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GENETIC DIVERSITY AND THE INFLUENCE OF TRADITIONAL AFRICAN FOODS ON THE VIRULENCE OF MUTANS STREPTOCOCCI ISOLATES FROM SOUTH AFRICAN CHILDREN

Cheryl Sam Toi

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree

of

Doctor of Philosophy

Johannesburg, 2005

DECLARATION

I, Cheryl Sam Toi declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

.....

.....day of....., 2005

The research reported in this thesis was carried out in the MRC/Wits Dental Research Institute, Johannesburg, South Africa

In memory of my father

Sam Sue Sun

1930-2004

and

dedicated to my mother Alice

whose encouragement and patience make all things possible.

PRESENTATIONS

The following presentations have arisen from material included in this thesis.

1. XXXIV Scientific Meeting of the South African Division of the IADR, Cape Town, 13-15 September 2000.

   Toi C, Cleaton-Jones PE. Restriction Fragment-Length Polymorphisms of Mutans Streptococci from Mother-Child Pairs.

2. XXXVI Scientific Meeting of the South African Division and XVI Meeting of the East and Southern Africa Division of the IADR, Durban, 15-17 August 2002.

   Toi CS, Cleaton-Jones PE. Acid production by Mutans Streptococci Growing on Traditional African Foods.

3. XXXVII Scientific Meeting of the South African Division of the IADR, Cape Town, 10-12 September 2003.

   Toi CS. Glucose Utilization and Extracellular Polysaccharide Production by Mutans Streptococci on Traditional African Staple Foods.

4. XXXVIII Scientific Meeting of the South African Division of the IADR, Pretoria, 2-4 September 2004.

   Toi C. Traditional African Foods and Glucosyltransferase Gene Expression in Mutans Streptococci.

ABSTRACTS AND PUBLICATIONS

The following abstracts and publications have arisen from material included in this thesis.

Abstracts

1. Toi CS, Cleaton-Jones PE. Restriction Fragment-length polymorphism of mutans streptococci from mother-child pairs. *J Dent Res* 2001; 80: Abstr no. 34 p 1365.

2. Toi CS, Cleaton-Jones PE. Acid production by mutans streptococci growing on traditional African foods. *J Dent Res* 2003; 82 (Spec Iss C): Abstr no. 24 p C614.

Publications

1. Toi C, Cleaton-Jones P. In vitro growth and acid production by mutans streptococci on traditional African staple foods. *Anaerobe* 2004; 10: 335-342.

2. Toi CS, Cleaton-Jones P, Fatti P. Characterization of *Streptococcus mutans* diversity by determining restriction fragment-length polymorphisms of the *gtfB* gene of isolates from 5-year-old children and their mothers. *Antonie van Leeuwenhoek* 2005; 88: 75-85.

ABSTRACT

Since the early 1980's, global trends in dental caries have indicated that 80% of the caries is present in approximately 20% of the population which suggests a variation in susceptibility to the disease. In Sub-Saharan Africa and in South Africa, caries prevalence has shown a downward trend in preschool and school children. The reasons for this decline are obscure and have not been attributed to dietary habit, oral hygiene, the use of fluoride dentrifices or to any public health prevention program. Furthermore, the numbers of mutans streptococci, a group of pathogens associated with dental caries, have remained similar in children with and without caries. This implies that good dental health is possible in the presence of high prevalence of mutans streptococci, but raises speculation that the decrease in dental caries, may be caused by a change in virulence of these strains. It is also unclear if these bacterial strains are acquired through inter-familial transmission or genetically altered by influences from the oral environment.

This thesis reports the first studies of gene expression and bacterial virulence in relation to traditional African-foods using clinical isolates from South African children. To establish the source of transmission, the phenotype and genotype of mutans streptococci strains from 31, five-year-old black, and coloured, children and their mothers living in Gauteng were characterized. The children were examined for caries, and plaque and salivary samples collected from both the children and their mothers. Samples were selectively cultured for mutans streptococci, biochemically differentiated and the genetic diversity of these isolates determined by PCR-RFLP of the *gtfB* and *gtfI* glucosyltransferase virulence genes. Phenotyping showed that *Streptococcus mutans* were 90% (155/172) prevalent, but the detection of *Streptococcus sobrinus* was low and comprised 10% (17/172) of the remaining

isolates. Twenty-six percent of *S. mutans* clinical isolates (41/155) did not metabolise melibiose and the *gtfA* gene encoding for the uptake of this sugar was absent in 26 of the 41 melibiose-negative strains.

GtfB gene polymorphisms in *S. mutans* clinical isolates from the two ethnic populations and from caries-free and caries-active 5-year-old children were similar (Principal Components Analysis). However high genetic diversity was observed in *S. mutans* isolates, with 23 different *gtfB* amplitypes shown by PCR-RFLP analysis. Sixteen different *gtfI* amplitypes were indicated in *S. sobrinus* clinical strains. The percentage match between *gtfB* amplitypes (*Hae*III enzyme digests) in the children and their mothers ranged from 3% to 9% in caries-free children and caries-active children, respectively. Identical *gtfB* amplitypes from melibiose-negative phenotypes were shared by four mothers and their children only.

To determine the growth and virulence response of the variant mutans streptococci genotypes, six laboratory reference strains (NCTC) and five, clinical isolates were challenged to traditional African staple foods and food combinations. The bacteria were exposed in batch culture for 16 hours to maize, samp, brown bread, maize+milk+sugar, maize+gravy, samp+beans, brown bread+margarine+peanut butter, 3% sucrose and a synthetic complex medium, BHI+3% sucrose. Results showed that growth was slow in maize (5.6 h) and samp (5.7 h) indicated by the long doubling time and the low number of generations (g = 4.2; g = 3.6). Sufficient lactic and acetic acid was produced to drop the pH to below 5.7, the 'critical' level for enamel demineralisation during the fermentation of brown bread (5.37), bread+margarine+peanut butter (5.51) and 3% sucrose (5.32). Water-insoluble extracellular polysaccharides produced was significantly lower (P<0.05) in samp and maize and

maize+milk+sugar, with most residual glucose found in BHI + 3% sucrose (1.61±0.52 mg/mL) and maize+gravy (0.51±0.61 mg/mL). Mean concentrations of extracellular protein ranged from a low of 0.015±0.007 mg/mL in samp+beans to a high of 0.29±0.16 mg/mL in BHI + 3% sucrose.

The inherent pH of individual foods closest to neutral was: milk (7.0) beans (6.07), samp (6.20), maize (6.82) and peanut butter (6.90). However, beans (5.7×10^{-3} [H^+]), milk (5.1×10^{-3} [H^+]) and peanut butter (4.0×10^{-3}[H^+]) showed more efficient buffering, which in combination with other food components, raised the inherent buffering capacity of the food. These mixed foods required a larger quantity of acid to be produced by bacterial metabolism to lower the pH to 5.7.

A preliminary study on the effect of single foods on *S. mutans gtfB* gene expression showed that mRNA *gtfB* transcripts were mostly inhibited in clinical isolates, but not in reference strains. The *gtfI* gene of all *S. sobrinus* test strains was expressed in 3% sucrose and BHI+3% sucrose, but the response differed in the remaining foods.The statistically significant association shown between mutans streptococci phenotype, *gtf* gene expression and the food challenge ($P<0.0001$), verifies that expressed phenotypic characteristics is dependent on gene expression.

The results presented in this thesis show that a high diversity of *gtf* genes exists in mutans streptococci clinical isolates from South African black African, and coloured, 5-year-old children and their mothers. However, no specific genotype was unique either to dental status or ethnic population, with children acquiring genotypes from other points of contact besides

the mother. Furthermore, the growth and virulence response (acidogenesis, water-insoluble ECP) of *S. mutans* and *S. sobrinus* genotypes and reference strains are subject to the dietary nutrients available. The inherent buffering capacity of maize, samp, maize+milk+sugar, maize+gravy and samp+beans, coupled to a low sucrose content, make these foods non-caries promoting, but the ability of test bacteria to remain viable on these nutrients, indicate that the mutans streptococci have a natural adaptive ability to assimilate other nutrients as a source of carbon when sucrose is limited. The control of *gtfB* and *gtfI* gene expression by traditional African foods suggests that virulence of the mutans streptococci are influenced more by the dietary environment than by genotype. Also, the difference in virulence properties between clinical and laboratory reference strains indicate an attenuation in virulence of wild-type strains.

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LIST OF ABBREVIATIONS

arbitrarily primed polymerase chain reaction	AP-PCR
ammonia	NH_3
base pairs	bp
basal synthetic medium	BSM
bovine serum albumin	BSA
brain heart infusion	BHI
carbon dioxide	CO_2
caries-active	c
caries-free	cf
colony-forming units	cfu
copy DNA	cDNA
cytosine	C
decayed surfaces	ds
decayed, missing and filled surfaces	dmfs
deoxyribose nucleic acid	DNA
dithiothreitol	DTT
Early Childhood Caries	ECC
enzyme commission	EC
ethylene diamine tetra-acetic acid	EDTA
extracellular polysaccharide	ECP
fructosyltransferase	FTF
gas-liquid chromatography	GLC
general linear models	GLM
glucosyltransferase	GTF
glucan binding proteins	GBP
guanine	G
hydrochloric acid	HCL
hydrogen	H_2
hydrogen peroxide	H_2O_2
hydroxyapatite	HA

internal diameter	ID
intracellular polysaccharides	IPS
kilobase	kb
kilodalton	kDa
magnesium chloride	$MgCl_2$
milliamperes	mA
microlitre	µL
micromole	µM
millimole	mM
milligram	mg
millilitre	mL
molar	M
molecular weight marker	MWM
multiple sugar metabolism	*msm*
mutans streptococci	MS
nanometer	nm
National Culture Type Collection	NCTC
nitrogen	N_2
optical density	OD
periodic acid-Schiff	PAS
phospho-transferase transport system	PTS
picogram	pg
Polymerase Chain Reaction Restriction Fragment-Length Polymorphism	PCR-RFLP
potassium chloride	KCL
randomly amplified polymorphic DNA	RAPD
restriction endonuclease	RE
restriction fragment-length polymorphism	RFLP
reverse transcription	RT
ribose nucleic acid	RNA
seconds	s
sodium chloride	NaCl
salt tris-EDTA	STE

sodium dodecyl sulphate-polyacrylamide gel electrophoresis	SDS-PAGE
total viable counts	TVC
tris-EDTA	TE
tris-borate-EDTA	TBE
trypticase, yeast, cystine, bacitracin	TYCSB
units	U
ultra violet	UV
unweighted pair-group matrix analysis	UPGMA
volt	V
World Health Organisation	WHO

CHAPTER 1

INTRODUCTION

1.1 Preface

Dental caries is the single most common chronic disease of all oral diseases (1) with prevalence beginning soon after tooth eruption in susceptible children, increasing with age. It is a localized, progressive demineralization of the hard tissues of the crown (enamel, dentine) and root (cementum, dentine) surfaces of teeth and is seldom life threatening but causes considerable pain. The demineralization is caused by acids produced by bacteria, particularly mutans streptococci and lactobacilli that act as secondary invaders and ferment dietary carbohydrates. This occurs within a gelatinous material called dental plaque, a biofilm that adheres to tooth surfaces and becomes colonized by bacteria. A significant constituent of dental plaque is the water insoluble ∞-(1,3)-rich glucans synthesized by the gtfB and C enzymes. These glucans facilitate the adherence and accumulation of stable biofilms that are mediated by a number of glucan binding proteins (2).

The oral cavity provides an ideal environment for the growth and survival of bacteria. The saliva, gingival crevicular fluid, and the host's diet provide a constant variety and supply of nutrients that affect the microbial ecology of the mouth, and are important factors in caries risk (3, 4). These nutrients are catabolized to acids which acidify plaque biofilms, inhibiting most of the species associated with enamel health while promoting the growth of aciduric bacteria such as mutans streptococci. Hence, an ecological shift in the composition and

metabolic activity of the biofilm on the tooth surface (5), coupled to the behaviour of individuals and societies (6), produces for dental caries.

1.2 Mechanism of dental caries

Dental caries is the progressive destruction of tooth enamel, dentine and cementum by action of bacteria in dental plaque. This destruction is caused by the demineralization of tooth hydroxyapatite by acids produced during carbohydrate metabolism by acidogenic bacteria. Two schools of thought exist for the level of 'critical pH', whereby a sufficient fall in plaque pH leads to the dissolution of enamel. The Swiss school is based on the intraoral plaque pH telemetry test, where dental caries is likely to occur at a pH of below 5.7 (7). The other is based on the Stephan curve where enamel demineralization is at pH 5.5 (8-10). The norm is, provided the pH remains below 5.7, the point at which saliva and the fluid phase of plaque is saturated with respect to calcium and phosphate (11), acid formation causes the dissolution of these compounds in the hydroxyapatite (HA) crystal structure:

$$Ca_{10}(PO_4)_6(OH)_2 \underset{\text{base [2]}}{\overset{\text{acid [1]}}{\rightleftharpoons}} 10\,Ca^{2+} + 6PO_4^{3-} + 2OH^-$$

With a consistent acid challenge over time, HA crystals are eroded that results in small increases in enamel porosity, but because the saliva is supersaturated with calcium and phosphate, the surface mineral becomes less porous than the subsurface and the lesion develop beneath an apparently intact enamel surface. This continues to grow before the visual appearance of the white spot lesion which develops into an overt lesion in which cavitation of the surface mineral layer occurs (12).

1.2.1 The microbiological course of dental caries

Bacteria rarely come into contact with a clean tooth surface. A pellicle is formed by the selective adsorption of salivary glycoprotein and proteins, bacterial components, polymers from gingival crevicular fluid onto the enamel surface (13, 14). This bacteria-free organic film occupies a critical position between the enamel surface and dental plaque, and colonizing microbes interact with this polymeric layer.

Caries is a plaque-associated infection and there are two main schools of thought on the role of plaque bacteria in the aetiology of caries. The Non-Specific Plaque Hypothesis proposed by Miller in 1890 (15) considers that any of the heterogeneous mixture of bacteria can play a role in disease aetiology. A new theory - the Specific Plaque Hypothesis that was developed after 1975 (16), proposes only a limited number of species are involved in causing disease. More recently, the Ecological Plaque Hypothesis proposed by Marsh (4) better describes the relationship between dental disease and shifts in the balance of the resident plaque microflora, mediated by factors such as nutrition and oral hygiene. This hypothesis succinctly implies that potentially cariogenic bacteria can be present in health, but at levels that are not clinically relevant.

The acquisition of oral bacteria by infants occurs by ecological succession, pioneer species (first bacterial colonizers) are succeeded by a climax community of high species diversity, in which allogenic succession occurs. In allogenic succession, factors of non-microbial origin are responsible for an altered pattern of community development (17, 18). For example, mutans streptococci and *Streptococcus sanguis* only appear in the mouth once teeth have erupted (19-21) and during a “window of infectivity” at 19-31 months where children are most likely to

acquire mutans streptococci species (22).

The microbial course of dental caries is best explained by Bowden's scheme (23) (Figure 1.1). Stage 1, represents the predominant pioneer species comprising primarily of streptococci acquired at birth (21, 24). Some bacteria such as the *Actinomyces, Lactobacillus and Rothia* are commonly isolated after tooth eruption (25) followed by colonization by *Streptococcus mutans*.

Stage 2 is dependant on the expansion of the *S. mutans* population, and on the interruption of the demineralization-remineralization balance leading to initial enamel dissolution. This may occur by loss of salivary buffer or through high carbohydrate (CHO) diets. Demineralization may now reach a point where a clinical white spot appears (Stage 3) and the levels of *Lactobacillus* increase. In Stage 4, loss of surface integrity and pitting occur. Throughout these stages, there are shifts in the bacterial community. In progressive lesions, strains of *Streptococcus mitis* and *Actinomyces viscosus* increase in numbers in association with *S. mutans* and *Lactobacillus*, whereas *Actinomyces naeslundii* and *Streptococcus sanguinis* are eliminated (23).

In summary, dental caries is a multi-factorial disease that develops over time from the interaction of cariogenic bacteria present in dental plaque; the diet, which provides a source of fermentable carbohydrate, and susceptible teeth of the host as well as the host immune response (26-28). These factors do not act in isolation but form parts of a group in promoting dental caries.

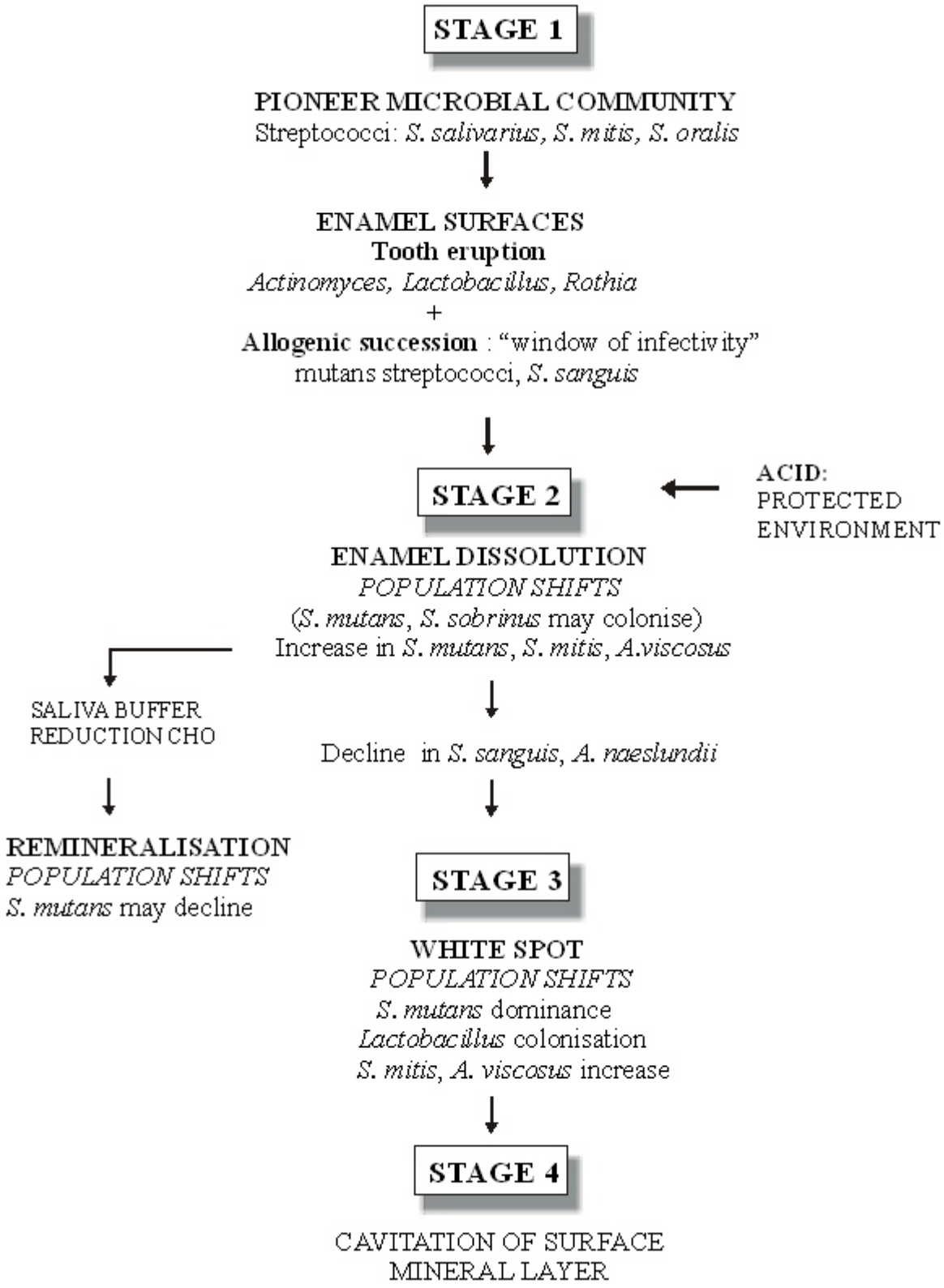

Figure 1.1 The microbial course of dental caries (23)

1.3 Global epidemiology of dental caries

Since the early 1980's, there has been a global decrease in dental caries among children (29-33), and in many industrialized countries 80% of the caries is present in approximately 20% of the population (34). Despite the decline in dental caries, there is still a problem for health service providers and dental treatment is costly. Some studies have attributed the decrease in caries to water fluoridation and application of fissure sealants (35, 36). Other studies have reported a higher incidence of caries in children not exposed to incident domestic water fluoridation (37, 38) to support the preventive role of fluoride.

1.3.1 Dental caries in Africa and South Africa

Studies on caries prevalence in Sub-Saharan Africa have shown a downward trend in 5-6 year-olds and 11-13 year-olds, while in the Middle-East and North Africa, caries prevalence has remained constant over a 30-year review period (39, 40). In South Africa, repeated cross-sectional epidemiological surveys of dental caries between 1981 and 1997 in Germiston, the largest industrial city in Africa, have also shown a downward trend of this disease in 2-5 year-old nursery school children (41). However, the most recent in 2002, cross-sectional study on pre-school children in the same nursery schools in Germiston, has indicated that in primary teeth, caries rates are on the increase in this low fluoride area (42). Although dental caries severity in South Africa is classified as low by World Health Organisation standards, the high level of untreated caries in all age groups is cause for concern (43) and South African children of 5-years and younger shows a prevalence of 46.6 % of untreated dental caries. This is due to lack of access to dental services through non-availability, or non-utilization of available services.

1.4 Factors influencing dental caries

1.4.1 Mutans streptococci

The mutans streptococci are normal resident oral micro-flora that colonises non-shedding surfaces such as pits and fissures of the occlusal surfaces of the teeth. They possess virulent cariogenic properties expressed only under specific environmental conditions. Some fermentable carbohydrates promote virulence such as acid and extracellular polymer production more than others, that can influence streptococcal strain selection and survival in the mouth.

1.4.1.1 Discovery of mutans streptococci as a cariogenic bacterium

In 1924, Clarke isolated the first organisms from human carious lesions and called them *Streptococcus mutans*, but because other investigators were unable to find this bacterium, this organism was soon forgotten (44). It was rediscovered in the 1960s (45), where it was demonstrated that caries could be experimentally-induced and transmitted in rodent models artificially-infected with strains resembling *S. mutans.* The name derives from the fact that cells can lose their coccal morphology and often appear as short rods or as cocco-bacilli. Once they sampled plaques from single carious sites or saliva from caries-active individuals, *S. mutans* were routinely associated with human dental caries. Reasons for the failure to isolate *S. mutans* in the past were probably due to low levels in plaque and saliva of individuals without dental caries.

The normal oral flora is composed of a variety of diverse microorganisms, dictated by the conditions and properties of the mouth.The streptococci have been isolated from all sites in

the mouth and comprise 28% of the cultivable micro-flora from supra-gingival dental plaque, 29% from the gingival crevice, 45% from the tongue and 46% from saliva (46). Early bacterial colonizers of infants are mainly streptococci, namely *S. sanguinis*, *S. oralis* and *S. mitis* biovar 1 (47, 48). These species comprise 96% of the streptococci and 56% of the initial microbial flora (49). Mutans streptococci and *S. sanguinis* appear in the mouth following tooth eruption (19, 20). To-day, the mutans streptococci group of bacteria are the most implicated in the aetiology of dental caries.

1.4.1.2 Classification of mutans streptococci

After *S. mutans* were collected from different sources, it became apparent that serological and genetic heterogeneity existed. Since 1970, eight serotypes (*a-h*) have been described, based on the serological specificity of carbohydrate antigens located in the cell wall (50, 51). Classification of these bacteria by Coykendall (52), sub-divided *S. mutans* into four subspecies: subspecies *mutans* (serotypes c,e,f), *sobrinus* (serotypes d,g), *rattus* (serotype b) and *cricetus* (serotype a). This was based on biochemical differences observed during the production of acid from raffinose, salicin or melibiose and sorbitol, aesculin hydrolysis, hydrolysis of arginine, and the production of hydrogen peroxide and bacitracin sensitivity. Differences also existed between clusters of these serotypes in the electrophoretic mobility of enzymes, in their profile of cell wall proteins, in peptidoglycan type, and in DNA base composition (46).

These subspecies were later elevated to species status as *S. mutans, S. sobrinus, S. cricetus, S. rattus* and *S. ferus* (53, 54). Subsequently, mutans streptococci have also been classified into five biotypes (I-V) according to their responses to biochemical tests (51, 52, 55). *Streptococcus*

mutans was assigned to those human isolates that resembled Clarke's original description and the representative strain of *S. mutans* is currently present in the National Collection of Type Cultures under the number NCTC 10449.

Recently, with the classification of oral streptococci by phenotypic characterization and phylogenetic determination of 16S rRNA sequences, seven distinct species of human and animal mutans streptococci have been described and are named according to their original source of isolation (56). These species are listed in Table 1.1 and have been designated into the 'Mutans group'. The 'mutans streptococci' designation generally refers to studies prior to reclassification when it was not possible to assign the newer name, or where a semi-selective medium was used eliminating organisms not in the mutans streptococci group (12).

1.4.1.3 Species most implicated in human caries

According to dental researchers, two species of the mutans streptococci, *S. mutans* and *S. sobrinus* are specifically implicated in enamel caries in early childhood caries (ECC) and in adults (22, 27, 48, 57-60). Studies on plaque samples from various parts of the world indicated that 70-100% of the human mutans streptococci strains can be classified as *S. mutans* (45). Children with early childhood caries have also shown more than one clonal type of this species that have been implicated in the presence of high sucrose consumption (61). *Streptococcus sobrinus* are isolated at a lower frequency than *S. mutans* while *S. cricetus* and *S. rattus* are recovered only rarely.

Table 1.1 Classification and differential characteristics of the mutans streptococci group (45, 46)

Species	Serotype/s	G+C DNA content (mol%)	Host
Streptococcus mutans	*c, e, f*	36-38	Human
Streptococcus sobrinus	*d, g*	44-46	Human
Streptococcus cricetus[a]	*a*	42-44	Human
Streptococcus ferus	*c*	43-45	Rat
Streptococcus rattus[a]	*b*	41-43	Human/Rodent
Streptococcus macacae	*c*	35-36	Monkey
Streptococcus downei	*h*	44-46	Monkey

[a] Strains predominant in only some parts of the world.

Other species of bacteria reported to be associated with stages in the development of caries are the *Lactobacillus* species, *L. casei*, *L. fermentum* and *L. acidophilus*. These species colonize teeth in low numbers but increase on the appearance of a incipient (white spot) lesion and increase to significant levels in cavities. The *Actinomyces* species that colonize the gingival crevice, are known to increase in numbers during gingivitis and have been associated with root surface caries. Of the Actinomyces species, *A. naeslundii* genospecies 2 is most associated with root lesions (62).

The cariogenic bacteria do not occur in isolation, but as caries progresses, a succession of bacterial communities are created with the numbers of specific genera and species increasing or decreasing through natural selection by environmental forces. The result is a different microbial composition at the advancing front of the lesion compared to the original surface

plaque (12).

1.4.1.4 Virulence mechanisms of mutans streptococci

Virulence is defined as the capacity of a pathogenic microorganism to cause disease (63). The mutans streptococci possess the following virulence factors that contribute towards the caries process (64, 65):

1. Aciduricity, or the ability to survive at low pH levels in cariogenic plaque.
2. Strong acidogenicity, the ability to produce large quantities of acid as a by-product of carbohydrate fermentation.
3. Ability to synthesize water-insoluble glucan polymers from dietary sucrose, promoting attachment to the tooth pellicle.

The synthesis of extracellular polysaccharides that facilitate bacterial attachment to teeth by forming plaque, are controlled by the *gtfB*, *gtfC*, *gtfD* and *ftf* genes that regulate the transcription and translation of the enzymes glucosyltransferase (GTF: EC 2.4.1.5) and fructosyltransferase (FTF: 2.4.1.10), respectively that catalyse the production of these glucans (66, 67). These enzymes, together with an accumulation of acid, such as lactic are produced within the plaque as bacteria metabolise and grow, resulting in enamel demineralization.

Streptococcus sobrinus differ in virulence from *S. mutans* in that it produces the enzyme GtfI that synthesizes water-insoluble glucans.This gene has a high sequence identity to GtfB but is primer dependent for polysaccharide synthesis, compared to the GtfB and GtfC enzymes which are not. Therefore, the presence of sucrose is important in the colonisation of teeth by *S. sobrinus* because it lacks lectin-like hydrophobic ligands, called adhesins (68). Another virulence characteristic of *S. sobrinus* is that it produces more acid, although animal

experiments have shown that *S. mutans* strains are more cariogenic (69, 70).

Following the recent application of molecular techniques, strains within a species could be differentiated on the basis of genetic variation allowing specific clonal types to be recognized. Kilian (71) showed that relatively few clones are found within species of pathogenic bacteria, and a limited number of these may be responsible for the majority of infections. Clones of some species persist for long periods at a site, whereas others are transient and are replaced by a distinct clone. The reasons for, and the mechanisms involved in this 'clonal turnover' are not yet understood, but suggestions are that this 'turnover' might play a role as an "immune-evasion" mechanism for the species.

Investigations in animal models have shown that clones of *S. mutans* vary in virulence (72) and the selection of more virulent clones within a host population can influence dominance by this species. Clone diversity and the clonal relationship between mutans streptococci species is still unknown in South African children and their mothers. Given the decrease in dental caries, but with high prevalence of mutans streptococci, it can be speculated that the decline in caries in South African populations is perhaps due to differences in virulence among the species of mutans streptococci present in these groups.

1.4.1.5 Global epidemiology of mutans streptococci

According to Bratthall (73), numerous studies have shown that only two of the five *mutans* group, *S. mutans* and *S. sobrinus* were present in all populations studied. In Icelandic school children, *S. mutans* (98%) appeared more prevalent than *S. sobrinus* (30.2%).The children infected with high levels of *S. mutans* had *S. sobrinus* strains as well (74). Also, persons with

both species generally had higher salivary levels of both bacteria than individuals with only one species. It is possible that the differences in attachment properties of these bacteria to the pellicle receptors on the tooth surface can result in different relations between these two strains (75), depending on local differences in the composition of diet. *Streptococcus mutans* serotype *c* is also more common than serotypes *e* and *f*. To-date, too few studies differentiating between *Streptococcus sobrinus* serotypes *d* and *g* have been done to formulate firm conclusions.

1.4.1.6 Prevalence of mutans streptococci in different populations

A review of the literature has shown that the isolation frequency of mutans streptococci in various populations have been dependent on methods used to cultivate these microorganisms. Variables that influenced results ranged from the cultivation medium, through methods of sampling, to the study subject (73). The general consensus was that saliva sampling, although approximate, is the preferred method for epidemiological studies, although more recent studies have indicated that screening plaque mutans streptococci was more effective than salivary mutans streptococci when caries experience was present at baseline (76).

Recent studies have shown that the isolation frequency of mutans streptococci, in relation to dental caries, differs between countries and populations. In Brazil (77), Uruguay (78), Latvia (79), Iceland (80) and Stockholm (81), preschool children with high counts of mutans streptococci had higher dmfs scores. In contrast, Afro-Caribbean children had lower levels of dental caries than Caucasian children living in the same South London boroughs, attending the same preschool care facilities and after controlling for age and caries experience, they also had lower salivary levels of mutans streptococci (82).

Closer to home, results from investigations in the Dental Research Institute have also shown a low, yet significant correlation between the levels of mutans streptococci and dental caries in 5-year-old children from KwaZulu (r^2=0.12 [12%]), Namibia (r^2=0.18-0.45 [18-45%]) (83) and in Gauteng (black African: r^2=0.05 [5%]; coloured: r^2= 0.12 [12%]) (84, 85). However, Bratthall (73) points out that mutans streptococci prevalence studies in different populations must be considered in relation to caries, socio-economic grouping, dietary habits, fluoridation of drinking water, age, gender and oral hygiene, since country-to-country differences have been demonstrated.

1.4.1.7 Acquisition of mutans streptococci

Dental decay in young children is collectively known as 'early childhood caries' or ECC (86). Early childhood caries is preceded by the acquisition of mutans streptococci after tooth eruption (20, 22) when enamel surfaces are available for stable colonisation. Acquisition occurs during a discrete period known as the 'window of infectivity' which is usually between eight and 52 months of age (20, 87). The initial acquisition of mutans streptococci by male and female children have been primarily through maternal than from paternal lines, or from extra-familial acquisition (88, 89).

The examination of transmission in different cultural groups have shown that mutans streptococci strains from Japanese and Swedish children were most similar to their mothers (90), whereas in Chinese families, the father played a more pronounced role as mutans streptococci source (91, 92). This suggests that the source of infection can vary from family to family and that the children's environment plays a role in the initial acquisition and transmission of mutans streptococci. Vertical transmission is not the only vector by which

mutans streptococci are perpetuated in human populations. Children of nursery school age contained identical genotypes of strains, suggesting that horizontal transmission may be another vector for acquisition of these organisms (93). Hence, dental caries is an infectious and transmissible disease which is strongly modified by diet (94). To-date, no such study has been reported in South African child populations.

1.4.2 Human dietary factors

In modern societies, current dietary recommendations emphasise the reduction of dietary fat and the increased consumption of starch-rich foods, which raises the frequency of a carbohydrate intake in the form of sugars and starches. This complex array of foodstuffs provides a source of exogenous nutrients to the oral micro-flora, and in particular the mutans streptococci. Variability in the frequency and type of food ingested can cause fluctuations in the stability of the bacterial ecosystem, contributing towards caries risk (3, 4). Carbohydrates influence the ecology of the mouth by increasing the proportions of mutans streptococci and lactobacilli in plaque, and decreasing acid-sensitive species. A shift to a homo-fermentative pattern of metabolism occurs, with lactic acid as the predominant fermentation product (45). Investigations have also suggested that the low pH generated from sugar metabolism rather than excess carbohydrate was responsible for population shifts in the oral micro-flora (95, 96).

While a strong sugar/caries relationship has been accepted for many years (97), some studies have shown that sugar consumption and caries prevalence are virtually independent of one another (98-101). Although poor oral hygiene in children consuming high-sugar foods does influence the salivary levels of mutans streptococci and are strongly related to a caries increment (102, 103), sugar-starch combinations too, are a complex problem and uncertainty

reigns (104, 105).

A change in population dietary patterns by the selection of low-fat, low sugar foods and widespread exposure to various fluoride vehicles have also altered the diet-dental caries dynamic that once existed (105, 106) and diet can have a limited influence on caries (3, 107). This implies that good dental health is possible with the presence of cariogenic factors in the diet, provided that there is good oral health care.

1.4.2.1 Inherent cariogenicity of foods

The methodology for assessing the cariogenic and erosive potential of foods was first laid down at the San Antonio conference in 1985 (108). These guidelines have subsequently been updated in a 1999 workshop, with new guidelines been drawn up (109), that provides a framework for the standardisation of future models. To-date, numerous studies have been done on evaluating the cariogenic potential of different foods. These have ranged from *in vivo* studies in humans (7, 110, 111) and experimental animals (112-115) to *in vitro* studies (115-119). Despite these various methods, there is no simple scientific method to predict the individual rate of caries incidence in children.

A review by Seow (28) on individual foods, reported that sucrose, glucose and fructose in drinks as well as in solids are probably the main sugars associated with early childhood caries. Sucrose, a widely used sugar is the only substrate used by bacteria to generate plaque dextrans which are essential for bacterial adherence (120). Coupled to this, the frequent eating of specific foods promotes caries risk by increasing plaque acidity and enhancing the establishment and dominance of the aciduric mutans streptococci (59). *In vitro* studies have

indicated that fructose and glucose are as cariogenic as sucrose in their abilities to drop the pH in plaque and to demineralise enamel (121, 122). Yet, raw starch produced only a small drop in plaque pH (123), but soluble starch and refined starch containing foods such as bread or biscuits cause a variable pH fall which may be as large as that caused by sugars (123, 124). Investigations made on dairy products, specifically milk and cheese, have shown no cariogenic effect, and findings concluded that these foods even exerted a protective function (28, 125).

1.4.2.2 Dietary factors and dental caries

Despite differences in the cariogenic potential of different foods, diet does have a limited influence on caries (100, 102), and the relationship between sugar consumption and caries is much weaker in the modern age of fluoride exposure than before (101). A systematic review on dietary sugars and caries, by Anderson and Curzon (126) concludes that no relationship was found between the quantity of sugar intake and dental caries, and there was only weak evidence to support an association between frequency of sugar consumption and dental caries.

In South Africa, studies on urban and rural black preschool children have also shown that nutrient intake was not strongly clinically relevant to caries prevalence and experience (127, 128). The numbers of mutans streptococci have remained similar in children with and without caries, and with low correlation to dental caries (84). This suggests that mutans streptococci strain selection and survival in the mouth can be influenced by fermentable carbohydrates, but few studies have shown association between strain diversity and virulence of mutans streptococci (60).

1.4.2.3 Dietary preferences of ethnic groups

Investigations on dietary patterns and caries have based studies on the typical westernised diet. Undoubtedly, high sucrose consumption is accompanied by higher mutans streptococcus counts in individuals, but some populations that have a minimum intake of refined sugars are still carriers of large numbers of these microorganisms. Urban black 5-year-old South African children also eat a mainly westernised diet (129), but cultural influences do play an important role in food preferences (130), and some traditional African food staples such as maize (131) and its combinations (132) have shown little potential to produce caries in rats.

Maize is not only the staple diet of Africa, but is also eaten in Mexico and Guatemala (133). The average human consumption of maize in South Africa was approximately 4250 000 tons in 1998, followed by wheat, rice and sorghum (134). Dietary surveys undertaken in South Africa between 1983 and 2000 in urban areas have indicated that foods commonly consumed by children between the ages of 1-9 were: maize porridge and dishes, sugar, brown bread, margarine and peanut butter and cooked samp (135). Some of these foods have been investigated in this study, and are presented in part B of this thesis.

1.5 Aims of the investigation

The decline in dental caries accompanied by the relatively high numbers of mutans streptococci in children with and without dental caries and an unchanged diet, strongly suggest that different mutans streptococci genotypes with a high possibility of attenuation in virulence, are harboured in South African nursery school children. The main purpose of this study was firstly to; investigate the genetic heterogeneity of *S. mutans* and *S. sobrinus* from children with and without caries, and to determine if mothers were the primary source of these genotypes; secondly, to determine if traditional African staple foods and food-combinations influenced the

virulence properties of these bacteria. The specific objectives of this study have been listed under two headings and are diagrammatically shown in Figure 1.2.

A. To determine the genetic diversity of mutans streptococci in two South African population groups

1. To determine the genetic diversity of *S. mutans* and *S. sobrinus* clinical isolates from 5-year-old children with and without caries, in the black African and coloured communities.
2. To determine if mothers are the primary source of mutans streptococci, by comparing differences between clinical isolates from mothers and their children.
3. To compare genotypes of local clinical isolates with historical mutans streptococci reference cultures from the National Culture Type Collection (NCTC).

B. Mutans streptococci virulence and traditional African foods

1. To determine the ability of mutans streptococci clinical strains (n=5), from urban black African and coloured children, and NCTC reference strains (n=6) to metabolize three traditional African staple foods and their combinations, by measurement of growth.
2. To determine the influence of traditional African foods on virulence properties, specifically acid production and the synthesis of water-insoluble extracellular polysaccharide.
3. To determine the cariogenic potential of traditional African staple and food combinations by measuring the inherent acidity and buffering capacity.
4. To ascertain if traditional African foods influence glucosyltransferase gene expression in *S. mutans* and *S. sobrinus* species.

1.6 Hypothesis

Streptococcus mutans and *Streptococcus sobrinus* clinical strains show diversity in both phenotype and genotype. They differ between ethnic groups and from laboratory reference strains and are acquired primarily from mothers, but vertical transmission via other sources does occur. These bacteria are constantly exposed to a traditional African diet and they respond by modulating their biochemical synthesis through adaptive mutation of their regulatory genes, resulting in an attenuation in virulence.

1.7 Plan of thesis

The order of this study is represented in the following flow diagram (Figure 1.2). Each objective of the investigation will be reported as follows:

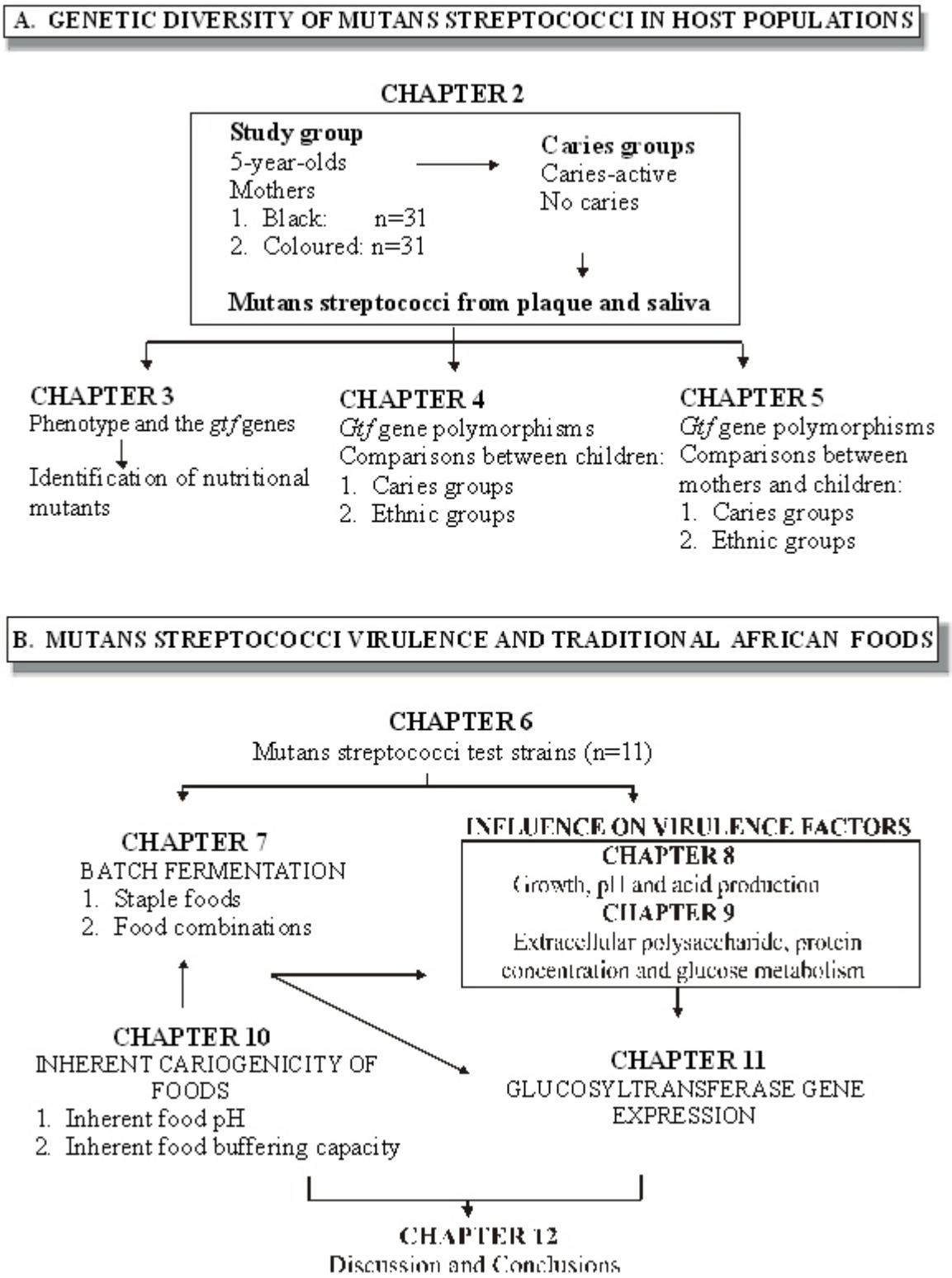

Figure 1.2 Flow diagram in order of study

PART A

GENETIC DIVERSITY OF MUTANS STREPTOCOCCI IN HOST POPULATIONS

CHAPTER 2

ETHNIC GROUP, CARIES STATUS AND MUTANS STREPTOCOCCI

2.1 General remarks

This chapter deals with the selection of the study population and the mutans streptococci clinical isolates that were used in Part A of this study, so is almost entirely methods. It does report the associations found between dental caries, mutans streptococci total viable count (TVC) and between ethnic population. Within each subsection, results will be given as shown in the following flowchart:

Chapter 2

Study population, selection of mutans streptococci clinical isolates and association with dental caries

↓

Chapter 3

↓

Biochemical grouping of phenotypes and association with the *gtf* genes

Chapter 4

↓

Gtf gene polymorphism and genetic variation of mutans streptococci isolates from 5-year-old children

Chapter 5

Interfamilial relationship between mutans streptococci isolates from children and their mothers

2.2 Ethics clearance

This study was approved by the University of the Witwatersrand's Committee for Research on Human Subjects (Medical), Clearance number M02-08-31 (Appendix I) prior to commencement, and informed consent was obtained from parents and assent from participating children.

2.3 Study population

A study group was selected during a 1995 dental caries surveillance study of 5-year-old children who were participants in the multi-institutional, longitudinal Birth-to-Ten (BTT) study (now the Birth-to-Twenty Study). This study aimed to determine the biological, environmental, economic and psycho-social factors associated with the survival and health of children living in an urban environment from birth to 10 years of age. Its future policies were to ensure that child health in a predominantly urbanised population is based on epidemiological evidence rather than ideology or tradition (136). This project was later extended to 20 years of age, where the focus on risk factors during adolescence, could be investigated (137). This multi-institutional study began in 1990 and will be completed in the year 2010. The role of the Dental Research Institute in the BTT project was to examine the prevalence of dental caries as well as nutrition intake in children from age 1 to age 5.

My study group for this thesis was from two South African populations. These were black African children and children from European-Malay-black descent, born between 23 April and 8 June 1990, and residing within the Gauteng region. For ease of reference, people of European-Malay-black descent will be referred using the South African term 'coloured'.

2.3.1 Selection of children for study

In 1995, 218 5-year-old children were dentally examined and sampled for microbiology at clinics within the contiguous cities of Soweto, Westbury, Noordgesig and Kliptown, (Appendix IV). For the current study, the experimental group comprised 31 children and their mothers of black African descent, the ethnic group that forms the majority of the total South African population (76.7%), and 31 were of coloured descent, an ethnic group comprising 8.9 % of the South African population (138). The remaining 156 children were excluded from the current sample because they had mutans streptococci levels that were below detectable limits.

2.4 Dental caries prevalence in children

Children's teeth were examined in good light using a dental mirror by a calibrated dentist (PC-J) calibrated to kappa=0.80, using WHO standards (139). Caries severity for primary teeth was recorded as dmfs (decayed, missing and filled surfaces) scores. The children were grouped into caries-free (cf) when a child's dmfs = 0, and caries-active (c) if the ds (untreated dental caries surface) ≥ 1. The prevalence of caries in children is shown in Table 2.1. Statistical analysis showed a significant difference in caries prevalence (P=0.01) (Chi-square test), but no significant difference in severity scores (Student's t-test) between the two ethnic groups was found.

Table 2.1 Dental caries prevalence and severity in five-year-old children by ethnic group

		Caries Prevalence*				Caries Severity	
		Caries-free		Caries-active		dmfs	ds
Population	N	n	%	n	%	($\bar{x}$ ± SD)	($\bar{x}$ ± SD)
Black African	31	12	39	19	61	4.1 ± 4.3	3.8 ± 4.0
Coloured	31	5	16	26	84	5.6 ± 7.1	7.4 ± 9.6
All children	62	17	27	45	73	4.7 ± 5.9	5.6 ±7.5

Caries-active = ds ≥ 1**;** **P* = 0.01

2.5 Microbiological sampling

Unstimulated saliva was collected from children and their mothers, by voiding into a sterile specimen container. A 10^{-1} dilution of the salivary sample was immediately made into a reduced transport fluid (140) (Appendix III). In the children, as part of a sub-study (84) plaque was removed with a sterile wooden toothpick from the occlusal surface of the maxillary right deciduous molar (tooth 55), the mandibular left molar (tooth 75), and the inter-proximal surfaces of teeth 75 and 85 of each child. The toothpicks were placed into sterile micro-centrifuge tubes containing one mL of reduced transport fluid. All samples were stored on ice, and processed within four hours after collection.

2.6 Culture methods

Samples were vortex-mixed for 15 seconds to disperse bacterial aggregates. Fifty µL volumes of 10^{-1} diluted saliva and plaque were inoculated onto a media selective for mutans streptococci; trypticase, yeast cystine bacitracin (TYCSB) agar (141) containing 15 % sucrose (Appendix III) using a spiral plater model D (Spiral Systems, Cincinnati, USA). Seeded plates were incubated at 37°C for 4 days in a gas mixture containing 10% H_2, 10% CO_2 and 80% N_2.

Thereafter, the plates were stood for two days at room temperature to enhance colony development and total viable counts (TVC) made. The distribution of mutans streptococci colony forming units (cfu) in children and their mothers are shown in Table 2.2.

Table 2.2 Prevalence of mutans streptococci (cfu × 10^5) in children and mothers by ethnic and caries group

	Black African				Coloured			
	n	5-year-olds	n	Mothers	n	5-year-olds	n	Mothers
Caries-free								
$\bar{x}$±SD	12	0.6 ± 1.8	11	10.6 ± 33.1	5	0.2 ± 0.5	3	1.7 ± 2.9
Min-Max		†-7.1		†-130.0		†-1.4		†-5.1
Caries-active								
$\bar{x}$±SD	19	0.7 ± 1.6	14	2.4 ± 4.0	26	113 ± 743	23	2.8 ± 4.3
Min-Max		†-8.5		†-11.0		†- 5100		0.003-16.0

† = 0.0002 ×10^5

2.7 Morphological and biochemical identification of isolates

Mutans streptococci strains were distinguished by their molar-tooth, granular frosted glass-like formation on TYCSB agar. Their growth is adherent on this agar and they produce extracellular glucan that surround the individual colonies (141, 142). The colonies were checked by gram staining, purified by subculture and biochemically confirmed. Purified subcultures were stored frozen at −20°C until used (Appendix III).

Clinical strains were differentiated into their biovars using the identification scheme of Shklair & Keene (55) shown in Table 2.3. This was supplemented by the aesculin hydrolysis test (56, 143, 144), and the production of hydrogen peroxide (52). Sterile, flat bottomed micro-

titration trays with lids (Nunc MicroWell™ plates) containing 200 µL of the test carbohydrate were prepared and inoculated with 50 µL of a 15×10^8 cfu/mL suspension of the purified bacterial culture. The fermentation plates were incubated at 37°C for 24-48 hours in an atmosphere of 10% carbon dioxide, 10% hydrogen and 80% nitrogen. Bile aesculin plates were streak inoculated with each test isolate and anaerobically incubated for 48 hours. A dichotomous recording was made for each carbohydrate: no fermentation = −ve and fermentation = +ve. A positive reaction was demonstrated by a colour change of the indicator dye from purple to yellow caused by the production of acid and a drop in pH.

To simplify the reading and interpretation of biochemical reactions, a numerical code was allocated to each carbohydrate assay as follows:
Mannitol = 1; Sorbitol = 2; Raffinose = 3; Melibiose = 4; NH_3 production = 5; Mannitol with bacitracin = 6; Aesculin hydrolysis = 7.

If the biochemical reaction was positive, i.e. demonstrated by acid production, the test bacterium was assigned the numerical code. For example, if only mannitol, sorbitol and raffinose were utilized, the biochemical profile would be 1230000. These codes were compared to codes of the selected reference strains and classified. The production of catalase and haemolysis on blood agar were only noted and not coded.

Table 2.3 Biochemical identification of mutans streptococci (55, 143-144)

Biotype	Serotype	Tests*						
		MAN	SOR	RAF	MEL	NH_3	Growth in bacitracin	AES
I	*c,e,f*	+	+	+	+	−	+	+
II	*b*	+	+	+	+	+	+	+
III	*a*	+	+	+	+	−	−	+/−
IV	*d,g, SL-1*	+	+/−	−	−	−	+	+/−
V	*e*	+	+	−	−	−	+	+
VI	*h*	+	−	−	+	−	−	−

*MAN = mannitol; SOR = sorbitol; RAF = raffinose; MEL = melibiose; NH_3 = ammonia from arginine hydrolysis; AES = aesculin hydrolysis; + = positive reaction; − = negative reaction; +/− = some strains positive, other strains negative.

2.8 Selection of mutans streptococci strains

2.8.1 Reference strains

Mutans streptococci laboratory reference strains were purchased from the National Collection of Type Cultures (NCTC) (PHLS, Colindale, London) and used as a control group. Prior to the commencement of RFLP analysis, the reference bacteria were biochemically confirmed and presence of the *gtfB*, *gtfI* and *gtfA* gene fragments were verified (Table 2.4).

Table 2.4 Biochemical characteristics of NCTC reference strains and PCR verification of the *gtfB*, *gtfI* and *gtfA* gene fragments

Species	Strain	Origin (146)	Serotype	Biotype (57)	Profile*	*gtfB*	*gtfI*	*gtfA*
S. mutans								
10449	Sims	Neotype	*c*	I	1234067	+	−	+
10923	LM-7	Bratthall	*e*	I	1234067	+	−	+
S. sobrinus								
12277	SL-1	Coykendall	*dg*	IV	1200060	−	+	−
10922	OMZ-176	Bratthall	*dg*	IV	1200060	−	+	−
10919	Z/AHT	Zinner	*a*	III	1200060	−	+	−
11061	OMZ-65	Bratthall	*dg*	IV	1200060	−	+	−

*Ferments: no fermentation response = 0; mannitol =1; sorbitol = 2; raffinose = 3; melibiose = 4; NH_3 from arginine = 5; resistant to bacitracin = 6; hydrolyses aesculin = 7

2.8.2 Clinical isolates

A total of 172, clinical isolates from children and their mothers were selected for investigation (Table 2.5) and were divided into two groups: caries-free and caries-active.The mutans streptococci isolates were biochemically defined and separated by phenotype as previously described and grouped into *S. mutans* and *S. sobrinus* species. These phenotypes are presented in Chapter 3.

Table 2.5 Population group and number of clinical isolates investigated by caries group

Population	Total	Clinical isolates			
		Caries-free		Caries-active	
		5-year-olds	Mothers	5-year-olds	Mothers
	n	n	n	n	n
Black African	80	15	15	33	17
Coloured	92	7	3	47	35
Total	172	22	18	80	52

Caries-active = ds $\geq$ 1

2.9 Association between mutans streptococci and caries

The relationship between the number of mutans streptococci and dental caries for each ethnic group were determined by the Spearman Rank correlation coefficient. A statistically significant association was found between dmfs and mutans streptococcus numbers (r=0.28; P=0.01), in coloured children only.

CHAPTER 3

MUTANS STREPTOCOCCI PHENOTYPE AND THE *gtfA* GENE

3.1 Introduction

The biochemical typing of clinical isolates is important to differentiate between bacterial species, their occurrence and modes of transmission. It enables the evaluation of certain strains associated with specific clinical disease conditions, such as whether individuals are colonized by one or multiple types of the microorganism.

The conventional method of differentiating between mutans streptococci is based on measurement of characteristics expressed by the bacteria, such as colonial morphology, micro-morphology, growth characteristics, biochemical properties, antibiotic resistance, utilization of compounds as the sole source of carbon for energy and growth, bacteriocin production and sensitivity to bacteriocins, serotype, and bacteriophage type (145, 146). However, these methods are currently being complemented by genotyping systems for use in investigating the ecology of bacteria in their natural habitats.These systems include molecular methods such as analysis by restriction fragment-length polymerase chain reaction (PCR-RFLP) (147, 148), real-time PCR (149) and randomly amplified polymorphic DNA (RAPD) analysis (150-152). Nevertheless, the preliminary isolation and identification of mutans streptococci from other oral streptococci using phenotyping is still necessary before these bacteria can be subjected to genotypic studies, where transmission or epidemiology is investigated (153, 154).

3.1.1 Biochemical considerations

Although the colonial morphology of mutans streptococci colonies growing on TYCSB is similar, species differentiation by observation only, leads to inaccuracies and is dependent on the trained eye of the examiner. Therefore, effective discrimination between mutans streptococci species depends on micro-morphology, specific growth characteristics and a battery of selected carbohydrate tests. A series of biochemical tests devised by Shklair and Keene (55), with supplementary assays was used in the current study. The methodology is described in 2.7. This scheme was used because of its simplicity for rapid identification and separation of mutans streptococci into its six biotypes. Additional tests such as the reduction of toxic hydrogen peroxide (H_2O_2) by oxidoreductase (catalase - EC 1.11.1.6) to water and oxygen (52), and the hydrolysis of aesculin to 6:7-dihydroxycoumarin producing a brown colouration with soluble ferric salt (56, 143, 144) were added.The reduction of H_2O_2 is specific to *Streptococcus sobrinus* identification, with variation in aesculin hydrolysis.

Studies on *Streptococcus mutans* and *S. sobrinus* clinical isolates have found biochemical properties different from commonly studied laboratory reference strains, classifying them into 'atypical' strains. A frequent occurrence is the failure of *S. mutans* strains to ferment melibiose (D-galactopyranosyl-∝-(1,6)-glucopyranose), a test frequently utilised in a number of typing schemes (155, 156).

3.1.2 Considerations in *gtf*A, *gtf*B and *gtf*I expression

The metabolism of melibiose and other sugars is controlled by the multiple sugar metabolism (*msm*) operon that includes the *gtf*A gene, necessary for the phosphorylation of sucrose (157-159). Inactivation of these segments leads to impairment of a normal sugar metabolism in *S.*

mutans, and hence a change in a 'typical' biochemical reaction during the fermentation of carbohydrates. The *gtf*A gene is absent in *S. sobrinus* strains and melibiose is not metabolised (56).

Examinations of biochemical reaction to various carbohydrates in association with chromosomal deletions have been done on melibiose-negative isolates (158, 160). Conclusions were that isolates unable to ferment this sugar lost the *gtf*A gene but the *msm* operon remained intact, indicating that melibiose phenotypes occur from various genetic events. More recently, Robinson *et al* (161) have shown that melibiose-negative strains have an incomplete insertion element (IS), IS*Smu*3, in place of the 18-kb stretch of chromosome encoding the *msm* and *gal* (galactosidase) operon that is consistent in strains derived from a common ancestor.

The co-transcribed *gtf*B and *gtf*C genes expressed in *S. mutans*, and *gtf*I genes in *S. sobrinus* encode for the production of extracellular enzymes known as glucosyltransferases. The glucosyltransferases form part of the glucan-binding proteins (GBPs) of the oral streptococci (2), and catalyse the production of glucose polymers, important in the attachment of bacteria to enamel surfaces and modification of permeability properties of dental plaque (162). Unlike the *gtf*A gene, these virulence genes are not susceptible to inactivation by environmental factors, but are rather influenced by the type and concentration of carbohydrate available to the bacterium as a carbohydrate source. They also can serve as differentiating features between *S. mutans* and *S. sobrinus* species, and as a means to determine genetic heterogeneity between strains (163).

3.1.3 Points to Consider

In South Africa, the distribution and prevalence of mutans streptococci strains in children from different populations have not yet been investigated, so information on strain evolution is not available. What is known (see Chapter 1) is that South African studies have shown a low caries prevalence and relatively high mutans streptococci levels in children, coupled in more recent times with an increase in caries (42). Therefore, an attenuation in virulence should be considered. Epidemiological studies at the molecular level would help determine if there is a 'change' in virulence, or if these *Streptococcus mutans* genotypes are acquired from other sources. This section of the thesis investigates the diversity of mutans streptococcus clinical isolates, from two South African child populations and their mothers at the phenotypic and genotypic level.

3.2 Aim

The object of this investigation was to:

1. Examine the prevalence of *Streptococcus mutans* and *Streptococcus sobrinus* phenotypes from children with and without caries in black African and coloured, 5-year-old children and their mothers.
2. Identify mutans streptococcus clinical isolates atypical to laboratory reference strains and their prevalence within the study groups.
3. Examine the association between *S. mutans* clinical isolates that are 'atypical' and the *gtfA* gene.

3.3 Materials and methods

Details explaining culture method and biochemical differentiation and have been described in Chapter 2, Subsections 2.6 to 2.7.

3.3.1 Recording of data

To identify mutans streptococci clinical isolates, the following numbering system was adopted:

1. To differentiate between isolates from 5-year-old children and mothers, the identification number is preceded by: c = child; m = mother. This is followed by B = black African and C = coloured to distinguish between the two ethnic populations.
2. Each child-parent set was given a case number following the relationship and population identification described in point 1.4. This consisted of five numerals.The fifth number was labelled according to the number of the isolate sampled from a family group. This ranged between one and five mutans streptococci isolates per case number. For example, isolate number cC04371 and mC04372. The first two letters identify the relationship and ethnic group and the case number of the two isolates is 0437. The last number indicates that isolate 1 was from the child and 2 was from the mother in this family pair.

3.3.2 Analysis of data

A numerical code was assigned to each isolate based on the response to each of the seven biochemical tests described in subsection 2.7. The biochemical code for each of the 172 clinical isolates investigated, was recorded and compared against the codes of the six laboratory reference strains (Table 2.3) and grouped into *S. mutans* and *S. sobrinus* species. The clinical isolates were further subdivided by caries and ethnic group, and examined for prevalence.

Streptococcus mutans phenotypes were grouped using the CLUSTER procedure of SAS (Version 9.1 for Windows, SAS Institute, Cary, NC: USA). The unweighted-pair group matrix analysis using arithmetic averages (UPGMA) was used to determine hierarchical clustering among strains tested (145, 164, 165). The distance between two clusters was calculated as the average distance between all pairs of objects in the different clusters and tree diagrams were generated to represent the results. Trees were depicted horizontally, with each row representing a case. Isolates with high similarity are adjacent to each other. The bivariate measure of association (strength) of the relationship between the clusters is measured using correlation in the CLUSTER procedure and is reported as r^2. $P<0.05$ was chosen as the critical indicator of statistical significance.

3.4 Results

3.4.1 Mutans streptococci species and caries

Streptococcus sobrinus were harboured by seven mothers and 10, 5-year-old children of which eight, had active-caries. Of the 172 clinical isolates investigated, 90% (155/172) of the isolates were biochemically characterised as *Streptococcus mutans* and the remaining 10% (17/172) was *S. sobrinus*. The *gtfB* and *gtfI* gene locus was amplified in all *S. mutans* and *S. sobrinus* isolates, respectively but the presence of the *gtfA* gene in *S. mutans* varied. The fermentation profile of all clinical isolates are shown in Appendix IV (Raw Data I).

3.4.1.1 *Streptococcus mutans* clinical isolates

The frequency distribution of clinical isolates based on fermentation reactions are presented in Table 3.1. *Streptococcus mutans* isolates that metabolised mannitol, sorbitol, raffinose and

melibiose were identical to *S. mutans* reference strains (Sims and LM-7) and are referred to as 'typical' isolates. Typical isolates comprised 70% (26/37) and 75% (88/118), from the caries-free and caries-active groups respectively.

Streptococcus mutans isolates that did not ferment raffinose and melibiose were atypical from *S. mutans* reference strains and comprised 26% (41/155) of the investigated isolates. Nineteen percent (29/155) of the 'atypical' isolates did not ferment both melibiose and raffinose and 8% (12/155) were melibiose negative phenotypes (Table 3.1). The *gtfA* region of the chromosome was absent in 63% (26/41) of the 'atypical' *S. mutans* isolates, but present in 89% (102/114) of typical clinical isolates. A two-way Analysis of Variance (ANOVA) indicated no significant difference between caries group and ethnic population but significant variation was found between *S. mutans* phenotype (P=0.0001).

Table 3.1 Frequency distribution of *Streptococcus mutans* clinical isolates (n =155) and the *gtfA* gene by caries and ethnic group. B=Black African; C=Coloured

Phenotype	Caries-free								Caries-active							
	B		C		Total		*gtfA* +ve		B		C		Total		*gtfA* +ve	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Typical	22	76	4	50	26	70.3	25	96	31	67	57	79	88	75	77	88
Mel+Raf -ve	6	21	3	37.5	9	24.3	1	11	12	26	8	11	20	17	6	30
Mel -ve	1	3	1	12.5	2	5.4	2	100	3	7	7	10	10	8	6	60
Total	29		8		37		28		46		72		118		89	

Mel = melibiose; Raf = raffinose

3.4.1.2 *Streptococcus sobrinus* clinical isolates

Streptococcus sobrinus strains were characterized by having a poor overall ability to ferment the range of carbohydrates. Clinical isolates that were able to produce acid from mannitol, sorbitol, were bacitracin resistant and possessed the *gtfI* gene were classified as *S. sobrinus*. As previously mentioned, these isolates comprised 10% of the bacteria investigated of which 14 (82%) were sampled from the caries-active group, and are shown in Table 3.2.

Table 3.2 Frequency distribution of *Streptococcus sobrinus* clinical isolates (n = 17) by caries and ethnic group

		Caries-free				Caries-active			
		Black African		Coloured		Black African		Coloured	
	N	n	%	n	%	n	%	n	%
S. sobrinus	17	1	5.9	2	11.8	4	23.5	10	58.8

3.4.2 Hierarchical clustering of *Streptococcus mutans* phenotypes

Hierarchical clustering of clinical isolates from 5-year-olds and their mothers are shown in Figure 3.1. Laboratory reference strains were included in the dendogram to compare the level of similarity between clinical isolates. For ease of reference *S. mutans* isolates from caries-free and caries-active children and their mothers are shown in two dendograms. However, no difference was shown when a combined analysis was made on both caries groups. These results are shown in Appendix V.

Streptococcus mutans clinical isolates from caries-free children and their mothers were grouped into three clusters. Twenty-seven isolates in cluster A were biochemically identical (r^2=1.00)

to *S. mutans* reference strains (Sims, LM-7). Cluster B comprised mainly of raffinose and melibiose negative phenotypes (shown in bold) and isolates in cluster C did not metabolise sorbitol, melibiose or raffinose. Cluster C differed from A by an average distance of 2.27. Similarly, *S. mutans* isolates from children with active caries and their mothers were divided in the same order described for isolates from the caries-free group. Four clusters (A-D) were identified by UPGMA in the caries-active sample. Eighty-three isolates were placed into cluster A, the major group biochemically similar by 98% (r^2=0.98) to laboratory reference strains (Figure 3.1). Cluster D comprised of four *S. mutans* isolates of which three (cB16502, mB16021 and cB16503), were able to produce ammonia from arginine hydrolysis and were least similar to reference strains (cluster A), differing by an average of 3.66.

3.4.3 Hierarchical clustering of *Streptococcus sobrinus* phenotypes

Streptococcus sobrinus clinical isolates were divided into two main clusters (A and B), separated by an average distance of 0.5 (Figure 3.2). Cluster A comprised 10 isolates similar (96%; r^2=0.96) to laboratory reference strains, whereas Cluster B consisted of seven isolates that did not ferment the carbohydrate, sorbitol. Clinical isolate cB16501, the only bacterium that hydrolysed arginine, was least similar to *S. sobrinus* reference strains, differing by an average distance of 3.00.Two sets of isolates from coloured mother-child pairs that were identical to each other, are shown in cluster A.

