Comparison of Extracellular Protein Profiles of Seven Serotypes of Mutans Streptococci Grown under Controlled Conditions

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Extracellular proteins produced by the four human commensal species of mutans streptococci were analysed. The organisms used were *Streptococcus mutans*, serotypes *c*, *e* and *f*, *Streptococcus cricetus*, serotype *a*, *Streptococcus rattus*, serotype *b*, and *Streptococcus sobrinus*, serotypes *d* and *g*. They were grown in continuous culture at different generation times and pH values in media containing either glucose or fructose to determine the extent of variation in extracellular protein production that could occur for an individual strain. The results for different organisms grown under the same conditions were then compared. The total amount of protein of molecular mass ≥ 60 kDa varied considerably with the growth conditions and with the strain. Generally more protein was present at a higher pH, conditions under which the organisms also form more lipoteichoic acid. With respect to individual protein components SDS-PAGE proved better than isoelectric focusing for detecting phenotypic responses by a particular strain to environmental changes and differences between the different strains. Differences in the molecular masses of protein components were particularly pronounced in the regions designated P1 (185–200 kDa), P2 (130–155 kDa) and P3 (60–95 kDa). Every strain produced at least one component in the P1 region that cross-reacted with antiserum to the purified protein from *S. mutans* serotype *c*, a protein which is indistinguishable from antigens B and I/II. Two components in the P2 region were dominant in the case of *S. cricetus* and *S. sobrinus* strains and showed glucosyltransferase (GTF) activity. GTF activity was also detected in the P3 region, particularly with *S. mutans* strains.

INTRODUCTION

The oral streptococci have been subjected to intensive study in the context of physiological characteristics potentially associated with their cariogenicity and the need to characterize strains for taxonomic purposes. Those streptococci originally classified as *Streptococcus mutans* have received particular attention, and the early evidence of genetic diversity based on serological (Bratthall, 1970) and other (Coykendall, 1971) parameters has led to their reclassification into five species (Coykendall, 1983; Schleifer *et al.*, 1984). The four species that are human commensals are *S. mutans*, comprising serotype *c*, *e* and *f* strains, *S. cricetus* (serotype *a*), *S. rattus* (serotype *b*), and *S. sobrinus* (serotypes *d* and *g*), all of which can be grouped under the collective name of mutans streptococci (Fitzgerald *et al.*, 1983; Coykendall, 1983).

The potential value of cellular protein profiles as a parameter for distinguishing between representative strains of mutans streptococci was evident from early work (Coykendall, 1971;

Abbreviations: D, dilution rate; FTF, fructosyltransferase; GTF, glucosyltransferase; IEF isoelectric focusing; LTA, lipoteichoic acid; RIE, rocket immunoelectrophoresis; SM units, arbitrary scanning microdensitometry units.

Simonson & Shklair, 1972), and has been borne out by subsequent studies on the protein components of individual strains (Hamada & Mizuno, 1974; Ellwood *et al.*, 1976; Russell, R. R. B., 1979*a*). More importantly, differences between the above four species were evident when the protein components of whole cells were examined by SDS-PAGE (Russell, R. R. B., 1976; Whiley *et al.*, 1982). The study of Whiley *et al.* (1982) was particularly valuable as computer analysis of scanned gels led to the construction of average linkage dendrograms, which reinforced the distinction between these and other species. The four species of human commensal mutans streptococci can also be distinguished on the basis of their extracellular glycosyltransferases separated by isoelectric focusing (IEF) (Tsumori *et al.*, 1983).

Comparison between the results of the different investigations is hindered by the different techniques that have been used, e.g. organisms were grown in different complex media for different periods of time and without pH control. Also, the cell fraction used for analysis has varied from the whole contents of disrupted cells (Simonson & Shklair, 1972), to the membrane fraction (Coykendall *et al.*, 1971), to SDS extracts of whole cells (Russell, R. R. B., 1979*a*) or cell wall preparations (Ellwood *et al.*, 1976). Except for the study on glycosyltransferases by Tsumori *et al.* (1983), and another earlier one on the use of IEF for distinguishing the protein profiles of serotypes *a*, *b* and *c* strains (Hamada & Mizuno, 1974), SDS-PAGE has been the procedure of choice. Up to 40 components were revealed in the above studies when components separated by SDS-PAGE were stained with a dye, but further identification of components on the basis of their molecular mass is generally not possible, as protein standards were not included.

