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Comparative Studies on the Effect of Growth Conditions on Adhesion, Hydrophobicity, and Extracellular Protein Profile of *Streptococcus sanguis* G9B

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***Streptococcus sanguis* G9B was grown in continuous culture at different generation times and pH values in media containing either glucose or fructose and differing in the concentrations of Na^+ and K^+. The growth pH, carbohydrate, and cation concentration each affected the yield of organisms, their ability to adhere to saliva-coated hydroxyapatite beads, and their hydrophobicity, as measured by adhesion to hexadecane. There was no correlation between adhesion to saliva-coated hydroxyapatite beads and hydrophobicity, the values for hydrophobicity varying between 44 and 83% for organisms that adhered poorly and between 24 and 75% for those that adhered effectively. For organisms grown in batch culture at pH 6.0 or 7.0 there was similarly no correlation between adhesion and hydrophobicity. The growth conditions also had a considerable influence on the production of extracellular protein. The total amount was greater at pH 7.5 than at other pH values, and there were also differences in the individual components in response to changes in generation time, pH, carbohydrate source, and cation concentration. Two protein bands were identified, namely, glucosyltransferase and protein P1 (also called antigen B or I/II). However, there was no correlation between a particular protein component and adhesion.**

The ability of bacteria to adhere to a surface is an important ecological factor, as is well illustrated by studies on the differential localization of organisms in the oral cavity (9). Considerable attention has been given to the adhesion of organisms to the pellicle coating the tooth surface, where the use of saliva-coated hydroxyapatite beads (SHA) provides a convenient model system (1, 5). Although there are differences of opinion on methodological details and interpretation of results (38), it is generally accepted that adhesion is a consequence of a cooperative effect involving physical factors and specific surface receptors, with particular attention currently being given to the role of hydrophobicity (9, 36, 48, 49, 59) and surface proteins (9, 37).

When studies are carried out on factors that may influence the adhesion of organisms to SHA, it is usual to compare batch-grown organisms of different genotypes (10) where the extent of genetic difference with respect to relevant characteristics is generally not known. Another approach, and the one followed in our studies, is to grow an organism under different but precisely defined growth conditions in a chemostat. An organism growing in a particular environment will express one of its genetically possible phenotypes, and it is well known that surface characteristics are particularly susceptible to phenotypic change in response to changes in the environment (7, 54). For organisms growing in dental plaque, pertinent aspects for investigation are the effects of generation time, growth pH, and carbohydrate source, and studies on oral streptococci and lactobacilli have shown how a number of their surface characteristics are affected by changing these growth parameters (27, 28). Another parameter, which has only recently been considered, concerns the effect of different concentrations of sodium and potassium ions on cell physiology through their influence on the proton motive force (PMF) (7, 29). This is particularly pertinent to the situation in dental plaque because of the relatively high potassium content of plaque fluid (53).

Our current studies were aimed at extending previous work on *Streptococcus sanguis* G9B that dealt with the effect of generation time, growth pH, and carbohydrate source on adhesion to SHA (46). Those studies showed that adhesion varied considerably depending on the growth conditions, and subsequent studies on *Streptococcus mutans* Ingbritt (4) and *Streptococcus salivarius* (58) have confirmed this effect. Also, in confirmation of other studies on batch-grown organisms (1), *S. sanguis* adhered more effectively than *S. mutans* under all chemostat growth conditions, although when results for different growth conditions were directly compared (28), the relative ability of the two species to adhere differed almost eightfold.

The above-described chemostat studies employed a standard medium which contained sodium and potassium ions in the molar ratio of 1.9:1.0, and in the present study we examined the potential effect of these ions on the ability of organisms to adhere to SHA by preparing media with higher and lower ratios. We also compared the adhesion results with those for hydrophobicity, as measured by adhesion to hexadecane (47), in view of the previously mentioned interest in the relation of hydrophobicity to adhesion to SHA. Proteins also contribute to adhesion through specific lectin-ligand interactions (9, 32), and because of the known effect of growth conditions on the production of individual proteins (19, 26), we also undertook an examination of extracellular protein profiles of organisms that had been grown in standard medium or in media with altered ion concentrations. The results of these studies indicate that conditions that lead to changes in adhesion or hydrophobicity (or both) can also lead to changes in the extracellular protein profiles. Two of the protein components have been identified, namely,

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glucosyltransferase and a component that cross-reacts with antiserum to *S. mutans* protein P1 (also called antigen B or I/II) (8).

MATERIALS AND METHODS

Organism. *S. sanguis* G9B was originally isolated from dental plaque and was available from previous studies (46).

Media. The medium employed in previous studies will be denoted "standard medium" to distinguish it from other, modified media. Standard medium consisted of the low-molecular-weight fraction of Trypticase (BBL Microbiology Systems, Cockeysville, Md.)-yeast extract medium that passed through Amicon hollow fiber filter H1P10 (21). 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 (21). The concentrations of Na^+ and K^+ in this medium were 125 and 66 mM, respectively, with contributions of 91 mM Na^+ and 13 mM K^+ from the organic components and 34 mM Na^+ and 53 mM K^+ from the added salts. A medium with an increased concentration of Na^+ ions, designated Na^+ medium, was prepared by adding 80 mM Na^+ salts and 7 mM K^+ salts to the organic components to give a total concentration of 171 mM Na^+ and 20 mM K^+. Conversely, K^+ medium contained 87 mM K^+ salts and no added Na^+ salts, to give a final concentration of 91 mM Na^+ and 100 mM K^+. The molar ratios of Na^+ to K^+ for standard, Na^+, and K^+ media were therefore 1.9:1, 8.6:1, and 0.9:1, respectively, but were different during growth at constant pH due to the addition of NaOH or KOH (see below).