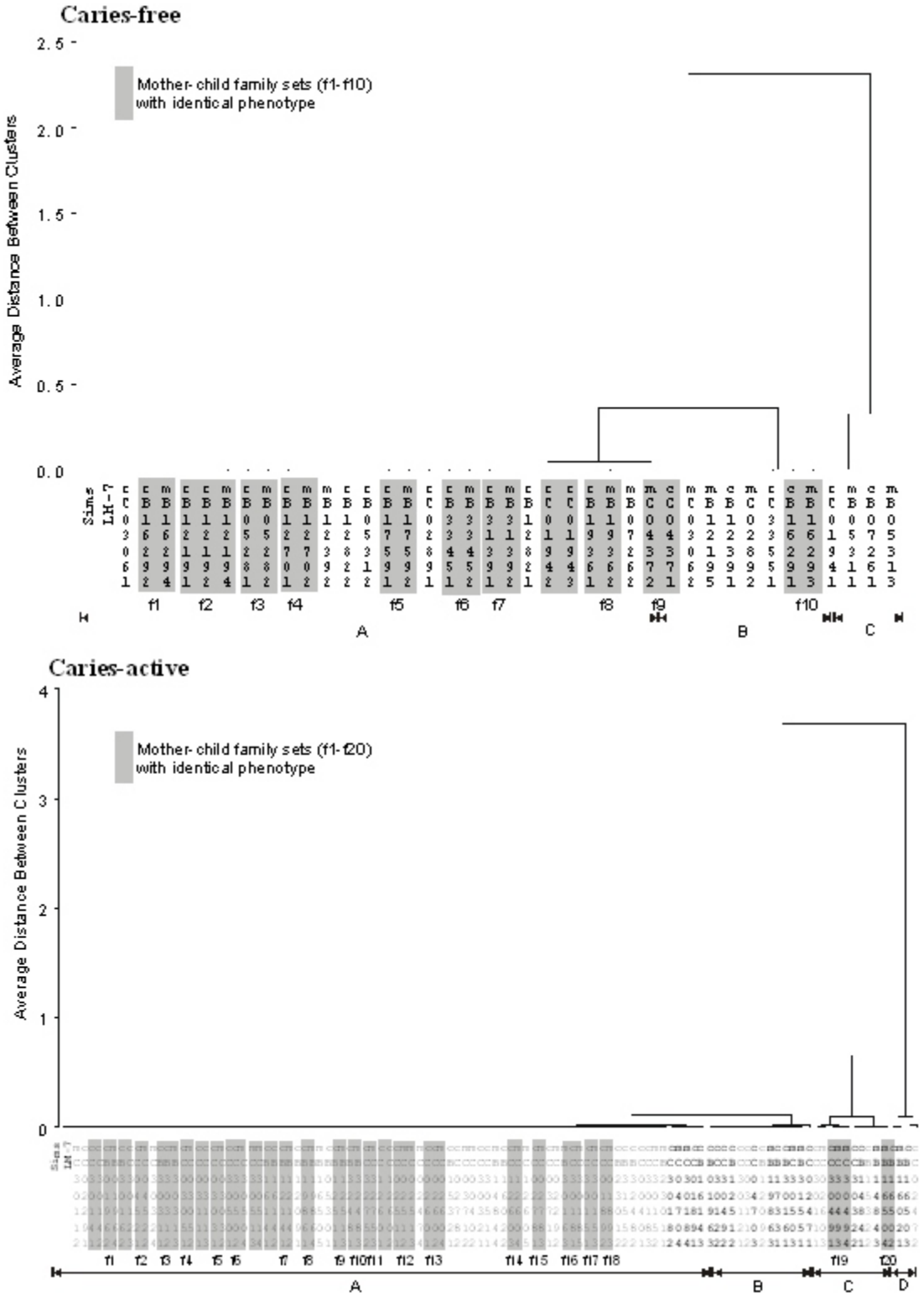

Figure 3.1 Dendogram produced by UPGMA clustering of *S. mutans* reference strains (Sims, LM-7) and clinical isolates from black African, and coloured, children with and without active-caries and their mothers. Melibiose-negative phenotypes are shown in bold. B = black African, C = coloured, c = child, m = mother.

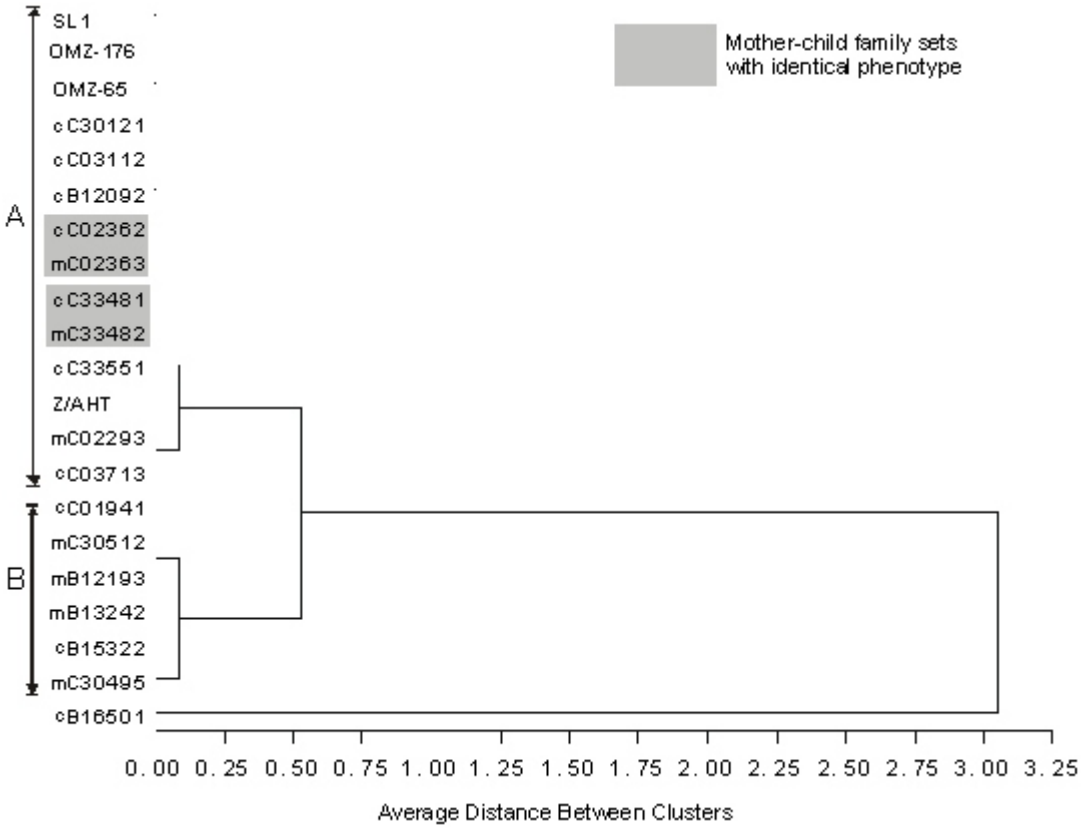

Figure 3.2 Dendogram produced by UPGMA clustering of *S. sobrinus* reference strains (SL-1, OMZ-176, OMZ-65, Z/AHT) and clinical isolates from black African and coloured children and their mothers. B = black African, C = coloured, c = child, m = mother.

3.5 Discussion

3.5.1 Phenotype and the *gtf* genes

Biochemical differentiation between the clinical isolates studied showed that 90% (155/172) were *S. mutans* strains, but 26% (41/155) of *S. mutans* isolates were melibose-negative phenotypes (Table 3.1). *Streptococcus sobrinus* strains comprised 10% of the test isolates and were mainly cultured from the caries-active children. Although *S. sobrinus* species comprised only a small number of the clinical isolates investigated in this study, this bacterium was isolated together with *S. mutans* strains and was prevalent in 5-year-old children with

active-caries. Some investigators (166-168) have found that the presence of both these species in some individuals show a significantly higher prevalence of dental caries, yet these microorganisms can also be widespread in populations with a low prevalence of dental caries (169). Therefore, the ratio in the isolation frequency between these two species is considered important especially when virulence mechanisms are studied. The higher numbers of *S. mutans* recovered from patients compared well with other studies where *S. mutans* accounted for 74 to 97% of the mutans streptococci, with *S. sobrinus* prevalent between 0.4 and 41% in preschoolers (45).

The PCR amplification of the *gtfA* gene confirmed loss in 63% (26/41)of the melibiose-negative phenotypes. Absence of the *gtfA* gene in melibiose-negative phenotypes was accompanied by a negative aesculin hydrolysis response in 56% (23/41) of *S. mutans* isolates. This finding was similar to work by Beighton and co-workers (155), who showed that melibiose-negative isolates were more unable to metabolise aesculin, than melibiose-positive strains. In this study, 19% of the melibiose-negative phenotypes did not ferment raffinose and concurs with investigations by Beighton *et al* (155) and Ushiro *et al* (160).

The percentage (26%) of melibiose-negative *S. mutans* clinical strains (41/155) was high compared to Ushiro *et al* (160) who found only 3% (6/222) of melibiose-negative phenotypes. Yet, Beighton *et al* (155) and Grönroos and Alaluusua (151) reported frequencies of 13% and 38.6% of melibiose negative phenotypes, respectively. Nevertheless, the frequency of isolation of these phenotypes show that they co-exist with melibiose-positive strains in the oral cavity.

The inability to ferment melibiose and the reduced ability to ferment raffinose is caused by

failure to transport these sugars across the cell membrane and by a lack of the enzyme ∝-galactosidase. This is apparently produced by loss and inactivity of the *aga* gene (156, 157, 160) which is part of the *msm* (multiple sugar metabolism) operon. However, the range of biochemical tests in the initial differentiation of test strains in this study, was not extended to measure ∝-galactosidase and ∝-glucosidase enzyme activity.

Confirmation of loss or inactivity of the *msm* (multiple sugar metabolism) operon that incorporates the *gtfA* codon was not incorporated into this investigation and lays ground for further study. As previously mentioned, studies by Robinson *et al* (2) showed that melibiose-negative strains have an incomplete insertion element (IS) IS*Smu3*, in place of the 18-Kb stretch of chromosome encoding the *msm* and *gal* operon that is consistent in strains derived from a common ancestor. They hypothesised that the mobility of IS elements is one mechanism that would contribute to the generation of diversity of RFLP patterns in *S. mutans.* The transmission of melibiose-negative phenotypes will be investigated and discussed in Chapter 5.

Although *S. sobrinus* strains are reportedly more cariogenic (170) compared to *S. mutans* and other caries-associated species (147), this bacterium was detected only at low levels (10%) in children with active caries. This is similar to other investigations where the percentage prevalence of *S. sobrinus* ranged between 2.8 % and 57.13 % (74, 142, 166, 170-172) and the detection of *S. mutans* was more numerous and in higher numbers than *S. sobrinus* (173, 174). In this study, *S. sobrinus* clinical isolates had a poor ability to ferment the selected range of carbohydrates, and in most cases, confirmation was made by PCR amplification of the *gtfI* gene.

3.5.2 Hierarchical clustering of *S. mutans* and *S. sobrinus*

The dendogram of *S. mutans* biotypes generated by the cluster analysis (UPGMA) clearly separated the clinical isolates by nature of their biochemical response to the test carbohydrates. The close clustering of identical isolates sampled from the same patient or a family-group suggests that in family sets, mothers are more than likely to be the major source of mutans streptococci infection in young children based on phenotype identification. According to Davey & Rogers, (175), infected children share at least one common biotype with the mother, compared to fathers or other members of the family.

The similar patterns found in the hierarchal clustering of *S. mutans* isolates from the caries-free and caries-active groups (Figure 3.1) suggests that clinical isolates from both the caries groups are biochemically the same. The isolates were not divided by ethnic group, but family groups were clustered closer together. The isolation of four, clinical isolates that hydrolysed arginine must be viewed with suspicion. According to Beighton *et al* (155), mutans streptococci isolates that appear to hydrolyse arginine are always mixed with catalase-positive gram-positive cocci.

Streptococcus sobrinus clinical isolates that did not produce acid from sorbitol (cluster B) (Figure 3.2) were clearly separated from cluster A where reference strains and similar clinical strains were grouped. Seventy percent (12/17) of *S. sobrinus* isolates were from the coloured population sample, where active-caries was more prevalent.

Clearly, the response of fresh bacterial isolates to test carbohydrates compared to laboratory reference strains is dependent on *gtfA* gene expression that can be influenced by external factors. According to Marsh (4), the variation in carbohydrate metabolism by mutans

streptococci strains can be a result of properties in the habitat. With regard to phenotyping, recent studies on the inter-familial transmission of mutans streptococci have replaced this method with molecular methods using genotyping - this is investigated and discussed in Chapter 5.

When considering virulence mechanisms, it is important to distinguish between *S. mutans* and *S. sobrinus* species. *Streptococcus mutans* clinical strains comprised a large percentage of the bacteria investigated, with some biochemical responses atypical from laboratory references, and from each other. These atypical fermentation patterns were evident when a loss or lack of expression of the *gtfA* gene occurred (Table 3.1) and imply that these reactions are primarily controlled by the *gtf*A gene. The instability of the *gtfA* gene does not appear to affect the survival of these bacteria, but studies on the prevalence of these variants may provide information on its contribution to caries, and the events that lead up to this genetic change. The influence of the nutrient environment derived from the host's diet on the stability of the *gtfA*, *gtfB* and *gtfI* virulence genes are examined in Chapter 11.

3.6 Conclusions

No difference was shown by the hierarchal clustering of mutans streptococci clinical isolates based on phenotype between the black African and coloured samples, or between the two caries groups. However, *S. mutans* isolates were clearly divided by phenotype into a group that was identical to laboratory references and those that were 'atypical', by not metabolising either melibiose or raffinose. The identical biochemical response of several clinical isolates from mother-child sets suggests that mothers are most likely to be the primary source of mutans streptococci species. The numbers of *S. sobrinus* were low, yet prevalent in children with

caries, confirming that this microorganism mostly colonize mouths with untreated dental caries.

Phenotypic typing systems detect expressed characteristics and depend on specific gene-expression. This is shown by the appearance of melibiose-negative phenotypes and the variable *gtfA* gene. The inconsistent response of mutans streptococci to carbohydrates can be attributed to alteration in gene expression that are sensitive to changes in the oral environment. Further investigation on the influence of nutrients from the host diet on 'typical' and 'atypical' strains and the transcription of the *gtfB* and *gtfI* genes is presented in Part B of this thesis.

CHAPTER 4

Gtf GENE POLYMORPHISMS IN CLINICAL ISOLATES FROM 5-YEAR-OLD CHILDREN

4.1 Preface

Given the shortcomings shown on differentiating between mutans streptococci clinical strains by biochemical methods described in Chapter 3, investigations on species identification and transmission and epidemiology of these microorganisms have been replaced by genotyping methods. Current systems described in the literature include T-RFLP (Terminal-Restriction Fragment-Length Polymorphisms) (176), PCR-RFLP (Polymerase Chain Reaction - Restriction Fragment-Length Polymorphisms) (147, 148, 177), real-time PCR (178) and RAPD (Randomly Amplified Polymorphic DNA) (152-154).

4.2 Introduction

A virulence property of *Streptococcus mutans* is its ability to produce extracellular polysaccharides (ECP) from sucrose that enhance the formation of plaque (66, 179, 180). Dental plaque provides conditions suitable for the attachment of bacteria to the tooth surface (45, 181), where they produce acids that promote early childhood caries (ECC) in infants and preschool children.

4.2.1 Glucosyltransferase and extracellular polysaccharide formation

The enzyme glucosyltransferase (EC 2.4.1.5) catalyses the formation of ECP from sucrose and

other carbohydrates. This enzyme is encoded by the related *gtfB*, the *gtfC* and *gtfD* genes in *S. mutans*, and the *gtfI* gene in *Streptococcus sobrinus* (182-186). *GtfB* produces a water-insoluble glucan, composed predominantly of ∝-1,3- linked glucose residues (184). *GtfD* makes primarily ∞-1-6 glucosidic linkages which are water soluble, and *gtfC* makes a polymer with the properties of both ∝-1,3 and ∝-1,6-linked glucosyl units (187). The GTF-I enzyme encoded by the *gtfI* gene, catalyse the formation of water-insoluble glucans (∝-1,3- linked glucose) (187).

4.2.2 Properties of *gtfB* and *gtfI* genes

The *gtf*B and *gtf*C genes are arranged in tandem, separated by only 198 bp in *S. mutans*. They share nucleotide sequence homology (182, 183), and are probably products of gene duplication (183). The *gtfD* gene is not closely linked to the *gtfBC* genes and is located elsewhere in the chromosome (188). Also, the GtfD enzyme shows less homology with GtfB and GtfC and was not considered for further study. The *gtfBC* gene coding region, span about 5000 bp, and both *gtfB* and *gtfC* are independently expressed (189, 190). A study on the *gtfB* gene cluster has found polymorphisms in *S. mutans* serotype *c* strains, indicating genetic heterogeneity among the same strains of mutans streptococci (191) which can serve as differentiating features between strains and as a means to determine genetic heterogeneity between isolates (168). The variant expression of the *gtf* genes is linked to the basal metabolism of the cell (192) with expression of the *gtfB* gene stimulated in the presence of sucrose (193). When exposed to different growth conditions, only during the various phases of growth is their significant change in expression (192). Investigations by Köhler and Krasse (72) have shown that strains of *S. mutans* vary in virulence and the selection of more virulent strains within a host population can influence dominance by this species.

PCR-RFLP analysis of *S. sobrinus* genotypes have suggested that this bacterium is more divergent than *S. mutans* (163). This divergence implies variation in the synthesis of ECP between *S. mutans* and *S. sobrinus* species. More recently, a study by Mattos-Graner *et al* (194) showed a high degree of *gtf* polymorphisms with varying expression levels of GtfB and GtfC isozymes in *S. mutans* isolates from young children. This points in the direction that the mutans streptococci possess different cariogenic properties, and the cariogenic potential should be evaluated based on genetic heterogeneity as the landmark.

4.2.3 Points to consider

Study on bacterial taxonomy, evolutionary mechanisms, phylogenetic relationships, population genetics and microbial epidemiology rely on the discrimination between bacterial strains (195). Therefore, it is necessary to identify mutans streptococci genotypes, considering the potential importance of the *gtfB* gene in cariogenesis and colonization (180). Interspecies differences exist genetically, as far as the *gtf* genes are concerned and information on the prevalence and variation of the *gtfB* gene in mutans streptococci isolates from South African children is still unknown.

Laboratory techniques used to differentiate between *S. mutans* and *S. sobrinus* species have targeted chromosomal DNA, 16S ribosomal DNA and specific genes. Studies over the past five years on the colonisation and transmission patterns of mutans streptococci in populations using genotyping, have used analysis methods mainly for chromosomal DNA (93, 196, 197). Subsequently, PCR-RFLP analysis of the *gtfB* and *gtfC* virulence genes by Mattos-Graner *et al* (194) have shown high diversity in isolates of *S. mutans* with different biofilm growth phenotypes. The high number of polymorphisms identified in the *gtf* genes allows for study on

the epidemiology of these microorganisms. To-date, no investigations have been done on the genetic diversity of mutans streptococci, targeting *gtf* gene polymorphisms on clinical isolates from South African children.

4.3 Aim

The objectives of this study were to determine using PCR-RFLP analysis:

1. G*tfB* and *gtfI* gene polymorphisms among *S. mutans* and *S. sobrinus* clinical isolates from children with active-caries and those without caries.
2. G*tfB* and *gtfI* gene polymorphisms among *S. mutans* and *S. sobrinus* clinical isolates from black African and coloured children.
3. Differences between clinical isolates with historical mutans streptococci reference strains of the National Culture Type Collection.

4.4 Materials and methods

4.4.1 Mutans streptococci clinical isolates

A total of 97 mutans streptococci clinical isolates from 5-year-old children were selected from the original sample group (97/172) that had been biochemically defined and discussed in Chapter 3. These bacteria were biochemically differentiated into *S. mutans* (n=87) and *S. sobrinus* (n=10), and expression of the glucosyltransferase genes *gtfB* and *gtfI* were verified.

4.4.2 DNA extraction

Bacterial DNA was prepared by growing the bacterial isolates in 5 mL of Brain Heart Infusion

broth (Biolab Diagnostics, Merck, Midrand, SA) supplemented with 3% sucrose to enhance bacterial growth and expression of the *gtfB* gene. The bacterial cells were grown until mid-log phase at 37°C, and after approximately eight hours, the cells were pelleted by centrifugation (1800 × *g*) at 4°C and washed with STE buffer (Appendix III). Chromosomal DNA was extracted using the phenol-chloroform method by Popovic *et al* (198) with some modification. The modification was to preserve DNA integrity by extending the lysis time for rupture of the streptococcal rigid cell walls. The cells were placed on a rotary shaker at 37°C for 45 minutes in 1 mL of 10 × TE buffer (pH 8) containing lysozyme (5 mg/ mL)(Boehringer Mannheim, GmbH, Germany) and RNAnase A (0.1 mg/mL) (Boehringer Mannheim, GmbH, Germany). Sodium dodecyl sulphate (SDS) was added to a final concentration of 1% and samples were incubated in a water-bath at 65°C for 15 minutes. Proteinase K (Roche Diagnostics, Indianapolis, USA) was added to a final concentration of 200 µg/mL and the samples re-incubated at 65°C for 2 hours.

DNA was extracted with two volumes of buffered phenol (pH 7.8) (ICN Pharmaceuticals, Costa Mesa, USA) followed by chloroform-isoamyl alcohol (24:1) extraction. The DNA was precipitated by adding 2.5 volumes of iced 3 M sodium acetate in ethanol and kept at −20°C for not less than two hours to increase recovery. DNA was recovered by centrifugation at 8000 × *g* for 5 minutes, washed twice with cold 95% ethanol, and dried at 37°C for 10 minutes. The precipitated DNA was resuspended in 300 µL TE buffer. The DNA template-stock solution was aliquoted and stored at −20°C until used.

4.4.3 Quantification, size and integrity of genomic DNA

The concentration of DNA was determined by measuring absorbancy on a spectrophotometer

(Ultrospec III, Pharmacia, Cambridge, England) at 260 nm (A_{260}) using a quartz cuvette. The ratio of the readings at 260 nm and 280 nm (A_{260}/ A_{280}) provided an estimate of DNA purity. The standard for pure DNA was set at a ratio of 1.7-1.9. The integrity and size of the preparation was checked on a 0.75% agarose gel (199), run for one hour on a submarine horizontal electrophoresis unit (CBC Scientific Company, Inc. Eel Mar, CA) in Tris-borate-EDTA (TBE) buffer containing 0.5 μg/ml^{-1} ethidium bromide (USB, Amersham, Buckinghamshire) (Appendix III). Intact DNA was visible at 21 kb (Figure 4.1).

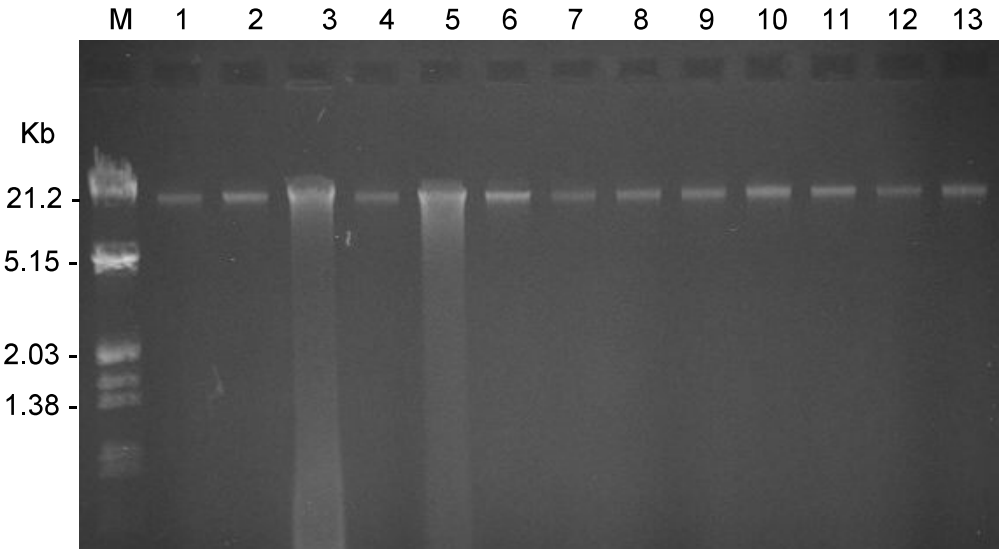

Figure 4.1 Extracted DNA of *Streptococcus mutans* clinical strains from black African, 5-year-old children and their mothers (Lanes 1-13), M = molecular weight marker (Lane 1).

4.4.4 PCR amplification

PCR amplification of the selected genes were achieved with primer specific oligonucleotides listed in Table 4.1. The *gtfB* and *gtfI* primers were designed by Shiroza *et al* (163) and the *gtfA* gene primers by Colby, Harrington and Russell (156). The 16S rRNA locus, common to *S.*

mutans strain NCTC 10449 was used as a control for the PCR reactions. The sequence used for rRNA primer design was from Bentley *et al* (200) (GenBank accession number X58303). The primer sequence for this locus was designed on Primer3 Software distribution (210) version 0.2 (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi.). Between 200 and 500 ng of chromosomal DNA was used as template to amplify the *gtfB* (4.6 kb), *gtfI* (4.7 kb) and 16S rRNA (1.3 kb) gene.

Table 4.1 Oligonucleotide primers used in this study

Gene	Primer	Sequence	Location*	Size	EPL
gtfB	GTFB-F961	5'-TCTAAGCAAGAAGCTGCTAGTAGT-3'	916-939	24 mer	4.6 kb
gtfC	GTFC-R5574	5'-TGGTTGAGATGTTGCTGAAGTTGC-3'	5551-5574	24 mer	
gtfI	GTFI-F293	5'-CGGATACAGACACTGCTAGTG-3'	293-313	21 mer	4.7 kb
	GTFI-R5021	5'-AATGGACTTAAAGCGCTAAACC-3'	5021-5042	22 mer	
gtfA	GTFA-F7157	5'-GGCTGTGATCTCATTCGTTT-3'	7157-7176	20 mer	554 bp
	GTFA-R7692	5'-AACCTGTGGAATACCTGGAG-3'	37985	20 mer	
16SrRNA	16S-F-36	5' -AGCGGGGGATAACTATTGGA-3"	36-55	20 mer	1.3 kb
	16S-R-1305	5'-CAAACTCTCGTGGTGTGACG-3'	1305-1325	20 mer	

*Nucleotide sequences were obtained from GenBank accession numbers M17361 (*gtfB*), D90213 (*gtfI*), M77351 (*gtfA*) and X58303 (16S rRNA).

Reactions were performed in a 25 µL reaction volume containing 2.5 µL of 10× reaction buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT) containing 2.0 mM $MgCl_2$, 1.0 U ExSel High Fidelity DNA polymerase (Southern Cross Biotechnology, Cape Town, SA), 300 µM each of dATP, dCTP, dGTP, dTTP (Roche Diagnostics, Mannheim, Germany) and 0.6 µM primer. The GeneAmp® PCR System 2400 (Perkin Elmer, PE Biosystems SA) was used for PCR amplification under the conditions shown in Table 4.2.

Table 4.2 PCR amplification cycles

Gene	Initial denaturation	Cycles	Denaturation	Primer annealing	Extension	Final extension
gtfB & gtfI	92°C - 2 min	10×	92°C - 10s	50°C - 30s	68°C - 3 min	68°C - 7 min
		20×	92°C - 10s	50°C - 30s	68°C - 3 min†	
gtfA	94°C - 2 min	10×	94°C - 25s	50°C - 30s	72°C - 1 min	72°C - 7 min
		15×	94°C - 25s	50°C - 30s	72°C - 1 min‡	
16S rRNA	94°C - 2 min	10×	94°C - 30s	52°C - 30s	72°C - 1 min	72°C - 7 min
		20×	94°C - 30s	52°C - 30s	72°C - 1 min‡	

†Plus 20 seconds per cycle; ‡ plus 5 seconds per cycle.

4.4.5 Detection of amplicons

Amplified gene fragments were detected by agarose gel electrophoresis (199). A 0.75%, 1% and 2% (w/v) agarose gel concentration was prepared for visualisation of the *gtfB*, *gtfI*, 16S rRNA and *gtfA* genes respectively (Figure 4.2). Ethidium bromide (0.5 μg/mL^{-1}) was incorporated into the gel for staining and detection of the nucleic acid zones. Ten μL of the PCR samples were mixed with 3 μL of 6 × gel loading buffer (Appendix III). A 100 bp low molecular DNA ladder (LMwm) and Lambda DNA/*Eco*R I + *Hin*dIII (Promega, Madison, USA), a high molecular weight marker (HMwm) were used to measure position of the base pairs. The samples were loaded into the wells and electrophoresed in Tris-Borate-EDTA (pH 8.0) running buffer (Appendix III) for 1.5 hours at 130 V and between 30-34 mA. The amplicons were visualised on a UV transilluminator (UVP, Inc., CA, USA) and photographed with a Polaroid DS-34 direct screen instant camera (Polaroid Corporation, MA, USA) using Fujifilm FP-3000B black and white film.

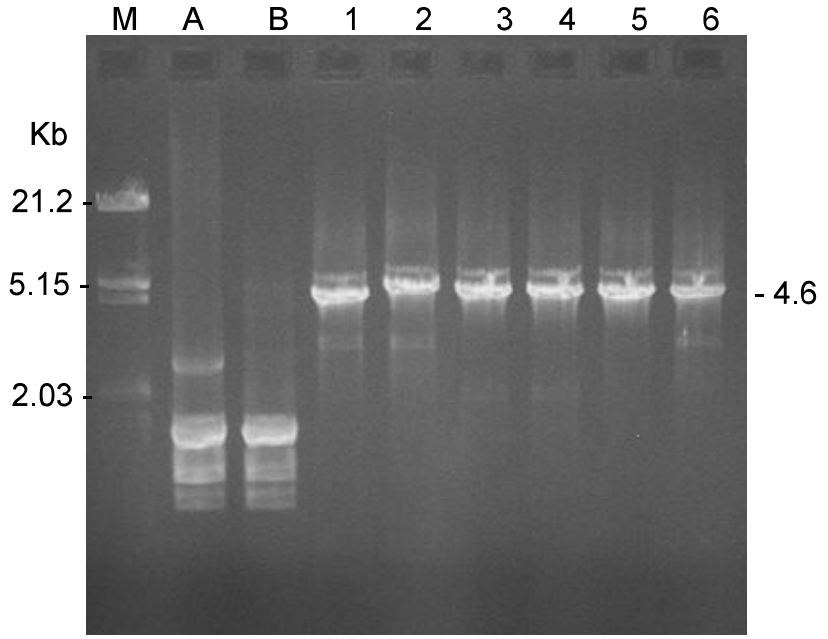

Figure 4.2 PCR amplicons of the *gtfB* gene (4.6 kb) (Lanes 1-6) of *S. mutans* clinical strains from coloured children and their mothers; Lanes: M = HMwm, 1 = mC03084, 2 = mC03083, 3 = cC03162, 4 = cC03161, 5 = mC03163 and 6 = Sims (NCTC 10449). Clinical strains not expressing the *gtfB* gene are indicated in lane A and B.

4.4.6 Enzyme restriction of *gtf* amplicons

The *gtfB* and *gtfI* genes were digested with *Hae*III and *Hin*fI enzymes(Promega, Madison, USA). The enzyme *Hae*III recognises and cuts at the 5'...GG▾CC...3' base sequence, whereas *Hin*fI enzyme cleaves at 5' ...G▾ANT C... 3' , where N means any one of four bases. Prior to RFLP analysis, the theoretical number of the fragment size of the *gtfB* and *gtfI* gene amplicon was determined by Gene Runner (Version 3.05, Hastings Software, Inc.) based on the sequence obtained from *S. mutans* strain GS5 (GenBank accession number M17361) and *S. sobrinus* strain 6715 (GenBank accession number D90213). The 5'...GG▾CC...3' sequence repeats at 12 positions in the *gtfB* gene, but eight visible fragments are produced that range from 133 to 901 bp in size (Figure 4.3). This 5'...GG▾CC...3' sequence repeats at nine positions in the *gtfI* gene,

but seven fragments are visible. The 5' ...G▾ANT C... 3' base sequence repeats at eight and 14 positions in the *gtfB* and *gtfI* genes respectively.

The enzyme digest was set up according to Gannon and Powell, (202), and the manufacturers instruction. Explained briefly, a 25 µL reaction volume was prepared using 16.3 µL of sterile, de-ionized water, 2.5µL of RE 10× buffer, 10 µg/µL of acetylated BSA, 5 µL of the DNA amplicon and 10 u/µL of the restriction enzyme. The mixture was incubated at 37°C for 2 hours in a water bath. The digests were resolved on a 2% agarose gel by electrophoresis, and photographed.

4.4.7 Measurement of RFLP patterns

Restriction fragment patterns of the *gtfB* and *gtfI* genes from clinical isolates were scanned with a photo-scanner (HP PhotoSmart S20, ©Lead Technologies, Inc., Haddonfield, USA) and peaks were measured by QuantiScan for Windows, Version 2 (Biosoft, Cambridge, UK).

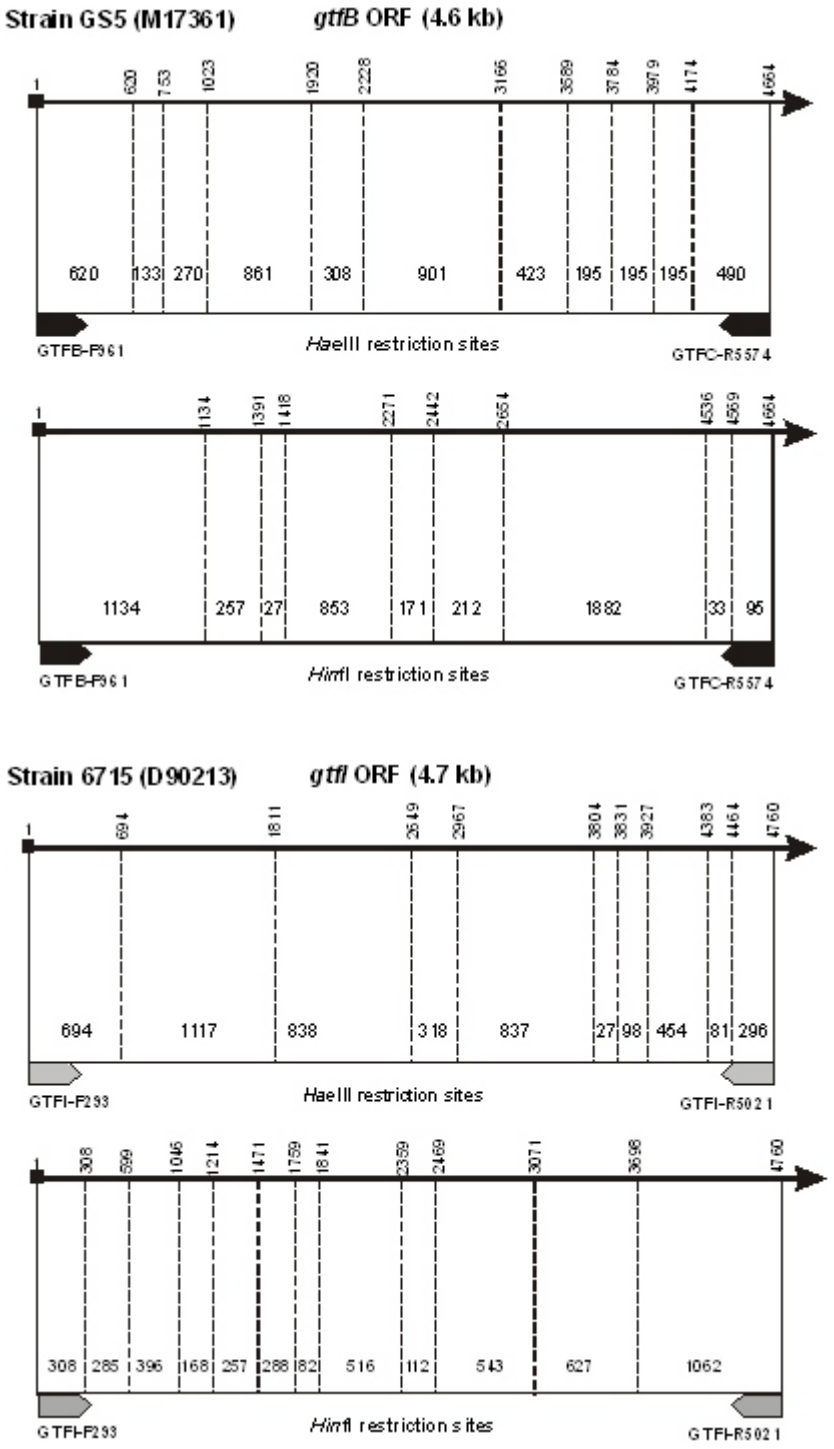

Figure 4.3 Chromosome locus of the *gtfB* and *gtfI* gene. Primer pairs used for specific amplification of *gtfB* and *gtfI* genes are presented as black and shaded arrows respectively.The restriction sites of the 4.6-kb *gtfB* and 4.7-kb *gtfI* amplicons are represented by the dashed lines: within them, the size of fragments are indicated.

4.4.8 Recording and analysis of data

For comparative analysis, restriction fragments of *gtf* gene amplicons that were different from mutans streptococci reference strains were investigated. The positions of peak banding were divided into lanes comprising of similar base pair values defined as follows: Lane 1 = 1001-3000 bp; Lane 2 = 801-1000 bp; Lane 3 = 601-800 bp; Lane 4 = 401-600 bp; Lane 5 = 201-400 bp. These lanes resulted in a fingerprint unique to the clinical isolate and were compared against NCTC reference strains. The original data showing the cut sites of the *gtfB* and *gtfI* genes and the division of the cut sites into their respective lanes are shown in Appendix IV - Raw Data II and III.

To differentiate between amplitypes, the difference between lanes was measured by the DISTANCE procedure of SAS (Version 9.1 for Windows, SAS Institute, Cary, NC: USA) and the Euclidean distance between the positions of peak banding was calculated. The unweighted pair-group method using arithmetic averages (UPGMA) (164, 165) was used for CLUSTER analysis and dendograms were computed to represent the results, with each row representing a genotype. The bivariate measure of association (strength) of the relationship between the isolates was measured using correlation (r^2) in the CLUSTER procedure and is reported as percent similarity. Difference between RFLP patterns of *gtf* amplicons from the two ethnic populations and caries groups were determined by the Principal Component Analysis. $P<0.05$ was accepted as the indicator of statistical significance.

4.5 Results

4.5.1 *Gtf* gene amplitype, ethnic group and caries

The Principal Components Analysis indicated no statistically significant differences in *gtfB* and *gtfI* amplitypes between mutans streptococci isolates from the black African and coloured population, or between the two caries groups which are shown in Appendix VIIb. Hence, further analysis of PCR-RFLP data was performed on the combined data.

4.5.2 *GtfB* gene polymorphisms of *Streptococcus mutans*

Between two and seven restriction fragments with molecular sizes ranging between 280 and 2000 bp were found in *Hae*III and *Hin*fI enzyme digests of the *gtfB* amplicons. In *Hae*III digests, DNA bands at 500 bp were common in clinical strains and *S. mutans* reference strains (Figure 4.4). Restriction fragments positioned at 1388, 920, 653 and 546 bp from clinical isolates were identical to *S. mutans* references (Sims, LM-7). Variation between peak bands occurred most often at the 0.6, 0.8 and 1.0-2.0 kb positions. The difference between amplitypes of *gtfB* genes produced by the two enzymes is shown in Figure 4.4. Bands located at the 0.8, 1.3 and 1.8 kb positions in *Hinf*I digests were common in Sims (NCTC 10449) and *S. mutans* clinical isolates. A total of 20 and 26 different *gtfB* amplitypes were shown after *Hae*III and *Hin*fI digestion, respectively.

4.5.3 Melibiose and raffinose-negative phenotypes

Biochemical identification showed that 17% (15/87) of *S. mutans* isolates did not ferment either melibiose and raffinose whereas 5% (5/87) were unable to metabolise melibiose. The

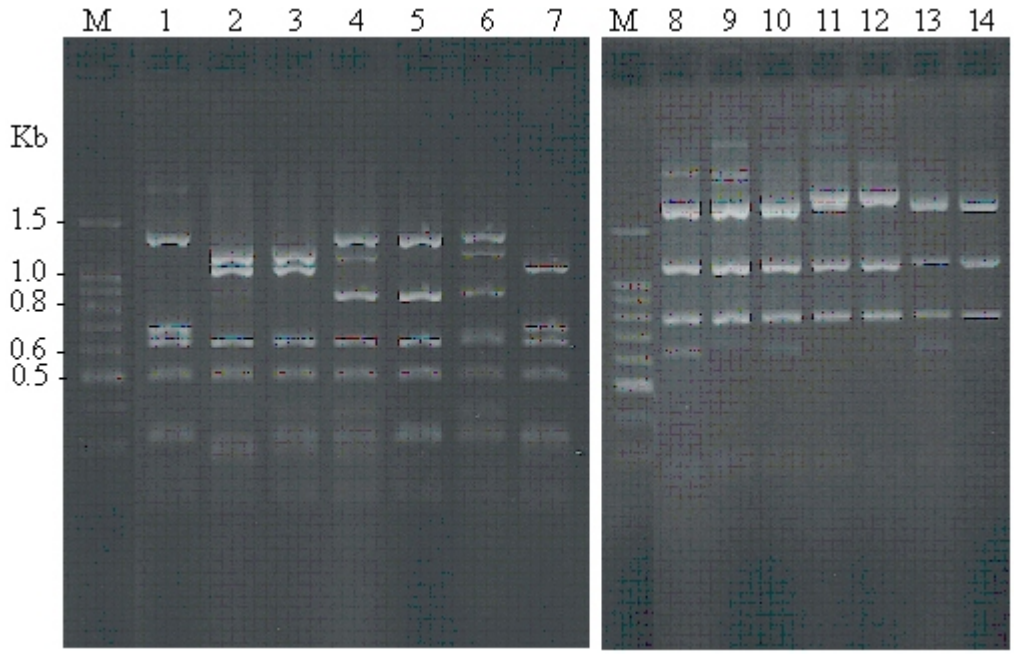

Figure 4.4 PCR-RFLP fingerprints of *gtfB* amplicons from *S. mutans* clinical isolates. Amplitypes from *Hae*III (Lanes 1-7) and *Hin*fI (Lanes 8-14) enzyme digests are shown as follows: M = LMwm, Lane 1&8 = cB12391, 2&9 = cB12512, 3&10 = cB31401, 4&11 = mB23012, 5&12 = Sims (NCTC 10449), 6&13 = cC02471, 7&14 = mC02892.

prevalence of melibiose-negative phenotypes were equally occurring in black African (n=11) and coloured (n=9) children. The remaining isolates (67/87) were biochemically identical to *S. mutans* reference strains. The distribution of melibiose and raffinose-negative phenotypes are shown on the dendograms in Figures 4.6 and 4.7, and are in shaded boxes.

4.5.4 *GtfI* gene polymorphisms of *Streptococcus sobrinus*

Between two and seven restriction fragments with molecular sizes ranging between 271 and 2000 bp were found in *Hae*III and *Hin*fI enzyme digests of *gtfI* amplicons from clinical isolates (n=20) and reference strains (SL-1, OMZ-176, Z/AHT). Peak bands positioned at 0.7 and 1.2 kb were common in reference and clinical strains in *Hae*III digests (Figure 4.5), and eleven

amplitypes were identified. Clinical isolates with DNA bands at the 1000, 900, 800, 600 and 400 bp positions were identical to laboratory reference SL-I (NCTC 12277) and OMZ-174 (NCTC 10922). In comparison, 12 amplitypes were apparent after *Hin*fI enzyme digestion, and bands located at the 1000 and 600 bp positions were common in clinical isolates and reference strains.

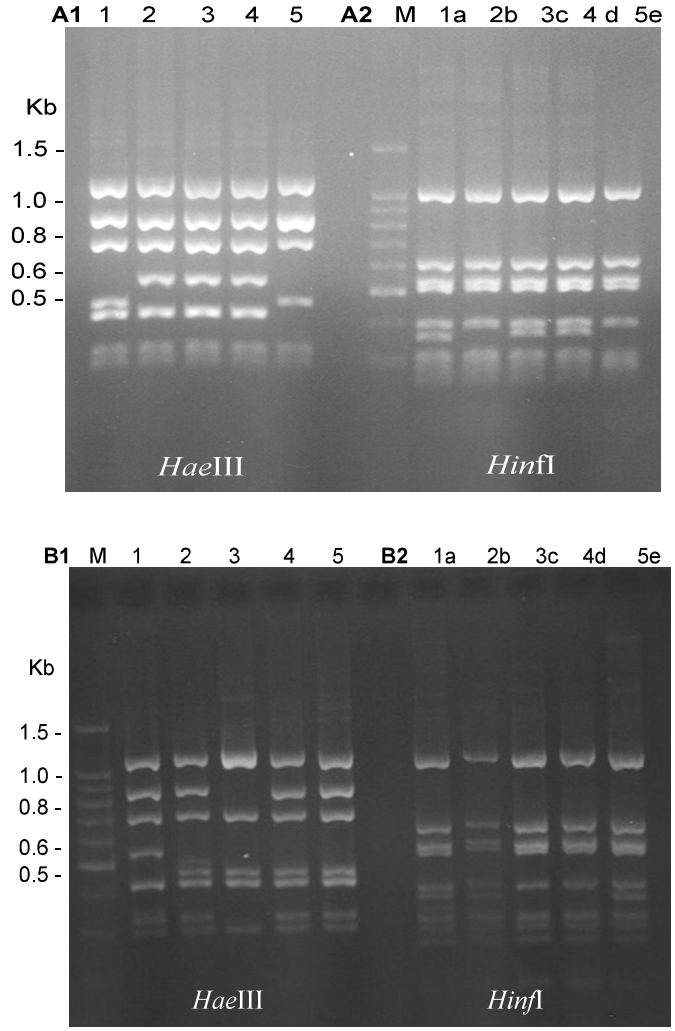

Figure 4.5 PCR-RFLP profiles of *gtfI* genes from *S. sobrinus* clinical isolates. Amplicons were digested with *Hae*III (A1 and B1), and *Hin*fI (A2 and B2) enzymes. Lanes A1 and A2: M = LMwm, 1 = cC02362, 2 = mC02363, 3 = SL-1 (NCTC 12277), 4 = OMZ-176 (NCTC 10922), 5 = Z/AHT (NCTC 10919); Lanes B1 and B2: M = LMwm, 1 = SL-1, 2 = mB12193, 3 = cB12092, 4 = cB16501, 5 = cC33551.

4.5.5 Hierarchical clustering of *Streptococcus mutans gtfB* amplitypes

The division of isolates by hierarchal clustering (UPGMA) of *gtfB* amplitypes (combined data) is shown in Figure 4.6 and 4.7. The separation of clusters was defined by differences in the number and position of bands above 1.0 kb. Cluster A comprised of *gtfB* amplitypes produced from *Hae*III (78%; r^2 =0.78*)* and *Hin*fI (76%; r^2=0.76) enzyme digests that were most similar to *S. mutans* reference strains and comprised 31% (28/89) and 47% (42/89), respectively of the total isolates investigated. The remaining clinical isolates were variant and differed from cluster A by an average distance ranging between 0.45-1.5 in *Hae*III digests and between 0.42-1.7 in *Hin*fI digests. The distribution of melibiose-negative phenotypes was randomised and not contained within any specific groups of amplitype. Dendograms showing the distribution of *gtfB* amplitypes from 5-year-old children by caries-group are shown in Appendix VI and VII.

4.5.6 Hierarchical clustering of *Streptococcus sobrinus gtfI* amplitypes

Dendograms of *gtfI* amplitype from *Hae*III and *Hin*fI enzyme digests are shown in Figure 4.8. Only a small number of clinical isolates (n=10) identified as *S. sobrinus* was investigated. Two clinical isolates only, were from caries-free coloured children (cC33551; cC01941) and the remaining test strains (n=8) were from children with active-caries. Similarly, the separation of clusters was defined by differences in the number and position of bands above 1.0 kb. One main cluster (A) was identified in both *Hae*III and *Hin*fI enzyme digests with amplitypes similar by 81% (r^2=0.81) and 76% (r^2=0.76), respectively, but differing by an average distance of approximately 0.41 between the remaining amplitypes.

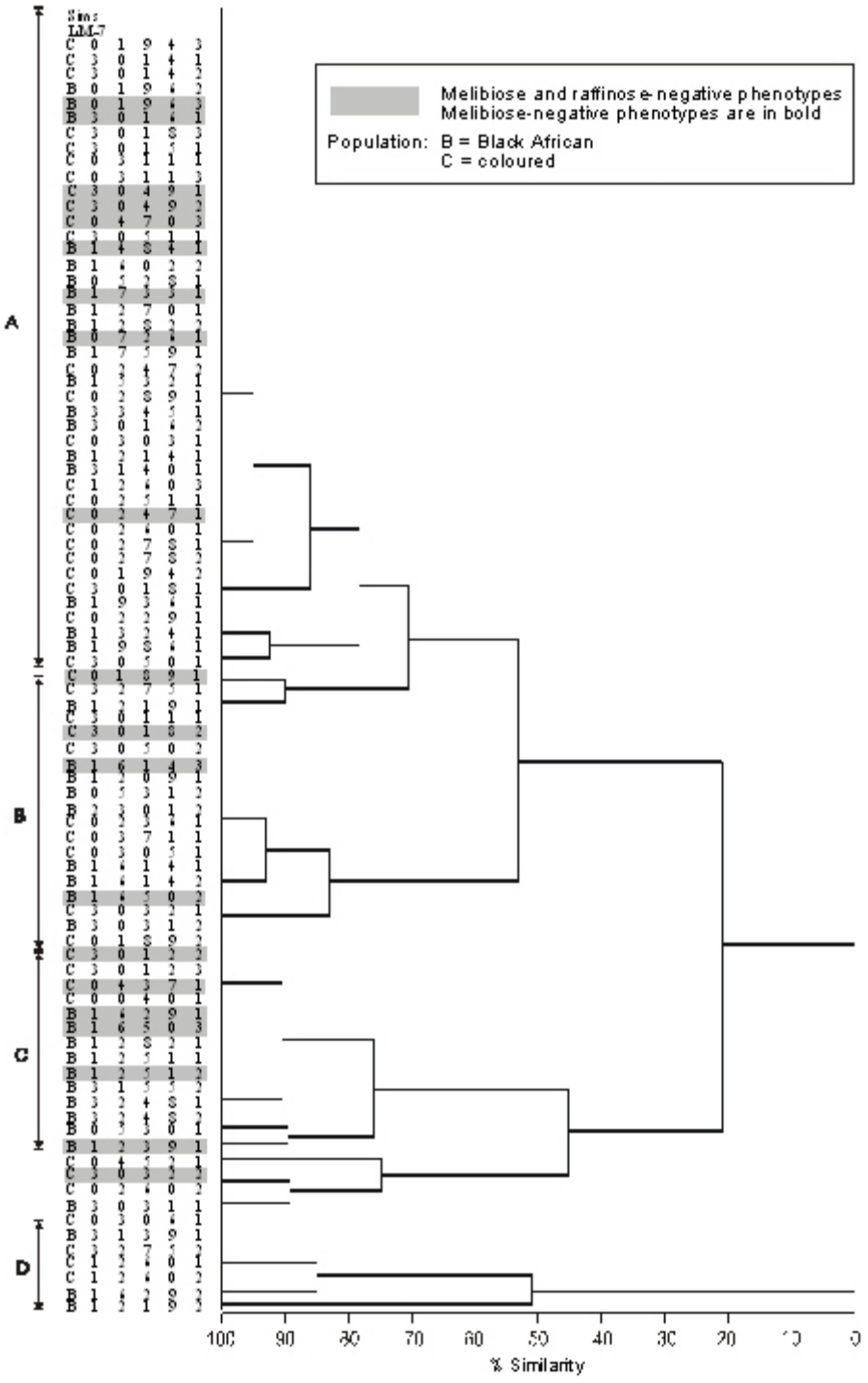

Figure 4.6 Dendogram produced by UPGMA clustering of *gtfB* amplitypes from *Hae*III enzyme digests of *S. mutans* reference strains (Sims, LM-7) and clinical isolates (n = 87) from black African, and coloured, 5-year-old children. Each isolated number is preceded by the ethnic population, followed by a four-digit number representing the family case number. The last digit is the isolate number.

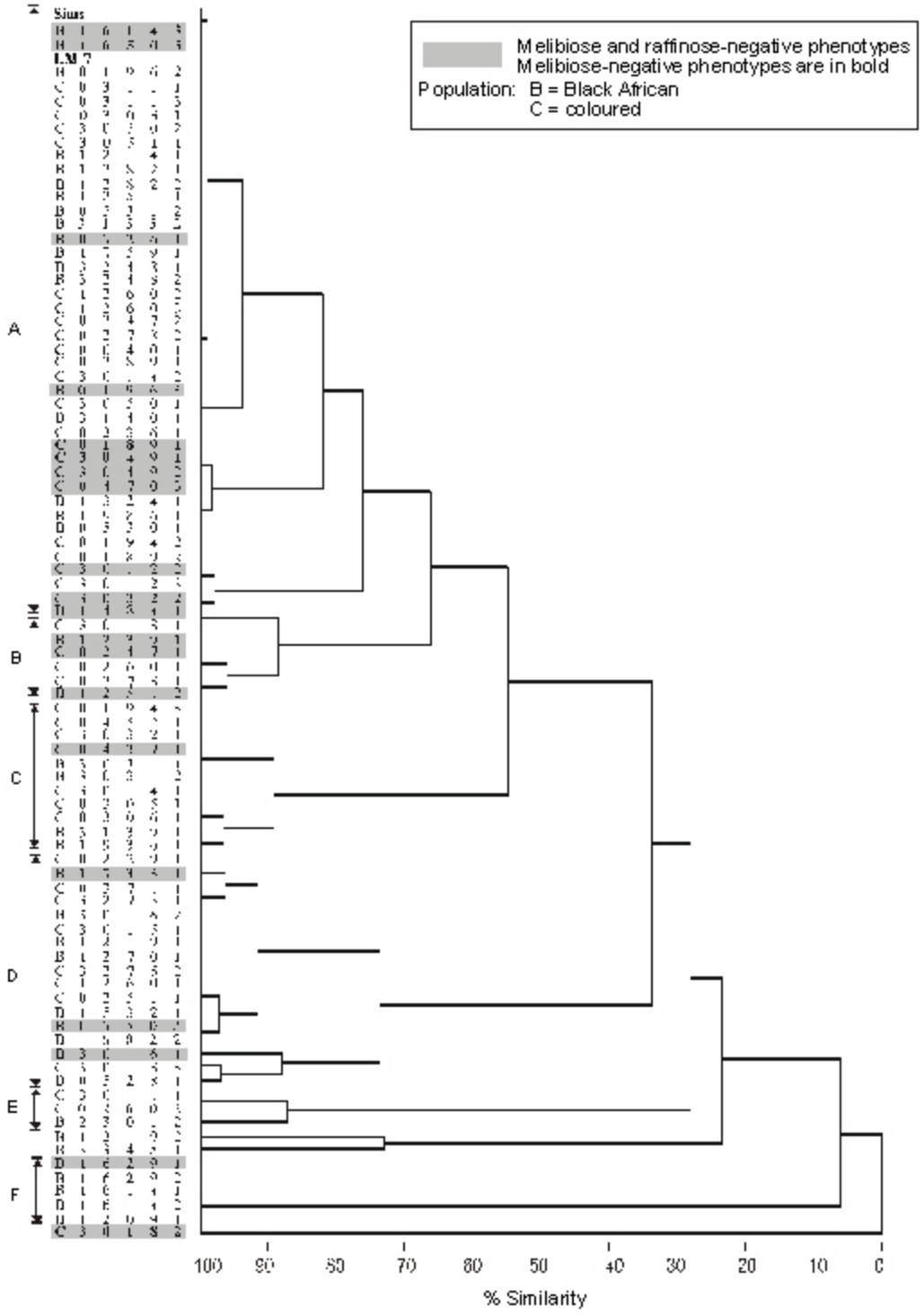

Figure 4.7 Dendogram produced by UPGMA clustering of *gtfB* amplitypes from *Hin*fI enzyme digests of *S. mutans* reference strains (Sims, LM-7) and clinical isolates (n = 87) from black African, and coloured, 5-year-old children. Each isolated number is preceded by the ethnic population, followed by a four-digit number representing the family case number. The last digit is the isolate number.

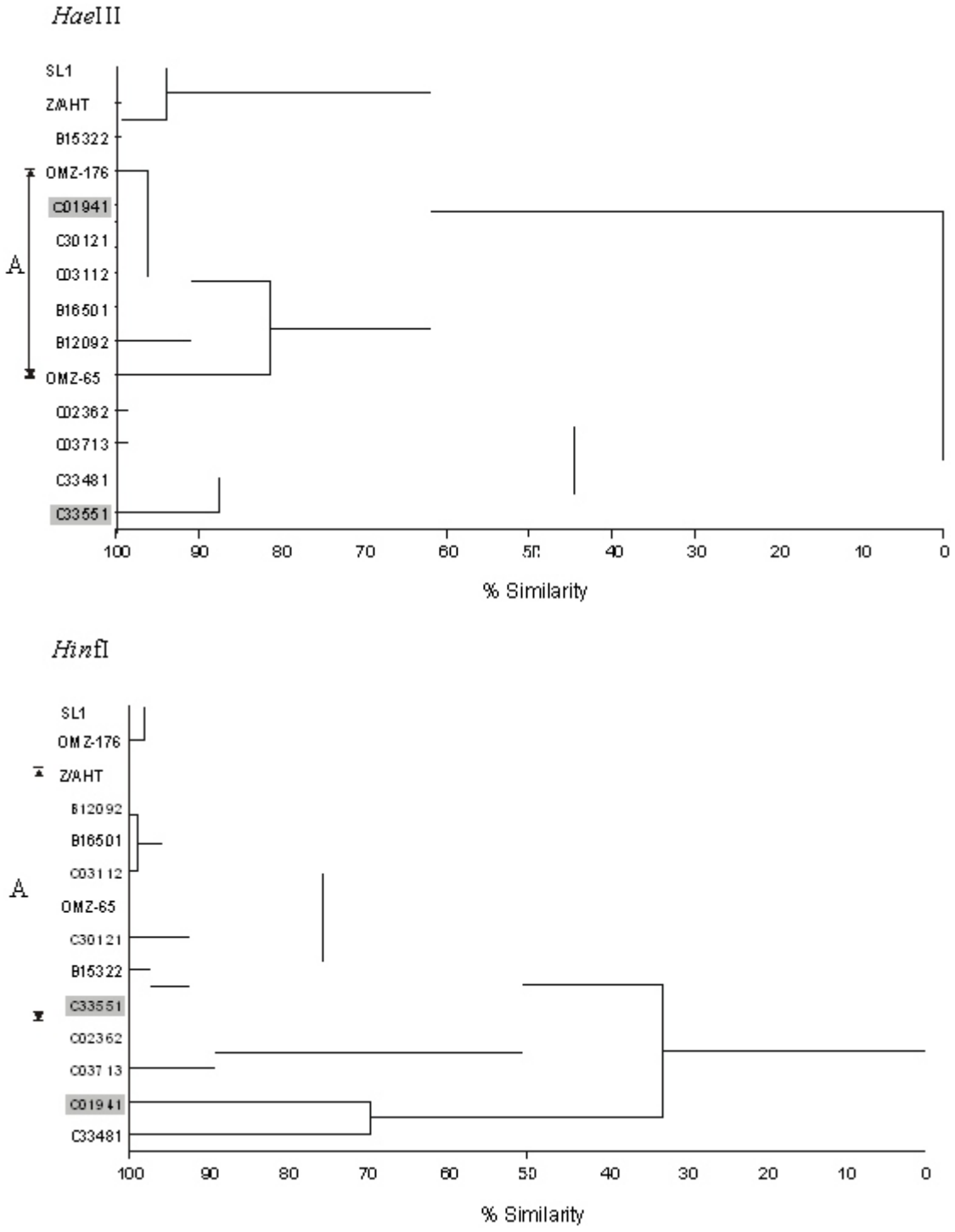

Figure 4.8 Dendogram of *gtfI* gene amplitypes produced by *Hae*III and *Hin*fI enzyme digestion of *S. sobrinus* reference strains (SL-1, Z/AHT, OMZ-176, OMZ-65) and clinical isolates (n = 10) from black African, and coloured children without and with active-caries. Isolates from caries-free children are in shaded boxes. B = black African, C = coloured.

4.5.7 Genetic diversity between *gtfB* gene amplitypes by ethnic and caries group

The variation between *gtfB* gene patterns by recognition sequence: 5'...GG▼CC...3' (*Hae*III) and 5'...G▼ANT C...3' (*Hin*fI), of clinical isolates from the two ethnic populations are shown in Table 4.3. The children harboured one to three distinct amplitypes. The number of amplitypes from children without and with active-caries ranged from 9 to 14 respectively. Thirty-two percent (28/87) of the isolates investigated had *gtfB* fingerprints identical to *S. mutans* type strain and unique amplitypes were different from reference strains.

Table 4.3 Comparison of *gtfB* RFLP patterns of *S. mutans* strains from black African and coloured, 5-year-olds by caries group, determined by *Hae*III and *Hin*fI enzyme digestion

	Caries-free				Caries-active			
	Black African		Coloured		Black African		Coloured	
	*Hae*III	*Hin*fI	*Hae*III	*Hin*fI	*Hae*III	*Hin*fI	*Hae*III	*Hin*fI
No of isolates	42				45			
No. of amplitypes	9	10	4	4	9	13	13	14
Unique amplitypes	6	8	1	2	3	8	7	9
% Diversity[a]	67	80	25	50	33	62	54	64
Identical amplitypes	3	2			6	5		
% match[b] *	75	25			15	42		

[a] % Diversity = (unique amplitypes/total amplitypes)×100.

[b] % Match = [identical amplitypes in black African and coloured children/{black African unique amplitype + coloured unique amplitype} − identical amplitypes}] × 100. *P<0.05

The number of *gtfB* amplitypes from black African children shown, was comparable in the two caries groups, but was higher in coloured children with active-caries. The percentage match (75%) between amplitypes (*Hae*III digests) from caries-free black African and coloured children was high compared with caries-active children (15%). *Hin*fI digests indicated differently, where

the percentage match (42%) was greater in caries-active children. A statistically significant association was shown in a percent match (P<0.0001) (Chi-square test) between caries groups and percent diversity between ethnic populations in *Hae*III digests (P<0.0001), but no association was indicated in amplitypes from *Hinf*I digests.

4.6 Discussion

Although PCR-RFLP analysis indicated several *S. mutans* and *S. sobrinus* amplitypes, the Principal Components Analysis indicated no significant difference between isolates from black African and coloured children or from caries-free and caries-active children. This suggests that mutans streptococci genotypes are not unique to specific racial or caries groups, and are more likely to be acquired from other sources through cross-infection.

The theoretical number and actual number of cuts resulting from *Hae*III and *Hin*fI enzyme digestion were in agreement, since the separation of DNA fragments is limited to the concentration of the resolving gel. DNA fragments with molecular weights below 2.0 kb were visualized on the 2% agarose gel which was sufficient to distinguish between *S. mutans* and *S. sobrinus* amplitypes. The efficacy of *Hae*III digests and RFLP techniques to discern between *S. mutans* and *S. sobrinus* species in different populations have shown good differentiation on chromosomal DNA (196) and in this study, has demonstrated that several amplitypes of the *gtfB* and *gtfI* virulence genes is found within *S. mutans* and *S. sobrinus* clinical isolates.

Melibiose-negative phenotypes were randomly distributed within the dendograms and comprised 23% (20/87) of *S. mutans* isolates from black African and coloured 5-year-olds. In comparison, other authors have reported percentages ranging from 3% in adults (160) to 38.6%

in children (151). Moveover, in this study nine of the S. *mutans* clinical isolates that were melibiose-negative had *gtfB* amplitype identical to references Sims and LM-7 (cluster A; Figure 4.6). However, melibiose-negative phenotypes were also apparent in amplitypes different from reference strains (cluster B-D). The isolation of melibiose-negative strains from both ethnic populations show a wide geographical and temporal distribution. This argues against the possibility that all of them are derived from a single common ancestor and can be acquired from a secondary source. The frequency of isolation of these phenotypes show that they co-exist with melibiose-positive strains in the oral cavity.

4.6.1 *GtfB* gene diversity of *Streptococcus mutans* isolates

PCR-RFLP patterns of the *gtfB* amplicons showed that 31% - 47% of isolates grouped into Cluster A of *Hae*III and *Hin*fI enzyme digests, respectively (Figures 4.6 and 4.7) were most similar to *S. mutans* reference strains. However, some 20-26 different amplitypes were found in this study after treatment with *Hae*III and *Hin*fI enzymes. Variation in genotype in *S. mutans* isolates from Alabama, China and Sweden have previously been reported by Li and co-workers (196). However, the cause of gene variation between different populations has not yet been established, but it is highly probable that factors such as inter-familial and contact with sources outside the family group, play a part in the transmission of this micro-organism. Results of this investigation are presented in Chapter 5.

To-date, studies done specifically on glucosyltransferase gene polymorphisms among *S. mutans* strains have found variation in these genes, but investigations have been restricted to using standard reference strains with small numbers of random clinical isolates (197, 203). Comparisons to other studies could not be easily made, since no investigations on *gtf* gene

polymorphisms have been described in different caries or ethnic groups. What has been reported is that DNA chromosomal profiles using the arbitrarily primed polymerase chain reaction (AP-PCR) characterized clinical isolates from three different populations (196) indicating genotypic differences between *S. mutans* species.

4.6.2 *GtfB* gene polymorphisms by ethnic and caries group

The high percentage match between *gtfB* amplitypes from caries-free black African and coloured children were probably influenced by the low numbers of caries-free children investigated. The differences found between *Hae*III and *Hin*fI digests can be attributed to the G+C mol% of a particular gene segment, since these enzymes recognise and cut at specific base sequence of GG▾CC and G▾ANTC, respectively.

4.6.3 *GtfI* gene diversity of *Streptococccus sobrinus* isolates

The prevalence of polymorphic variation within the 5'...GG▾CC...3' and 5'...G▾ANTC...3' base sequences of *gtfI* amplicons, shown by a smaller number of amplitypes (*Hae*III: n=11; *Hin*fI: n=12) was low compared to the *gtfB* gene. This may have been attributed to the low numbers of *S. sobrinus* clinical isolates investigated in this study, and in future larger numbers of this species should be incorporated. Recent studies by Klein *et al* (197) showed only one genotype of *S. sobrinus* in their study on clinical isolates from Brazilian nursery school children. In comparison, an earlier study on *gtf* heterogeneity by Shiroza *et al* (163) suggested that *S. sobrinus* is more divergent from laboratory references than *S. mutans* species.

4.7 Conclusions

The recovery level of *S. sobrinus* was low and larger numbers have to be examined in future to

extrapolate more meaningful information since a complexity of *gtf* genes coding for the enzyme glucosyltransferase does exist (197) that affects glucan binding and therefore sucrose-dependent adherence (204, 205). The genetic variation in the *gtfB* and *gtfI* genes in this study verifies this complexity and implies an attenuation in virulence of these microorganisms. The cause for change in these genes is still unclear, but the implication is that variant genotypes brought about by mutation, can be caused by a response of the microorganism to the habitat (195). The oral cavity is a dynamic environment influenced by the passage of different foods and the level of oral hygiene practised by the host. In order to understand the path of natural adaptation of these bacterial species to a challenging environment, the effect of dietary nutrients on these bacteria is discussed in part B of this thesis.

The PCR-RFLP method showed a diversity in genotype of *S. mutans* and *S. sobrinus* isolates from two South African 5-year-old child populations, which were unspecific to ethnic population or caries status. The glucosyltransferase genes are important in the pathogenesis of the mutans streptococci and share a common function, and similarity of a pattern of expression may be the easiest available means of attributing function on a genomic scale. The variation in homology of the *gtf* genes in this study, suggests differences in extracellular polysaccharide production between strains and caries can be influenced not only by the source of mutans streptococci, but also by the presence of a specific genotype.

CHAPTER 5

Gtf GENE POLYMORPHISMS IN CLINICAL ISOLATES FROM CHILDREN AND THEIR MOTHERS

5.1 Introduction

Since the mutans streptococci are predominantly found in the mouth, transmission is likely via the saliva. Studies have shown that a minimum infective dose of mutans streptococci are necessary for implantation (206), enhanced by repeated inoculation (45). In children, mutans streptococci are acquired via vertical and horizontal transmission (207). The primary oral colonization by *Streptococcus mutans* coupled with caries-promoting feeding behaviours results in levels of these organisms exceeding 30% of the total cultivable plaque flora which in turn results in rapid demineralization of tooth structure (208).

