MICROBIAL ECOLOGY IN HEALTH AND DISEASE VOL. 2: 231–239 (1989)

Adaptive Changes in a Strain of *Streptococcus mutans* during Colonisation of the Human Oral Cavity

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When a bacterial strain undergoes a change in environment, its ability to colonise a new niche is optimised over time by the process of natural selection. In this study, a readily identifiable strain of *Streptococcus mutans* was recovered from supragingival dental plaque of human subjects 3 y after implantation. Fifty fresh isolates were compared to the original laboratory strain that was used to infect these subjects. It was found that the fresh isolates adhered less well than the laboratory strain to saliva-coated hydroxyapatite. The fresh isolates also produced more acetoin and less lactic acid, and had a greater cell yield during aerobic growth in glucose-containing medium. During growth in the presence of sucrose, the fresh isolates produced slightly greater amounts of plaque than the laboratory strain. No differences were observed between fresh and laboratory adapted strains with regard to bacteriocin production, antigenic composition, nutrient requirements, growth rates, or resistance to tetracycline. These results indicate that environmental factors operating in the oral cavity of human subjects select variants of *S. mutans* that are better adapted to proliferate *in vivo*. Identification of these factors is important to our general understanding of the ecology of *S. mutans* and to the development of therapeutic methods.

KEY WORDS—*Streptococcus mutans*; Adaptation; Implantation.

INTRODUCTION

Indigenous strains of viridans streptococci have been shown, largely through the use of rodent models, to change *in vivo* and adapt to their oral environment over time.[1,2,3,13,14,20] The property best studied in this regard is antigenic variation where evidence suggests that salivary antibodies periodically select for variants with altered cell surface antigens. A more complete understanding of the natural ecology of these bacteria, particularly in humans, has been hampered by the lack of reliable identifying markers that would enable us unambiguously to determine the relationship of divergent strains isolated over time from a subject.

In the case of mutans streptococci, a variety of stable genetic markers, such as resistance to antibiotics, has been available in laboratory strains for some time. However, such strains have invariably lacked the ability to infect and persistently colonise the human oral cavity for sufficient periods of time to study their ecology and natural history.[22,24,26,31,32]

Recently, a strain of *S. mutans* called strain JH1005 has been characterised.[17] It is a derivative of a serogroup *c* strain that was isolated from the plaque of a healthy adult and maintained in the laboratory since 1979. JH1005 possesses two distinctive genetic markers, a spontaneous mutation to tetracycline resistance and the hyper-production of a bacteriocin-like molecule that is unusual in the breadth of its antibacterial spectrum. It has been found to readily infect and persist on the teeth of clinical subjects for prolonged periods.[18]

The present study takes advantage of this combination of properties to investigate the adaptive changes that occur during infection and prolonged colonisation by *S. mutans*. It compares the laboratory-adapted strain of JH1005 to JH1005 isolates recovered from the human oral cavity 3 y following infection. A number of phenotypic properties are examined that are likely to be important in the adaptation of this organism to its natural habitat.

MATERIALS AND METHODS

Microorganisms and media

Saliva samples and supragingival plaque pooled from the left maxillary and mandibular molars were obtained from three adult male volunteers who had been infected for over 3 y with strain JH1005.[20] The samples were dispersed by vortex-mixing in phosphate buffered saline, and serial 10-fold dilutions

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0891–060X/89/040231–09 $05.00

were seeded on Mitis Salivarius agar containing 3·5 μg/ml bacitracin (MSB)[16]. Isolated colonies that appeared on this medium following incubation for 3 d were chosen randomly and purified once on the selective medium. Glycerol stabs of 17 independent isolates from both subjects 1 and 2, and 16 independent isolates from subject 3, yielding 50 isolates in total, were prepared by mixing equal volumes of an overnight culture grown in a chemically defined medium[27] with glycerol, and stored at −40°C. These stabs were used to inoculate additional cultures as needed.

The laboratory strain of JH1005 used to colonise the clinical subjects was stored in glycerol at the time of infection. At the time that plaque samples were processed for recovery of isolates adapted to growth *in vivo*, the glycerol stab of the laboratory strain was sampled and treated in an identical fashion to obtain ten control isolates. These were also stored as glycerol stabs.