Growth conditions. Batch cultures were grown in a Multigen fermentor with automatic pH controller (New Brunswick Scientific Co., Inc., Edison, N.J.) and gassed with 95% N_2–5% CO_2. Cultures were monitored for alkali addition and grown to stationary phase. The alkali added was 6 M NaOH for organisms growing in standard or Na^+ medium and 6 M KOH for those in K^+ medium, which increased the final concentration of Na^+ by 230 mM and of K^+ by 280 mM.

Organisms in continuous culture were grown in a Bio-Flo chemostat of 325-ml capacity (New Brunswick Scientific Co.) as described previously (21, 46). The dilution rate (D) was maintained between 0.1 and 0.5 h^{-1}, corresponding to mean generation times between 7 and 1.4 h, respectively. The pH was maintained at the required value by adding 2 M NaOH to cultures in standard or Na^+ medium and 2 M KOH to cultures in K^+ medium. The increase in the relevant ion concentrations for cultures grown at $D = 0.1$ h^{-1} approximated 25 mM for those at pH 5.5, 70 mM at pH 6.5, and 120 mM at pH 7.5. Accordingly, the ratio of Na^+ to K^+ for Na^+ medium increased from 9.8:1 at pH 5.5 to 12.1:1 at pH 6.5 and 14.6:1 at pH 7.5. The respective values for K^+ medium were 0.73:1 (pH 5.5), 0.54:1 (pH 6.5), and 0.41:1 (pH 7.5).

Adhesion assay. Assays of the ability of labeled organisms to adhere to SHA and of organisms grown under different conditions to inhibit adhesion of standard labeled organisms were carried out as previously described (4, 46). Isotherms of adsorption were determined for organisms grown in batch culture at pH 6.0 and 7.0 in standard medium containing glucose and [*methyl*-^{3}H]thymidine. In each case the maximum value for bound cells approximated 10% of that of free cells, a value similar to that obtained previously (46). Organisms grown in continuous culture were tested for their ability to inhibit the adhesion of labeled organisms grown at pH 6.0, and those grown in batch culture were compared with labeled organisms grown at pH 7.0. In each case a constant amount of labeled organisms (8.8×10^9 cells per ml) was employed, and increasing amounts of unlabeled cells were added so that the latter represented 33, 50, 67, and 75% of the total. Each experiment was carried out in triplicate and the result was compared with those of experiments carried out at the same time with unlabeled organisms grown under the same conditions as the labeled organisms. A comparison of results confirmed the previous conclusion (4, 46) that values were reproducible, both for the triplicate samples and for experiments carried out on different days. For example, the values for four different experiments on the inhibition by 75% unlabeled organisms grown at pH 6.0 were 58 to 62%.

Hydrophobicity. To assay the comparative hydrophobicities of organisms grown under different conditions, suspensions were prepared in 0.01 M Tris hydrochloride buffer (pH 7.0) (optical density of ca. 1.0 at 600 nm with a 1-cm light path), and 1.2 ml was vortexed with 0.2 ml of hexadecane for 2 min (47). The percent reduction in the optical density (600 nm) of the aqueous phase from six samples was determined, and the mean was calculated.

Protein components of culture fluid. After centrifugation, the supernatant from each culture fluid was filtered through a 0.2-μm Millipore filter, and 100 ml of filtrate was dialyzed for 24-h periods against 4 liters of distilled water containing 0.01% sodium azide (twice), and then against 4 liters of distilled water (once). The indiffusible fraction was freeze-dried and dissolved in 1 ml of 0.22-μm-filtered distilled water. Analysis of the protein components was then carried out on 10 to 50 μl of the concentrate. The reported comparative studies were performed on the same volume of each sample (generally 40 μl).

IEF in polyacrylamide gels. Concentrated culture fluid was subjected to isoelectric focusing (IEF) on Ampholine thin-layer gels (pH 4.0 to 6.5; LKB, Bromma, Sweden) by previously described procedures (19). Protein standards with pI values of 4.15 (glucose oxidase), 4.55 (soybean trypsin inhibitor), 5.20 (β-lactoglobulin A), 5.85 (bovine carbonic anhydrase B), and 6.55 (human carbonic anhydrase B) were purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Gels were fixed, stained with 0.1% Coomassie brilliant blue R-250, and dried as previously described (19).

For the quantitative analysis of stained gels, bands were scanned at 590 nm with a Gelman ACD scanning microdensitometer (Gelman Sciences Proprietary Ltd., Sydney, Australia), and peak areas were measured by means of an attached digital integrator.

SDS-PAGE. Culture fluid samples to be examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were prepared as described previously (19) by dilution in solubilizing buffer containing 2% SDS, addition of 5% 2-mercaptoethanol, and heating to 100°C for 2 min immediately before application to the gel. PAGE was carried out essentially by the procedure of Laemmli (30, 31) as employed in previous studies (19). The separating gel contained 7.5% acrylamide (acrylamide-to-bisacrylamide ratio, 30:0.08 [wt/wt]) in 0.375 M Tris hydrochloride buffer (pH 8.6) and 0.1% SDS. The stacking gel contained 5% acrylamide in 0.1 M Tris hydrochloride buffer (pH 6.8). Samples were run at 60 V through the stacking gel and at 120 V through the separating gel with Tris-glycine-SDS buffer. Standards of known molecular mass were ferritin (220 kilodaltons [kDa]), phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), and ovalbumin (43 kDa) (all obtained from

Pharmacia Fine Chemicals). Separated proteins were fixed and stained with 0.1% Coomassie brilliant blue R-250. For the quantitative analysis of stained gels, bands were scanned at 590 nm with a Gelman ACD scanning microdensitometer as described above.