5.1.1 Acquisition and interfamilial transmission of mutans streptococci

In the 1960s, studies on laboratory animals (209) suggested that children acquire oral microbes primarily from their mothers. Since then, numerous studies (20, 22, 210) indicated that the primary care-giver of the infant, usually the mother is the main reservoir of mutans streptococci that infect the child. This occurs from approximately 8 months old, increasing only after the first tooth has erupted. The fidelity of transmission between mother and child using methods such as bacteriocin profiles, serotyping and genotyping (91, 211-213) has also demonstrated that mothers are the main source of mutans streptococci for their infants. Furthermore, mothers with high levels of *S. mutans* tend to have children who have high levels

of this bacterium (214-216), and maternal salivary levels of mutans streptococci infection may influence infection rates of mutans streptococci in their children at 4-5 years (210). However, current studies indicate that the child's environment plays a role in the initial acquisition and transmission of mutans streptococci in addition to the oral condition of the children (87).

Demographic studies by Li and Caufield (88) using a chromosomal DNA fingerprinting technique, have shown that the conservation of mutans streptococci within mother-child pairs was gender and race specific. They hypothesised that genetic, environmental and behavioural differences between African American families and white families could be contributing factors to the variation in mutans streptococci transmission (217). This suggests that patterns in mutans streptococci transmission are subject to the cultural group and habits of the population. Kozai *et al* (90) showed that 51.4% of mutans streptococci genotypes found in Japanese children were also identified in their mothers, but the remaining genotypes were from fathers and others. Hence, sources other than the mother are potential transmitters of mutans streptococci.

5.2 Aim

Demographic studies have shown genotypic differences between mutans streptococci clinical strains from mothers and their children. To-date, no attempt has been made to determine the transmission and diversity of mutans streptococci genotypes in South African children and their mothers, or between different ethnic groups. The object of this study was to determine if mothers serve as the primary source of *S. mutans* and *S. sobrinus* species, and if different ethnic populations are a contributing factor to the variation in the transmission and acquisition of mutans streptococci. This study investigates *gtfB* and *gtfI* gene polymorphisms of clinical

isolates from 5-year-old children and their mothers within the black African and coloured communities.

5.3 Materials and methods

5.3.1 Selection of mother-child family groups

A total of 47, 5-year-old children and their mothers from the original study group were investigated. One hundred and twenty-nine *S. mutans* clinical isolates (Table 5.1) and 17, *S. sobrinus* isolates from 23 black African, and 24 coloured families were studied.

Table 5.1 *Streptococcus mutans* isolates (n = 129) from caries-free (n = 33) and caries-active (n = 96) children and their mothers in two South African populations

	Caries-free			Caries-active		
	Mother/child pairs	No. of isolates		Mother/child pairs	No. of isolates	
		children	mothers		children	mothers
	n	n	n	n	n	n
Black African	11	13	14	12	18	15
Coloured	3	3	3	21	33	30

The extraction of bacterial DNA, quantification, measurement of size and integrity of genomic DNA, PCR amplification, detection of *gtf* amplicons, PCR-RFLP of amplicons, measurement of restriction fragment pattern and recording and the analyses of data have been described in Chapter 4, sub-sections 4.4.1 to 4.4.8.

5.4 Results

The Principal Component Analysis showed no significant association in *GtfB* amplitype between ethnic population, caries-group or by relation (mother-child) (Appendix XII and XIII). For this reason presentation of the data in this chapter was grouped, but for completeness, results of the Cluster analysis defined by caries and racial groups are presented in Appendix VIII-XI. In this study, seven mothers harboured *S. sobrinus* in their saliva, but this bacterium was detected in only two of their children (Figure 5.3). In comparison, *S. mutans* was isolated from all participants harbouring *S. sobrinus*.

5.4.1 Genetic diversity of *gtfB* genes

A dendogram of relationships derived from a similarity matrix (UPGMA) of *gtfB* gene patterns produced by *Hae*III enzyme digestion is shown in Figure 5.1. A total of 23 *gtfB* amplitypes was identified of which 13 were common to mothers and their children. Seventeen, mother-child family sets (f1-f17) had identical amplitypes.Thirty-four percent (44/129) of the amplitypes were identical (r^2=1.0) to *S. mutans* reference strains (cluster A) with the remaining amplitypes varying from laboratory strains by an average distance of 0.52-1.38. Families with *gtfB* profiles identical (f1-f10) to *S. mutans* reference strains are grouped into cluster A. Seven families (f11-f17) shared the same amplitype, but these were divergent from reference strains.

*Hin*fI digests of *gtfB* amplicons are shown in Figure 5.2. In comparison to *Hae*III profiles, 33 different *gtfB* patterns were shown, of which 12 were common in mothers and children. Forty-seven (36%) of the isolates were identical (r^2=1.00) to *S. mutans* reference strains (cluster A). The remaining isolates differed from reference strains by an average distance of 0.42-1.7. Isolates from families f1 to f11 were most similar (r^2=1.0) to Sims (cluster A), and f12 to f23

were divergent from *S. mutans* reference strains. An analysis of variance (ANOVA) between RFLP's resulting from *Hae*III and *Hin*fI enzyme restriction showed that patterns were significantly different from each other (F=3.36; $P<0.0001$).

The hierarchal clustering of *GtfB* amplitypes separated by caries group and ethnic population is shown in Appendix VIII - XI. *Streptococcus mutans* isolates that were identical to reference bacteria (cluster A) in the pooled analysis shown in Figures 5.1 and 5.2, were also grouped into cluster A when comparisons were made by caries and population division.

5.4.2 Melibiose-negative phenotypes

Thirty-four, *S. mutans* melibiose-negative phenotypes were distributed between mothers and children. These phenotypes comprised 26% (34/129) of clinical isolates, 10 of which were melibiose-negative and the remaining 24 that did not ferment either melibiose and raffinose. The distribution of melibiose-negative isolates are shown in figures 5.1 and 5.2. Melibiose-negative phenotypes that shared identical RFLP patterns were common in two families (f4, f6) in *Hae*III digests (Figure 5.1) and in four families (f12, f13, f20, f23) in *Hin*fI enzyme digests (Figure 5.2). Forty-four percent (15/34) and 50% (17/34) of the melibiose-negative isolates were grouped into cluster A in *Hae*III and *Hin*fI digests, respectively showing RFLP fingerprints identical or very similar (63%) to *S. mutans* reference strains. The remaining melibiose-negative phenotypes were randomly distributed among genetically variant amplitypes that clustered farther away from laboratory type strains in the dendograms. Comparisons (ANOVA) between the biochemical profiles of *S. mutans* clinical isolates and *gtfB* amplitypes of *Hin*fI digests, showed a statistically significant difference (F=2.26; P=0.02).

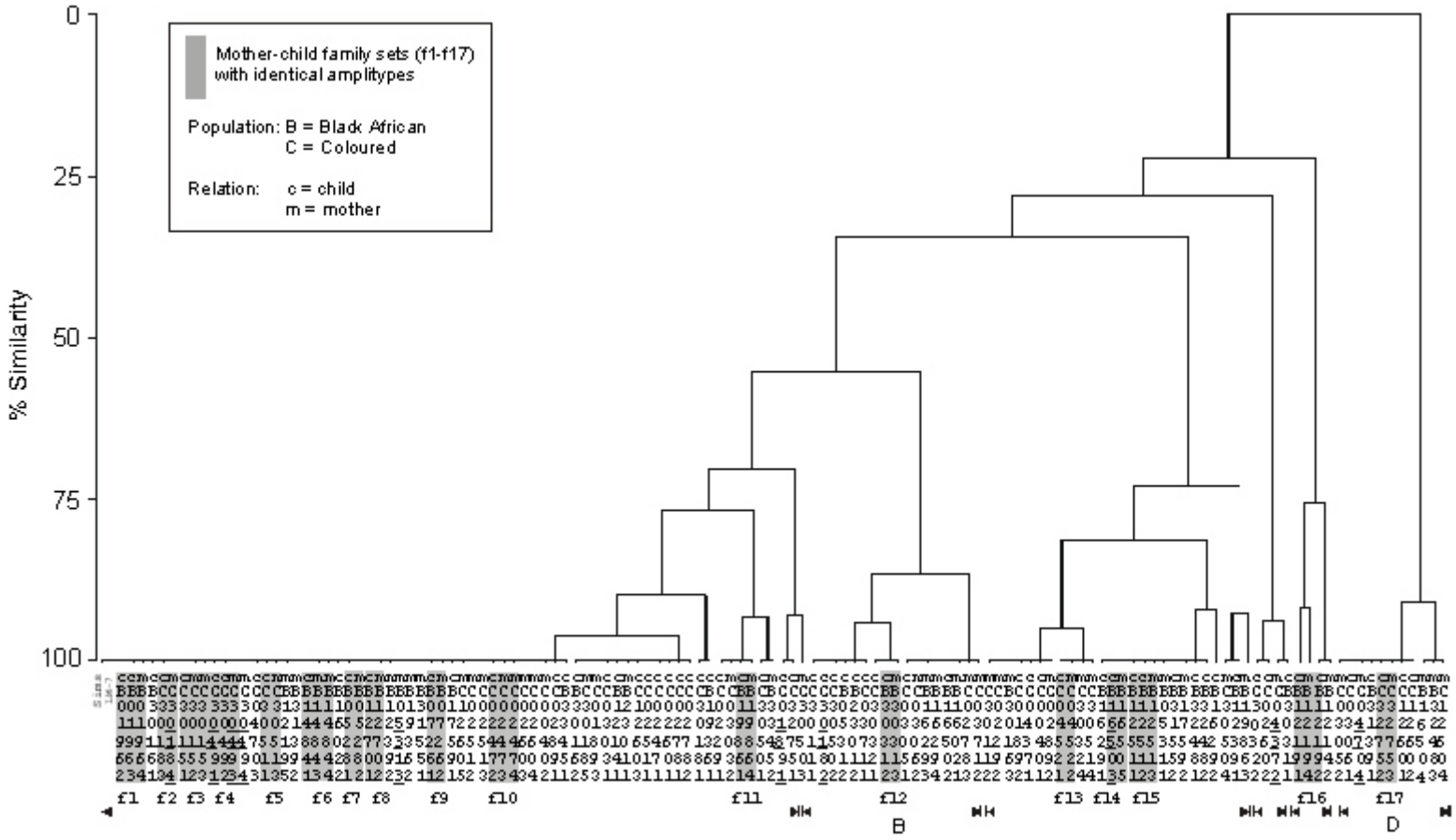

Figure 5.1 Dendogram of *gtfB* amplitypes produced by *Hae*III digestion of *S. mutans* reference strains (Sims, LM-7) and clinical isolates (n = 129) from black African and coloured children and their mothers. The distribution of melibiose and raffinose-negative phenotypes are shown in bold (n = 24) and melibiose-negative phenotypes (n = 10) are in underlined bold.

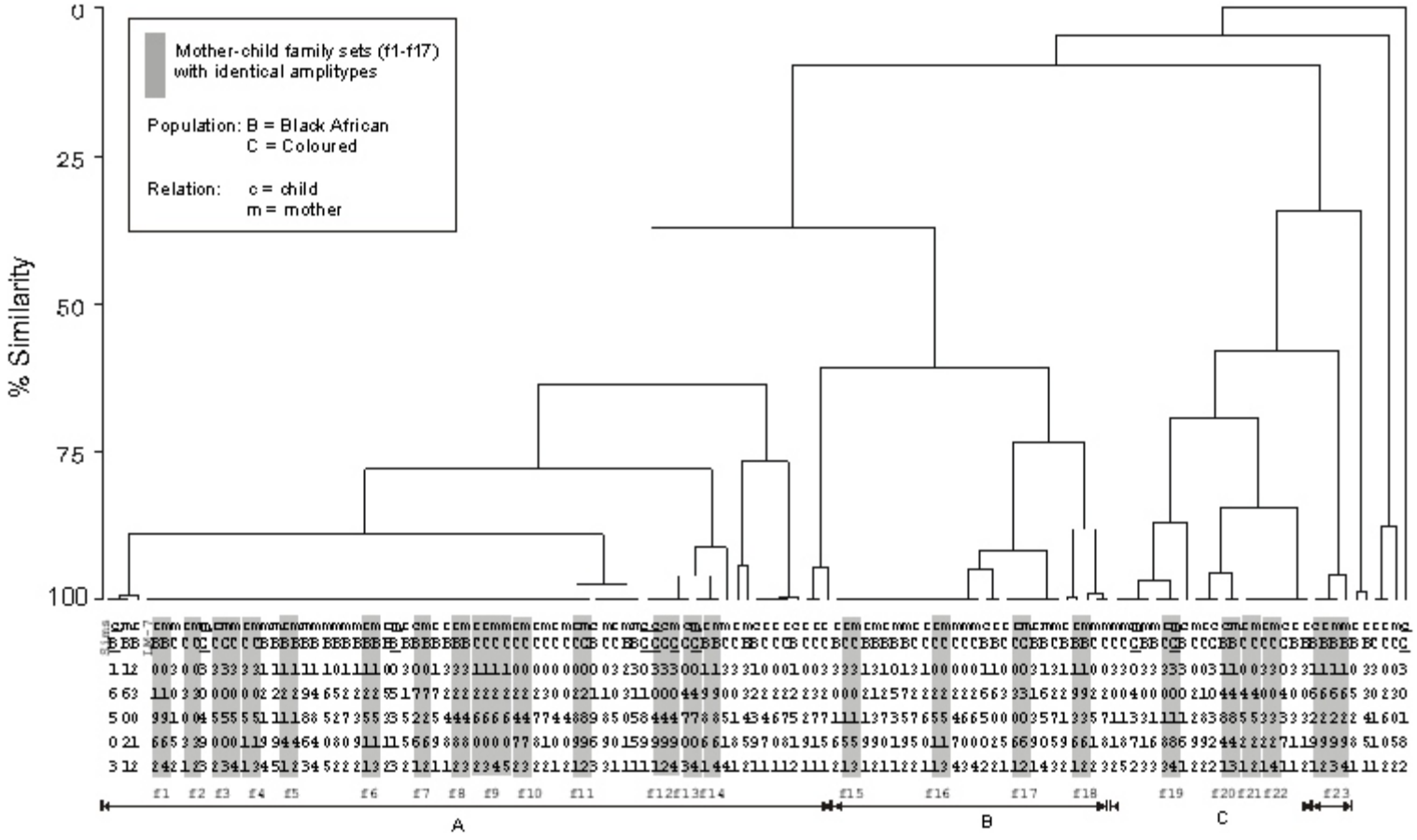

Figure 5.2 Dendogram of *gtfB* amplitypes produced by *Hin*fI digestion of *S. mutans* reference strains (Sims, LM-7) and clinical isolates (n = 129) from black African and coloured children and their mothers. The distribution of melibiose and raffinose-negative phenotypes (n = 24) is in bold and melibiose-negative phenotypes (n = 10) are in underlined bold.

5.4.3 Genetic diversity of *gtfI* genes

The relationship between *S. sobrinus* clinical isolates demonstrated by *gtfI* gene amplitypes from *Hae*III and *Hin*fI enzyme digests are shown in Figure 5.3. A total of 17, *S. sobrinus* isolates were investigated of which 10 were from children and seven from mothers. However, only two mother-child pair of isolates were evident of which mother-child pair cC02362 and mC02363 were identical to each other in both enzyme digests.

Sixteen amplitypes were shown in *Hae*III digests and UPGMA analysis separated clinical isolates into five different clusters. Cluster A comprised the majority of isolates (n=7) that was 81% (r^2=0.81) similar to reference strains OMZ-176 (NCTC 10922) and OMZ-65 (NCTC 11061). The association between amplitypes ranged from a low of 20% (r^2=0.20) to 100%. Amplitypes that were different from reference strains differed by an average distance of 0.34-0.87. In comparison, 14 amplitypes were indicated in *Hin*fI enzyme digests. Similarly, clinical isolates were divided into five clusters of which cluster A was the major group, comprising of 10 clinical isolates that were 71% (r^2=0.71) similar to each other. The remaining groups of isolates differed by an average distance of approximately 0.53 from cluster A.

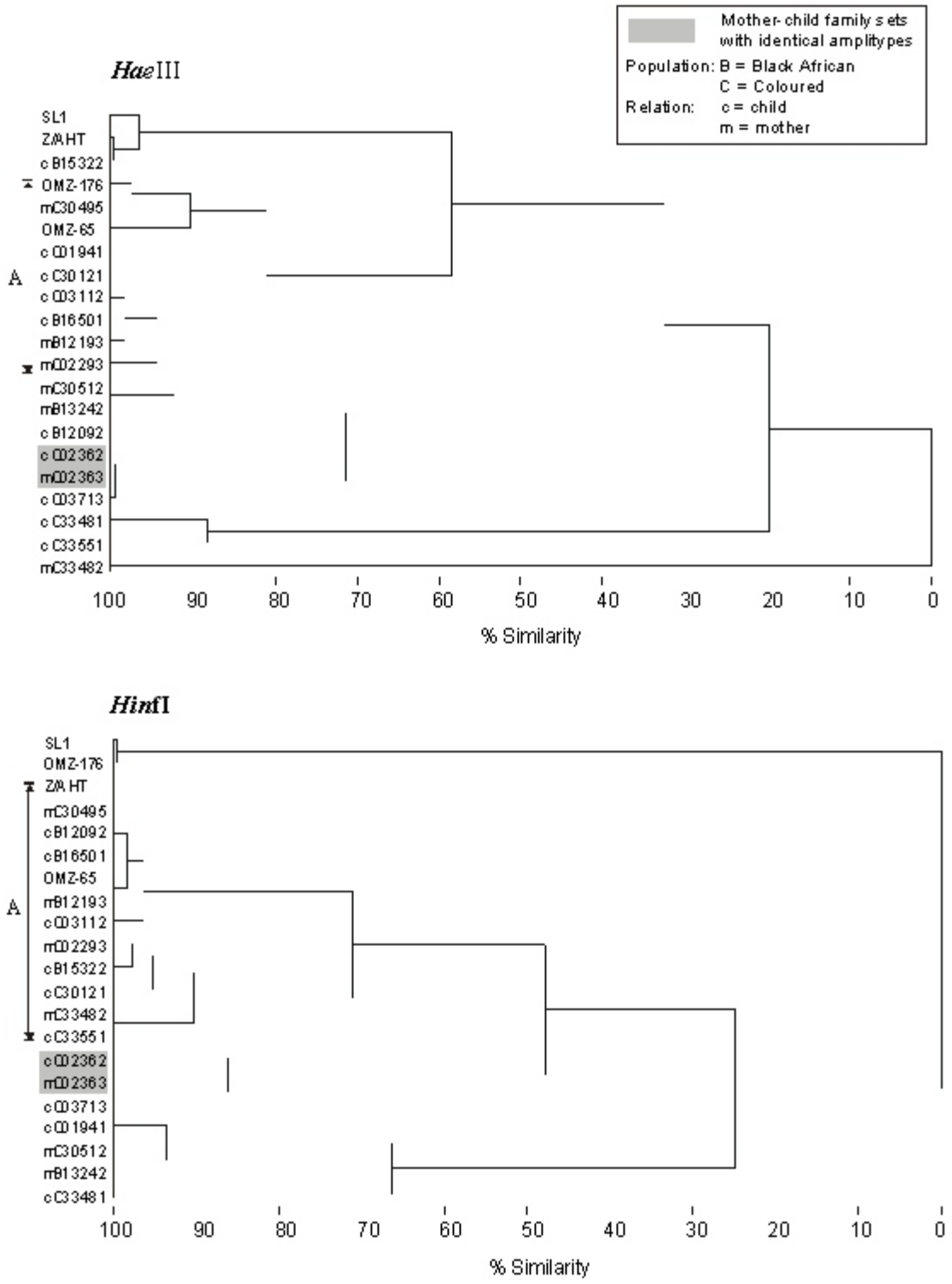

Figure 5.3 Dendogram of *gtfI* amplitypes produced by *Hae*III and *Hin*fI enzyme digestion of *S. sobrinus* reference strains (SL-1, Z/AHT, OMZ-176, OMZ-65), and clinical isolates (n = 17) from black African and coloured children and their mothers.

5.4.4 Genetic diversity between *gtfB* gene amplitypes in mother-child pairs

The variation between *gtfB* gene fingerprints by recognition sequence: 5'...GG▼CC...3' (*Hae*III) and 5'...G▼ANT C...3' (*Hin*fI), of clinical isolates from mother-child sets are shown in Table 5.2. A statistically significant difference was found in % match (P=0.02)(Chi-square test) of amplitypes between the two caries groups, but no significant difference between the two ethnic populations or in % diversity of genotypes between mothers and children was shown.

Table 5.2 Comparison of *gtfB* RFLP patterns of *S. mutans* strains from mother-child pairs by caries group, determined by *Hae*III and *Hin*fI enzyme digestion

	Caries-free				Caries-active			
	Mother		Child		Mother		Child	
	*Hae*III	*Hin*fI	*Hae*III	*Hin*fI	*Hae*III	*Hin*fI	*Hae*III	*Hin*fI
No of Families	14				33			
No. of amplitypes	7	8	10	10	14	15	15	21
Unique amplitypes	4	4	7	6	5	4	6	10
% Diversity[a]	57	38	70	60	36	27	40	48
Identical mother-child pairs	3	4			9	11		
% match[b] *	38	67			45	37		
TVC/mL ($\bar{x}$ ± SD) ×10^5	9.7 ± 31.1		0.6 ± 1.8		2.9 ± 4.4		3.4 ± 12.3	

[a] % Diversity = (unique amplitypes/total amplitypes)×100.

[b] % Match = [identical amplitype sets in mother-child pairs/{mother's unique amplitype + children's unique amplitype} − identical amplitype sets in mother-child pairs}] ×100.*P<0.05

The salivary concentration of mutans streptococci per millilitre of saliva was greater in the mothers of caries-free children and in caries-active 5-year-olds (Table 5.2), but no statistically significant difference or association between total viable count and caries severity or

prevalence (Spearman correlation coefficient) was shown. No comparison between mothers and their children were made for *gtfI* amplitypes, since two mother-child sets were identified, with the remaining isolates (n=13) unrelated.

5.5 Discussion

The number of caries-free children was low (30%) in this study group and in future, larger numbers of high-risk, caries-free children should be investigated for the prediction of caries based on mutans streptococci virulence. Nevertheless, no statistically significant difference was shown in mutans streptococci total viable count between children and their mothers in the two caries groups. The dental health of mothers was not examined in this study and it is unknown whether the mothers of children with active-caries also had dental caries, and if this would have had any influence on mutans streptococci genotype and transmission.

5.5.1 Ethnic population, caries status and *gtf* diversity

Because the diet of the black African is more fibre rich with fewer refined carbohydrates than the coloured population, it was anticipated that exposure to different carbohydrate sources would have an effect on *gtfB* and *gtfI* gene variation since this gene is responsible for the production of glucosyltransferase that synthesize glucans. Also, active-caries were higher in coloured children (n=21) than black African 5-year-olds (n=12). However, speculation was disproved by the Principal Components Analysis of RFLP patterns where isolates were not separated into significant groups by ethnicity or caries grouping, suggesting that these variables have no influence on *S. mutans* genotypes. Subsequently, Mattos-Graner *et al* (194) have found that inherent *gtf* gene polymorphisms influence the formation of biofilms, and hence plaque, to a lesser degree compared to the amount of gtfC and gtfB isozymes produced. Their results

implied that factors that control the expression of the *gtf* genes that encode these enzymes may account more for virulence differences among *S. mutans* strains.

5.5.2 Melibiose-negative phenotypes and RFLP's

Melibiose-negative phenotypes with identical *gtfB* amplitypes were shown in four children and their mothers, are indicated in Figures 5.1 and 5.2 (*viz, Hae*III:f4 and f6; *Hin*fI: f12, f13, f20, f23). The total number of melibiose-negative phenotypes found in mother-child pairs were considered low and the distribution of these isolates by amplitype varied according to the restriction enzyme used. Hence, melibiose-negative strains do not have identical *gtfB* genotype. The melibiose-negative isolates grouped into mother-child pairs comprised only 26% (9/34) of the melibiose-negative isolates and the source of the remaining phenotypes (n=25) could not be attributed to mother-child transmission.This implies that infection from other contacts is possible or the synthesis of melibiose is reliant on expression of the *gtfA* gene which is influenced by the oral environment. Hence, the effect of factors such as dietary nutrients should be examined. The influence of traditional African dietary nutrients on *gtfA* gene expression is discussed in Chapter 11.

5.5.3 *GtfI* gene diversity of *Streptococcus sobrinus* from children and mothers

Only two, mother-child pairs of identical, *S. sobrinus* isolates were apparent and suggest that these bacteria are not always transmitted from mother to child. All of the caries-active children harbouring *S. sobrinus* were infected with *S. mutans* as well. Other investigators have shown that the prevalence of dental caries in different populations is higher in young children harbouring both these strains than those with either *S. mutans* or *S. sobrinus* alone (74, 167, 168, 218). The variation in amplitype numbers produced by *Hae*III (n=16) and *Hin*fl (n=14)

enzyme digests was marginal. This can be attributed to differences in the G+C content of the *gtfI* gene fragment. Because *Hae*III and *Hin*fI recognises and cleaves only the particular base sequence of GG▾CC and G▾ANTC respectively, the outcome of DNA restriction fragments is dependent on the G+C mol% of a particular gene segment. However, a study on *gtf* heterogeneity by Shiroza *et al* (163) suggested that *S. sobrinus* are more divergent than *S. mutans* strains.

5.5.4 *GtfB* gene diversity of *Streptococcus mutans* from children and mothers

The absence of significant variation shown by the Principal Components Analysis, between *S. mutans* isolates from mothers and children was expected since mothers are a primary source of these microorganisms. The percentage diversity of amplitypes from 5-year-old children was high in both caries groups (Table 5.2) and the percentage match of amplitypes from mother-child pairs between the two caries groups was statistically significant. Yet, besides the 67% match shown in *Hin*fI digests (caries-free), these percentages accounted for less than 50% of the total, suggesting that more than half of the genotypes identified were acquired from sources other than the mother.

In other investigations, the match in genotype between mother-child pairs in different population groups have shown differences. In Swedish families, 24% of 3-year-old children had identical genotypes to their mothers, none showed their fathers' genotypes, and 44% harboured genotypes that did not match those of any family members (89). In Chinese families 36-45 % of similar mother-child pairs were observed in nursery school children (91, 219) compared with 71% observed in an American population (88). In Japan, 31.4% of the genotypes harboured by 0- to 11-year-old children matched genotypes detected in their fathers,

while 30.5% of the children studied carried genotypes not found in their parents (220). The extent of *S. mutans* diversity show that additional genotypes become part of the established flora over time. These comparisons indicate that differences in genotype can be attributed to cultural practices that may influence the degree of contact between children and their parents and other individuals.

Comparison between *S. mutans* species from father and sibling was not done, since in South Africa, women mostly are the primary care-givers to children and extended members of the family in these two ethnic groups. Therefore, it is possible that different strains may have been acquired by horizontal transmission between siblings and children in playgroups (90). Nevertheless, this does not rule out the possibility that the variant genotypes may also have been acquired through father-child transmission.

The differences found in *gtfB* gene profiles verifies the complexity of this virulence gene and hence of *S. mutans* strains, yet the conditions responsible for polymorphic differences is still unclear and further investigations on the polymorphic loci and the nature of the mutations needs to be done. Nevertheless, this variance has the potential to influence glucan binding (191) and consequently sucrose-dependent adherence (204). However, a problem encountered with cross-sectional studies of this nature is that transient infection with *S. mutans* genotypes are possible, so it was impossible to determine if the genotypes present were those that were persistent after initially acquisition, or whether there was an increase in diversity over time, acquired from other sources. In a longitudinal study, Klein *et al* (197) showed that genotypes acquired from maternal or alternative sources may show effective persistence in the mouth. In this study, identical *gtfB* amplitypes were shown in the two caries groups but a greater number

of genotypes were found in both mothers and children in the caries-active group. In future, larger numbers of isolates need to be sampled from caries-free children, for investigation and comparison. However, the % match in the caries-active group accounted for less than 50% suggesting that children acquired some 60% of *S. mutans* genotypes from another source.

5.6 Conclusions

Results from this study suggest that the PCR-RFLP analysis approach of investigating the *gtfB* gene, provides a useful tool in microbial epidemiology by targeting a virulence gene that is important in the pathogenesis of this microorganism. It has the ability to differentiate between *S. mutans* genotypes and to trace the source of infection, but does not identify the virulence potential of strains, nor does it target specific amplitypes for being part of more virulent *S. mutans* strains.

The mother-child route of transmission of *S. mutans* strains are the same in the two South African ethnic populations as well as in caries-free and caries-active children. The divergent genotypes are either acquired from sources such as other members of the family or from play-groups, or these bacteria have genetically adapted to challenges in the oral environment. PCR-RFLP of the virulent *gtfI* gene of *S. sobrinus* showed different amplitypes, and that mother to child transmission of these bacteria is low, indicated by the small number of mother-child pairs with identical amplitypes. The differences in correlation values of dendograms produced by *Hae*III and *Hin*fI enzyme digests verify that the base sequences of the *gtfB* and *gtfI* gene fragments are not homologous in *S. mutans* and *S. sobrinus* species. It is highly probable that variation between these gene segments is brought about by adaption of these virulence genes to changes in the oral environment over time.

PART B

MUTANS STREPTOCOCCI VIRULENCE AND TRADITIONAL AFRICAN FOODS

CHAPTER 6

MUTANS STREPTOCOCCI TEST STRAINS

6.1 General remarks

Chapter 6 deals with the selection of the mutans streptococci test isolates and reference strains that were used in Part B of this study. Chapter 7 explains the reasons for selecting the Batch Culture technique and the methodologies used for the preparation of the food challenges and the method of batch culture. No results are reported in these two chapters, so are entirely methods. Within each subsection, results will be given as shown in the following flowchart:

Chapter 8

Growth, pH and acid production

⬇

Chapter 9

Extracellular polysaccharide, protein concentration and glucose metabolism

⬇

Chapter 10

Inherent acidogenicity and buffering capacity of traditional African foods

⬇

Chapter 11

Gtf gene expression and traditional African foods

6.2 Introduction

The mutans streptococci are normal resident oral micro-flora that colonizes non-shedding surfaces such as pits and fissures of the occlusal surfaces of the teeth. However these surfaces are susceptible to food impaction providing all of the necessary nutrients required for mutans streptococci growth and acid production. They possess virulent cariogenic properties expressed only under specific environmental conditions. Although some studies found that diet can have a limited influence on caries (3, 34) some fermentable carbohydrates promote virulence such as acid and extracellular polymer production more than others, that can influence streptococcal strain selection and survival in the mouth.

Previous studies on urban and rural black preschool children in South Africa have postulated that nutrient intake was not clinically relevant to caries prevalence and experience other than in susceptible individuals (127, 128). In addition, the numbers of mutans streptococci have remained similar in children with and without caries, and with low correlation to dental caries (84). Yet, the survival of this microorganism in the oral cavity is direct evidence that all of the necessary nutrients required for growth is being provided.

Investigations of mutans streptococci glucosyltransferase gene polymorphisms reported in Chapter 4 and 5 have shown a diversity in genotype of clinical isolates from the black African and coloured urban population. The percentage match in amplitype (Chapter 4) between caries-free and caries-active children were statistically significant ($P<0.0001$) suggesting that similar *Streptococcus mutans* amplitypes are found in both caries groups. Comparisons of *gtfB* amplitypes between mothers and their children also showed that about 50% of the genetic variation found in clinical strains from children was attributed to acquisition from sources

other than the mother (Chapter 5).

The investigation of other sources of mutans streptococci was not included in this thesis, so the extent to which these sources contributed towards genetic diversity remains unknown. Also, it could not be determined if the genotypes shown in Chapter 4 and 5 were those that were persistent after initial acquisition, or whether there was an increase in diversity over time. The impact of the oral environment can influence strain selection and survival in the mouth. For example, a change in fermentable carbohydrate in the human diet can play a contributing part in the balance of the oral microflora. Variability in the frequency and type of food ingested can cause fluctuations in the stability of this ecosystem, resulting in enrichment within the microbial community of the pathogens due to the imposition of major ecological pressures and contributing towards caries risk (3, 4).

Little is known about the association between strain diversity and the virulence of mutans streptococci (60) in different environments, moreover these virulence attributes are not static or constitutively produced entities, but are strongly influenced by environmental conditions that control expression and modulation of the *gtf* genes (221). Factors that control *gtf* gene expression still need to be identified, since the production of GtfB and GtfC enzymes may account for differences in virulence among infecting *S. mutans* strains (194).

6.2.1 Points to consider

The genetic variation found within the *gtfB* and *gtfI* genes (Chapter 4 and 5) that encode for the glucosyltransferase enzymes that catalyse the production of extracellular polysaccharides necessary for bacterial adhesion, emphasises that the *gtf* gene sequence is divergent. The

implication is that this virulence property is not in a condition of stasis. However, Mattos-graner and co-workers (194) hypothesised that biofilm formation in *S. mutans* was independent of the GtfB and GtfC genotype and there is a need to identify factors that control *gtf* gene expression. Factors in the environment, such as dietary nutrients change conditions in the oral habitat that may influence gene expression and modulation of *gtf* genes. This still has to be proven. In the two South African populations investigated, some of the clinical strains may have been acquired from contact with fathers, siblings and other children, yet it is also possible that the genetic diversity found within clinical isolates may have risen from adaptation to the traditional African diet. The glucan formed as a by-product from these dietary substrates play a critical role in the pathogenesis of this ubiquitous disease.

To-date, *in-vitro* studies on the influence of traditional African staple foods, such as maize and their food combinations on the virulence of mutans streptococci have not yet been investigated. In addition, the identification of specific variants to be 'more or less cariogenic' has yet to be determined at the level of gene expression. This section of study focuses on the influence of traditional African staple and combination foods on the virulence of mutans streptococci reference and clinical strains by their response to these food challenges

6.3 Selection of reference strains and clinical isolates

6.3.1 Mutans streptococci reference strains

The mutans streptococci reference strains selected for this study were obtained from the National Collection of Type Cultures (NCTC) (Colindale, London) (Table 6.1). These strains were described in Chapter 2, sub-section 2.8.1.

6.3.2 Mutans streptococci clinical strains

Five clinical strains that were collected and identified from two previous studies investigating the influence of food challenges on plaque pH (111), and the prevalence of acidogenic bacteria in 5-year-old South African children (84) was examined. Two of the clinical isolates were collected from the plaque of a 5-year-old coloured child with active-caries (c) and the remaining isolates were from three, 12-year-old caries-free (cf) children of black and coloured descent. The code assigned to these clinical isolates for this study was 953012A (c), 953012B (c), 942036 (cf), 942037 (cf) and 942007 (cf) (Table 6.1).

6.3.3 Biochemical verification of strains

The identity of the test bacteria was confirmed by methods described in Chapter 2, sub-section 2.7. Similarly, a numerical code was allocated to each carbohydrate assay. If the carbohydrate was metabolized by the bacterium, the test strain was assigned the corresponding numerical code (Table 6.1). The selection of wild-type strains were also based on their atypical fermentation reaction to melibiose, raffinose and aesculin hydrolysis, compared with the control reference strains.

6.3.4 Genetic verification of strains

Verification of the selected clinical test strains was checked by PCR amplification of the *gtfB* and *gtfI* virulence genes that encode the GTF-I and GTF-SI enzymes, and the *gtfA* gene which is part of the *msm* (multiple sugar metabolism) operon responsible for the uptake and metabolism of melibiose and other sugars (159, 160) (Table 6.1). The bacterial cultures were kept frozen at -20°C in a cryo-protective fluid containing glycerol and fortified with foetal calf serum (Appendix III), until analysed.

Table 6.1 Biochemical characteristics of mutans streptococci reference strains, clinical isolates and PCR verification by amplification of the *gtfB*, *gtfI* and *gtfA* gene fragments

Strain number	Strain / source of origin (*serotype*)	* Profile no.	*gtfBC*	*gtfI*	*gtfA*
Streptococcus mutans					
NCTC 10449	Sims (*c*)	1234067	+	−	+
NCTC 10923	LM-7 (*e*)	1234067	+	−	+
Streptococcus sobrinus					
NCTC 12277	SL-1 (*dg*)	1200060	−	+	−
NCTC 10922	OMZ-176 (*dg*)	1200060	−	+	−
NCTC 10919	Z/AHT (*a*)	1200060	−	+	−
NCTC 11061	OMZ-65 (*dg*)	1200060	−	+	−
Clinical Isolates					
Caries-free:					
942036	12-year-old, black	1200000	−	+	−
942037	12-year-old, black	1234060	+	−	+
942007	12-year-old, coloured	1200067	+	−	−
Active-caries:					
953012A	5-year-old, coloured	1234067	+	−	+
953012B	5-year-old, coloured	1200060	−	+	−

* Biochemical numeric coding: 1 = mannitol; 2 = sorbitol; 3 = raffinose; 4 = melibiose; 5 = NH_3 from L-arginine; 6 = bacitracin resistant; 7 = aesculin hydrolysis; + = present; − = not present.

CHAPTER 7

BATCH CULTURE

7.1 Introduction

The traditional method of studying microbial growth has been batch culture, in which a volume of medium is inoculated with a small number of organisms and their growth followed over time (222). Batch culture examines microbial growth kinetics, whereby all substrates are added at the beginning of the fermentation, and the consumption of the growth-controlling substrate or the increase in biomass concentration is monitored as a function of time. Inherent in this system is that the cell's environment and hence the cell's composition and physiological state change during the experiment (223).

7.1.1 Growth phases in batch culture

In order to monitor microbial physiology within a controlled environment, it is necessary to understand bacterial population growth and death. The growth of bacteria during batch culture comprises five stages. Growth commences with lag, whereby the microorganisms adapt to the new environment by synthesizing new enzymes. A longer lag phase occurs when cells are starved or when the growth medium is not 'rich'. This is followed by exponential growth or log phase where the cells multiply and the rate of division is determined by the composition of the medium and environmental conditions. When the concentration of one or more nutrients becomes limiting and falls to a low level, this is accompanied by a decline in the growth rate, termed late log phase. The stationary phase ensues and can be caused by nutrient deprivation

or the accumulation of inhibitory products of metabolism. The organisms are still viable and some use endogenous reserves for maintenance. Death due to cell lysis or effects of toxic metabolites occurs at a variable time after the onset of stationary phase (222). In the case of mutans streptococci species, endogenous reserves are sourced from intracellular polysaccharides produced by these microorganisms, and are available in dental plaque.

7.1.2 Growth kinetics and influence of the environment

Bacterial growth kinetics, defined by Monod (224), is the relationship between the specific growth rate (μ_{max}) of a microbial population and the substrate concentration (K_s). In the natural environment, microorganisms grow mostly in mixtures of substrates (225). Growth may also be controlled by two or more nutrients at the same time (226, 227), and kinetic properties of a cell might change due to adaptation (221, 228). Similarly, the oral environment provides a variety of mixtures of substrates sourced from dietary carbohydrates that are available to cariogenic bacteria at intermittent times.

7.1.3 Laboratory cultivation techniques versus environmental conditions

Various experimental systems of laboratory culture methods are available. These include the following major systems.

7.1.3.1 Suspended-culture systems

According to a review by Kovárová-Kovar and Egli (223), microbial growth kinetics of suspended cells have been investigated in the laboratory in batch, continuous-culture or fed-batch culture systems. Fed-batch culture has never been routinely used to estimate growth-rate and substrate limitation. In batch-culture, either the consumption of the growth-controlling

substrate or the increase in biomass concentration is monitored as a function of time. However, inherent in this system is that the cell's environment and hence the cell's composition and physiological state change during the experiment. In continuous culture, the growth-controlling substrate is kept at equilibrium concentrations, independently of culture density or time. This allows a stable environment for growth and hence the same physiological state. In comparison with closed batch-culture, this allows for more precise and reproducible data. However, due to the constancy of environmental control, this system represents artificial growth conditions and is not appropriate to study some microbial kinetic phenomena as they occur in nature. In this respect, simulation of the natural environment probably resides somewhere between the closed batch-culture and open continuous culture systems.

7.1.3.2 Biofilm culture systems

The biofilm model systems were developed for the study of bacterial interaction during plaque development, and to replace *in vivo* studies of natural plaque. The major oral biofilm culture systems in current use are: Chemostat-based systems, where experimental surfaces are submerged in chemostats; Growth-rate-controlled biofilm fermenter that gives controlled media-limited outgrowth rates of thin filter-deposited biofilms, analogously to the chemostat; 'artificial mouths'; the constant-depth film fermenter and the multiplaque artificial mouth (229). The general consensus was that each culture system had its strengths, limitations and difficulties, with no one system better than the others for all purposes. Selection of the appropriate system depends on the specific problems addressed.

The non-biofilm culture systems have formed the cornerstone of oral microbiology. According to Sissons (229), the most common plaque model is a batch culture of a single oral organism.

This is not a biofilm, has no genotypic or spatial heterogeneity and a changing bacterial environment far removed from that of plaque. Within these limitations, it can be a valid approach to the study of behaviour relevant to that in plaque biofilms of the organism involved.

7.1.4 Emphasis of this Batch culture study

The emphasis of this investigation is on the growth and growth-limited biodegradation kinetics of suspended (planktonic) heterotrophic mutans streptococci strains where the substrates are available in the bulk liquid. Mixed-substrate growth or threshold concentrations can also be applied to the conditions prevailing in biofilms (229). By necessity rather than choice, this investigation has been restricted to classical kinetics where the model system is structured according to the microorganism used, substrates and growth conditions in batch culture.

7.2 Selection of staple foods and food combinations

The most common staple foods and food combinations for urban black children (137) were studied, and are listed as follows:

Staple foods (Appendix II):

1. maize[1]
2. samp[2] (coarsely ground maize)
3. brown bread[3]

Food combinations (Appendix II):

4. maize-meal (soft) + milk[4] + sugar [5](refined carbohydrate and dairy product)
5. maize-meal (stiff) + gravy [6](refined carbohydrate + fat + protein)
6. samp + beans[7] (refined and complex carbohydrate)
7. brown bread + margarine[8] + peanut butter[9] (refined carbohydrate + fat)

These foods were compared against a reference control comprising of a complex synthetic medium, Brain Heart Infusion broth (BHI)(Biolab Diagnostics, Midrand, SA) containing 3% sucrose and a synthetic basal medium with 3% sucrose (Appendix III) .

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[1] Ace super maize meal (Delmas Milling Co, Randfontein, SA)

[2] Induna samp (Delmas Milling Co., Randfontein, SA)

[3] Albany brown bread (Albany bakeries™, Germiston, SA)

[4] Spar full cream milk (Spar SA (Pty) Ltd, Pinetown)

[5] Selati white sugar (Transvaal Sugar Ltd, Malelane, SA)

[6] All Gold Tomato and Onion mix (Langeberg Food Processors, Bellville, SA)

[7] Spar Kidney Beans (Spar South Africa (Pty) Ltd, Pinetown, SA)

[8] Stork Country Spread (Unifoods (Pty) Ltd, Durban, SA)

[9] Black Cat Crunchy peanut butter (Langeberg Food Processors, Bellville, SA)

7.3 Preparation of staple and combination foods

The staple foods, *viz* maize meal and samp were cooked by adding water and steamed. Food combinations were cooked by microwave (Sharp, model R-4A52, Osaka, Japan). Maize was cooked into a soft porridge with milk and sugar added to taste, whereas maize with tomato and onion gravy, and samp with beans was cooked into a stiff consistency. A slice of brown bread was buttered with margarine and peanut butter. All foods were homogenized (Heidolf, DIAX 900, Kelheim, Germany) to form a slurry (Figure 7.1). The homogenate was incorporated as a 2% solution (w/v) into a basal synthetic medium (BSM) for carbohydrate utilization (230) (Appendix III) and filtered through a muslin cloth. The pH of the medium was buffered to pH 7.0 and sterilized at 115°C for 15 minutes.



Figure 7.1 Staple foods (maize, brown bread and samp) and preparation of foods by homogenization for the incorporation into a basal synthetic test medium.

A synthetic complex culture medium, Brain Heart Infusion broth (BHI) (Biolab Diagnostics, Midrand, SA) was prepared according to manufacturers instruction and 3% sucrose was added.

7.3.1 Preliminary study on basal synthetic medium

A preliminary study was made to determine if BSM with no added sugar (carbon source), had any inhibitory affect on the growth of the test microorganisms. This medium is used when the ability of bacteria to use various carbon sources or, with the addition of a suitable carbon source, or growth factor or vitamin requirement is investigated (230). It is an iso-osmotic medium, comprising of essential salts (KCL) and trace elements ($MgSO_4$) required for enzymatic reactions, co-factors and protein constituents.The ammonium dihydrogen phosphate serves as the sole source of nitrogen.

A suspension equivalent to a MacFarlane's 0.5 nephelometer standard (15×10^8 /mL) was prepared from *S. mutans* reference strain NCTC 10449. One millilitre was inoculated into 50 mL of BSM and incubated at 37°C with gentle shaking for 16 hours. At commencement of the study (baseline) and after every two hours, 50 µL samples were removed and plated onto 5% blood agar for total viable counts. Results showed that total viable counts remained the same ($\bar{x} = 3\times10^7$ cfu/mL) during 16 hours of incubation. After 24 and 36 hours the TVC decreased to a mean of 1.6×10^3 cfu/mL and 20 cfu/mL, respectively.

7.4 Preparation of test bacteria for batch fermentation

The reference and clinical mutans streptococci strains were resuscitated from frozen, verified as pure cultures on blood agar, and subcultured twice to obtain vigour. These were grown till mid-log phase in 45 mL of BHI broth with 3% sucrose, in sterile Nunc^R polypropylene tubes

at 37°C for approximately16 hours. The cells were harvested when they reached an optical density (OD) of 0.8-1.0 at 550 nm, pelleted by centrifugation (1000×*g*) for 10 minutes, washed, and resuspended in phosphate buffered saline (Appendix III) and inoculated into a SuperSpinner fermenting flask (B. Braun Biotech, Melsungen, Germany) containing 300 mL of the prepared food culture medium (Figure 7.2) .



Figure 7.2 SuperSpinner fermentation flask containing the mutans streptococcus test bacterium and 300 mL of the traditional African food challenge.

7.5 Batch culture

The flask was incubated at 37°C and was constantly stirred (30 rpm) and purged by an anaerobic gas mixture comprising of 10% hydrogen, 10% carbon dioxide and 80% nitrogen (Figure 7.3) that was pre-filtered through a sterile MILLEX®-FG_{50} filter (Millipore S.A., Molsheim, France). Two millilitre samples were aseptically removed with a sterile syringe at commencement of the experiment, and after every two hours for a total time of 16 hours, for the following determinations:

1. Growth rate
2. pH
3. Analysis of acids
4. Residual glucose and glucose consumption
5. Extracellular water-insoluble polysaccharide
6. Extracellular protein
7. Expression of virulence genes

Each of these methodologies are presented and discussed in Chapters 8, 9 and 11.



Figure 7.3 Batch culture system used to determine the effect of traditional African foods on *S. mutans* and *S. sobrinus* references and clinical isolates. The food challenge was seeded with the test bacterium, incubated at 37°C and purged by an anaerobic gas mixture.

CHAPTER 8

GROWTH, PH AND ACID PRODUCTION

8.1 Introduction

The growth rate of a microbial population depends on environmental factors such as temperature, pH, water activity (a_w), oxygen tension, redox potential (*E*h) and the availability of nutrients (224). When the concentration of an essential nutrient or substrate is limited, the specific growth rate is decreased, although there is an excess of all other essential components.

8.1.1 Source of nutrients in the oral habitat

In the oral environment, nutrients are made available by bacteria mainly from the metabolism of endogenous substrates present in saliva that contains amino acids, peptides, proteins and glycoproteins, vitamins and gases (231). However, the composition of saliva is influenced by exogenous nutrients that are supplied by the transient passage of foods in the form of dietary carbohydrates (232). The host's diet also supplements the substrates available from saliva. Therefore, a constant source of carbon is available to the oral bacteria, and the likelihood of a nutrient deficient habitat is minimised. The current literature indicates that the type of diet can influence bacterial cell surfaces, adhesion and the proportions of different bacterial species in biofilm development (229, 233). In addition, continual changes in the mouth can be accommodated by alterations in the pattern of the gene expression. Therefore, the mutans streptococci are able to modify and regulate their metabolic function according to environmental challenges.

8.1.2 Types of foods that influence bacterial ecology

The different foodstuffs consumed provides a variety of nutrients of which fermentable carbohydrates are known to influence the ecology of the mouth. The general concept is that carbohydrate excess is associated with dental caries, whereas carbon substrates in limited quantities, is a natural state.

An update on the nomenclature of carbohydrates and their dental effects (234) has shown that in numerous investigations, sucrose appears to be the most cariogenic dietary constituent, and is the only substrate used for the formation of glucans essential for bacterial adherence (235). A review by Seow (28) has also indicated that the monosaccharides, glucose and fructose which are present in honey and fruit, are as cariogenic as sucrose in their abilities to cause a pH drop and to demineralise enamel.

Starch is found in a wide range of foods including bread, pasta, potatoes, rice and other cereals, and in a hydrolysed form, they may have potential to produce dental caries. However, raw starch causes only a small drop in plaque pH (123), and *in vitro* studies showed that *Streptococcus mutans* does not appear to produce acid from starches (234). According to Sheiham (97), foods rich in starch, without the addition of sugars, play only a small role in coronal dental caries.

Some foods, such as dairy products like milk and cheese have a protective function on teeth (236, 237). These foods have a natural buffering capacity produced by proteins (casein) which can also adsorb onto the tooth surface and reduce cellular adhesion of mutans streptococci (238). Also, Drummond *et al* (239) observed an increased salivary flow rate and elevated pH

in the mouth during the ingestion of cheese.

8.1.3 Effect of nutrients on pH and acid production

The mean pH for unstimulated whole saliva is in the range 6.75-7.25 (240), a neutral condition ideal for microbial growth, but given the frequent consumption of different foods, this balance is easily shifted. In the 1940s, Stephan found that the mixed bacteria in dental plaque produced a rapid drop in pH following a sugar rinse and a slow pH return towards baseline level. This pH decrease was inversely and clearly related to caries activity and became a corner stone of plaque and mixed-bacterial involvement in the cause of dental caries (241).

Depending on the frequency of a carbohydrate intake, plaque bacteria are exposed to varying challenges of low pH. The consumption of sugar-containing snacks or drinks between meals can result in the enhanced growth of aciduric species such as the mutans streptococci and *Lactobacillus* species and the inhibition of other bacterial species. Such a change in the bacterial composition of plaque and the ability of the mutans streptococci to form acids as end products, predisposes a surface to dental caries. If sufficient quantities of acids are produced to decrease the pH to 5.7, this pH is considered the 'critical pH', the level at which demineralization of dental enamel occurs (7).

The type of acid produced is also determined by the complexity of a nutrient medium. Some single carbon sources support a much higher specific growth rate. For example, at low glucose concentrations, the major part is used by *S. mutans* as a carbon source for cell production, with the main fermentation product being acetic and formic acid. At higher concentrations, all the glucose utilised is converted to lactic acid and the carbon required for energy and growth is

derived from another source in the culture medium (117, 242).

To-date, a considerable amount of data has demonstrated the role of sucrose and glucose in the generation of acids by mutans streptococci (234, 243), but very little is known concerning the role that specific foods and combination of foods play in growth rate and acid production, and their contribution to the formation of more cariogenic plaque. A summary of studies on some foods investigated *in vitro*, is shown in Table 8.1. No current literature is available on the *in vitro* effect of traditional African staple and combination foods.

Table 8.1 Summary of *in vitro* studies on the effect of foods on mutans streptococci

Author	Food	Study method	Results
Carlsson & Griffith, 1974 (117)	glucose	Continuous culture	Lactate formed in glucose surplus; acetate and formate formed in glucose limitation
Newman *et al*, 1976 (244)	sucrose, glucose	Batch culture	ECP mostly produced in sucrose at uncontrolled pH
Donoghue *et al*, 1983 (245)	sucrose, glucose	Artificial mouth	High TVC when *S. mutans* was the initial coloniser
Duguid R, 1985 (246)	glucose, fructose, sucrose	Batch culture	Lactic acid and a pH drop produced from sucrose, but at low monosaccharide concentrations, a pH rise and lactic-acid consumption occurred
Vacca-Smith *et al*, 1994 (247)	milk	Hydroxyapatite beads	Reduced adherence of *S. mutans*. Effect mediated by the *k*-casein fractions
Vacca-Smith & Bowen 1995 (67)	milk	Hydroxyapatite beads	Reduced adherence of GTF. Reduced expression of adsorbed GTF activity

8.1.3.1 Strain-related acid production

Investigations in acid production at low pH between oral streptococci have shown that the mean velocity of acid production by *S. mutans* and *Streptococcus sobrinus* was significantly higher than other oral streptococci (248). Yet, very few examinations on strain differences in acid production have been made. A study by de Soet, and co-workers (248) emphasised the need for recognition of the significant physiological differences that occur within single species. To-date, no comparisons have been made on acid production between mutans streptococci reference and clinical strains on 'preferred' foods of African children.

8.2 Aim

The object of this investigation was to measure the growth rate and acids produced by mutans streptococcus reference strains and clinical isolates, during the fermentation of traditional African staple foods and food combinations. The order of this study was to:

1.1 Investigate the specific growth rate on single nutrients in comparison to a combination of nutrients.

1.2 Investigate the ability of mutans streptococci test bacteria to produce acids and to decrease environmental pH to levels at which enamel demineralization occurs.

1.3 Investigate the differences between mutans streptococci reference and clinical strains. in growth and acid production.

8.3 Materials and methods

8.3.1 Seeding of culture and measurement of growth rate

A purified single colony was raised in 50 mL BHI containing 3% sucrose and incubated

overnight at 37°C. The cells were harvested by centrifugation at 2000 rpm (648× *g*) and washed with phosphate buffered saline to remove the growth medium. The size of the initial inoculum was estimated by total viable count (TVC) and the turbidity measured and compared against a MacFarlane's turbidity standard on a Pharmacia LKB Ultrospec® III spectrophotometer (Pharmacia LKB Biochrom Ltd, Cambridge, England) at 550 nm, before the seed culture was inoculated into the fermenting flask. Thereafter, bacterial population growth was monitored every two hours, by measuring optical density (OD). The viability of test bacteria was estimated by TVC, to monitor progression of the growth stages. Samples from the fermentation flask were plated onto 5% horse blood agar using Tryptone-soy agar base (Appendix III), with a spiral plater Model D (Don Whitley Scientific Ltd, Shipley, England) (Figure 8.1). After 48 hours of anaerobic incubation at 37°C in a candle jar, the test microorganisms were counted.



Figure 8.1 Spiral plater used for monitoring total viability of mutans streptococci test strains.

8.3.2 pH and analysis of fermentation acids

The pH of the cell-free culture supernatant was measured with a Sentron 2001 pH system (SENTRON, Roden, The Netherlands) that had been pre-calibrated with phosphate buffer solution (pH 7) and biphthalate (pH 4) (J. T. Baker, Deventer, Holland). One millilitre aliquots of sample were distributed into micro-centrifuge tubes (Elkay, Costelloe, Galway) containing 50µL of 0.3 M oxalic acid (Appendix III) to stop further metabolism, and stored at -20°C, until analysed for acids. Samples were prepared and analysed for fermentation acids, when the pH of the culture media dropped to its lowest point, or first reached a pH of 5.7, the critical point for the demineralization of tooth enamel.

8.3.2.1 Preparation of volatile and non-volatile acids

Culture supernatant of the test bacteria were extracted and derivatized according to methods described in the Supelco Bulletin No. 856B (249). Volatile acids were prepared by ether extraction and non-volatile acids by methylation which are described in Appendix III (H). Caproic and benzoic acid was included during the extraction of volatile and non-volatile acids, respectively, as internal reference standards (Appendix III).

8.3.2.2 Analysis of fermentation acids by gas-liquid chromatography

The derivatized samples were measured for acids by a Shimadzu GC8AIT (Shimadzu Corp., Kyoto, Japan) gas-liquid chromatograph with a thermal conductivity detector, linked to a Shimadzu C-R1B chromatopac integrator. A 1.5-m glass column (ID, 2.5 mm) packed with 10% SP-1000/1% H_3PO_4 on 100/120 Chromosorb W AW was used as the stationary phase. This system can detect pyruvic, lactic, oxalic, methyl melonic, melonic, fumaric, succinic, acetic, formic, propionic, I-butyric, butyric, I-valeric, valeric, I-caproic and heptanoic acids.

The temperature of the injector/detector and column temperature was set at 180°C and 120°C, respectively, and current at 90 mA with an attenuation of 1. Helium was used as the carrier gas, with flow rate set at 20 mL per minute at 2.5 kPa.

The gas-chromatograph was pre-calibrated with volatile (cat. no. 46975-U) and non-volatile (cat. no. 4-6985) standard controls (Supelco Inc., Bellefonte, PA, USA) (Appendix III) and the integrator was set to record and calculate retention time (R_t), concentration and area. Fifteen µl of the prepared test samples were injected into the column with an SGE Series II syringe (SGE, Ringwood, Australia) and samples were allowed to elute for between 10 and 30 minutes.

8.3.2 Analysis of data

Data were analysed with SAS (Version 9.1, SAS Institute, Cary, NC: USA), and GraphPad Prism for Windows (Version 4, GraphPad Software, Inc. San Diego, CA, USA). Mutans streptococci total viable counts (TVC) were log transformed to symmetrize the data. Log transformation is used to stabilize the variance in bacterial count in each food challenge so that each group is approximately equal. The General Linear Models Procedure (GLM) was used to determine differences between the food-challenges, bacterial test strain and time on TVC, pH, and acid concentration. Multiple comparisons were made by Tukey's Studentized Range and correlation was ascertained from the Error SSCP matrix. The influence of food and bacterial strain on doubling time and cell generation was determined by a two-way analysis of variance (ANOVA) and the relationship between acid concentration and change in pH was determined by the Spearman correlation coefficient. The critical level for statistical significance was set at $P<0.05$.

8.3.3 Calculation of doubling time and cell generations

Bacterial growth kinetics in batch culture were calculated according to Doelle (250). The mean doubling time *(t_d)* and number of generations (*g*) in microbial populations were calculated by using the following equations:

1. Doubling time (hours): $t_d = \frac{t \text{ (time)}}{n \text{ (number of cell divisions)}}$

where: $t_d = \frac{t = (t_1 - t_0)}{n = 3.32 (\log N_t - \log N_0)}$

(3.32=1/log 2; N_t = number of cells present at time *t*; N_0 = number of cells at zero time)

2. Number of generations: $g = \frac{t}{t_d}$

8.4 Results

8.4.1 Bacterial viability, growth and time

Mean averages in TVC of bacterial test strains challenged to staple and mixed-foods are shown in Figure 8.2. The variation in log TVC was significantly influenced more by the bacterial strain ($F=29.70$; $P<0.0001$), than food-type ($F=19.69$; $P<0.0001$), or time ($F=3.24$; $P=0.001$). This was indicated by the probability values shown specifically in samp, samp+beans, maize and maize+gravy (Table 8.2). The strength of the relation between the food and TVC of the test bacteria is shown by r^2 (Pearson product-moment correlation).

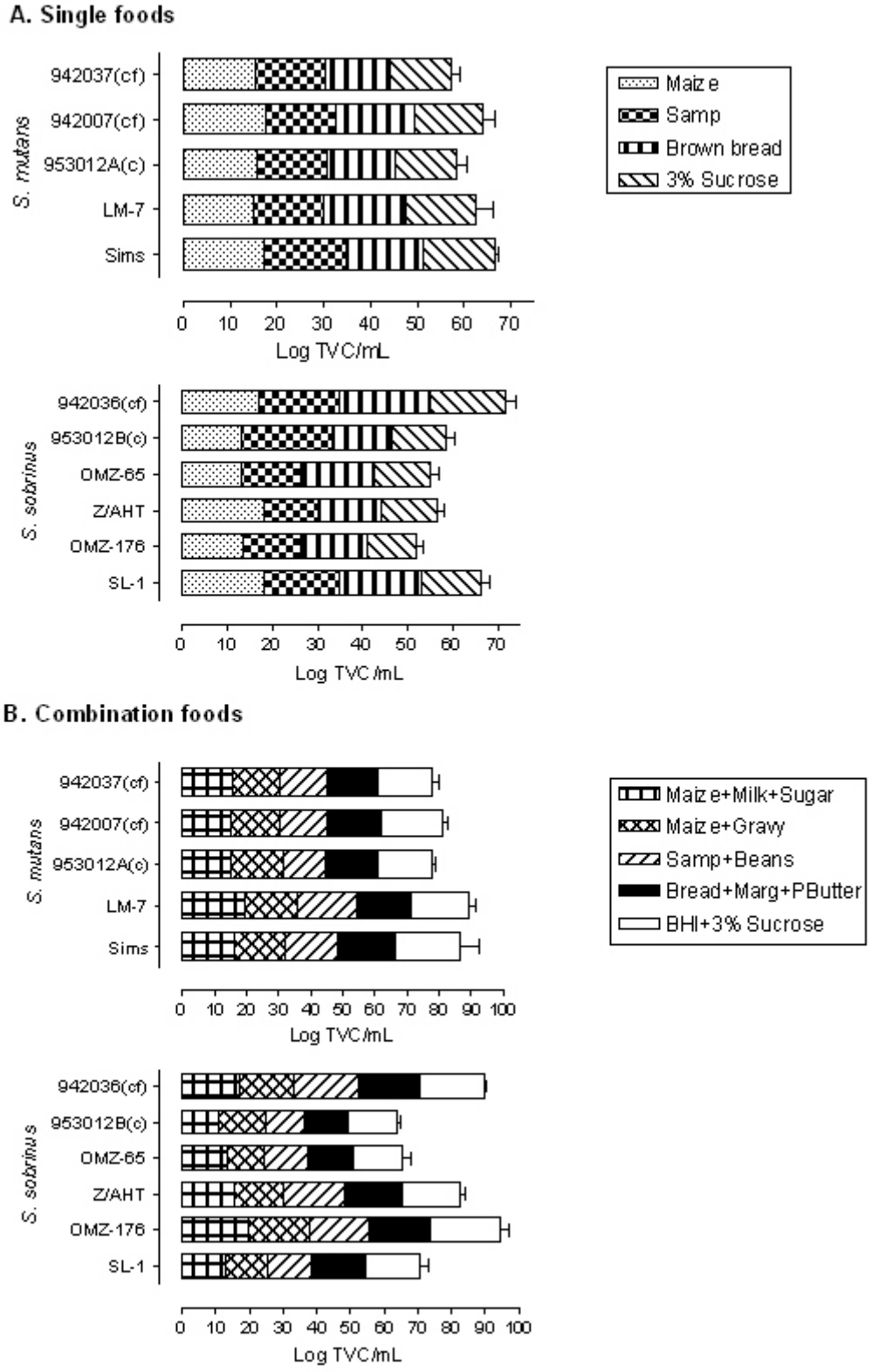

Figure 8.2 Distribution of log transformed TVC ($\bar{x} \pm$ SD) of *S. mutans* and *S. sobrinus* reference strains and clinical isolates grown in batch culture for 16 hours on traditional African foods and complex synthetic medium.

Table 8.2 Probability values of GLM tests for TVC and pH between mutans streptococci test bacteria by food challenge

Food	TVC			pH	
	F	P^*	r^2†	F	P^*
Staple					
Maize	27.07	<0.0001	0.77	10.32	<0.0001
Samp	66.46	<0.0001	0.88	2.62	0.008
Brown Bread	10.51	<0.0001	0.54	2.42	0.01
Combination					
Maize+Milk+Sugar	18.09	<0.0001	0.67	1.97	0.05
Maize+Gravy	33.28	<0.0001	0.79	1.51	0.15
Samp+Beans	28.34	<0.0001	0.76	4.68	<0.0001
Bread+Margarine+P Butter	6.6	<0.0001	0.43	1.83	0.07
Control					
3% Sucrose	5.18	<0.0001	0.37	4.63	<0.0001
BHI+3% Sucrose	6.49	<0.0001	0.42	0.79	0.64

$^*P<0.05$; † = strength of association (Pearson product-moment correlation) is shown by r^2

Tukey's test showed that the TVC in BHI+3% sucrose was significantly greater ($P<0.05$) than other foods, with no differences found in staple foods or food mixtures. A significant decrease ($F=8.20$; $P<0.0001$) in viable bacteria occurred over time in 3% sucrose (Figure 8.3), that was not apparent in other food challenges. A decrease in bacterial numbers during the lag phase of growth in BHI+3% sucrose, bread+margarine+peanut butter and samp+beans (Figure 8.3) was shown. Baseline counts above 10^{10}/ml (BHI+3% sucrose) implies clumping and the serial dilution of aggregates.

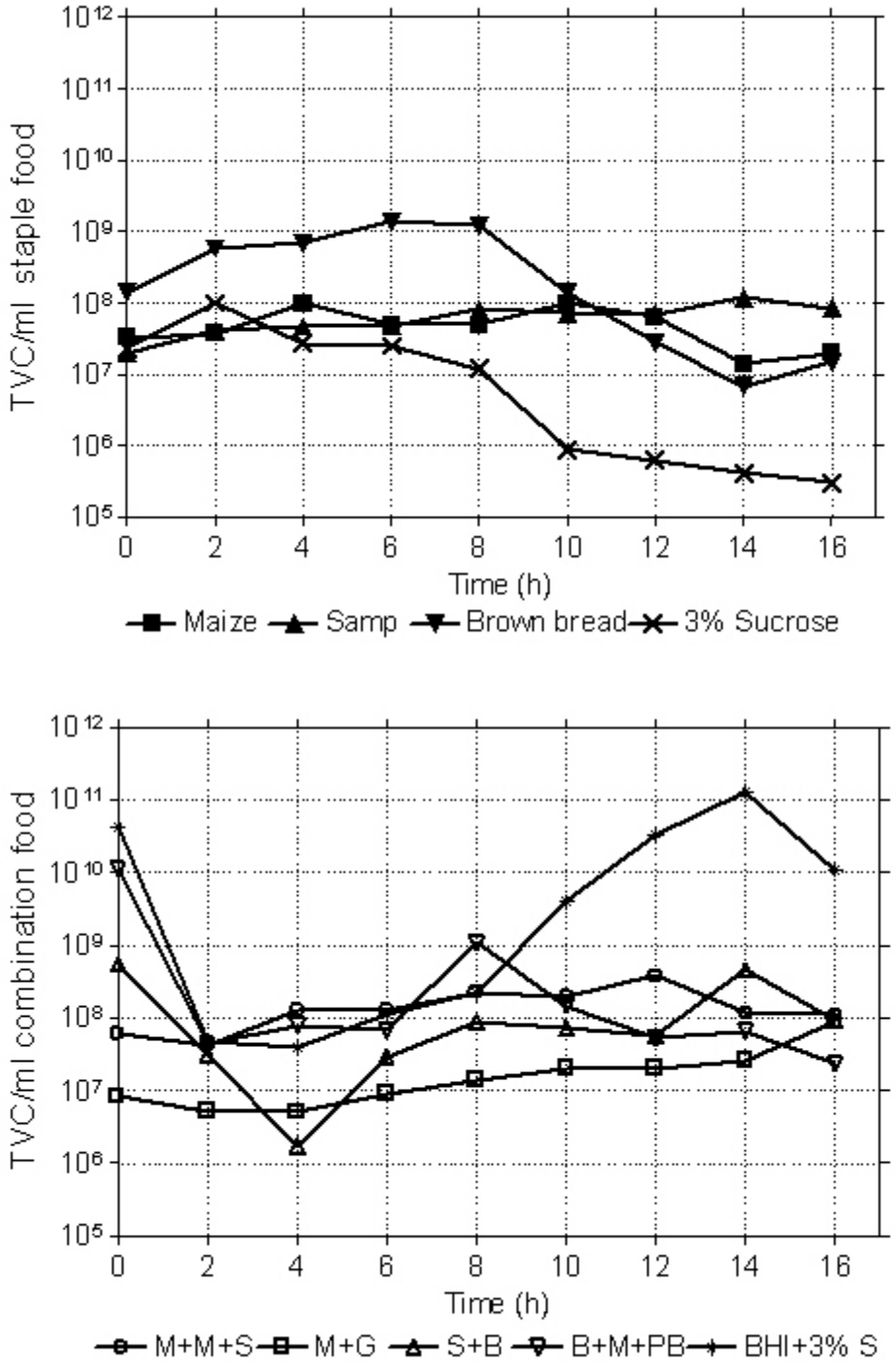

Figure 8.3 Total viable count ($\bar{x}$) of mutans streptococcus test strains by food challenge, over 16 hours of batch fermentation. M+M+S = maize+milk+sugar, M+G = maize+gravy, S+B = samp+beans, B+M+PB = brown bread+margarine+peanut butter, BHI+3%S = Brain Heart Infusion broth+3% sucrose.

