Influence of Growth Conditions on Adherence of *Streptococcus mutans* Ingbritt to Saliva-Coated Hydroxyapatite

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Streptococcus mutans Ingbritt grown under standardized conditions adhered less effectively to saliva-coated hydroxyapatite beads than did *Streptococcus sanguis* G9B, and there was competition for binding. The results with Ingbritt were influenced by the generation time, the pH of growth, and the carbohydrate source as shown by studies on organisms grown in continuous culture.

The adherence of bacteria to pellicle coating the tooth surface is the important first stage in plaque formation, and there has been considerable interest in the relative adherence of different bacteria and the mechanism involved (4, 7). Saliva-coated hydroxyapatite (SHA) beads (3) have proved particularly useful and relevant for obtaining such information, as is shown by the number of reports of results obtained with *Streptococcus mutans* and *S. sanguis* (2, 3, 5, 6, 12-14, 17, 18).

The work by Rosan and colleagues (2, 17) showed that strains of *S. sanguis* adhered much more effectively than did those of *S. mutans*. However, according to usual procedures, these organisms had been grown to stationary phase without pH control, and growth conditions are known to affect surface polymers (9-11). In a subsequent collaborative study with Rosan and colleagues (16) on *S. sanguis* G9B grown under the defined conditions obtainable in continuous culture, it was shown that the adherence capacity could vary severalfold depending on generation time and pH. This report describes a similar study on *S. mutans* Ingbritt and also provides a comparison with *S. sanguis* G9B. The methods used for determining the adsorption isotherms and the binding competition between cells grown under different conditions were those described previously (16).

S. mutans Ingbritt was grown under previously described conditions (9-11). Following defined procedures (16), standard reference cells were grown at pH 6.0 in a Multigen Fermenter (New Brunswick Scientific Co., New Brunswick, N.J.), gassed with 95% N₂-5% CO₂ and collected at the end of exponential phase. To obtain labeled cells, cultures were grown in the presence of 2 μ Ci of [methyl-³H]thymidine per ml (16).

The adsorption isotherm for the adherence of these labeled organisms to SHA beads is shown in Fig. 1 and indicates a saturation of binding sites. However, at the inflexion point, only 4.2% of cells were bound compared with 17.6% of *S. sanguis* G9B grown under the same conditions. Figure 1 also provides the results of an experiment in which unlabeled *S. sanguis* G9B cells (Multigen; pH 6.0) were added to SHA beads at a concentration sufficient to saturate binding sites (5.7 mg/ml), unbound cells were removed by washing (16), and labeled *S. mutans* Ingbritt was added in increasing amounts. The resultant adsorption isotherm showed a decrease in the binding capacity of *S. mutans* Ingbritt suggestive of competition between *S. sanguis* and *S. mutans*.

Confirmatory evidence for competition was sought by following the procedure (16) of incubating SHA beads with mixtures of unlabeled and labeled cells in which the labeled cells remained constant at 1.25 mg/ml and the unlabeled cells represented 33, 50, 67, and 75% of the total (0.62 to 3.75 mg/ml). The results in Fig. 2 provide evidence of competition between unlabeled *S. sanguis* G9B and labeled *S. mutans* Ingbritt. A comparison of these results with those obtained with unlabeled *S. mutans* Ingbritt (Fig. 2) also confirms that *S. sanguis* G9B binds more effectively to SHA beads than does *S. mutans* Ingbritt. These results were obtained with unheated saliva (16); using saliva heated at 60°C, a different strain of *S. mutans*, and organisms grown without pH control, competition between the two species was not observed (2). The heating of saliva does not affect the adherence of *S. sanguis* G9B to SHA beads (17), but was shown to have an effect on the adherence of other strains of *S. sanguis* and also *S. mutans* strains (5).

FIG. 1. Isotherms of adsorption of [^3H]thymidine-labeled cells of *S. mutans* Ingbritt grown with glucose in batch culture at pH 6.0. Results are compared for adsorption to SHA beads (●) and to SHA beads precoated with *S. sanguis* G9B (■).

Figure 2 also includes the results of growing *S. mutans* Ingbritt without pH control, namely, in a flask in an anaerobic jar or in a Multigen with gassing. Both of these cultures were much less effective in inhibiting the adherence of labeled *S. mutans* Ingbritt than were the cells grown at pH 6.0. For example, with 75% unlabeled cells (Fig. 3), the organisms grown in the Multigen without pH control were only 40% as effective as those grown with pH control. The comparable value for *S. sanguis* G9B is 80% (16). The poorer binding capacity of *S. mutans* grown without pH control when compared with *S. sanguis* cannot be accounted for simply on the basis of the final pH of the cultures, which was 4.28 for strain Ingbritt (average of 9 cultures) compared with 4.42 for G9B (average of 10 cultures).