Bacteriocin assays

The antibacterial spectra of the bacteriocin-like activity of fresh and laboratory isolates were tested by a cross-streaking method. Samples (0·05 ml) of the glycerol stabs were streaked evenly down the center of brain–heart infusion agar plates and allowed to dry. Sterile toothpicks were used to cross-streak single colonies of ten test strains consisting of mutans streptococci strains FA1, BHT-2, MT3, and LM7, *S. salivarius* strain SS2, *S. sanguis* strain KJ3, *S. mitis* strain 26, *Actinomyces viscosus* strain T6, *Capnocytophaga sputigena* strain 4, and *Actinobacillus actinomycetemcomitans* strain Y4. Following incubation in candle jars for 2 d at 37°C., the streaks of the test strains were examined for zones of growth inhibition at their intersection with the streak of the JH1005 isolate.

Quantitative measurements of bacteriocin production were performed by growing Todd–Hewitt broth (Difco) cultures, inoculated from glycerol stabs, at 37°C. for 24 h. Cells were removed by filtration through 0·2 μ Millipore membranes, and serial two-fold dilutions of the culture liquors were prepared using fresh Todd–Hewitt broth. Twenty μl of diluted samples were transferred to sterile microtiter wells to which were added 100 μl of molten (42°C) brain-heart infusion broth containing 0·7 per cent (w/v) Bactoagar (Difco) and 10^4 colony forming units of the *S. mutans* indicator strain Jo. The plates were sealed and incubated overnight at 37°C. The bacteriocin titre was taken as the inverse of the highest dilution of culture liquor which prevented visible growth of the indicator strain.

Adhesion to saliva-treated hydroxyapatite

The attachment of fresh and laboratory isolates to saliva-treated hydroxyapatite was measured using the methods of Gibbons and Etherden.[15] Cells for the assay were grown in Todd–Hewitt broth supplemented with 4 μCi of ^{3}[H]thymidine (New England Nuclear Corp., Boston, MA). The cells were washed three times and re-suspended in 0·05 M KCl buffer containing 1 mM potassium phosphate (pH 6), 1 mM $CaCl_2$ and 0·1 mM $MgCl_2$. Spheroidal hydroxyapatite beads (5 mg; BDH, Poole, England) were treated with whole, clarified saliva for 1 h to form an experimental pellicle, and then treated with bovine serum albumin (2 mg/ml) for 30 min to block naked regions of the hydroxyapatite. Radiolabelled cells (5×10^6) were then continuously mixed with the hydroxyapatite beads for 1 h at room temperature (RT). The beads were washed three times to remove unattached cells, and radioactivity remaining associated with the beads was measured by scintillation counting. Values for cpm/cell, as determined by liquid scintillation and Petroff Hauser counts of the original cell suspensions, were used to calculate the number of attached cells/mg of hydroxyapatite.

Cell surface antigenic composition

Formalin-killed cells of JH1005 laboratory isolate number 1, emulsified with Freund's Incomplete Adjuvant (Sigma Chemical Co., St Louis, MO), were used to immunise two female New Zealand White rabbits as described by Beem and coworkers.[1] Lancefield extracts[25] of fresh and laboratory strains were prepared from 250 mg (wet weight) of cells grown in chemically defined medium and washed twice with 0·85 per cent (w/v) saline. The cells were re-suspended in 0·05 N HCl and placed in a boiling water bath for 10 min. After cooling to RT, the cell suspensions were adjusted to pH 9 with 2 N NaOH, and cellular debris removed by centrifugation. The antiserum and Lancefield extracts were reacted by double diffusion and immunoelectrophoresis using 1·2 per cent (w/v) agarose in Veronal buffer (pH 9·6) as described by Howell *et al.*[20]

Twenty-one independent monoclonal antibodies prepared against and reactive with cell surface

antigens of *S. mutans* strain Ingbritt[28] were screened for their reactivity with a single laboratory strain of JH1005. Eight reactive antibodies were then tested for reactivity with all of the fresh and laboratory isolates using solid-phase radioimmunoassay.[29] Polyvinyl chloride microtitre plates were coated by exposure to 5×10^9 cells/ml in PBS for 18 h at 4°C. The plates were washed with PBS and blocked with 5 per cent (v/v) newborn calf serum (NBCS) in PBS for 1 h at 20°C. Plates were washed with PBS and incubated for 2 h with 50 µl of $\log_{10}$ dilutions of hybridoma supernatants. Plates were again washed with 0·5 per cent NBCS (v/v) in PBS and reacted with (^{125}I)-polyvalent goat anti-mouse immunoglobulin (50 000 cpm/well). After 16 h, plates were washed extensively with PBS, air dried, and the wells excised and counted by scintillation spectroscopy.