8.4.2 Bacterial growth kinetics

The mean doubling time (t_d) of the test bacteria ranged from 2.2 - 5.7 hours in 3% sucrose and samp, respectively (Figure 8.4). The food challenge accounted for 24.11% of the total variance (F=3.68; *P*=0.001), whereas the influence of the test strains was insignificant (F=1.26; *P*=0.27) (two-way ANOVA). The number of cell generations was lowest in samp and maize, samp+beans and maize+gravy compared to the synthetic control medium. Similarly, the food challenge accounted for 23.7% of the total variance in the number of cell divisions (F=3.64; *P*=0.001); mutans streptococci strain had no significant effect (F=1.39; *P*=0.20).

8.4.2.1 Mutans streptococci strain and doubling time

The effect of test foods on doubling time and the number of cell divisions is shown in Table 8.3. Although food-type accounted more for the variation in growth rate than the bacterial test species, the doubling time and number of generations of *S. mutans* NCTC 10449 was low in maize (t_d=17.5; *g*=0.92) and samp (t_d=18.4; *g*=0.86) compared to other test strains. In comparison, *S. sobrinus* reference NCTC 12277 showed a low doubling time and more cell divisions when challenged to maize (t_d=1.9; *g*=8.2) and samp (t_d=3.2; *g*=5.0).

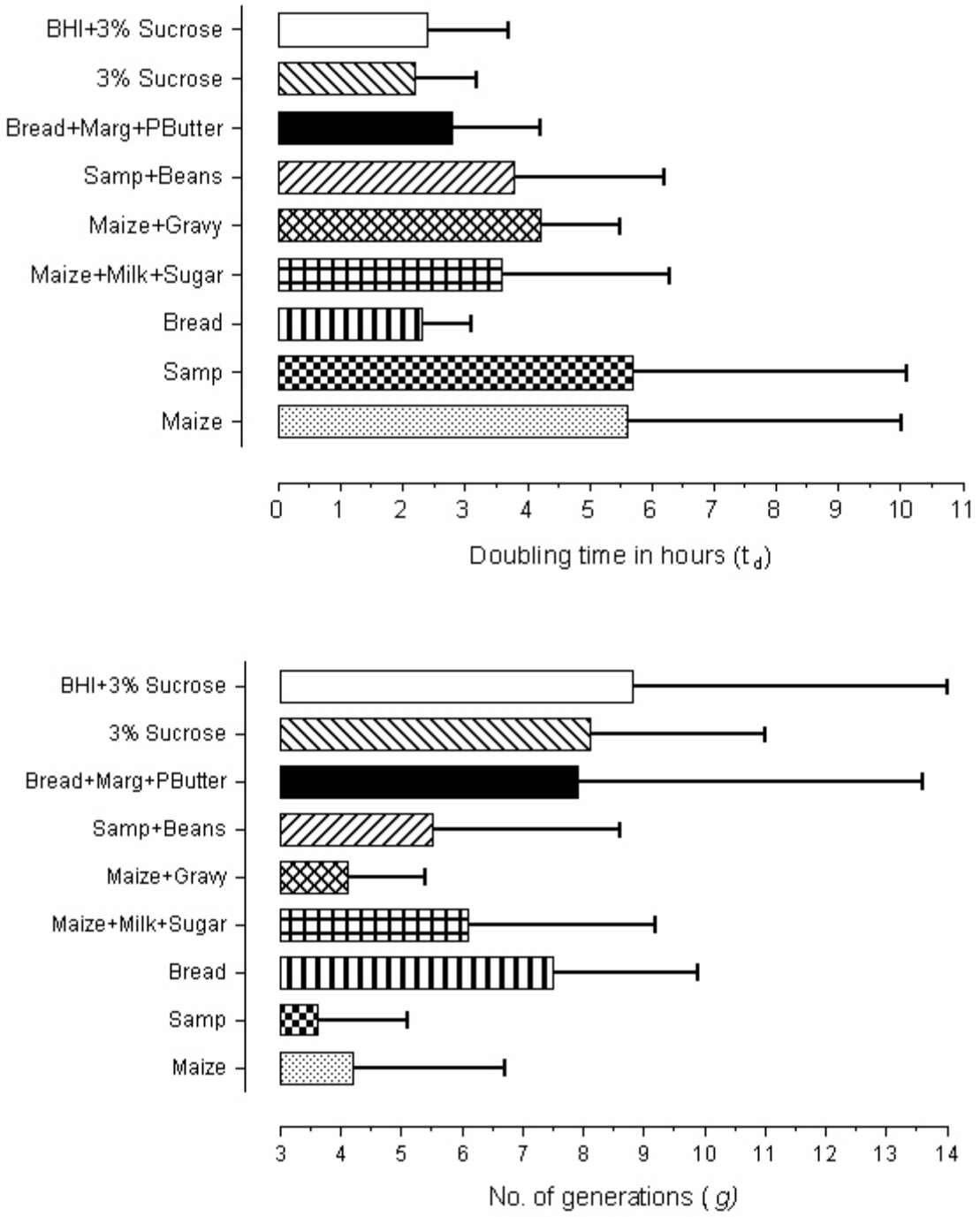

Figure 8.4 Growth response of mutans streptococci test strains (n = 11) to traditional African food challenges (n = 9) (P<0.001). Doubling time (t_d) in hours ($\bar{x}$ ± SD) and number of generations (g)($\bar{x}$ ± SD) after 16 hours of batch culture.

Table 8.3 Comparison of doubling time (t_d) in hours and number of divisions (g) of mutans streptococci reference and clinical strains after 16 hours in batch culture**.** A=staple foods, B=combination foods**,** C=controls

Food	*Streptococcus mutans*										*Streptococcus sobrinus*											
	Sims (10449)		LM-7 (10923)		953012A (c)		942037 (cf)		942007 (cf)		SL-1 (12277)		OMZ-176 (10922)		Z/AHT (10919)		OMZ-65 (11061)		942036 (cf)		953012B (c)	
	t_d	g	t_d	g	t_d	g	t_d	g	t_d	g	t_d	g	t_d	g	t_d	g	t_d	g	t_d	g	t_d	g
A																						
Maize	7.5	0.92	5.2	3.1	2.8	5.8	2.0	7.6	6.6	2.3	1.9	8.2	5.6	2.8	7.4	2.1	6.9	2.3	2.5	6.2	3.2	5.0
Samp	18.4	0.86	4.4	3.6	3.8	4.2	4.4	3.6	3.7	4.2	3.2	5.0	7.9	2.0	2.4	6.5	4.3	3.6	5.4	2.9	5.3	3.0
Bread	4.1	3.9	1.8	8.5	2.4	6.7	1.2	2.5	3.0	5.3	2.4	6.8	2.6	6.0	2.3	6.8	1.8	8.6	1.5	10.4	2.3	6.8
B																						
M+M+S	1.4	11.31	2.7	5.9	4.16	3.85	2.2	7.35	3.61	4.43	1.48	10.81	2.9	5.6	3.77	4.25	1.12	1.44	4.7	3.4	1.88	8.51
M+G	4.3	3.73	3.4	4.66	6.08	2.63	4.8	3.32	3.88	4.12	5.7	2.81	3.1	5.2	6.02	2.66	3.74	4.28	2.53	6.34	2.72	5.88
S+B	1.3	12.7	2.0	8.01	3.19	5.0	5.1	3.17	3.37	4.73	2.35	6.81	5.4	2.97	2.56	6.26	9.91	1.16	3.64	4.4	3	5.34
B+M+PB	0.7	22.92	1.4	11.83	2.62	6.1	5.1	3.12	2.46	6.51	3.24	4.94	2.6	6.19	1.47	10.87	3.39	4.72	5.13	3.12	2.32	6.9
C																						
3% S	5.0	3.1	1.1	14.4	2.1	7.6	2.2	7.0	1.4	11.4	2 .0	7.6	3.0	5.2	2.2	7.2	1.9	8.1	1.8	8.5	1.8	8.7
BHI+3% S	0.8	21	1.7	9.0	2.2	7.4	4.9	3.2	2.2	7.0	1.5	10	1.1	15.2	2.2	7.1	1.8	8.8	3.9	4.0	3.7	4.3

M+M+S=maize+milk+sugar, M+G=maize+gravy, S+B=samp+beans, B+M+PB=bread+margarine+peanut butter, 3% S=3% sucrose, BHI+3% S=Brain Heart Infusion broth+3% sucrose

8.4.3 pH and bacterial growth

The change in pH of the culture medium was influenced more by the test foods (F=63.47; *P*<0.0001) over time (F=115.33; *P*<0.0001), than by mutans streptococci test strains (F=8.13; P<0.0001). The association between TVC and pH for each food is shown in Figure 8.5.

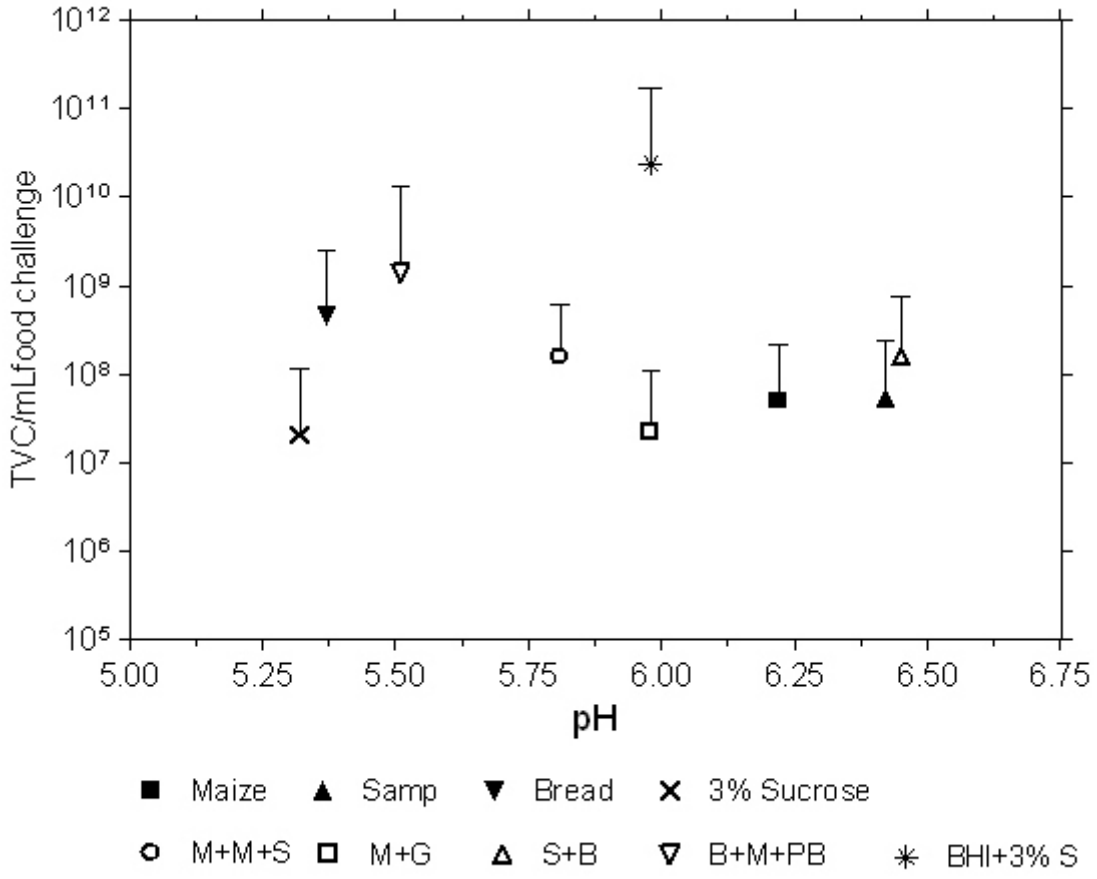

Figure 8.5 TVC ($\bar{x}$ ± SD) and pH of mutans streptococci test strains exposed to traditional African foods in batch culture. M+M+S = maize+milk+sugar, M+M = maize+gravy, S+B = samp+beans, B+M+PB = bread+margarine+peanut butter, BHI+3%S = Brain Heart Infusion+3% sucrose.

Spearman correlation indicated a low, statistically significant relationship between a decrease in pH and cell numbers (*r*=-0.14; *P*<0.0001). A high TVC with a low pH was evident in brown

bread and bread+margarine+peanut butter compared to the control medium where a high bacterial count was accompanied by a near neutral pH value (6.0).

8.4.3.1 Change in pH

The mean values for pH, maximum drop in pH units and the minimum pH reached after each food challenge are reported in Table 8.4. The least change in pH was in maize, samp and samp+beans, but the variation was more significant (2-way ANOVA) between test strains grown in in maize, samp+beans, and 3% sucrose (Table 8.4). The mean values for pH and maximum drop in pH units for each food-type and bacterial species is shown in Table 8.5. In brown bread and 3% sucrose, enough acids were produced by all clinical strains to reduce the pH to below 'critical point', compared with reference strains where the responses varied.

Table 8.4 Mean pH ($\bar{x}$), standard deviation (SD), minimum pH, maximum drop in pH units, and probability values for mutans streptococci strains, following batch culture. n=99

Food	$\bar{x}$	SD	min	max drop	F	P^*
Staple						
Maize	6.22	0.54	4.72	2.28	10.32	<0.0001
Samp	6.42	0.27	5.94	1.06	2.62	0.01
Brown bread	5.37	0.99	4.15	2.85	2.42	0.01
Combination						
Maize+Milk+Sugar	5.81	0.94	4.16	3.04	1.97	0.05
Maize+Gravy	5.97	0.71	4.54	2.66	1.51	0.15
Samp+Beans	6.45	0.4	5.7	1.5	4.68	<0.0001
Bread+Margarine+Peanut Butter	5.51	0.93	4.12	3.08	1.83	0.07
Control						
3% sucrose	5.32	1.05	3.68	3.32	4.63	<0.0001
BHI + 3% sucrose	5.98	0.97	4.08	3.12	0.79	0.64

$^*P<0.05$

Table 8.5 Mean and standard deviation (mean ± SD) in pH and maximum drop in pH units (max) produced by mutans streptococci reference (NCTC) and clinical strains, by food challenge over 16 hours of batch culture

Food	*Streptococcus mutans*										*Streptococcus sobrinus*											
	Sims (10449)		LM-7 (10923)		942037 (cf)		942007 (cf)		953012A (c)		SL-1 (12277)		OMZ-176 (10922)		Z/AHT (10919)		OMZ-65 (11061)		942036 (cf)		953012B (c)	
	mean ± SD	max	mean ± SD	max	mean ± SD	max	mean ± SD	max	mean ± SD	max	mean ± SD	max	mean ± SD	max	mean ± SD	max	mean ± SD	max	mean ± SD	max	mean ± SD	max
Staple																						
Maize	6.2±0.3	1.06	6.5±0.3	0.78	6.3±0.4	1.12	5.2±0.8	2.28	6.4±0.2	0.72	5.7±0.6	1.77	6.6±0.2	0.69	6.4 ±0.3	0.85	6.3±0.3	0.95	6.4±0.3	0.84	6.5±0.2	0.8
Samp	6.3±0.3	0.95	6.6±0.2	0.65	6.6±0.2	0.65	6.3±0.3	1.06	6.3±0.3	0.85	6.4±0.3	0.9	6.6±0.2	0.54	6.4±0.2	0.77	6.4±0.3	0.82	6.3±0.3	1.01	6.5±0.2	0.6
Bread	5.1±1.0	2.61	5.1 ±1.1	2.62	5.5±0.9	2.26	5.0±1.0	2.8	5.2±1.0	2.69	5.3±1.1	2.85	6.4±0.3	0.97	5.3±1.0	2.66	6.1±0.6	1.77	5.0±0.9	2.65	5.2±1.0	2.7
Combination																						
M+M+S	5.4±1.1	2.94	5.2±1.0	2.7	5.7±1.0	2.84	6.2±0.6	2.14	5.8±0.9	2.46	6.1±0.5	1.36	6.0±0.8	2.27	5.5±1.0	2.74	5.9±1.1	2.7	5.5±1.0	2.36	6.6±0.3	0.8
M+G	5.8±0.8	1.97	6.2± 0.8	1.93	6.1±0.8	2.33	6.4± 0.6	1.94	6.1±0.5	1.39	6.0±0.5	1.52	6.1±0.5	1.4	5.5±0.9	2.46	5.5±1.0	2.45	6.0±0.7	2.33	6.1±0.5	1.2
S+B	6.7±0.2	0.67	6.1±0.5	1.27	6.4±0.3	0.95	6.5±0.2	0.65	6.7±0.3	0.67	6.8±0.3	0.77	6.3±0.5	1.2	6.1±0.4	1.18	6.5±0.3	0.85	6.1±0.5	1.3	6.7±0.3	0.8
B+M+PB	6.1±0.7	1.78	5.4±0.8	2.24	5.7±1.1	2.54	4.9±1.1	2.88	6.0±0.8	2.01	5.9±0.5	1.51	5.6±0.7	1.88	5.6±0.8	2.2	5.0±1.1	2.76	5.2±0.9	2.48	5.2±1.1	2.8
Controls																						
3% S	6.3±0.3	0.96	5.3±0.8	2.1	5.6±0.8	2.47	4.7 ±1.2	2.93	4.9±1.2	3.02	5.2±1.0	2.63	6.4±0.2	0.63	5.6±0.8	2.27	4.8±1.0	2.74	5.3±0.8	2.24	4.3±1.1	3.1
BHI+3%S	6.0±1.1	2.7	6.0 ±0.9	2.09	6.48±1.0	3.12	5.87±1.1	2.47	6.1±0.8	1.97	6.3±1.1	2.64	6.4±0.7	1.51	5.6 ±1.0	2.36	5.7±0.9	2.45	5.6±1.0	2.19	5.8±1.0	2.3

M+M+S=maize+milk+sugar, M+G=maize+gravy, S+B=samp+beans, B+M+PB=bread+margarine+peanut butter, 3% S=3% sucrose, BHI+3% S=Brain Heart Infusion broth+3% sucrose

8.4.3.2 pH and time

The fermentation of bread, bread+margarine+peanut butter and 3% sucrose, decreased the pH to below the 'critical' pH level of 5.7 after six hours (Figure 8.6), whereas a fall in pH in BHI+3% sucrose, maize+milk+sugar and maize+gravy occurred after 10 hours. There was insufficient acid to reduce the pH to below 5.7 after exposure to maize, samp and samp+beans.

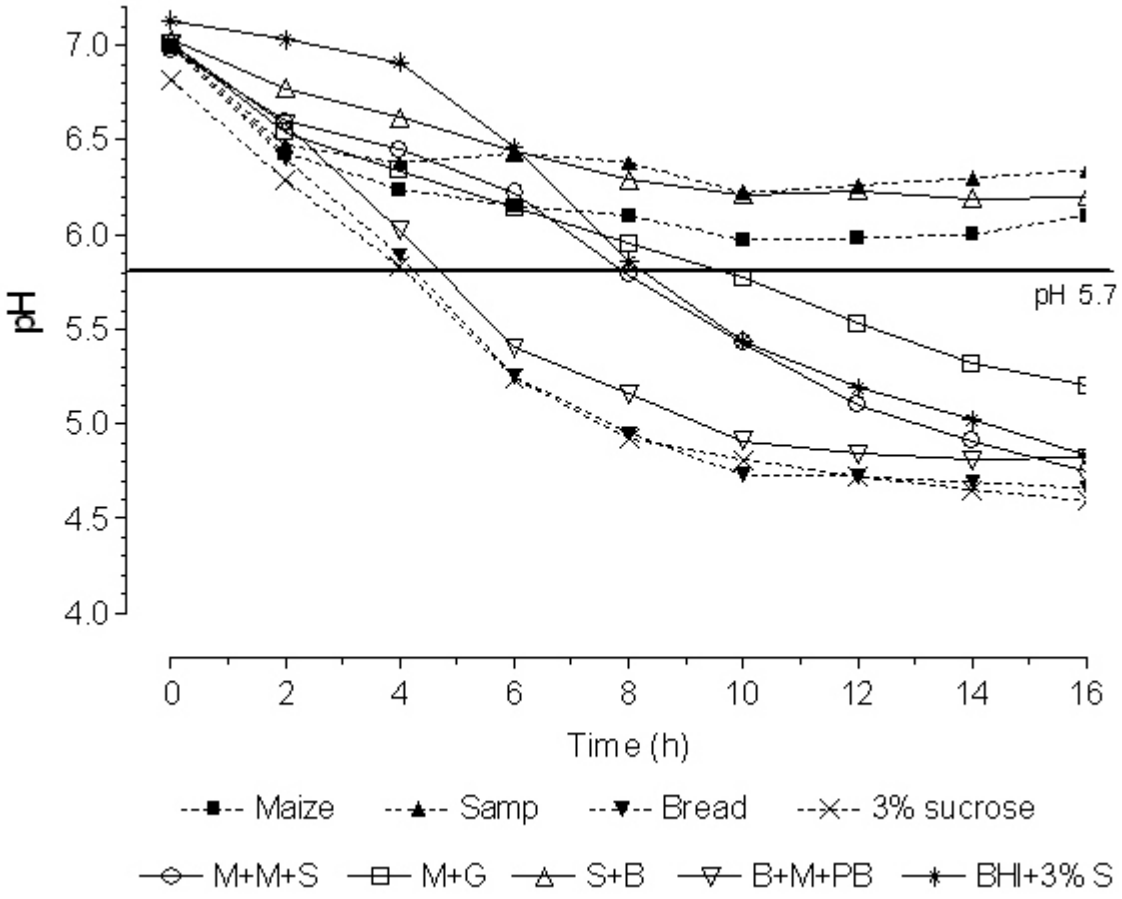

Figure 8.6 Mean decrease in pH produced by mutans streptococci test strains in batch fermentation of foods over 16 hours. M+M+S = maize+milk+sugar, M+G = maize+gravy, S+B = samp+beans, B+M+PB = bread+margarine+peanut butter, BHI+3% S = Brain Heart Infusion broth+3% sucrose.

8.4.4 Acids produced

Lactic, acetic and formic acid were the main acids formed as fermentation by-products (Figure 8.7). The influence of the food challenge on the production of lactic acid was significantly greater ($F=29.79$; $P=<0.0001$), compared to test strains ($F=4.91$; $P<0.0001$). However, mutans streptococci strain differences had a stronger influence on acetate concentration ($F=17.78$; $P<0.0001$). Comparisons between foods showed that the least amounts of lactic acid were produced in samp, maize, samp+beans and maize+gravy compared with 3% sucrose and the control medium (Table 8.6). Concentrations of acetic acid were significantly low in BHI+3% sucrose and maize, whereas the small quantities of formic acid was not statistically significant.

As a percentage of the total acid end-product, lactic acid was less than 20% in staple and combination foods, but comprised 64% in BHI+3 % sucrose. This correlated significantly with a decreasing pH ($r=-0.12$; $P=0.04$). The quantities of lactate produced in samp+beans, samp and maize were small, but the percentage concentration of acetic acid was higher and ranged between 34% - 95%, with most acid being formed in bread+margarine+peanut butter. More lactic acid was produced by strains NCTC 11061, 942007(cf) and 942036 (cf), whereas acetic acid was produced by strains NCTC 10919, 10449 and 942037(cf).

Table 8.6 Probability values of GLM tests for acids produced between mutans streptococci strains for food challenges. Mean, standard deviation (SD), and maximum (max) concentrations of lactic, acetic and formic acid in mM/mL

		Lactic				Acetic				Formic			
Food	n	mean ± SD	max	F	*P*	mean ± SD	max	F	*P*	mean ± SD	max	F	*P*
Staple													
Maize	34	0.87 ± 1.58	8.48	1.26	0.31	7.59 ± 13.20	46.9	67.34	<0.0001	0.12 ± 0.35	1.37	2.92	0
Samp	33	0.50 ± 0.80	2.24	9.54	<0.0001	16.41 ± 24.91	87.1	90.43	<0.0001	0.09	2.1	0.91	0.54
Brown Bread	35	3.45 ± 2.64	8.45	9.45	<0.0001	8.45 ± 21.62	86.5	44.04	<0.0001	0.06 ± 0.20	0.84	0.83	0.61
Combination													
Maize+Milk+Sugar	37	1.10 ± 1.22	4.44	3.39	0.006	10.19 ± 0.91	54.7	5.59	0	0.46 ± 1.71	9.6	1.07	0.42
Maize+Gravy	36	0.94 ± 2.37	13.5	3.94	0.003	13.29 ± 18.92	66.9	21.66	<0.0001	0.08 ± 0.30	1.59	1.86	0.11
Samp+Beans	37	0.46 ± 1.06	4.15	27.2	<0.0001	15.42 ± 21.23	67.5	104.1	<0.0001	0.59 ± 2.69	15.1	0.96	0.5
Bread+Marg+P Butter	38	2.64 ± 3.30	13	21.89	<0.0001	21.65 ± 29.13	97.7	7.82	<0.0001	0.12 ± 0.32	1.41	1.77	0.13
Control													
3% Sucrose	34	3.92 ± 5.44	4.52	0.74	0.69	12.42 ± 4.94	18.5	2.46	0.04	1.59 ± 6.57	31.66	0.95	0.51
BHI+3% Sucrose	38	12.23±10.72	35.8	7.47	<0.0001	4.11 ± 5.18	18.8	2.95	0.01	0.14 ± 0.23	0.92	2.03	0.1

* $P<0.05$

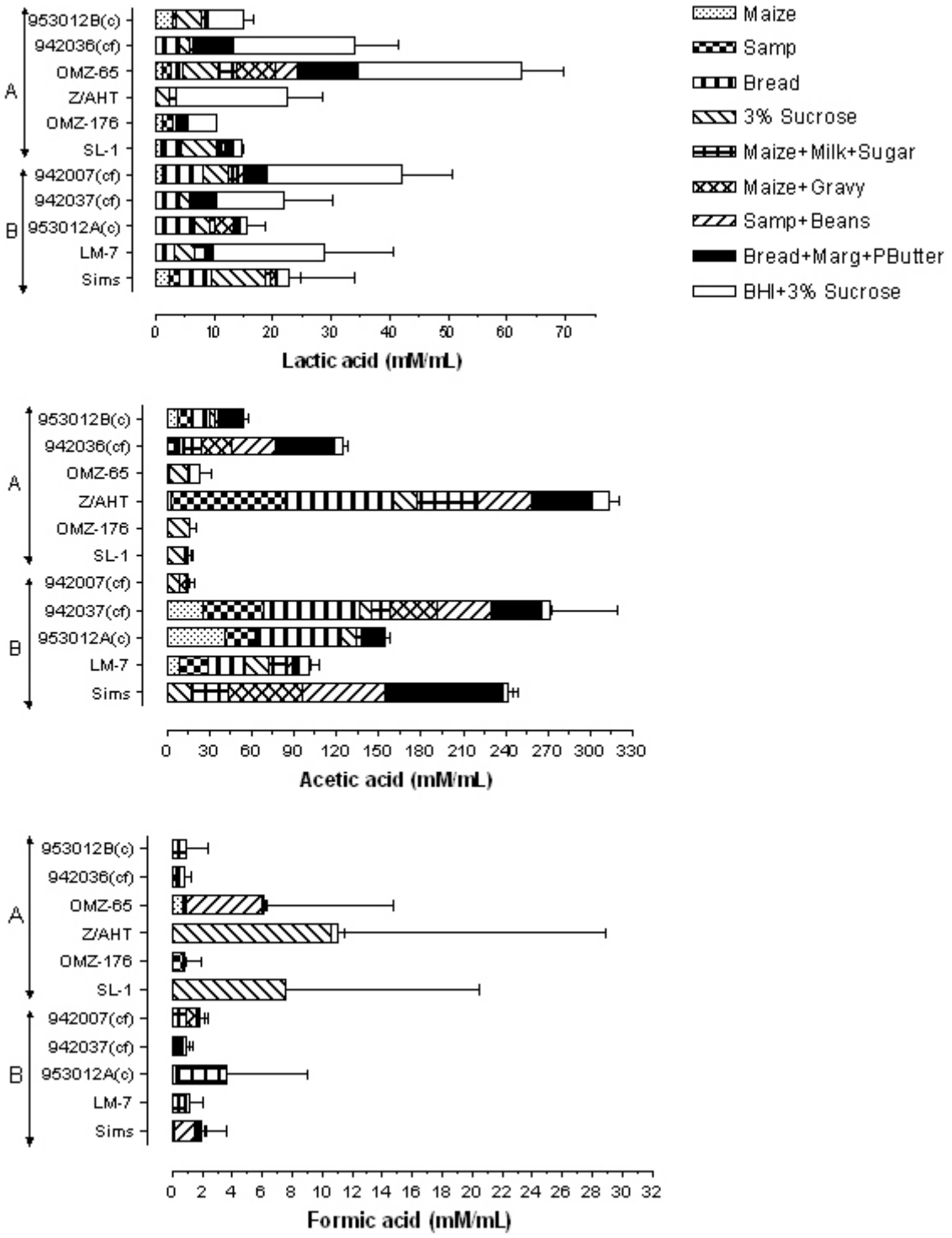

Figure 8.7 Concentration ($\bar{x} \pm$ SD) of lactic, acetic and formic acid produced by *S. sobrinus* (A) and *S. mutans* (B) clinical and reference strains grown in batch culture for 16 hours, on traditional African foods and a complex synthetic medium.

8.4.5 Relationship between acid concentration and pH

The association between acid concentration and pH is shown in Figure 8.8. A statistically significant increase in lactic acid was sufficient to decrease the pH of the culture medium to below 5.7 in bread+margarine+peanut butter (r=-0.60; P<0.0001), 3% sucrose (r=-0.59; P=0.0002) and brown bread (r=-0.4; P=0.02). Significant correlations between acetic acid and pH were also found in maize+milk+sugar (r=-0.37; P=0.03), maize+gravy (r=-0.63;P=0.0001), bread+margarine+peanut butter (r=0.37; P=0.03), maize (r=0.40; P=0.01), and 3 % sucrose (r=0.41; P=0.02). Yet, the decrease in pH was above 5.7 in foods other than 3 % sucrose and bread+margarine+peanut butter. No relationship between acids and pH during the fermentation of samp or samp+beans was shown. The low concentrations of formic acid were statistically insignificant.

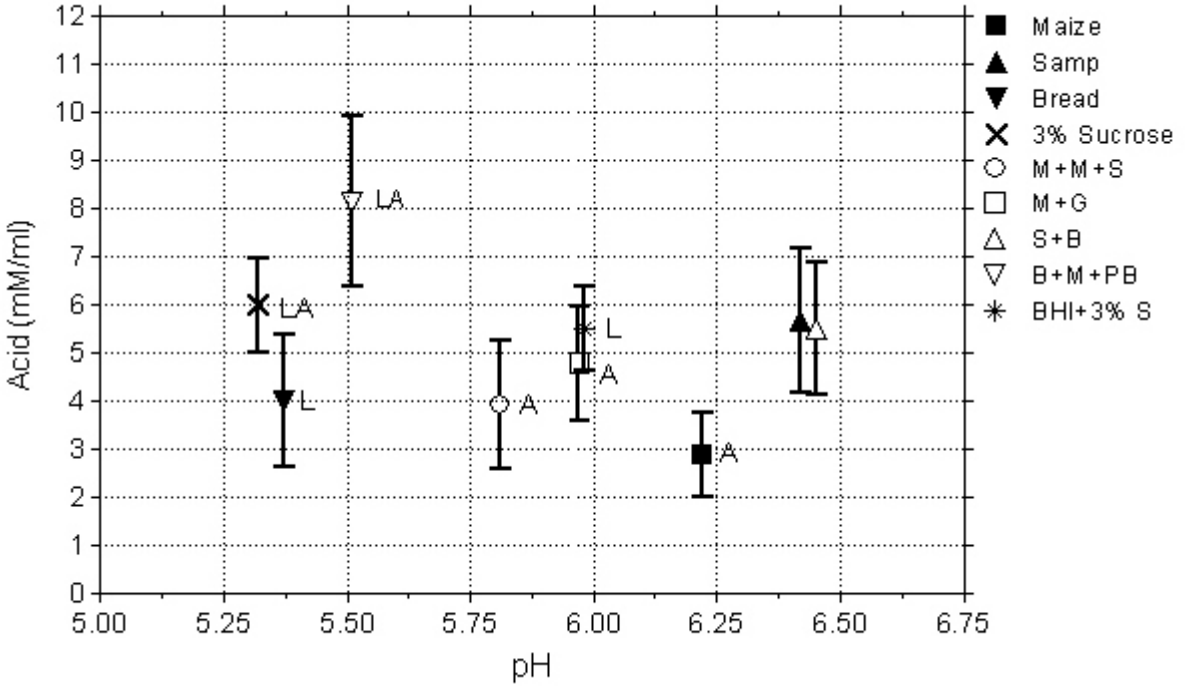

Figure 8.8 Concentration ($\bar{x} \pm$ SD) of lactic (L) and acetic (A) acid associated with change in pH by food challenge. M+M+S = maize+milk+sugar, M+G = maize+gravy, S+B = samp+beans, B+M+PB = bread+margarine+peanut butter, BHI+3% S = Brain Heart Infusion broth+3% sucrose. LA = lactic and acetic acid.

8.5 Discussion

8.5.1 Relationship between population growth, time and strain

The control medium, Brain Heart Infusion broth and 3% sucrose, provided all factors necessary for the optimal growth of test strains shown by the classical growth curve (Figure 8.3). This culture broth has been long documented as suitable for the cultivation of fastidious bacteria such as streptococci, pneumococci and meningococci (251). Pilot studies on the basal synthetic medium showed that this medium served as an inert support medium, and in no way

influenced mutans streptococci growth and metabolism, independent of the carbohydrates incorporated and tested.

The significant differences in growth response between test strains suggests that variation within strains of the same species exists in their ability to metabolise these foods. Hence, these strains are able to change themselves phenotypically according to the growth environment. The phenotype of these test strains is shown by their biochemical response to sugars in Chapter 6. According to Bowden and Hamilton (252), metabolic adaptation of bacteria is usually transient and expressed only during periods of environmental stress.

The sharp decline in cell counts in BHI, samp+beans and bread+margarine+peanut butter during lag phase (Figure 8.3), can be attributed to an adjustment in the growth properties of cells to the nutrients available. In the clinical context, the prevalence of mutans streptococci strains on fissures and approximal surfaces of teeth can change, where the concentrations of different nutrients may vary from site to site and also from time to time in the oral cavity.

Measurement of bacterial growth kinetics showed that the influence of the food challenge on doubling time and the number of generations was significantly stronger than the test strain. Yet, strain variation in response to the food challenges was demonstrated by differences in doubling time and the number of cell divisions (Table 8.3). For example, NCTC 10449 (*S. mutans*) produced 0.92 and 11.31 cellular divisions in maize and maize+milk+sugar, respectively after 16 hours of fermentation compared to the wild-type strain 942037(cf) where 7.6 and 7.5 generations were produced in the same foods.

The long doubling times in samp and maize and the decrease in the number of generations compared to the control medium, suggests that these bacteria required more time to adapt to the available nutrients in order to metabolise these foods as a carbon energy source. In the healthy mouth, the mutans streptococci grow relatively slow in the plaque biofilm. Similarly, this was also reflected by the relatively long doubling time (0.8h) of the bacteria in the synthetic control medium growing in conventional liquid culture. However, the number of generations in this culture fluid was numerous compared to some of the staple foods and food-mixtures.

Clinical isolates differed from laboratory type strains in their ability to adapt. This may be caused by exposure of these wild-type strains to frequent pulses of the same nutrient (ie. maize and samp) in the host's diet. These bacteria have long-term adaption and the cells can eventually lose their high affinity for glucose. Kinetic studies on *S. mutans* have shown that the phosphoenolpyruvate-mediated phosphotransferase (PEP-PTS) system is a high affinity scavenger system activated under substrate-limited conditions (253). Hence these bacteria are able to metabolise other available carbon substrates. However, on reintroduction to sucrose substrates, the proton motive force is reactivated and transports carbohydrates as the free sugar. In this way the mutans streptococci have the advantage over the fluctuating 'feast and famine' conditions in the oral cavity.

As expected, the bacterial population doubled in the shortest time during fermentation of 3% sucrose, but bacterial viability was short-lived after 8-10 hours of batch fermentation, (Figure 8.3). This decrease in viable cells was probably caused by depletion of the sugar, since the

basal synthetic medium (Appendix III) to which the 3% sucrose was incorporated, does not contain other fermentable carbohydrates. In comparison, growth in maize and samp was static, with microorganisms utilising carbon catabolic enzyme systems that were expressed through the absence of sugars. According to Carlsson (254), when sugars are limiting growth, concentration rather than type of a particular nutrient becomes the controlling factor in nutrition, since the mutans streptococci have the ability to synthesize a different uptake system with higher affinity for the nutrient

8.5.2 Relationship between pH and growth rate

The fermentation of food challenges containing simple sugars or refined starches (bread; bread+margarine+peanut butter), produced the most acid in the least amount of time sufficient to decrease the pH to below 5.7. A low pH was also accompanied by an increase in logarithmic growth of the test organisms. Evidently, the sucrose content in bread is the favourite carbon energy source of mutans streptococci, and this sugar is used for cellular metabolism before other carbohydrates. Numerous studies have indicated that sucrose is the most cariogenic sugar (7, 234, 255-257), but foods that contain soluble and refined starches such as bread also cause a variable fall in pH which may be as large as that caused by sugars (123, 124). Animal studies have also suggested an increase in cariogenicity of starch-sugar mixes (258). Hence, in the oral cavity, repeated conditions of low pH together with proliferation of mutans streptococci with the inhibition of non-aciduric organisms, increases the likelihood of caries.

The clinical effect of a sucrose rinse on plaque bacteria, is a rapid fall in plaque pH to around

5.0 and a slow return to resting level close to neutrality. This decrease is caused by the production of lactic acid by bacterial metabolism and is illustrated in the Stephan curve (10). An investigation on the plaque acidogenic response to carbohydrates in three South African ethnic groups, demonstrated a sharp drop in pH after three minutes, to levels between 6.22 and 5.87 following a 10% sucrose rinse (111). In batch culture, a pH drop to below 5.7 was observed in 3% sucrose, brown bread and bread+margarine+peanut butter, but only after six hours of fermentation.

The delay in pH response to sucrose, indicates that investigations in batch culture may not mimic changes found *in vivo* plaque pH studies or in artificial plaque biofilms, since factors such as salivary buffering and flow in the host, play significant. The complexity of the oral environment is difficult to emulate *in vitro*, but the SuperSpinner fermenting flask allowed the manipulation and evaluation of independent variables under a constant and controlled conditions, thus eliminating confounding variables that could be presented in biofilms or *in situ* studies.

Some foods are caries-protective and the long time period required to lower the pH in maize+milk+sugar is attributed to the natural buffering capacity of milk proteins, since little acidogenicity in plaque pH has been found after milk rinses (259-261). Dairy products such as milk and cheese have been reported to be non-cariogenic (28) and *in vivo* investigations in animals, have shown that ingestion can protect the teeth against caries (236, 237). The inherent buffering ability of some of the traditional African foods may be an explanation for the maintenance of neutral pH during batch culture. Results of this investigation are presented and discussed in Chapter 10.

In comparison to foods containing sucrose, a neutral pH and growth stasis was found during the fermentation of the complex carbohydrates; maize, samp and samp + beans. This corroborated results from previous investigations where it was shown that raw starch caused only a small drop in plaque pH (123), and that cooked, staple starchy foods had low cariogenic potential, and caries caused by heat treated starch was less than that caused by sugars (234, 257).

Samp is comprised of hard, dry, coarsely ground maize kernels, with bran and germ partly removed, whereas maize meal is ground by dry milling techniques in which nutritional additives such as vitamins are incorporated. Samp also contains a high percentage of crude fibre, and requires a longer cooking time to facilitate digestibility compared to refined maize, which is more glutinous when cooked. The low ability of samp to sustain bacterial growth may be due to the partial removal of germ that decreases the mineral, protein (18.4%), sugar (10.8%) and fat content (33%) and approximately 3.7% of protein is lost in the removal of bran (262). The maintenance of cell viability in this investigation can probably be attributed to the cooking process, whereby these foods are degraded and change in form. This increases the retention and allows salivary amylase to provide metabolic substrates such as maltose, maltotriose, dextrins, and small amounts of glucose available to cariogenic bacteria (263). Both samp and maize have little potential to facilitate the course of dental caries, but this study suggests that they provide sufficient nutrients to maintain viability of the mutans streptococci.

8.5.3 Acid anions, pH and strain

Bacterial strains produced high concentrations of lactic acid during the fermentation of foods containing sucrose or refined starches (brown bread, bread+margarine+peanut butter). This

was accompanied by a pH of 5.7 and below. Foods such as maize, samp and samp+beans, that is sucrose limited, produced more acetic acid with minimal pH change. The decrease in pH by lactic acid is attributed to its higher dissociation constant (pK_a) of 3.08, compared with formic (3.75) and acetic (4.76) so the decrease in pH by this acid would be lower than other carboxylic acids.

Clearly mutans streptococci test strains were able to produce mixed-acid fermentation in all the food challenges (Figure 8.7). Studies on mixed-acid fermentation on resting plaque, showed that when conditions are strictly anaerobic, formate and acetate is produced when sugar limited the growth at pH 7.0 (264), but at pH 5.5, streptococcal cells also produced lactic acid under glucose-limiting and strictly anaerobic conditions (265). This investigation showed that larger quantities of acetic acid together with smaller amounts of lactic and formic acid was produced, during the bacterial metabolism of foods with limited sugars (maize+gravy; samp+beans), and minimal pH changes. This result suggests that the concentration of lactic acid is directly proportional to a decrease in pH. Work by Duguid (246) showed that *S. mutans* was capable of consuming previously-formed lactic acid and converted it to lactic, acetic and formic acid when sugar concentrations were low. Hence, part of the high acetic acid concentrations in this study may have been a by-product of lactic acid metabolism. The physiological course of sucrose and glucose limitation, is that the cellular concentrations of fructose 1,6-diphosphate (FDP) and glyceraldehyde-3-phosphate (GAP) are low. This inhibits lactic dehydrogenase (LDH) and releases the negative inhibition of pyruvate-formate lyase resulting in the formation of acetate and formate (266).

8.6 Conclusion

The traditional starch African staple foods, such as maize and samp and their food-mixtures, (maize+gravy, maize+milk+sugar, samp+beans) are not caries-promoting. Maize and samp as single foods clearly do not provide the nutrients necessary for optimal mutans streptococcus growth. However, the mutans streptococci physiologically adapt to metabolise nutrients available in these foods, allowing them to remain viable. This is more evident in clinical wild-types than in laboratory reference strains. Brown bread, in comparison contains the nutrients wheat, yeast and sugar that afford easily assimilated carbon sources.

The response in growth and acid production by mutans streptococci test strains to bread and bread+margarine+peanut butter, was similar to 3% sucrose. This points to bread as a food most likely to promote dental caries. It has also been ranked third on the scale as a cariogenic risk indicated by plaque pH studies (267). To validate these conclusions, the synthesis of water-insoluble extracellular polysaccharides from these foods are presented in Chapter 9.

CHAPTER 9

EXTRACELLULAR POLYSACCHARIDE, PROTEIN CONCENTRATION AND GLUCOSE METABOLISM

9.1 Introduction

The mutans streptococci metabolise sugars that result in the synthesis of glucan and fructan which contribute towards the plaque matrix. These glucans mediate the initial adherence to the tooth surface (268, 269), facilitate bacterial accumulation on smooth surfaces and serve as a reservoir for metabolisable polysaccharides outside of the cell (45, 270-275). The formation of these extracellular polysaccharides (ECP) is catalysed by the constitutive enzyme glucosyltransferase (GTF; EC 2.4.1.5) (271, 276) secreted from the bacterial cell into the culture medium, and initiates the transfer of glucose or fructose units directly from sucrose to the growing polymer chains.

In addition to glucosyltransferase, three extracellular glucan-binding proteins (Gbp) have shown association with the accumulation of *Streptococcus mutans* in plaque, that contribute to cariogenicity. These are; GbpA (277), a 59.0 kDa protein contributing to plaque biofilm development (278, 279), GbpB (280), a 41.3 kDa protein associated with peptidoglycan synthesis in cell wall formation (281) and GbpC (72.0 kDa) (282), a wall-anchored surface protein observed only in cells grown under stress conditions (283). These proteins are not catalysts, but in the presence of different forms of glucans, they aggregate the bacterial cells. They are mostly secreted into the culture supernatant, but in some strains, they remain cell

associated (281). Some of these proteins modulate virulence and can act as protective immunogens (2).

The simultaneous measurement of glucosyltransferase and the three glucan binding proteins produced under different environmental conditions have not yet been reported in the literature. What has been established by Mattos-Graner *et al* (281) is that GbpB produced by *S. mutans* clinical isolates and laboratory strains showed various distributions between cells and culture medium, since binding of bacterial cells by these proteins, are influenced by various forms of glucan polymers formed in the culture supernatant. There is also significant variability in the ability of *S. mutans* clinical isolates containing distinct genotypes to form biofilms *in vitro.* Furthermore, an investigation by Mattos-Graner and co-workers (194) have indicated that *S. mutans* biofilm formation is influenced more by the amounts of GtfC and GtfB produced, suggesting that factors that control expression of GtfB and GtfC may be responsible for differences in virulence among infecting *S. mutans* strains.

9.1.1 Composition of glucosyltransferase

The glucosyltransferases are glucan-binding proteins which catalyse the synthesis of glucan. *Streptococcus mutans* produce at least three glucosyltransferase (Gtf) enzymes. The GtfB (162-kDa) enzyme that catalyses the formation of an insoluble glucan composed predominantly of ∝-1,3-linked glucose moieties, which has been termed mutan. GtfD (155-kDa) produces a soluble dextran made up of highly branched ∝-1,6- linked glucose residues, and GtfC (149-kDa) that produces a polymer with the properties of the two glucans (45, 187). The GtfC enzyme appears to play a crucial role in colonization of smooth enamel surfaces by *S. mutans* (205).

Collectively these enzymes are termed GTF-S, GTF-I, and GTF-SI that synthesise soluble and insoluble glucans, respectively. These enzymes are constitutive and are secreted and adsorbed to the pellicle where they can interact with glucan binding proteins (GBP) present on the cell surfaces, where attachment to tooth enamel is encouraged. Glucosyltransferase can also be found in cell-associated forms (284), but little is known about the activities of these enzymes when bound to bacterial cell surfaces (285).

9.1.2 Glucan synthesis

The metabolism of sucrose by *S. mutans* is best illustrated by Hamada *et al* (64) in Figure 9.1. Sucrose is split into the monosaccharides, glucose and fructose by glucosyltransferase, invertase or fructosyltransferase which are fermented to produce acids.

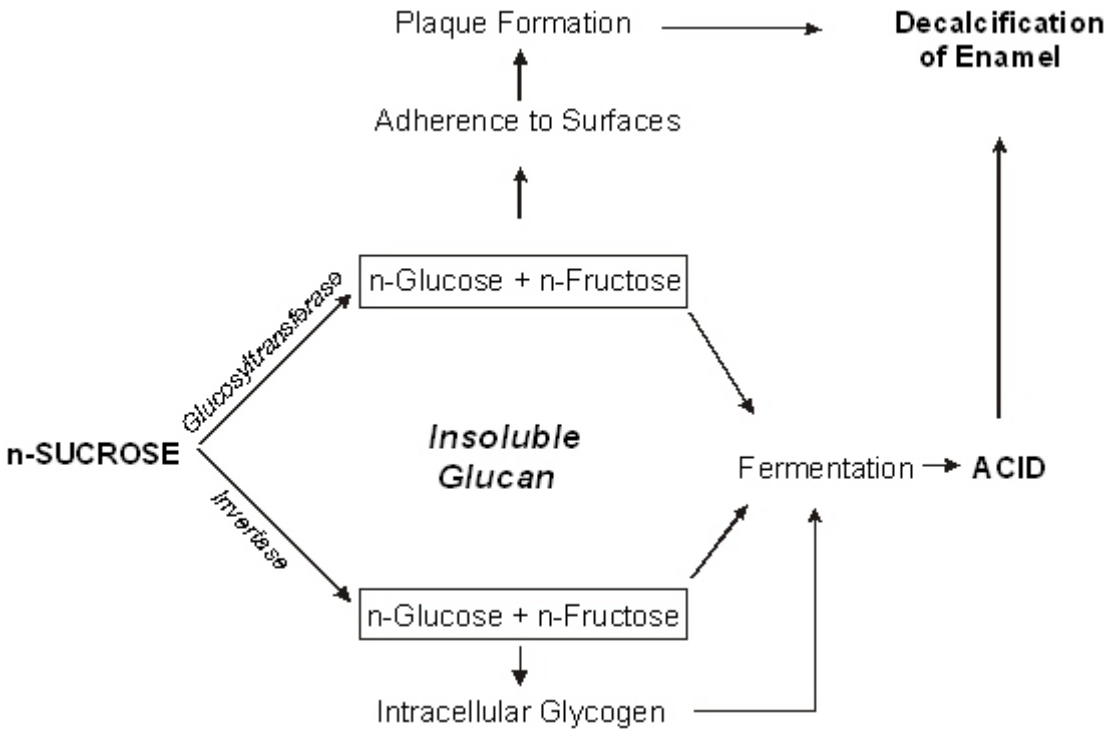

Figure 9.1 Utilization of sucrose by *Streptococcus mutans* and pathogenesis of dental caries.

A continuous and an excess supply of sugars from the human diet permits the synthesis of ECP as well as intracellular glycogen-like polysaccharides (IPS) which is a storage type of iodophilic glucan which is used by the microorganism when exogenous sugars are limited in the diet (286). So in periods of 'starvation' the mutans streptococci are still able to synthesize acids from IPS stored in the bacterial cell. Hence, the location of glucosyltransferase varies with the culture conditions and the strain of mutans streptococci. The presence of cell-associated glucosyltransferases are influenced by the composition of the growth medium.

Spinell and Gibbons (287) showed that in some growth media containing sucrose, all of the enzyme activity was found in the culture fluid and only small amounts were cell-associated. On the addition of small amounts of sucrose or dextran, a significant amount of glucosyltransferase became cell-bound. It is not yet clear how different nutrients contribute to polymer production and has yet to be fully elucidated. Recent studies have suggested that the structure of glucans synthesized by enzymes adsorbed to the surface is different to that produced by the same enzyme in solution (288). This suggests that the quality of polysaccharides produced is also determined by the dietary substrate. Studies have shown that the *S. mutans* plaque prepared from sucrose-containing cultures had markedly enhanced demineralization potential compared with glucose-grown plaque (68). This was produced by the presence of water-insoluble polysaccharides that altered the diffusion properties of plaque (14), and permitted easier permeation of dietary carbohydrates into the biofilm.

9.1.3 Role of sucrose in glucan synthesis

To-date, the general consensus is that sucrose is the carbon substrate of choice, facilitating the synthesis of soluble and insoluble extracellular glucan. It is unique in that the bond between

the glucose and fructose moieties has sufficient energy on cleavage to support the synthesis of polysaccharide (253), making this sugar ideal for bacterial adhesion and eventual plaque formation.

According to Wennerholm *et al* (289), the levels of mutans streptococci respond rapidly to changes in the quantity of sucrose in the diet. This is a result of the direct uptake of sugar where 90% enters glycolysis, leading to acid generation, but the distinctive feature of *S. mutans* is the extracellular enzymes that degrade sucrose polymers and synthesize glucans. However, the role of sugar in caries aetiology is complex because sugar is rarely eaten in its pure form. The cariogenicity of sugar-containing foods can be modified by factors such as the amount and type of carbohydrates (sucrose vs other sugars, sugar/starch combinations), protective components (proteins, fats, calcium, phosphate, fluoride) and physical and chemical properties (liquid vs solid, retentiveness, solubility, pH, buffering capacity, sialogogue properties (290). Yet, foods rich in starch without the addition of sugars were found to play a small role in coronal dental caries (97).

According to Moynihan (234) on the nomenclature of carbohydrates and their dental effects, hydrolysed starch products pose a threat to dental health, whereas intrinsic sugars, found in fresh fruit and vegetables; starch based staple foods; and milk sugars and the polyols are less harmful to teeth. Yet, in the modern age of fluoride exposure, the relationship between sugar consumption and caries is much weaker, but controlling the dietary intake of sugar remains a justifiable part of caries prevention (101).

9.1.4 Points to consider

The staple foods of the South African population comprise mainly of starches. Results in Chapter 8 showed a low growth rate and minimal change in pH during the fermentation of these complex carbohydrates (samp, maize, maize+milk+sugar and samp+beans). Yet, these foods were able to sustain microbial cell viability, indicating that the mutans streptococci test strains are able to metabolise available nutrients. The current literature (14) shows that sucrose is the primary substrate involved in the synthesis of soluble and insoluble extracellular glucans, and that the glucosyltransferases have a high affinity for sucrose. The question raised is: are the mutans streptococci able to produce extracellular ECP necessary for cell adhesion, from traditional African staple foods which contain marginal amounts of sucrose and would the addition of other nutrient components to the staple food influence glucan production? Also, are the resultant end-products required for adhesion, influenced by different *S. mutans* and *S. sobrinus* genotypes. To-date, no such studies have been reported on the production of ECP by mutans streptococci from the fermentation of traditional African staple and combination foods and if these foods contribute towards attachment to the tooth pellicle that is necessary for dental caries.

This section of study does not differentiate between glucosyltransferase, GbpA, GbpB and GbpC protein fractions, since the extent of experimental work falls beyond the scope of this thesis. Future research into the genes encoding for glucan binding proteins and their response to dietary changes in the oral environment is still necessary. However, a preliminary study was incorporated into my study to demonstrate the ratio of extracellular glucosyltransferase to glucan binding protein produced by some *S. mutans* laboratory reference strain and clinical isolate, within the total protein measured.

The focus of this section of study was to show if traditional African foods influence the production of extracellular proteins and encoding of the glucosyltransferase gene by the mutans streptococci. Emphasis was placed on the enzyme glucosyltransferase and not on the action of non-enzymic glucan binding proteins, since these proteins are also dependant on the nature of the polysaccharides produced.

9.2 Aim

The objective of this investigation was to determine if traditional African staple and combination foods facilitate the production of water-insoluble extracellular polysaccharides by mutans streptococci reference and clinical strains. The order of this study was to:

1. Measure the quantity of water-insoluble ECP produced during the fermentation of staple foods in comparison to mixed foods.
2. Estimate the quantities of glucose metabolised to water-insoluble ECP produced.
3. Relate the concentration of cell-free glucosyltransferase by measurement of extracellular proteins secreted into the culture medium over time.
4. Determine any differences between mutans streptococci reference and clinical strains in glucose metabolism, cell-free glucosyltransferase and water-insoluble ECP production.

9.3 Materials and methods

9.3.1 Collection of samples

A one millilitre sample of culture supernatant was collected at the commencement of batch-

culture (baseline), and after every two hours, beginning at four hours of incubation. These were frozen at -20°C until analysed.

9.3.2 Quantification of water-insoluble ECP

The bacterial cells were removed by centrifugation at 5000 × g for five minutes at 4°C. Two volumes of absolute ethanol were added to the culture supernatant and the mixture was allowed to precipitate for four hours at 4°C (66). The precipitated ECP was collected by centrifugation at 8000 × *g* for 10 minutes at 4°C, suspended in 2 M H_2SO_4 and total carbohydrate was determined by the phenol-sulphuric acid method of Dubois *et al* (291) (Appendix III) using glucose as a standard. This method was modified by reducing the test sample and reagent size. To estimate the quantity of ECP produced by the test bacteria, the polysaccharides that naturally occurred in the food challenges and synthetic culture medium, were measured at baseline and subtracted from the final concentration at each time interval.

9.3.3 Measurement of sucrose conversion to glucose

The quantity of residual glucose in the culture supernatant is an indication of the amount of glucose used during the extracellular synthesis of sucrose resulting in the transfer of glucose units to an acceptor, which is usually the glucan polymer. Hence, the glucose remaining in the culture supernatant after growth of the organisms was determined by the UV-method for the enzymatic determination of D-glucose (R-BIOPHARM GmbH, Darmstadt, Germany). The protocol followed was as per instructions provided by the kit (Appendix III). A reagent blank containing distilled water, and the bacteria-free food challenge was included in each experimental assay.

9.3.3.1 Principle of method of UV analysis

D-Glucose is phosphorylated to D-glucose-6-phosphate (G-6-P) in the presence of the enzyme hexokinase (HK) and adenosine -5'- triphosphate (ATP) with the simultaneous formation of adenosine-5'-diphosphate (ADP). In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidized by nicotinamide-adenine dinucleotide phosphate (NADP) to D-gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH). The amount of NADPH formed in this reaction is stoichiometric to the amount of D-glucose. The increase in NADPH is measured by means of its absorbance at 340 nm (292).

9.3.3.2 Methodology

For each set of analyses, a standard curve using D-glucose as standard, with concentrations ranging between 0.001 mg/mL to 2.0 mg/mL was constructed. A one millilitre sample was removed from the fermentation flask at baseline and at 4, 8, 12 and 16 hours of culture. Twenty-five µL of test sample was added to 150 µL of Solution 1 (Appendix III) and 475 µL of distilled water, mixed, and after 3 minutes, absorbancies (A_1) were read at A_{340}. Five micro-litres of Solution 2 (Appendix III) was added and the reaction was allowed to continue for 15 minutes and the absorbence (A_2) was read a second time. The absorbence ($\triangle A$) differences were determined for both reagent blank and samples using the following calculation: $\triangle A = (A_2 - A_1)_{sample} - (A_1 - A_2)_{blank}$

The glucose concentration of test samples was interpolated from the glucose standard curve on GraphPad Prism Version 4 (GraphPad Software, Inc, San Diego, CA, USA) for windows, using linear regression analysis. An increase or decrease in glucose concentration during the

batch culture of each food was determined by the subtraction of glucose present at baseline level.

9.3.4 Determination of extracellular protein concentration

To relate the amount of glucosyltransferase secreted into the culture medium by the test bacteria, the extracellular protein content was determined by the Lowry method (293) using the protocol from Scopes (294) (Appendix III). Bovine serum albumin was used to construct the standard curve. Bacterial cells were removed from the culture supernatant by centrifugation (Hettich, Tuttlingen, Germany) for 15 minutes at 1400×*g*.

9.3.5 Preliminary investigation of extracellular proteins and glucosyltransferase

Because the glucosyltransferase fraction was not extracted and purified from the culture supernatant, it was necessary to establish that part of the protein present was indeed glucosyltransferase. To determine the range and size of proteins present in the culture supernatant, proteins were visualised using SDS-PAGE electrophoresis. The molecular weight of the proteins were visually compared against a standard molecular weight marker (MBI Fermentas, St. Leon-Rot, GMBH). The *in situ* determination of glucosyltransferase activity was used to determine the molecular weight of this enzyme, compared to the glucan binding proteins.

9.3.5.1 Bacterial test strains

An *S. mutans* clinical isolate from a child with active-caries [933012A(c)] and a laboratory reference strain, Sims (NCTC 10449), was used as a check on cell-free extracellular protein and the glucosyltransferase fraction.

9.3.5.2 Preparation of samples

To remove interfering low molecular weight solutes, 400 µL of an eight hour culture supernatant was concentrated in a Ultrafree®-MC (30 000 NMWL) centrifugal filter unit (Millipore, Bedford, USA) by centrifuging at 4000×*g* for 25 minutes at 20°C. The samples were prepared for electrophoresis by combining 100 µL of the culture supernatant with an equal volume of 2X treatment buffer (Appendix III) and reduced by incubation at 60°C for 5 minutes to form SDS-protein micelles.

9.3.5.2 Discontinuous gel electrophoresis

Samples were loaded in quantities ranging between 30 and 40 µL onto 1.5 mm, 7.5% (w/v) acrylamide gels containing 0.1% (w/v) SDS and electrophoresed according to the system of Laemmli (295). The molecular mass of proteins were estimated from a plot which related the migration distances for five marker proteins (Combithek^{R} - Boehringer Mannheim, Cat No 1317474) to their log M_r. The markers were α_2-macroglobulin (170 kDa), β-galactosidase (116.4 kDa), fructose-6-phosphate kinase (85.2 kDa), glutamate dehydrogenase (55.5 kDa) and aldolase (39.2 kDa).

The samples were first run through a 4% (w/v) stacking gel at 25 mA (60 V) for 30 minutes, on a Hoeffer SE 260 Dual cooled vertical slab gel electrophoresis unit (Hoeffer, San Francisco, USA) (Figure 9.2). The current was changed to 45 mA (120 V) and run for two hours on the separating gel. The 8x10 mm gels were fixed in a glass dish containing a mixture of 50% methanol with 7.5% acetic acid for 30 minutes and stained using the polychromatic silver staining method of Sammons *et al* (296) (Appendix III). The gels were washed in three changes of distilled water (one hour per wash), and incubated with agitation in the silver stain

for 45 minutes with a ratio of 1:3 (gel:stain volume). The gels were rinsed thoroughly with distilled water to remove silver on the gel surface, and the reducing solution was added in a ratio of 1:5.5 and placed on a platform shaker for 8 minutes. The reducing solution was poured off and the colour enhancer added in the ratio of 1:5.5. The gel was incubated with shaking for one hour and replaced twice more to give full colour development.

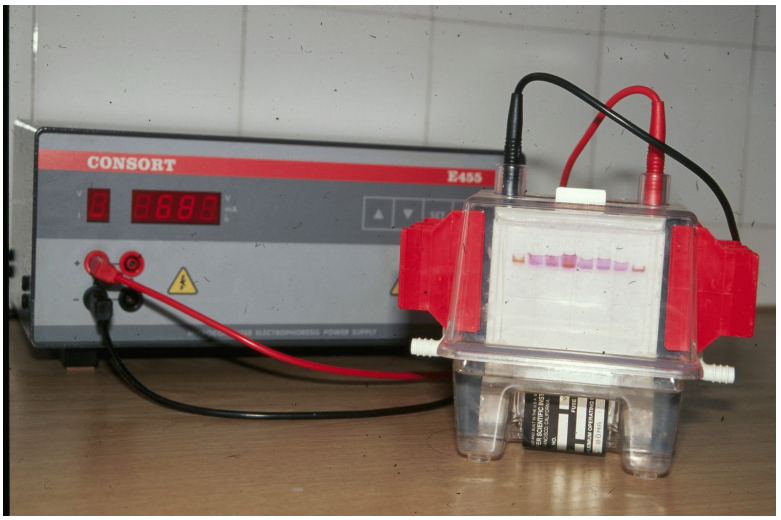

Figure 9.2 SDS-PAGE electrophoresis of total proteins from culture supernatant sampled from batch culture.

9.3.6 Determination of glucosyltransferase activity

In situ glucosyltransferase activity was determined by incubating the gels in 250 mL of 10 mM sodium acetate/acetic acid buffer (pH 6.0) containing 3% sucrose (w/v) and Triton X-100 (1% v/v) (66, 297) at 37°C for 16 hours, in a shaking incubator.

Gels were stained for carbohydrate with periodic acid-schiff (PAS) reagent by a modification of the method of Kapitany and Zebrowski (298). Briefly, the gel was fixed with 12.5% acetic

acid (v/v) for one hour, oxidized with 1% periodic acid (v/v) for two hours, then washed against 15% acetic acid (v/v) for two hours. The gel was transferred into Schiffs reagent (Appendix III) and stored at 4°C in the dark until bands had developed colour to sufficient intensity, and washed in several changes of distilled water.

9.3.7 Analysis of data

Data were analysed with SAS (Version 9.1 for Windows, SAS institute, Cary, NC, USA). The concentration of water-insoluble ECP produced, total extracellular protein and glucose were recorded as μg and mg per mL of sample, respectively. The General Linear Models procedure (GLM) was applied to determine the influence of the food challenges and mutans streptococci test strains on ECP, extracellular protein and glucose concentration. and comparisons were made with Tukey's studentized range test. The association between water-insoluble ECP produced, glucose utilized and the total protein concentration in the culture supernatant was determined by the Spearman correlation coefficients. The level of statistical significance was set at $P < 0.05$.

9.4 Results

9.4.1 Effect of foods on water-insoluble ECP

The mean concentration of water-insoluble polysaccharides produced by *Streptococcus mutans* and *Streptococcus sobrinus* test strains by food-type are shown in Table 9.1 and 9.2, respectively. Overall, the influence of the food challenge ($F=68.80$, $P<0.0001$) was significantly greater on ECP production than the bacterial test strains ($F=8.97$, $P<0.0001$) or

time (F=2.96, P=0.003). The effect of mutans streptococci test strains and time on the variation of ECP, differed in each food challenge (GLM procedure) (Table 9.3). Significantly low volumes of water-insoluble ECP (P<0.05) were produced during the fermentation of samp, maize and maize+milk+sugar that did not increase significantly from baseline levels, compared to the remaining test foods and the control medium.

Comparison between ECPs' produced by the bacterial test strains, significantly varied in all food challenges. This was particularly evident in maize+milk+sugar and samp, accounting for 92% and 88%, respectively of the variance. The period of time taken to produce ECP in maize and 3% sucrose was statistically inconsequential. However, an increase in water-insoluble glucan occurred after four hours in BHI+3% sucrose, bread and bread+margarine+peanut butter.

Table 9.1 Mean concentration (mean ± SD) of water-insoluble ECP (mg/mL), (n = 8)[a], extracellular protein (P) (µg/mL) (n = 8)[a] and residual glucose (G) (mg/mL) (n = 5)[a] of *Streptococcus mutans* test strains, after 16 hours of batch culture

		Streptococcus mutans														
		Sims			LM-7			942037 (cf)			942007 (cf)			953012A (c)		
		ECP	P	G	ECP	P	G	ECP	P	G	ECP	P	G	ECP	P	G
Maize	mean	0.30	28.76	0.005	0.31	35.05	0.01	0.24	1.69	0.01	0.65	50.29	0.01	0.44	32.40	0.01
	SD	0.08	3.79	0.01	0.09	4.70	0.02	0.06	1.58	0.01	0.20	10.57	0.01	0.11	3.17	0.01
Samp	mean	0.28	27.68	0.004	0.33	50.56	0.008	0.13	0.81	0.004	0.48	43.80	0.004	0.56	33.93	0.05
	SD	0.09	3.68	0.009	0.09	4.95	0.009	0.04	0.29	0.01	0.06	6.46	0.01	0.12	3.44	0.09
Brown bread	mean	2.71	38.98	0.03	0.72	40.59	0.04	3.65	11.57	0.12	0.73	57.10	0.05	0.94	39.30	0.09
	SD	0.35	3.69	0.07	0.35	10.09	0.07	0.40	3.11	0.06	0.06	8.01	0.07	0.25	3.25	0.08
Maize+milk+sugar	mean	1.14	29.96	0.03	0.20	7.64	0.24	0.23	9.46	1.26	0.06	34.98	1.34	0.03	22.69	0.53
	SD	0.31	8.24	0.02	0.08	5.53	0.14	0.08	5.49	1.15	0.05	7.33	0.80	0.04	1.93	0.38
Maize+Gravy	mean	1.22	18.45	0.29	1.86	15.36	0.33	0.38	3.24	1.32	0.36	14.38	1.01	0.08	38.70	0.29
	SD	0.78	1.1	0.56	0.41	5.10	0.56	0.14	1.66	0.45	0.16	6.26	0.55	0.09	5.13	0.58
Samp+Beans	mean	0.31	19.26	0.002	0.92	13.24	0.01	0.54	0.11	0.09	0.29	26.54	0.14	0.7	16.71	0.18
	SD	0.3	3.02	0.004	0.61	2.70	0.01	0.16	0.16	0.09	0.19	3.69	0.10	0.69	1.38	0.12
Bread+Marg+P Butter	mean	3.17	32.97	0.05	1.86	33.69	0.06	2.9	36.52	0.65	1.93	57.63	0.15	0.66	62.02	0.22
	SD	1.78	4.45	0.12	0.1	11.59	0.11	0.92	14.36	0.77	0.47	11.86	0.11	0.43	5.21	0.16
3% sucrose	mean	0.25	14.58	0.17	1.48	16.34	1.36	0.32	20.83	0.77	0.34	26.65	0.63	0.29	24.70	0.72
	SD	0.35	0.62	0.11	1.24	1.39	0.82	0.33	2.09	0.62	0.58	2.80	0.38	0.47	2.18	0.45
BHI+3% Sucrose	mean	1.49	353.3	1.77	0.91	174.1	1.95	1.37	165.7	1.4	0.96	200.3	1.77	1.50	472.7	1.50
	SD	0.54	15.97	0.50	0.23	32.08	0.53	0.47	41.16	0.31	0.27	42.09	0.60	0.70	30.79	0.36

BHI=Brain Heart Infusion; cf=caries-free; c=caries; [a]number of determinations per mean value.