The present study was aimed at complementing and extending the previous work on strains representative of the four species and the seven serotypes by using both SDS-PAGE and IEF and by including protein standards. Further, the examination was concerned with those proteins preferentially released into the culture fluid, which was expected to reveal fewer components. Growth conditions were also controlled because of the profound effects induced by relatively minor differences in the environment on the production of surface and extracellular components by oral streptococci (Knox & Wicken, 1984, 1985). To achieve these requirements, organisms were grown in continuous culture under different conditions to determine the extent of variability of extracellular protein production for each strain, and then the results from different strains for a particular growth condition were compared.

The proteins that exhibited GTF activity and those that reacted with antiserum prepared against protein P1 from strain Ingbritt were identified. P1 has been shown to be indistinguishable from the previously described antigens B and I/II (Forester *et al.*, 1983). Comparative studies were also made of the effect of growth conditions on the production of lipoteichoic acid (LTA) as previous studies on *S. rattus* strain BHT (Jacques *et al.*, 1979*b*) and *S. mutans* strain Ingbritt (Jacques *et al.*, 1979*a*; Hardy *et al.*, 1981) showed that the amounts of both cellular and extracellular LTA could differ several-fold in response to changes in growth conditions.

METHODS

Organisms. The strains chosen for study were representative of the different species and serotypes, and were obtained from the following sources: *S. mutans* strains Ingbritt, serotype *c* (B. Krasse, University of Göteborg, Fack, Sweden), LM7, serotype *e* (L. A. Thomson, NIDR, Bethesda, Md, USA) and OMZ175, serotype *f* (D. Bratthall, University of Göteborg); *S. cricetus* strain AHT, serotype *a* (R. J. Fitzgerald), *S. rattus* strain BHT, serotype *b*; *S. sobrinus* strains B13, serotype *d* (S. Edwardsson, University of Lund, Malmö, Sweden) and 6715, serotype *g* (R. J. Fitzgerald).

The culture of strain Ingbritt, which has also been used in several previous studies (Jacques *et al.*, 1979*a*; Hardy *et al.*, 1981; Knox *et al.*, 1982; Forester *et al.*, 1983), was obtained in 1971 and is now designated Ingbritt 162 to distinguish it from a culture obtained from Professor Krasse in 1973, which is designated Ingbritt 175 and is more hydrophobic (unpublished data). Independent confirmation of the serotyping of the strains was obtained in other studies with Professor D. Bratthall for those of serotypes *a*, *b*, *d*, *e* and *f* (Knox *et al.*, 1983), and in our laboratory for those of serotypes *c* and *g* (unpublished data).

Medium. The medium consisted of the low *M*₁ fraction of trypticase (BBL Microbiology Systems)/yeast extract medium that passed through an Amicon hollow fibre filter H1P10; glucose or fructose was added to a final concentration of 0.5%, and the medium was then sterilized by membrane filtration (Jacques *et al.*, 1979*a*).

Growth conditions. Organisms were grown in continuous culture in a Bio-Flo chemostat of 325 ml capacity (New Brunswick Scientific) as described by Jacques *et al.* (1979*a, b*). Experiments were done at either constant pH (6.0) and $D = 0.1\text{--}0.5\text{ h}^{-1}$, or constant D (0.1 h^{-1}) and pH 5.5, 6.5 or 7.5.

Protein components of culture fluid. The supernatant fluid from each culture fluid was filtered through a $0.22\text{ }\mu\text{m}$ Millipore filter and 100 ml of the filtrate dialysed for 24 h periods against 4 l distilled water containing 0.01% sodium azide (twice), followed by 4 l distilled water (once). The indiffusible fraction was freeze-dried, redissolved in 1 ml distilled water, filtered as described above, and 10–50 μl samples were used for analysis of the protein components. For comparison of different samples the same volume was used (generally 40 μl).

IEF. Concentrated culture fluid was subjected to IEF on Ampholine thin-layer gels (pH range 4.0–6.5; LKB) as described by Hardy *et al.* (1981). 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. Gels were fixed, stained with 0.1% Coomassie brilliant blue R-250 and dried as described by Hardy *et al.* (1981). Duplicate samples were examined on at least two occasions to confirm the reproducibility of the results.

For the quantitative analysis of stained gels, bands were scanned at 590 nm using a Gelman ACD scanning microdensitometer, and peak areas measured by an attached digital integrator. The maximum variation between duplicate experiments generally did not exceed 10%.