Growth yields and fermentation end-products

Duplicate 3 ml cultures in chemically defined medium containing 0·5 per cent (v/v) glucose were inoculated from glycerol stabs. The cultures were incubated anaerobically in an atmosphere of 80 per cent nitrogen, 10 per cent carbon dioxide, and 10 per cent hydrogen, or aerobically on a rollerdrum (New Brunswick Scientific, Edison, NJ) operating at 50 rpm. After 48 h at 37°C., the terminal pH and dry weight of each culture was measured. Non-metabolised glucose was measured using the glucose oxidase method (Sigma Chemical Co., St Louis, MO). Ethanol and acetoin concentrations in cell-free culture liquors were assayed by gas–liquid chromatography,[5] and lactic acid was measured using ion chromatography (Dionex Corporation, Marlton, NJ).

Plaque production

Duplicate cultures of chemically defined medium containing 2 per cent (w/v) sucrose were inoculated from glycerol stabs and incubated anaerobically for 3 d. The cultures were centrifuged at $12\,000 \times \boldsymbol{g}$ for 20 min and the dry weights of the resulting pellets, washed twice by centrifugation with distilled water, were taken as a measurement of total mass produced. Tenacious plaque production was measured by decanting the cultures and adding an equal volume of sterile, distilled water. The tubes were placed on a rollerdrum operating at 50 rpm for 5 min and then decanted. This procedure was repeated. The dry weight of plaque adherent to the walls for each culture tube was measured.

Minimum growth requirements

Concentrated stock solutions of amino acids, vitamins, nucleosides, and salts were prepared and stored as described by Carlsson.[4] When preparing a medium, the appropriate amounts of stock solutions were mixed and glucose and ammonium acetate were added in dry form. The pH was adjusted to 7·0 and water was added to give the final volume of the medium. The medium was sterilised by vacuum filtration through 0·2 µ membranes and stored at −80°C until used. The nutritional requirements were determined initially for three fresh and three laboratory isolates in an atmosphere of 80 per cent nitrogen, 10 per cent hydrogen, and 10 per cent carbon dioxide. Each strain was subcultured six times in each medium using a 1 per cent inoculum to rule out carryover growth. The cell yields were estimated throughout the experiment by measuring the optical densities of the cultures at 550 nm. The minimum growth requirements determined in this manner were tested for their ability to support the growth of the remaining laboratory and fresh isolates.

Data analysis and statistical methods

The fresh isolates from the three subjects were randomly distributed for analysis of the various phenotypic properties. Laboratory-adapted isolates were evenly distributed throughout the series of samples, as shown in the Figures. Results of the analyses were initially grouped by subject and analysed for significant differences by analysis of variance. If the overall variance ratio (F) test indicated significant differences ($p < 0{\cdot}05$) among the three groups, then pairwise comparisons were made using the LSD test to determine which of the groups differed significantly ($\alpha < 0{\cdot}05$) from the others. If no significant differences were observed, the results from the three subjects' isolates were pooled and compared to the results of the laboratory adapted strains using the Mann–Whitney U test.

RESULTS

Isolation and identification of fresh isolates

Saliva samples taken from three adult subjects infected for 3 y with strain JH1005 contained between 10^3 and 3×10^4 colony forming units of mutans streptococci/ml. Supragingival plaque samples yielded colonies on MSB medium with

uniform morphology. Fifty freshly isolated colonies as well as ten clones from the original laboratory strain grew on brain-heart infusion agar containing 1 μg/ml tetracycline. When cross-streaked against a battery of ten test strains, all of the isolates, both fresh and laboratory, exhibited the same pattern of growth inhibition: mutans streptococci, *S. salivarius*, and *S. sanguis* strains were inhibited, but no effect was observed on the growth of the Gram-negative species tested. The glycerol stabs were tested for purity by Gram stain and by streaking samples on BAP and BHI. These tests indicated that one of the laboratory isolates was contaminated. This strain was discarded. The fifty fresh isolates and the nine remaining laboratory isolates, verified as to their identity and purity, were compared in the following manner.

