added with CaCl₂ at a concentration of 0.05 M and shaken; good flocculation occurred.

In another experiment, the dispersed cells were treated with trypsin (40°C, 15 min, pH 8.2). After the treatment, the cells were washed twice with deionized water and suspended in 0.05 M CaCl₂; the suspension was shaken, and the cells formed flocs.

These results show that reflocculation of the cells induced by mineral salts is not dependent on whether the cells are living or non-living.

**Substrate utilization.** Organic compounds utilized by the bacterium as a carbon source were as follows: glucose, fructose, galactose, mannose, sucrose, lactose, starch, glycerol, mannitol, pyruvate, malate, glutamate, and gelatin. The following compounds were not utilized: formate, acetate, butyrate, lactate, succinate, citrate, methyl alcohol, ethyl alcohol, n-butyl alcohol, and cellulose. The results show that the bacterium can grow on a relatively wide variety of organic compounds, including polymeric substances such as starch or gelatin. Organic acids and alcohols were poorly utilized. The ability of this bacterium to utilize various kinds of organic substances may be a factor for making the bacterium predominant in activated sludge.

**Metabolic activity.** The uptake of glucose and glutamate by the bacterium is shown in Fig. 1, where the absorption curves for glucose and glutamate are plotted per gram of the initial cell dry weight. The absorption curve of glucose is almost linear for 6 hr, and the average rate of glucose uptake was 188 mg/g (dry weight) of the cells per hr. On the other hand, the rate of glutamate uptake decreased after 2 hr. The rate of glutamate uptake in the first 2 hr was 79.6 mg/g (dry weight) of the cells per hr. These results show that the flocs of the bacterium have high purification activity compared to that of naturally activated sludge (14).

**DISCUSSION**

Although flavobacteria have often been isolated as the predominant bacteria of activated sludge (1, 6, 14), there have been few reports concerning floc formation by these bacteria. McKinney and Horwood (7) isolated a Flavobacterium species with the ability to form flocs, but they did not ascertain the predominance of such a bacterium in activated sludge.

In contrast to Zoogloea (4, 13; Anderson and McCoy, Bacteriol. Proc., p. 162, 1963; and Bergey's Manual of Determinative Bacteriology, 7th ed.), the Flavobacterium species dealt with herein is unique in that it can flocculate in spite of the lack of capsular material or gelatinous matrix. As in the case of Z. ramigera (2, 3), the Flavobacterium species accumulates a large amount of PHB when grown on glucose. However, floc formation is independent of the accumulation of PHB, since the flocs formed on peptone did not accumulate the polymer. The ability to accumulate PHB, however, may have a close relationship to the purification process, since recent investigations have shown that the assimilatory process is mainly responsible for the rapid removal of organic wastes in the activated sludge stabilization (14).

Although floc formation by growing cells was somewhat different from that by dispersed cells, the data show clearly that cations play an important role in the mechanism of floc formation in general. The fact that floc formation by cations
is reversible, i.e., disintegration of flocs in deionized water and reflocculation of the dispersed cells by cations, suggests strongly that the process of flocculation is physicochemical rather than physiological. The fact that dead cells can flocculate in the presence of cations is additional evidence to support this view. With respect to the mechanism of floc formation by bacteria, McKinney (8) presented a theory, according to which the bacterial surfaces undergo direct chemical reactions, thus forming peptide linkages, salt linkages, and ester linkages. This theory, however, has not been widely accepted. Of three linkages suggested by McKinney, peptide and ester linkages must be abandoned in the case of the bacterium studied here, because these linkages are formed by enzymatic reactions and are rigid chemical bonds. The evidences that the electric charge of the cell surface is negative and the process of floc formation is physicochemical suggest that the mechanism of floc formation in this Flavobacterium species is as follows: negatively charged surfaces of adjacent cells are bridged by ionic bonds intermediated by cations. At any rate, the results of this study show clearly that bacterial flocculation can occur without zoogloea formation.

The bacterium utilized a relatively wide variety of organic compounds, including polymeric substances such as starch and gelatin. Moreover, metabolic activity of the bacterium was high compared to that of naturally activated sludge. These properties, along with floc-forming ability, may be factors for making the bacterium predominant in activated sludge.

LITERATURE CITED