Glucosyltransferases. After SDS-PAGE, unfixed gels were incubated in the presence of 1% Triton X-100 with sucrose and raffinose (50). Fructosyltransferase will form polymers from either carbohydrate, but glucosyltransferases will form polymers only from sucrose. After the gels had been fixed in 25% isopropanol–10% (vol/vol) acetic acid overnight and then washed in 10% acetic acid, polysaccharide was detected by the periodic acid-Schiff reaction as previously described (19).

Electroblotting. The electroblotting procedure used here (3) has been applied to the detection of proteins in the culture fluid that react with antiserum prepared by injecting rabbits with protein P1 from *S. mutans* Ingbritt (8). After SDS-PAGE, proteins were transferred to nitrocellulose with a Hoefer Transphor model TE50 (Hoefer Scientific Instruments, San Francisco, Calif.). The blot was subjected to a preliminary incubation with 5% bovine serum albumin in 0.85% NaCl–50 mM Tris hydrochloride buffer (pH 8.8) containing 100-fold-diluted preimmune serum. The blot was then incubated with the same buffer containing 500-fold-diluted immunoglobulin G fraction prepared from P1 antiserum (kindly provided by H. Forester). The two-step reaction and the use of the immunoglobulin G fraction increased the specificity of the reaction and minimized the potential for nonspecific binding. The next stage of the reaction employed alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Calbiochem-Behring Australia Proprietary Ltd.) followed by Fast Red TR Salt (no. F1500) (Sigma Chemical Co., St. Louis, Mo.) (R. R. B. Russell and H. Forester, unpublished procedure).

Detection of LTA. The relative amounts of lipoteichoic acid (LTA) in certain culture fluids were estimated by rocket immunoelectrophoresis with antiserum prepared against *Lactobacillus casei* LTA (22). This antiserum reacts with the common polyglycerophosphate backbone.

RESULTS

Yield of strain G9B. Organisms were grown at $D = 0.1\ h^{-1}$ in continuous culture in standard, Na^+ or K^+ medium with either limiting glucose or limiting fructose and at pH 5.5, 6.5, or 7.5. A comparison of the yields of organisms (Table 1) shows that in all but one instance (K^+ medium, pH 6.5), the yield in glucose was greater than in fructose, the respective median values being 1.18 and 0.80 mg/ml. A similar effect was obtained when organisms were grown in standard medium at constant pH (6.0) and at D between 0.1 and 0.5 h^{-1}; the respective median values for glucose- and fructose-grown organisms were 1.11 and 0.85 mg/ml.

TABLE 1. Yields of strain G9B grown in continuous culture at $D = 0.1\ h^{-1}$ in different media at different pH values

Medium	Carbohydrate	Yield (mg/ml) at pH:		
		5.5	6.5	7.5
Standard	Glucose	0.92	1.36	1.31
	Fructose	0.75	1.13	1.06
Na^+	Glucose	0.74	1.18	1.08
	Fructose	0.67	1.05	0.61
K^+	Glucose	0.73	1.20	1.32
	Fructose	0.54	1.24	0.80

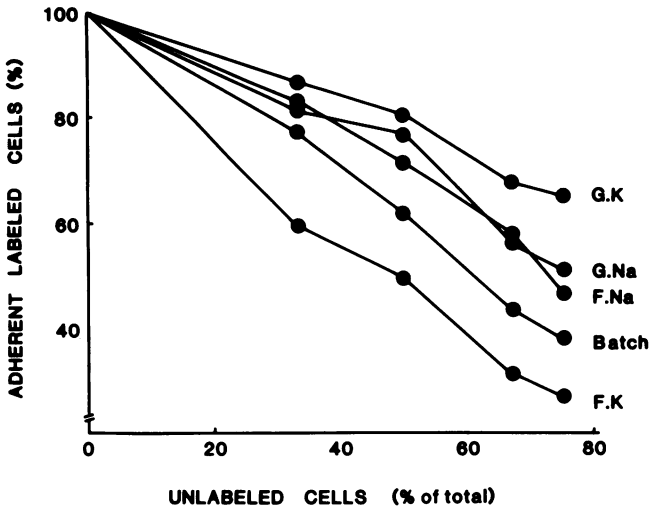

FIG. 1. Inhibition of adhesion to SHA of [^{3}H]thymidine-labeled batch-grown cells grown at pH 6.0 in glucose-containing standard medium by increasing proportions of homologous cells (Batch) and chemostat-grown organisms. The latter were grown at pH 5.5 with limiting glucose (G) or fructose (F) in K^+ medium (K) or Na^+ medium (Na).

The results in Table 1 also provide evidence that a change from Na^+ to K^+ medium could influence the yield of cells, particularly at pH 6.5 and 7.5 where higher values were obtained in K^+ medium irrespective of the carbohydrate source.

Adhesion and hydrophobicity of strain G9B grown in continuous culture in Na^+ and K^+ media. In previous studies on strain G9B grown in standard medium (46) it was shown that carbohydrate source, D, and particularly growth pH can affect the ability of organisms to adhere to SHA. To confirm and extend these results, organisms were grown at $D = 0.1\ h^{-1}$ in Na^+ and K^+ media containing limiting glucose or fructose at pH 5.5, 6.5, and 7.5.