Adaptive changes in adhesion

The ability of fresh and laboratory isolates to adhere to saliva-coated hydroxyapatite was compared. The average of triplicate samples for each strain from two independent trials was determined. The results are shown in Figure 1, expressed as the number of adherent cells per mg of hydroxyapatite. Isolates from the three subjects did not differ significantly in their attachment, though on average they adhered less well to saliva-treated hydroxyapatite than did the laboratory-adapted isolates. The mean attachment levels obtained were 0·89 (±0·29 S.D.) × 10^6 cells/mg of hydroxyapatite for the fresh isolates and 1·22 (±0·22 S.D.) × 10^6 cells/mg for the laboratory isolates: this difference was significant by the Mann–Whitney U test to $p < 0{\cdot}001$.

Cell surface antigenic changes

Lancefield extracts of the fresh and laboratory isolates were reacted in double-diffusion plates with rabbit antiserum prepared against the first laboratory isolate of JH1005. All of the isolates gave identical patterns consisting of one major (group antigen) and one minor precipitin line. No spurs were observed at the intersections of precipitin lines formed by extracts of adjacent fresh and laboratory strains. Also, immunoelectrophoresis of extracts of the fresh and laboratory isolates revealed two precipitin lines. No differences between the extracts were observed by measurements of the electrophoretic mobility of these lines. Nor were there any obvious differences noted in the densities of the precipitin lines.

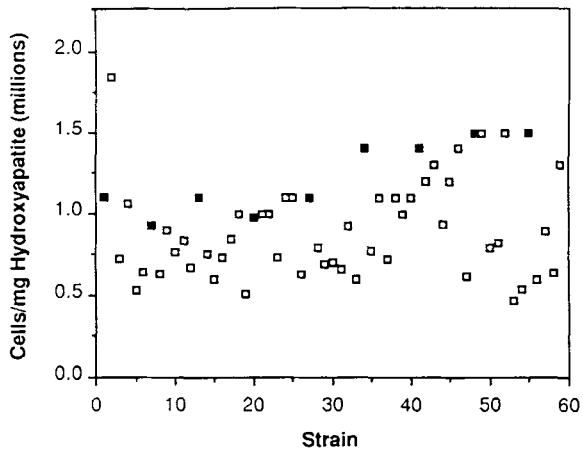

Figure 1. The attachment of 50 fresh (□) and nine laboratory (■) adapted isolates to saliva-treated hydroxyapatite. Each point represents the average of two independent trials

Binding activity was detectable at a concentration of <2 ng/ml for eight of the monoclonal preparations. These eight antibodies were then tested for reactivity with all of the fresh and laboratory isolates using solid-phase radioimmunoassay. No differences could be detected in terms of quantity and avidity of reactivity of the antibodies to the fresh and laboratory isolates.

Changes in growth and metabolism

Fresh and laboratory adapted isolates grew at approximately the same rate in chemically defined medium containing glucose, with a doubling time of 1·3 h during anaerobic cultivation and 1·8 h during aerobic cultivation. Aerobically grown glucose-containing cultures of the fresh isolates had a significantly higher pH than did the laboratory isolates (Figure 2). The mean values from two independent trials were pH 5·38 (±0·18 S.D.) and pH 5·19 (±0·18 S.D.), respectively. The higher pH of the fresh isolate cultures was found to correlate with increased cell yields as measured by their dry weights (Figure 3). The mean values obtained from two independent trials were 1·41 (±0·29 S.D.) and 1·04 (±0·12 S.D.) optical density units for the fresh and laboratory adapted isolates, respectively. The differences in pH and cell yields were significant by the Mann–Whitney U test to $p < 0{\cdot}01$.

The observed differences were not the result of changes in glucose consumption, since both laboratory and fresh isolates used virtually all of the glucose provided in the medium. The differences

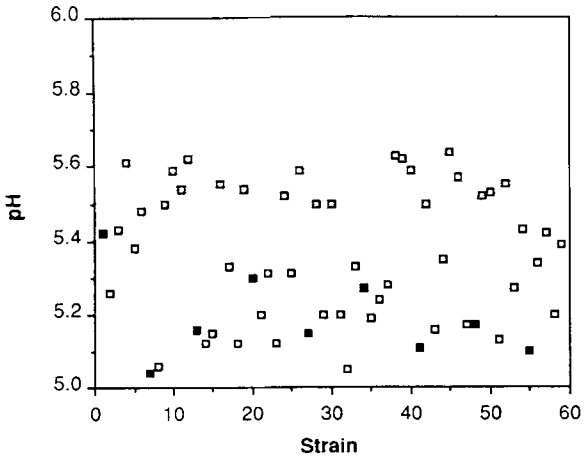

Figure 2. Terminal pHs of 50 fresh (□) and nine laboratory (■) adapted isolates grown aerobically in chemically defined medium containing glucose