Table 9.2 Mean concentration (mean ± SD) of water-insoluble ECP (mg/mL), (n = 8)[a], extracellular protein (P) (µg/mL) (n = 8)[a] and residual glucose (G) (mg/mL) (n = 5)[a] by *Streptococcus sobrinus* test strains, after 16 hours of batch culture

		Streptococcus sobrinus																	
		SL-1			OMZ-176			Z/AHT			OMZ-65			942036 (cf)			953012B (c)		
		ECP	P	G	ECP	P	G	ECP	P	G	ECP	P	G	ECP	P	G	ECP	P	G
Maize	mean	0.24	34.64	0.005	0.94	50.11	0.01	0.15	6.35	0.006	1.35	49.53	0.005	0.17	1.07	0.005	0.69	0	0.005
	SD	0.10	3.98	0.01	0.20	5.83	0.01	0.07	2.71	0.01	1.07	5.15	0.01	0.10	1.94	0.01	0.22	0	0.01
Samp	mean	0.13	31.7	00.004	0.45	51.1	0.01	0.04	9.66	0.004	0.32	48.92	0.004	0.12	9.5	0.004	0.1	1.5	0.19
	SD	0.61	2.44	0.01	0.13	5.35	0.01	0.02	4.04	0.01	0.10	6.41	0.01	0.04	1.86	0.01	0.03	1.49	0.11
Brown bread	mean	2.04	58.6	0.08	2.27	55.62	0.09	2.78	20.58	0.03	0.8	48.44	0.06	3.23	12.67	0.03	3.51	31.38	0.08
	SD	0.67	6.72	0.07	0.05	3.97	0.04	0.64	3.04	0.07	0.17	2.73	0.08	1.45	1.70	0.07	0.32	4.14	0.11
Maize+Milk+Sugar	mean	0.01	18.83	0.04	0.05	23.13	0.11	0.28	17.85	0.02	0.11	15.81	0.16	0.28	9.41	0.40	0.01	19.73	0.46
	SD	0.03	1.54	0.02	0.05	2.10	0.07	0.11	5.12	0.02	0.07	10.45	0.21	0.11	3.19	0.23	0.03	1.32	0.33
Maize+Gravy	mean	0.04	34	0.28	0.08	30.04	0.32	0.29	13.22	0.29	0.17	21.97	0.36	0.30	23.77	0.87	0.42	27.11	0.27
	SD	0.06	5.62	0.58	0.10	3.02	0.56	0.13	5.24	0.57	0.05	4.03	0.55	0.15	9.63	0.41	0.95	1.79	0.59
Samp+Beans	mean	0.66	17.96	0.003	0.67	12.96	0.01	0.33	15.17	0.002	0.37	21.3	0.02	0.47	4.81	0.005	0.84	20.61	0.29
	SD	0.60	0.97	0.004	0.52	3.70	0.01	0.07	8.88	0.004	0.16	2.97	0.01	0.81	2.07	0.006	0.73	2.64	0.40
Bread+Marg+PB	mean	0.47	58.25	0.06	0.47	65.83	0.05	1.86	34.68	0.05	2.16	44.52	0.06	2.57	30.52	0.07	1.21	102	0.48
	SD	0.49	5.28	0.12	0.45	8.01	0.12	0.40	20.91	0.12	0.20	25.96	0.12	1.10	7.70	0.11	0.88	10.87	0.30
3% Sucrose	mean	0.35	15.98	0.06	0.77	16.09	0.08	1.88	15.38	0.08	1.24	19.62	0.12	2.52	19.85	0.02	0.34	18.9	0.07
	SD	0.44	0.97	0.06	0.68	0.79	0.08	1.40	0.90	0.06	0.81	1.37	0.17	0.71	1.48	0.02	0.43	1.15	0.05
BHI+3% Sucrose	mean	0.44	256.6	1.74	1.04	495.1	1.97	3.49	57.45	1.12	0.74	227.3	1.52	0.74	146.6	1.51	1.01	515.7	1.43
	SD	0.13	55.32	0.60	1.03	48.85	0.54	0.44	7.84	0.18	0.35	51.49	0.57	0.28	30.94	0.45	0.41	12.20	0.77

PB=Peanut butter; BHI=Brain Heart Infusion; cf=caries-free; c=caries; [a]number of determinations per mean value.

butter (P<0.05), but was produced only after eight hours in maize+milk+sugar and maize+gravy.

Table 9.3 Baseline, mean ± standard deviation (SD) and range of water-insoluble ECP (mg/mL) (n = 88), and the influence of mutans streptococci test strains over time

Food	Mean Baseline	Mean ± SD	Min-Max	Test strain		Time	
				F	*P*	F	*P*
Staple							
Maize	0.37	0.49±0.49	0.02-3.70	9.45	†	1.03	0.41
Samp	0.23	0.26±0.19	0.01-0.71	47.89	†	2.41	0.03
Brown Bread	1.47	2.13±1.23	0.10-4.00	39.66	†	3.26	0
Combination							
Maize+Milk+Sugar	0.1	0.22±0.33	0.00-1.57	25.22	†	4.7	0
Maize+Gravy	0.29	0.47±0.66	0.00-2.88	42.01	†	3.64	0
Samp+Beans	0.34	0.60±0.47	0.00-1.80	33.95	†	0.91	0.5
Bread+Marg+P Butter	1.08	1.75±1.19	0.04-5.50	26.79	†	3.89	0
Controls							
3% Sucrose	0.64	0.89 ± 1.04	0.00- .77	9.42	†	0.72	0.67
BHI+3% Sucrose	0.73	1.24 ± 0.91	0.09-4.38	28.58	†	4.92	†

† = P<0.0001

9.4.1.1 Comparison between staple and food combinations

Tukey's Studentized range test showed no significant difference in ECP production between samp, maize, maize+gravy, samp+beans, maize+milk+sugar, whereas more was produced in brown bread , bread+margarine+peanut butter, BHI+3% sucrose and 3% sucrose, compared to the remaining test foods (P<0.05).

9.4.2 Effect of foods on glucose metabolism

The measure of food metabolised by test bacteria indicated by glucose levels in the culture supernatant was influenced more by food-type (F=96.23, $P<0.0001$) than by mutans streptococci test strain (F=5.95; $P<0.0001$). The mean baseline levels of glucose (mg/mL) at commencement of this study and residual glucose after 16 hours of batch culture are shown in Table 9.4.

The test bacteria could account for 79%, 68% and 82% of the variation in 3% sucrose, samp and maize+gravy, respectively. Time did not play a significant role in the metabolism of glucose in samp, samp+beans and bread+margarine+peanut butter. Yet, a significant increase in the quantity of measured glucose after four hours of fermentation in maize+milk+sugar and maize+gravy was shown, compared with eight hours in BHI+3% sucrose and 3% sucrose.

9.4.2.1 Comparison between staple and food combinations

Significant differences ($P<0.05$) in the amount of glucose metabolised was shown between maize and its combinations (maize+milk+sugar, maize+gravy), whereas no variation was indicated in bread and bread+margarine+peanut butter. The assimilation of glucose from the synthetic control medium was higher than other test foods, yet maize+gravy, maize+milk+sugar and bread+margarine+peanut butter were comparable to 3% sucrose.

Table 9.4 Baseline, mean ± standard deviation (SD) and range of glucose (mg/mL) (n = 55), and the influence of mutans streptococci strains over time

Food	Mean Baseline	Mean ± SD	Min-Max	Test strain		Time	
				F	*P*	F	*P*
Staple							
Maize	0.02	0.007±0.01	0-0.03	1.17	0.34	23.6	†
Samp	0.02	0.03±0.07	0-0.28	8.37	†	0.22	0.92
Brown Bread	0.16	0.06±0.07	0-0.23	2.06	0.05	19.9	†
Combination							
Maize+Milk+Sugar	0.01	0.42±0.61	0-2.19	6.78	†	4.36	0
Maize+Gravy	1.32	0.51±0.61	0-1.88	7.64	†	26.1	†
Samp+Beans	0.01	0.07±0.15	0-0.98	2.79	0.01	1.42	0.24
Bread+Marg+P Butter	0.27	0.17±0.31	0-1.66	3.08	0.01	2.23	0.1
Controls							
3% Sucrose	0.02	0.37±0.53	0-2.03	11.8	†	8.01	†
BHI+3% Sucrose	1.01	1.61 ± 0.52	0.77-2.27	2.52	0.02	12.5	†

† = $P<0.0001$

9.4.3 Effect of foods on extracellular protein

The GLM procedure showed that the variation in protein concentration was significantly influenced more by the food-type (F=263.82, $P<0.0001$) than by mutans streptococci test strains (F=16.21, $P<0.0001$). Time was not a significant factor. The degree of influence of the test bacteria on protein concentration by food challenge is shown in Table 9.5. Mutans streptococci strains accounted for 98% and 96% of the variances in samp, maize and BHI+3% sucrose, respectively compared to the remaining foods ($P<0.05$). A change in the concentration of extracellular proteins occurred after four hours of batch fermentation in all food challenges, excepting for samp+beans.

9.4.3.1 Comparison between staple and food combinations

No significant difference was shown between staple foods and food-combinations. The concentration of extracellular protein was significantly higher ($P<0.05$) in the control medium compared to other test foods. The quantities produced in bread+margarine+peanut butter was greater than other foods, whereas the amount of protein from 3% sucrose was low ($P<0.05$) compared with BHI+3% sucrose and bread+margarine+peanut butter.

Table 9.5 Baseline, mean ± standard deviation (SD) and range of extracellular protein (mg/mL) (n = 88), and the influence of mutans streptococci strains over time

Food	Mean Baseline	Mean ± SD	Min-Max	Test strain		Time	
				F	*P*	F	*P*
Staple							
Maize	0.021	0.027±0.022	0-0.054	181.31	†	3.38	0
Samp	0.022	0.029±0.020	0.0005-0.056	291.06	†	7.51	†
Brown Bread	0.03	0.039±0.018	0.011-0.061	115.71	†	4.82	0
Combination							
Maize+Milk+Sugar	0.013	0.019±0.009	0.007-0.031	25.22	†	4.7	0
Maize+Gravy	0.017	0.021±0.012	0.003-0.043	42.01	†	3.64	0
Samp+Beans	0.014	0.015±0.007	0-0.026	33.95	†	0.91	0.5
Bread+Marg+P Butter	0.067	0.047±0.025	0.023-0.099	26.79	†	3.89	0
Control							
3% Sucrose	0.018	0.020 ±0.004	0.015-0.027	79.11	†	5.56	†
BHI+3% Sucrose	0.243	0.291±0.160	0.057-0.531	168.2	†	2.81	0.01

† = $P<0.0001$

9.4.4 Cell-free culture supernatant proteins

9.4.4.1 *Streptococcus mutans* reference strain (Sims)

The SDS-PAGE profile of extracellular proteins from BHI+3% sucrose grown cultures of *S. mutans* (Sims: NCTC 10449) after 16 hours of batch culture is shown in Figure 9.2. A polypeptide fragment of approximately 150 kDa was identified in the cell-free culture supernatant, and assumed to be glucosyltransferase. This was confirmed by the *in situ* determination of glucosyltransferase activity in sucrose (Figure 9.3). Polypeptides of molecular weight less than 30 kDa were not resolved on the large-pore gels.

The 150 kDa protein fragment was shown in the supernatant of both single and mixed foods. The intensity of this protein band appeared greatest in the control BHI+3% sucrose (Lane 1, b). A protein band of approximately 250 kDa (Lane 1 a) was evident only in the culture supernatant of *S. mutans* (NCTC 10449) that had been challenged to BHI+3% sucrose. Visual comparison based on band intensity, suggested that the concentration of the glucosyltransferase fragment was smaller than the molecular weight marker that comprised of 0.02 mg/mL protein. Proteins ranging between 41-58 kDa (Lane 1 c) were found in some of the food challenges. A polypeptide with glucosyltransferase activity was identified as a major band at approximately 150 kDa, on the PAS stained gel (Figure 9.3).

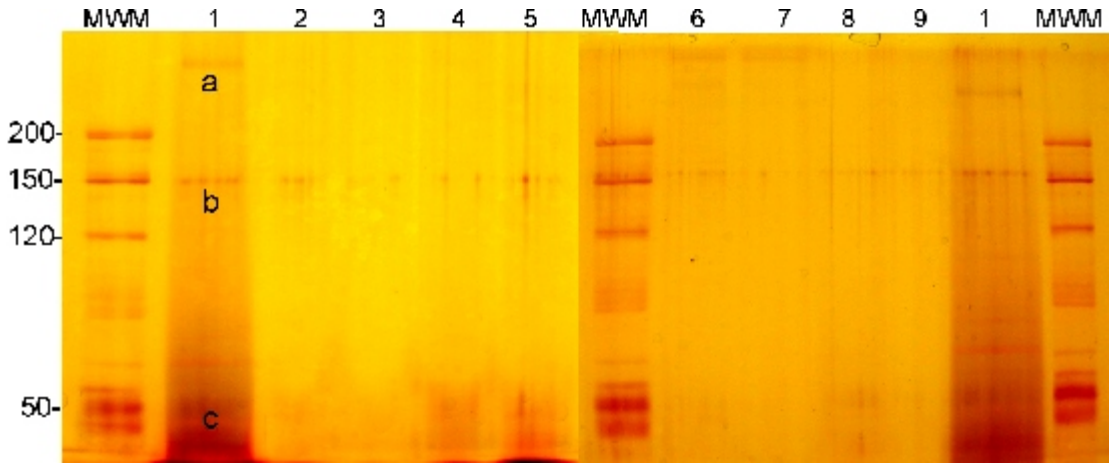

Figure 9.3 Silver stained SDS-PAGE of extracellular protein from a 16-hour culture supernatant of *S. mutans* (Sims) grown in food-combinations: Lane 1 = BHI+3% sucrose, Lane 2 = maize+milk+sugar, Lane 3 = maize+gravy, Lane 4 = samp+beans, Lane 5 = bread+margarine+peanut butter, and single foods: Lane 6 = maize, Lane 7 = samp, Lane 8 = brown bread, Lane 9 = 3% sucrose, MWM = molecular weight marker.

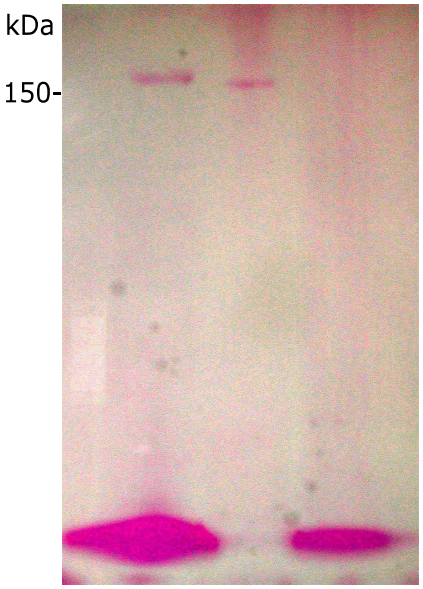

Figure 9.4 A 16-hour culture supernatant of *S. mutans* reference strain (Sims) grown in BHI+3% sucrose and activity stained for glucosyltransferase (155 kDA) with PAS reagent.

9.4.4.2 *Streptococcus mutans* clinical strain 953012A(c)

Protein bands of approximately 144 kDa (Lane 1 b) were identified in the culture supernatant of clinical strain 953012a challenged to single foods and food mixtures (Figure 9.4). An additional protein band of 150 kDa (Lane 1 a) was found only in BHI+3% sucrose. Proteins ranging between 41-58 kDa (Lane 1 c) were found in all food challenges. Comparison between protein band intensities showed higher concentrations of the glucosyltransferase protein in mixed foods compared to single foods.

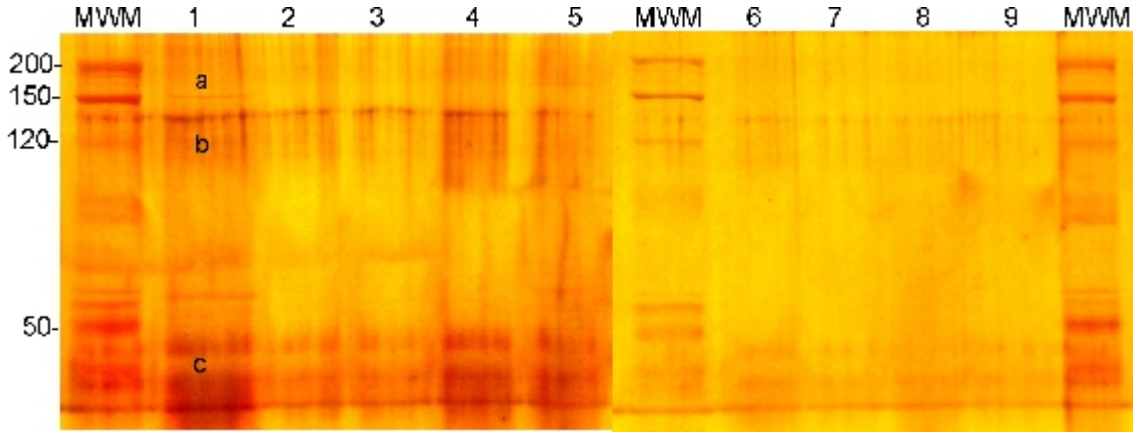

Figure 9.5 Silver stained SDS-PAGE of extracellular protein from a 16-hour culture supernatant of *S. mutans* clinical isolate 953012A(c) grown in food-combinations: Lane1 = BHI+3% sucrose, Lane 2 = maize+milk+sugar, Lane 3 = maize+gravy, Lane 4 = samp+beans, Lane 5 = bread+margarine+peanut butter; and single foods: Lane 6 = maize, Lane 7 = samp, Lane 8 = brown bread, Lane 9 = 3% sucrose, MWM = molecular weight marker.

9.4.5 Association between water-insoluble ECP, glucose and extracellular protein

The mean ratio between the concentration of water-insoluble glucans, glucose and total protein, over time are shown in Figure 9.5. No statistical significance was found between the amount of water-insoluble glucans produced and glucose metabolised. However, statistically significant associations were shown between ECP and total protein, in maize (r=0.53; P<0.0001), samp (r=0.70; P<0.0001), brown bread (r=-0.54; P<0.0001), maize+gravy (r=-0.52; P<0.0001), and bread+margarine+peanut butter (r=-0.60; P<0.0001). The negative r values suggested that a significant increase in ECP was accompanied by a decrease in total protein. Statistically significant, but weak correlation was shown between glucose and extracellular protein in maize+milk+sugar (r=0.29; P=0.03), maize+gravy (r=-0.29; P=0.03), bread+margarine+peanut butter (r=0.51; P<0.0001) and 3% sucrose (r=0.29; P=0.03).

Mean concentrations of water-insoluble ECP, glucose and extracellular proteins after 16 hours of batch fermentation are shown in Figure 9.6. The least amount of ECP was produced in samp (0.03 mg/mL), maize (0.13 mg/mL) and maize+gravy (0.16 mg/mL), with residual glucose highest in 3% sucrose (0.62 mg/mL), BHI+3% sucrose (0.54 mg/mL) and maize+milk+sugar (0.31 mg/mL). The concentration of extracellular protein was highest in BHI+3% sucrose (0.048 mg/mL), but a decreased from baseline levels were found in bread+margarine+peanut butter (-0.02 mg/mL).

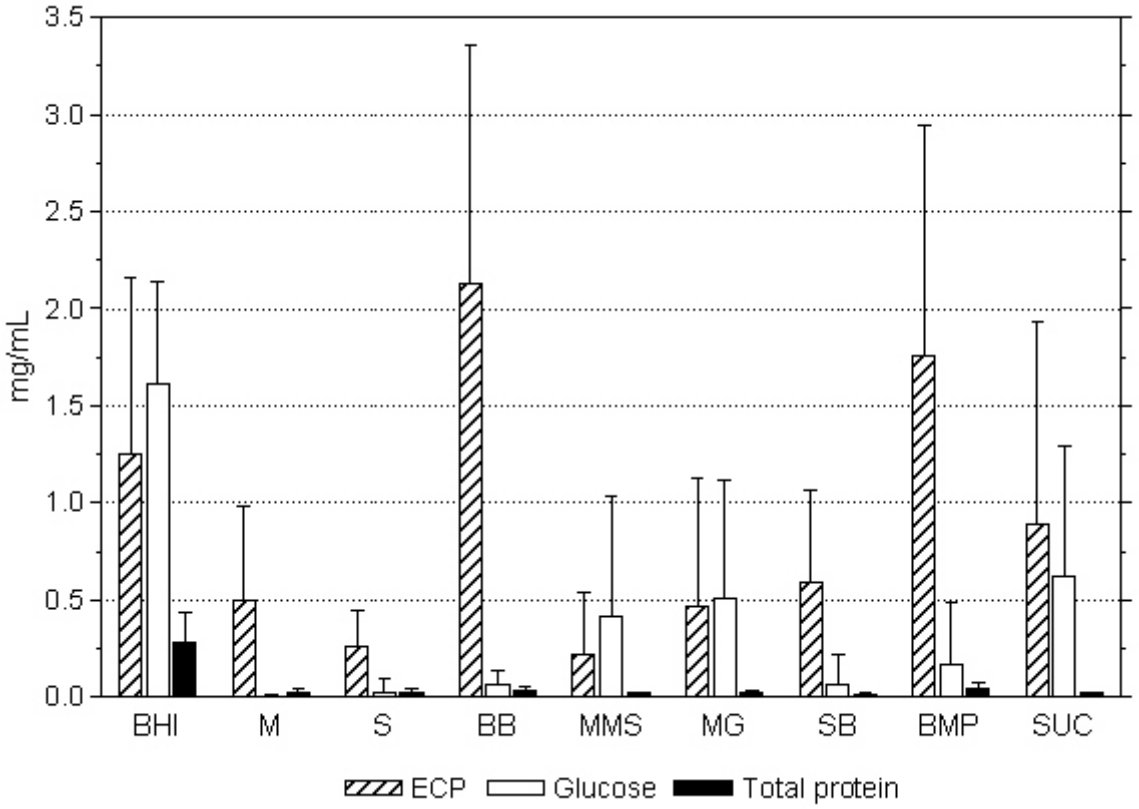

Figure 9.6 Concentration ($\bar{x}$±SD) of water-insoluble ECP (n=88), glucose (n=55) and total protein (n=88). BHI = Brain heart infusion broth+3% sucrose, M = maize, S = samp, BB = brown bread, MMS = maize+milk+sugar, MG = maize+gravy, SB = samp+beans, BMP = bread+margarine+peanut butter, SUC = 3% sucrose.

9.4.6 Comparison between mutans streptococci strains

Tukey's Studentized Range test indicating bacterial test strains that responded more virulently to the food challenges are shown in Table 9.6. The test strains are ranked in order of importance, and ratios indicate the strength of the producing bacterium to the remaining strains. In maize, maize+gravy and samp+beans, significant amounts of ECP were produced mainly by *S. mutans* and *S. sobrinus* reference strains, but clinical isolates produced more metabolites in samp. In foods containing refined starches such as brown bread and bread+margarine+peanut butter, most extracellular glucans were produced by clinical strains.

The conversion of sucrose to glucose units and the assimilation of glucose in all food challenges, excepting for BHI+3% sucrose, was significant in clinical isolates. No differences were shown between strains, in maize and brown bread.

Significant concentrations of extracellular proteins from clinical isolate 942007(cf) were apparent in all food challenges. The yield from clinical isolates in bread+margarine+peanut butter, 3% sucrose and BHI+3% sucrose was high compared with NCTC reference strains which produced more protein in maize and samp.

9.4.7 Effect of food challenges on cellular adherence

A clumping of bacterial cells with adherence to the glass bottom and sides of the BioSpinner flask was observed in BHI+3% sucrose, 3% sucrose, brown bread and bread+margarine+peanut butter, after four hours of fermentation. No adherence was observed in the remaining test foods, but finer bacterial growth was noted, with settlement of the cells at the bottom of the flask, after six hours of batch culture.

Table 9.6 Comparison of mutans streptococci test strains (n = 11) ranked in order of importance for each food challenge. The ratios indicate the strength of the producing bacterium to the remaining strains.

	Water-insoluble ECP		**Glucose**		**Extracellular protein**	
Food	Strain	ratio	Strain	ratio	Strain	ratio
Maize	OMZ-165	1:9	NS		942007(cf)	1:9
	OMZ-176	1:6			OMZ-65	1:9
					OMZ-176	1:7
Samp	943012A(c)	1:8	943012B(c)	1:10	942007(cf)	1:8
	942007(cf)	1:8			OMZ-176	1:7
	OMZ-176	1:8			OMZ-65	1:7
					Z/AHT	1:4
Brown Bread	942037(cf)	1:8	NS		942007(cf)	1:10
	953012B(c)	1:6			LM-7	1:6
	942036(cf)	1:6			953012B(c)	1:2
Maize+Milk+Sugar	Sims	1:10	942007(cf)	1:8	942007(cf)	1:10
	Z/AHT	1:6	942037(cf)	1:6	OMZ-65	1:9
	942036(cf)	1:6			Sims	1:9
Maize+Gravy	LM-7	1:10	942037(cf)	1:8	942036(cf)	1:8
	Sims	1:9	942007(cf)	1:8	OMZ-65	1:8
					942007(cf)	1:8
					LM-7	1:6
					Z/AHT	1:4
Samp+Beans	LM-7	1:2	953012B(c)	1:6	942007(cf)	1:10
					OMZ-65	1:9
					Z/AHT	1:8
					942036(cf)	1:6
Bread+Marg+P Butter	Sims	1:6	942037(cf)	1:7	942007(cf)	1:10
	942037(cf)	1:4			943012B(c)	1:9
	942036(cf)	1:4				
3% Sucrose	942036(cf)	1:8	LM-7	1:9	953012A(c)	1:5
	Z/AHT	1:6	942037(cf)	1:6	942007(cf)	1:3
	LM-7	1:1	953012A(c)	1:5	942037(cf)	1:1
BHI+3% Sucrose	Z/AHT	1:10	OMZ-176	1:1	OMZ-65	1:9
	953012A (c)	1: 4	LM-7	1:1	942007(cf)	1:9
	Sims	1: 4			942037(cf)	1:6

NS = no significant variation between strains

9.5 Discussion

9.5.1 The influence of foods on water-insoluble ECP, sucrose metabolism and extracellular proteins

The food challenges significantly influenced the bacterial synthesis of water-insoluble ECP that was coupled to the amount of glucose metabolised and extracellular protein concentration. Also, the influence of the test bacteria on these variables over time, was small yet statistically significant.

In single and mixed-foods, the low quantity of water-insoluble glucan produced (Figure 9.5) in samp ($\bar{x}$=0.26 mg/mL) and maize+milk+sugar ($\bar{x}$=0.22 mg/mL) suggested that these foods do not enhance the formation of dental plaque. At baseline level, only small amounts of glucose were detected in maize, samp, maize+milk+sugar and samp+beans (Table 9.4), but the final concentration of glucose in samp, maize+milk+sugar, and samp+beans in relation to extracellular ECP production showed that the glucans produced were synthesized from the glucose content only, present in these foods. In comparison, the residual glucose concentration in maize, brown bread, maize+gravy and bread+margarine+peanut butter in relation to extracellular ECP production, implies that some of the primary glucans formed may have been used as a carbon substrate for normal cell function.

Maize and samp are composed primarily of starch, which consists of two glucose polymers: amylose, a linear molecule and amylopectin, a branched form. Other carbohydrates are simple sugars present as glucose, sucrose and fructose in amounts that vary from one to three percent of the kernel (262). The glucose polymers are not readily soluble in oral fluids and have a low

diffusion rate in plaque. They must be broken down to maltose by salivary amylase before they can be synthesised by the plaque bacteria, and most starch is cleared from the mouth before it can be assimilated (14), a factor that reduces its potential to promote caries.

In vivo studies on the cariogenic potential of maize have shown that caries in rats was almost zero when fed on maize starch (114) and cooked maize and beans (132), compared with sucrose and glucose. The response to cooked maize by mutans streptococcus strains in batch culture, explains part of why these foods are not conducive to caries development.

Although investigations on diets consisting mainly of starch have found it to be non-caries promoting, especially in people of developing countries, exposure to high heat gelatinises and cleaves it into small oligosaccharides, that can be transported and metabolized by bacteria in the oral cavity (105, 133, 299, 300). Hence, starch in a cooked form, is more easily fermented by human dental plaque (104, 123, 124). However, Schmid *et al* (131) showed that both cooked and uncooked maize were non-cariogenic in rats, providing that sucrose was not added.

Hydrolysed starch have relatively long glycosidic chain length, and are metabolised more slowly than simple sugars and for this reason, uptake is longer. This accounted for the 8 hours required for glucans to be produced from maize+gravy and maize+milk+sugar, and implied that when the source of simple dietary sugars is limited, the mutans streptococci are able to adapt at metabolising longer chain and branched chain saccharides.

The quantity of water-insoluble glucan formed in brown bread and bread+margarine+peanut

butter was similar to 3% sucrose and BHI+3% sucrose. This suggests that the nutrients in these foods are as caries-promoting as sucrose, and the sucrose content in test foods with refined starches, enhanced glucan formation compared to the cooked starches of staple foods. When sucrose is added to a cooked starch, the starch brings sucrose into closer contact with the tooth surface for a longer period than if the food were only a sucrose food (301). Glucose was first metabolised in food challenges where sucrose was added or where trace amounts were present, but the influence of the test strains on the variation in glucan production strongly suggests that the effect was strain specific. The short time (4 hours) taken for test bacteria to produce water-insoluble ECP in the synthetic control medium and bread, was indicative of the speed at which simple sugars are metabolised. Previous studies (14, 17, 45) have indicated that sucrose is the carbon substrate of choice and its uptake for biomass and energy formation occupies a key position in bacterial metabolism in the mouth.

Larger quantities of residual glucose recovered after 16 hours in batch culture was measured in foods where sucrose was added (BHI+3% sucrose, 3% sucrose, milk+maize+sugar) (Figure 9.3). However, the ratio of glucose metabolized to the quantity of water-insoluble ECP produced was not directly proportional, suggesting that other nutrients together with sucrose contribute towards glucan formation. The small amount of water-insoluble glucan produced in 3% sucrose illustrates this point. Notably, the production of water-insoluble ECP was higher in brown bread and its combination, compared to the synthetic control medium.

The hydrolysis of maize and samp during the cooking process released sufficient monosaccharides, indicated by glucose baseline levels (Table 9.4). Staple foods in combination, such as maize+gravy increased the amount of sugar available for bacterial cell

metabolism. Yet, the quantity of water-insoluble glucan produced by the bacteria appeared dependent on the food mixture. For example, less water-insoluble ECP from the fermentation of maize+milk+sugar was formed compared with samp+beans. The small quantity of ECP produced from maize+milk+sugar can be attributed to the casein proteins in milk which act as inhibitors for adherence and expression of the glucosyltransferase enzyme (67). Bowen and Pearson (302) have also shown that milk given in combination with high concentrations of sucrose seem to exert a protective effect.

The adherence of mutans streptococci cells to the fermentation flask, and cellular clumping observed during growth, was particularly evident in the BHI+3% sucrose and 3% sucrose challenge. This correlates with its ability to assimilate sucrose for the synthesis of adherent water-insoluble glucans resulting in adhesion to tooth enamel surfaces. Interestingly, the adherence in bread and its combination was similar to the control media and shows that refined carbohydrates solicits a response that is similar to sucrose exposure.

The fine growth and settlement to the bottom of the culture flask of bacterial cells challenged to maize, samp and some of their mixtures indicate that the nutrients available from these foods, are not conductive for glucan production. Consequently, the test bacterium retains very weak cell-associated glucosyltransferase activity that prevents adherence since glucosyltransferase at the cell surface requires cell-bound glucan to bind extracellular glucosyltransferase to the surface glucan (303). This was also shown by the small quantity of extracellular protein found in the culture supernatant of these foods. In comparison, the higher concentration of cell-free protein shown in foods with added sucrose such as; BHI+3% sucrose, bread+margarine+peanut butter and 3% sucrose suggests a higher concentration of

extracellular glucosyltransferase, but this need to be experimentally confirmed. Hence, the quantity of water-insoluble glucan produced is dependent on the supporting carbohydrate substrate available.

The preliminary investigation separating extracellular glucosyltransferase (150 kDa) from glucan binding protein (41-58 kDa), showed a somewhat higher concentration of glucosyltransferase (judged by visual estimation of band intensity) in BHI+3% sucrose and mixed-foods compared with single foods on SDS-PAGE. Moreover, protein banding between 41-58 kDa was more evident in the culture supernatant of *S. mutans* clinical isolate 953012A(c), suggesting that more glucan binding proteins are produced by clinical wild-type strains exposed to traditional African foods than by standard laboratory strains. The extra protein fraction located at approximately 250 and 150 kDa in BHI+3% sucrose, from NCTC 10449 and clinical isolate 953012A (c), respectively showed no activity *in situ*. These preliminary results show the glucosyltransferase and glucan binding fractions formed part of the total protein that was measured in the culture supernatant of all food challenges. Future research on the influence of traditional African foods on the ratio of glucosyltransferase to glucan binding proteins, and the concentration of each protein and its effect on adhesion should be elucidated.

A weakness in this study is that the extracellular protein fractions were undefined. Hence, the quantity of glucosyltransferase and its contribution towards water-insoluble ECP synthesis remains unknown. Also, the effect of traditional African foods on the production and action of glucan binding proteins could not be specified. What this study does confirm, is that glucosyltransferase is present in the cell-free form when exposed to traditional African foods,

but sucrose is still the critical factor that determines the quantity of this enzyme and the development of adherence to smooth surfaces. The mechanism and measurement of adherence to various solid supports was beyond the scope of this study and further work on mutans streptococci adherence to tooth enamel surfaces exposed to traditional African foods, is ground for future research.

9.5.2 Variation between mutans streptococci test strains

Comparison between NCTC laboratory reference strains and clinical isolates clearly show that different responses were solicited in different foods. Results made it very difficult to determine which one of the test strains was the most virulent (Table 9.6). However, clinical isolate 942007(cf), a *S. mutans* melibiose-negative phenotype, appeared more able to produce water-insoluble ECP from maize and samp compared with *S. mutans* references and clinical isolates. Clearly, there are differences in response between clinical isolates and reference strains; for example, *S. mutans* strains 10923 (LM-7) and 10449 (Sims) were the most virulent producers of water-insoluble ECP in maize+gravy compared with clinical isolates. This suggests that clinical isolates maybe less virulent because of regular exposure to this food in the diet.

The metabolism of sucrose, measured by endpoint glucose showed that this carbohydrate was not all converted to water-insoluble ECP, but was required by the bacterium for normal cell function. The assimilation of glucose did not vary significantly between test strains, but extracellular protein concentrations in all food challenges was greater in the culture supernatant of clinical strain 942007(cf), but appeared not to be significantly associated with ECP production.

The varying amounts of end-product produced by the test bacteria showed that they do not respond similarly to the same nutrients, and adjust their cellular composition and metabolic function (304). They switch between two or more transport systems for different sugars such as the phosphoenolpyruvate-mediated phosphotransferase (PEP-PTS) system which operates principally under substrate-limited conditions and are repressed under sugar excess conditions (17). In the mouth, the mutans streptococci are less limited to nutrient deficiencies and is kept in steady state, since the primary source of nutrients is the endogenous supply of host proteins and glycoproteins from saliva and gingival crevicular fluid, which is supplemented with nutrients from the host's diet. Whereas, in batch culture, the test bacteria respond only to growth-controlling substrates.

The effect of different nutrients on mutans streptococci metabolism in oral biofilms has been shown (223, 228, 233). According to Marsh (13), bacteria growing on biofilm display properties that are different from cells growing in conventional liquid culture, such that genes associated with ECP synthesis can be up-regulated on a surface (305, 306). Preliminary results on the expression of these genes in test bacteria growing in suspended culture, are presented and discussed in Chapter 11.

9.6 Conclusion

The quantity of water-insoluble glucan produced by mutans streptococcus test bacteria in the food challenges are not as important as quality of the polysaccharides determined by the type of carbohydrate substrate serving as a carbon source. Apparently, the heavy deposit of cells observed on the wall of the culture vessel in BHI+3% sucrose, 3% sucrose, bread and bread+margarine+peanut butter suggests that these substrates facilitate plaque formation.

Future studies using biofilm technology could help elucidate the formation of plaque by these foods.

Mutans streptococci test strains responded more vigorously to foods containing sucrose, indicating a preference for this carbon source. Exposure to traditional African foods with no sucrose or refined carbohydrate solicited a stress response that differed between test bacteria. This reaction is usually transient and expressed only during a change in condition such as sugar depletion (252). Such nutrient deficiencies control the expression and modulation of the activities of factors involved in pathogenesis such as water-insoluble polysaccharide production. Maize and samp as single foods, clearly does not provide these necessary requirements for optimal growth. Brown bread, in comparison contains wheat flour, yeast and sugar which afford easily assimilated carbon sources.

The different response to test foods between clinical isolates and NCTC reference strains suggests that clinical strains phenotypically adapt to frequent exposure of specific nutrients in the host's diet and to possible nutrient deficiencies, compared to laboratory reference strains. Clear differences in virulence between clinical test strains isolated from children with and without caries could not be compared because of the small number of isolates investigated.

CHAPTER 10

INHERENT ACIDOGENICITY OF TRADITIONAL AFRICAN FOODS

10.1 Preface

Dental erosion without bacterial involvement is caused by chemical, biological and behavioural factors (307). These risk factors for enamel erosion depend on eating patterns, with increased consumption of acidic foods and beverages (308).

10.2 Introduction

Damage to tooth structure by chemical means is caused by etching of the tooth surface by acid, especially if exposure is long and repeated over time, added to bacterial fermentation risk. One factor is the inherent pH, as well as the buffering capacity of different foods. The periodic increase in organic acids such as lactate, after the consumption of common dietary sugars and starches, result in the under-saturation of calcium and phosphate and therefore net loss of mineral (263). The current literature indicates that acidic food and drink, such as soft drinks and fruit juices can soften hard tissue (307). When solid foods are ingested, salivary clearance is longer. Even an hour after ingestion, foods such as bread, chocolate and bananas have shown to take much longer to clear than a sucrose solution with carbohydrate residues present (309). Hence, the long clearance time and the ability of these foods to buffer the production of acids formed by cariogenic bacteria in plaque, will determine the speed in the development of dental caries.

The buffering effect of foods can be defined as the ability of the food to resist a change in pH, brought about by the production of lactic acid by the mutans streptococci through synthesis of sucrose, that may add to the effects of the existing pH. The buffering capacity of a foodstuff is determined by its acid-base equilibrium, which attenuates pH changes upon exposure to acid or alkali. Traditional African foods have not been characterized relative to their buffer capacities or inherent pH. By evaluating these two variables, the effect of acid anions produced by mutans streptococci by fermentation of these foods, and their eventual effect on enamel demineralization can be determined.

10.3 Aim

The object of this investigation was to characterise the following:

1.1 Inherent pH of traditional African foods

1.2 Buffering capacity of traditional African foods

1.3 Effect of inherent pH and buffering effect on acid production by mutans streptococcus strains in traditional African staple foods, food-mixtures and their components.

10.4 Materials and methods

10.4.1 Determination of buffering capacity

One gram of the prepared portions of the individual foods and food mixtures was homogenized in 10 mL distilled water and initial pH values were measured. A 0.1 M solution of lactic acid and sodium bicarbonate were prepared for titration of all samples. Lactic acid was selected as the titratable acid, since this is the major organic acid produced as a by-product of fermentation

by mutans streptococci. Each sample was titrated slowly with either acid or base from a burette (Bürette Digital II, Brand GMBH+CO, Wertheim, Germany), with thorough stirring, until the system reached an end-point of pH 5.7, which was monitored by a Sentron 2001 pH meter (SENTRON, Roden, The Netherlands) (Figure 10.1).For comparison, a 10% sucrose solution was included as a positive control.



Figure 10.1 Sentron pH system and digital burette used for determination of the inherent buffering capacity of test-foods and food components.

Results were reported as the volume (mL) required to lower or increase pH to 5.7, the pH defined as critical for demineralization of enamel (7), and then converted to active hydrogen

[H^+] and hydroxyl [OH^-] ions (310, 311). Measurements were made in triplicate and the mean values recorded.

10.5 Results

10.5.1 Inherent pH of food

The following staple food, food combinations and food components had inherent pHs below 5.7: brown bread, maize+gravy, tomato and onion gravy and margarine. The remaining foods were close to neutral pH ranging between 5.96 (10% sucrose) and 7.00 (milk). A one-way analysis of variance (ANOVA) showed a statistically significant difference between the inherent pH of the foods tested ($F=45.14$; $P<0.0001$).

10.5.2 Inherent food buffering capacity

Maize+milk+sugar had the lowest buffering capacity, requiring the least amount of acid titrant (0.21 mL) for food combinations, whereas more acid was required in samp+beans (0.47 mL) (Table 10.1). For individual foods, soft maize (0.05 mL) had the lowest buffering capacity while beans (0.60 mL) showed the highest. The buffering capacity of the 10% sucrose control medium (0.07 mL) was comparable to soft maize and both foods showed a low ability to buffer compared with other individual foods tested. In foods with inherently low pH values below 5.7, gravy required the most bicarbonate titrant (0.69 mL), with brown bread (0.16 mL) requiring the least amount of bicarbonate ions.

Table 10.1 Inherent pH (n = 3)[a] and food buffering capacity (mean ± SD) to pH 5.7, of test foods and mixtures in volumes, active H^+ and OH^- concentration

Food	Inherent pH	Buffer capacity, mL		Concentration	
		0.1M lactic acid	0.1M Sodium bicarbonate	$[H^+]$ M/L	$[OH^-]$ M/L
Tomato and onion gravy	4.30±0.0		0.69±0.01		6.5×10^{-3}
Margarine	4.40±0.40		0.38±0.005		3.7×10^{-3}
Brown Bread	4.76±0.24		0.16±0.01		1.6×10^{-3}
Maize+Gravy	4.9±0.001		0.41±0.01		3.9×10^{-3}
Sugar (10%)	5.96±0.0	0.07±0.01		7.0×10^{-4}	
Beans	6.10±0.01	0.6±0.006		5.7×10^{-3}	
Bread+Marg+P Butter	6.20 ±0.08	0.44±0.02		4.2×10^{-3}	
Samp	6.20±0.04	0.16±0.02		1.6×10^{-3}	
Maize (soft)	6.67±0.03	0.05±0.03		5.0×10^{-4}	
Maize (stiff)	6.82±0.08	0.17±0.01		1.7×10^{-3}	
Samp+Beans	6.80±0.50	0.47± 0.03		4.5×10^{-3}	
Peanut Butter	6.90±0.30	0.42±0.01		4.0×10^{-3}	
Maize+Milk+Sugar	6.98±0.0	0.21±0.001		2.0×10^{-3}	
Milk	7.00 ±0.07	0.54±0.02		5.1×10^{-3}	

[a]Number of determinations per mean value.

10.6 Discussion

10.6.1 Inherent pH and buffering capacity

The inherently higher buffering capacity of individual foods like beans (5.7×10^{-3} $[H^+]$ M/L) and the low buffering ability of samp (1.6×10^{-3} $[H^+]$ M/L) showed that these foods in combination had a relatively higher buffering capacity, and more acid (4.5×10^{-3} $[H^+]$ M/L) was required to

drop the pH to the critical 5.7 level. This means that the fermentation of staple foods and food combinations that possess a high buffering capacity, requires a greater concentration of acid anions, to lower the pH to critical point, and hence more acid has to be produced by the test bacteria in order to bring about change in the environmental pH. Because of the low intrinsic buffering capacity of sucrose and soft maize, plaque pH is more likely to decrease rapidly. This explains why short-term exposure of plaque to constant sucrose rinses showed the greatest pH-lowering capacity (312).

The inherently basic pH of milk and peanut butter, and their high degree of resistance to a fall in pH can be attributed to total nitrogen, non-protein nitrogen and phosphates in milk (313, 314), and the high protein content of peanut-butter. These food components in combination, allowed for a higher buffering effect than for single foods. A review of studies on the effects of milk on cariogenicity, dental caries and mutans streptococci have suggested that milk is not cariogenic (28) and that there is little acidogenicity in plaque pH after a milk rinse (259, 261). Even after prolonged exposures, milk has a low potential to demineralize tooth enamel (315).

Although brown bread was found to be inherently acidic, it required a relatively small quantity of bicarbonate to increase the pH. This low buffering capacity, suggests that an adequate salivary flow rate and an efficient salivary buffering system can return the oral environment to a favourably neutral pH soon after bread is eaten. Fortunately, most staple food and food components are eaten in combination.

10.6.2 Effect of inherent pH and buffering capacity on acid production

The low buffering capacity of maize was comparable to sucrose (Table 10.1), but bacterial

strains, 942007 (cf) and NCTC 12277 only, were able to decrease the pH of the maize culture medium (Table 9.7) to the level that produce enamel demineralization. In comparison, samp and samp+beans with its ability to resist acidity, prevented a noticeable drop in pH for all mutans streptococci strains.

Although bread+ margarine+ peanut butter required a high titratable quantity of acid to reduce the pH, there was a decrease in pH below critical point, produced by strains 942007(cf) (pH 4.95), 953012B(c) (pH 5.17), NCTC 11061 (pH 4.96) and 10923 (pH 5.39). This may have been caused by the sucrose ingredient of bread. The pH values shown for test bacteria challenged to maize+milk+sugar was variable but overall negligible.

The longest time taken to reduce the pH to below 5.7 by test microorganisms, was observed in maize+milk+sugar compared with other food mixtures containing some sucrose ingredient (*viz* bread, maize+milk+sugar, bread+margarine+peanut butter and maize+gravy). This demonstrates the prolonged buffering capacity of milk over time.

10.7 Conclusion

The decrease in plaque pH after a food challenge is dependant on the inherent pH and buffering capacity of individual food components. The presence of sucrose, even in small amounts decreases the overall buffering effect and predisposes the food to a rapid drop in pH when exposed to acids. Food components high in protein content, *viz*. beans (7.10 g), milk (7.9 g) and peanut butter (24.4 g) (Appendix II) have a higher buffering capacity compared to foods composed mainly of refined starches.

Chapter 8 suggests that the metabolism of traditional African foods as a source of carbon energy is variable between mutans streptococci strains, and hence the production of acids varies. Therefore, a drop in pH of the nutrient medium in the form of foods, is dependent on the rate of conversion by the bacterium and the quantity of its acid by-product. This implies that a food with high buffering capacity will require exposure to higher concentrations of acid in order to decrease the pH. Consequently, the long time taken for the bacteria to metabolise maize, samp and samp+beans, prevented large quantities of acid from being produced, and the high inherent buffering capacity of these foods allowed only a slight decrease in pH.

In the oral environment other physiological systems such as salivary buffering and salivary flow rate would influence natural buffering. In batch culture, no natural buffering systems were incorporated, but an evaluation of these independent variables could be measured under constant and controlled conditions.

CHAPTER 11

Gtf GENE EXPRESSION AND TRADITIONAL AFRICAN FOODS

- A SPECULATIVE STUDY-

11.1 Preface

The regulation of gene expression within a cell is controlled by gene transcription, the process in which a gene's DNA sequence serves as a template for mRNA synthesis. Control of gene expression is through the regulation of the transcription rate; that is the rate at which RNA polymerase transcribes the gene into molecules of messenger RNA (mRNA). Most of the regulation of gene expression in bacteria occurs at the level of transcription. Gene transcription levels change in response to a wide variety of signals that occur during normal physiological function, and in response to disease and other factors such as a change in the environment. A change in transcription level cause variations in the steady-state levels of individual mRNAs (316).

In bacteria, the most common form of control over the synthesis of enzymes is through induction and repression where processes such as the fermentation of sugars is induced by the specific sugar or the production of its metabolite (317). Results in Chapter three showed different *Streptococcus mutans* biotypes, suggesting that the response of a bacterium to sugars is dependant on the expression of the encoding gene.

11.2 Introduction

The literature indicates that expression of the glucosyltransferase B/C operon of *Streptococcus mutans* is influenced by environmental conditions, such as pH , carbon source and whether the bacteria are attached to the surfaces (193, 285, 305, 318, 319). Studies conducted on *S. mutans* reference strains exposed to chemically defined culture medium with sucrose and glucose as carbohydrate sources, have suggested that the type and amount of these sugars and the environmental pH have a major influence on transcription of the *gtfBC* genes when the organisms were grown in biofilms (318). In batch culture, the synthesis of GTFs by *S. mutans* was shown to be regulated by the addition of sucrose. A twofold increase in enzyme synthesis was recorded by Hudson and Curtiss (193), whereas Wexler *et al* (285) found a threefold increase in activity from the *gtfBC* promoter when sucrose was added to slowly growing glucose-limited *S. mutans* cells in continuous culture. This suggests that the transcription of the exo-polysaccharide machinery of *S. mutans* is influenced by the environment, and hence conditions of the oral habitat.

In vivo, *Streptococcus mutans* are exposed to a variety of sugar concentrations and alternative carbon sources provided in the diet. To-date, no investigations have been made on *gtf* gene expression in mutans streptococci reference and clinical strains, during exposure to traditional African staple foods and mixtures.

11.2.1 Points to consider

Results from Chapter 9 showed that relatively low, mean concentrations of total protein (range = 0.02-0.05 mg/mL) was present in the culture supernatant of all the foods tested compared to the control medium, BHI+3% sucrose (0.29 mg/mL). However, the quantity of water-insoluble

ECP produced by the mutans streptococci test species, significantly varied between the food challenges. This points to a possible change in the transcription level of the *gtfB* and *gtfI* gene that encode for glucosyltransferase (a protein) that catalyse the production of glucans. The significant differences shown between test bacteria exposed to the same food challenge implied varying degrees of virulence in ECP production. In the clinical context, this means that plaque formation and bacterial adherence to tooth surfaces is determined by the food type and the mutans streptococci strain present.

11.3 Aim

The object of this investigation was to determine:

1.1 The effect of traditional African foods on expression of the *gtfB*, *gtfI* and *gtfA* genes of mutans streptococci reference strains and clinical isolates.

1.2 The difference in gene expression between mutans streptococci reference strains and clinical isolates.

11.4 Materials and methods

11.4.1 Sampling of mutans streptococci test bacteria

Twenty millilitre samples of bacterial culture were sterile sampled at six and eight hours during the batch fermentation of each test strain in each food challenge (Chapter 8). The bacterial cells were pelleted by gentle centrifugation at 1000 × *g* for 10 minutes in 50 mL centrifuge tubes (Elkay, Basingstoke, England), and the culture supernatant discarded. The bacterial cells were frozen and stored at -20°C until analysed.

11.4.2 Verification of virulence genes

Prior to the analysis of gene transcripts, the presence of the *gtfB, gtfI* and *gtfA* genes encoding for glucosyltransferase was verified in chromosomal DNA by PCR. DNA was extracted from the test bacteria as previously described in Chapter 4, from 8 hour cultures that were raised in Brain Heart Infusion broth containing 3% sucrose. The 16S *rRNA* gene was used as a experimental control.

11.4.3 Extraction of RNA

The cells were washed with 10 mL STE (Appendix III), pelleted and the cell walls pre-treated with lysozyme (15 mg/mL) (Boehringer Mannheim, GmbH, Germany) in 10× TE buffer (Appendix III) at 37°C for 30 minutes. Total RNA was prepared by cellular extraction using the hot phenol method (320).

Protein was removed by incubation in PK buffer (Appendix II) with 200 µg/mL of proteinase K for 30 minutes. For RNA extraction, one millilitre of 10× RNA lysis solution (Appendix II) was added to the mixture, followed by 1 mL of acid phenol (Sigma, St. Louis, USA) that was pre-warmed to 65°C. The tubes were inverted several times and incubated for 10 minutes in a water-bath, and centrifuged at 10 000 × *g* for 5 minutes at 4°C. The upper phase was transferred to a new tube containing hot phenol, re-incubated and centrifuged as before. The upper phase was transferred to a mixture of phenol: chloroform: isoamyl alcohol (chloropan) (Appendix II), vortexed, centrifuged, and then repeated in chloroform: isoamyl alcohol. The RNA was then precipitated in 0.3 M sodium acetate and alcohol at -20°C for two hours and the RNA finally treated with DNase (321). The pellet was resuspended in 100µL of DNase buffer (Appendix III).Ten units of DNase I (Roche Diagnostics GmbH, Mannheim, Germany) was added and the

mixture incubated at room temperature for 15 minutes. The chloropan and chloroform: isoamyl alcohol extraction was repeated, and the RNA collected by ethanol precipitation. The supernatant was removed and the pellet was washed with 1 mL, 80 % ethanol to remove salts and trace amounts of organic solvents present in the RNA sample. The RNA sample was air-dried for 15 minutes. The dried pellet was suspended in 300 µL of sterile water and stored at -20°C for short-term storage.

11.4.4 Quantification and integrity of RNA

The concentration of RNA was determined by measuring absorbance on an Ultrospec III spectrophotometer (Pharmacia LKB Biochrom Ltd, Cambridge, England). The samples were diluted in 10 mM Tris-Cl buffer (pH 7.5), and the ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provided an estimate of the purity of RNA, which was set between 1.9 and 2.1. The integrity and size distribution of total RNA was checked by denaturing agarose gel electrophoresis and ethidium bromide staining prior to analysis (322).

11.4.5 Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR analysis was used to identify transcripts of the *gtfB, gtfI* and *gtfA* genes of the mutans streptococci reference and clinical test strains. The gene specific oligonucleotide primers used for PCR-RFLP of bacterial DNA, were used in this study and have been presented in Chapter 4 (Table 4.1). Similarly, the 16S *rRNA* gene transcript was used as an internal control of RNA quality and a check for amplification efficiency. A mock RT reaction without the RT enzyme was run in parallel with every RT-PCR reaction, to check if contamination with genomic DNA had occurred.

A two-step RT-PCR analysis was selected for reverse transcription and detection of the *gtfB* (4.6 kb), *gtfI* (4.7 kb), and 16S rRNA (1.5 kb) genes. The cDNA template was first synthesized from total RNA by reverse transcription using ImProm-II™ Reverse transcriptase (Promega, Madison, USA) (323). RNA (1 pg-1 µg) was combined with the gene specific primers (0.5 µM) and heated for 5 minutes at 70°C. After quick chilling, the reverse transcription mix (Appendix III) was added to the primer/RNA mix and annealed at 25°C for 5 minutes. First strand synthesis was carried out at 42°C for 60 minutes and inactivated at 70°C for 15 minutes. Analysis was continued by PCR amplification using ExSel High Fidelity DNA polymerase (Southern Cross Biotechnology, Johannesburg, SA). Ten µL of the RT reaction was added to the PCR mix containing 0.2 mM of dATC, dCTP, dGTP and dTTP; 0.6 µM of gene specific primers and 1.5 mM of $MgCl_2$.

The following cycling protocol was used for reverse transcription and amplification of the *gtfB* and *gtfI* gene transcripts: cDNA was denatured at 92°C for 2 minutes, followed by 10 cycles at 92°C for 10 seconds. Primer annealing was set at 50°C for 30 seconds and 68°C for 3 minutes. This cycle was repeated another 20 times with an increase in 20 seconds per cycle during elongation and followed by a final extension at 68°C for 7 minutes. DNA contamination was checked by omitting reverse transcriptase in the negative control reaction. As a check on the performance of the analysis, a positive control was incorporated into each of the assays by inclusion of total RNA extracted from a 24-hour broth culture of *S. mutans* reference NCTC 10449 (Sims) and *S. sobrinus* reference NCTC 12277 (SL-1), raised in Brain Heart Infusion containing 3% sucrose.

The OneStep RT-PCR kit (Qiagen, GmbH) was used to identify transcripts of the 554 bp, *gtfA*

gene. The master mix was prepared according to the manufacturers instruction (Appendix III), and between 1 pg and 2 µg of RNA template was added to the reaction. The cycling programme comprised of reverse transcription at 50°C for 30 minutes, initial PCR activation at 95°C for 15 minutes, and 30 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and elongation at 72°C for 1 minute. Final extension was at 72°C for 10 minutes.

11.4.6 Detection of gene transcripts

Gene transcripts were detected by agarose gel electrophoresis (199). A 0.75% and 2% (w/v) gel for *gtfB*, *gtfI* and *gtfA,* genes respectively, containing ethidium bromide (0.5 µg/mL) was used to resolve the cDNA. Ten µL of the PCR samples were mixed with 3 µL of 6 × gel loading buffer (Appendix III - /blue/orange 6X loading dye). A 100 bp DNA ladder and Lambda DNA/*Eco*R I + *Hin*d III (Promega, Madison, USA) molecular weight markers were used. The samples were loaded into the wells and electrophoresed in Tris -Borate-EDTA (pH 8.0) running buffer (Appendix III) for 1.5 hours at 130V and 30-34 mA. The amplified DNA bands shown in figure 11.2, were visualised with a UV transilluminator (UVP, Inc, CA, USA) and photographed with a Polaroid DS-34 direct screen instant camera (Polaroid Corporation, MA, USA).

11.4.7 Restriction fragment length polymorphisms of transcribed genes

As a verification check on *gtf* gene transcripts, PCR amplified gene transcripts were digested with *Hae*III restriction endonuclease, previously described in Chapter 4, Section 4.4.6, and the patterns compared against genomic DNA *gtf* gene fragments.

11.4.8 Recording and analysis of data

The visible banding of gene transcripts on agarose gels was recorded as positive gene expression, if no bands were detected, the RT-PCR procedure was repeated a second time to eliminate the possibility of false negatives, and indicated as absent. A quantitative study of the genes expressed was not included in this investigation. The Cochran-Mantel-Haenszel Statistics was used to determine the general association between the food challenge and expression of the *gtf* genes, as well as the association between expression of the *gtfB* and *gtfA* genes. Comparisons between restriction fragment-length polymorphisms of the transcribed genes were analysed using the Distance Procedure (SAS - Version 9.1 for Windows, SAS Institute, Cary, NC: USA) and the unweighted-pair group matrix analysis, using arithmetic averages (UPGMA).

11.5 Results

11.5.1 Verification of virulence genes in test strains

The presence of the *gtfB* and *gtfI* genes, encoding the enzymes GTF-I and GTF-SI were verified in *S. mutans* and *S. sobrinus* test bacteria and are presented in Table 11.1. The *gtfA* gene was present in all *S. mutans* test strains, other than clinical isolate 942007(cf). The *gtfB* and *gtfI* codons are unique to *S. mutans* and *S. sobrinus* species, respectively.

Table 11.1 Genes encoding for the enzymes GTF-I and GTF-SI (*gtfB*, *gtfI*), control of sucrose phosphorylase (*gtfA*) and protein synthesis (16S *rRNA*)

Species	*gtfB*	*gtfI*	*gtfA*	16S *rRNA*
Streptococcus mutans				
NCTC 10449 (type)	+	−	+	+
NCTC 10923	+	−	+	+
953012A(c)	+	−	+	+
942037(cf)	+	−	+	+
942007(cf)	+	−	−	+
Streptococcus sobrinus				
NCTC 12277	−	+	−	+
NCTC 10922	−	+	−	+
NCTC 10919	−	+	−	+
NCTC 11061	−	+	−	+
953012B(c)	−	+	−	+
942036(cf)	−	+	−	+

+ = gene expressed; − = gene not expressed

11.5.2 RNA integrity

The hot phenol preparation and treatment with RNase provided sufficient RNA of good quality, necessary for RT-PCR analysis for most of the samples treated. A check on the integrity of isolated RNA on denaturing agarose gels, showed the presence of intact 16S and 23S *rRNA* ribosomal RNA bands (Figure 11.1).

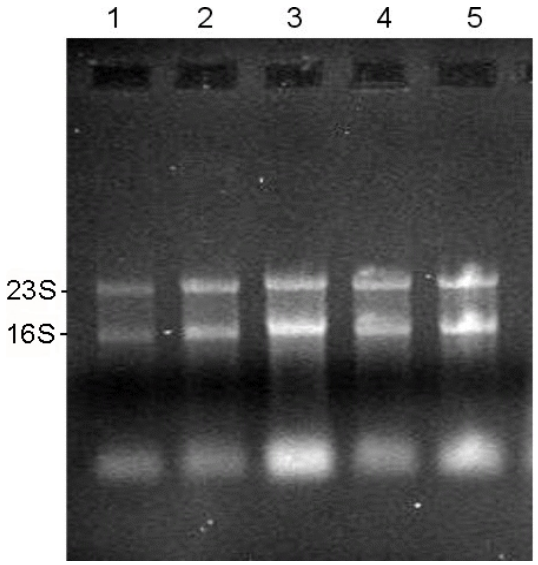

Figure 11.1 Total RNA extracted from mutans streptococci strains on formaldehyde agarose gel. Lane 1 = Sims, Lane 2 = LM-7, Lane 3 = 953012A (c), Lane 4 = 942036(cf), Lane 5 = 942007(cf).

11.5.3 Expression of gene transcripts in food challenges

Expression of the *gtfB* and *gtfI* genes was shown as banding at 4.6 and 4.7 kb, respectively after PCR of the gene transcripts (Figure 11.2).

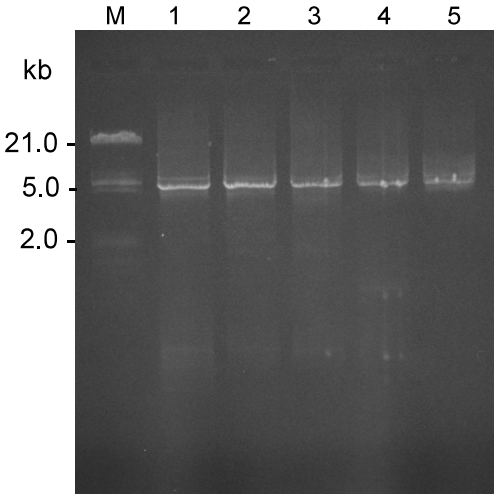

Figure 11.2 *GtfI* gene transcripts (4.7 kb) of *S. sobrinus* strains synthesized from total RNA after RT-PCR, following a 3% sucrose challenge. Lane M = Mwm, Lane 2 = OMZ-176, Lane 3 = Z/AHT, Lane 4 = OMZ-65, Lane 5 = 953012B (c).

11.5.3.1 Expression of the *gtfB* and *gtfA* genes in *Streptococcus mutans*

The influence of the food challenges on *gtf* gene expression by *S. mutans* test bacteria are shown in Table 11.2. Between 80%-100% of the bacteria expressed the *gtfB* gene in maize+milk+sugar, samp+beans, bread+margarine+peanut butter and BHI+3% sucrose. However, after a 3% sucrose challenge, NCTC references - Sims and LM-7, were the only bacteria that expressed the *gtfB* gene. Exposure of clinical strains to maize, samp and brown bread on *gtfB* expression was mainly inhibitory. Comparison between *S. mutans* clinical strains showed that *gtfB* expression in isolate 942037(cf), was inhibited by all foods excepting for BHI+3% sucrose (1/9).

Apart from isolate 942007 (cf), the *gtfA* gene was transcribed in all test bacteria in eight of the nine food challenges (89%) investigated. The basal medium containing 3% sucrose inhibited transcription of both *gtfB* and *gtfA* gene in *S. mutans* clinical isolates.

Table 11.2 Expression of *gtfB* and *gtfA* genes by *S. mutans* reference (NCTC) and clinical strains after exposure to traditional African foods

	Sims (10449)		LM-7 (10923)		953012a (c)		942037 (cf)		942007 (cf)		% ratio (food)	
Food	*gtfB*	*gtfA*	*gtfB*	*gtfA*	*gtfB*	*gtfA*	*gtfB*	*gtfA*	*gtfB*	*gtfA*	*gtfB*	*gtfA*
Maize	+	+	+	+	−	+	−	+	+	−	3/5	4/5
Samp	+	+	+	−	−	+	−	+	−	−	2/5	3/5
Brown bread	+	−	+	+	−	+	−	+	−	−	2/5	3/5
3% Sucrose	+	+	+	+	−	−	−	−	−	−	2/5	2/5
M+M+S	+	+	+	+	+	+	−	+	+	−	4/5	4/5
M+G	+	+	+	+	+	+	−	+	−	−	3/5	4/5
S+B	+	+	+	+	+	+	−	+	+	−	4/5	4/5
B+M+PB	+	+	+	+	+	+	−	+	+	−	4/5	4/5
BHI+3% S	+	+	+	+	+	+	+	+	+	−	5/5	4/5
% ratio (strain)	9/9	8/9	9/9	8/9	5/9	8/9	1/9	8/9	5/9	0/9		

M+M+M = maize+milk+sugar, M+G = maize+gravy, S+B = samp+beans B+M+PB = brown bread+margarine+peanut butter, BHI+3% S = Brain Heart Infusion+3% sucrose. + = gene expressed; − = gene not expressed.

11.5.3.2 Expression of the *gtfI* gene in *Streptococcus sobrinus*

The *gtfI* gene was transcribed by all *S. sobrinus* test bacteria (6/6) challenged to 3% sucrose and BHI+3% sucrose (Table 11.3). Samp and maize+milk+sugar were foods that were most inhibitory to the *gtfI* gene.Comparisons between reference and clinical strains showed that reference bacteria SL-1 and OMZ-176 expressed the *gtfI* gene in 78% (7/9) and 89% (8/9) of the foods, respectively, whereas OMZ-65 and clinical isolate 942036(cf) were least able to express this gene in any of the staple or food-mixtures.

Table 11.3 Expression of *gtfI* genes by *S. sobrinus* reference (NCTC) and clinical strains after exposure to traditional African foods

Food	SL-1 (12277)	OMZ-176 (10922)	Z/AHT (10919)	OMZ-65 (11061)	953012B (c)	942036 (cf)	% ratio (food)
Maize	+	+	+	−	+	−	4/6
Samp	−	+	−	−	−	−	1/6
Brown bread	−	+	+	−	+	−	3/6
3% Sucrose	+	+	+	+	+	+	6/6
M+M+S	+	−	−	−	−	−	1/6
M+G	+	+	+	−	−	−	3/6
S+B	+	+	−	−	−	−	2/6
B+M+PB	+	+	−	−	−	−	2/6
BHI+3% S	+	+	+	+	+	+	6/6
% ratio (strain)	7/9	8/9	5/9	2/9	4/9	2/9	

M+M+M = maize+milk+sugar, M+G = maize+gravy, S+B = samp+beans B+M+PB = brown bread+margarine+peanut butter, BHI+3% S = Brain Heart Infusion+3% sucrose. + = gene expressed; − = gene not expressed.

11.5.4 Influence of food challenge and phenotype on *gtf* gene transcription

The Cochran-Mantel-Haenszel Statistics showed no statistically significant association between the food challenge and expression of the *gtf* gene, or between test strains. However, a significant association was indicated between *gtfB* and *gtfA* gene transcription and the food challenge ($P<0.0001$). A statistical significant correlation was also shown between phenotype, *gtf* gene expression and the food challenge ($P<0.0001$).