The adsorption isotherm was determined for [^{3}H]thymidine-labeled organisms grown in batch culture at pH 6.0 in glucose-containing standard medium, and the ability of increasing proportions of chemostat-grown organisms to inhibit the adhesion of these labeled organisms was then determined. Typical results are shown in Fig. 1, which compares the values for homologous unlabeled, batch-grown cells with those obtained for organisms grown at $D = 0.1\ h^{-1}$ and pH 5.5 in glucose or fructose and in either Na^+ or K^+ medium. Table 2 lists the values obtained with all of the chemostat-grown cultures, expressed as percent inhibition of adhesion of labeled organisms in the presence of 75% unlabeled organisms. Inhibition varied between 35 and 73%, compared with 58 to 62% for four different experiments with homologous unlabeled, batch-grown organisms. Confirming previous studies (4, 46), organisms grown in batch culture without pH control were less effective, giving a value of 48%.

Because of the potential effect of chain length on the results of adhesion experiments, the observations recorded for each chemostat run were examined. In each case there was a predominance of chains of two to six cells, and there

TABLE 2. Properties of *S. sanguis* G9B grown at $D = 0.1\ h^{-1}$ in Na^+ or K^+ medium at different pH values with either glucose or fructose

pH	Carbohydrate	Medium	Inhibition of adhesion[a] (%)	Hydrophobicity (%)
7.5	Glucose	Na^+	36	83
		K^+	35	58
	Fructose	Na^+	69	48
		K^+	66	24
6.5	Glucose	Na^+	51	56
		K^+	32	44
	Fructose	Na^+	59	40
		K^+	69	66
5.5	Glucose	Na^+	49	73
		K^+	35	67
	Fructose	Na^+	53	67
		K^+	73	75

[a] By 75% unlabeled cells.

was no relation between minor variations in the microscopic appearance and the values for adhesion.

Organisms were also examined for their hydrophobicity, and the results for the chemostat-grown cultures are included in Table 2. There is no apparent correlation between the degree of hydrophobicity and the adhesion results. Thus, rearrangement of the results into three groups of four cultures shows that hydrophobicity values varied between 44 and 83% for organisms inhibiting adhesion by 32 to 36%, between 40 and 73% for organisms inhibiting by 49 to 59%, and between 24 and 75% for organisms inhibiting by 66 to 73%. It is also significant that the organisms grown in batch culture without pH control gave a much higher hydrophobicity value (>95%) than the organisms grown at pH 6.0 (47%), yet were less effective in inhibiting adhesion.

Adhesion and hydrophobicity of strain G9B in batch culture at pH 7.0. Comparative studies were also carried out on cultures grown to stationary phase at pH 7.0 in standard, Na^+, or K^+ medium containing 2% glucose or fructose. Organisms were examined for their ability to inhibit the adhesion of [^{3}H]thymidine-labeled organisms grown in standard medium at the same pH. The values obtained with 75% unlabeled organisms show that the growth conditions had a minimal effect on the results (Table 3). However, values for hydrophobicity (Table 3) range from 40 to 82% and bear no relation to the ability of organisms to inhibit adhesion.

Extracellular protein profiles of cultures grown in continuous culture in standard medium. Culture fluids from strain G9B grown in standard medium containing limiting glucose or fructose were examined for their protein components by

TABLE 3. Properties of batch-grown cultures of *S. sanguis* G9B grown at pH 7.0 in different media

Carbohydrate	Medium	Inhibition of adhesion[a] (%)	Hydrophobicity (%)
Glucose	Standard	82	60
	Na^+	72	53
	K^+	78	40
Fructose	Na^+	75	48
	K^+	67	82

[a] By 75% unlabeled cells.

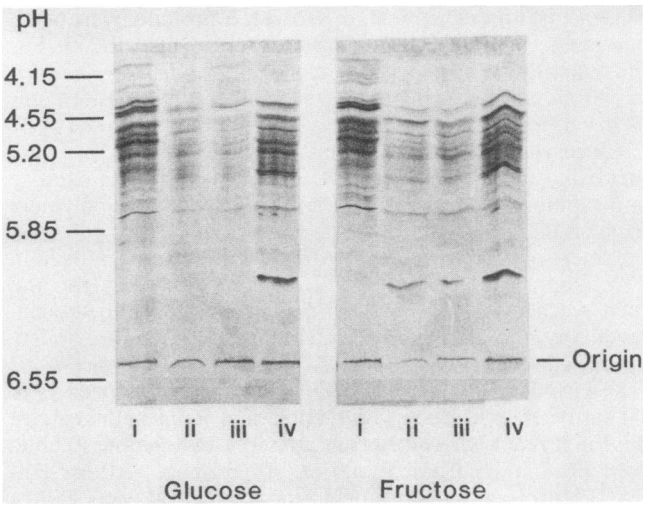

FIG. 2. IEF of extracellular proteins of organisms grown in continuous culture in limiting glucose or fructose either at a constant pH (6.0) and $D = 0.1\ h^{-1}$ (lanes i) or $0.5\ h^{-1}$ (lanes ii) or at a constant D ($0.1\ h^{-1}$) and pH 5.5 (lanes iii) or 7.5 (lanes iv). The migration distances of proteins of known pI values are given.