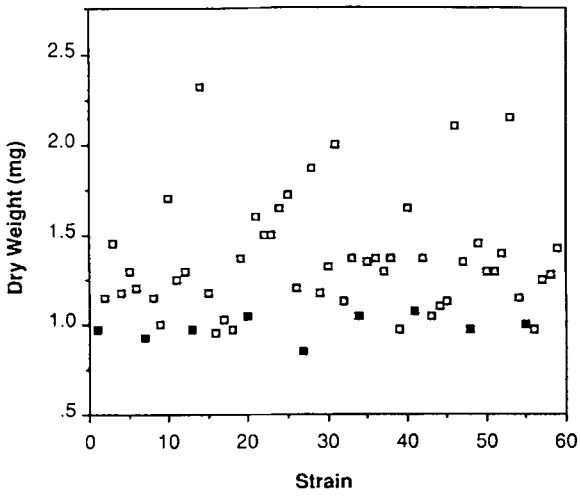

Figure 3. Cell yields of 50 fresh (□) and nine laboratory (■) adapted isolates grown aerobically in chemically defined medium containing glucose

did reflect an apparent change in pyruvate dissimilation. The spent culture liquors of the fresh isolates contained significantly more acetoin than laboratory strains (Figure 4). The mean value determined for cultures of the fresh isolates was 4·6 (±1·4) mM acetoin compared to 3·3 (±0·7) mM for the laboratory strains. The difference was significant at $p<0·01$. Ion chromatographic analysis of the culture liquors indicated a concomitant decrease in lactic acid production by the fresh isolates. No other significant differences were observed in the types or amounts of end-products formed by aerobically-grown strains. No differences were detected between fresh and laboratory-adapted strains when grown anaerobically in glucose-containing medium.

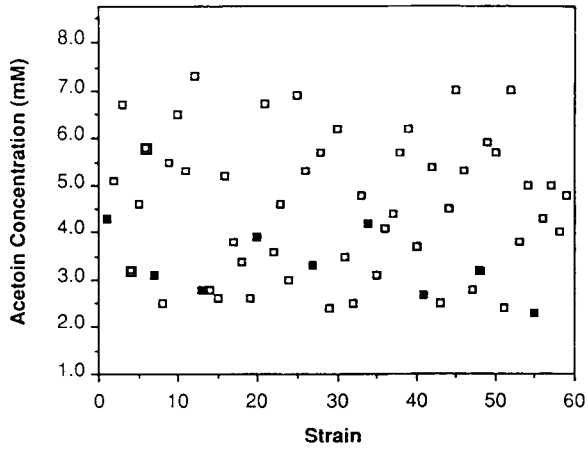

Figure 4. Acetoin production by 50 fresh (□) and nine laboratory (■) adapted isolates during aerobic growth in chemically defined medium containing glucose

Changes in plaque production

Laboratory isolates of JH1005 produced an average of 2·20 (±0·19 S.D.) mg total mass per ml of culture when grown in chemically defined medium containing 2 per cent (w/v) sucrose (Figure 5). Fresh isolates produced an average of 2·50 (±0·25) mg under the same conditions. This difference was significant at $p<0·05$. The amounts of adherent plaque produced by the laboratory and fresh isolates were 0·64 (±0·09) and 0·70 (±0·15) mg, respectively (Figure 6). Thus, the proportion of adherent plaque to total mass was approximately the same, 30 per cent, for both the laboratory and fresh isolates.

Other phenotypic properties

Fresh and laboratory isolates did not differ with regard to several other properties that were examined. These included the spectrum and activity of their bacteriocin-like product, and their ability to metabolise glucose, mannitol, galactose, or ribose. Under conditions of anaerobic cultivation, growth of all the fresh and laboratory isolates could be achieved in a minimal medium containing glucose,

