

# Bacterial Flocculation and Production of Poly- $\beta$ -Hydroxybutyrate

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Experiments with a number of bacteria isolated from activated sludge have shown that flocculation is independent of the presence of poly- $\beta$ -hydroxybutyrate (PHB) in the cells. Several strains gave flocculent growth without any PHB detectable. Other strains, producing PHB in varying amounts, utilized this compound as an endogenous substrate, and after its disappearance the floc structure remained unchanged. The PHB content of various samples of activated sludge was found to be negligible.

Crabtree et al. (2, 3) reported that dispersed cells of *Zoogloea ramigera* I-16-M were devoid of poly- $\beta$ -hydroxybutyrate (PHB), whereas flocculating cultures accumulated large amounts of this material. The PHB could be dissimilated endogenously and this resulted in deflocculation. Since capsule or "gum" polysaccharides were not present in the cells, Crabtree thought the flocculation to be closely associated with the intracellular accumulation of PHB.

Tezuka (6) observed that flocs of a strain of *Flavobacterium* accumulated a large amount of PHB when grown on glucose. On peptone, however, the flocs did not contain this polymer. In 1969 Angelbeck and Kirsch (1) reported the presence of 1% of PHB in both aggregated and nonaggregated cells of *Z. ramigera* Z.R.C. Deinema and Zevenhuizen (4) demonstrated that the formation of cellulose fibrils by gram-negative bacteria is one of the causes of bacterial flocculation.

In the present investigation the flocs of a number of bacteria and samples of activated sludge were analyzed for PHB.

## MATERIALS AND METHODS

A number of floc-forming bacteria were isolated from activated sludge. Seven representative strains were selected, and their PHB and floc production were compared with those of *Z. ramigera* I-16-M isolated by Crabtree et al. (2). The identification and cultivation of the floc-forming bacteria have been described previously (4).

Casitone-glucose medium contained 1 g of casitone (Difco), 5 g of glucose, 0.35 g of yeast extract (Difco), and 1,000 ml of tap water, pH 6.9. Arginine medium (3) contained 1 g of arginine hydrochloride, 2 g of  $K_2HPO_4$ , 1 g of  $KH_2PO_4$ , 0.2 g of  $MgSO_4 \cdot 7H_2O$ , 7

mg of yeast extract (Difco), and 1,000 ml of tap water, pH 6.9.

The PHB content of the cells was estimated by the method of Stockdale (5).

## RESULTS AND DISCUSSION

Four strains of floc-forming bacteria were grown in the arginine medium. The composition of this culture solution was derived from the medium of Crabtree et al. (3), the concentration of arginine being doubled to obtain a higher yield. The results recorded in Table 1 demonstrate a good floc formation and a low amount of PHB in the cells, the latter being not surprising as the C/N ratio of arginine is only 1.3. In the experiments described by Crabtree et al. (3), strain I-16-M did not flocculate in the arginine medium. This was thought to be due to the absence of PHB in the cells. However, the flocs of the four strains used in the present investigation were solubilized upon enzymatic treatment with cellulase, indicating that the flocculation depended on the production of cellulose fibrils (4) and was not correlated with

TABLE 1. Percentage of PHB in bacterial flocs grown in arginine medium

Strain	Floc formation <sup>a</sup>	% PHB
I-16-M <sup>b</sup>	++	2.1
Z-8	++	1.6
109	++	1.3
212	+	1.2

<sup>a</sup>Symbols: ++, flocs in a clear supernatant; +, flocs and suspended bacteria in supernatant.

<sup>b</sup>I-16-M is identical with *Zoogloea ramigera* ATCC 19623.

TABLE 2. Percentage PHB in bacterial flocs grown on casitone-glucose

Strain	Genus	Mechanism of flocculation	No. of days of incubation at 30 C					Floc structure after 46 days
			4	7	14	21	46	
I-16-M <sup>a</sup>	<i>Zoogloea</i>	Cellulose fibrils	42.2	33.9	24.2	9.2	0	Unchanged
Z-8	<i>Achromobacter</i>	Cellulose fibrils	16.1	13.2	2.4	0	0	Unchanged
109	<i>Achromobacter</i>	Cellulose fibrils	29.7	27.4	18.4	16.5	10.8	Unchanged
212	<i>Achromobacter</i>	Cellulose fibrils	13.1	8.1	1.6	1.5	0	Unchanged
X-1	<i>Achromobacter</i>	Cellulose fibrils	0	0	0	0	0	Unchanged
315a <sup>b</sup>	<i>Pseudomonas</i>	?	5.3	2.1	0	0	0	Unchanged
329	<i>Pseudomonas</i>	?	18.1	21.0	32.0	35.0	ND <sup>c</sup>	Unchanged
93	<i>Flavobacterium</i>	?	2.0	2.0	1.2	0.5	0	Unchanged

<sup>a</sup> I-16-M is identical with *Zoogloea ramigera* ATCC 19623.

<sup>b</sup> Glucose was substituted by the same amount of glycerol.

<sup>c</sup> Not determined, cells dead.

TABLE 3. Percentage of PHB in activated sludge samples

Origin of sewage	% PHB
Amsterdam, domestic sewage . . . . .	0.0-0.2
Ede, domestic sewage . . . . .	0.0-0.2
Zeist, domestic sewage . . . . .	0.0-0.2
Potato sewage . . . . .	0.5-2.0
Dairy sewage . . . . .	0.1

the PHB production in the cells.

A casitone-glucose medium, with a C/N ratio of about 14, which might be more favorable for PHB accumulation, was used as a second medium for bacterial floc formation (Table 2). After 4 days the cells were washed in 0.005 M phosphate buffer, pH 6.9, and left shaking for 6 weeks at 30 C. Several strains of bacteria accumulated a large amount of PHB in their cells, but this disappeared with time as a normal endogenous reserve material. Much difference was observed in the time necessary for the consumption of the intracellular PHB; strain I-16-M utilized all of its PHB (42.2%) within 42 days, whereas in strain 109 only two-thirds of the initial 29.7 disappeared in the same period of time. In the flocs of X-1 no PHB could be detected, and strain 329 could not use it as an endogenous substrate. The amount of PHB in the cells of strain 329 did not decrease, and its percentage apparently increased due to respiration of other cell components, for instance, lysis products of dead cells. The cells of strain 329 were slowly dying during this experiment, and after 46 days no more viable cells were present in the flocs. Strain 315<sup>a</sup> was cultivated with glycerol, as glucose gave rise to

the formation of an unknown organic acid which acidified the culture solution and stopped the cell growth. From the results given in Table 2, it follows that the presence of PHB in the cells is not responsible for floc formation since after disappearance of the PHB the floc structure remained unchanged.

For comparison, the percentage of PHB in the flocs of various activated sludge samples was determined (Table 3). It appeared to be very low. Only with potato sewage, with a C/N ratio of about 6, the amount increased slightly, but a value of 12, as found by Crabtree et al. (3), was never observed.

From the experiments described, it can be concluded that PHB is often a reserve material in bacterial cells but plays no essential role in the mechanism of flocculation.

#### LITERATURE CITED

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