IEF and SDS-PAGE. The differences are illustrated in Fig. 2 (IEF) and 3 (SDS-PAGE), which compare culture fluids from organisms grown either at a constant pH (6.0) and $D = 0.1\ h^{-1}$ (lanes i) or $0.5\ h^{-1}$ (lanes ii), or at a constant D ($0.1\ h^{-1}$) and pH 5.5 (lanes iii) or 7.5 (lanes iv). The first point to be noted is that there are differences in the total amounts of protein in the culture fluids, with greater amounts being present at the lower dilution rate (lanes i in Fig. 2 and 3) and at the higher pH (lanes iv in Fig. 2 and 3). The differences in the gels shown in Fig. 2 were quantitated by scanning microdensitometry. After allowing for differences in the yields of organisms, it was calculated that the total amounts

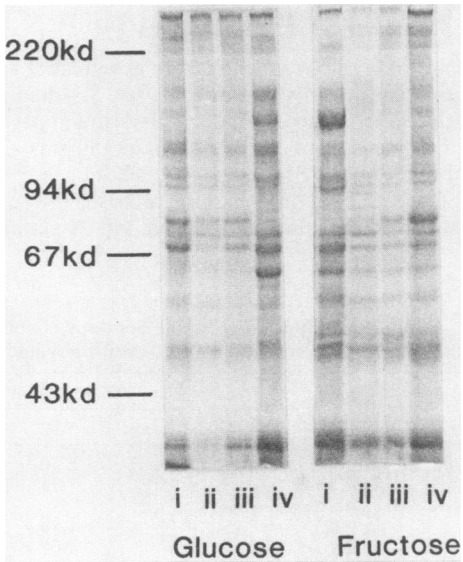

FIG. 3. SDS-PAGE of extracellular proteins of organisms grown under the same conditions as in Fig. 2. The migration distances of proteins of known molecular mass are given on the left.

of protein for those grown at pH 6.0 and $D = 0.1\ h^{-1}$ in glucose and fructose were, respectively, 2.4 and 1.6 times the amounts present at $D = 0.5\ h^{-1}$. A similar comparison of organisms grown at $D = 0.1\ h^{-1}$ and different pH values indicated that the amounts present at pH 7.5 in glucose and fructose were, respectively, 1.9 and 2.6 times the amounts at pH 5.5.

The results of IEF (Fig. 2) show numerous protein components with pI 4.1 to 5.7. A comparison of lanes i and iv for glucose- and fructose-grown organisms, where the total amounts are similar, shows differences in the relative amounts of several components; for example, there are differences in the region of the standard protein of pI 4.55 and in the region estimated to be pI 5.4. However, the component that is well differentiated from the others is that with pI of ca. 6.0, which was present when organisms were grown at $D = 0.1\ h^{-1}$ and pH 7.5 (lane iv) in glucose or fructose but was not detectable in the culture fluid of organisms grown at pH 6.0 (lane i) in glucose or fructose. This component was also present in the other two fructose-grown cultures (lanes ii and iii), which contained less protein in general, but was not detectable in the comparable glucose-grown cultures.

A similar comparison of the results of SDS-PAGE (Fig. 3) also shows that there are differences in the protein profiles for culture fluids where the total amounts are similar. This is more obvious in lanes i and iv for glucose- and fructose-grown organisms, where differences in the protein profiles for the four culture fluids are particularly evident with respect to protein components of approximately 50 kDa and in two regions, 60 to 80 kDa and 130 to 155 kDa, where glucosyltransferase activity was subsequently detected (see below). There are also components outside this range, and those at the upper end (≥185 kDa) include proteins reacting with antiserum to *S. mutans* protein P1 (see below).

Extracellular protein profiles of cultures grown in continuous culture in Na^+ and K^+ media. Culture fluids from organisms grown in Na^+ and K^+ media were examined by SDS-PAGE, and differences were quantitated by scanning microdensitometry. At pH 5.5 or 6.5 the change from Na^+ to K^+ medium had a minimal effect, but there were significant changes at pH 7.5, as shown by the results for glucose-grown (Fig. 4a) and fructose-grown (Fig. 4b) organisms. For glucose-grown organisms (Fig. 4a) there are major differences in the 130- to 155-kDa region, where glucosyltransferase activity was subsequently detected (see below). For fructose-grown organisms (Fig. 4b) the differences were primarily in the greater amounts of components of <90 kDa that were produced by organisms in K^+ medium.

Growth at pH 7.5 in the K^+ medium also resulted in the excretion by organisms of greater amounts of LTA than in Na^+ medium. As determined by rocket immunoelectrophoresis the results, expressed as centimeters per 50 μg of cells, were 0.98 and 0.65, respectively, for glucose-grown cultures and 1.48 and 1.04, respectively, for fructose-grown cultures.

Identification of protein components. The protein components of representative culture fluids were separated by SDS-PAGE, and the gels were then tested for glucosyltransferase activity. All samples gave a reaction with sucrose but not with raffinose, which is consistent with the presence of glucosyltransferase. Polysaccharide was associated with proteins in the 130- to 155-kDa region and, to a much lesser extent, with proteins in the 70- to 80-kDa region. This is illustrated by the results for culture fluid from organisms grown in standard medium with limiting glucose at $D = 0.1\ h^{-1}$ and pH 7.5 (Fig. 5A).