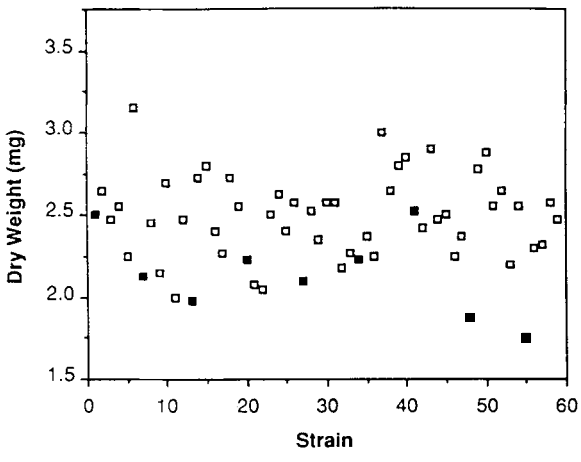

Figure 5. Total mass production by 50 fresh (□) and nine laboratory (■) adapted isolates during growth in chemically defined medium containing sucrose

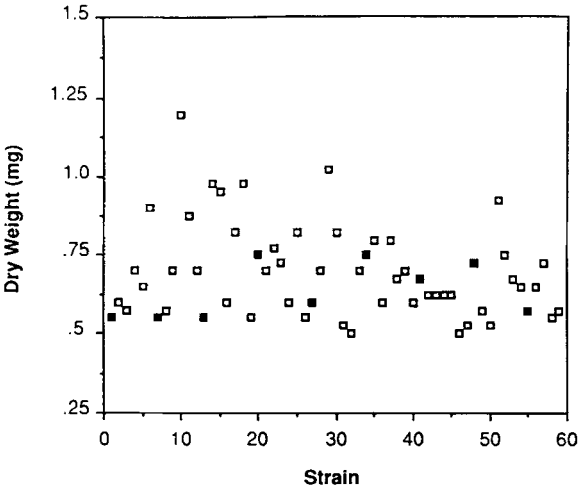

Figure 6. Tenacious plaque production by 50 fresh (□) and nine laboratory (■) adapted isolates during growth in chemically defined medium containing sucrose

seven vitamins (biotin, riboflavin, thiamine, pantothenic acid, p-aminobenzoic acid, pyridoxine, and nicotinic acid), five salts (K_2PO_4, $MgSO_4$, $FeSO_4$, $MnSO_4$, and NaCl), and five amino acids (cysteine, glutamic acid, lysine, arginine, and histidine).

DISCUSSION

Gibbons and Qureshi[14] demonstrated that *S. mutans* undergoes physiological changes and antigenic variations during colonisation of gnotobiotic rats. Since similar variants were not detected among isolates derived from laboratory stock cultures, the authors concluded that environmental factors operating in the oral cavity select variants that are better adapted to proliferate *in vivo*. Evidence from several studies suggests that *S. mutans* also undergoes adaptive changes in laboratory animals and humans who harbor complex microbiotas.[9,10,20] We have been able to directly confirm this hypothesis by making use of a reliably marked strain that is also capable of persistently colonising the human oral cavity.

Fresh isolates of strain JH1005 were recovered and characterised using methods that minimised their opportunity to adapt to laboratory conditions. Laboratory stock isolates were recovered using identical methods in order to assure that the immediate histories of the fresh and laboratory isolates were the same. Thus, phenotypic differences that were observed between fresh and laboratory adapted isolates are likely to be due to changes that have occurred at the genetic level as part of the natural microevolutionary process.

The selective adhesion of bacteria to surfaces has been shown to be an important ecological factor.[8,12] The natural distribution of mutans streptococci in the oral cavity can largely be explained by its predilection for attaching to tooth surfaces. In the present study, we found that *S. mutans* adapted to growth *in vivo*, exhibited reduced adhesion to saliva-coated hydroxyapatite. A similar result has been reported for the attachment of *S. salivarius* to epithelial cells following adaptation of this organism to growth in the gnotobiotic rat.[20] These findings could be interpreted as indicating that, at least in certain cases, selective adherence is most important to initial colonisation; once attached to the desired tissue, other factors, such as glucan production in the case of *S. mutans*, probably assume greater importance for the continued maintenance of the organism in its ecological niche.

Previously, antigenic drift has been demonstrated to occur in viridans streptococci colonising the oral cavities of rodents.[1,2,3,13,14] In one case, both the strain and serotype antigens of mutans streptococci were found to vary within a period of several months following infection of gnotobiotic rats.[2] Although saliva from the clinical subjects in this study could be shown to contain antibodies reactive with the serotype antigen of strain JH1005 (data not shown), none of the fresh isolates recovered from these subjects three years after infection had altered antigenic composition as determined by double

diffusion, immunoelectrophoresis, and radioimmunoassay using monoclonal antibodies. This discrepancy might reflect the differences between rodent and human hosts and simple and complex microfloras. Mutans streptococci constitute a much greater proportion of the plaque flora in conventional rats than in humans,[30] and therefore probably provide a greater immunogenic challenge. Since little is known of the biological role of cell surface antigens, we cannot speculate about the ecological significance of their resistance to antibody-induced drift during prolonged colonisation of the human oral cavity.

