Chemostat Studies of the Effect of Environmental Control on *Streptococcus sanguis* Adherence to Hydroxyapatite

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Received 4 May 1981/Accepted 27 August 1981

Streptococcus sanguis is a major component of early dental plaque. The ability of *S. sanguis* to adhere to salivary pellicle appears to involve specific bacterial surface receptors. The nature of these receptors is still not known; however, the component molecules may be subject to environmental control as has been shown for teichoic acids and certain proteins. To study these environmental effects, a chemostat was employed to vary the growth conditions of *Streptococcus sanguis* strain G9B. This strain has been used extensively to study the adhesion of [³H]thymidine-labeled batch-grown cells to saliva-coated hydroxyapatite beads. The effects of dilution rate, pH, and carbon source on adhesion were studied with a competition assay in which the labeled batch cells were used as a reference standard. In this assay, cells from the chemostat were harvested and compared for their ability to inhibit adhesion of labeled cells relative to unlabeled control batch-grown cells. Subsequent studies used chemostat grown cells labeled with [³H]thymidine as a reference standard so that results were internally controlled and reflected only the particular alteration in environment which was studied. These results indicated that when glucose was used as a growth-limiting substrate, cells grown at relatively high dilution rates ($D = 0.5 \text{ h}^{-1}$; mean generation time = 1.4 h) behaved similarly to batch-grown cells and appeared to compete for the same binding sites. Cells grown at $D = 0.1 \text{ h}^{-1}$ (mean generation time = 7 h) no longer competed with either batch-grown cells or chemostat cells grown at $D = 0.5 \text{ h}^{-1}$. Moreover, adsorption isotherms of such slow-growing cells ($D = 0.1 \text{ h}^{-1}$) suggested that binding was no longer specific. When fructose was used as the growth-limiting carbohydrate, cells grown at $D = 0.1 \text{ h}^{-1}$ did not show this loss of specificity and competed nearly as well as control batch-grown glucose cells. However, the effect of pH appeared to be independent of carbohydrate source, because cells grown in either glucose or fructose at pH 5.5 at $D = 0.1 \text{ h}^{-1}$ lost the ability to compete with reference batch or chemostat cells grown at $D = 0.5 \text{ h}^{-1}$. This effect was very sharp, since cells grown in the pH range from 6 to 7.5 at $D = 0.5 \text{ h}^{-1}$ competed nearly as well as control cells. A similar effect of pH was found for batch cultures grown with excess glucose. These studies reinforce the idea that the environment can profoundly affect the bacterial surface and consequently the ability of the organism to adhere, a property which appears to be a primary event in some infectious diseases and in dental plaque formation.

Several studies have shown that the initiation of dental plaque is associated with the deposition of *Streptococcus sanguis* which subsequently forms microcolonies on the tooth surface (2, 3, 23, 25). Although the percentage of *S. sanguis* tends to decrease as plaque matures (7), it continues to be a major species in most supragingival dental plaque and is found in very large numbers in attached subgingival plaque (15, 19). In addition to adhering to tooth surfaces, part of its persistence in plaque may also be associated with its ability to interact with a

variety of other oral organisms, particularly filaments which become more prominent as plaque matures (12, 14, 17, 18). Thus, *S. sanguis* may play a pivotal role in plaque initiation and could be an important factor in both dental caries and periodontal disease.

Recent in vitro studies of plaque formation indicate that surface components of *S. sanguis* interact with salivary glycoproteins which form a coating or pellicle on tooth or hydroxyapatite surfaces (4, 6). This interaction can often be described by the Langmuir adsorption isotherm

(1) which allows calculation of a relative affinity (K_a) and the maximum number of binding sites (N) for a particular bacterial strain. The product of the affinity and number of binding sites ($K_a N$) can be used to estimate the relative ability of various strains to adhere. The model used for these determinations employs saliva-coated hydroxyapatite beads (SHA) which are equilibrated with radioactively labeled bacteria. This method allows precise estimation of the number of free bacteria and the number bound, parameters which form the basis for calculation of the isotherm.