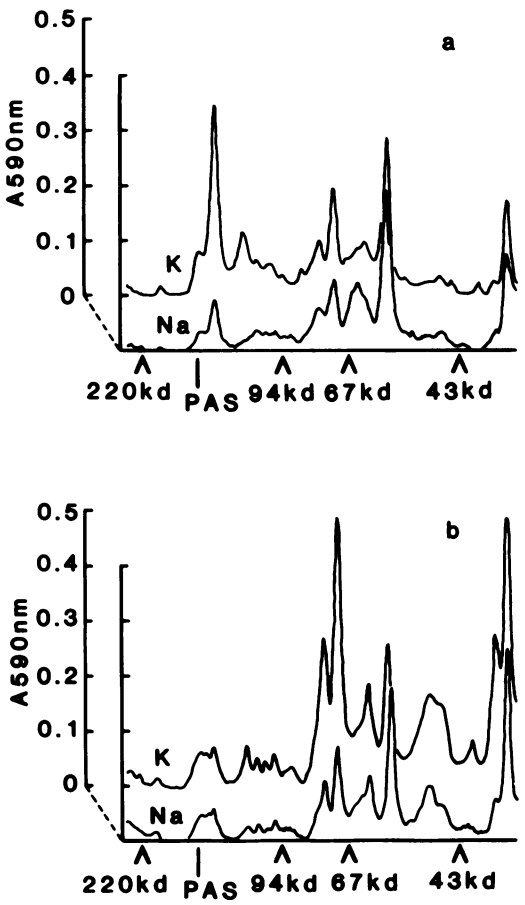

FIG. 4. Microdensitometry scans after SDS-PAGE of the extracellular protein components produced by organisms grown in continuous culture ($D = 0.1\ h^{-1}$) at pH 7.5 with limiting glucose (a) or fructose (b). In each case results are given for organisms grown in K^+ and Na^+ media. Molecular masses are given for protein standards, and the position of major band giving a reaction with periodic acid-Schiff reagent (PAS) is shown. A590nm, Absorbance at 590 nm.

Culture fluids were also tested for the presence of protein components reacting with the immunoglobulin G fraction of rabbit antiserum prepared against protein P1 from *S. mutans* Ingbritt. The protein components in duplicate samples of culture fluid were separated by SDS-PAGE, and one sample was subjected to electroblotting and then examined for specific reaction with antibody. The results in Fig. 5B were obtained with organisms grown in standard medium with limiting glucose at $D = 0.2\ h^{-1}$ and pH 6.0 and compare SDS-PAGE (lane i) with immunoblotting (lane ii). The latter result shows that reactivity is primarily associated with a component of 185 kDa, with a significant reaction by proteins of higher (220 kDa) and lower molecular mass. The P1 protein from *S. mutans* Ingbritt also has a molecular mass of 185 kDa and is readily degraded to lower-molecular-mass components (8).

DISCUSSION

The current studies confirm and extend the previous work on oral streptococci which indicated that changes in generation time, pH, and carbohydrate source can cause major changes in an organism's surface characteristics and the

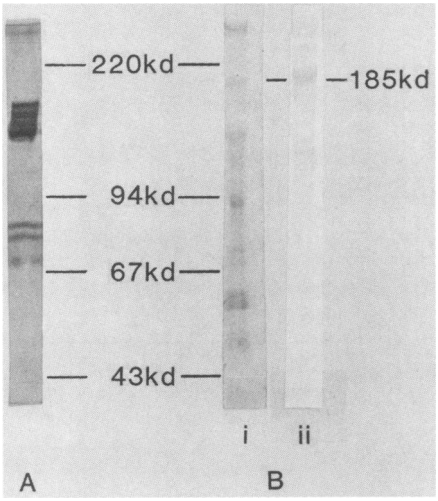

FIG. 5. Examination of proteins separated by SDS-PAGE for specific reactivities. (A) Culture fluid from organisms grown in standard medium with limiting glucose at $D = 0.1\ h^{-1}$ and pH 7.5 was tested for glucosyltransferase reactivity and shows a major and several minor reactive components. (B) Culture fluid from organisms grown in standard medium with limiting glucose at $D = 0.2\ h^{-1}$ and pH 6.0 was tested for component reactivity with antiserum to *S. mutans* protein P1 after electroblotting. Lane i shows the control gel stained with Coomassie brilliant blue, and lane ii shows that there is a major serologically reactive component of 185 kDa and several minor lower-molecular-mass components.

production of extracellular proteins and LTA (4, 19, 21, 22, 26, 46, 61).

Growth of oral organisms in a chemostat has proved to be a valuable tool for obtaining information that enables a prediction of their potential response to the environmental changes that can occur in dental plaque (16, 27, 55). An important feature is that calculations of the rates of growth of plaque organisms indicate that they grow at rates much slower than can be achieved under normal batch growth conditions (17) but which can be attained in the chemostat. The range of pH values examined, 5.5 to 7.5, is also relevant to oral ecology, and growth with limiting carbohydrate is pertinent because evidence indicates that plaque organisms are generally limited in their growth rate by the availability of carbohydrate (16, 56).

The additional parameter examined in the current study concerned the effect of Na^+ and K^+ ions. The medium designated K^+ medium contained 100 mM K^+, but this concentration was increased to 125 to 220 mM depending on the amount of KOH added to maintain the required pH. Similarly for Na^+ medium, which contained 171 mM Na^+, the final concentration was 196 to 291 mM depending on the final pH. To relate these values to those in dental plaque, it is necessary to know the concentration of the ions in plaque fluid. Published values (53), namely, 35.1 ± 9 mM Na^+ and 61.5 ± 13.5 mM K^+, understate the potential range, as each sample analyzed was a pool of 10 to 40 individual samples in order to obtain a sufficient volume of plaque fluid. Recent analyses (G. M. Mears and K. W. Knox, unpublished data) on aqueous extracts of two plaque samples from 32 individuals provided the basis for calculations of the probable ion concentrations in the respective plaque fluids and indicated that in several instances the concentration of each cation was two to three times the reported average values. Such plaque fluid values for K^+ (120 to 180 mM) would generally fall within the range for K^+ medium (125 to 220 mM), but those for Na^+ (70 to 105 mM) would be below those for Na^+ medium (196 to 291 mM).