Adaptation of *S. mutans* from the rich environment of laboratory culture medium to the more stringent conditions of the oral cavity was expected to select for certain changes in central intermediary metabolism. *S. mutans* colonising the human oral cavity is estimated to undergo only two or three doublings per day.[11] This represents a small fraction of the growth potential for this organism *in vitro*. The basis for this difference undoubtedly is attributable to the transitory supply of essential nutrients *in vivo*. The ability of *S. mutans* to survive prolonged periods of starvation and to make efficient use of nutrients when they become available are obvious selective advantages. Isolates of JH1005 adapted to growth *in vivo* have an increased dependence on heterofermentative pathways during aerobic cultivation as evidenced by increased levels of acetoin produced for a given amount of glucose consumed. As a result of this change, cultures of JH1005 adapted to growth *in vivo* were less acidic (which has also been reported for *S. mutans* colonising the oral cavity of gnotobiotic rats)[14] and had increased cell yields. This latter is presumably due to increased NADH and ATP production associated with heterofermentative pathways such as that mediated by pyruvate dehydrogenase. Since pyruvate dehydrogenase in this organism is thought to function only under aerobic conditions,[6,19] these findings suggest that *in vivo* exposure of *S. mutans* to oxygen can act as a selective pressure.

Variation in colonial morphology is commonplace with mutans streptococci, particularly the transition between rough and smooth colony types. This transition occurs readily both during *in vitro* and *in vivo* growth and has been correlated with changes in levels of glucan and plaque production.[14,21] In the present study, we observed no variations in colony morphology. This result probably reflects the fact that the fresh isolates were recovered from plaque taken from smooth surfaces where it is expected that persistence is heavily dependent on maintaining a firm attachment, such as that afforded by glucan production. We did observe a slight (12 per cent) increase in total mass production by fresh isolates of JH1005. This difference may be a pleiotropic effect: the higher terminal pH of cultures of the fresh isolates probably allows for greater activity of glucosyl transferase, which in one case was shown to have a pH optimum of ca. 6·5.[7] This possibility was not investigated further.

Bacteriocin production by *S. mutans* is generally thought to be a stable phenotypic property.[23] In this study, we observed no differences between the fresh and laboratory adapted isolates in the spectrum of activity or the quantitative production of the bacteriocin-like molecule of JH1005. The nutritional requirements of this strain also appear to be a stable phenotypic property under the conditions that we studied them. Strain JH1005 had the same vitamin and salts requirements as strain JC2 described by Carlsson[4] but required several additional amino acids (glutamic acid, lysine, arginine, and histidine). The results of this study suggest that the nutrient requirements are supplied *in vivo* by the host, diet, and microflora to the extent that they do not act as observable selective pressures. The limiting factor for the growth of *S. mutans in vivo* is therefore likely to be the availability of a fermentable carbon source.

The model system developed for these studies has shown itself capable of identifying certain adaptive changes in *S. mutans* that occur when it is introduced from the laboratory back into its natural habitat, the human oral cavity. These changes are of interest both from the standpoint of increasing our basic understanding of the ecology of this organism in the human host and from the practical standpoint of developing replacement therapy, vaccines, and other therapeutic methods. In the case of replacement therapy, an effector strain that has been preadapted to conditions manifested in the human oral cavity would probably colonise more readily and with greater efficiency. For this study, we chose to examine phenotypic properties which are virulence related and which we felt would be subject to significantly different selective pressures during *in vitro* and *in vivo* cultivation. The finding that no statistical differences were observed between the isolates obtained from three different subjects with regard to these phenotypic properties argues in favour of the general nature of the selective pressures being investigated. Except in certain cases (e.g., antigenic drift),

the usefulness of this model is limited to identifying selective pressures that effect phenotypic properties which change in a measureable fashion during the transition of *S. mutans* from the oral cavity to the laboratory or back. There are undoubtedly important selective pressures that would not be revealed because they do not induce such changes.

ACKNOWLEDGEMENTS

This work is supported by grants DEO4529 and DEO7009 from the National Institute of Dental Research.

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