SDS-PAGE. Samples of concentrated culture fluid were diluted in solubilizing buffer containing 2% (w/v) SDS to which 5% (w/v) 2-mercaptoethanol was added and heated to $100\text{ }^{\circ}\text{C}$ for 2 min immediately before application to the gel (Hardy *et al.*, 1981). Gel electrophoresis was done essentially by the procedure of Laemmli (1970) as used in previous studies (Hardy *et al.*, 1981). The separating gel contained 7.5% (w/v) acrylamide [ratio of acrylamide to bisacrylamide 30:0.08 (w/w)] in 0.375 M-Tris/HCl buffer (pH 8.6) and 0.1% SDS. The stacking gel contained 5% acrylamide in 0.1 M-Tris/HCl buffer (pH 6.8). Duplicate samples were run at 60 V through the stacking gel and at 120 V through the separating gel with Tris/glycine/SDS buffer. Molecular mass standards obtained from Pharmacia were ferritin (220 kDa), phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa) and ovalbumin (43 kDa). Separated proteins were fixed and stained with 0.1% Coomassie brilliant blue R-250. All samples were examined on at least two occasions to confirm the reproducibility of the results. For the quantitative analysis of stained gels, bands were scanned at 590 nm using a Gelman ACD scanning micro-densitometer as described above. The maximum variation generally did not exceed 10%.

Glycosyltransferase. After SDS-PAGE, unfixed gels were incubated in the presence of 1% (v/v) Triton X-100 with sucrose and raffinose (Russell, R. R. B., 1979*d*). Fructosyltransferase (FTF) will form polymers from either carbohydrate but GTF will form polymers only from sucrose. After the gels had been fixed overnight in 2-propanol/acetic acid/water (5:2:13, by vol.) followed by washing in 10% (v/v) acetic acid, polysaccharide was detected by the periodic acid-Schiff reaction (Hardy *et al.*, 1981).

Electroblotting. This procedure (Burnette, 1981) was used to detect proteins that react with rabbit antiserum prepared against protein P1 from *S. mutans* Ingbritt (Forester *et al.*, 1983). After SDS-PAGE, proteins were transferred to nitrocellulose using a Hoefer Transphor model TE50. The blot was subjected to a preliminary incubation with 5% (w/v) bovine serum albumin in 0.85% NaCl/50 mM-Tris/HCl buffer (pH 8.8) containing 100-fold diluted pre-immune serum. The blot was then incubated with the same buffer containing 500-fold diluted IgG fraction prepared from P1 antiserum (kindly provided by Dr H. Forester, United Dental Hospital, Sydney). The two step reaction and the use of the IgG fraction increased the specificity of the reaction and minimized the potential for non-specific binding. The next stage of the reaction used alkaline-phosphatase-conjugated goat anti-rabbit IgG (Calbiochem-Behring Australia) followed by Fast Red TR salt (no. F1500, Sigma) (R. R. B. Russell & H. Forester, unpublished procedure).

Estimation of LTA. Organisms were extracted with hot aqueous phenol, and the LTA contents of both the extract and the corresponding culture fluid were estimated by rocket immunoelectrophoresis (RIE) (Jacques *et al.*, 1979*a, b*). All tests were done with the same collection of antiserum prepared against *Lactobacillus casei* LTA, which reacts with the polyglycerophosphate 'backbone' common to most LTAs. A standard amount of *L. casei* LTA was included in each experiment. Previous studies showed that results are reproducible under the defined conditions, with a coefficient of variation of 7.7% (Jacques *et al.*, 1979*b*).

Values for different cultures were compared by expressing results as rocket height per 50 μg cells or, in the case of culture fluids, cell equivalents.

RESULTS

Protein components of S. mutans strains of serotypes c, e and f

Culture fluids from *S. mutans* strains Ingbritt 162 (serotype *c*), LM7 (serotype *e*), and OMZ175 (serotype *f*) grown with limited glucose or fructose at different dilution rates and pH values were concentrated and examined by both SDS-PAGE and IEF. In an attempt to improve resolution

Fig. 1. SDS-PAGE of extracellular proteins of cultures of strain LM7 grown in limiting glucose or fructose. Organisms were grown at pH 6.0 and $D = 0.1 \text{ h}^{-1}$ (i), 0.2 h^{-1} (ii), 0.35 h^{-1} (iii) or 0.5 h^{-1} (iv), or at $D = 0.1 \text{ h}^{-1}$ and pH 5.5 (v), 6.5 (vi) or 7.5 (vii). The migration distances of proteins of known molecular mass (kDa) are given on the left, and the calculated molecular mass of culture fluid components on the right.

by IEF, the broad range gel (pH 3.5–9.5) used in previous studies on strain Ingbritt 162 (Hardy *et al.*, 1981) was replaced by a narrow range gel (pH 4.0–6.5). However, for each strain the numerous bands between pI 4.5 and 5.5 were insufficiently separated to detect differences due to a change in growth conditions (data not shown).