These experiments have shown that strains of *S. sanguis* have a higher $K_a N$ than other oral streptococci (1) and thus may explain why *S. sanguis* is among the earliest colonizers of the tooth surface. In addition to quantifying the interaction between the streptococci and the SHA, the isothermal plots can indicate whether specific interactions occur or whether cells merely continue to pile up on the SHA surface, eventually occupying all available sites (16). Our studies suggested that many of the interactions between *S. sanguis* and the SHA were specific, involving bacterial surface components (receptors) and specific salivary glycoproteins. Although the specific surface components of *S. sanguis* involved in adherence have not yet been identified, preliminary studies suggested that the degree of adherence and, by implication, the receptors could be altered by changes of environmental conditions (Appelbaum et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, B104, p. 34).

A number of recent studies with chemostat-grown cells have shown that growth rate, substrate, and pH can have profound effects on the quantities of surface components such as lipoteichoic acids and proteins (9, 10, 11). Moreover, these changes were also reflected in other surface-associated properties, e.g., immunogenicity and sensitivity to lytic enzymes (8, 13). If adherence were associated with either lipoteichoic acid (20, 21) or proteins as suggested by other investigators (Liljemark and Bloomquist, IADR Abstr. 1979, p. 201), then this property also should be affected by environmental changes. In this report we show with chemostat cultures that growth rate, carbohydrate substrate, and pH can exert profound effects on the ability of a reference strain of *S. sanguis* to adhere to SHA.

MATERIALS AND METHODS

Organism. *S. sanguis* strain G9B was originally isolated from dental plaque (22). It is a serotype 1 strain and contains, in addition to the group H teichoic acid, the *c* and *d* protein surface antigens. Organisms

for study were drawn from either lyophilized or frozen (-70°C) cultures.

Medium. Organisms were grown in the low-molecular-weight fraction of Trypticase (BBL Microbiology Systems)-yeast extract medium that passed through an Amicon hollow fiber filter H1P10 (10). Except in some preliminary studies where the basal medium was sterilized by autoclaving, glucose or fructose was added to a final concentration of 2% for batch culture experiments or 0.5% for continuous culture experiments, and the medium was then sterilized by membrane filtration (10).

Batch cultures. For the experiment described in Fig. 2, the control culture was grown for 15 h without pH control. The labeled cells required for this experiment were grown in the presence of [methyl- ^3H]thymidine (specific activity, 20 Ci/mmol; New England Nuclear Corp., Boston, Mass.) at a final concentration of 2 $\mu\text{Ci/ml}$ (1). The other cultures described in Fig. 2 and all batch culture standards for chemostat experiments were grown in a Multigen Fermenter with automatic pH controller (New Brunswick Scientific Co., New Brunswick, N.J.) and gassed with 95% N_2 -5% CO_2 . Cultures were monitored for alkali addition and grown until the end of the exponential phase, which was 16 to 23 h depending on the pH.

Control and labeled cells required for the chemostat studies were grown at pH 6.0, with the concentration of labeled thymidine being the same as described above. Cultures were harvested by centrifugation ($7,000 \times g$, 15 min, 4°C), washed twice with 0.85% NaCl, and stored in buffered KCl (1). The specific activity of cells was approximately 250,000 cpm/ 1.5×10^9 cells.

Continuous culture. Cultures were grown in a BioFlo chemostat of 325-ml capacity (New Brunswick Scientific Co.) under the same conditions as described previously for *S. mutans* strains (10, 11). The generation time of organisms in continuous culture is related to the dilution rate per hour (D) by the formula generation time = $\ln 2/D$. The 0.5% glucose used in the chemostat is a limiting concentration; cultures were checked daily to ascertain that at least 99% of the glucose was being utilized (10). Cultures were examined routinely for purity, and organisms were stored frozen until required for the adherence assay.

For [^3H]thymidine-labeled cultures, organisms were grown at $D = 0.1$ and 0.5 h^{-1} (equivalent to generation times of 7 and 1.4 h, respectively). To the culture growing at $D = 0.1 \text{ h}^{-1}$ 0.5 mCi of [^3H]thymidine was added, and then 0.1 mCi was added at the end of each hour for 5 h to compensate for displacement of labeled compound. After a total of 7 h, the collected culture and the contents of the culture vessel were combined, harvested, washed, and stored frozen in buffered KCl; the specific activity was 120,000 cpm/ 6×10^9 cells.

The same procedure was followed for obtaining labeled cultures grown at $D = 0.1 \text{ h}^{-1}$, pH 5.5 and 7.0. For the culture grown at $D = 0.5 \text{ h}^{-1}$ and pH 6.0, 0.1-mCi amounts were also added on five occasions, but at 20-min intervals, and the culture was harvested after a total of 3.6 h, or approximately 2.5 generation times.