The work of Ellwood and colleagues has highlighted the potential ecological significance of a high K^+ ion concentration through the enhancement of synthesis of glucosyltransferases (23, 34) and rates of acid production (34, 35); conversely, high Na^+ concentrations inhibit enzyme synthesis (24) and acid production (35). This differential effect is explained on the dual basis that PMF plays an important role in membrane activities, including sugar uptake (16, 25, 29), and that the expulsion of Na^+ ions dissipates PMF (7, 29). The mechanism by which PMF is coupled to protein export remains to be clarified, but an important indicator is the recent observation that enhancement of glucosyltransferase secretion by *S. salivarius* in response to increased K^+ concentration is accompanied by an increase in the proportion of octadecenoic acid in membrane lipids essentially at the expense of octadecanoic acid (33).

The influence of environmental factors on microbial activity is clearly indicated by the differences in cell yields. Previous studies showed that a change of carbohydrate source from glucose to fructose influenced the yields of *S. mutans* Ingbritt (19), and the lower yields of fructose-grown *S. sanguis* under all but one growth condition confirm this effect. While in each case the culture was carbohydrate limited, it has been pointed out that such cultures may be either carbon or energy limited, and yields of organisms are less in the latter case (54).

A change from K^+ to Na^+ medium also frequently results in a reduction of cell yield, with the greatest effect being obtained at pH 7.5. At this pH there is also the greatest difference in the relative concentrations of Na^+ and K^+ in the two media because of the high amount of the relevant cation supplied through the addition of NaOH or KOH. The results may therefore reflect the previously discussed contribution of PMF to cellular activity and the effect of high concentrations of Na^+ on PMF. The addition of alkali to maintain a constant pH is an essential feature of chemostat studies, and the effects achieved at different pH values could be at least partly due to differences in the concentrations of Na^+ and K^+.

The yields of cells obtained under different growth conditions can be expressed as Y_s values, or grams of cells synthesized per mole of carbon substrate assimilated. The range of values obtained in the current study is illustrated by those for organisms growing at pH 7.5, namely, 47.5 and 38.9 for organisms in glucose-containing K^+ and Na^+ media, respectively, and 28.8 and 22.0 for the corresponding fructose-grown cultures. Reported Y_s values for glucose-grown oral streptococci vary from 30 to 50, compared with theoretical values of 19 to 25 (25). The current results would indicate that variations in the concentrations of Na^+ and K^+ in different media could partially account for the observed differences.

Adhesion is a very important factor in plaque formation, and most studies employ SHA as their model system. Both low- and high-affinity sites have been described (9, 11, 12, 14, 40, 42), and a distinction has been drawn (12) between specific neuraminidase-sensitive receptors (10, 32) that are mainly responsible for high-affinity binding sites and hydrophobic interactions associated with the total number of sites. Although results are open to different interpretations (38), it seems appropriate to use high cell concentrations ($\geq 10^9$/ml) when comparing adhesion and hydrophobicity.

The current studies extend the previous observations on

the effect of growth rate, pH, and carbohydrate source on the adhesion to SHA of *S. sanguis* (46) and *S. mutans* (4). The variability in hydrophobicity under different growth conditions has also been observed with chemostat-grown cultures of *S. mutans* and *S. milleri* (43) and with batch-grown cultures of several organisms (47). A comparison of the current results for adhesion and hydrophobicity fails to provide evidence for a direct correlation. Thus, organisms that adhered poorly (32 to 36% inhibition) gave values for hydrophobicity between 44 and 75%, and organisms that adhered very well (66 to 73% inhibition) gave values from 24 to 75%. For batch cultures grown at a constant pH of 7.0, where all organisms adhered very well (67 to 82% inhibition), the values for hydrophobicity also covered a wide range, namely, 40 to 82%. Of equal significance is the observation that organisms grown under the usual batch culture conditions without pH control were considerably more hydrophobic than those grown with pH control, whereas there were only minor differences in adhesion. This situation could have influenced the results in other studies in which comparisons between adhesion and hydrophobicity have been based on organisms grown in batch culture without pH control. For example, the majority of fresh isolates from dental plaque were classified as hydrophobic on the basis of values of >75% (59), and values for different *S. sanguis* strains were reported as >80% (10, 60). The latter studies also showed that strains of similar hydrophobicity could differ twofold in their adhesion to SHA (10, 60).

The concomitant loss of hydrophobicity and adhesion to SHA on treating *S. sanguis* with hydrophobic bond-disrupting agents points to a role for hydrophobicity in adhesion (41), but there remain the questions of the significance of the absolute level and varying degrees of hydrophobicity and whether current methods in fact provide a quantitative measure of hydrophobicity. Further, as others have also pointed out (10, 12, 41, 42), adhesion would depend on a cooperative effect of hydrophobic bonding and complementary macromolecules, and one parameter cannot be taken in isolation. The observed lack of correlation between adhesion and hydrophobicity is therefore not unexpected. The need to consider other parameters is well illustrated by studies on a mutant of *S. sanguis* in which loss of ability to adhere was associated with the loss of fimbriae, the concomitant loss of hydrophobicity, and the suspected loss of proteins involved in adhesion that are believed to be associated with the fimbriae (13). Other studies on *S. sanguis* have also invoked a role for LTA associated with surface "fibrils" in the interaction of this organism with salivary glycoproteins (20).

Cell surface proteins and LTA have the potential to contribute to adhesion through their relative nonspecific properties of hydrophobicity and charge. The contribution of charge (44) now receives less attention, with the greater emphasis being placed on hydrophobicity. However, a strong indication that both parameters should be considered is given by a recent study on two mutants of *S. sanguis* in which values for both the net surface charge and hydrophobicity were different from those for the respective parent strains (58). The negative charge on the bacterial cell surface would be contributed to by both LTA and the individual proteins. Published values for isolated streptococcal surface proteins mostly fall within the range of pI 3.7 to 5.0 (58), and the current studies on extracellular proteins show that the majority have pI values between 4.5 and 5.5.