The results obtained by SDS-PAGE confirmed and extended the previous observation obtained with strain Ingbritt 162 (Hardy *et al.*, 1981), that this procedure is an effective means for distinguishing changes in protein profiles induced by changes in growth conditions. Thus, Fig. 1 provides a comparison of the culture fluids of strain LM7 with limiting glucose or fructose at pH 6.0 and $D = 0.1$ – 0.5 h^{-1} or at $D = 0.1 \text{ h}^{-1}$ and pH 5.5–7.5. To aid in the comparison of components of different molecular masses from the different strains, those in the ranges 185–200 kDa, 130–155 kDa and 60–95 kDa are designated P1, P2 and P3 respectively. Thus, for strain LM7, the major components are in the P2 and more particularly the P3 regions, and the greatest differences are seen with respect to the P3 component of 67 kDa. P1 was a minor component but was readily detectable by its reaction with antiserum to P1 from *S. mutans* Ingbritt 162 (see below).

Protein components of S. cricetus strain AHT

Culture fluids from *S. cricetus* strain AHT were also examined by SDS-PAGE and IEF. By IEF up to 17 bands could be discerned between pI 4.5 and 5.5 but they were insufficiently resolved for detailed analysis (data not shown). SDS-PAGE provided satisfactory separation of components; Fig. 2 shows results for cultures grown with glucose or fructose at pH 6.0 and $D = 0.1$ – 0.5 h^{-1} or at $D = 0.1 \text{ h}^{-1}$ and pH 5.5–7.5. The growth conditions had a major effect on the amount of two components in the P2 region with estimated molecular masses of 150 kDa (P2a) and 130 kDa (P2b), but there were also considerable differences in the amounts of a P3 component (67 kDa). Lower molecular mass components (< 67 kDa) were more prominent in cultures grown with limiting fructose at higher pH values (Fig. 2, lanes v and vi).

Fig. 2. SDS-PAGE of extracellular proteins of cultures of strain AHT grown in limiting glucose or fructose. Cultures were grown at pH 6.0 and $D = 0.1 \text{ h}^{-1}$ (i), 0.2 h^{-1} (ii) and 0.5 h^{-1} (iii) or at $D = 0.1 \text{ h}^{-1}$ and pH 5.5 (iv), 6.5 (v) and 7.5 (vi). Molecular masses (kDa) are given for protein standards (left) and culture fluid components (right).

Protein components of S. sobrinus strains of serotypes d and g

For comparison of culture fluids from *S. sobrinus* strains B13 (serotype *d*) and 6715 (serotype *g*), SDS-PAGE again proved to be more useful than IEF. The protein profiles obtained by SDS-PAGE showed the influence of D and, more particularly, pH on the production of individual components, components P2a (150 kDa) and P2b (130 kDa) being prominent. In general, the effects were similar to those obtained with *S. cricetus* strain AHT, although under a given condition the protein profiles differed between the two species. Further studies on the effect of growth conditions on the production of P1, P2a and P2b by strain B13 are described later.

Protein components of S. rattus strain BHT

The studies on this serotype *b* strain showed that both SDS-PAGE and IEF were effective in distinguishing changes in protein profiles induced by changes in D or growth pH. Under a given condition, the profile obtained by SDS-PAGE could be distinguished from that obtained for other serotypes (see below).

To extend the information on extracellular protein components, an examination was undertaken of those components in strain BHT culture fluid that were retained by an XM300 membrane, which had previously been shown to retain LTA but not deacylated LTA (Jacques *et al.*, 1979*a, b*). After SDS-PAGE and IEF, gels were analysed by scanning microdensitometry. The scans in Fig. 3, which illustrate the range of differences observed, were obtained for organisms grown in limiting fructose at $D = 0.1 \text{ h}^{-1}$ and either pH 5.5 or 7.5. Differences in SDS-PAGE profiles of protein of molecular mass $\geq 60 \text{ kDa}$ are apparent, organisms grown at the lower pH producing less of the higher molecular mass ($> 95 \text{ kDa}$) components. The results of IEF showed that the major component produced during growth at each value had a pI value estimated to be 5.0. However, the other components detected in cultures grown at pH 7.5 were present in only minor amounts at pH 5.5.