Adherence assay. The adherence assay followed the previously described modification (1) of the method developed by Clark and co-workers (4), with increasing concentrations of labeled cells being added to 40

mg of SHA beads. Stimulated saliva from several donors was pooled and clarified as described previously (1), but was not heat inactivated. Both dilution and washing were performed in a hypotonic phosphate buffer (2 mM) at pH 6.8 which contained 1 mM CaCl_2 and 50 mM KCl (1). Radioactive determinations of cells adsorbed to beads, free cells, and specific activity were performed as described previously (1). Control studies showed no difference in adsorption isotherms between the results for saliva obtained in Sydney and Philadelphia. Cell suspensions were adjusted to the required concentration by measuring optical density in a Corning Colorimeter 252 with a 252/600 filter and calculating dry weight from a calibration curve obtained with a batch culture; an optical density of 0.825 corresponded to 1.0 mg/ml (6×10^9 cells per ml).

Competition assays. [^3H]thymidine-labeled cells from batch or chemostat cultures were used as reference cells for competition assays as described below. Increasing amounts of unlabeled test cells with a final concentration of 0.5 to 3.2 mg/ml were added to a fixed concentration (1.0 mg/ml) of labeled reference cells in the presence of SHA beads (1). All experiments were carried out in triplicate. In the previous report (1) results were plotted in terms of the ratio of labeled to unlabeled cells, namely 1:1, 1:2, and 1:3. In the present study results are expressed relative to the percentage of unlabeled cells, so that the above ratios convert to 50, 67, and 75% unlabeled cells, with a resultant change from a curvilinear to a linear plot. All tests included homologous unlabeled batch cells (control cells) grown at the same time and under similar conditions to labeled cells. For chemostat-grown cells, the control was the culture collected before adding [^3H]thymidine.

RESULTS

Effect of growth rate. The method employed for comparing organisms grown under different conditions for their adherence properties is illustrated by the results in Fig. 1. Increasing concentrations of unlabeled organisms grown in glucose at pH 6.0 and $D = 0.1, 0.2$, and 0.4 h^{-1} were added to labeled cells grown in batch culture at pH 6.0. The results show that organisms grown at $D = 0.2$ or 0.4 h^{-1} were as effective as the control batch-grown unlabeled cells, whereas organisms grown at $D = 0.1 \text{ h}^{-1}$ were much less effective. In the subsequent experiments, comparisons are expressed as percent inhibition of the adherence of labeled cells when unlabeled cells represented 75% of the total (or a ratio of labeled to unlabeled cells of 1:3).

Effect of pH of growth on batch cultures. Figure 2 shows the results of a study in which the adherence of organisms grown in batch culture at different pH values was compared with that of control cells grown without pH control; the final pH of such uncontrolled cultures was 4.6. It is apparent that growth at a constant pH of 5.5 resulted in a marked loss of

FIG. 1. Inhibition of adherence to SHA beads of [^3H]thymidine-labeled cells grown at pH 6.0 by increasing proportions of unlabeled batch-grown cells (○) and by organisms grown in continuous culture in limiting (0.5%) glucose at pH 6.0 and $D = 0.1 \text{ h}^{-1}$ (■), 0.2 h^{-1} (●), and 0.4 h^{-1} (▲). The results are the means of triplicate experiments.

ability of these cells to compete with labeled batch cells in the adherence assay. Although the pH effect was not as great in the usual unregulated batch culture, it was thought desirable to use both labeled and unlabeled batch-grown cells maintained at pH 6 for control cells in subsequent experiments.

Effect of pH of growth on continuous cultures. Organisms were grown at $D = 0.1 \text{ h}^{-1}$ in limiting glucose or fructose at pH 5.5 to 7.5 (Fig. 3). At pH >6.0 slow-growing cells in fructose adhered better than glucose-grown cells; the trend was the same in each case and comparable to the results seen in batch culture (Fig. 2), namely, that maximal adherence occurred at pH 6.0 to 6.5, and minimal adherence took place at pH 5.5. Both fructose- and glucose-grown cells tended to form long chains at low pH.

