Commensal Bacteria and Yeast Adhesion

(an *in vitro* study using epithelial cells)

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DECLARATION

This thesis is the original work of the author.

Karen H. Jaun

Summary

Candida albicans is an opportunistic pathogen of increasing importance and candidosis is one of the most common infections of the oral mucosa. One of the important factors in the successful colonization and infection of the oral mucosa by C. albicans is the ability of the yeast to adhere to host surfaces. Since the superficial cells of the oral mucosa are colonized by members of the oral commensal flora, it is likely that they play a part in the adherence and subsequent colonization of the mucosa by Candida. However, there is little information in the literature on this topic and, therefore, the present study was carried out.

Sixteen strains of streptococci and three strains of lactobacilli were isolated from the oral cavity, and along with four type strains, were examined for their effect on the adherence of C. albicans MRL 3153 to HeLa cells.

Firstly, the effect of the bacteria on yeast adhesion was examined using candida cells cultured in defined media containing glucose, sucrose or galactose and bacteria grown in Todd Hewitt broth. These growth conditions were used since it is known that yeasts cultured in galactose are more adherent than yeasts grown in medium containing sucrose or glucose. In these experiments, pretreatment of the HeLa cells with each bacterial strain produced a reduction in the subsequent adhesion of the yeast within a range of 23-63% implying that both bacteria and yeasts compete for the same epithelial cell receptor. However, the type of carbon source used in the growth of C. albicans had little effect on the ability of the streptococci and lactobacilli to inhibit adherence of the yeast to HeLa cells. These results suggested that there may be more than one adhesin expressed on the yeast cell surface depending on the carbon source used in its growth.

In the next series of experiments, the bacteria were cultured in a semi-defined medium containing either glucose, sucrose or galactose

while the yeasts were cultured in a defined medium containing 50 mM glucose. Generally, it was found that the carbon source used for growth of the bacteria did affect their ability to cause subsequent inhibition of yeast adhesion to HeLa cells. Most strains had a reduced inhibitory effect when grown in sucrose or galactose, while six isolates completely lost their inhibitory activity on the adherence of C. albicans to HeLa cells. These results suggested that the carbon source used in the growth of the bacteria affected the synthesis of some cell surface component which was important in adhesion.

Since it was clear from these experiments that commensal oral bacteria were able to inhibit yeast adhesion to buccal cells, it was decided to investigate the possible mechanisms involved.

Since lipoteichoic acid (LTA) has been shown to mediate the attachment of a number of streptococci to epithelial cells the next step was to extract this polymer from ten of the bacterial strains investigated previously, using the hot phenol/water extraction method. These crude preparations of LTA from seven streptococcal and three lactobacillus strains inhibited adherence of C. albicans to buccal epithelial cells up to a maximum of 48%.

In order to see if the inhibitory effect of the crude extracts could be enhanced, LTA samples from four strains of bacteria were purified by column chromatography and examined for their effect on yeast adhesion. Purified LTA from L. casei NCTC 6375 inhibited adherence of C. albicans GDH 2346 by 16% more than the crude polymer. However, with Strep. salivarius D66 and NCTC 7366 and Strep. mitior C32, purified LTA had less of an inhibitory effect on yeast adhesion than the crude polymer. It was concluded that LTA is involved in mediating adherence of L. casei to epithelial cells. However, in the case of the streptococci, it is

unlikely that LTA is the major adhesin. With these organisms it may be required to form a complex with some other cell surface component for adherence to occur.

Further investigations involved treating LTA with ammonium hydroxide to discover which part of the polymer was responsible for inhibiting yeast adhesion. Since deacylated LTA had no effect on yeast adhesion it was reasonable to assume that the inhibitory effect of the polymer was due to the lipid portion of the molecule binding to receptors on epithelial cells and therefore blocking subsequent adherence by candida.

It is known that in some oral streptococci, LTA is contained within, or complexed with, surface fibrils following its excretion from the cells. The ten strains of bacteria used in the LTA assays were examined by negative staining to see whether they possessed any fibrillar structure. All strains of streptococci apart from Strep. salivarius NCTC 7366 showed the presence of fibrils. No such structures were found on the surface of the lactobacillus strains.