Fig. 3. Microdensitometry scans after (a) SDS-PAGE and (b) IEF of the XM300 retentate of culture fluids of strain BHT grown with limiting fructose at $D = 0.1 \text{ h}^{-1}$ and either pH 5.5 or 7.5. The migration distances for proteins of known molecular mass (a) or isoelectric point (b) are shown.

Table 1. Amounts of extracellular protein $\geq 60 \text{ kDa}$ produced by cultures grown at $D = 0.1 \text{ h}^{-1}$ at pH 5.5 or 7.5 with limiting glucose or fructose

The protein components in culture fluids were separated by SDS-PAGE followed by scanning microdensitometry. The total area under the peaks of proteins of molecular mass $\geq 60 \text{ kDa}$ was then calculated and expressed in scanning microdensitometry (SM) units per $100 \mu\text{g}$ dry wt cells. The results are those for typical gels; the maximum variation between replicates was 10%.

Species	Serotype	Strain	Protein (SM units per $100 \mu\text{g}$ dry wt cells)			
			Glucose		Fructose	
			pH 5.5	pH 7.5	pH 5.5	pH 7.5
<i>S. mutans</i>	c	Ingbritt 162	270	242	372	311
	e	LM7	210	246	230	407
	f	OMZ175	76	96	167	368
<i>S. cricetus</i>	a	AHT	528	1124	369	878
<i>S. sobrinus</i>	d	B13	160	242	209	275
	g	6715	290	352	147	314
<i>S. rattus</i>	b	BHT	—	—	269	195

From comparative analyses on the XM300 diffusates, it was estimated that over 90% of the pI 5.0 component was retained by the membrane. The effect of growth conditions on the amount of this component was determined by scanning microdensitometry. For organisms grown in limiting fructose at pH 6.0, the relative amounts at $D = 0.5, 0.2$ and 0.05 h^{-1} were in the proportions 1.0:1.4:2.7. Values also increased with increasing pH (at constant $D = 0.1 \text{ h}^{-1}$), the relative amounts at pH 5.5, 6.5 and 7.5 being in the proportions 1.0:1.35:1.5.

Fig. 4.

Fig. 5.

Fig. 4. SDS-PAGE of extracellular proteins produced by cultures of serotypes *a-g* strains grown with limiting glucose at pH 6.0 and $D = 0.2 \text{ h}^{-1}$. The migration distances for proteins of known molecular mass are shown on the left, and components designated P1, P2 and P3 are identified on the right.

Fig. 5. Western electroblots of culture fluids of serotypes *a-g* strains that had been separated by SDS-PAGE (Fig. 4) and examined for reactivity with antiserum prepared against purified P1 from serotype *c* strain Ingbritt. The molecular mass values refer to the major P1 components of strains of serotype *c* (185 kDa), *d* (200 kDa) and *a* (> 200 kDa).

Comparisons of total protein production

Comparisons of the total amounts of extracellular protein of molecular mass $\geq 60 \text{ kDa}$ were made by scanning microdensitometry of gels after SDS-PAGE and expressing the total area under the peaks as arbitrary scanning microdensitometry (SM) units per 100 μg dry wt of cells. There were major differences in the amount of protein produced by the same strains grown under different conditions and by different strains grown under the same conditions (Table 1), particularly for *S. mutans* OMZ175 and *S. cricetus* AHT.

Comparison of protein components of different strains grown under different conditions

The results of IEF and SDS-PAGE were compared for culture fluids from each organism under the same condition. IEF was of limited value in distinguishing between the different serotypes, except that *S. rattus* strain BHT displayed a characteristic profile (Fig. 3*b*). However, differences were distinguishable when SDS-PAGE was used, particularly in the regions designated P1 (185–200 kDa), P2 (130–155 kDa) and P3 (60–95 kDa), as shown by comparing cultures grown in limiting glucose at pH 6.0 and $D = 0.2 \text{ h}^{-1}$ (Fig. 4).

A component in the P1 region was present in substantial amounts only in the culture fluid from *S. mutans* serotype *c*, but it was serologically detectable in all cases (see below).