Effect of dilution rate at constant pH. Organisms grown at pH 6.0 at different dilution rates in limiting glucose or fructose were examined for their ability to inhibit the adherence of labeled cells grown in batch culture with glucose at pH 6.0. The slowest growing cultures ($D = 0.1 \text{ h}^{-1}$) were less effective competitors than those growing at the intermediate rates of 0.2 to 0.4 h^{-1} for both carbon sources, albeit the effect of slow

FIG. 2. Comparison of the ability of organisms grown in batch culture at different pH values to inhibit the adherence of [^3H]thymidine-labeled cells to SHA beads; the labeled and unlabeled control cells were grown without pH control. The results given are those where unlabeled cells were 75% of the total cells.

growth was more pronounced with glucose (Fig. 4). The result with fructose at $D = 0.5 \text{ h}^{-1}$ may reflect a variation that was noted in the adherence properties of different cultures grown with this carbohydrate at pH 6.0. Indeed, when the competition assay was repeated with organisms grown in limiting fructose at pH 6.5, the inhibition of adherence at $D = 0.5 \text{ h}^{-1}$ was the same as for cells grown at $D = 0.4$ and 0.6 h^{-1} (70 to 73%).

Comparison of batch- and chemostat-grown cultures. To examine the effect of growth conditions in more detail, adsorption isotherms of [^3H]thymidine-labeled batch-grown cells were compared to those of labeled cells grown in glucose at $D = 0.1$ and 0.5 h^{-1} . These curves (Fig. 5) indicate that cells grown at the higher dilution rate saturate the SHA at approximately the same cell concentration as batch-grown cells. In contrast, the slower-growing cells do not show saturation kinetics but the number of cells adsorbing appears to be directly proportional to the number of free cells. These data suggest some rather profound modifications in surface associated with the growth rate in glucose.

Since most of our studies were done using batch-grown cells as a reference, it was of interest to determine whether these relationships hold when cells grown at $D = 0.1 \text{ h}^{-1}$ are

FIG. 3. Effect of pH of growth in continuous culture at $D = 0.1 \text{ h}^{-1}$ with limiting glucose or fructose on the ability of cells to inhibit adherence to SHA beads of [^3H]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.

FIG. 4. Effect of dilution rate in continuous culture at pH 6.0 with limiting glucose or fructose on the ability of cells to inhibit adherence to SHA beads of [³H]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.

used as reference cells for competition assays. Unlabeled chemostat cells were grown in glucose at $D = 0.1 \text{ h}^{-1}$; after a sufficient number of cells were collected, [³H]thymidine was added, and labeled cells were collected. The competition assays (Fig. 6) confirmed the observation that unlabeled slow-growing chemostat cells do

not compete with labeled batch-grown cells (upper two curves), whereas the control unlabeled batch-grown cells show their usual competition. When labeled $D = 0.1 \text{ h}^{-1}$ cells were used as the reference strain, a slight competition was observed with $D = 0.1 \text{ h}^{-1}$ cells (internal control, $P < 0.01$). A greater degree of competition was noted between unlabeled batch-grown cells and slow-growing labeled chemostat cells ($P < 0.001$), although the absolute values for adherence were low (this would be expected from isotherms shown in Fig. 5). Although the results were significant, they were not normalized because it would tend to amplify the differences.

DISCUSSION

These experiments demonstrate clearly that environmental alterations have profound effects on the ability of *S. sanguis* to adhere to SHA. The effects of growth rate are particularly striking because of the change in adsorption isotherm from the typical curvilinear pattern to a linear pattern suggesting nonspecific adsorption of the cells (Fig. 5). This change is reiterated in the competition assays where cells grown at low dilution rates in glucose fail to compete with cells grown more rapidly. Moreover, this effect was repeated by using labeled chemostat cells versus unlabeled chemostat cells so that the experiments were completely internally controlled.

The competition between labeled chemostat cells and the unlabeled chemostat or unlabeled batch-grown cells may be of particular interest

FIG. 5. Adsorption isotherms for [³H]thymidine-labeled cells grown with glucose in batch culture at pH 6.0 (■), and in continuous culture at $D = 0.5 \text{ h}^{-1}$, pH 6.0 (▲) and $D = 0.1 \text{ h}^{-1}$, pH 7.0 (●).