Since some strains of oral streptococci and lactobacilli are known to excrete substantial amounts of LTA into the surrounding environment, culture supernatants from ten strains of bacteria were examined for the presence of LTA by rocket immunoelectrophoresis. Only two strains, Strep. salivarius M65 and MU289 were found to excrete LTA. The supernatants from these two strains also inhibited the adherence of C. albicans GDH 2346 to buccal cells. Therefore, it would appear that extracellular LTA may be involved in the inhibition of yeast adhesion to epithelial cells by some strains of oral streptococci.

Since there is evidence that the colonization and infection of the oral mucosa by candida may be prevented by growth inhibiting factors

produced by the oral commensal flora, it was decided to screen the bacterial strains for this activity using an agar disc screening method. All strains of Strep. salivarius partially inhibited the growth of both C. albicans GDH 2346 and MRL 3153 as did cell suspensions of Strep. sanguis NCTC 7864 and L. casei NCTC 6375, suggesting that some product inhibitory to yeast growth was secreted by the bacteria.

Although these studies were carried out in vitro it is possible that in vivo yeast adhesion may be blocked either by soluble LTA or by fibril-bound polymer.

ABBREVIATIONS

A_{280}	absorbance of a solution in a cell of 1 cm light path at wavelength 280 nm.
BHI	Brain heart infusion
DEAE-sephacel	Diethylaminoethyl sephacel
EDTA	ethylenediaminetetraacetate
h	hours
LTA	Lipoteichoic acid
M	molar
mM	millimolar
MEM	Minimum Essential Medium
MRS	deMan, Rogosa and Sharp
N	normal
NS	not significant
P	probability
PBS	phosphate buffered saline
PI	partial inhibition
RIE	rocket immunoelectrophoresis
rpm	revolutions per minute
SE	standard error
THB	Todd Hewitt broth
v/v	volume per volume
V	volts
w/v	weight per volume
YNB	Yeast Nitrogen Base

INTRODUCTION

I. The Oral Environment

The mouth harbours many microorganisms in an ecosystem of considerable complexity. Until quite recently the oral cavity was regarded as a single habitat for micro-organisms but it is now clear that the teeth, gingival crevice, tongue, other mucosal surfaces and saliva all form different habitats or sites where micro-organisms multiply under a variety of environmental conditions. Each habitat contains its own characteristic population usually consisting of many different microbial species. These species may interact in a variety of ways with other species occupying the same site, thus producing a dynamic micro-system affected by many changes throughout the life of the host.

A. Development of the oral flora

A comprehensive review of the development of the oral flora from birth has been carried out by Socransky and Manganiello (1971) in which the authors emphasize the variability of the flora in different parts of the mouth.

At birth the oral cavity is usually sterile, but rapidly acquires organisms from the mother and the environment. The early oral microflora after birth is mainly aerobic and facultatively anaerobic. However, most organisms fail to become established, possibly due to the fact that in the newborn the mucosa is unable to bind organisms (Ofek *et al*, 1977). In their study of the colonization of the oral cavity of newborn infants, Ofek *et al* demonstrated that LTA and streptococci adhered poorly to oral mucosal cells, suggesting that binding sites are developed or unmasked during the first few days after birth.

The eruption of teeth introduces other habitats, namely, the smooth surfaces and the pits and fissures of teeth and also the gingival cervice area. At this stage the oral flora begins to take on the character-

istics of the adult microflora. Streptococcus sanguis appears shortly after the first teeth erupt (Carlsson et al, 1970) and with the eruption of more teeth, Strep. mutans appears (Berkowitz et al, 1975). The development of 'anaerobic niches', as a result of reducing conditions created by the original inhabitants or by anatomical features such as gingival crevices, leads to a gradual shift from a predominantly aerobic-facultative flora to a facultative-anaerobic flora in which organisms such as Veillonella and Actinomyces predominate (Morhart and Fitzgerald, 1980).