The P2 region was dominant in the *S. cricetus* strain (serotype *a*) and to a lesser extent in the *S. sobrinus* strains (serotypes *d* and *g*), where there were two prominent components, designated P2a (150 kDa) and P2b (130 kDa).

Within the P3 region a 90 kDa component was characteristic of *S. sobrinus* strains (serotypes *d* and *g*). A 67 kDa component was a major product of strains of *S. cricetus* (serotype *a*) and *S. mutans* (serotypes *c*, *e* and *f*), and a component of intermediate molecular mass (78–80 kDa) was dominant in *S. rattus* (serotype *b*).

Characteristics of P1 components

The protein components of a representative culture fluid from each strain were separated by SDS-PAGE (see Fig. 4), transferred by electroblotting to nitrocellulose membranes, and examined for reactivity with rabbit antiserum prepared against purified P1 from strain Ingbritt 162 (Forester *et al.*, 1983). In each case there were reactive components, though the reactions by *S. rattus* (serotype *b*) and *S. sobrinus* (serotype *g*) were weak (Fig. 5). Purified P1 has a molecular mass of 185 kDa, and all strains except *S. sobrinus* (serotype *d*) produced a similar component. Characteristic patterns were shown by *S. cricetus* (serotype *a*), which produced a component > 200 kDa, and by *S. sobrinus* (serotype *d*), where a component of 200 kDa predominated. For the three strains of *S. mutans*, there were numerous bands of less than 185 kDa, particularly with the serotype *c* strain, and evidence for a 200 kDa component for the serotype *e* and, particularly, *f* strains.

The relative amounts of P1 produced by *S. mutans* strains Ingbritt 162 (serotype *c*), LM7 (serotype *e*) and OMZ175 (serotype *f*) and *S. sobrinus* strain B13 (serotype *d*) under different growth conditions were quantified by microdensitometry after SDS-PAGE. In each case the amount produced by glucose- or fructose-grown organisms increased as *D* increased (at constant pH = 6.0), and as the growth pH decreased (at constant *D* = 0.1 h⁻¹), as shown for strain B13 in Fig. 6.

Characteristics of P2 and P3 components

GTF activity was tested in culture fluids from organisms grown at *D* = 0.2 h⁻¹ and pH 6.0 in glucose and fructose. The strains representing the four species were AHT, BHT, Ingbritt 162, LM7 and 6715. After separation of protein components by SDS-PAGE, the gels were tested for the ability to form polysaccharide from sucrose and raffinose. All gave a reaction in both the P2 and P3 regions with sucrose, but not raffinose, which is consistent with the presence of GTF (data not shown). The reaction was greater in the P2 region for strains AHT and 6715, and in the P3 region for the two *S. mutans* strains Ingbritt 162 and LM7. In previous studies with strain Ingbritt 162 (Knox *et al.*, 1982) dextran-binding protein also migrated in the P3 region.

The production of components in the P2 region was particularly affected by growth conditions. A comparison of the gels and the results of scanning microdensitometry showed that for both glucose- and fructose-grown organisms the maximum amounts were obtained at higher pH values, particularly in the case of BHT. Generally, an increase in *D* also led to an increase in the amount of P2.

For strains of *S. cricetus* and *S. sobrinus* where the P2 component was subdivided into P2a and P2b, the relative amounts of individual components were also influenced by the growth conditions. For example, the ratio of P2a:P2b based on peak areas for strain B13 grown in limiting glucose increased from 0.8 to 2.4:1.0 as the pH rose from 5.5 to 7.5 (at *D* = 0.1 h⁻¹) and from 0.5 to 2.5:1.0 as *D* increased from 0.2 to 0.5 h⁻¹ (at pH 6.0). Fructose-grown cultures frequently gave lower ratios, for example, 0.3:1.0 at pH 6.0 and *D* = 0.5 h⁻¹ compared with 2.5:1.0 for glucose-grown cultures of strain B13.

Effect of growth conditions on LTA production

Previous studies on strains BHT (Jacques *et al.*, 1979*b*) and Ingbritt 162 (Jacques *et al.*, 1979*a*; Hardy *et al.*, 1981) showed that growth at a higher pH resulted in the production of more LTA and that carbohydrate source could also influence the results. Examination of the additional strains in the current study indicated that they generally responded in a similar manner. The results in Table 2 are expressed as the sum of the values for cellular and extracellular LTA, as estimated by RIE, for cultures grown at pH 5.5 and 7.5 with limiting glucose or fructose. The results are uniformly higher at the higher pH but the response to a change in carbohydrate is variable.