FIG. 6. Competition assays employing [^3H]thymidine-labeled cells grown at pH 6.0 with glucose in batch culture and in a chemostat at $D = 0.1 \text{ h}^{-1}$. The competing unlabeled cells were grown under the same conditions.

even though in absolute terms the reduction in adherence was low. Assuming that the isotherms of rapid-growing (batch) and slow-growing cells do reflect nonspecific and specific binding, respectively, then competition of unlabeled cells grown at $D = 0.1 \text{ h}^{-1}$ (which do not seem to bind to any specific site on SHA) with homologous labeled cells should show no change or a slight decrease in labeled cells adhering. Indeed, based on isotherms shown in Fig. 4, if two different labels were used in this competition assay, an additive effect would be expected because there is a direct proportion between the free and bound cells in this case. Just such effects have been shown previously with interactions between *S. sanguis* serotype 1 and 2 strains and with *S. sanguis* and *Streptococcus mutans* (1). In contrast, if unlabeled cells with a rather high affinity for SHA (batch or rapidly growing chemostat cells, $D = 0.5 \text{ h}^{-1}$) were competing with labeled cells binding non-specifically (slow growing, $D = 0.1 \text{ h}^{-1}$), it would be expected that certain numbers of sites would be rendered unavailable for binding, and distinct inhibition of the labeled cells would be observed. This is exactly what was observed in competition between the batch-grown and labeled slow-growing chemostat cells (Fig. 6).

The observation (Fig. 4) that cells grown in fructose at $D = 0.1 \text{ h}^{-1}$ compete better than glucose grown cells at $D = 0.1 \text{ h}^{-1}$ indicates that substrate as well as growth rate can affect cell surface factors involved in adherence. These results are consistent with the idea that specific cell surface polymers are responsible for adhesion and with the fact that some surface poly-

mers have been shown to be responsive to a variety of environmental influences (9). These effects of changes in growth rate and substrate on adhesion, presumably through effects on surface macromolecules, support the idea that shifts in metabolism can result from altered growth rate as well as changes in substrate (5, 8, 24). Our studies suggest the importance of such metabolic shifts on surfaces and their interaction with the environment.

The diminished competition and the nearly linear isotherm of the cells grown at low pH (Fig. 2) also suggest that a major surface change has occurred. Since the changes are similar to effects of growth rates, it is possible that the same surface receptor is affected. However, caution must be exercised with this interpretation because it was observed that cells grown at low pH tend to form much longer chains. Chain formation not only can affect an assay based on radioactive labeling (1), but also suggests some change in a surface property which inhibits cell separation. Indeed, it would be predicted that if specific sites were involved in attachment, a long chain of cells would not compete as effectively as the same number of singlets or pairs of cells; the latter arrangement is usually seen with this strain in batch or rapidly growing cells. Thus, the pH effect could represent an artifact which calls attention to the importance of determining chain length in studies of adherence. However, competition experiments similar to those shown in Fig. 6 were also performed with ^3H -labeled cells grown at $D = 0.1 \text{ h}^{-1}$, pH 5.5. The resultant curves were nearly identical to those shown in Fig. 6. These observations sug-

gest that for the time being exact conclusions as to the effects on surface associated with pH should be held in abeyance, at least until the specific receptors are identified.

The exact nature of the surface components involved in adherence is still not known. Assuming that it is one or a combination of the surface-associated polymers known to be responsive to the environment, then lipoteichoic acids and proteins become prime candidates. Proponents of both theories have presented circumstantial evidence (20, 21; Liljemark and Bloomquist, IADR Abstr. 1979, p. 269) as well as arguments supporting their ideas, but as yet, no direct, incontrovertible evidence has been published. The results of our studies suggest that the chemostat may offer unique opportunities to gain such evidence by looking for differences in the spectrum of surface-associated polymers in cells showing significant variation in adherence resulting from only a single change in environment, i.e., growth rate, substrate, pH, etc. The identification and eventual isolation of such surface components may prove to be of importance in unraveling the mechanisms of microbial adhesion not only in dental plaque formation, but also in other infectious diseases where adherence may be the initial pathological event.

ACKNOWLEDGMENTS

We thank Virginia Palmer, Lois Argenbright, and Rosemary Ann Brown for their excellent technical assistance and Martha J. Pina for her help in typing the manuscript.

This work was supported by Public Health Service grants DE-03180 and DE-02623 (to B.R.), DE-04174 and DE-04175 (to K.W.K. and A.J.W.), and DE-05164 (to B.A.) from the National Institute of Dental Research.

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