B. Factors affecting the development of the oral flora

i. Nutrients

In order to grow oral bacteria must be able to utilize metabolic factors present in the diet or metabolic products released by other micro-organisms in the same or related sites. In addition an endogenous supply of nutrients is provided by saliva and crevicular fluid. Saliva contains nitrogenous material, vitamins and dissolved CO_2 which will stimulate the growth of many oral bacteria (Marsh and Martin, 1984).

Superimposed upon this background level of nutrients is the complex array of foodstuffs ingested periodically. Fermentable carbohydrates, and in particular sucrose, play an important role in the ecology of the mouth in terms of acid production and polymer synthesis. Evidence obtained from in vivo studies using rodents suggests that sucrose-induced glucan synthesis promotes the formation of plaque by Strep. mutans (Hamada and Slade, 1980). It has been shown that the plaque proportions of Strep. mutans in humans can be generally increased or decreased greatly by an increased or decreased intake of dietary sucrose (de Stoppelaar et al, 1970).

ii. Anaerobiosis

A factor which may limit the growth rates of organisms present

in the mouth is the oxidation-reduction potential(Eh). Values for Eh have been shown to range from +60mV to +310mV for the tongue, saliva and attached gingiva, to Eh levels as low as -200mV for the coronal plaque and -360mV for the gingival crevice area (Socransky and Manganiello, 1971).

The two main factors that govern the Eh of a particular site are the local oxygen concentration and the reducing ability of the micro-organisms themselves. Organisms such as streptococci and neisseria are thought to play a vital role in early coronal plaque formation by creating local anaerobic conditions favourable to the subsequent establishment of more anaerobic organisms (Morhart and Fitzgerald, 1976).

iii. pH value

The metabolism of a micro-organism is often dependent on pH, and bacteria inhibited by low pH value may not survive the acid conditions which can occur in dental plaque or in the plaque which forms on the fitting surface of upper dentures (Morhart and Fitzgerald, 1980). Denepitiya et al (1980) have shown that only lactobacilli and yeasts were able to grow at pH 4.5 but as the pH was increased to a value of 7.0, more organisms were able to proliferate. They concluded that pH was an important variable in determining the microbial composition of the oral flora.

C. The microbial flora of different parts of the mouth

The oral cavity consists of a variety of both hard and soft tissues which can be colonized by a mixture of oral micro-organisms. It is widely recognized that qualitative and quantitative differences exist in the composition of the microbial flora colonizing different sites (Gibbons and van Houte, 1975).

The predominant cultivable organisms found on the surface of the cheek and tongue are the 'viridans' group of streptococci. Streptococcus

salivarius accounts for 20-25 percent of the organisms isolated from the tongue (Carlsson, 1967; van Houte et al, 1971) followed by Strep. mitior, which is the predominant species found on the buccal mucosa, and then Strep. sanguis (Morhart and Fitzgerald, 1980).

The microbial community which forms on teeth is called dental plaque and unlike mucosal surfaces where the microbial load is continually decreased by shedding of epithelial cells, the tooth surface is non-renewable. Thus, in stagnation areas thick deposits of plaque can accumulate. The distribution of bacteria colonizing the tooth surface is reported by Babb and Hamada (1982) who found that the predominant three groups of micro-organisms present were streptococci, Veillonella and Actinomyces.

D. Oral streptococci

Facultative streptococci form the most numerous single group in the oral cavity. A survey by Socransky and Manganiello (1971) showed that streptococci represented a high percentage of the organisms present on the dorsal surface of the tongue, saliva, dental plaque and the gingival crevice.

The most abundant of the oral streptococci are those referred to collectively as the "viridans" group which includes Strep. sanguis, Strep. mitior, Strep. salivarius, Strep. milleri and Strep. mutans. Historically, these have been classified by a variety of criteria over the years and considerable confusion has surrounded their taxonomy and nomenclature. In addition to α-haemolytic varieties, from which the name "viridans" is derived, some of the streptococci in this group may display either complete (β) haemolysis or be totally non-haemolytic. Unlike many other streptococcal species, few "viridans" streptococci possess the cell wall antigens used in Lancefield grouping.

i. Streptococcus sanguis

This organism was first described by White and Niven (1946) who isolated it from the blood and heart tissue of several patients with infective endocarditis.