A contribution of LTA to surface hydrophobicity is indicated by studies on group A streptococci (39). Such a role is not negated by a lack of correlation between hydrophobicity and total cellular LTA (37), as only the fraction on the surface would be contributing. Evidence for surface proteins contributing to hydrophobicity is also indirect but is supported by reports that the loss of M protein by group A streptococci (57) and of surface proteins by *S. sanguis* mutants coincides with decreased hydrophobicity (58). Hydrophilic strains of *S. sanguis* release a number of proteins that are not detectable in the culture fluids of the hydrophobic strains (36), and a correlation between loss of hydrophobicity and protein secretion has also been observed with *S. mutans* strains (36; K. W. Knox, L. N. Hardy, L. J. Markevics, and A. J. Wicken, manuscript in preparation).

While the surface proteins would, therefore, contribute to the overall charge and hydrophobicity of the cell surface, the known specificity of adhesion would reside in their ability to provide specific lectin-like interaction with components of plaque (9). Individual reactive proteins have been isolated from *S. sanguis* (9, 32), but, despite considerable recent progress (2), difficulties remain in identifying and quantitating the range of proteins exposed on the cell surface. As extracellular proteins would be transient surface components and can be more readily estimated, it is pertinent to regard variations in the amounts of individual extracellular proteins under different growth conditions as an indicator of the extent of variation that could be occurring with surface proteins.

The current studies have shown that growth conditions can have a profound effect on the total amount of extracellular protein and the relative amount of individual components. The effects of generation time (*D*), pH, and carbohydrate source have been observed in other studies on *S. mutans* Ingbritt serotype *c* (19) and related organisms from serotypes *a* through *g* (L. N. Hardy, K. W. Knox, A. J. Wicken, and D. J. Fitzgerald, manuscript in preparation). The additional parameter studies on this occasion, namely, the effect of Na^+ and K^+ ions, have also proved to be of major importance.

The differences observed under different growth conditions are an expression of the organism's phenotypic response to changes in the environment, although the reasons are more difficult to define. As shown by the results in Fig. 4, the production of individual proteins is generally depressed in Na^+ medium, and this could be accounted for in terms of the previously discussed role of PMF in cellular activities. The effects of growth pH could also be influenced by the previously mentioned differences in cation concentrations, coupled with potential effects on the internal pH of the organisms and the pH difference across the cell membrane, which in turn affects PMF. The effects of carbohydrate source and generation time are probably secondary to other fundamental changes which are not yet understood. The complexity of the situation with regard to generation time was shown in a recent review (18), which concluded that it is rare for there to be no effect of generation time on enzyme synthesis. Most frequently the rate of synthesis increases as the generation time increases from, say, 1 to 14 h or is maximal at intermediate values; less frequently there is an increase in activity as generation time decreases, and only rarely is activity minimal at intermediate values.

A comparison of the extracellular protein profiles with the results for adhesion of organisms grown in standard medium (46) or the current modified media provides no correlation between changes in a specific protein component and surface properties. Nevertheless, the changes with respect to spe-

cific components distinguished on the basis of their molecular weights or, to a lesser extent, their pIs are potentially of ecological significance in view of other studies on proteins with comparable physical and also biological properties. These are the proteins with molecular masses as estimated by SDS-PAGE of 185, 130 to 155, and 60 to 80 kDa and pIs of 4.5 and 5.4.

The 185-kDa protein is probably identical to that of pI 5.4 on the basis of the known properties of such a protein isolated from *S. mutans* Ingbritt and designated P1 (8). Further, the *S. sanguis* component cross-reacts with antiserum to *S. mutans* P1. The latter protein is identical to the previously described antigens B and I/II (8), and the results confirm previous serological studies on the occurrence of antigen B in *S. sanguis* (51). The interest in this antigen derives from its potential use in a caries vaccine (summarized in reference 8), and the view that it could play a role in adhesion is supported by two recent studies on *S. mutans* which showed that the amounts in different strains paralleled differences in adhesion (36) and that specific antibody partially inhibited adhesion (6).

Protein components in the region of 130 to 155 kDa show glucosyltransferase activity, as do components of lower molecular mass. G9B is a serotype 1 strain, and a higher molecular mass of 174 kDa has been reported for the enzyme from a serotype 2 strain (15). That study also showed that the enzyme is readily degraded to a component of lower molecular mass, and a comparable situation could account for the results in the current study. Variations in glucosyltransferase activity would certainly influence plaque accumulation but would not be expected to influence adhesion as measured in this study.

Loss of proteins of 120 kDa and lower (92 and 86 kDa) molecular masses has been associated with loss of adhesion (45). However, emphasis has been given to the component of 86 kDa (45) because of its possible relation to an adhesion of 70 to 90 kDa that was isolated from *S. sanguis* (22). As this component also has a pI of ca. 4.5, the variability in the production of the components of 60 to 80 kDa and pI ca. 4.5 may reflect, at least in part, differences in the amount of the same component. Components in the same molecular weight range have also been identified on the surface of *S. sanguis* G9B (2) and in the culture fluid of other strains (36). More specifically, there are reports that indicate the frequent occurrence of an 86-kDa surface-associated protein (2), another protein of 80 kDa (52), and an extracellular component of 60 kDa (52). While such comparisons of physical properties do have value, this is limited in the absence of more specific evidence, such as the serological tests on the 185-kDa component (8).

The variability in the production of individual protein components under different growth conditions points to an important effect of the environment on the bacterial cell surface, which is also reflected in the differences in adhesion and hydrophobicity. The conditions of growth are therefore a crucial factor in defining surface properties and extrapolating the results to the circumstances in vivo.

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