Fig. 6. Quantification by scanning microdensitometry after SDS-PAGE of the P1 component in the culture fluid of strain B13 grown (a) at constant pH (6.0) and different dilution rates (D), or (b) at constant dilution (0.1 h^{-1}) and different pH values. The results with both glucose- and fructose-grown cultures are the means of at least three determinations and error bars represent two SDs. □, Glucose; ▨, fructose.

Table 2. Total amount of LTA (cellular and extracellular) produced by cultures grown at $D = 0.1 \text{ h}^{-1}$ at pH 5.5 or 7.5 with limiting glucose or fructose

The LTA content of aqueous phenol extracts of organisms and of the corresponding culture fluids were estimated by RIE, using antiserum to *L. casei* LTA. Values were calculated as rocket height in cm per 50 μg cells or, for culture fluids, cell equivalents, and the sum of the two values was determined to obtain the total LTA production. The results are those for typical experiments, the maximum variation between replicates being 15%.

Species	Serotype	Strain	Rocket height (cm per 50 μg cells)			
			Glucose		Fructose	
			pH 5.5	pH 7.5	pH 5.5	pH 7.5
<i>S. mutans</i>	<i>e</i>	LM7	2.1	9.2	3.7	7.5
	<i>f</i>	OMZ175	1.7	6.3	3.4	8.0
<i>S. cricetus</i>	<i>a</i>	AHT	10.0	31.8	16.0	23.0
<i>S. sobrinus</i>	<i>d</i>	B13	2.3	5.3	2.3	9.9
	<i>g</i>	6715	3.4	11.0	1.4	6.6

DISCUSSION

These studies provide further evidence of the genetic differences between the four species of human commensal mutans streptococci and also reinforce the importance for comparative studies of defining the growth conditions. The ones chosen were relevant to those in the oral cavity with respect to availability of two dietary carbohydrates, the pH range (5.5–7.5), and the choice of generation times between 1.4 h ($D = 0.1 \text{ h}^{-1}$) and 7 h ($D = 0.1 \text{ h}^{-1}$). The influence of carbohydrate source and growth pH on a particular strain is evident from the results for total protein (Table 1) and total LTA (Table 2). When strains grown under the same conditions are compared *S. cricetus* AHT generally produced considerably more LTA and extracellular protein than strains of *S. mutans* and *S. sobrinus* but less LTA than *S. rattus* BHT (Jacques *et al.*, 1979b).

Of the two procedures that were used for separating proteins, IEF was the less effective even when the relatively narrow pH range of 4.0–6.5 was used. Only in the case of *S. rattus* were the individual protein components sufficiently resolved for comparative studies, with a component of pI 5.0 predominating. In contrast, SDS-PAGE proved particularly valuable and enabled differences to be discerned between culture fluids of the same organism grown under different

conditions and different organisms grown under the same conditions. While silver staining would detect additional components (McBride *et al.*, 1984, 1985), dye-binding enabled comparison with previous results (Hardy *et al.*, 1981), and focused attention on the major components. This is illustrated by the results in Fig. 4, where it is apparent that there are marked differences in the relative amounts of individual proteins in the P2 and P3 regions produced by the different strains grown under the same conditions. Differences in components in the P1 region were more apparent after electroblotting. As expected, the profiles of protein components differentially released into the culture fluid were less complex than those from whole cell extracts (Russell, R. R. B., 1976; Whiley *et al.*, 1982).

Protein P1 from *S. mutans* serotype *c* strain Ingbritt 162 was shown (Forester *et al.*, 1983) to be indistinguishable in its physicochemical and serological properties from two other proteins previously isolated from serotype *c* strains, namely, antigens B (Russell, R. R. B. *et al.*, 1979*c*) and I/II (Russell, M. W. & Lehner, 1978; Russell, M. W. *et al.*, 1980). There is serological evidence for the widespread occurrence of this protein among mutans streptococci (Russell, R. R. B., 1980; Moro & Russell, 1983; Ayakawa *et al.*, 1985), and this is confirmed by the current studies. In addition, it is apparent that a serological reaction is frequently given by proteins of different molecular mass. While a component of 185 kDa was present in the culture fluid of most strains, one of 200 kDa was prominent in the case of *S. sobrinus* serotype *d*. In this strain, too, unlike the others, there was very little reactive material of molecular mass < 185 kDa. Previous studies on P1 from strain Ingbritt 162 (Forester *et al.*, 1983) suggest that such low molecular mass fractions result from the instability of the protein.