The natural habitat of Strep. sanguis is the oral cavity and it is, in fact, one of the most common and numerous streptococci found in the mouth (Carlsson, 1965; 1967). It appears in the mouth after the first teeth erupt and disappears when the teeth are lost. This is reflected in adhesion studies showing that Strep. sanguis adheres firmly to the pellicle coated enamel (van Houte et al, 1970). However, Strep. sanguis is also recovered in high numbers from the surface of buccal epithelial cells (Gibbons and van Houte, 1971) indicating that it can adhere to both hard and soft tissues.

ii. Streptococcus mitior

Some confusion has existed in the past regarding the naming of this organism, and a number of texts still use the designation Strep. mitis. The name Strep. mitior originates from Schottmuller, 1903 (Hardie and Bowden, 1974). Unlike most other streptococci, this organism lacks rhamnose as a component of its cell wall, and has been defined essentially by the absence of a number of biochemical activities useful for the identification of other streptococci.

A study carried out by Liljemark and Gibbons (1972) found Strep. mitior to be the predominant species colonizing the non-keratinized human oral mucosa. They averaged from 76 to 89% of the total flora cultivable from cheek, lip and ventral tongue surfaces, and represented 34, 40 and 18% of the streptococci in dental plaque, in saliva and on the tongue dorsum, respectively.

iii. Streptococcus salivarius

Andrews and Horder isolated this organism from the saliva of

patients with oral disease in 1906. It is defined by its ability to synthesize large amounts of extracellular levan from sucrose or melitose (Niven et al, 1941). This property results in the production of characteristic large, mucoid colonies on sucrose agar.

Streptococcus salivarius is found on the dorsal surface of the tongue (Krasse, 1954; Gibbons et al, 1976), and is one of the earliest colonizers of the oral cavity in infants. A study carried out by Carlsson et al (1970a)found that Strep. salivarius established itself in the oral cavity of children within 24-48 hours after birth. It has been shown to preferentially attach to keratinized oral epithelial cells in vitro, reflecting its preference for the tongue surface (Sklavanou and Germaine, 1980).

E. Lactobacilli

Lactobacilli are gram-positive, non-motile, non-sporing rods. The lactobacilli may be split into two groups on the basis of glucose fermentation: homofermentative species which produce principally lactic acid and heterofermentative species which produce less lactic acid and a considerable number of other end products (principally acetic acid and ethanol) including gas (Hardie, 1983).

In a healthy mouth, lactobacilli are found on the teeth, on the oral mucosa and on the tongue (van Houte et al, 1972). Their numbers vary according to circumstances but it is probable that some are present in all human cavities soon after birth, although not in significant proportions. During the first two years of life lactobacilli occur only transiently and in very low numbers (Carlsson et al, 1975). Among children of up to five years of age the lactobacilli that occur in the mouth are predominantly L. casei (Crossner, 1984).

Lactobacilli are aciduric and a favourable environment for the growth of these organisms is created by the acid produced from a frequent intake of easily fermentable carbohydrate (van Houte, 1980). Long-term acidic conditions are created when there are sites in the mouth where carbohydrate and lactobacilli can be retained (van Houte, 1980). In the edentulous mouth retention sites are virtually absent and the lactobacilli counts are extremely low. However, once artificial dentures are inserted the counts rise (Kleinberg, 1982).

The role of lactobacilli in dental caries has been widely studied (for reviews, see Ellen, 1976; van Houte, 1980). They are present in large numbers in the mouths of caries-active persons, which is why they were previously thought to be the causative micro-organism in dental caries. At the present time they are believed to be involved in the spread of dental caries after cavitation has occurred (Ikeda et al, 1973).

II. Candida albicans and other pathogenic Candida species

A. Historical aspects

Several yeast species of the genus Candida are endogenous, opportunistic pathogens. Normally they reside harmlessly as commensals in human and animal hosts; but they can take advantage of defects in the host defence mechanisms to cause infections in a wide range of tissues.