11.5.6 Restriction fragment-length polymorphisms of gene transcripts

Fingerprints of some *gtfI* gene transcripts produced by *Hae*III enzyme restriction, are shown

in Figure 11.3. A dendogram generated by the cluster analysis (UPGMA) of fingerprints from *gtfB* and *gtfI* gene transcripts is shown in Figure 11.4. One major cluster (cluster A) was evident in both mutans streptococci species. Amplitypes from *gtfB* gene transcripts (*S. mutans*) in cluster A was 87% (r^2=0.87) similar to each other, but *gtf*I gene transcripts (*S. sobrinus*)was similar by 50% (r^2=0.50). Overall, the similarity between amplitypes of *gtfB* transcripts was higher (69%) than *gtfI* gene transcripts (30%).

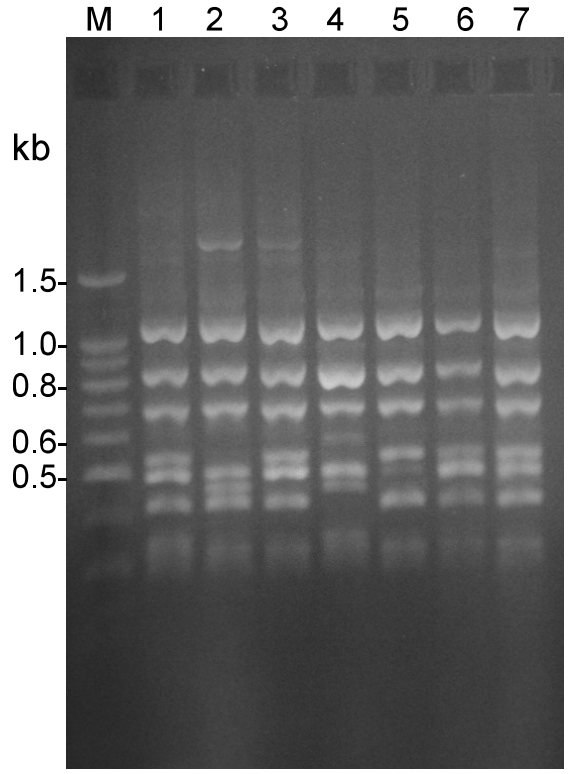

Figure 11.3 *Hae*III enzyme digests of *gtfI* gene transcripts from *S. sobrinus* reference and clinical strains grown in BHI+3% sucrose (Lanes 1-5) and 3% sucrose (Lanes 6-7). Lane M = molecular weight marker, Lane 1 = SL-1, Lane 2 = OMZ-176, Lane 3 = Z/AHT, Lane 4 = OMZ-65, Lane 5 = 953012B(c), Lane 6 = SL-1, Lane 7 = 953012B(c).

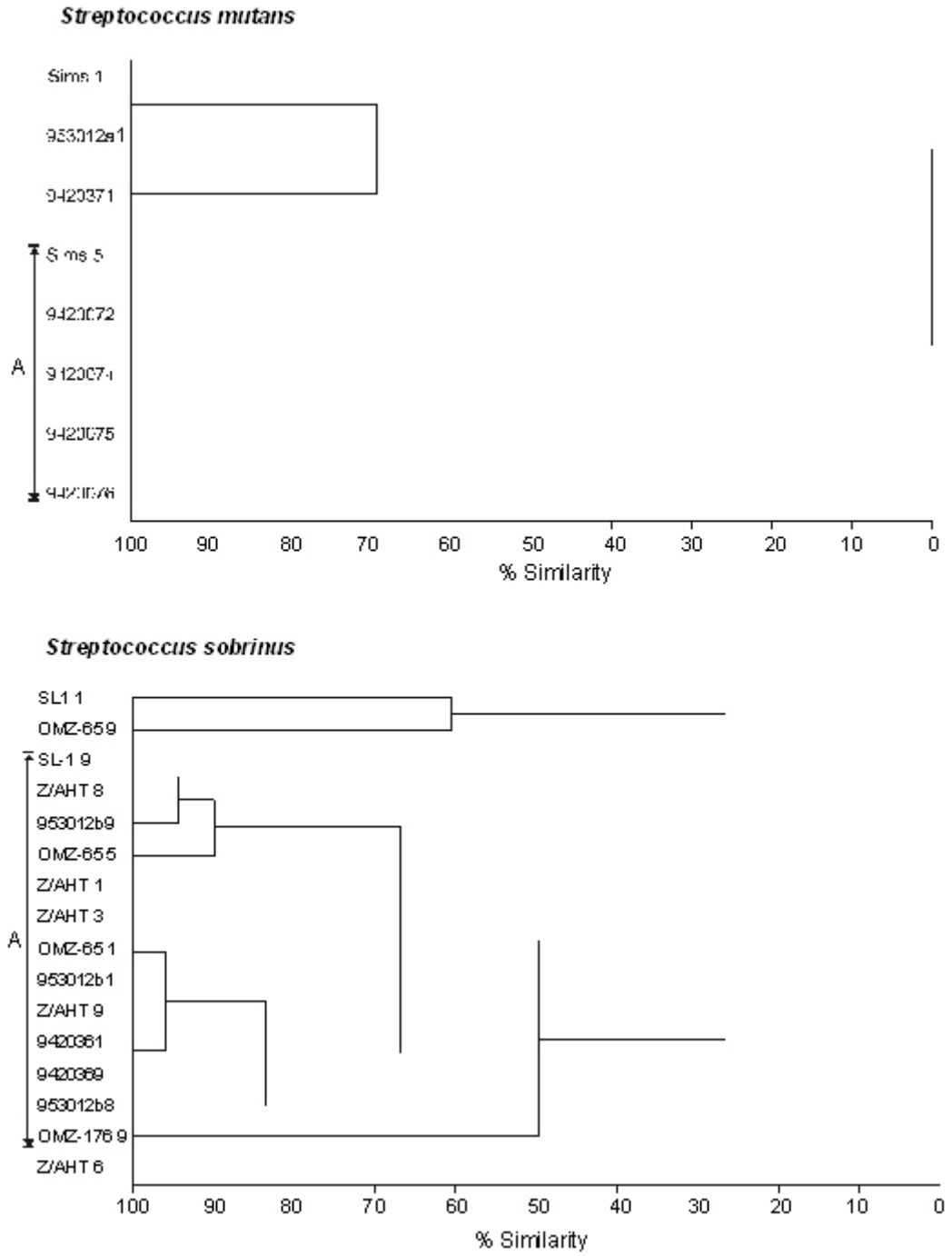

Figure 11.4 Dendogram of PCR-RFLP (*Hae*III) of *gtfB* and *gtfI* gene transcripts from *S. mutans* and *S. sobrinus* clinical and reference strains exposed to food challenges. The last numeral of each case number specifies the food-type: 1 = BHI+3% sucrose, 2 = maize+milk+sugar, 3 = maize+gravy, 4 = samp+beans, 5 = bread+margarine+peanut butter, 6 = maize, 7 = samp, 8 = brown bread, 9 = 3% sucrose.

11.5.6 *Gtf* gene expression and water-insoluble ECP production

The relationship between the quantity of water-insoluble polysaccharide formed and *gtfB* and *gtfI* gene transcription, by food challenge is shown in Figures 11.5 and 11.6, respectively. The cumulative quantity of water-insoluble ECP produced by *S. mutans* test bacteria that expressed the *gtfB* gene was high in all test foods, excepting in bread, compared with the test microorganisms where *gtfB* gene transcription was undetected.

Transcription of the *gtfI* gene by *S. sobrinus* test bacteria challenged to brown bread, 3% sucrose and BHI+3% sucrose showed high water-insoluble ECP production, compared to the remaining foods. Yet, a large amount of glucan was also produced in bread and bread+margarine+peanut butter by reference and clinical strains where expression of the *gtfI* gene was not detected in either food.

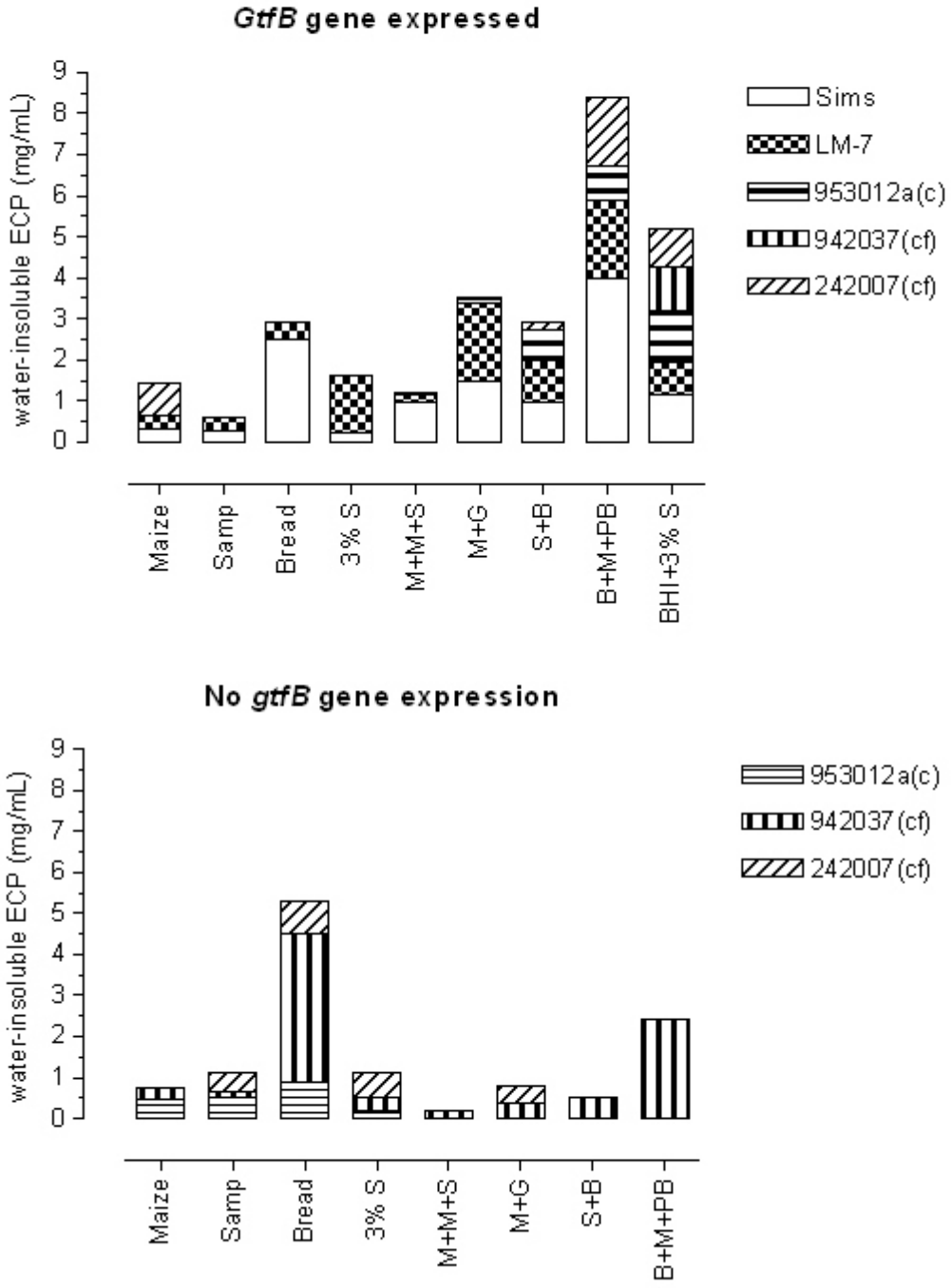

Figure 11.5 Mean cumulative concentration of water-insoluble ECP produced, and *gtfB* gene expression by *S. mutans* test bacteria at 8 hours of batch culture. 3% S = 3% sucrose, M+M+S = maize+milk+sugar, M+G = maize+gravy, S+B = samp+beans, B+M+PB = bread+margarine+peanut butter.

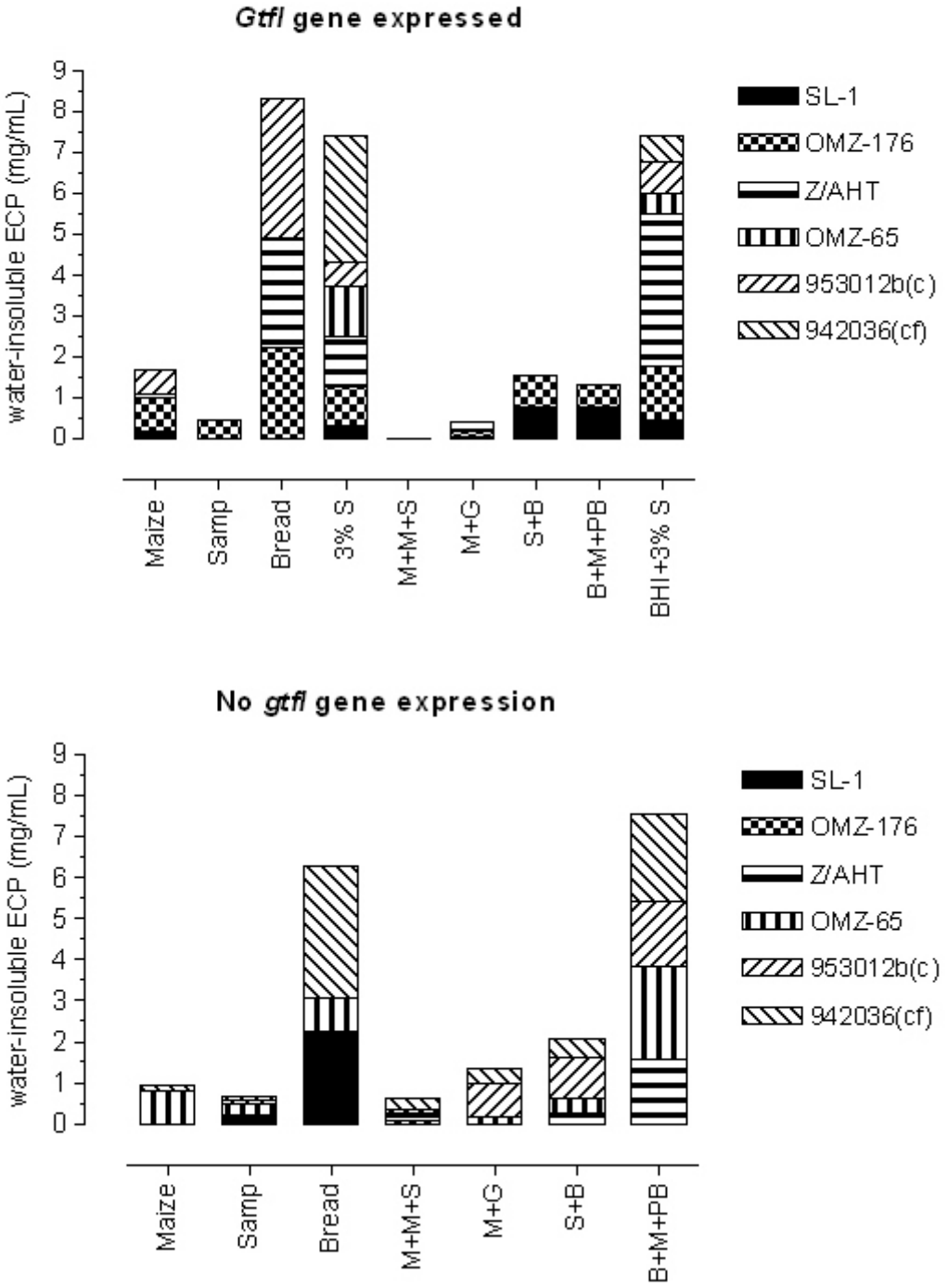

Figure 11.6 Mean cumulative concentration of water-insoluble ECP produced, and *gtfI* gene expression by *S. sobrinus* test bacteria at 8 hours of batch culture. 3% S = 3% sucrose, M+M+S = maize+milk+sugar, M+G = maize+gravy, S+B = samp+beans, B+M+PB = bread+margarine+peanut butter.

11.6 Discussion

The RT-PCR method was proven effective in showing the effect of traditional African foods on the transcription of glucosyltransferase genes. Preliminary experiments to optimise the RT-PCR method indicated that the "two-step" RT-PCR system provided more sensitive detection of RNA molecules and amplification of RT-PCR products with lengths of more than 1 kb.

11.6.1 Effect of traditional African foods on gene expression

As expected, the *gtfB* gene was expressed by all test bacteria in BHI+5% sucrose that provided all nutrients necessary for cell function. However, the basal synthetic medium containing 3% sucrose did not support either *gtfB* or *gtfA* gene expression by *S. mutans* clinical strains. Results showed by Fujiwara *et al* (354) indicated that sucrose reduced *gtfB* and *gtfC* mRNA expression of *S. mutans* MT8148, suggesting the presence of independent promoters. However, according to Hudson and Curtiss (193), expression of the *gtfBC* operon is increased by the presence of sucrose and is followed by a rapid decline in expression over time. In this study, bacteria were examined for gene expression after eight hours of fermentation and failure to detect gene expression in the isolates could be attributed to a rapid decline in expression. This could explain the water-insoluble glucans present in the culture supernatant where *gtfB* and *gtfI* gene expression was undetected at 8 hours. Hence, future studies should incorporate time-interval analysis of gene expression. Alternatively, the clinical isolates required additional growth factors other than sucrose, that were not available in the basal synthetic medium (Appendix III).

This study examined bacteria in a planktonic state with continuous stirring, which did not

allow easy settling of the bacterial cells onto the surface of the culture flask. Expression of the *gtfB* gene is increased in *S. mutans* cells bound to acquired tooth pellicles, and in the natural state, clinical test strains are components of plaque or saliva. The prevention of cell adherence and the quantity and kind of nutrient available can influence the state of the cell indirectly through alteration of the characteristics of the environment (233). Goodman and Gao (192) have found that *gtf* expression occurs at specific growth phases and have suggested a possible cell-density-dependent component regulating *gtfBC*, raising the possibility that intercellular signalling (quorum sensing) may be involved in regulating *gtf* expression.

Verification of the *gtfA* gene showed this gene was absent in clinical strain 942007(cf) (Table 11.1) and prior investigations also identified this bacterium as a melibiose-negative phenotype (Table 6.1). However, the *gtfB* gene was expressed during exposure of this isolate to maize, maize+milk+sugar, maize+gravy and bread+margarine+peanut butter. This suggests that loss of the *gtfA* gene does not necessarily affect *gtfB* gene transcription or the metabolism of nutrients from traditional African foods. However, it does indicate that loss of the *gtfA* gene is not detrimental to cell function.

The variation in *gtfB* expression by *S. mutans* clinical isolates in response to different food challenges, indicates that differences at the DNA gene expression level exist within the same species. Chia and co-workers (191), showed genetic variations in the *gtfB* gene in *S. mutans* serotype c, reflecting a complexity in genes coding for the glucosyltransferases. Furthermore, differences in phenotype within the same strain should be considered since this can serve as an initial indication and reason for inconsistent reaction to various carbohydrates. This study

compares well with a study by Li and Burne (306), where the type and amount of carbohydrate influence the transcription of the extracellular polysaccharide mechanisms of *S. mutans*.

The transcription *gtfI* mRNA by *S. sobrinus* test bacteria in the presence of sucrose (3% sucrose, BHI+3% sucrose) implies that this species has a preference for sucrose as a carbon source, that enhances its capacity to produce water-insoluble glucans. The percentage of *gtfI* gene expression ranged between 22% in clinical isolates [942036(cf)] to 89% in reference strains [OMZ-176] suggesting that wild-type strains are less likely to produce glucans from these foods, especially if the sucrose content is low. This classically illustrates that provided easily assimilated growth substrates are available, *S. sobrinus* species does not expend energy synthesizing the enzymes for a less efficient pathway.

11.6.2 RFLP of transcribed *gtf* genes

Polymorphisms produced by *HaeIII* enzyme digests of the amplified gene transcripts from *S. mutans* and *S. sobrinus* test bacteria were evident for some of the test bacteria (eg. Sims, OMZ-65, 953012b). These differences were shown between transcripts of the same strain exposed to different food challenges, suggesting that adaptive genetic changes had occurred. The difference between profile patterns of *S. mutans* and *S. sobrinus* is consistent with the fact that evolutionary, *S. mutans* (G+C content, 36%-38%) is distant from *S. sobrinus* (G+C content, 44%-46%) (324).

11.7 Conclusion

Expression of the *gtfI* gene in *S. sobrinus* references and clinical strains were enhanced by the

sucrose component of the food challenges. This suggests *S. sobrinus* strains are more virulent in the presence of sucrose, facilitating in biofilm formation and hence, the formation of dental plaque. In comparison, expression of the *gtfB* gene in *S. mutans* clinical strains are induced by other nutrients besides sucrose, compared to laboratory references. This suggests that *S. mutans* are more able to express pathogenicity under environmental conditions where nutrients other than sucrose are available.

The effect of maize and samp and its food-mixtures on clinical isolates was mainly inhibitory on *gtf* expression, but appears to be strain dependent. Compared with *S. mutans* clinical isolates, the *gtfB* gene was not inhibited in *S. mutans* reference strains exposed to all food challenges. This shows a higher predisposition in expressing virulence properties by laboratory references compared to wild-type strains. The loss in virulence expression by clinical isolates points to a natural adaptation, through constant exposure to a specific nutrient environment. Hence, this preliminary investigation showed that the ability of mutans streptococcus strains to grow as plaque biofilms are affected by an attenuation in virulence, brought about by nutrients in the traditional African diet that can be considered factors that control *gtf* gene expression.

CHAPTER 12

GENERAL DISCUSSION AND CONCLUSIONS

12.1 Caries status and the study population

The focus of this study was on children from two ethnic groups that comprise the majority of the South African population that are considered to be at risk to dental caries. Equal numbers of children were randomly selected from the two communities, but the percentage prevalence of coloured children with active-caries (84%) was higher compared to black Africans (61%) indicating that the coloured children in the study sample are more predisposed to dental caries. Reasons for the higher rate of caries in the coloured children studied could be attributed partly to the high numbers of mutans streptococci found, where a low yet significant association (7%) with dmfs was shown. However, it must be borne in mind that other variables such as the host-immune response, salivary flow rate and clearance, frequency of eating, and socioeconomic factors, to name a few, does influence the course of dental caries.

A disadvantage to this study was that the number of caries-free children investigated was small and because more clinical isolates were from children with untreated caries, results were skewed. In future, comparative studies between caries groups should comprise equal numbers of children with and without dental caries.

12.2 Mutans streptococci phenotype and the *gtfA* gene

Seventy-four percent (114/155) of clinical isolates that were isolated from both caries groups were biochemically similar to *Streptococcus mutans* reference strains. Twenty-six percent

(41/155) were 'atypical' because they did not ferment either melibiose or raffinose. Failure to detect the *gtfA* gene suggested loss or absence of this gene in 26 of the 41 isolates from both caries groups. Some biochemical profiles of melibiose-negative strains were similar to *S. sobrinus* and were clustered close to this species on the phylogenetic tree, but PCR amplification of the *gtfB* codon confirmed that these isolates were *S. mutans*. A lack of the enzyme ∝-galactosidase produces this inability to ferment melibiose or raffinose, but enzymic tests were not incorporated into the battery of biochemical tests in this study. The inclusion of ∝-glucosidase, β-glucosidase and ∝-galactosidase enzyme activity would have provided a more comprehensive series of tests, that would have indicated some genetic relationship to local deletion or polar mutations in concurrent loss of enzyme activity.

The prevalence of *S. sobrinus* was low (10%) (17/172) compared to *S. mutans* strains, and these bacteria were mainly harboured by caries-active children. However, *S. sobrinus* was not significantly associated with caries and did not play a major role in dental caries in this investigation. A study by Becker *et al* (147) showed similar results. They detected *S. sobrinus* in only 9 of 30 subjects and the same conclusion was drawn. The frequent observation of melibiose-negative *S. mutans* strains in the sample population suggests that melibiose-negative strains have no ecological advantage and they co-exist with melibiose-positive strains in the mouth (161). The distribution of melibose-negative phenotypes in caries-free and caries-active children shows that these strains are not specific to caries and cannot be considered to be more virulent than 'typical' *S. mutans* strains. Melibiose-negative strains were also not limited to any one of the ethnic populations studied, and the source of these bacteria was in some cases from the mother.

12.3 *Gtf* gene diversity of mutans streptococci from children

Comparisons between *gtfB* and *gtfI* amplitype by caries and ethnic groups showed no difference between population or caries. However, a low number of caries-free, coloured children were investigated and results could have been influenced by the small sample group that may be not a true reflection of this population. Coykendall (324) reported genetic heterogeneity in *S. mutans*. Similarly, the number of distinct amplitypes detected by *gtfB* (n=20) and *gtfI* (n=11) gene polymorphisms in *S. mutans* and *S. sobrinus* test bacteria, respectively indicates the high oral colonization by strains of distinct virulence. The gene encoding for glucosyltransferase controls the production of extracellular glucans, and genetic diversity suggests a variation in virulence between *S. mutans* strains (325). However, the high prevalence of dental caries in the coloured population observed in this study could not be explained by *gtf* gene diversity, since similar genetic variation was shown in *S. mutans* isolates from black African children. However, factors that control the transcription and translation of the *gtf* codons appear to be more important in contributing towards plaque formation than mutans streptococci genotype (194).

Data on the genetic diversity of *S. sobrinus* are scant and reason for this could be that relatively small numbers of these microorganisms are isolated from caries-active individuals. In my study, *S. sobrinus* were present in 32% (10/31) of children. Yet, only 23% (7/31) of the mothers were colonized by *S. sobrinus.* In contrast, Klein and co-workers (197) found *S. sobrinus* in 75% of children investigated and 37.5% of their mothers harboured this bacterium. Differentiation of *S. sobrinus* genotypes by the investigation of *gtfI* gene polymorphisms has not been studied yet in different ethnic populations. Eleven different amplitypes of the *gtfI* gene were shown in black African and coloured 5-year-old children, whereas 16 distinct

genotypes from AP-PCR analysis, were detected in Brazilian nursery school children at time of acquisition (197).

12.4 Inter-familial transmission of MS between mothers and their children

The percentage match in *gtfB* amplitype from *S. mutans* isolates between children and their mothers ranged from 38% (caries-free) to 45% (caries-active). This means that 55% of amplitypes were acquired from alternative sources or some strains express virulence-associated characteristics that promote their colonization and survival (326, 327).The acquisition of *S. mutans* genotypes by children from unrelated sources such as play-groups and nursery schools are possible, but the literature reports a low number of genotypes that were shared between children at nursery schools (87, 93, 197). This current study did not investigate fathers or siblings and acquisition of *gtf* amplitypes from these sources is purely speculative. However, new genotypes and a gain-loss pattern of mutans streptococci strains were reported in children and their mothers over periods of 7 years (328) and 20 months (197), which may partly explain the transience of mutans streptococci genotypes. In South African black African and coloured child populations, the percentage of mutans streptococci genotypes acquired from mothers is influenced by cultural habits and has been discussed in detail in Chapter 5.

Only two children with active-caries and their mothers had matching *gtfI* amplitypes, with the remaining strains transitory (mothers: n=4; children: n=8). Unlike *S. mutans,* this implies that *S. sobrinus* strains are not frequently transmitted from mothers to their children although this species is seldom found without *S. mutans* (59). The period of colonization by this microorganism and the source of transmission is unclear in the literature, but it can be speculated that dominance by *S. mutans* over *S. sobrinus* is dependent on the available

carbohydrate and resting pH of the oral environment.

12.5 PCR mediated typing and *Hae*III vs *Hin*fI enzyme restriction

Over the past decade, genomic fingerprinting of microorganisms by PCR amplification of sites comprising repetitive elements which may occur in varying numbers and at different positions of the genome, has been employed to investigate the relatedness of bacteria (329). Compared to traditional methodologies, PCR is specific, sensitive and fast. Yet, the robustness of PCR based fingerprinting is limited by the method of DNA preparation, how differences between profiles were scored and which similarity coefficient is used for profile analysis.

The correlation values between clusters of the same *gtfB* amplitypes produced by *Hae*III and *Hin*fI enzyme restriction was not significantly different (Fisher's Exact Test) from each other. According to Colby *et al* (156), a range of restriction enzymes is required to clarify if any two isolates are indeed identical since a restriction enzyme may allow a particular grouping of strains whereas another enzyme may yield entirely different grouping (330). Characterization of mutans streptococci clinical strains by PCR-RFLP has the advantage of targeting specific virulence genes as an alternative to whole DNA genotyping. However, biases can be introduced during cell lysis, DNA extraction and PCR amplification. These were reduced by the incorporation of experimental controls. Most importantly, RFLP analysis of the *gtf* genes showed that similar phenotypes were genetically different, and at the level of gene expression, the cariogenic potential between mutans streptococcus clinical strains is likely to differ.

12.6 Melibiose-negative phenotype and *gtfB* gene diversity

Dendograms generated by UPGMA showed that the distribution of melibiose-negative

phenotypes were not specific to a caries or an ethnic population, neither did they all share the same *gtfB* fingerprint. Melibiose-negative strains from nine children and their mothers, shared the same *gtfB* amplitype indicating common origin. The variation in *gtfB* amplitype shown in melibiose-negative phenotypes in this study, is in contrast to Colby *et al* (156) who reported similar ribotype patterns in melibiose-negative isolates. The evolution and genetic mechanism for generation of these atypical *S. mutans* strains remains unclear and speculation is that loss of function of the *msm* gene and the capacity to utilize the range of sugars predominantly of plant origin, are an ancient relic from a free-living ancestor of streptococci, with no selective advantage in the human mouth (161).

12.7 Mutans streptococci virulence, dental caries and ethnic group

Repetitive DNA elements in the *gtfB* and *gtfI* genes of *S. mutans* and *S. sobrinus* have shown that these elements are associated with DNA polymporphisms in the chromosome. This has provided epidemiological information such as extent of transmission - especially between mothers and their children; source and reservoir in a South African community and level of pathogenicity of these microorganisms, indicated by the variant mutans streptococci clinical strains prevalent in South African children and mothers. However, it was difficult to determine if these gene polymorphisms had arisen over a period of time, or if these strains had been introduced through exposure to other infected sources. Disease isolates are often diverse and many genotypes are only recovered once, whereas comparison with closely related lineages not associated with dental caries may be of more value.

Fujiwara *et al* (203) demonstrated a shift in virulence among clinical strains of *S. mutans* where inter-strain difference of the *gtfB* was limited, but *gtfC* showed significant inter-strain

variations. A decreased in glucan binding suggested that *gtfC* is critical in sucrose-dependent adherence (204, 205). In my study, a shift in virulence was evident, shown by variation in amplitype of the *gtfB* gene.

12.8 Mutans streptococci response to traditional African foods

12.8.1 The role of traditional African foods in growth and acid production

Constant exposure to a human diet has made *S. mutans* especially versatile in its utilization of a variety of carbohydrates, ranging from monosaccharides (glucose, galactose and fructose), disaccharides (maltose, lactose and sucrose), and trisaccharides (raffinose) to high-molecular-weight dextran, fructan and starch (331). In this study, maize and samp and their food mixtures (maize+gravy, maize+milk+sugar, samp+beans) did not encourage logarithmic growth of the test bacteria (Figure 8.2) and bacterial stasis was observed. Low concentrations of lactic acid were produced (Table 8.9) and pH did not decrease to below 6.0. However, on close examination of the individual test strains, the cellular division of *S. mutans* (Sims -NCTC 10449) was low and growth of *S. sobrinus* (SL-1 -NCTC 12277) reference strain was high in maize and samp suggesting a more virulent bacterium. As expected, vigorous bacterial growth was apparent in BHI+3% sucrose, 3% sucrose and bread+margarine+peanut butter with low mean pH values. Most lactic acid was produced in the control medium, but because of the pH buffers incorporated, the overall pH did not fall to below 5.7, the 'critical' level for enamel demineralization. Comparisons between clinical strains, in response to the food challenges, showed that isolate 942007(cf) (*S. mutans*), a melibiose-negative phenotype, and isolate 953012B(c), an *S. sobrinus* wild-type, responded more favourably to the food challenges in growth rate and acid production. This suggests a more virulent genotype, and confirms the

hypothesis that loss of the *msm* operon in melibiose-negative phenotypes does not impair bacterial cell function. It has been suggested that in an animal model, *S. sobrinus* could be more acidogenic than the other species of mutans streptococci (332).

The growth stasis and entry into stationary phase of test bacteria exposed to maize and samp is probably a stress response produced by nutrient starvation. In this case, a lack of essential elements required for energy and normal cell-function occurred. However, after 16 hours of batch culture these bacteria were viable and were still able to survive the limited carbohydrate (sugar) condition. Cell survival of *S. mutans*, which is manifested as a stress response to nutrient starvation has been attributed to the induction of starvation-specific proteins (333) that is regulated by individual nutrient components (334). In the mutans streptococci stress proteins are induced mainly by sugar starvation responses where alternative carbon sources are utilized. This stress response to glucose-starvation also appears common to *Escherichia coli* and *Bacillus subtilis* where general stress proteins (GSP) are up-regulated (335, 336). According to Titgemeyer and Hillen (337), bacteria use carbon sources in a strictly controlled hierarchical manner by sophisticated mechanisms that enable them to sense the nutritional situation, and adjust their catabolic capacities by regulatory responses that fall under the term carbon catabolite repression.

Bread and bread+margarine+peanut butter were the only foods where a low pH and high cell density resulted (Figure 8.3). Not only are these foods caries-promoting, but the clinical implication is that the mutans streptococci are capable of rapid plaque acidification following the ingestion of refined carbohydrates other than pure sucrose. This acid-tolerant growth can be ascribed to at least three, up-regulated stress-related proteins in *S. mutans*: the transcription

elongation factor, GreA; the ATPase protease, ClpL; and the single-stranded DNA-binding protein, Ssb (338).The investigation of stress induced proteins was beyond the scope of this study, but lays ground for further research into different carbohydrate sources, and mutans streptococci protein induction.

12.8.2 The role of traditional African foods in ECP production, protein concentration and glucose metabolism

The low concentration of water-insoluble ECP produced in maize, samp and maize+gravy implies that these foods do little to enhance dental plaque formation. Brain Heart Infusion containing 3% sucrose, brown bread and bread+margarine+peanut butter promoted ECP formation and hence, plaque formation, shown by the clumping and adherence of bacterial cells to the fermentation flask. The production of ECP by mutans streptococci favours the presence of sucrose and refined carbohydrates compared with starches. In hydrolysed starches, the released monosaccharides are first used for normal cell function before glucan synthesis. This may explain the low levels of residual glucose and water-insoluble ECP. However, an increase in ECP was significantly associated by a decrease in extracellular protein.

The differences in response to the food challenges between clinical isolates and laboratory reference strains suggest that mutans streptococci clinical strains metabolically adapt to frequent exposure of specific foods in the host's diet. This response is controlled by gene expression and hence, the genetic adaptation to metabolise these nutrients may decrease the virulence properties of these bacteria.

12.9 Inherent food buffering and dental caries development

Foods containing sucrose decrease the inherent buffering effect which cause a rapid drop in pH on exposure to acids produced by bacteria. In comparison, foods with a high protein content take a longer time to drop the pH. Hence, foods such as beans, milk and peanut butter require higher concentrations of acid over a longer period of time before demineralization of dental enamel can occur. However, this effect would depend on the duration of exposure by foods to the tooth surface.

12.10 The role of traditional African foods in *gtf* gene expression

The speculative investigation on the transcription and expression of the *gtfB* gene in *S. mutans* clinical strains compared to NCTC reference strains were dependant on other essential nutrients besides sucrose. However, transcription of the *gtfI* gene in *S. sobrinus* strains were facilitated in the presence of sucrose (3% sucrose; BHI+3% sucrose) in all test bacteria. This suggests that *S. sobrinus* species are more virulent when sucrose is a food component. Regarding *S. mutans* clinical isolates, the *gtfB* gene was not expressed in samp, brown bread or 3% sucrose, and isolate 943027(cf) expressed this gene in BHI+3% sucrose only. The specific m-RNA targets were not measured in this investigation, but a quantitative analysis of bacterial gene expression using real-time RT-PCR has shown that the expression ratio of the *gtfB, gtfC* and *gtfD* genes coincide with the optimal ratio that induces sucrose-dependent cellular adhesion(354). Using real-time RT-PCR will be a powerful tool in determining gene expression changes in mutans streptococci virulence exposed to traditional African foods in future studies.

12.10.1 Association between *gtfB* gene transcription and ECP production

Gene transcription in four, of the five *S. mutans* test strains challenged to samp+beans (0.60 mg/mL), bread+margarine+peanut butter (1.75 mg/mL) and BHI+3% sucrose (1.24 mg/mL), produced water-insoluble ECP. *GtfB* expression in *S. mutans* reference strains (Sims, LM-7) produced ECP in samp, brown bread and 3% sucrose, with concentrations ranging between 0.26-2.13 mg/mL. Although the transcription of *gtfB* genes encoding for glucosyltransferase remained undetected in clinical isolates exposed to these foods, water-insoluble ECP was measured in the culture supernatant (0.13-3.65 mg/mL) over 16 hours of batch culture. *Streptococcus sobrinus* clinical isolates also produced water-insoluble ECPs when challenged to samp, brown bread and 3% sucrose.

Irrespective of RT-PCR test repeats, experimental error cannot be ruled out in this preliminary investigation, since RNA is relatively unstable due to the presence of ribonucleases (Rnases), that break down RNA molecules. Although all recommended precautions were taken during the experimental work, changes can occur during handling of the sample and isolation of the RNA. Two major types of artifacts can develop - down regulation of genes and enzymatic degradation of RNA results in an artificial reduction of both nonspecific and specific mRNA species. Prokaryotic mRNAs are highly unstable with an average half-life of about 3 minutes for fast growing bacteria. Sometime the bacterial mRNA begins to degrade while it is still been translated and since mRNAs are very rapidly turned over in bacteria, gene-expression studies are made even more difficult (322).

12.10.2 Regulation of *gtf* gene expression

The differences in *gtf* gene transcription between the test microorganisms after exposure to

traditional African food challenges appears to be an adaptive response to different dietary carbohydrates. This is important in the context of mutans streptococci pathogenesis and dental caries. It must be kept in mind that the mutans streptococci species are normal oral flora that become pathogenic when the opportunity arises. Current research has found that many species of streptococci have evolved peptide pheromone quorum-sensing systems that probably help them adapt to and survive host-imposed fluctuations in the immediate environment and coincidently regulate the expression of virulence factors that promote their pathogenicity (340). In *S. mutans*, a peptide pheromone quorum-sensing system controls genetic competence and the system functions optimally when the cells are living in actively growing biofilm (339). These quorum-sensing systems are primarily made of small soluble signal peptides that are detected by neighbouring cells via a histidine kinase/response regulator pair that modulate physiologic homeostasis and adaptation to environmental conditions in response to fluctuations in population density (340). Because quorum sensing is density dependant, a larger amount of the auto-inducer is produced when there is a high cellular growth rate that stimulates the bacteria to switch on genes that are involved with pathogenesis. This is illustrated by the high growth rate, the expression of the *gtfB* and *gtfI* genes and water-insoluble ECP production by test bacteria in BHI+3% sucrose, compared to the single food, samp.

12. 11 Principal findings of the study

1. The *gtfB* and *gtfI* genes of *S. mutans* and *S. sobrinus* clinical isolates, encoding for glucosyltransferase is genetically diverse, but are not different between South African, black African or coloured populations or between caries-free and caries-active children.

2. A high *gtfB* gene diversity and the percentage match between *S. mutans* isolates from children and their mothers suggests that mothers serve less as a source of *S. mutans,* and acquisition is from alternative sources.
3. *Streptococcus sobrinus* clinical strains were harboured by 10% of the caries-active sample population, suggesting that this species is often infective in untreated caries, and the emergence of melibiose-negative *S. mutans* phenotypes may be more important in dental caries development.
4. Bacterial test strains were least able to grow, produce acid or form water-insoluble extracellular polysaccharides in maize, samp and their combinations: maize+gravy, maize+milk+sugar and samp+beans suggesting that these traditional African foods are not caries-promoting.
5. Foods with a high protein component are more resistant to a drop in pH produced by acids and are less caries promoting than refined carbohydrates.
6. Gene expression of the *gtfB* and *gtfI* gene is facilitated in the presence of sucrose, but other nutrients beside sucrose control *gtf* gene expression specifically in *S. mutans* clinical strains. Hence, the induction and repression of *gtf* gene expression in *S. mutans* and *S. sobrinus* strains is not only limited by the available carbon source supplied by traditional African dietary nutrients, but is also specific to clinical wild-types.
7. The significant difference in response to the food challenges between clinical isolates and NCTC reference strains suggest that clinical strains genetically adapt to frequent exposure of specific foods eaten by the individual depending on the carbon source, compared to laboratory strains.
8. The traditional African diet can be considered a factor that controls *gtf* gene expression in mutans streptococci, thereby changing its ability to display virulence properties.

12.12 Strengths, weakness and opportunities for future studies

The mutans streptococci, colonize the mouth at infancy as normal commensals but become pathogenic only when environmental conditions become favourable and more virulent clones of *S. mutans* within the host population become dominant. However, the extent of virulence in mutans streptococci bacterial isolates cannot be predetermined, since this study showed that clinical strains responded differently to the environment. The survival ability of these microorganisms is to rapidly adapt to the environment by natural genetic competence, and the ability of these cells to bind to and to take up exogenous DNA might be an important mechanism for the horizontal transfer of genes (341).

Besides the influence of dietary carbohydrate and cariogenic bacteria, dental caries is also influenced by factors such as the host immune system (342-344), salivary buffering capacity and salivary clearance (345-348), socio-economics (349), and the oral hygiene of individuals. These conditions differ between countries and populations caused by dissimilar lifestyles and must be considered during epidemiological investigations. Future microbial studies on caries aetiology should focus on caries-free children where a change in genotype, their acquisition and dominance of new genotypes will serve as indicators during the development of this disease.

In addition to virulence factors investigated in this thesis, other properties of the microorganism may influence virulence. It has been suggested that *S. mutans* and *S. sobrinus* are proteolytic at low levels (350), producing two extracellular metalloproteases capable of degrading both gelatin and collagen-like substances (351). Furthermore, collagen mediates adhesion of *S. mutans* to human dentine and may play a role in the pathogenesis of root surface

caries (352). However, no correlation has been shown between the level of enzyme activity and the collagen loss of dentine (353).

This study is the first to comprehensively report on the genetic variation of *S. mutans* and *S. sobrinus* clinical strains from 5-year-old children in two populations of Gauteng, South Africa. No other investigations have targeted the *gtf* virulence genes encoding for glucosyltransferase, as a means to study mutans streptococcus genetic diversity and prevalence in populations.

Results on the effect of traditional African foods on mutans streptococci clinical isolates compared with laboratory reference strains, suggests that these carbohydrates are able to control *gtfB* and *gtfI* gene expression. However, these are preliminary findings only, and expanding this section of study was beyond the scope of this thesis, but could form a thesis in itself.

The batch culture technique used in this study does not simulate the growth of bacteria in biofilm as it occurs *in vivo*. However, it allowed easy comparison between traditional African staple foods and food-mixtures on specific virulence properties of the mutans streptococci in the planktonic phase. The extracellular proteins, comprising of the enzyme protein glucosyltransferase and glucan binding proteins were not differentiated in this study, and was assumed to comprise either of these protein fractions. Although the presence and activity of glucosyltransferase was verified by *in situ* methods and protein electrophoresis, further investigation by purifying and measuring each of these proteins is necessary to determine the specific role of each protein in cell attachment to the tooth pellicle.

The distribution of variant genotypes within the black African and coloured ethnic populations, coupled to the attenuation in expression of virulence properties during exposure to different dietary nutrients points in the direction of natural genetic transformation. Also, the ability of the cells to bind to and to take up exogenous DNA may be an important mechanism for horizontal gene transfer. Hence, DNA from virulent strains of *S. mutans* or *S. sobrinus* may transform a non-virulent strain to virulence or vice versa. This natural genetic competence has already been reported in four well-studied organisms; *Streptococcus pneumoniae*, *Bacillus subtilis, Neisseria gonorrhoeae* and *Haemophilus influenzae* (339). The development of competence and expression of the uptake machinery is regulated in response to cell-cell signalling and/or nutritional conditions that might also aid in the conversion of mutant alleles to wild-type or functional alleles. The process of transformation of genes, both natural and artificial lays new ground for further investigation into mutans streptococci virulence.

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KEY TO APPENDICES

Obs	Observation
caseno	Case number
relat	Relation
ds	Decayed surfaces
dmfs	Decayed, missing and filled surfaces
mstvc	Mutans streptococci, total viable count
h1-h7	*Hae*III restriction enzyme digest band positions
n1-n7	*Hin*fI restriction enzyme digest band positions
b	Black African
c	Coloured
c	Child
m	Mother
ref	Reference strain
area:	2 = Crosby
	4 = Jeppe
	5 = Kliptown
	8 = Noordgesig
	9 = Parkhurst
	10 = Rosettenville
	11 = Westbury
	24 = Soweto
expt	Experiment: *viz* food challenge:
	1. Brain Heart Infusion + 3% sucrose

2. Maize + milk + sugar
3. Maize + gravy
4. Samp + beans
5. Brown bread + margarine + peanut butter
6. Maize
7. Samp
8. Brown bread
9. BSM + 3% sucrose

tvc	Total viable count
tprot	Total protein
lac	Lactic acid
acet	Acetic acid
form	Formic acid
glu	Glucose
ECP	Extracellular polysaccharide

APPENDIX I

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Toi

CLEARANCE CERTIFICATE **PROTOCOL NUMBER** M02-08-31

PROJECT Genetic Diversity and Influence of Traditional African Foods on the Virulence of Mutaus Streptococci in South African Children

INVESTIGATORS Mrs C Toi

DEPARTMENT Dental Research Institute, Wits Medical School

DATE CONSIDERED 02-08-21

DECISION OF THE COMMITTEE Approved "Bacteria were isolated by Mrs Toi from specimens collected under clearances 35/2/89, 3/11/89, 8/11/89, 42/3/90-studies in which she was

Unles otherwise specified the ethical clearance is valid for 5 years but may be renewed upon application
This ethical clearance will expire on 30 July 2007.

DATE 02-08-22 **CHAIRMAN** .....(Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached where applicable.

c c Supervisor: Prof P Cleaton-Jones
Dept of Dental Research Institute, Wits Medical School
Works2\lain0015\HumEth97.wdb\M 02-08-31
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DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. I agree to a completion of a yearly progress form. I/we agree to inform the Committee once the study is completed.

DATE 27/08/02 SIGNATURE .....

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX II

COMPOSITION OF TRADITIONAL AFRICAN FOODS AND SUPPLIERS OF FOODS USED IN THE STUDIES

1. Staple foods

1. **ACE SUPER MAIZE MEAL** enriched with vitamins (Delmas Milling Co, Randfontein, SA).

   Nutritional information per 400 g meal

Nicotinamide	10 mg 50 % RDA
Riboflavin	1 mg 55 % RDA

2. **INDUNA SAMP** (coarsely ground maize) (Delmas Milling Co, Randfontein, SA)

   Stamp mielies

3. **ALBANY BROWN BREAD** with calcium, sliced (Albany bakeries™, Germiston SA)

Nutritional information:	100 g	2 slices (± 75 g)
Energy	950 KJ	665 KJ
Protein	8.4 g	5.9 g
Total Fat	1.5 g	1.1 g
Carbohydrates	46.5 g	32.5 g
Dietary Fibre	4.7 g	3.3 g
% of RDA for adults and children older than 10 years:		
	2 slices (± 75g)	8 slices (± 300g)
Folic Acid	22 %	88 %
Magnesium	15 %	60 %
Iron	10 %	39 %
Vitamin B1	11 %	43 %
Vitamin B2	5 %	19 %

Vitamin B6	8 %	30 %
Calcium	6 %	24 %

Ingredients: Brown bread flour, water, yeast, salt, sugar, soya flour, vegetable fat, emulsifiers, preservatives (calcium propionate, sodium diacetate), flour improvers..

B. Combination food composition

1. **SPAR FULL CREAM MILK** (Spar SA (Pty) Ltd, Pinetown, SA)

Nutritional information per 250 mL serving.

Energy	663 KJ
Carbohydrates (lactose)	12.1 g
Proteins	7.9 g
Milk fat	8.5 g

Percentage RDA (for persons 4 years and older) per 250 mL portion of Spar Full Cream Milk:

Calcium	38 %
Phosphate	30 %
Vitamin B2	23 %
Proteins	15 %

2. **SELATI WHITE SUGAR** (Transvaal Sugar Ltd, Malelane, SA)

Cane sugar

3. **ALL GOLD TOMATO AND ONION MIX** (Langeberg Food Processor, Bellville, SA)

Ingredients per 410 g can

Tomatoes

Onions

Cane sugar

Corn Starch

Salt

Herbs

Garlic

Spices

4. **SPAR KIDNEY BEANS** (Spar South Africa (Pty) Ltd, Pinetown, SA).

Nutritional value of dry beans. Composition of dry beans (100 g cooked)

Water	70 %
Kilojoules	405,00
Protein	7.10 g
Fat	0.30 g
Carbohydrates	17.10 g
Fibre	5.10 g
Calcium	19.00 mg
Phosphorus	89.00 mg
Iron	1.70 mg
Sodium	16.00 mg
Potassium	400 mg
Magnesium	33.00 mg
Thiamine	0.14 mg
Riboflavin	0.07 mg
Cholesterol	0.00 mg

Source: NRIND Food Composition Tables - Second Edition (1986)

5. **STORK COUNTRY SPREAD MARGARINE (Medium Fat Spread)** (Unifoods (Pty) Ltd, Durban SA)

Nutritional information per serving of	30 g	Per 100 g
Energy	557 KJ	1856 KJ
Total fat	15 g	50 g
Trans fatty acids (max)	0 g	1 g
Sodium (max)	194 mg	648 mg
Vitamin A (21% RDA)	207 µg RE	691 µg RE
Vitamin D (31% RDA)	2 µg	6 µg
Vitamin E (15% RDA)	2 mg ∞TE	5 mg ∞TE

(RDA=recommended daily allowance)

Ingredients:

Vegetable oils and fats

Moisture (47 %), salt

Milk solids

Emulsifiers

Preservative: sodium benzoate

Flavourant

Colourant: beta carotene

Vitamins A, D & E

6. **BLACK CAT CRUNCHY PEANUT BUTTER** (Langeberg Food Processors, Bellville, SA)

Nutritional facts per 100 g

Energy	2570 KJ
Total Oil	51.0 g
Protein	24.4 g
Carbohydrate	16.5 g
Dietary Fibre	5.5 g
Magnesium	157.0 mg
Niacin	31.9 mg
Vitamin E	8.8 g

Serving size 2tbs (32 g) % of RDA serving

Protein	13.9 %
Magnesium	16.7 %
Niacin	56.7 %
Vitamin E	28.0 %

APPENDIX II

RAW DATA I

Bacterial test strain, food challenge (1-9), time interval (hrs), TVC (cfu/mL), total protein (µg/mL), lactic, acetic acid and formic acid (mM/mL), glucose (mg/mL) and water insoluble extracellular polysaccharides (mg/mL).

Obs	strain	expt	tvc	pH	tprot	lac	acet	form	glu	ECP
1	10449	1	600000	7.20	352.69	.	.	.	.	0.566
2	10449	1	1100000	7.00	322.24	.	.	.	0.447	1.002
3	10449	1	18000000	6.95	369.29	.	.	.	.	1.439
4	10449	1	290000000	6.44	356.97	0.00	0.00	0.00	0.492	1.706
5	10449	1	37000000000	5.51	339.17	1.51	3.62	0.38	.	2.102
6	10449	1	370000000000	4.86	356.11	3.64	2.55	0.26	0.000	2.199
7	10449	1	1.4E12	4.61	359.36	3.07	8.50	0.20	.	1.310
8	10449	1	120000000000	4.50	370.83	.	.	.	0.002	1.560
9	10449	2	510000000	7.20	15.11	.	.	.	.	0.768
10	10449	2	22000000	6.37	24.01	.	.	.	0.027	0.711
11	10449	2	28000000	6.02	42.32	0.67	.	.	.	1.148
12	10449	2	56000000	4.78	28.83	1.16	27.58	0.05	0.052	1.229
13	10449	2	15000000	4.26	36.33	1.16	23.91	0.00	.	1.431
14	10449	2	3700000	4.34	28.29	0.77	28.99	0.00	0.021	0.913
15	10449	2	2200000	4.45	34.62	0.75	.	.	.	1.326
16	10449	2	200000	4.41	30.17	0.62	.	.	0.018	1.569
17	10449	3	8000000	7.20	16.82	.	.	.	.	0.697
18	10449	3	4500000	6.47	19.05	.	.	.	0.000	1.504
19	10449	3	11000000	5.90	19.90	0.72	.	.	.	0.816
20	10449	3	9100000	5.26	17.34	0.76	39.28	0.00	0.000	2.879
21	10449	3	4500000	5.25	19.21	0.72	66.87	0.00	.	0.849
22	10449	3	8000000	5.26	18.19	0.66	51.79	0.00	0.000	0.679
23	10449	3	7300000	5.27	19.39	0.74	.	.	.	0.897
24	10449	3	16000000	5.23	17.68	0.73	.	.	0.000	1.415
25	10449	4	3400000000	7.20	19.22	.	.	.	.	0.986
26	10449	4	6100000	6.69	18.19	0.00	.	.	0.000	1.294
27	10449	4	390000000	6.62	16.82	0.00	.	.	.	0.946
28	10449	4	420000000	6.54	18.02	0.00	67.53	3.84	0.000	0.768
29	10449	4	1400000	6.53	21.61	0.00	47.59	0.00	.	0.873
30	10449	4	1600000	6.53	25.72	0.00	57.73	0.00	0.000	0.453
31	10449	4	510000	6.56	17.34	0.00	.	.	.	0.477
32	10449	4	530000	6.56	17.16	0.00	.	.	0.000	0.428
33	10449	5	120000000000	7.20	28.46	.	.	.	.	0.752
34	10449	5	7200000	6.69	41.63	0.00	.	.	0.000	4.917
35	10449	5	15000	6.28	33.93	0.00	.	.	.	5.176
36	10449	5	10000000000	5.95	31.71	0.00	73.84	0.00	0.000	5.499
37	10449	5	180000000	5.67	36.67	0.62	81.61	0.00	.	2.725
38	10449	5	23000000	5.55	29.31	0.50	95.25	0.94	0.000	1.957
39	10449	5	160000000	5.44	29.31	0.51	.	.	.	1.989
40	10449	5	14000000	5.42	32.73	0.42	.	.	0.000	2.361
41	10449	6	50000000	7.00	28.89	.	.	.	.	0.184
42	10449	6	38000000	6.23	26.68	.	.	.	0.000	0.325
43	10449	6	28000000	6.17	26.27	.	.	.	.	0.366
44	10449	6	37000000	6.17	24.46	2.59	0.00	0.00	0.000	0.396
45	10449	6	50000000	5.94	28.08	1.52	0.00	0.00	.	0.270

Obs	strain	expt	tvc	pH	tprot	lac	acet	form	glu	ECP
46	10449	6	40000000	5.99	28.68	2.39	0.00	0.00	0.000	0.219
47	10449	6	27000000	5.97	37.12	.	.	.	.	0.241
48	10449	6	27000000	6.05	29.89	.	.	.	0.000	0.373
49	10449	7	29000000	7.00	20.85	.	.	.	.	0.295
50	10449	7	38000000	6.05	30.49	.	.	.	0.000	0.313
51	10449	7	23000000	6.16	25.07	.	.	.	.	0.385
52	10449	7	5000000	0.19	30.89	2.24	0.00	0.00	0.000	0.188
53	10449	7	30000000	6.15	25.67	2.10	0.00	0.00	.	0.320
54	10449	7	29000000	6.34	31.10	0.92	0.00	0.00	0.000	0.236
55	10449	7	42000000	6.35	27.08	.	.	.	.	0.375
56	10449	7	37000000	6.34	30.29	.	.	.	0.000	0.132
57	10449	8	53000000	7.00	31.50	.	.	.	.	2.272
58	10449	8	36000000	5.42	37.73	.	.	.	0.000	2.716
59	10449	8	33000000	4.62	42.15	5.11	0.32	0.44	.	2.287
60	10449	8	32000000	4.39	38.73	5.84	0.84	0.02	0.000	3.077
61	10449	8	23000000	4.41	43.35	5.18	0.00	0.00	.	3.077
62	10449	8	7300000	4.50	37.93	.	.	.	0.000	3.026
63	10449	8	4900000	4.45	41.75	.	.	.	.	2.836
64	10449	8	4900000	4.46	38.73	.	.	.	0.000	2.375
65	10449	9	18000000	7.00	13.64	.	.	.	.	0.150
66	10449	9	5900000	6.50	15.02	.	.	.	.	0.000
67	10449	9	4700000	6.37	13.77	.	.	.	.	0.802
68	10449	9	5300000	6.24	14.27	0.43	11.57	0.00	.	0.000
69	10449	9	9100000	6.04	14.27	1.79	13.50	0.00	.	0.171
70	10449	9	2800000	6.17	15.02	3.09	24.88	0.00	.	0.234
71	10449	9	2800000	6.15	14.77	.	.	.	.	0.879
72	10449	9	2000000	6.10	15.14	.	.	.	.	0.000
73	10449	9	2200000	6.05	15.39	32.29	16.72	0.00	.	0.000
74	SL1	1	78000	7.20	266.46	.	.	.	.	0.433
75	SL1	1	4100000	7.20	206.880	.	.	.	0.471	0.408
76	SL1	1	9100000	7.00	153.610	.	.	.	.	0.543
77	SL1	1	70000000	6.80	303.810	.	.	.	0.447	0.591
78	SL1	1	50000000	6.31	308.550	1.10	0.00	0.0	.	0.471
79	SL1	1	28000000	5.28	239.760	1.33	0.00	0.0	0.383	0.315
80	SL1	1	85000000	4.75	311.310	1.68	0.00	0.0	.	0.210
81	SL1	1	130000000	4.56	262.780	.	.	.	0.000	0.534
82	SL1	2	18000000	7.00	21.170	.	.	.	.	0.000
83	SL1	2	18000000	6.52	19.980	.	.	.	0.039	0.000
84	SL1	2	2400000	6.21	17.220	0.76	0.00	0.0	.	0.000
85	SL1	2	160000	5.98	19.320	0.77	0.03	0.0	0.023	0.000
86	SL1	2	10000	5.68	17.620	0.44	0.00	0.0	.	0.000
87	SL1	2	15000	5.65	18.540	.	.	.	0.040	0.000
88	SL1	2	48000	5.65	16.830	.	.	.	.	0.000
89	SL1	2	12000	5.64	19.980	.	.	.	0.065	0.092
90	SL1	3	630000	7.00	29.610	.	.	.	.	0.067
91	SL1	3	270000	6.30	43.040	.	.	.	0.017	0.102
92	SL1	3	250000	5.97	31.320	.	.	.	.	0.019
93	SL1	3	110000	5.74	40.350	.	.	.	0.000	0.000
94	SL1	3	610000	5.79	28.640	.	.	.	.	0.000
95	SL1	3	450000	5.65	28.640	0.59	0.00	0.0	0.000	0.000
96	SL1	3	770000	5.53	37.677	0.85	0.05	0.0	.	0.000
97	SL1	3	200000	5.48	32.790	0.71	0.24	0.0	0.000	0.143
98	SL1	4	9100000	7.20	17.560	.	.	.	.	0.423
99	SL1	4	260000	6.82	19.050	.	.	.	0.000	1.364
100	SL1	4	350000	6.79	17.560	.	.	.	.	1.412
101	SL1	4	81000	6.72	16.720	.	.	.	0.000	0.000
102	SL1	4	120000	6.54	19.470	0.00	0.00	0.0	.	0.485

Obs	strain	expt	tvc	pH	tprot	lac	acet	form	glu	ECP
103	SL1	4	260000	6.56	16.930	0.08	0.00	0.0	0.000	0.070
104	SL1	4	81000	6.50	17.990	0.09	0.00	0.0	.	0.241
105	SL1	4	81000	6.43	18.410	.	.	.	0.000	1.284
106	SL1	5	3700000	7.00	64.930	.	.	.	.	1.010
107	SL1	5	2500000	6.33	58.740	.	.	.	0.025	0.905
108	SL1	5	3200000	5.80	56.890	.	.	.	.	1.183
109	SL1	5	50000000	5.64	50.700	.	.	.	0.000	0.058
110	SL1	5	77000000	5.56	55.240	1.24	6.25	0.0	.	0.043
111	SL1	5	23000000	5.54	53.380	1.42	0.00	0.0	0.000	0.035
112	SL1	5	42000000	5.49	60.600	1.18	0.00	0.0	.	0.067
113	SL1	5	28000000	5.51	65.550	.	.	.	0.000	0.427
114	SL1	6	3100000	7.00	25.870	.	.	.	.	0.184
115	SL1	6	24000000	5.81	34.710	.	.	.	0.000	0.271
116	SL1	6	92000000	5.23	33.110	.	.	.	.	0.155
117	SL1	6	72000000	5.67	36.320	0.72	0.00	0.0	0.000	0.123
118	SL1	6	940000000	5.32	34.510	0.00	0.00	0.0	.	0.400
119	SL1	6	560000000	5.39	38.330	1.66	0.00	0.0	0.000	0.196
120	SL1	6	37000000	5.39	35.920	.	.	.	.	0.286
121	SL1	6	100000000	5.38	38.330	.	.	.	0.000	0.330
122	SL1	7	1000000	7.00	33.510	.	.	.	.	0.147
123	SL1	7	32000000	6.58	27.080	.	.	.	0.000	0.211
124	SL1	7	31000000	6.61	30.690	.	.	.	.	0.133
125	SL1	7	33000000	6.46	34.510	.	.	.	0.000	0.194
126	SL1	7	20000000	6.13	30.690	0.00	0.00	0.0	.	0.098
127	SL1	7	17000000	6.10	34.310	0.00	0.00	0.0	0.000	0.143
128	SL1	7	16000000	6.16	31.100	0.00	0.00	0.0	.	0.055
129	SL1	7	16000000	6.21	31.700	.	.	.	0.000	0.040
130	SL1	8	14000000	7.00	64.450	.	.	.	.	1.765
131	SL1	8	170000000	6.39	71.080	.	.	.	0.108	2.682
132	SL1	8	430000000	5.24	58.630	2.77	0.00	0.0	.	3.077
133	SL1	8	1200000000	5.24	61.440	5.28	0.00	0.0	0.000	1.573
134	SL1	8	26000000	4.39	53.600	2.85	0.00	0.0	.	2.532
135	SL1	8	91000000	4.52	54.810	.	.	.	0.016	1.822
136	SL1	8	11000000	4.44	51.590	.	.	.	.	1.033
137	SL1	8	14000000	4.15	53.200	.	.	.	0.002	1.873
138	SL1	9	7200000	6.91	17.270	.	.	.	.	0.310
139	SL1	9	11000000	6.50	17.270	.	.	.	.	0.588
140	SL1	9	910000	5.94	15.390	.	.	.	.	1.406
141	SL1	9	570000	5.06	14.890	6.75	6.68	22.5	.	0.292
142	SL1	9	470000	4.50	15.140	5.53	15.96	0.0	.	0.000
143	SL1	9	500000	4.28	15.520	5.95	14.81	0.0	.	0.227
144	SL1	9	530000	4.44	16.150	.	.	.	.	0.130
145	SL1	9	53000	4.44	17.020	.	.	.	.	0.177
146	SL1	9	56000	4.40	15.140	.	.	.	.	0.000
147	10922	1	460000000000	7.20	494.200	.	.	.	.	0.462
148	10922	1	65000000	6.98	455.440	.	.	.	0.299	0.627
149	10922	1	96000000	6.90	406.99	.	.	.	.	0.573
150	10922	1	570000000	6.19	481.42	.	.	.	0.281	3.551
151	10922	1	3200000000	5.69	527.19	4.510	0.22	0.00	.	0.794
152	10922	1	830000000	5.70	564.51	4.950	0.00	0.00	0.295	0.889
153	10922	1	530000000	5.82	499.97	4.620	0.00	0.00	.	0.481
154	10922	1	260000000	5.78	531.31	.	.	.	0.280	0.944
155	10922	2	70000000	7.00	19.78	.	.	.	.	0.000
156	10922	2	140000000	6.73	21.84	.	.	.	0.099	0.002
157	10922	2	160000000	6.61	22.25	.	.	.	.	0.049
158	10922	2	170000000	6.39	24.11	.	.	.	0.114	0.136
159	10922	2	1500000000	5.67	25.14	.	.	.	.	0.061

Obs	strain	expt	tvc	pH	tprot	lac	acet	form	glu	ECP
160	10922	2	3400000000	5.41	24.72	0.530	0.00	0.25	0.184	0.049
161	10922	2	1300000000	5.03	21.42	0.740	0.64	0.06	.	0.013
162	10922	2	1100000000	4.73	25.75	0.360	0.00	0.00	0.127	0.125
163	10922	3	26000000	7.00	29.61	.	.	.	.	0.283
164	10922	3	28000000	6.60	30.83	.	.	.	0.145	0.160
165	10922	3	29000000	6.40	30.83	.	.	.	.	0.069
166	10922	3	42000000	6.02	28.15	0.020	0.00	0.00	0.112	0.012
167	10922	3	91000000	5.83	36.45	0.000	0.00	0.10	.	0.010
168	10922	3	91000000	5.60	26.93	0.000	0.00	0.00	0.000	0.000
169	10922	3	140000000	5.61	27.17	.	.	.	.	0.000
170	10922	3	850000000	5.65	30.35	.	.	.	0.000	0.072
171	10922	4	110000000	7.00	16.30	.	.	.	.	0.423
172	10922	4	21000000	6.65	12.27	.	.	.	0.024	1.155
173	10922	4	14000000	6.46	14.60	.	.	.	.	1.238
174	10922	4	36000000	6.02	5.93	.	.	.	0.000	0.266
175	10922	4	55000000	6.08	14.60	.	.	.	.	0.314
176	10922	4	45000000	6.05	16.51	0.050	0.00	0.00	0.000	0.328
177	10922	4	31000000	5.95	9.10	0.050	0.00	0.00	.	0.160
178	10922	4	67000000	5.80	14.39	0.000	0.00	0.00	0.000	1.445
179	10922	5	15000000	7.00	64.93	.	.	.	.	1.010
180	10922	5	110000000	6.03	55.86	.	.	.	0.000	0.986
181	10922	5	310000000	5.16	78.33	0.870	0.00	0.00	.	0.130
182	10922	5	27000000	5.15	77.09	0.950	0.00	0.00	0.000	0.099
183	10922	5	1100000000	5.12	62.25	2.410	0.00	0.00	.	0.128
184	10922	5	360000000	5.20	63.90	.	.	.	0.000	0.221
185	10922	5	270000000	5.26	58.74	.	.	.	.	0.147
186	10922	5	170000000	5.16	65.55	.	.	.	0.000	1.036
187	10922	6	200000	7.00	36.51	.	.	.	.	0.856
188	10922	6	810000	6.63	52.38	.	.	.	0.017	0.714
189	10922	6	610000	6.58	50.81	.	.	.	.	0.875
190	10922	6	1400000	6.44	51.49	.	.	.	0.000	0.856
191	10922	6	610000	6.35	48.36	1.750	0.00	0.72	.	0.736
192	10922	6	810000	6.40	54.84	0.700	0.00	1.26	0.000	1.202
193	10922	6	1000000	6.31	53.05	1.040	0.00	0.00	.	0.999
194	10922	6	1000000	6.44	53.50	.	.	.	0.000	1.270
195	10922	7	410000	7.00	38.30	.	.	.	.	0.306
196	10922	7	200000	6.64	52.16	.	.	.	0.016	0.497
197	10922	7	410000	6.64	53.10	.	.	.	.	0.512
198	10922	7	200000	6.49	54.39	.	.	.	0.000	0.555
199	10922	7	210000	6.48	55.06	1.900	0.00	2.10	.	0.546
200	10922	7	210000	6.46	52.83	1.190	0.00	0.00	0.000	0.605
201	10922	7	210000	6.47	50.59	1.630	0.00	0.00	.	0.258
202	10922	7	210000	6.49	52.38	.	.	.	0.000	0.333
203	10922	8	200000	7.00	46.12	.	.	.	.	2.265
204	10922	8	2100000	6.64	57.52	.	.	.	0.071	2.272
205	10922	8	2300000	6.51	58.19	.	.	.	.	2.280
206	10922	8	2800000	6.39	56.40	.	.	.	0.067	2.208
207	10922	8	4900000	6.15	56.40	0.900	0.00	0.00	.	2.242
208	10922	8	13000000	6.12	56.63	0.970	0.00	0.00	0.061	2.369
209	10922	8	2200000	6.13	58.42	0.430	0.00	0.00	.	2.313
210	10922	8	7100000	6.03	55.29	.	.	.	0.080	2.244
211	10922	9	390000	6.79	14.70	.	.	.	.	1.958
212	10922	9	310000	6.56	15.73	.	.	.	.	1.270
213	10922	9	110000	6.44	15.11	.	.	.	.	0.662
214	10922	9	220000	6.45	16.34	0.000	13.15	0.00	.	0.553
215	10922	9	200000	6.47	16.14	0.000	13.86	0.00	.	0.169
216	10922	9	38000	6.32	17.17	0.000	19.43	0.00	.	0.015