Studies on antigen B or I/II have primarily been done on products isolated from *S. mutans* serotype *c* strains. The antigen may play a role in microbial adhesion (Douglas & Russell, 1984; McBride *et al.*, 1985) but its potential as a caries vaccine is hampered by the possibility of adverse tissue reactions (reviewed by Cohen *et al.*, 1983). Whether the serologically related proteins from other species display similar properties is not known.

P2 components of 150 kDa (P2a) and 130 kDa (P2b) were dominant in the case of *S. cricetus* (serotype *a*) and to a lesser extent with the serotype *d* and *g* strains of *S. sobrinus*. Within the P3 region, which spanned 60–95 kDa, there were sufficient differences to distinguish each of the species and each of the serotypes. Thus the molecular mass of the major component differed from 90 kDa for *S. sobrinus* (serotypes *d* and *g*) to 78–80 kDa for *S. rattus* (serotype *b*) and about 67 kDa for *S. cricetus* (serotype *a*) and *S. mutans* (serotypes *c* and *e*).

The two species that produced greater amounts of the 130 and 155 kDa components, namely *S. cricetus* and *S. sobrinus*, also gave a strong reaction for GTF activity in the P2 region, whereas for *S. mutans* activity was apparently greater in the P3 region. The detection of glucan formed by GTF in the P3 region could be due to the presence of glucan-binding protein, as one of 74 kDa has been isolated from *S. mutans* serotype *c* (Russell, R. R. B., 1979*b*; Russell, R. R. B. *et al.*, 1983) and is known to migrate in the P3 region (Knox *et al.*, 1982). However, the binding protein also has FTF activity (Russell, R. R. B. *et al.*, 1983), and such activity was not detected in the present study.

The detection of GTF activity in the P2 region is consistent with previous studies on mutans streptococci, particularly those on *S. mutans* serotype *c*. Russell, R. R. B. (1981) reported activity associated with components of 132, 140 and 150 kDa, which were further differentiated when it was shown that the high molecular mass enzyme formed insoluble glucan and a lower molecular mass enzyme (140 kDa) formed soluble glucan (Kurumitsu & Wondrack, 1983). Studies on *S. sobrinus* serotype *g* strain 6715 also showed that the formation of water-insoluble glucan was associated with the higher molecular mass component (Furuta *et al.*, 1983).

Other proteins within the P2 and P3 regions have not been identified but it is known that mutans streptococci produce a cross-reactive 'extracellular common protein' of 60 kDa (Schöller *et al.*, 1982) and proteins of molecular mass 130–135 kDa that interact with salivary proteins (Ogier *et al.*, 1984).

As well as differences between strains, individual strains also showed major phenotypic differences in extracellular protein profiles that reflected changes in the environment, such as carbohydrate present, generation time and growth pH. These results reinforce earlier studies

which showed that growth conditions affected protein production by strain Ingbritt 162 (Hardy *et al.*, 1981) and the surface and extracellular characteristics of oral streptococci in general (Knox & Wicken, 1984, 1985). However, the present studies show that, in such disparate species as *S. mutans* and *S. cricetus*, the phenotypic differences with respect to the major extracellular proteins are secondary to the inter-species differences.

Growth conditions had a major effect on the amounts of extracellular P1. As with *S. mutans* strain Ingbritt 162 (Forester *et al.*, 1983; Knox & Wicken, 1984), the amounts produced by the other *S. mutans* serotypes and *S. sobrinus* increased with increasing dilution rate or decreasing growth pH, and growth in fructose promoted formation of extracellular P1 relative to growth in glucose.

The production of components within the P2 and P3 regions is similarly influenced by the growth conditions. In particular, the relative amounts of P2a and P2b could differ several-fold, as shown by *S. sobrinus* strain B13. Components P2a and P2b probably correspond to the GTFs that synthesize insoluble and soluble glucan respectively (Furuta *et al.*, 1983; Kurumitsu & Wondrack 1983), and differences in the relative amounts of the two enzymes produced by *S. sobrinus* strain 6715 grown under different conditions have been reported (Walker *et al.*, 1984).

These studies on the group of oral organisms now referred to as mutans streptococci have provided further evidence for genetic differences between strains representing four species and seven serotypes. The results also illustrate the range of phenotypic responses that can occur as a consequence of changes in the environment and point to the need to take account of growth conditions when comparing the characteristics of different strains of bacteria.

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