The effect of pH was examined in greater detail by growing organisms in continuous culture at a dilution rate of 0.1 h^{-1} (7-h generation time) and at either pH 5.5, 6.5, or 7.5; the growth-limiting (0.5%) carbohydrate was either glucose, fructose, or sucrose (9, 10). Organisms were then tested by the standard procedure for their ability to inhibit the adherence of labeled organisms grown in the Multigen at pH 6.0. The results obtained with 75% unlabeled cells (Fig. 3) confirm that the adherence properties of organisms are influenced by the pH of growth, with the general trend being for an increase in adherence with an increase in the pH of growth.

Whether generation time also influenced adherence properties was examined by growing organisms in continuous culture at pH 6.0 and at dilution rates of 0.1, 0.3, and 0.5 h^{-1} corre-

FIG. 2. Inhibition of adherence to SHA beads of [^3H]thymidine-labeled cells of *S. mutans* Ingbritt grown at pH 6.0 by increasing proportions of unlabeled *S. mutans* Ingbritt grown at pH 6.0 (●), grown in a Multigen with no pH control (○), grown in an anaerobe jar (▲), and by *S. sanguis* G9B grown in a Multigen at pH 6.0 (■). The error bars on the results for unlabeled *S. mutans* Ingbritt grown at pH 6.0 represent 1 standard deviation either side of the mean of the results of five experiments reported in this paper, in which these cells were included in each day's inhibition studies as a reference control.

sponding to generation times of 6.9, 2.3, and 1.4 h, respectively. The results for glucose (Fig. 4) indicate that adherence increases as the generation time decreases, with the fastest-growing cells giving results comparable to those for batch-grown cells. A recent report (1) comparing *S. mitis* grown in batch and continuous cultures concluded that growth in continuous culture with limiting glucose led to a marked decrease in the production of surface components required for aggregation. However, the results in Fig. 4 for batch and continuous cultures grown at pH 6.0 indicate that the adherence properties of *S. mutans* Ingbritt are only minimally affected by this change.

The results in Fig. 3 and 4 also permit a comparison of the effects of different carbohydrates on adherence properties. Organisms grown with limiting sucrose do not produce significant amounts of glucans, and any effect of glucans would be minimal as evidence now indicates that they are not involved in the initial attachment mechanism (18). This situation would account for the similarity in the results for glucose and sucrose. On the other hand, growth

FIG. 3. Comparison of the ability of *S. mutans* Ingbritt cells grown in batch culture with and without pH control and cells grown at 7-h generation time at different pH values to inhibit the adherence of [3 H]thymidine-labeled cells to SHA beads. The labeled cells were grown at pH 6.0. The results given are those where unlabeled cells were 75% of the total.

in fructose at high dilution rate and pH 6.0 enhances adherence properties, and these are conditions where the increased production of lipoteichoic acid and certain proteins also occurs (9, 10).

Lipoteichoic acid had been proposed as a factor involved in adherence because of its negative charge (15), and evidence for the interaction

between lipoteichoic acid in the surface of *S. sanguis* and salivary glycoprotein has recently been reported (8). However, the specificity of adherence generally accords with bacterial surface proteins functioning as lectins and reacting with salivary glycoproteins (4, 7); also, a protein-containing component that blocks adherence has been isolated from *S. sanguis* (13).

The importance of surface components in adherence, coupled with the known effects of growth conditions on surface components (9–11) and adherence properties (14, 16), points to the need to consider growth conditions when studying adherence. As shown by the current studies and those on *S. sanguis* (16), the pH of growth is particularly important, and it would seem desirable that where comparative studies on adherence employ organisms grown in batch culture, the pH should be kept constant. This would also enable the growth rate to be monitored so that organisms could be collected at the same phase of growth.

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FIG. 4. Effect of dilution rate in continuous culture at pH 6.0 with limiting glucose, fructose, or sucrose on the ability of *S. mutans* Ingbritt to inhibit adherence to SHA beads of [3 H]thymidine-labeled cells grown in batch culture with glucose at pH 6.0. The results given are those where unlabeled cells were 75% of the total.

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