Obs	strain	expt	tvc	pH	tprot	lac	acet	form	glu	ECP
217	10922	9	16000	6.16	16.76	.	.	.	.	0.000
218	10922	9	17000	6.29	16.34	.	.	.	.	0.846
219	10922	9	10000	6.29	16.55	.	.	.	.	1.432
220	10923	1	140000000	7.20	97.13	.	.	.	.	0.367
221	10923	1	130000000	6.91	172.73	.	.	.	0.459	0.971
222	10923	1	150000000	6.48	189.50	1.876	.	.	.	0.892
223	10923	1	210000000	5.44	197.99	23.68	8.64	0.14	0.419	0.959
224	10923	1	300000000	5.22	181.42	28.15	0.03	0.20	.	1.130
225	10923	1	500000000	5.20	185.66	22.86	13.69	0.20	0.483	1.084
226	10923	1	3400000	5.11	190.51	.	.	.	.	0.955
227	10923	1	1000000	5.17	178.18	.	.	.	0.468	0.940
228	10923	2	36000000	7.00	2.74	.	.	.	.	0.094
229	10923	2	760000000	5.85	20.93	.	.	.	0.374	0.365
230	10923	2	720000000	4.85	6.58	0.00	1.17	0.08	.	0.195
231	10923	2	1800000000	4.63	6.78	2.22	27.70	0.00	0.355	0.165
232	10923	2	630000000	4.30	6.18	3.49	12.51	2.24	.	0.198
233	10923	2	700000000	4.40	5.97	.	.	.	0.209	0.162
234	10923	2	34000000	4.40	5.37	.	.	.	.	0.211
235	10923	2	30000000	4.40	6.58	.	.	.	0.248	0.238
236	10923	3	12000000	7.00	21.85	.	.	.	.	1.065
237	10923	3	4500000	6.73	17.71	.	.	.	0.179	2.405
238	10923	3	3400000	6.67	16.25	0.00	0.00	0.00	.	2.301
239	10923	3	7700000	6.59	16.98	0.00	0.00	0.00	0.154	1.849
240	10923	3	2800000	6.28	19.90	0.00	0.00	0.00	.	1.869
241	10923	3	39000000	5.52	8.21	.	.	.	0.000	1.882
242	10923	3	71000000	5.07	14.05	.	.	.	.	1.630
243	10923	3	42000000	5.09	7.96	.	.	.	0.000	1.878
244	10923	4	80000000	7.00	17.56	.	.	.	.	0.474
245	10923	4	80000000	6.32	11.22	.	.	.	0.021	1.621
246	10923	4	180000000	5.96	12.06	.	.	.	.	1.594
247	10923	4	100000000	5.87	9.74	0.00	3.29	0.20	0.000	0.275
248	10923	4	430000000	5.73	11.43	1.15	1.87	0.00	.	0.420
249	10923	4	77000000	5.75	15.87	0.00	2.22	0.00	0.000	0.501
250	10923	4	4400000000	5.73	12.70	.	.	.	.	0.800
251	10923	4	17000000	6.06	15.24	.	.	.	0.000	1.711
252	10923	5	380000000	7.00	61.96	.	.	.	.	1.065
253	10923	5	440000000	5.62	33.26	0.00	14.94	0.00	0.028	2.405
254	10923	5	190000000	5.03	31.64	0.00	0.00	0.14	.	2.301
255	10923	5	76000000	4.76	28.41	2.73	0.82	0.03	0.000	1.849
256	10923	5	10000000	4.95	29.62	.	.	.	.	1.869
257	10923	5	790000	4.88	28.61	.	.	.	0.000	1.882
258	10923	5	430000	4.88	29.22	.	.	.	.	1.630
259	10923	5	120000	4.87	26.79	.	.	.	0.000	1.878
260	10923	6	11000000	7.00	38.52	.	.	.	.	0.226
261	10923	6	3900000	6.52	44.56	.	.	.	0.024	0.487
262	10923	6	2200000	6.39	30.69	.	.	.	.	0.337
263	10923	6	1800000	6.22	33.38	0.00	10.78	0.00	0.000	0.289
264	10923	6	1200000	6.26	31.36	0.00	7.61	0.00	.	0.321
265	10923	6	1600000	6.28	34.72	0.00	9.67	0.00	0.000	0.239
266	10923	6	2600000	6.34	31.14	.	.	.	.	0.201
267	10923	6	2800000	6.75	36.06	.	.	.	0.000	0.361
268	10923	7	6700000	7.00	42.77	.	.	.	.	0.376
269	10923	7	10000000	6.55	54.84	.	.	.	0.006	0.290
270	10923	7	3500000	6.66	49.03	.	.	.	.	0.391
271	10923	7	1200000	6.63	53.05	.	.	.	0.000	0.207
272	10923	7	2200000	6.35	51.04	0.00	14.11	0.00	.	0.296

Obs	strain	expt	tvc	pH	tprot	lac	acet	form	glu	ECP
273	10923	7	4900000	6.47	54.39	0.00	16.17	0.00	0.000	0.398
274	10923	7	4000000	6.53	43.88	0.00	30.36	0.00	.	0.454
275	10923	7	820000	6.62	55.51	.	.	.	0.000	0.199
276	10923	8	160000000	7.00	29.13	.	.	.	.	0.399
277	10923	8	190000000	5.54	27.12	.	.	.	0.009	0.738
278	10923	8	230000000	4.48	30.92	2.26	25.76	0.00	.	0.537
279	10923	8	39000000	4.44	50.14	3.17	21.61	0.00	0.000	0.101
280	10923	8	170000000	4.41	45.90	4.43	26.15	0.00	.	1.086
281	10923	8	13000000	4.38	48.58	.	.	.	0.000	0.939
282	10923	8	1800000	4.44	41.20	.	.	.	.	1.101
283	10923	8	810000	4.41	51.71	.	.	.	0.000	0.858
284	10923	9	74000000	6.70	14.02	.	.	.	.	0.000
285	10923	9	76000000	6.50	14.39	.	.	.	.	0.286
286	10923	9	70000000	5.80	15.39	.	.	.	.	2.243
287	10923	9	91000000	5.10	17.15	3.18	16.99	0.00	.	3.767
288	10923	9	55000000	4.60	16.90	3.02	18.05	0.00	.	0.863
289	10923	9	410000	4.80	16.90	3.73	15.76	0.00	.	1.251
290	10923	9	54000	4.88	17.15	.	.	.	.	2.865
291	10923	9	42000	4.84	18.02	.	.	.	.	0.925
292	10923	9	4000	4.83	17.15	.	.	.	.	1.095
293	11061	1	1100000	7.20	103.12	.	.	.	.	0.325
294	11061	1	19000000	6.44	239.03	.	.	.	0.431	0.426
295	11061	1	74000000	5.87	248.60	.	.	.	.	1.003
296	11061	1	2600000	5.36	231.96	25.01	5.17	0.05	0.362	0.292
297	11061	1	39000000	4.96	227.62	35.80	17.01	0.00	.	1.212
298	11061	1	480000	5.00	261.14	22.15	0.12	0.00	0.000	1.020
299	11061	1	1100000	5.00	256.58	.	.	.	.	0.874
300	11061	1	370000	4.90	250.20	.	.	.	0.000	0.743
301	11061	2	410000	7.00	0.00	.	.	.	.	0.017
302	11061	2	1200000	6.72	28.56	.	.	.	0.502	0.005
303	11061	2	3300000	6.86	24.00	.	.	.	.	0.117
304	11061	2	410000	6.64	24.46	.	.	.	0.218	0.138
305	11061	2	1600000	5.96	22.86	4.20	0.11	0.00	0.048	0.130
306	11061	2	200000	4.69	7.58	2.35	0.00	0.00	.	0.200
307	11061	2	220000	4.44	6.89	4.44	0.11	0.00	.	0.101
308	11061	2	120000	4.30	12.14	2.35	0.00	0.00	0.003	0.147
309	11061	3	390000	7.00	21.85	.	.	.	.	0.194
310	11061	3	160000	6.40	27.94	.	.	.	0.247	0.212
311	11061	3	100000	5.89	28.43	1.69	0.00	0.54	.	0.140
312	11061	3	41000	5.33	19.17	4.68	0.00	0.08	0.197	0.227
313	11061	3	81000	4.79	18.93	13.46	0.00	0.00	.	0.166
314	11061	3	20000	4.56	17.95	.	.	.	0.001	0.134
315	11061	3	40000	4.57	20.63	.	.	.	.	0.222
316	11061	3	61000	4.55	20.88	.	.	.	0.000	0.102
317	11061	4	410000	7.00	23.07	.	.	.	.	0.076
318	11061	4	390000	6.75	20.88	.	.	.	0.028	0.445
319	11061	4	460000	6.54	23.80	3.16	0.00	0.09	.	0.442
320	11061	4	370000	6.15	26.24	3.78	0.25	0.00	0.019	0.361
321	11061	4	160000	6.30	19.41	4.15	0.00	15.10	.	0.331
322	11061	4	340000	6.32	20.63	.	.	.	0.000	0.260
323	11061	4	350000	6.37	19.66	.	.	.	.	0.561
324	11061	4	280000	6.37	16.73	.	.	.	0.000	0.515
325	11061	5	790000	7.00	108.37	.	.	.	.	2.168
326	11061	5	3000000	4.80	35.40	6.83	0.00	0.00	0.015	2.444
327	11061	5	3700000	4.60	37.45	13.03	0.00	0.33	.	2.210
328	11061	5	710000	4.50	35.86	11.22	0.00	0.00	0.000	2.234
329	11061	5	2000000	4.29	32.44	.	.	.	.	2.362

Obs	strain	expt	tvc	pH	tprot	lac	acet	form	glu	ECP
330	11061	5	300000	4.24	35.86	.	.	.	0.000	1.927
331	11061	5	300000	4.30	30.61	.	.	.	.	1.938
332	11061	5	140000	4.32	40.19	.	.	.	0.000	1.971
333	11061	6	200000	7.00	37.18	.	.	.	.	0.654
334	11061	6	810000	6.22	52.83	.	.	.	0.000	0.692
335	11061	6	610000	6.17	49.02	.	.	.	.	0.901
336	11061	6	200000	6.30	51.04	1.60	0.00	0.66	0.000	0.938
337	11061	6	410000	6.05	51.26	1.16	0.00	0.00	.	0.818
338	11061	6	810000	6.19	51.71	1.15	0.00	1.37	0.000	2.213
339	11061	6	1000000	6.22	50.37	.	.	.	.	3.702
340	11061	6	410000	6.21	52.83	.	.	.	0.000	0.898
341	11061	7	2400000	7.00	36.51	.	.	.	.	0.319
342	11061	7	410000	6.22	52.16	.	.	.	0.000	0.410
343	11061	7	810000	6.27	50.37	.	.	.	.	0.118
344	11061	7	410000	6.29	53.27	.	.	.	0.000	0.281
345	11061	7	200000	6.18	53.50	1.11	0.00	0.32	.	0.347
346	11061	7	200000	6.23	52.83	1.66	0.00	0.00	0.000	0.373
347	11061	7	190000	6.24	51.49	1.76	0.00	0.00	.	0.410
348	11061	7	610000	6.26	41.20	.	.	.	0.000	0.270
349	11061	8	300000	7.00	42.99	.	.	.	.	0.854
350	11061	8	9300000	6.57	48.13	.	.	.	0.000	1.097
351	11061	8	16000000	6.46	47.91	.	.	.	.	0.779
352	11061	8	37000000	6.34	51.26	.	.	.	0.000	0.554
353	11061	8	32000000	5.86	47.46	0.00	5.23	0.00	.	0.621
354	11061	8	33000000	5.73	50.37	1.32	0.00	0.00	0.000	0.874
355	11061	8	38000000	5.41	47.91	4.24	0.00	0.00	.	0.732
356	11061	8	120000000	5.23	51.49	.	.	.	0.000	0.874
357	11061	9	9800000	6.78	17.38	.	.	.	.	0.245
358	11061	9	610000	5.89	19.64	.	.	.	.	1.166
359	11061	9	570000	5.39	18.82	.	.	.	.	0.719
360	11061	9	330000	4.63	20.05	4.62	17.52	0.00	.	2.169
361	11061	9	490000	4.26	18.41	6.55	8.17	0.00	.	1.879
362	11061	9	41000	4.16	19.85	5.91	13.89	0.00	.	2.358
363	11061	9	220000	4.10	19.85	.	.	.	.	0.947
364	11061	9	34000	4.11	22.32	.	.	.	.	0.110
365	11061	9	44000	4.04	20.26	.	.	.	.	1.533
366	10919	1	1100000	7.00	58.67	.	.	.	.	3.436
367	10919	1	45000000	6.80	72.41	.	.	.	1.472	3.555
368	10919	1	100000000	5.63	58.46	17.68	5.51	0.90	.	3.543
369	10919	1	140000000	4.99	53.47	11.82	12.15	0.28	1.046	4.377
370	10919	1	160000000	4.72	44.52	23.94	18.83	0.00	.	3.261
371	10919	1	23000000	4.78	54.51	.	.	.	0.996	3.363
372	10919	1	19000000	4.82	60.96	.	.	.	.	2.784
373	10919	1	12000000	4.80	56.59	.	.	.	1.129	3.636
374	10919	2	19000000	7.00	10.38	.	.	.	.	0.045
375	10919	2	7400000	6.26	18.92	.	.	.	0.000	0.207
376	10919	2	5400000	5.95	26.20	.	.	.	.	0.336
377	10919	2	1700000	5.48	11.42	0.00	67.20	0.00	0.001	0.290
378	10919	2	2900000	5.07	16.63	0.00	54.70	0.00	.	0.371
379	10919	2	2200000	4.51	18.50	3.67	9.10	0.00	0.028	0.320
380	10919	2	1000000	4.35	20.37	.	.	.	.	0.333
381	10919	2	1600000	4.26	20.58	.	.	.	0.032	0.328
382	10919	3	5000000	7.00	1.02	.	.	.	.	0.194
383	10919	3	2500000	6.05	16.63	.	.	.	0.066	0.321
384	10919	3	3400000	5.87	13.30	.	.	.	.	0.017
385	10919	3	2200000	5.41	11.84	0.00	37.68	0.00	0.045	0.383
386	10919	3	1000000	4.74	15.59	0.00	31.88	0.00	.	0.419

Obs	strain	expt	tvc	pH	tprot	lac	acet	form	glu	ECP
387	10919	3	2300000	4.54	16.63	0.00	27.10	0.00	0.036	0.358
388	10919	3	6300000	4.62	14.13	.	.	.	.	0.403
389	10919	3	5800000	4.62	16.63	.	.	.	0.000	0.287
390	10919	4	2300000000	7.00	2.27	.	.	.	.	0.234
391	10919	4	40000000	6.09	29.11	.	.	.	0.000	0.222
392	10919	4	30000000	6.13	27.24	.	.	.	.	0.319
393	10919	4	40000000	6.10	15.38	.	.	.	0.000	0.302
394	10919	4	40000000	5.82	11.22	0.00	35.12	0.00	.	0.405
395	10919	4	60000000	5.87	12.46	0.00	42.03	0.00	0.000	0.362
396	10919	4	30000000	5.88	11.42	0.00	37.42	0.00	.	0.434
397	10919	4	470000000	6.05	12.26	.	.	.	0.000	0.327
398	10919	5	1500000	7.00	78.03	.	.	.	.	1.132
399	10919	5	4000000	6.02	55.76	.	.	.	0.000	1.558
400	10919	5	25000000	5.61	29.74	0.00	42.22	0.00	.	1.599
401	10919	5	1500000000	5.27	23.70	0.00	41.68	0.00	0.000	1.986
402	10919	5	22000000	4.93	21.00	0.00	41.38	0.00	.	2.232
403	10919	5	41000000	4.80	23.29	.	.	.	0.000	2.233
404	10919	5	100000000	4.90	23.29	.	.	.	.	2.258
405	10919	5	800000	5.00	22.66	.	.	.	0.000	1.907
406	10919	6	200000000	7.00	0.00	.	.	.	.	0.028
407	10919	6	870000000	6.45	8.72	.	.	.	0.000	0.146
408	10919	6	200000000	6.44	5.80	.	.	.	.	0.155
409	10919	6	45000000	6.25	7.89	0.00	2.74	0.00	0.000	0.149
410	10919	6	39000000	6.15	7.26	0.00	2.72	0.00	.	0.195
411	10919	6	39000000	6.20	6.43	0.00	2.24	0.00	0.000	0.206
412	10919	6	20000000	6.24	7.68	.	.	.	.	0.221
413	10919	6	21000000	6.30	7.05	.	.	.	0.000	0.066
414	10919	7	.	.	0.00	.	.	.	.	0.029
415	10919	7	180000	6.26	11.01	.	.	.	0.000	0.043
416	10919	7	180000	6.31	9.34	.	.	.	.	0.034
417	10919	7	200000	6.29	10.59	0.00	82.03	0.00	0.000	0.045
418	10919	7	22000	6.32	10.17	0.00	75.51	0.00	.	0.075
419	10919	7	61000	6.23	12.67	0.00	87.10	0.00	0.000	0.072
420	10919	7	51000	6.27	11.42	.	.	.	.	0.018
421	10919	7	85000	6.34	12.05	.	.	.	0.000	0.036
422	10919	8	43000	7.00	14.34	.	.	.	.	1.277
423	10919	8	1400000	5.95	22.87	.	.	.	0.000	3.210
424	10919	8	1600000	5.82	22.45	.	.	.	.	2.941
425	10919	8	5100000	4.60	21.62	0.00	66.64	0.00	0.000	3.315
426	10919	8	1200000	4.40	19.33	0.00	82.12	0.00	.	2.977
427	10919	8	1200000	4.34	22.25	0.00	78.85	0.00	0.000	2.731
428	10919	8	2300000	4.42	23.29	.	.	.	.	2.948
429	10919	8	1300000	4.41	18.50	.	.	.	0.000	2.821
430	10919	9	5500000	6.77	14.14	.	.	.	.	0.382
431	10919	9	1200000	6.48	14.77	.	.	.	.	0.977
432	10919	9	1200000	6.26	15.39	.	.	.	.	0.141
433	10919	9	370000	6.05	15.02	1.50	21.67	31.66	.	2.642
434	10919	9	81000	5.79	15.14	2.10	20.51	0.00	.	2.078
435	10919	9	42000	5.24	15.27	3.41	7.17	0.00	.	0.529
436	10919	9	160000	4.77	15.89	.	.	.	.	3.544
437	10919	9	37000	4.68	15.39	.	.	.	.	2.890
438	10919	9	50000	4.50	17.40	.	.	.	.	3.726
439	13ah	1	2600000	7.00	494.20	.	.	.	.	0.462
440	13ah	1	2200000	6.71	516.06	.	.	.	2.264	0.525
441	13ah	1	400000	6.37	510.28	.	.	.	.	0.644
442	13ah	1	2200000	5.86	532.55	.	.	.	2.263	1.423
443	13ah	1	130000	5.11	505.95	5.14	0.00	0.00	.	1.332

Obs	strain	expt	tvc	pH	tprot	lac	acet	form	glu	ECP
444	13ah	1	770000	4.79	523.690	8.04	0.00	0.00	0.854	1.222
445	13ah	1	410000	4.79	525.950	4.69	0.00	0.00	.	1.415
446	13ah	1	290000	4.75	516.880	.	.	.	0.774	1.072
447	13ah	2	23000	7.00	21.030	.	.	.	.	0.000
448	13ah	2	1500000	6.71	21.034	.	.	.	0.850	0.000
449	13ah	2	120000	6.59	19.320	.	.	.	.	0.000
450	13ah	2	41000	6.49	20.640	.	.	.	0.721	0.000
451	13ah	2	12000	6.49	19.720	0.63	0.00	0.00	.	0.000
452	13ah	2	4100	6.56	19.850	0.25	0.00	2.55	0.395	0.000
453	13ah	2	6100	6.20	19.320	0.90	7.79	0.00	.	0.000
454	13ah	2	100000	6.20	16.960	.	.	.	0.328	0.072
455	13ah	3	750000	7.00	29.610	.	.	.	.	0.283
456	13ah	3	1500000	5.99	25.220	.	.	.	0.000	2.768
457	13ah	3	890000	5.77	28.640	0.09	0.15	0.00	.	0.127
458	13ah	3	13000000	5.81	24.980	0.08	0.15	0.00	0.000	0.063
459	13ah	3	3000000	5.90	27.170	0.14	0.45	0.00	.	0.017
460	13ah	3	800000	5.89	25.460	.	.	.	0.009	0.000
461	13ah	3	470000	5.98	28.880	.	.	.	.	0.000
462	13ah	3	1100000	5.91	26.930	.	.	.	0.010	0.110
463	13ah	4	20000	7.00	17.140	.	.	.	.	0.423
464	13ah	4	410000	7.03	20.320	.	.	.	0.226	1.694
465	13ah	4	410000	6.91	24.120	.	.	.	.	1.804
466	13ah	4	810000	6.88	21.160	.	.	.	0.070	0.196
467	13ah	4	20000	6.46	21.800	.	.	.	.	0.276
468	13ah	4	41000	6.46	23.910	0.00	0.64	0.00	0.980	0.279
469	13ah	4	35000	6.30	17.990	0.02	0.00	0.00	.	0.338
470	13ah	4	41000	6.30	18.410	0.00	0.25	0.00	0.162	1.674
471	13ah	5	23000	7.00	86.230	.	.	.	.	1.010
472	13ah	5	300000	6.29	100.140	.	.	.	0.729	2.795
473	13ah	5	2400000	4.91	107.950	.	.	.	.	2.023
474	13ah	5	1400000	4.52	122.350	.	.	.	0.662	0.576
475	13ah	5	1000000	4.48	96.730	0.63	9.12	0.00	.	0.454
476	13ah	5	400000	4.38	95.020	0.25	7.42	0.00	0.679	0.626
477	13ah	5	200000	4.30	108.440	0.90	7.99	0.00	.	0.454
478	13ah	5	880000	4.19	99.170	.	.	.	0.052	1.780
479	13ah	6	3200000	7.00	0.000	.	.	.	.	0.316
480	13ah	6	610000	6.56	0.000	.	.	.	0.000	0.658
481	13ah	6	300000	6.60	0.000	.	.	.	.	0.660
482	13ah	6	100000	6.49	0.000	.	.	.	0.000	0.763
483	13ah	6	200000	6.25	0.000	8.48	4.16	0.00	.	0.433
484	13ah	6	200000	6.25	0.000	0.00	10.46	0.00	0.000	0.869
485	13ah	6	710000	6.30	0.000	0.00	7.21	0.00	.	0.874
486	13ah	6	200000	6.35	0.000	.	.	.	0.000	0.951
487	13ah	7	150000000	7.00	0.000	.	.	.	.	0.014
488	13ah	7	360000000	6.47	3.930	.	.	.	0.168	0.083
489	13ah	7	370000000	6.54	3.480	.	.	.	.	0.072
490	13ah	7	740000000	6.59	0.000	.	.	.	0.225	0.094
491	13ah	7	650000000	6.40	1.200	0.00	14.40	0.00	.	0.030
492	13ah	7	660000000	6.45	1.650	0.00	7.86	0.00	0.281	0.009
493	13ah	7	1200000000	6.39	1.200	1.84	8.25	0.00	.	0.020
494	13ah	7	810000000	6.44	0.510	.	.	.	0.280	0.075
495	13ah	8	410000	7.00	21.490	.	.	.	.	2.715
496	13ah	8	810000	5.85	34.490	.	.	.	0.232	3.651
497	13ah	8	4900000	4.93	31.980	0.00	14.70	0.00	.	3.527
498	13ah	8	23000000	4.51	32.210	0.00	14.08	0.00	0.009	3.589
499	13ah	8	1600000	4.62	32.210	0.00	8.18	0.00	.	3.651
500	13ah	8	200000	4.38	31.300	.	.	.	0.002	3.651

Obs	strain	expt	tvc	pH	tprot	lac	acet	form	glu	ECP
501	13ah	8	200000	4.34	34.030	.	.	.	.	3.651
502	13ah	8	200000	4.51	33.350	.	.	.	0.000	3.651
503	13ah	9	370000	6.77	18.610	.	.	.	.	0.360
504	13ah	9	110000	5.43	16.340	.	.	.	.	0.280
505	13ah	9	850000	4.02	18.820	.	.	.	.	0.879
506	13ah	9	410000	3.81	18.610	2.95	11.13	0.00	.	0.269
507	13ah	9	890000	3.70	19.440	5.65	9.46	0.00	.	1.221
508	13ah	9	750000	3.91	19.020	3.97	6.40	0.00	.	0.000
509	13ah	9	60000	3.85	19.440	.	.	.	.	0.000
510	13ah	9	58000	3.72	19.230	.	.	.	.	0.000
511	13ah	9	2000	3.68	20.670	.	.	.	.	0.091
512	13eg	1	9000000	7.00	494.200	.	.	.	.	1.160
513	13eg	1	10000000	6.89	465.540	.	.	.	1.883	0.796
514	13eg	1	41000000	6.52	475.640	.	.	.	.	0.794
515	13eg	1	47000000	6.01	497.090	.	.	.	1.809	2.150
516	13eg	1	170000000	5.65	424.100	1.07	0.00	0.00	.	1.929
517	13eg	1	10000000	5.45	513.380	1.05	0.00	0.00	1.364	1.566
518	13eg	1	7700000	5.30	434.200	1.13	0.00	0.00	.	2.702
519	13eg	1	33000000	5.03	477.09	.	.	.	1.422	0.945
520	13eg	2	950000	7.00	19.78	.	.	.	.	0.000
521	13eg	2	4200000	6.62	25.34	.	.	.	0.356	0.033
522	13eg	2	5000000	6.48	21.22	.	.	.	.	0.079
523	13eg	2	6800000	6.21	24.72	.	.	.	0.604	0.001
524	13eg	2	630000	5.41	23.90	0.96	0.00	0.00	.	0.000
525	13eg	2	7800000	4.99	21.01	0.42	0.00	0.49	1.019	0.000
526	13eg	2	7200000	4.64	22.46	0.56	12.02	9.60	.	0.000
527	13eg	2	6800000	4.54	23.07	.	.	.	0.682	0.094
528	13eg	3	27000000	7.00	29.61	.	.	.	.	0.230
529	13eg	3	8900000	5.61	38.16	.	.	.	0.046	0.148
530	13eg	3	37000000	5.63	43.77	0.13	4.73	0.00	.	0.094
531	13eg	3	44000000	5.72	37.18	0.01	0.00	0.00	0.033	0.000
532	13eg	3	25000000	5.91	44.74	0.06	0.00	0.00	.	0.000
533	13eg	3	17000000	6.07	34.49	.	.	.	0.029	0.000
534	13eg	3	7100000	6.15	38.64	.	.	.	.	0.000
535	13eg	3	16000000	6.23	43.04	.	.	.	0.010	0.182
536	13eg	4	810000	7.00	16.30	.	.	.	.	0.098
537	13eg	4	200000	6.99	16.51	.	.	.	0.186	1.497
538	13eg	4	60000	0.60	14.81	.	.	.	.	1.500
539	13eg	4	160000	6.60	18.41	.	.	.	0.316	0.000
540	13eg	4	81000	6.68	15.87	.	.	.	.	0.287
541	13eg	4	280000	6.56	15.87	0.00	0.00	0.00	0.265	0.238
542	13eg	4	1400000	6.42	16.93	0.00	0.00	0.00	.	0.170
543	13eg	4	2600000	6.35	18.99	0.16	0.00	0.00	0.139	1.701
544	13eg	5	5200000	7.00	64.93	.	.	.	.	1.022
545	13eg	5	1600000	6.79	57.30	.	.	.	0.266	1.132
546	13eg	5	2800000	6.42	58.33	.	.	.	.	0.902
547	13eg	5	2900000	6.22	56.27	.	.	.	0.466	0.177
548	13eg	5	47000000	5.26	66.37	1.66	12.16	0.00	.	0.327
549	13eg	5	70000000	5.15	71.53	1.25	12.39	0.00	0.068	0.266
550	13eg	5	110000000	5.04	60.39	0.12	19.16	0.00	.	0.284
551	13eg	5	9000000	4.99	61.01	.	.	.	0.110	1.173
552	13eg	6	40000000	7.00	25.11	.	.	.	.	0.687
553	13eg	6	900000	6.44	34.27	.	.	.	0.026	0.377
554	13eg	6	2000000	6.31	33.60	.	.	.	.	0.413
555	13eg	6	14000000	6.30	34.94	.	.	.	0.000	0.463
556	13eg	6	7600000	6.32	32.48	0.00	45.47	0.00	.	0.440
557	13eg	6	7100000	6.28	34.05	0.00	31.44	0.00	0.030	0.407

Obs	strain	expt	tvc	pH	tprot	lac	acet	form	glu	ECP
558	13eg	6	6100000	6.29	31.14	0.00	46.86	0.00	.	0.354
559	13eg	6	9600000	6.32	33.60	.	.	.	0.001	0.347
560	13eg	7	1500000	7.00	28.01	.	.	.	.	0.399
561	13eg	7	9000000	6.26	34.94	.	.	.	0.207	0.555
562	13eg	7	1400000	6.25	34.94	.	.	.	.	0.677
563	13eg	7	3500000	6.23	32.93	0.00	20.12	0.00	0.030	0.420
564	13eg	7	3800000	6.15	40.53	0.00	31.81	0.00	.	0.707
565	13eg	7	5000000	6.16	33.82	0.00	12.53	0.00	0.001	0.617
566	13eg	7	3000000	6.17	33.15	.	.	.	.	0.625
567	13eg	7	500000	6.22	33.15	.	.	.	0.000	0.484
568	13eg	8	19000000	7.00	33.15	.	.	.	.	0.470
569	13eg	8	9400000	6.18	40.31	.	.	.	0.205	1.228
570	13eg	8	9800000	5.14	44.11	6.22	86.53	0.00	.	0.801
571	13eg	8	10000000	4.68	39.64	5.61	60.74	0.00	0.101	1.059
572	13eg	8	500000	4.47	41.20	7.92	34.67	0.78	.	0.822
573	13eg	8	300000	4.38	39.41	.	.	.	0.017	0.964
574	13eg	8	200000	4.31	40.08	.	.	.	.	1.200
575	13eg	8	300000	4.34	36.51	.	.	.	0.000	1.035
576	13eg	9	7200000	6.83	20.88	.	.	.	.	0.787
577	13eg	9	3400000	6.42	22.32	.	.	.	.	0.000
578	13eg	9	2300000	6.22	24.18	.	.	.	.	0.092
579	13eg	9	1500000	4.93	23.97	1.56	12.00	0.00	.	0.000
580	13eg	9	870000	4.18	25.83	3.86	10.80	0.00	.	0.000
581	13eg	9	130000	4.07	27.47	2.76	7.62	0.00	.	0.000
582	13eg	9	180000	3.85	25.21	.	.	.	.	0.447
583	13eg	9	37000	3.88	25.00	.	.	.	.	1.296
584	13eg	9	43000	3.81	27.47	.	.	.	.	0.000
585	2007	1	17000000	7.20	103.37	.	.	.	.	0.325
586	2007	1	35000000	7.11	219.19	.	.	.	2.206	1.170
587	2007	1	130000000	6.60	238.80	.	.	.	.	0.981
588	2007	1	430000000	5.34	198.21	29.98	0.82	0.00	2.233	1.182
589	2007	1	2200000000	5.04	231.96	25.50	2.00	0.00	.	1.021
590	2007	1	500000000	4.88	212.35	13.34	2.01	0.02	2.212	0.942
591	2007	1	440000000	4.73	196.61	.	.	.	.	1.087
592	2007	1	360000000	4.75	202.09	.	.	.	2.224	1.008
593	2007	2	3000000	7.00	32.57	.	.	.	.	0.063
594	2007	2	2400000	6.41	44.51	.	.	.	1.342	0.016
595	2007	2	1200000	6.63	44.02	.	.	.	.	0.001
596	2007	2	2000000	6.51	26.24	.	.	.	1.581	0.042
597	2007	2	1200000	6.47	42.08	.	.	.	.	0.114
598	2007	2	4900000	6.09	30.38	1.14	0.20	0.26	2.167	0.134
599	2007	2	22000000	5.66	29.40	0.51	0.00	0.00	.	0.053
600	2007	2	26000000	4.86	30.62	2.60	0.00	0.00	1.588	0.096
601	2007	3	1600000	7.00	0.00	.	.	.	.	0.067
602	2007	3	1800000	6.83	21.26	.	.	.	1.400	0.475
603	2007	3	2000000	6.80	17.39	.	.	.	.	0.438
604	2007	3	2200000	6.73	16.70	.	.	.	1.420	0.539
605	2007	3	1200000	6.39	16.47	.	.	.	.	0.441
606	2007	3	1700000	6.29	15.11	1.48	0.00	0.45	0.762	0.407
607	2007	3	13000000	5.71	14.65	2.36	12.03	1.59	.	0.299
608	2007	3	21000000	5.06	13.51	3.21	2.60	0.00	0.134	0.209
609	2007	4	6300000	7.00	23.07	.	.	.	.	0.076
610	2007	4	1600000	6.60	27.46	.	.	.	0.257	0.142
611	2007	4	3700000	6.49	26.24	.	.	.	.	0.129
612	2007	4	2600000	6.35	27.70	0.87	0.06	0.13	0.138	0.259
613	2007	4	750000	6.37	33.79	0.71	0.05	0.00	.	0.633
614	2007	4	5600000	6.37	21.36	0.92	0.00	0.00	0.174	0.305

Obs	strain	expt	tvc	pH	tprot	lac	acet	form	glu	ECP
615	2007	4	20000000	6.44	27.21	.	.	.	.	0.297
616	2007	4	3400000	6.45	25.51	.	.	.	0.097	0.466
617	2007	5	12000000	7.00	61.96	.	.	.	.	1.537
618	2007	5	41000000	5.42	64.25	.	.	.	0.270	1.552
619	2007	5	35000000	4.30	50.36	4.12	0.00	0.00	.	2.089
620	2007	5	410000000	4.12	77.16	3.65	0.17	0.00	0.076	1.599
621	2007	5	10000000	4.25	37.93	4.60	0.00	0.57	.	2.905
622	2007	5	4500000	4.21	58.89	.	.	.	0.071	2.250
623	2007	5	6900000	4.23	48.65	.	.	.	.	1.712
624	2007	5	4900000	4.31	61.81	.	.	.	0.044	1.800
625	2007	6	32000000	7.00	29.13	.	.	.	.	0.787
626	2007	6	44000000	4.87	50.14	.	.	.	0.000	0.507
627	2007	6	25000000	4.80	66.02	1.00	0.00	0.00	.	0.888
628	2007	6	88000000	4.85	59.31	0.00	0.00	0.00	0.000	0.957
629	2007	6	0	4.85	49.70	1.94	0.00	0.00	.	0.594
630	2007	6	32000000	4.73	50.59	1.90	0.00	0.00	0.001	0.403
631	2007	6	56000000	4.73	49.03	.	.	.	.	0.623
632	2007	6	50000000	4.72	48.36	.	.	.	0.000	0.462
633	2007	7	7100000	7.00	28.01	.	.	.	.	0.452
634	2007	7	3200000	6.32	47.46	.	.	.	0.000	0.547
635	2007	7	3400000	6.28	45.00	.	.	.	.	0.385
636	2007	7	4200000	6.29	46.57	0.00	0.00	0.00	0.000	0.505
637	2007	7	1200000	5.94	45.00	0.00	0.00	0.00	.	0.541
638	2007	7	4200000	5.98	46.12	0.00	0.00	0.00	0.000	0.538
639	2007	7	2600000	6.04	45.00	.	.	.	.	0.443
640	2007	7	3100000	6.06	47.24	.	.	.	0.000	0.462
641	2007	8	40000000	7.00	37.62	.	.	.	.	0.662
642	2007	8	61000000	5.20	58.86	.	.	.	0.083	0.840
643	2007	8	96000000	4.50	59.76	6.25	0.00	0.03	.	0.781
644	2007	8	58000000	4.40	62.22	8.45	0.37	0.00	0.000	0.743
645	2007	8	12000000	4.20	57.30	6.67	0.80	0.02	.	0.773
646	2007	8	2400000	4.32	59.31	5.74	0.00	0.00	0.000	0.841
647	2007	8	4500000	4.33	60.43	.	.	.	.	0.701
648	2007	8	2500000	4.40	61.32	.	.	.	0.000	0.763
649	2007	9	15000000	6.70	21.50	.	.	.	.	0.396
650	2007	9	910000000	6.28	23.15	.	.	.	.	1.453
651	2007	9	4100000	5.65	25.21	.	.	.	.	1.213
652	2007	9	360000	4.26	29.54	1.58	8.90	0.00	.	0.000
653	2007	9	2900000	4.05	27.27	3.15	9.06	0.00	.	0.035
654	2007	9	1200000	3.87	28.92	8.47	6.41	0.00	.	0.000
655	2007	9	810000	3.93	29.12	.	.	.	.	0.000
656	2007	9	590000	3.87	27.89	.	.	.	.	0.000
657	2007	9	320000	3.77	27.27	.	.	.	.	0.000
658	2036	1	150000000	7.00	104.31	.	.	.	.	0.087
659	2036	1	110000000	6.80	177.31	.	.	.	2.236	0.632
660	2036	1	580000000	5.59	171.49	10.24	3.30	0.92	.	0.833
661	2036	1	610000000	4.97	148.22	27.19	3.35	0.44	1.468	0.909
662	2036	1	240000000	4.92	162.49	24.77	12.05	0.11	.	0.937
663	2036	1	200000000	4.81	160.42	21.53	4.02	0.10	1.575	0.908
664	2036	1	190000000	4.82	93.43	.	.	.	.	0.840
665	2036	1	37000000	4.84	155.35	.	.	.	1.276	0.767
666	2036	2	16000000	7.00	4.86	.	.	.	.	0.055
667	2036	2	450000000	6.46	12.93	.	.	.	0.512	0.282
668	2036	2	490000000	6.01	9.92	.	.	.	.	0.313
669	2036	2	500000000	4.64	12.93	0.00	12.79	0.00	0.590	0.426
670	2036	2	18000000	4.82	5.80	0.00	14.95	0.00	.	0.282
671	2036	2	12000000	4.68	8.99	0.00	7.83	0.00	0.443	0.349

Obs	strain	expt	tvc	pH	tprot	lac	acet	form	glu	ECP
672	2036	2	410000	4.78	7.48	.	.	.	.	0.254
673	2036	2	200000	4.78	12.36	.	.	.	0.443	0.255
674	2036	3	3300000	7.00	4.11	.	.	.	.	0.031
675	2036	3	1000000	6.22	21.94	.	.	.	1.218	0.426
676	2036	3	9600000	6.22	27.75	.	.	.	.	0.484
677	2036	3	33000000	6.48	34.32	.	.	.	0.875	0.398
678	2036	3	81000000	6.24	34.88	.	.	.	.	0.394
679	2036	3	57000000	5.66	23.81	0.00	13.84	0.00	0.560	0.257
680	2036	3	37000000	5.00	21.75	0.00	14.17	0.00	.	0.202
681	2036	3	5900000	4.67	21.56	0.00	33.82	0.00	0.350	0.205
682	2036	4	30000000	7.00	0.00	.	.	.	.	0.291
683	2036	4	170000000	6.36	7.11	.	.	.	0.344	0.556
684	2036	4	330000000	5.95	5.42	0.00	35.15	0.00	.	0.490
685	2036	4	100000000	5.78	5.23	0.00	25.70	0.00	0.006	0.472
686	2036	4	280000000	5.81	5.42	1.77	35.88	0.00	.	0.489
687	2036	4	440000000	6.05	5.05	.	.	.	0.000	0.548
688	2036	4	550000000	5.87	4.67	.	.	.	.	0.445
689	2036	4	470000000	5.70	5.61	.	.	.	0.000	0.478
690	2036	5	67000000	7.00	49.33	.	.	.	.	0.082
691	2036	5	270000000	5.53	26.25	4.87	40.45	0.00	0.064	2.385
692	2036	5	160000000	4.85	28.69	5.59	43.11	0.00	.	3.587
693	2036	5	140000000	4.71	27.56	9.31	40.51	0.00	0.000	2.492
694	2036	5	210000000	4.62	30.19	7.28	40.98	0.00	.	2.931
695	2036	5	46000000	4.52	27.00	.	.	.	0.008	2.475
696	2036	5	33000000	4.58	28.13	.	.	.	.	3.189
697	2036	5	31000000	4.69	27.00	.	.	.	0.000	3.424
698	2036	6	7200000	7.00	5.61	.	.	.	.	0.066
699	2036	6	82000000	6.38	1.67	.	.	.	0.000	0.117
700	2036	6	190000000	6.46	0.00	.	.	.	.	0.086
701	2036	6	280000000	6.17	0.00	0.00	0.00	0.00	0.000	0.325
702	2036	6	69000000	6.16	0.17	0.00	0.00	0.00	.	0.183
703	2036	6	4900000	6.18	1.10	0.00	0.00	0.00	0.000	0.229
704	2036	6	6100000	6.26	0.00	.	.	.	.	0.095
705	2036	6	5600000	6.31	0.00	.	.	.	0.000	0.266
706	2036	7	11000000	7.00	8.24	.	.	.	.	0.062
707	2036	7	57000000	6.33	9.36	.	.	.	0.000	0.157
708	2036	7	86000000	6.34	8.99	.	.	.	.	0.111
709	2036	7	74000000	6.16	7.86	0.00	6.27	0.00	0.000	0.189
710	2036	7	78000000	5.99	7.30	0.00	5.14	0.69	.	0.113
711	2036	7	47000000	5.99	9.92	0.00	3.22	0.00	0.000	0.141
712	2036	7	40000000	6.16	11.80	.	.	.	.	0.122
713	2036	7	61000000	6.14	12.55	.	.	.	0.000	0.068
714	2036	8	1300000000	7.00	9.36	.	.	.	.	0.816
715	2036	8	7100000000	4.75	12.36	2.98	7.33	0.84	0.000	4.008
716	2036	8	14000000000	4.35	14.80	2.97	3.20	0.10	.	4.008
717	2036	8	12000000000	4.53	11.99	5.86	5.95	0.00	0.000	4.008
718	2036	8	1300000000	4.41	14.05	4.79	7.12	0.00	.	0.959
719	2036	8	150000000	4.44	12.55	.	.	.	0.000	4.008
720	2036	8	10000000	4.53	14.05	.	.	.	.	4.008
721	2036	8	10000000	4.51	12.18	.	.	.	0.000	4.008
722	2036	9	110000000	6.85	21.29	.	.	.	.	2.495
723	2036	9	110000000	6.33	18.20	.	.	.	.	3.415
724	2036	9	210000000	5.82	20.47	.	.	.	.	3.112
725	2036	9	170000000	5.10	20.05	0.88	9.97	0.00	.	3.115
726	2036	9	64000000	4.80	19.23	1.73	7.57	0.00	.	3.175
727	2036	9	3300000	4.70	17.79	2.07	7.60	0.00	.	1.426
728	2036	9	1900000	4.75	19.85	.	.	.	.	2.133

Obs	strain	expt	tvc	pH	tprot	lac	acet	form	glu	ECP
729	2036	9	1400000	4.67	19.23	.	.	.	.	2.131
730	2036	9	550000	4.61	22.53	.	.	.	.	1.743
731	2037	1	610000	7.20	102.62	.	.	.	.	0.367
732	2037	1	16000000	7.15	100.56	.	.	.	1.375	1.241
733	2037	1	11000000	7.13	176.93	.	.	.	.	1.279
734	2037	1	49000000	7.06	165.11	.	.	.	1.303	1.445
735	2037	1	1000000000	6.71	193.07	3.07	5.47	0.40	.	1.430
736	2037	1	160000000	6.35	195.70	8.91	5.56	0.23	1.864	1.945
737	2037	1	24000000	5.47	191.38	12.29	6.10	0.33	.	1.718
738	2037	1	13000000	4.08	200.39	22.53	7.52	0.00	1.501	1.532
739	2037	2	5500000	7.00	0.00	.	.	.	.	0.094
740	2037	2	7000000	6.34	16.30	.	.	.	2.190	0.240
741	2037	2	7100000	6.19	13.68	.	.	.	.	0.164
742	2037	2	4900000	5.98	13.68	0.00	12.12	0.00	2.159	0.254
743	2037	2	4700000	5.65	12.36	0.00	15.00	0.00	.	0.201
744	2037	2	3500000	4.83	8.24	1.31	10.14	0.00	1.921	0.357
745	2037	2	410000	4.40	4.86	.	.	.	.	0.186
746	2037	2	1800000	4.16	6.55	.	.	.	0.000	0.307
747	2037	3	6500000	7.00	0.00	.	.	.	.	0.096
748	2037	3	2000000	6.55	4.29	.	.	.	1.460	0.577
749	2037	3	2000000	6.46	5.61	.	.	.	.	0.499
750	2037	3	3900000	6.39	3.73	.	.	.	1.318	0.411
751	2037	3	8100000	6.39	3.73	.	.	.	.	0.395
752	2037	3	4500000	5.76	2.04	0.00	47.01	0.00	0.635	0.382
753	2037	3	810000	5.03	3.54	0.00	28.37	0.00	.	0.326
754	2037	3	2200000	4.67	2.98	0.00	26.22	0.00	1.872	0.362
755	2037	4	3500000	7.00	0.00	.	.	.	.	0.183
756	2037	4	4300000	6.55	0.17	.	.	.	0.217	0.706
757	2037	4	2600000	6.43	0.00	.	.	.	.	0.556
758	2037	4	3500000	6.25	0.35	.	.	.	0.153	0.571
759	2037	4	2200000	6.08	0.00	0.00	41.16	0.00	.	0.552
760	2037	4	1400000	6.05	0.00	0.00	37.45	0.00	0.018	0.637
761	2037	4	810000	6.09	0.35	0.00	37.50	0.00	.	0.605
762	2037	4	810000	6.10	0.00	.	.	.	0.046	0.533
763	2037	5	6300000	7.00	64.91	.	.	.	.	1.065
764	2037	5	3700000	6.75	49.89	.	.	.	1.277	2.135
765	2037	5	11000000	6.41	39.57	.	.	.	.	2.874
766	2037	5	16000000	5.97	32.82	.	.	.	1.659	3.619
767	2037	5	9800000	4.93	29.25	3.39	97.74	1.41	.	3.673
768	2037	5	7300000	4.73	25.87	3.31	1.18	0.71	0.014	3.358
769	2037	5	0	0.46	24.75	5.57	5.90	0.00	.	3.313
770	2037	5	8700000	4.54	25.12	.	.	.	0.027	3.355
771	2037	6	12000000	7.00	0.00	.	.	.	.	0.128
772	2037	6	25000000	6.54	4.29	.	.	.	0.000	0.258
773	2037	6	7400000	6.46	2.04	.	.	.	.	0.305
774	2037	6	3700000	6.27	0.35	0.00	26.74	0.00	0.000	0.238
775	2037	6	6300000	5.98	3.73	0.00	28.08	0.00	.	0.300
776	2037	6	1800000	5.88	0.73	0.00	22.17	0.00	0.000	0.247
777	2037	6	900000	6.00	1.67	.	.	.	.	0.173
778	2037	6	1000000	5.90	0.73	.	.	.	0.018	0.233
779	2037	7	6700000	7.00	0.73	.	.	.	.	0.096
780	2037	7	10000000	6.55	0.17	.	.	.	0.000	0.166
781	2037	7	3500000	6.66	0.00	.	.	.	.	0.106
782	2037	7	1200000	6.63	0.00	.	.	.	0.000	0.080
783	2037	7	2200000	6.35	0.00	0.00	42.61	0.00	.	0.193
784	2037	7	4900000	6.47	0.00	0.00	38.30	0.00	0.000	0.101
785	2037	7	4000000	6.53	0.00	0.00	45.69	0.00	.	0.144

Obs	strain	expt	tvc	pH	tprot	lac	acet	form	glu	ECP
786	2037	7	820000	6.62	0.54	.	.	.	0.004	0.125
787	2037	8	1300000	7.00	4.86	.	.	.	.	2.715
788	2037	8	6300000	6.38	14.24	.	.	.	0.141	3.822
789	2037	8	69000000	5.70	15.18	1.86	69.39	0.00	.	3.867
790	2037	8	3500000	4.98	12.74	3.13	67.04	0.00	0.185	3.867
791	2037	8	300000	4.77	11.99	7.67	69.21	0.00	.	3.867
792	2037	8	32000	4.79	11.43	.	.	.	0.068	3.867
793	2037	8	30000	4.74	10.67	.	.	.	.	3.649
794	2037	8	30000	4.79	11.43	.	.	.	0.038	3.569
795	2037	9	2700000	6.97	21.50	.	.	.	.	0.000
796	2037	9	3100000	6.35	17.38	.	.	.	.	0.464
797	2037	9	1400000	6.18	18.82	.	.	.	.	0.986
798	2037	9	940000	6.04	18.61	0.88	9.97	0.00	.	0.000
799	2037	9	850000	5.71	20.67	1.73	7.57	0.00	.	0.375
800	2037	9	460000	5.36	22.53	2.07	7.60	0.00	.	0.255
801	2037	9	38000	5.08	22.73	.	.	.	.	0.160
802	2037	9	340000	4.60	22.12	.	.	.	.	0.606
803	2037	9	23000	4.50	23.15	.	.	.	.	0.000

APPENDIX III

MEDIA AND REAGENTS USED IN THIS STUDY

A. Transport media

A.1 Reduced transport media (140)

Salt solution No. 1

0.6%	*di*-Potassium hydrogen phosphate (K_2HPO_4)	0.9 g
Distilled water (H_2O): make to		150 mL

Salt solution No. 2

1.2%	Sodium chloride (NaCl)	1.8 g
1.2%	Ammonium sulphate $(NH_4)_2SO_4$)	1.8 g
0.6%	Potassium dihydrogen phosphate (KH_2 PO_4)	0.9 g
0.25	Magnesium sulphate ($MgSO_4$)	0.75 g
Distilled H_2O: make to		150 mL

Combine the following:

Salt solution No.1		75.0 mL
Salt solution No. 2		75.0 mL
0.1 M	Ethylene diamine tetra-acetic acid (EDTA)	10.0 mL
8%	Sodium carbonate anhydrous (Na_2CO_3)	5.0 mL
1%	Dithiothreitol (DTT) (freshly prepared)	20.0 mL
Distilled H_2O: to make		1000 mL
pH	8±0.2	

Sterilize by filtration (0.22 μm pore size)

B. Dilution media

B.2 Peptone water (buffered)

Peptone from meat	10.0 g
NaCl	5.0 g
di-Sodium hydrogen orthophosphate (Na_2HPO_4)	9.0 g
Potassium dihydrogen orthophosphate (KH_2PO_4)	1.5 g

Distilled H_2O: make to	1000 mL

C. Agar media

C.1 Trypticase, yeast, cystine, sucrose, bacitracin agar (141)

Tryptone	15.0 g
Yeast extract	5.0 g
L-Cystine	0.2 g
Sodium sulphite (Na_2SO_4)	0.1 g
NaCl	1.0 g
di-Sodium hydrogen orthophosphate (Na_2HPO_4)	2.0 g
Sodium hydrogen carbonate ($NaHCO_3$)	2.0 g
Sodium acetate ($NaC_2H_3O_2$)	1.2 g
Sucrose	50.0 g
Agar	15.0 g
Addition: Sucrose (final content = 18%)	130 g
Distilled H_2O: make to	1000 mL

Method:

Autoclave basic media and sucrose separately at 115°C for 15 minutes. Add sterile sucrose to the basic media and stir. Cool media to approximately 55°C and add bacitracin (200 U/1000 mL). Stir well and dispense.

C.2 Blood agar - 5 %

Tryptone soy agar (Biolab)	38.0 g
Horse blood	50.0 mL
Distilled water: make to	1000 mL

D. Media for biochemical fermentation

D.1 Fermentation broth base

Purple broth base (Difco)	24.0 g
Thioglycollate medium without dextrose or indicator	12.0 g

Carbohydrates (0.5 % w/v)	5.0 g
Distilled H_2O: make to	1000 mL

D.2 Purple broth base (Difco)

Beef extract	1.0 g
Peptone	10.0 g
NaCl	5.0 g
Brom-cresol purple	0.015 g

D.3 Carbohydrates

Composition per liter of fermentation broth

Mannitol	5.0 g
Sorbitol	5.0 g
Raffinose	5.0 g
Melibiose	5.0 g
Bacitracin broth (bacitracin in mannitol broth)	200 U

Carbohydrates were added to basal media before autoclaving.

D.4 Arginine broth

Tryptone	5.0 g
Yeast extract	5.0 g
di-Potassium hydrogen orthophosphate (K_2HPO_4)	2.0 g
L-Arginine monohydrochloride	3.0 g
Glucose	0.5 g
Distilled H_2O: make to	1000 mL

The media was dispensed in sterile, flat bottomed micro-titration trays with lids and filled with 200µl of each fermentation broth.

D.5 Bile aesculin agar

Bile aesculin agar (Biolab)	62.5 g
Distilled H_2O: make to	1000 mL

E Freezing and storage of bacterial cells (-70°C)

E.1 Freezing medium

15% sterile glycerol

20% foetal calf serum

65% Brain heart infusion broth used for bacterial cells)

The pelleted bacterial test cells were suspended in the storage medium, and aliquoted into micro-tubes (Elkay, Galway, Ireland) and stored at -20°C.

F Reagents for DNA extraction

F.1 STE buffer

1 M Tris-HCl (100 mM) [pH 8]	10.0 mL
0.5 M EDTA (10 mM) [pH 8]	2.0 mL
3 M NaCl (15 mM)	5.0 mL
Distilled H_2O: make to	100 mL

F.2 10×TE (Tris-EDTA) buffer

1 M Tris-HCl [pH 8]	10.0 mL
0.5 M EDTA [pH 8]	2.0 mL
Distilled H_2O: make to	100 mL

F.3 Chloroform-isoamyl alcohol

Chloroform ($CHCl_3$)	24 parts
isoamyl alcohol	1 part

F.4 Agarose gel electrophoresis

F.4.1 10×TBE (Tris-borate-EDTA) buffer

Boric acid	55.0 g
Tris base	108.0 g
0.5 M EDTA [pH 8]	40.0 mL
Distilled H_2O: make to	1000 mL

F.4.2 Agarose gel

Agarose D-1 low EEO (Pronadisa, Madrid, Spain)	0.75%-2%

Agarose was mixed with 1×TBE buffer in a volumetric flask and the weight was noted. The mixture was heated in a microwave oven, and judged to be dissolved when the solution became optically clear. The mixture was re-weighed and topped up with distilled water to the original weight to maintain the correct agarose concentration. The agarose was cooled to approximately 55°C and poured into a submarine horizontal gel unit to form a 30 mm thick gel.

F.4.3 Ethidium bromide

Ethidium bromide (USB, Amersham, Buckinghamshire)	10 mg/mL

An appropriate quantity from a 10 mg/ml ethidium bromide stock, was added to the cooled agarose to form a final concentration of 0.3 µg/ml prior to pouring the gel.

F.4.4 10×gel-loading buffer

Bromophenol blue (Saarchem)	0.25%
Xylene cyanol (ACE)	0.25%
Orange G (Saarchem)	0.25 %
glycerol	30%

G Batch culture medium and complex synthetic medium

G.1 Basal synthetic media for carbohydrate utilisation (234)

Composition: g/L

ammonium dihydrogen orthophosphate ($NH_4H_2PO_4$)	1.0 g
potassium chloride (KCl)	0.2 g
magnesium sulphate ($MgSO_4$)	0.2 g
Distilled H_2O: make to	1000 mL

Add:

20 g (w/v) [2 %] of the following staple food combinations and single foods to the Basal synthetic medium.

1.1 Maize-meal (soft)

1.2 Samp

1.3 Brown bread

1.4 Maize- meal (soft) + milk + sugar (refined carbohydrate + dairy)

1.5 Maize-meal (stiff) + gravy [tomato & onion] (refined carbohydrate + fat + protein)

1.6 Samp + kidney beans (refined and complex carbohydrates)

1.7 Bread + margarine + peanut butter (refined carbohydrate + fat)

1.8 3 % sucrose (w/v)

G.2 Phosphate buffered saline (pH 7.5)

NaCl	8.0 g
Potassium chloride (KCl)	0.2 g
KH_2PO_4	0.12 g
di-Sodium orthophosphate (Na_2PO_4)	0.91 g
Distilled H_2O: make to	1000 mL

H Reagents for analysis of volatile and non-volatile acids

H.1 0.3 M oxalic acid

Oxalic acid ($H_2C_2O_4.2H_2O$)	9.46 g
Distilled H_2O: make to	250 mL

H.2 Preparation of volatile acids by ether extraction

Reagents:

A. Volatile acids standards mix (# 4-6975) (Supelco, Inc, Bellefonte, PA, USA)

B. Internal Standard: caproic acid, 1.26 mL in 100 mL distilled water

C. 50 % H_2SO_4

D. Ether

E. Silica gel with indicator (dried in oven)

One mL of culture supernatant and standard acid mix were acidified with 20 µL of 50

% aqueous H_2SO_4 to which 1 mL of ether was added. Samples were mixed, centrifuged and the ether layer removed to a microcentrifuge tube. Traces of water was removed by adding silica gel crystals.

H.3 Preparation of non-volatile acids by methylation

Reagents:

A. Non-volatile acids standard mix (# 4-6985) (Supelco, Inc, Bellefonte, PA, USA)
B. Internal Standard: benzoic acid, 0.12 g in 100 mL distilled water
C. 50 % aqueous H_2SO_4
D. Methanol
E. Chloroform

One mL of culture supernatant and standard acid mix were acidified with 0.4 ml H_2SO_4 and 1 mL methanol. Samples were derivatised by standing overnight at room temperature. One mL of chloroform was added to the mixture and the sample centrifuged. The top aqueous phase was discarded and the chloroform phase removed into clean microcentrifuge tubes

I Measurement of water-insoluble ECP (291)

Reagents:

A. 1 % phenol
B. H_2SO_4
C. D-glucose

A standard curve using glucose as standard, with concentrations ranging between 0.1 mg/mL to 4 mg/mL was constructed. Five hundred µL of phenol and 2.5 mL H_2SO_4 were added to 100 µL samples, mixed and allowed to stand at room temperature for 25 minutes. Measurement were made at 485 nm on a spectrophotometer, and the concentrations of the samples were determined from the standard curve by plotting the sugar concentration *vs* the absorbance. The amount of colour produced at a constant

phenol concentration was proportional to the amount of sugar present.

J Measurement of glucose

The UV-method for the enzymatic determination of D-glucose (R-BIOPHARM GmbH, Darmstadt, Germany), Cat.No. 0716251, was used. The protocol followed was as per instruction provided by the kit.

Solution 1:	NADP	110 mg
	ATP	260 mg
	Magnesium sulphate	
	Triethanolamine buffer	
	Distilled water	45 mL
	pH	7.6
Solution 2:	Hexokinase	320 U
	Glucose-6-phosphate dehydrogenase	160 U

K Measurement of extracellular proteins (293)

Reagents:

A.	Solution A :	
	Na_2CO_3 (sodium carbonate)	10.0 g
	NaOH (sodium hydroxide)	2.0 g
	Distilled H_2O; make to	500 mL
B.	Solution B:	
	$CuSO_4 \cdot 5H_2O$ (copper sulphate)	0.5 g
	$Na_3C_6H_5O_7 \cdot 2H_2O$ (sodium citrate)	1.0 g
	Distilled H_2O: make to	50.0 mL
C.	Solution C:	
	Folin-Ciocalteu phenol reagent (Merck, Germany)	10.0 mL
	Distilled H_2O	10.0 mL

D. Solution D:

Solution A	50.0 mL
Solution B	0.5 mL

Solution D (2.5 mL) was added to 100 µL of sample, vortexed and allowed to stand for 10 minutes at toom temperature. Two hundred and fifty µL of solution C was added and mixed. After 20-30 minutes the samples were read at A_{750}.The concentration of the unknown samples were interpolated on a standard curve using bovine serum albumin as standard.

L Reagents for SDS-PAGE eletrophoresis (Laemmli buffer system) (295)

L.1 Acrylamide/Bis (30% T, 2.67% C)

Acrylamide	54.4 g
N'N'-bis-methylene-acrylamide	1.6 g
Distilled H_2O: make to	200 mL

Solution was filtered and stored at 4°C in the dark.

L.2 1.5 M Tris-HCl pH 8.8 (Resolving buffer)

Tris base	36.3 g
Distilled H_2O: make to	200 mL

Solution was adjusted to pH 8.8 with HCL.

L.3 0.5 M Tris-HCL pH 6.8 (Stacking buffer)

Tris base	3.0 g
Distilled H_2O: make to	50 mL

Solution was adjusted to pH 6.8 with HCL.

L.4 10% Sodium dodecylsulphate

Sodium dodecylsuphate (SDS)	5.0 g
Distilled H_2O: make to	50 mL

L.5 Initiator (APS)

Ammonium persulphate $(NH_4)_2S_2O_8$	0.1 g
Distilled H_2O: make to	1.0 mL

L.6 2X treatment buffer

Tris (0.5 M Tris-Cl, pH 8.8)	2.5 mL
SDS (10% w/v)	4.0 mL
Glycerol	2.0 mL
2-mercaptoethanol	1.0 mL
Bromophenol blue 0.05% (w/v)	0.2 mL
Distilled H_2O: make to	10 mL

L.7 Tank buffer (pH 8.3) (0.025 M Tris, pH 8.3, 0.192 M glycine, 0.1% SDS)

Tris-base	12.1 g
Glycine	56.6 g
SDS	4.0 g
Distilled H_2O: make to	4.0 L

L.8 NNN'N'-tetramethylethylenediamine (TEMED)

L.9 Butan-1-ol AR

L.10 Preparation of 1.5 mm Laemmli gels

	Separating gel	Stacking gel
Acrylamide/Bis	6.6 mL	1.3 mL
Resolving buffer	5.0 mL	
Stacking buffer		2.5 mL
10% SDS	0.2 mL	0.1 mL
10% $(NH_4)_2S_2O_8$ (APS)	0.05 mL	0.05 mL
TEMED	0.005 mL	0.005 mL
Distilled H_2O	9.7 mL	6.1 mL
Final volume	20 mL	10 mL

The acrylamide/bis, resolving buffers, 10% SDS and distilled water were combined and

degassed for 5 minutes. The catalysts APS and TEMED were added to the mixture. The solution was poured into the gel caster and overlaid with butan-1-ol to facilitate polymerisation. The gel was allowed to set for 1 hour after which the stacking gel was combined in the same manner and the solution poured over the separating gel. A comb to form 10 wells was inserted into the gel sandwich and left to polymerise for 45 minutes. The final size of the gel was 8X10 cm. Prior to use, the wells were rinsed thoroughly with distilled water.

L.11 **Polychromatic silver stain** (296)

Silver stain:	
Silver nitrate	0.48 g
Distilled H_2O	250 mL
Reducing solution:	
NaOH	30 g
Distilled H_2O	1 L
Immediately before use, add 37% formaldehyde	7.5 mL
Colour enhancer:	
Na_2CO_3	70.5 g
Distilled H_2O: make to	10 L

M Glucosyltransferase activity

M.1 **Schiff's reagent** (298)

Pararosaniline chloride	1 g
Distilled H_2O	200 mL
Potassium metabisulphite	2 g
Hydrochloric acid	2 mL
Activated charcoal	2 g

Dissolve the pararosaniline in the boiling distilled water and cool the mixture to 50°C, then add the metabisulphite. Mix well and cool the mixture to 25°C. Add hydrochloric acid and activated charcoal. Mix thoroughly and stand in the dark at 4°C for 24 hours. Filter, and store in a dark bottle at 4°C.

N RNA extraction

N.1 STE buffer

Tris-HCL(pH 8)	100 mM
EDTA (pH 8)	10 mM
NaCl	150 mM

Stored at room temperature.

N.2 10 × TE buffer

Tris-HCL(pH 8)	100 mM
EDTA (pH 8)	10 mM
Distilled water	1000 mL

Stored at room temperature.

N.3 Protein kinase (PK) buffer

Tris (pH 7.5)	50 mM
EDTA	5 mM
SDS (w/v)	0.5 %

N.4 RNA lysis solution

Sucrose	0.5 M
Sodium acetate (pH 5.2)	10 mM
SDS	1 %

N.5 Chloropan

Phenol: Chloroform: Isoamyl alcohol	25:24:1

N.6 Chloroform: isoamyl alcohol 24:1

N.7 DNase buffer (321)

Sodium acetate	0.1 M
$MgSO_4$ (pH 5.0)	5 mM

O RT-PCR

O.1 Reverse Transcription mix

	Final concentration	
Nuclease-Free water		7.3 µL
ImProm-II 5× Reaction Buffer		4.0 µL
$MgCl_2$ (25 mM)	1.5 mM	1.2 µL
dNTP Mix, 10 mM each dNTP	0.5 mM	1.0 µL
Recombinant Rnasin® Ribonuclease Inhibitor	20 U	0.5 µL
ImProm-II™ Reverse Transcriptase		1.0 µL
Final Volume		15.0 µL

P Suppliers of culture media and chemical reagents

1. Saarchem, Biolab
   Merck (SA) (Pty) Ltd
   1 Friesland Drive
   Longmeadow Business Estate, Modderfontein

2. ACE
   Associated Chemical Enterprises c.c.
   30 Mandy Road, Reuven Estates
   Glenvista

3. Difco
   The Scientific Group (Pty) Ltd
   Elevation Close, Waterfall Park,
   Bekker Street, Midrand

4. USB
   AEC-Amersham (Pty) Ltd
   15th & Virginia Street,
   Marlboro, Sandton

5. Pronadisa
   Separation Scientific (SA) (Pty) Ltd
   Honeydew Centre, Beyers Naude drive,
   Honeydew

6. Promega
   Whitehead Scientific (Pty) Ltd.
   P O Box 194,
   Brackenfell

7. Qiagen/Abgene
   Southern Cross Biotechnology (Pty) Ltd
   P O Box 1799,
   Pinegowrie

8. Sigma-Aldrich S.A.(Pty) Ltd
   CNR Kelly and Ackerman Streets
   Southern Life Industrial Park
   Unit 16/17
   Jet park

9. Roche molecular chemicals
   Roche Diagnostics (Pty) Ltd
   9 Will Scarlet Road, Randburg

10. Supelco
Anatech Instruments (Pty) Ltd
Meadowbrook Office Park
Jacaranda Avenue, Olivedale

11. Fermentas
Inqaba Biotec Industries (Pty) Ltd
P O Box 14356
Hatfield, Pretoria

APPENDIX IV

RAW DATA I

Patient number, ethnic group, area, fermentation profile, caries group, total viable count, *gtf* and 16s rRNA genes of mutans streptococci isolates from 5-year-old children (c) and their mothers (m).

Obs	caseno	race	area	relat	profile	ds	dmfs	mstvc	gtfA	gtfBC	gtfI	16s
1	1501941	c	5	c	1000000	0	0	500	0	0	1	1
2	1501942	c	5	c	1234007	0	0	500	1	1	0	1
3	1501943	c	5	c	1234007	0	0	500	0	0	0	1
4	1530111	c	5	c	1034007	2	2	2400	1	1	0	1
5	2530112	c	5	m	1234067	2	2	1000	1	1	0	1
6	1502291	c	5	c	1234067	2	2	100	0	1	0	1
7	2502292	c	5	m	1234007	2	2	99000	0	1	0	1
8	2502293	c	5	m	1230060	2	2	99000	0	0	1	1
9	1530141	c	5	c	1234067	1	1	29000	1	1	0	1
10	1530142	c	5	c	1234067	1	1	29000	1	1	0	1
11	1501962	b	5	c	1234067	6	6	1900	1	1	0	1
12	1501963	b	5	c	1200067	6	6	1900	0	1	0	1
13	2501964	b	5	m	1234067	6	6	26000	1	1	0	1
14	1530161	b	5	c	1200007	6	6	510	0	1	0	1
15	1530162	b	5	c	1234067	6	6	510	1	1	0	1
16	2530163	b	5	m	1234007	6	6	8700	1	1	0	1
17	1530181	c	5	c	1234007	9	1	930	1	1	0	1
18	1530182	c	5	c	1230007	9	1	930	1	1	0	1
19	1530183	c	5	c	1234000	9	1	930	1	1	0	1
20	2530184	c	5	m	1230060	9	1	1000	0	0	0	1
21	2530185	c	5	m	1234007	9	1	1000	1	0	0	1
22	1530151	c	5	c	1234007	1	1	980	1	1	0	1
23	2530152	c	5	m	1234000	1	1	18000	1	1	0	1
24	2530153	c	5	m	1234007	1	1	21000	1	1	0	1
25	1501891	c	5	c	1230060	31	1	1400	0	1	0	1
26	1501892	c	5	c	1234007	31	1	1400	1	1	0	1
27	2501893	c	5	m	1234007	31	1	8900	1	1	0	1
28	1530121	c	5	c	1200060	2	2	2000	0	0	1	1
29	1530122	c	5	c	1200067	2	2	22000	0	1	0	1
30	1530123	c	5	c	1234067	2	2	22000	0	1	0	1
31	1504521	c	11	c	1234067	9	9	20	1	1	0	1
32	2504522	c	11	m	1234067	9	9	260	1	1	0	1
33	1530321	c	11	c	1234060	6	6	3200	1	1	0	1
34	1530322	c	11	c	1000060	6	6	830	0	1	0	0
35	2530324	c	11	m	1234067	6	6	4700	1	1	0	1
36	1504371	c	11	c	1200060	0	0	26000	0	1	0	1
37	2504372	c	11	m	1230067	0	0	30	1	1	0	1
38	1530311	b	11	c	1234067	9	9	80	1	1	0	1
39	1530312	b	11	c	1234067	9	9	1000	1	1	0	1
40	2530313	b	11	m	1234067	9	9	1100000	1	1	0	0
41	1503051	c	8	c	1234067	21	1	180	1	1	0	1
42	2503052	c	8	m	1234067	21	1	700	1	1	0	1

Obs	caseno	race	area	relat	profile	ds	dmfs	mstvc	gtfA	gtfBC	gtfI	16s
43	1503111	c	8	c	1234067	25	25	7100	1	1	0	1
44	1503112	c	8	c	1200060	25	25	7100	0	0	1	1
45	1503113	c	8	c	1234067	25	25	26000	1	1	0	1
46	2503062	c	8	m	1200060	0	0	800	0	1	0	1
47	1503031	c	8	c	1234067	2	2	19	1	1	0	1
48	2503032	c	8	m	1234067	2	2	670000	1	1	0	1
49	1530491	c	11	c	1030567	1	1	2200	1	0	0	1
50	1530492	c	11	c	1200067	1	1	40	1	1	0	1
51	2530493	c	11	m	1030007	1	1	24000	1	0	0	1
52	2530494	c	11	m	1030007	1	1	24000	1	0	0	1
53	2530495	c	11	m	1030067	1	1	24000	0	0	1	1
54	1504703	c	11	c	1200060	21	1	220	0	1	0	1
55	2504704	c	11	m	1230007	21	1	810000	1	1	0	1
56	1530501	c	11	c	1234067	14	14	1300	1	1	0	1
57	1530502	c	11	c	1234067	14	14	1100	1	1	0	1
58	2530503	c	11	m	1200007	14	14	280000	1	1	0	1
59	2530504	c	11	m	1234067	14	14	280000	1	1	0	1
60	1530511	c	11	c	1234060	7	7	1300	1	1	0	1
61	2530512	c	11	m	1000060	7	7	4100	0	0	1	1
62	2530513	c	11	m	1234067	7	7	4100	1	1	0	1
63	2530514	c	11	m	1234067	7	7	4100	1	1	0	1
64	1516291	b	24	c	1200067	0	0	20	0	1	0	1
65	1516292	b	24	c	1234067	0	0	110000	1	1	0	1
66	2516293	b	24	m	1200067	0	0	110000	0	1	0	1
67	2516294	b	24	m	1234067	0	0	110000	1	1	0	1
68	1516141	b	24	c	1234067	2	2	140000	1	1	0	1
69	1516142	b	24	c	1234067	2	2	140000	1	1	0	1
70	1516143	b	24	c	1230060	2	2	140	0	1	0	1
71	1512191	b	24	c	1234067	0	0	400	1	1	0	1
72	1512192	b	24	c	1234067	0	0	430	0	1	0	1
73	2512193	b	24	m	1000060	0	0	220	0	0	1	1
74	2512194	b	24	m	1234067	0	0	160	1	1	0	1
75	2512195	b	24	m	1200060	0	0	830	0	1	0	1
76	1512141	b	24	c	1234067	8	8	43000	1	1	0	1
77	2512142	b	24	m	1234067	8	8	8100	1	1	0	1
78	1512091	b	24	c	1234067	6	6	200000	1	1	0	1
79	1512092	b	24	c	1200060	6	6	200000	0	0	1	1
80	1531391	b	24	c	1234060	0	0	240	0	0	0	1
81	2531392	b	24	m	1234060	0	0	24000	1	1	0	1
82	1513241	b	24	c	1234067	4	4	43000	1	1	0	1
83	1519861	b	24	c	1234067	6	6	100	1	1	0	1
84	2519863	b	24	m	1200060	6	6	4	0	0	0	1
85	2519864	b	24	m	1234067	6	6	26000	1	1	0	1
86	1514841	b	24	c	1000060	4	4	40000	0	1	1	1
87	2514843	b	24	m	1000500	4	4	120000	0	1	0	1
88	2514844	b	24	m	1234060	4	4	390	1	1	0	1
89	1516501	b	24	c	507	4	4	20	0	0	1	1
90	1516502	b	24	c	507	4	4	30	1	1	0	1
91	1516503	b	24	c	30507	4	4	11000	1	1	0	1
92	2516504	b	24	m	1000500	4	4	30000	0	1	0	1
93	2516505	b	24	m	1234067	4	4	30000	1	1	0	1
94	1516022	b	24	c	1234060	4	4	8100	0	1	0	1
95	2516021	b	24	m	507	4	4	33000	1	1	0	1
96	1505281	b	24	c	1234067	0	0	16000	1	1	0	1
97	2505282	b	24	m	1234067	0	0	100	1	1	0	1
98	1505301	b	24	c	1234067	1	1	40	1	1	0	1

Obs	caseno	race	area	relat	profile	ds	dmfs	mstvc	gtfA	gtfBC	gtfI	16s
99	1517331	b	24	c	1200060	2	2	3100	0	0	0	1
100	1512701	b	24	c	1234067	0	0	83000	1	1	0	1
101	2512702	b	24	m	1234067	0	0	41000	1	1	0	1
102	1512391	b	24	c	1200060	0	0	6100	0	1	0	1
103	2512392	b	24	m	1234067	0	0	22000	1	1	0	1
104	1512821	b	24	c	1234060	0	0	41	1	1	0	1
105	1512822	b	24	c	1234067	0	0	61	1	1	0	1
106	1512511	b	24	c	1234067	5	5	210000	1	1	0	1
107	1512512	b	24	c	1200067	5	5	210000	1	1	0	1
108	2512513	b	24	m	1234067	5	5	770000	1	1	0	1
109	2531402	b	24	m	1234007	8	8	150000	0	1	0	1
110	2505311	b	24	m	1000067	0	0	670000	1	1	0	1
111	1505312	b	24	c	1234067	0	0	41000	1	1	0	1
112	2505313	b	24	m	1030560	0	0	670000	1	1	0	1
113	1519361	b	24	c	1234007	0	0	100	1	1	0	1
114	2519362	b	24	m	1234007	0	0	390000	1	1	0	1
115	2523011	b	24	m	1234000	14	18	1100000	1	1	0	1
116	1523012	b	24	c	1234007	14	18	180	1	1	0	1
117	2531551	b	24	m	1200000	8	12	630000	0	1	0	1
118	1531552	b	24	c	1234007	8	12	2400	0	1	0	1
119	1507261	b	24	c	1000067	0	0	2700	0	1	0	1
120	2507262	b	24	m	1234007	0	0	710000	1	1	0	1
121	1517591	b	24	c	1234067	0	0	200	1	1	0	1
122	2517592	b	24	m	1234067	0	0	180000	1	1	0	1
123	1532481	b	24	c	1234067	1	1	13000	1	1	0	1
124	1532482	b	24	c	1234007	1	1	100	1	1	0	1
125	2532483	b	24	m	1234067	1	1	41000	1	1	0	1
126	1532752	c	8	c	1234067	3	4	2500000	1	1	0	1
127	2532753	c	8	m	1234067	3	4	61000	1	1	0	1
128	1512601	c	8	c	1234067	1	10	6000000	1	1	0	1
129	1512602	c	8	c	1234067	1	10	6000000	1	1	0	1
130	1512603	c	8	c	1234060	1	10	41	1	1	0	1
131	2512604	c	8	m	1234060	1	10	220000	0	1	0	1
132	2512605	c	8	m	1234060	1	10	220000	0	1	0	1
133	1502511	c	8	c	1234067	1	1	500	1	1	0	1
134	2502512	c	8	m	1234067	1	1	200000	1	1	0	1
135	2502513	c	8	m	1234067	1	1	200000	0	1	0	1
136	1502471	c	8	c	1204000	2	2	1800	0	1	0	1
137	1502472	c	8	c	34060	2	2	61	1	1	0	1
138	2502474	c	8	m	1234067	2	2	200000	1	1	0	1
139	1502601	c	8	c	1234067	10	14	1100	1	1	0	1
140	1502602	c	8	c	1234067	10	14	1100	1	1	0	1
141	2502603	c	8	m	1034060	10	14	1600000	1	1	0	1
142	2502604	c	8	m	1234067	10	14	1600000	1	1	0	1
143	1515321	b	24	c	1234067	14	14	3900	1	1	0	1
144	1515322	b	24	c	1000060	14	14	850000	0	0	1	1
145	1502361	c	2	c	1234067	5	6	41000	0	1	0	1
146	1502362	c	2	c	1200060	5	6	810	0	0	1	1
147	2502363	c	2	m	1200060	5	6	350000	0	0	1	1
148	1502781	c	2	c	1234060	2	21	370000	1	1	0	1
149	1502782	c	2	c	1234067	2	21	240	1	1	0	1
150	2502783	c	2	m	1234060	2	21	760000	1	1	0	1
151	1503711	c	2	c	1234060	1	1	1400000	1	1	0	1
152	2503712	c	2	m	1234067	1	1	1000000	1	1	0	1
153	1503713	c	2	c	1230060	1	1	41000	0	0	1	1
154	1500401	c	2	c	1234007	3	3	390	1	1	0	1
155	2500402	c	2	m	1234067	3	3	590000	1	1	0	1

Obs	caseno	race	area	relat	profile	ds	dmfs	mstvc	gtfA	gtfBC	gtfI	16s
156	1502891	c	2	c	1234067	0	0	20	1	1	0	1
157	2502892	c	2	m	1200060	0	0	510000	0	1	0	1
158	1533451	b	4	c	1234067	0	0	710000	1	1	0	1
159	2533452	b	4	m	1234067	0	0	13000000	1	1	0	1
160	1533481	c	9	c	1200060	4	4	510000000	0	0	1	1
161	2533482	c	9	m	1200060	4	4	280000	0	0	1	1
162	1533551	c	10	c	1200060	0	12	140000	0	0	1	1

RAW DATA II

Cut sites of *gtfB* genes by enzyme *Hae*III (h1-h7) and *Hin*fI (n1-n7), of clinical isolates and NCTC reference strains.

Obs	caseno	race	relat	h1	h2	h3	h4	h5	h6	h7	n1	n2	n3	n4	n5	n6	n7
1	1501941	c	c	3420	1221	912	746	470	443	346	1202	806	702	593	450	357	341
2	1501942	c	c	1217	738	448	349	0	0	0	2524	1387	852	0	0	0	0
3	1501943	c	c	1420	949	648	502	340	54	0	2762	1593	1040	579	493	173	0
4	1530111	c	c	1622	1142	875	345	324	0	0	591	523	382	307	0	0	0
5	2530112	c	m	1137	644	438	326	202	0	0	2089	1282	785	0	0	0	0
6	1502291	c	c	1333	910	720	648	308	193	0	1484	1022	742	622	530	340	0
7	2502292	c	m	1190	659	438	341	0	0	0	2484	1387	907	0	0	0	0
8	2502293	c	m	1221	926	758	758	478	443	352	1610	1184	691	593	443	352	316
9	1530141	c	c	1247	859	631	505	319	0	0	1873	1163	811	0	0	0	0
10	1530142	c	c	1300	897	631	515	0	0	0	1750	1245	1174	811	0	0	0
11	1501962	b	c	1018	850	618	499	316	0	0	1873	1162	803	0	0	0	0
12	1501963	b	c	1018	896	631	510	326	0	0	1837	1332	1162	803	0	0	0
13	2501964	b	m	1287	878	638	499	326	0	0	1873	1174	811	0	0	0	0
14	1530161	b	c	1200	891	634	381	295	193	102	2812	1407	1022	730	600	392	0
15	1530162	b	c	1149	1055	818	621	481	302	189	1484	1041	765	590	230	200	0
16	2530163	b	m	2573	1305	648	502	301	0	0	2666	1593	1022	783	0	0	0
17	1530181	c	c	974	793	400	269	0	0	0	2527	1960	1317	1055	914	732	492
18	1530182	c	c	2608	1179	805	134	94	0	0	2122	944	818	709	635	315	116
19	1530183	c	c	1361	929	662	513	308	202	0	2762	1456	1022	743	600	290	0
20	2530184	c	m	3181	1305	872	621	481	381	301	2355	1458	1022	743	600	53	0
21	2530185	c	m	1699	1142	831	645	500	477	143	2056	1404	780	112	0	0	0
22	1530151	c	c	1233	850	631	516	408	333	0	1855	1140	795	0	0	0	0
23	2530152	c	m	1287	887	631	516	333	0	0	1892	1174	810	0	0	0	0
24	2530153	c	m	1233	850	631	516	408	333	0	1855	1140	795	0	0	0	0
25	1501891	c	c	1184	930	886	647	0	0	0	1269	960	684	511	469	359	0
26	1501892	c	c	1190	674	438	341	259	0	0	2407	1303	865	0	0	0	0
27	2501893	c	m	1304	1169	896	709	561	123	99	1979	1234	1005	872	147	127	0
28	1530121	c	c	1117	858	713	543	417	317	0	1149	1053	673	628	548	413	311
29	1530122	c	c	1190	659	438	326	211	0	0	2296	1387	907	0	0	0	0
30	1530123	c	c	1190	674	438	341	211	0	0	2333	1387	907	0	0	0	0
31	1504521	c	c	752	572	505	365	0	0	0	4906	2149	1054	0	0	0	0
32	2504522	c	m	752	572	505	164	0	0	0	5093	2070	1094	0	0	0	0
33	1530321	c	c	896	586	505	357	0	0	0	4384	2149	1093	0	0	0	0
34	1530322	c	c	1550	469	384	331	0	0	0	5289	2231	810	0	0	0	0
35	2530324	c	m	716	558	356	191	0	0	0	5094	2149	1179	0	0	0	0
36	1504371	c	c	1550	716	558	169	0	0	0	4906	2149	1224	0	0	0	0
37	2504372	c	m	572	0	0	0	0	0	0	5491	2149	672	0	0	0	0
38	1530311	b	c	558	480	0	0	0	0	0	4384	2231	1179	0	0	0	0
39	1530312	b	c	811	558	492	182	0	0	0	4906	2317	1136	0	0	0	0
40	2530313	b	m	810	550	450	0	0	0	0	4906	2320	780	0	0	0	0
41	1503051	c	c	1700	1080	882	381	308	246	0	2335	1786	0	0	0	0	0
42	2503052	c	m	1985	1237	1134	419	0	0	0	2075	0	0	0	0	0	0
43	1503111	c	c	1030	868	645	516	333	0	0	1855	1162	802	0	0	0	0
44	1503112	c	c	1063	810	680	541	417	0	0	985	597	518	390	310	0	0
45	1503113	c	c	1030	868	645	516	333	0	0	1855	1162	802	0	0	0	0
46	2503062	c	m	2294	1134	801	555	489	0	0	2386	1864	0	0	0	0	0
47	1503031	c	c	1167	1070	832	623	513	356	314	1767	1138	816	0	0	0	0
48	2503032	c	m	1178	832	647	605	513	349	317	1844	1175	861	0	0	0	0

Obs	caseno	race	relat	h1	h2	h3	h4	h5	h6	h7	n1	n2	n3	n4	n5	n6	n7
49	1530491	c	c	1375	899	607	495	377	302	0	1284	968	761	340	273	378	0
50	1530492	c	c	1285	854	708	597	471	282	246	1353	948	746	370	226	0	0
51	2530493	c	m	1190	840	635	508	419	346	311	1711	1162	833	0	0	0	0
52	2530494	c	m	1352	869	607	463	287	251	188	1339	958	753	378	0	0	0
53	2530495	c	m	1084	811	650	358	278	98	0	1022	640	542	411	335	237	0
54	1504703	c	c	1423	899	607	463	282	185	0	1231	910	715	319	294	359	0
55	2504704	c	m	1631	1472	1083	191	0	0	0	1339	1156	938	715	0	0	0
56	1530501	c	c	1178	1080	824	686	617	500	332	2237	1825	1150	816	0	0	0
57	1530502	c	c	1167	824	599	504	370	342	302	1864	1150	825	0	0	0	0
58	2530503	c	m	990	840	623	513	339	308	0	1748	1188	833	0	0	0	0
59	2530504	c	m	1201	686	629	508	342	0	0	1748	1188	833	0	0	0	0
60	1530511	c	c	1167	824	623	508	423	419	346	1786	1138	816	0	0	0	0
61	2530512	c	m	1399	1160	597	455	251	185	0	1312	929	738	374	307	0	0
62	2530513	c	m	1070	990	629	513	346	0	0	1711	1162	833	0	0	0	0
63	2530514	c	m	618	463	292	181	0	0	0	1258	929	723	618	366	333	210
64	1516291	b	c	4830	4025	2511	1259	798	279	173	7446	3133	1546	203	0	0	0
65	1516292	b	c	3354	2329	1288	1203	798	158	0	8345	3279	1581	0	0	0	0
66	2516293	b	m	4941	4025	2494	1259	817	274	73	5795	3205	1581	213	0	0	0
67	2516294	b	m	6205	4118	2438	1259	817	336	166	7794	3133	2383	1581	208	61	0
68	1516141	b	c	5171	2438	1318	874	368	162	0	7446	3354	1732	213	0	0	0
69	1516142	b	c	5291	4310	2552	1318	855	351	170	7446	3354	1693	50	0	0	0
70	1516143	b	c	1199	976	918	375	356	0	0	1300	972	556	481	377	0	0
71	1512191	b	c	1260	1106	999	629	862	0	0	1802	1586	651	0	0	0	0
72	1512192	b	c	1710	1271	1117	862	635	354	0	2455	2065	1722	1016	0	0	0
73	2512193	b	m	1112	885	727	505	470	433	327	1112	669	584	555	424	386	330
74	2512194	b	m	1319	1127	1047	999	878	653	367	1691	1175	817	0	0	0	0
75	2512195	b	m	1225	846	617	490	314	278	213	1753	1144	817	0	0	0	0
76	1512141	b	c	1742	1260	862	617	490	374	314	1769	1165	809	0	0	0	0
77	2512142	b	m	1742	1138	1018	854	617	494	311	1802	1165	817	0	0	0	0
78	1512091	b	c	5171	4310	1348	855	344	286	177	8157	3591	1732	233	45	0	0
79	1512092	b	c	1101	719	470	428	307	0	0	1067	635	555	406	317	301	271
80	1531391	b	c	1488	1357	1214	0	0	0	0	1462	1186	0	0	0	0	0
81	2531392	b	m	1260	854	617	490	385	268	311	1786	1165	795	0	0	0	0
82	1513241	b	c	1095	922	695	661	529	520	0	2010	1289	896	700	0	0	0
83	1519861	b	c	1096	923	695	661	529	320	286	2010	1288	895	709	0	0	0
84	2519863	b	m	1127	1037	683	623	490	308	273	1660	1133	817	0	0	0	0
85	2519864	b	m	1096	923	695	661	529	320	286	2010	1288	895	709	0	0	0
86	1514841	b	c	1411	941	688	545	336	221	0	3136	1911	1355	932	0	0	0
87	2514843	b	m	1411	941	688	545	336	221	0	3136	1911	1355	932	0	0	0
88	2514844	b	m	1153	961	702	545	342	297	229	1930	1328	951	0	0	0	0
89	1516501	b	c	1101	849	719	465	428	327	304	1078	642	561	406	320	268	0
90	1516502	b	c	1943	1294	846	329	0	0	0	1146	678	0	0	0	0	0
91	1516503	b	c	1943	1180	789	0	0	0	0	1557	846	0	0	0	0	0
92	2516504	b	m	2200	1646	0	0	0	0	0	2171	1394	564	0	0	0	0
93	2516505	b	m	1275	1164	702	545	339	294	224	1970	1355	951	0	0	0	0
94	1516022	b	c	1078	876	712	470	433	334	307	1034	622	549	402	370	323	301
95	2516021	b	m	1805	1224	846	330	0	0	0	1420	894	255	0	0	0	0
96	1505281	b	c	1310	944	668	515	315	206	0	3193	2283	1762	1133	762	212	0
97	2505282	b	m	1323	900	661	515	321	206	0	1762	1339	1133	774	218	0	0
98	1505301	b	c	1397	1262	709	674	534	294	219	1950	1341	932	731	0	0	0
99	1517331	b	c	1052	817	694	471	422	328	297	1162	1024	789	670	0	0	0
100	1512701	b	c	1388	929	646	494	312	258	197	1931	1183	798	228	160	0	0
101	2512702	b	m	1415	929	621	514	306	268	201	2048	1230	814	228	0	0	0
102	1512391	b	c	1289	695	639	501	336	0	0	2299	1697	1151	800	643	0	0
103	2512392	b	m	1471	965	659	514	318	205	0	1970	1279	847	0	0	0	0
104	1512821	b	c	1336	1169	684	523	289	209	0	2009	1279	847	0	0	0	0
105	1512822	b	c	1442	947	684	514	325	278	209	2048	1254	847	232	163	0	0

Obs	caseno	race	relat	h1	h2	h3	h4	h5	h6	h7	n1	n2	n3	n4	n5	n6	n7
106	1512511	b	c	1500	1042	684	533	337	278	213	2216	1357	899	0	0	0	0
107	1512512	b	c	1142	1049	639	514	321	0	0	2859	2299	1739	1179	830	692	296
108	2512513	b	m	1443	1003	671	532	318	201	0	2173	1330	899	241	176	0	0
109	2531402	b	m	1336	1169	684	523	318	209	0	2008	1330	899	261	210	0	0
110	2505311	b	m	1821	1055	696	522	175	0	0	1821	1055	696	522	175	0	0
111	1505312	b	c	1455	825	541	387	281	204	166	1880	1197	836	269	0	0	0
112	2505313	b	m	1634	940	607	410	298	213	171	1795	1170	827	275	0	0	0
113	1519361	b	c	1328	895	637	516	396	322	282	2392	1874	1040	696	166	0	0
114	2519362	b	m	1258	875	630	494	393	319	279	2324	1695	1236	1025	666	168	0
115	2523011	b	m	1289	1156	886	639	508	340	0	2930	1893	1180	830	0	0	0
116	1523012	b	c	1707	969	0	0	0	0	0	834	0	0	0	0	0	0
117	2531551	b	m	1289	875	647	514	337	288	0	2413	1893	1180	830	0	0	0
118	1531552	b	c	1580	1300	692	481	246	0	0	1998	1109	855	0	0	0	0
119	1507261	b	c	1587	968	634	461	236	134	0	1880	1211	846	266	0	0	0
120	2507262	b	m	1565	954	616	461	334	243	140	1816	1183	836	278	0	0	0
121	1517591	b	c	1587	996	625	467	339	250	136	1837	1170	827	254	0	0	0
122	2517592	b	m	1732	1041	644	410	198	117	0	1800	1163	765	300	0	0	0
123	1532481	b	c	1863	1087	702	488	339	0	0	2016	1269	866	0	0	0	0
124	1532482	b	c	1587	1372	692	481	246	0	0	1998	1109	850	0	0	0	0
125	2532483	b	m	1836	1072	467	0	0	0	0	1816	1225	846	0	0	0	0
126	1532752	c	c	1900	1453	1165	386	0	0	0	1830	1734	798	0	0	0	0
127	2532753	c	m	1920	1431	1148	406	0	0	0	1824	126	568	210	0	0	0
128	1512601	c	c	1890	1432	1148	404	0	0	0	1919	1260	708	149	137	0	0
129	1512602	c	c	1856	1432	1148	550	0	0	0	2019	1369	885	0	0	0	0
130	1512603	c	c	1390	1260	963	687	540	321	210	1923	1260	885	0	0	0	0
131	2512604	c	m	1431	1148	403	0	0	0	0	1887	126	886	0	0	0	0
132	2512605	c	m	1471	991	696	533	0	0	0	1979	1296	910	0	0	0	0
133	1502511	c	c	1419	1032	866	630	517	322	0	1769	1025	666	530	183	0	0
134	2502512	c	m	1373	925	651	505	0	0	0	2497	1847	1055	696	522	180	0
135	2502513	c	m	1078	935	644	528	322	0	0	1769	1025	686	545	180	0	0
136	1502471	c	c	1336	1184	907	663	403	332	533	2327	1804	1194	830	659	0	0
137	1502472	c	c	1390	950	677	533	316	201	0	1818	1278	885	0	0	0	0
138	2502474	c	m	1391	980	677	552	0	0	0	1689	1125	686	0	0	0	0
139	1502601	c	c	1909	1358	905	665	522	322	282	1695	1426	1070	686	530	178	0
140	1502602	c	c	1419	522	298	0	0	0	0	433	344	234	0	0	0	0
141	2502603	c	m	1230	856	623	500	315	0	0	1769	1025	686	161	0	0	0
142	2502604	c	m	1021	856	630	517	315	0	0	1577	1011	657	178	0	0	0
143	1515321	b	c	1314	925	637	522	0	0	0	1769	1025	666	537	342	0	0
144	1515322	b	c	1102	1021	695	630	505	380	312	1647	1070	666	522	161	0	0
145	1502361	c	c	1400	904	592	483	0	0	0	1974	1840	1191	818	0	0	0
146	1502362	c	c	1672	1271	1202	0	0	0	0	1072	795	754	697	671	0	0
147	2502363	c	m	1672	1272	1206	0	0	0	0	1045	707	671	0	0	0	0
148	1502781	c	c	1929	1340	853	601	469	345	294	2356	1756	1164	999	799	0	0
149	1502782	c	c	1283	1125	840	601	469	294	201	1735	1150	808	0	0	0	0
150	2502783	c	m	1929	1283	840	584	469	303	198	2301	1715	1137	789	0	0	0
151	1503711	c	c	1264	828	592	469	290	204	0	1715	1123	780	639	0	0	0
152	2503712	c	m	1301	1141	853	592	469	366	290	1906	1191	808	0	0	0	0

Obs	caseno	race	relat	h1	h2	h3	h4	h5	h6	h7	n1	n2	n3	n4	n5	n6	n7
153	1503713	c	c	1672	1271	0	0	0	0	0	1050	710	697	0	0	0	0
154	1500401	c	c	1124	610	0	0	0	0	0	2094	1249	857	0	0	0	0
155	2500402	c	m	1124	885	609	0	0	0	0	2094	1248	857	0	0	0	0
156	1502891	c	c	1031	853	601	476	294	262	201	1975	1220	818	0	0	0	0
157	2502892	c	m	1062	704	639	520	335	0	0	1804	1194	840	0	0	0	0
158	1533451	b	c	1060	909	681	525	413	328	283	3097	2052	1845	1490	1115	786	505
159	2533452	b	m	1361	1334	953	681	612	525	324	3292	2540	1960	1186	810	232	0
160	1533481	c	c	1510	1405	1227	1141	927	831	794	1173	1024	802	0	0	0	0
161	2533482	c	m	1826	1524	1444	4130	7121	6115	2103	1162	1024	774	0	0	0	0
162	1533551	c	c	2073	1793	1392	1173	927	832	795	1165	1120	646	0	0	0	0
163	10449	ref		1388	920	653	546	0	0	0	1972	1210	830	0	0	0	0
164	10923	ref		1573	1389	1064	954	626	404	325	1972	1210	830	0	0	0	0

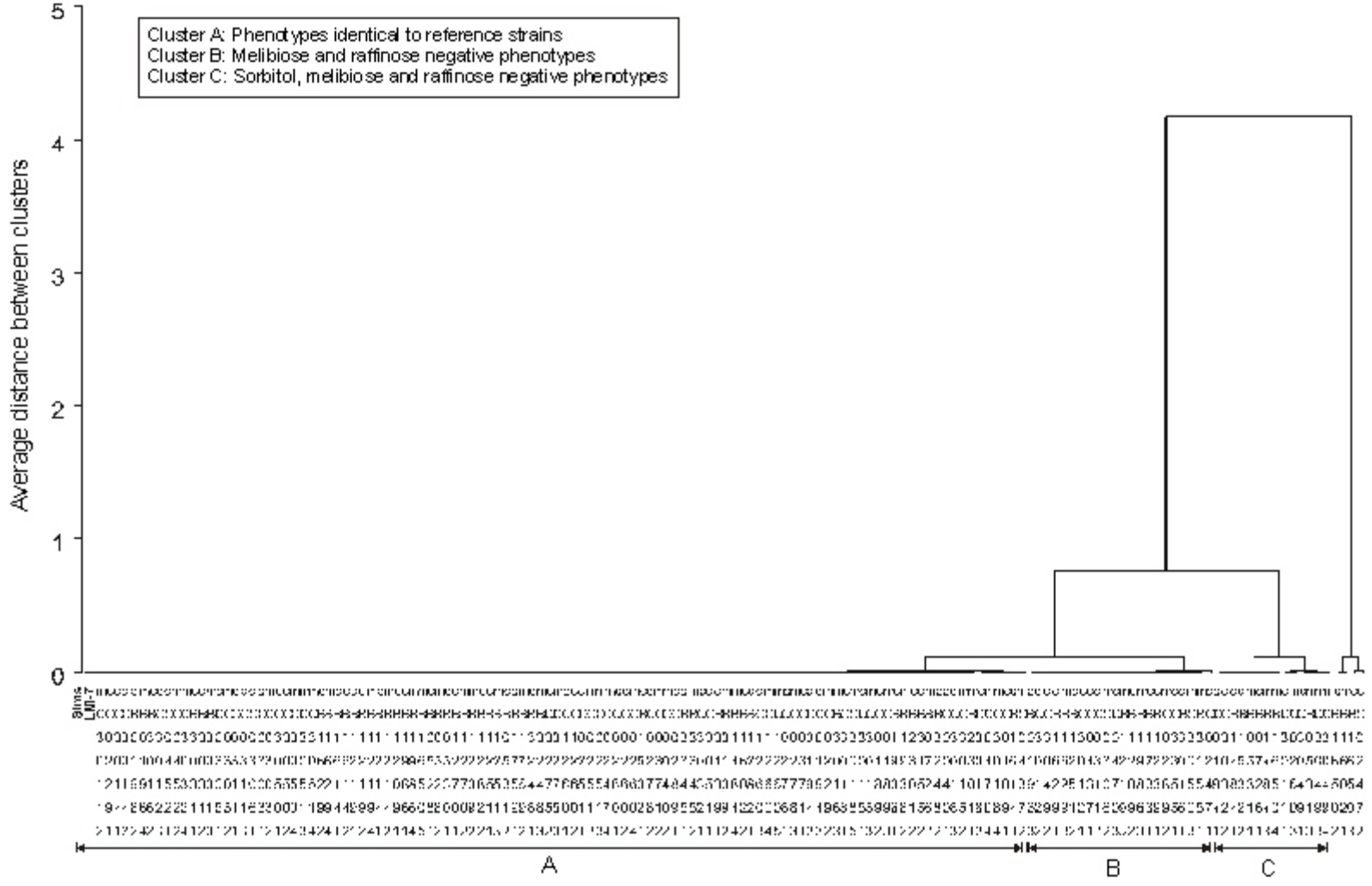
RAW DATA III

Cut sites of *gtfI* genes by enzyme *Hae*III (h1-h7) and *Hin*fI (n1-n7), of clinical isolates and NCTC reference strains.

Obs	caseno	race	relat	h1	h2	h3	h4	h5	h6	h7	n1	n2	n3	n4	n5	n6	n7
1	1501941	c	c	3420	1221	912	746	470	443	346	1202	806	702	593	450	357	34
2	2502293	c	m	1221	926	758	758	478	443	352	1610	1184	691	593	443	352	316
3	1530121	c	c	1117	858	713	543	417	317	0	1149	1053	673	628	548	413	311
4	1503112	c	c	1063	810	680	541	417	0	0	985	597	518	390	310	0	0
5	2530495	c	m	1084	811	650	358	278	98	0	1022	640	542	411	335	237	0
6	2530512	c	m	1399	1160	597	455	251	185	0	1312	929	738	374	307	0	0
7	2512193	b	m	1112	885	727	505	470	433	327	1112	669	584	555	424	386	330
8	1512092	b	c	1101	719	470	428	307	0	0	1067	635	555	406	317	301	271
9	1516501	b	c	1101	849	719	465	428	327	304	1078	642	561	406	320	268	0
10	1515322	b	c	1102	1021	695	630	505	380	312	1647	1070	666	522	161	0	0
11	1502362	c	c	1672	1271	1202	0	0	0	0	1072	795	754	697	671	0	0
12	2502363	c	m	1672	1272	1206	0	0	0	0	1045	707	671	0	0	0	0
13	1503713	c	c	1672	1271	0	0	0	0	0	1050	710	697	0	0	0	0
14	1533481	c	c	1510	1405	1227	1141	927	831	794	1173	1024	802	0	0	0	0
15	2533482	c	m	1826	1524	1444	4130	7121	6115	2103	1162	1024	774	0	0	0	0
16	1533551	c	c	2073	1793	1392	1173	927	832	795	1165	1120	646	0	0	0	0
17	12277	ref		1007	796	669	528	417	330	0	997	597	528	507	388	365	301
18	10922	ref		1018	813	676	528	417	323	0	997	604	528	507	392	365	310
19	10919	ref		1060	796	704	453	320	0	0	1008	604	534	507	388	304	0
20	11061	ref		1095	965	842	713	464	324	0	1064	634	548	527	401	314	0

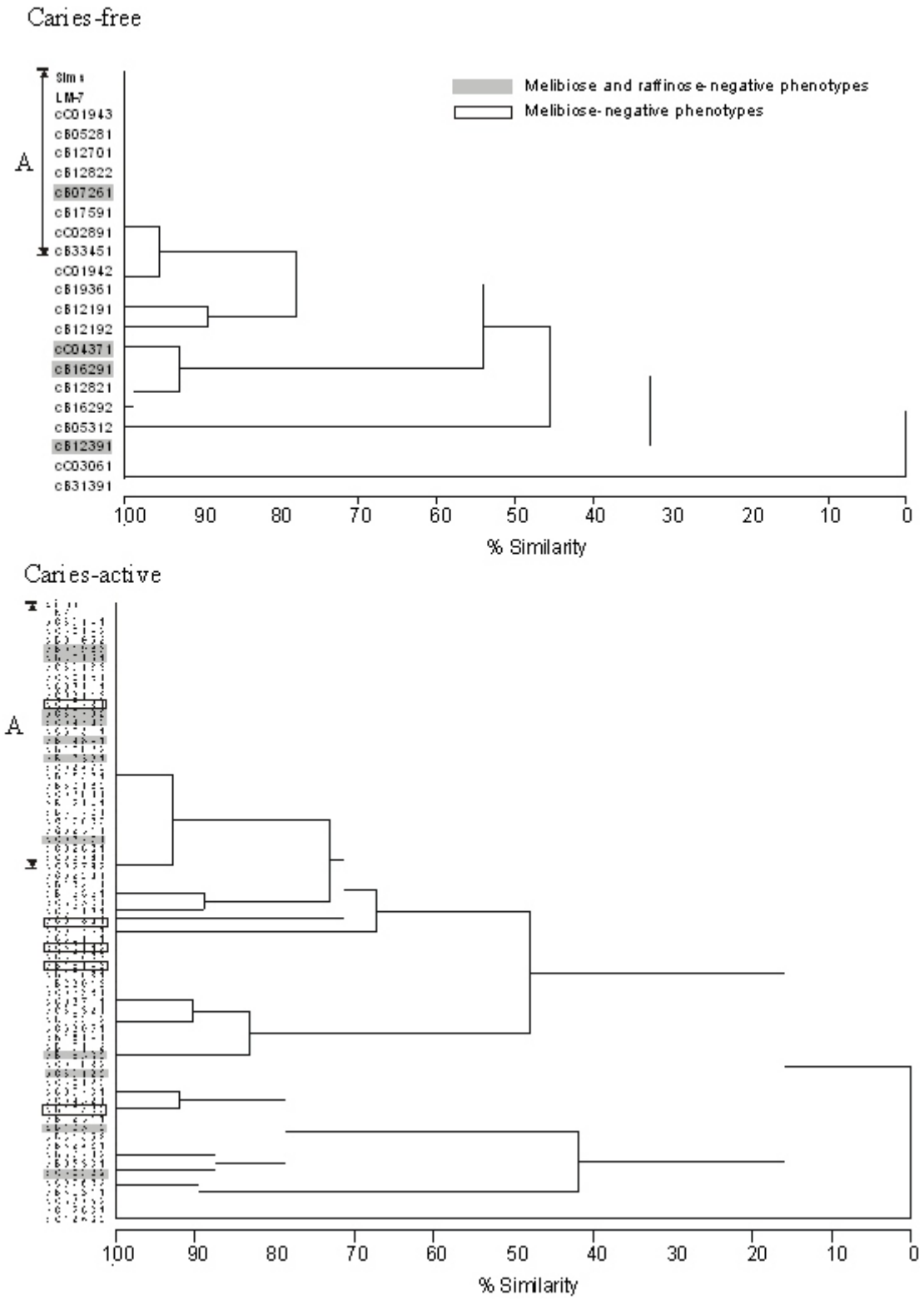
APPENDIX V

Dendogram of phenotypes produced by UPGMA clustering of *S. mutans* reference strains (Sims, LM-7) and clinical isolates (n = 172) from black African and coloured children with and without active-caries, and their mothers. B = black African, C = coloured, c = child, m = mother.



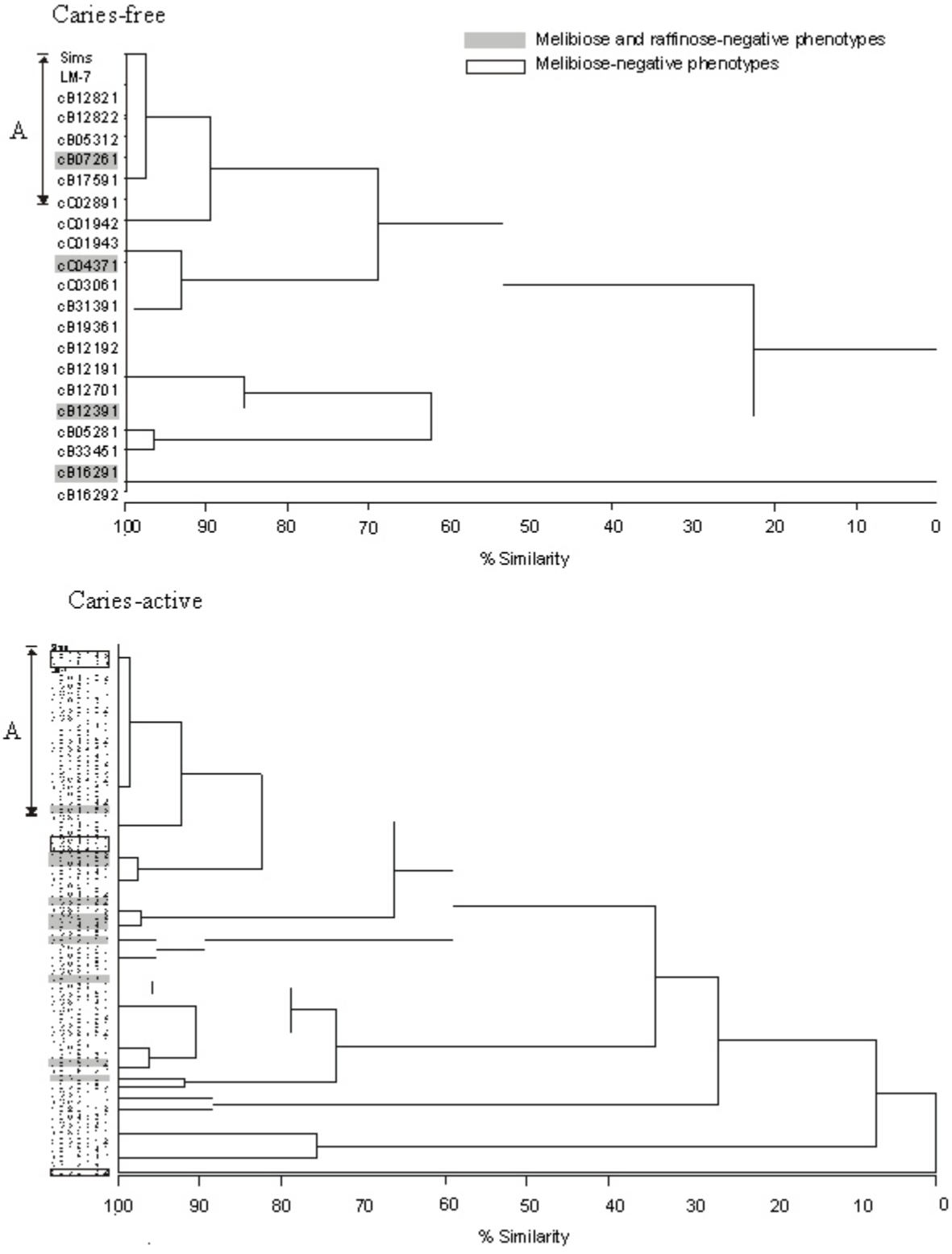
APPENDIX VI

Dendogram of *gtfB* amplitypes produced by *Hae*III digestion of *S. mutans* reference strains (Sims, LM-7) and clinical isolates from black African and coloured children without and with active-caries. B = black African, C = coloured, c = child.



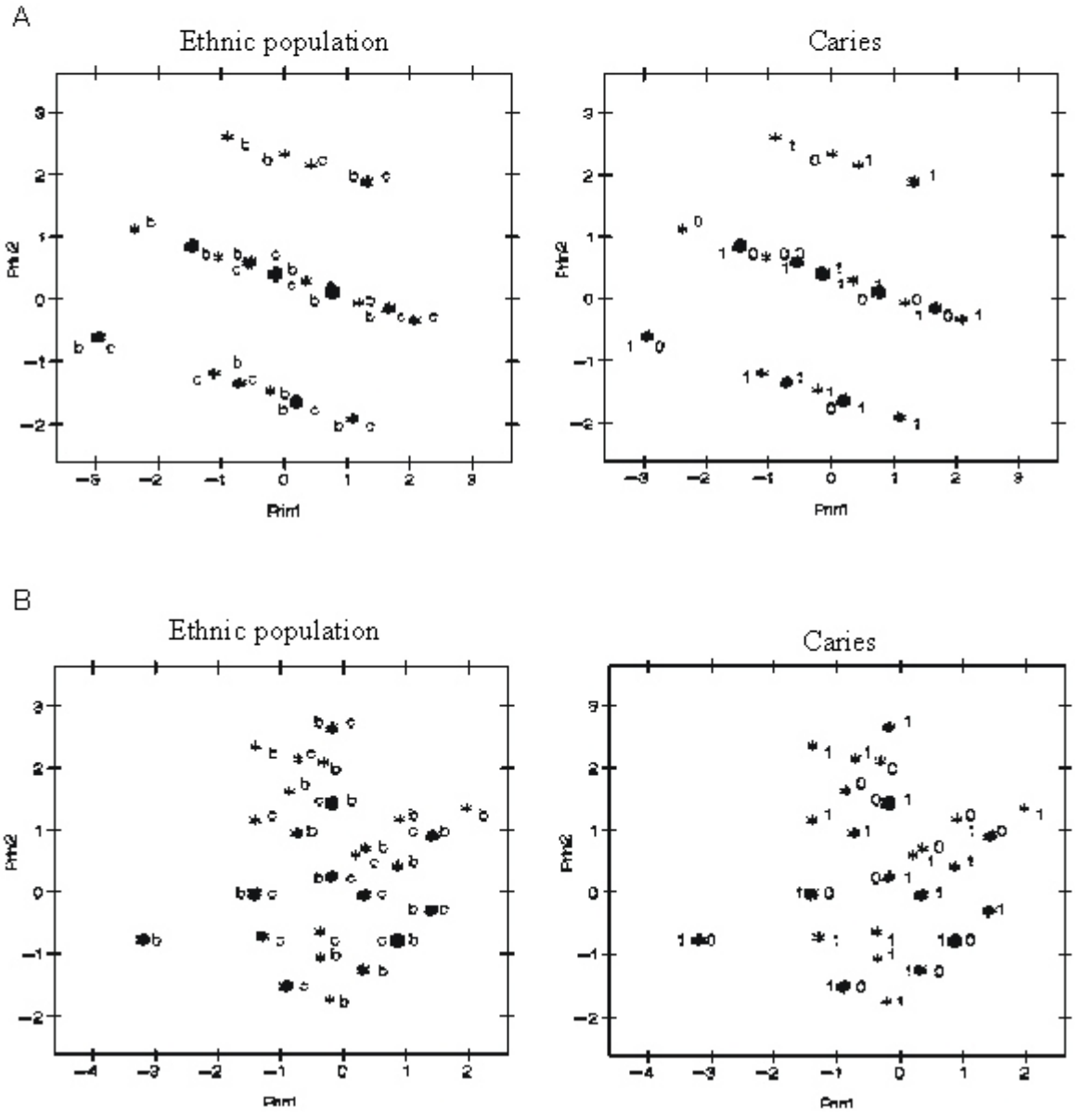
APPENDIX VII

Dendogram of *gtfB* amplitypes produced by *Hin*fI digestion of *S. mutans* reference strains (Sims, LM-7) and clinical isolates from black African and coloured children without and with active-caries. B = black African, C = coloured, c = child.



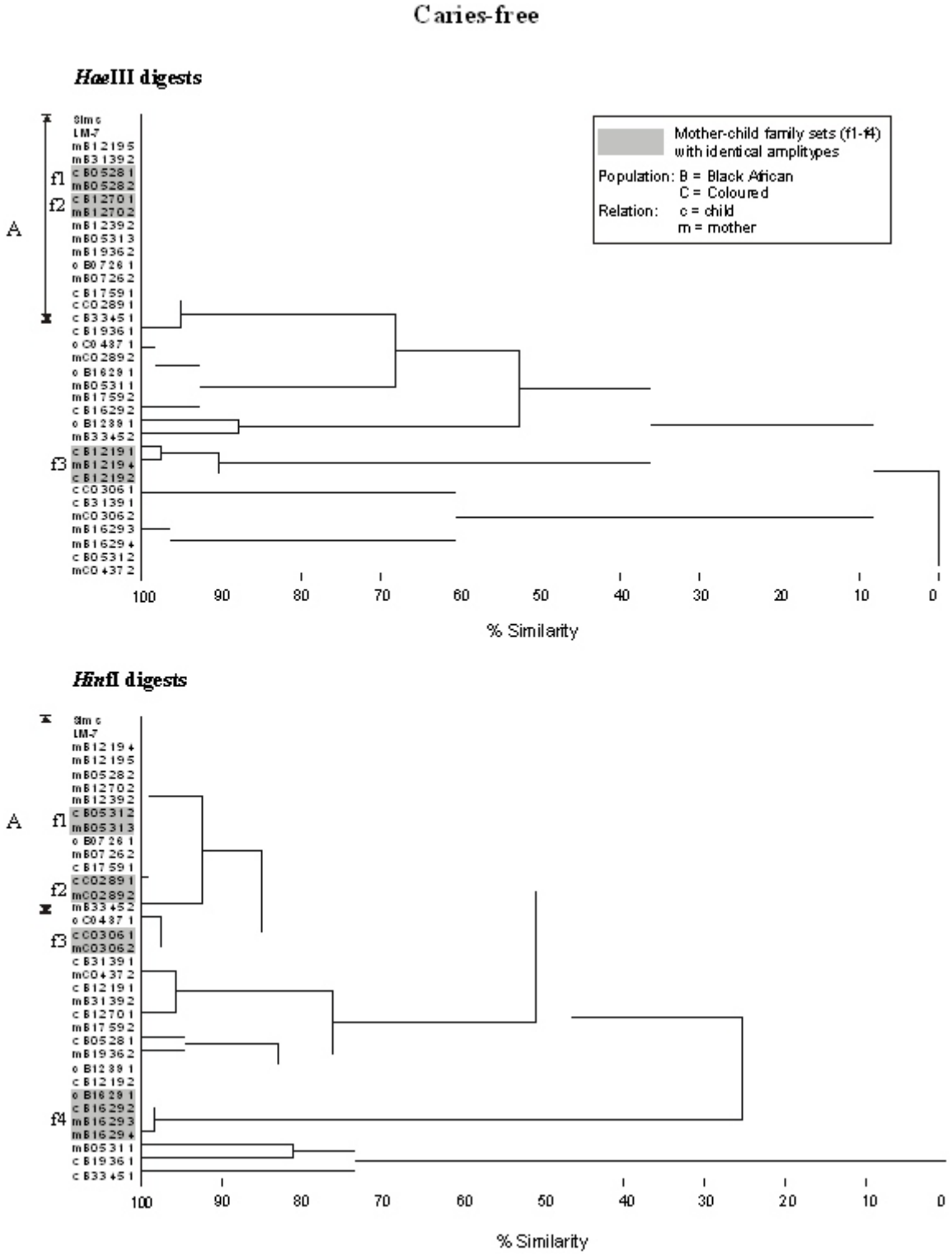
APPENDIX VIIb

Plot of the first two Principal Components: *gtfB* amplitypes produced by *Hae*III (A) and and *Hin*fI (B) enzymes, defined by ethnic (b=black African; c=coloured) and caries group (0=caries-free; 1=caries active), of 5-year-old children.



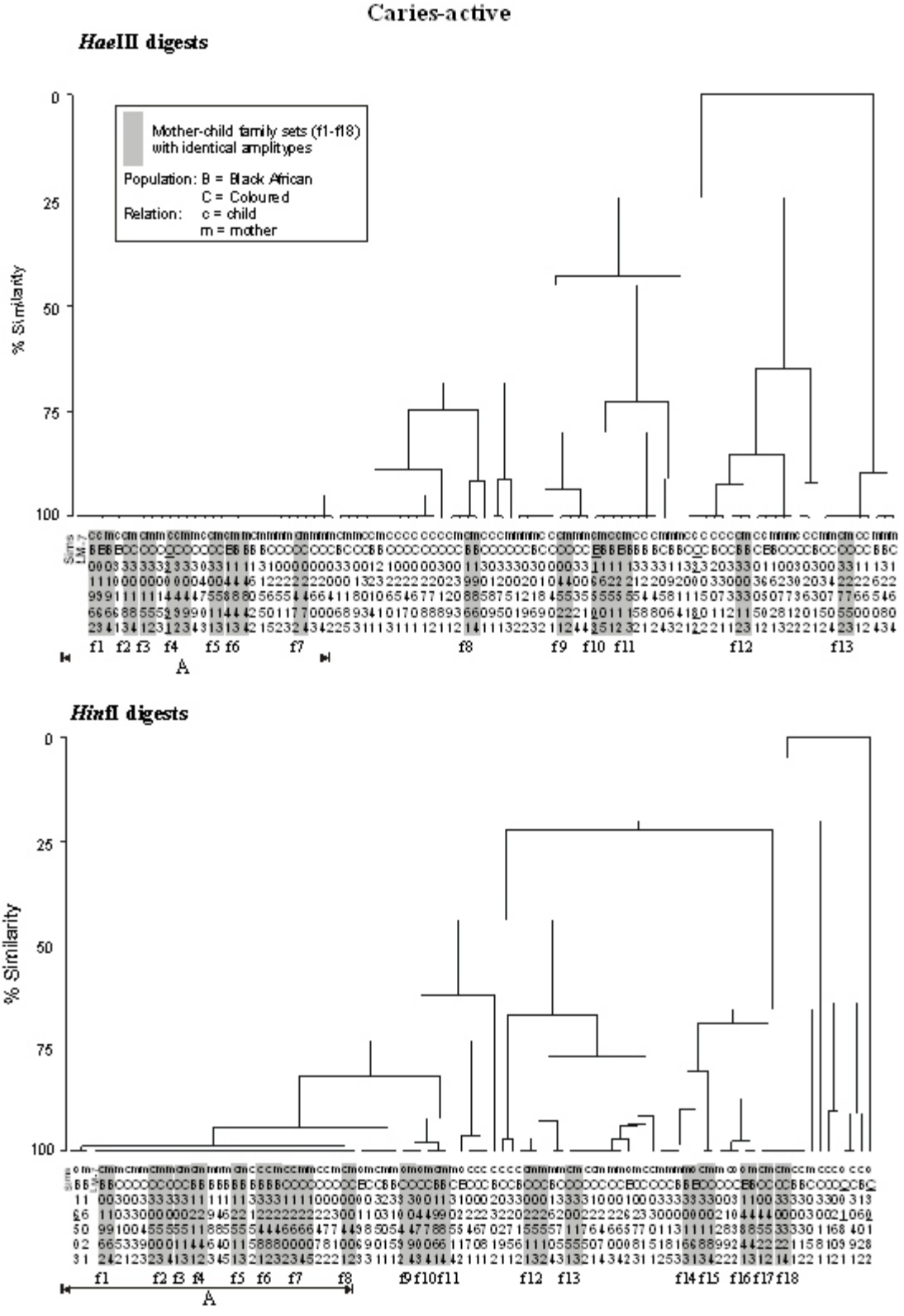
APPENDIX VIII

Dendogram of *gtfB* amplitypes from *S. mutans* reference strains (Sims, LM-7) and clinical isolates from caries-free black African and coloured children, and their mothers. The distribution of melibiose and raffinose-negative phenotypes are in bold.



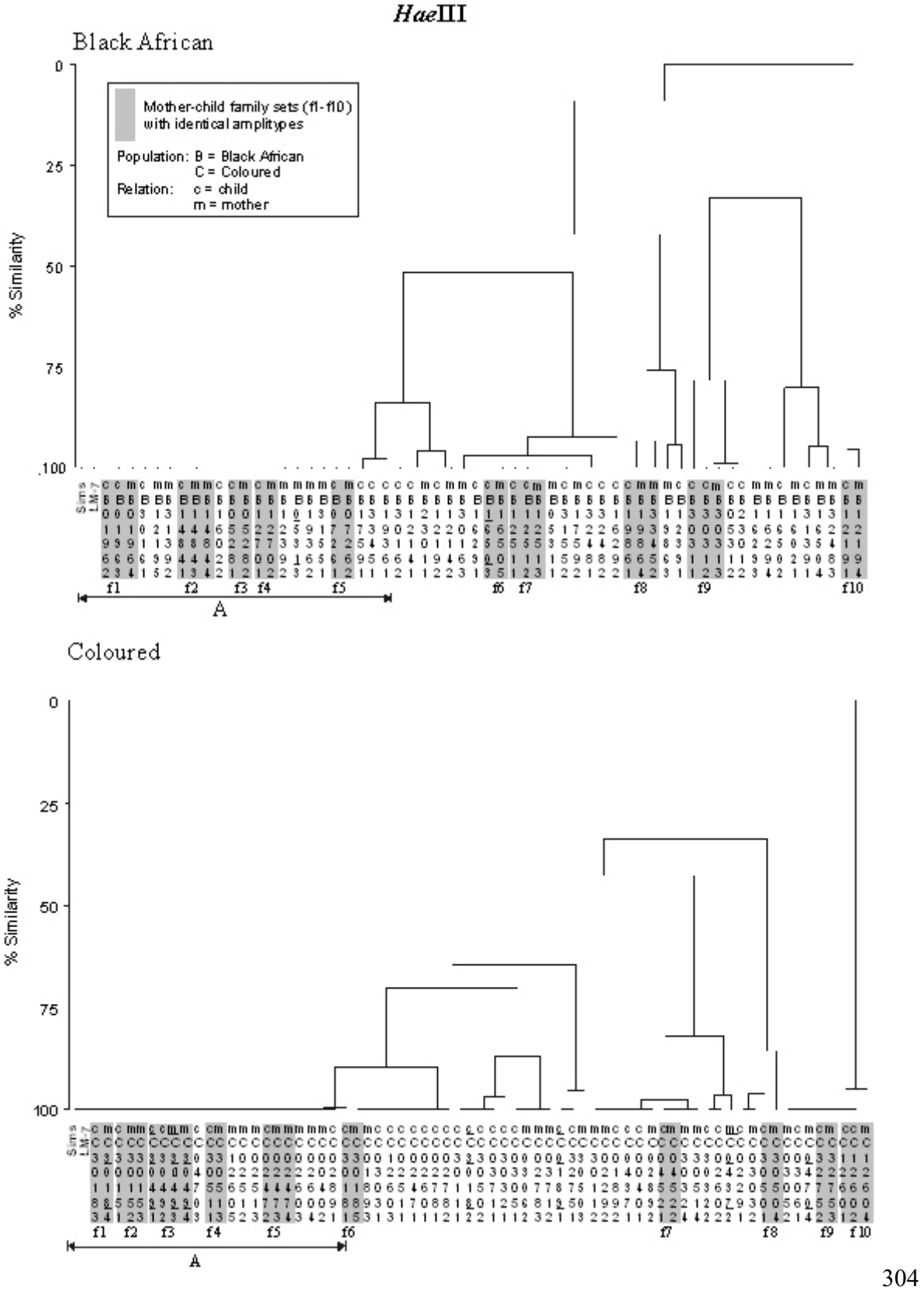
APPENDIX IX

Dendogram of *gtfB* amplitypes from *S. mutans* reference strains (Sims, LM-7) and clinical isolates from caries-active, black African and coloured children, and their mothers.The distribution of melibiose and raffinose-negative phenotypes are in bold and melibiose-negative isolates are in underlined bold.



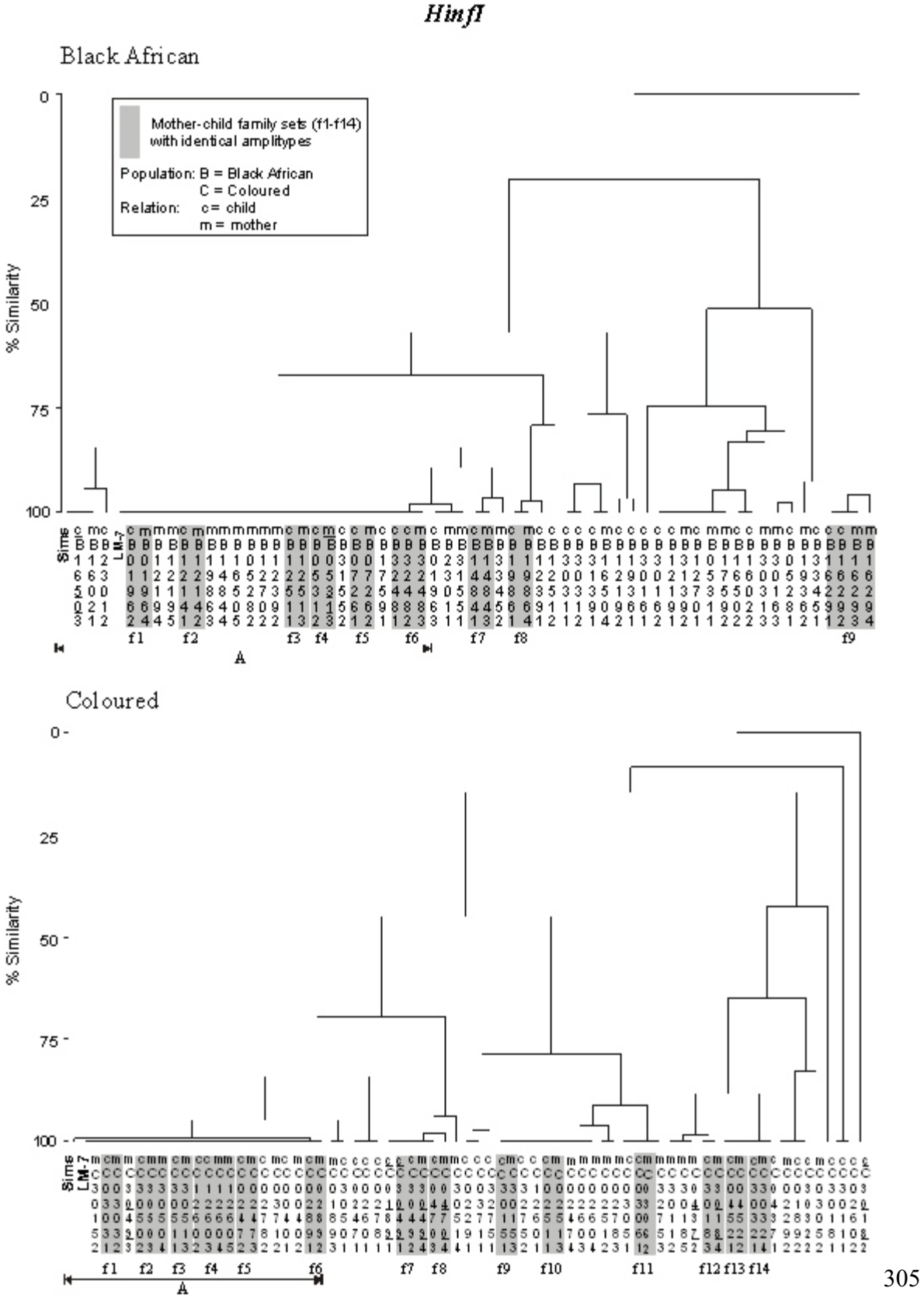
APPENDIX X

Dendogram of *gtfB* amplitypes (*Hae*III) from *S. mutans* reference strains (Sims, LM-7) and clinical isolates from black African and coloured children, and their mothers. The distribution of melibiose and raffinose-negative phenotypes are shown in bold and melibiose-negative isolates are in underlined bold.



APPENDIX XI

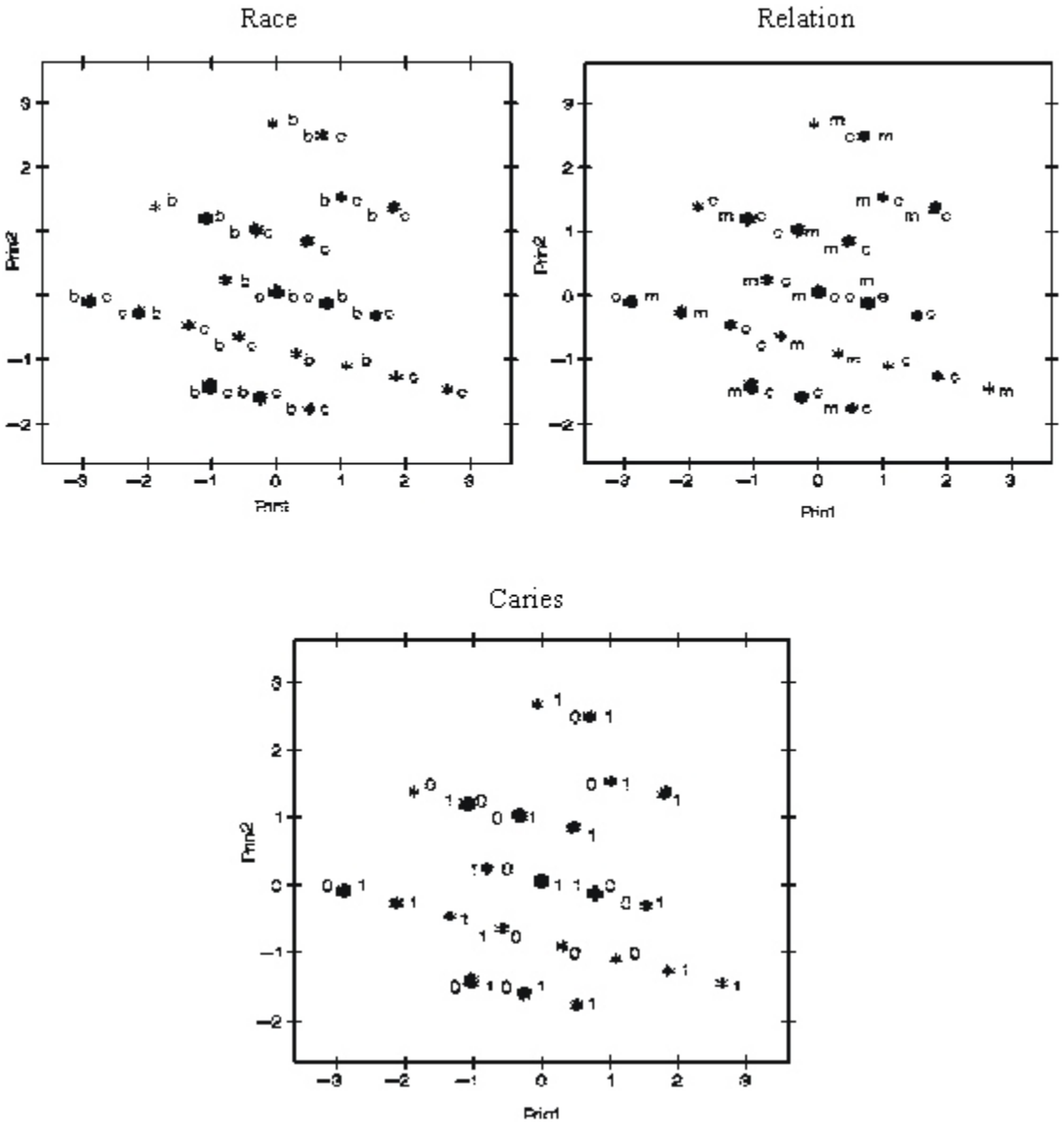
Dendogram of *gtfB* amplitypes (*Hin*fI) from *S. mutans* reference strains (Sims, LM-7) and clinical isolates from black African and coloured children, and their mothers. The distribution of melibiose and raffinose-negative phenotypes are shown in bold and melibiose-negative isolates are in underlined bold.



APPENDIX XII

Plot of the first two Principal Components: *gtfB* amplitypes produced by *Hae*III enzymes, defined by race (b = black African; c = coloured), relation (m = mother; c = child) and caries (0 = caries-free; 1 = caries-active).

***Hae*III**



APPENDIX XIII

Plot of the first two Principal Components: *gtfB* amplitypes produced by *Hin*fI enzymes, defined by race (b = black African; c = coloured), relation (m = mother; c = child) and caries (0 = caries-free; 1 = caries-active).