In man the most common manifestations of candidosis are superficial lesions affecting the oral and vaginal mucosa, although the literature contains reports of candida infections of almost all tissues. Oral and vaginal forms of candidosis were commonly known as 'thrush' due to the similarity of the white speckled appearance of these lesions to the breast of a thrush.

As a clinical entity, thrush has been recognized since antiquity. Hippocrates described two cases of oral thrush (Odds, 1979) and Pepys mentioned the disease in his diary for 1665 (Winner and Hurley, 1964).

For almost a century following its discovery, the "thrush fungus" was the subject of grossly confused taxonomic studies. However, the differences were finally settled and a new genus, Candida, was proposed at the Third International Congress for Microbiology in 1939 (Martin and Jones, 1940).

B. Biological Aspects

i. Morphology

Yeasts of the genus Candida are classified among the fungi imperfecti since they cannot form a sexual stage. All pathogenic Candida species multiply primarily by the production of buds from ovoid blastospores (yeast cells). The principal characteristic of this genus is the ability to produce pseudohyphae (chains of elongated yeast cells) and C. albicans can appear not only as blastospores and pseudohyphae, but also as true hyphae and as large, refractory chlamydospores (Odds, 1979). Pseudomycelia resemble true mycelia but the mode of formation is different. True hyphae arise as branches of existing hyphae or by germination of spores. Mitotic cell division occurs within the extending hypha and septa are formed at intervals along the hypha. Pseudohyphae are formed by end-to-end aggregation of elongated blastospores and are distinguished from true hyphae by constrictions of the pseudohyphae at septal joints (Odds, 1979).

ii. Growth and nutrition

Most pathogenic yeasts are able to grow in the pH range of 3 to 8 and at temperatures ranging from 20°C to 40°C. They require biotin, thiamine or other vitamins of the B complex for growth, and a nitrogen source other than nitrate. End products of Candida metabolism of dietary

sugars include carboxylic acids (Samaranayake et al, 1981) and acidic proteases (Germaine et al, 1978). Reports on anaerobic growth of C. albicans are contradictory. Szawatkowski and Hamilton-Miller (1978) have observed anaerobic growth of C. albicans, whereas other authors have reported the inability of the yeast to grow anaerobically (Montplaisir et al, 1976).

C. Distribution of Candida in Nature

Although yeasts are distributed ubiquitously in terrestrial and aquatic habitats, yeasts which are involved in human candidosis have a natural distribution which is restricted more or less to man and other warm blooded animals. Candida albicans has been recovered from a far wider range of animal hosts than any other species. Recoveries of C. albicans from samples of soil, plants and the atmosphere are extremely rare, and have been achieved only from sources such as farms and hospital wards where human or animal contamination is probable (Odds, 1984).

D. Oral Candidosis

It is well known that Candida infections are most often found in persons debilitated by other conditions. Wilson, in 1962, and Odds (1979) stated that "C. albicans is a better clinician and can discover abnormalities in persons much earlier in the course of the development of such abnormalities than we can with our chemical tests".

i. Factors predisposing to candidosis

In man, oral infection by the yeast C. albicans appears to be primarily related to host susceptibility and several predisposing factors are recognized. In the oral cavity, mechanical, environmental and host-related factors may all influence the rate and density of colonization with C. albicans. Prolonged and extensive use of broadspectrum antibiotics,

immunosuppressive drugs and cancer chemotherapy have increased the number of Candida-induced oral infections (Bodey, 1984). Diabetes has also been associated with an increase in the rate and density of colonization of C. albicans (Tapper-Jones et al, 1981).

Oral "thrush" has become a common complication among patients with acute leukaemia. The frequency of oral candidosis in 1000 leukaemic patients was estimated to be 16% whereas it was 7% in patients with solid tumours (Edwards et al, 1978; Kirkpatrick and Alling, 1978).

Of the nutritional factors implicated, diets rich in carbohydrates have been suggested as a factor predisposing to candidosis (Gentles and La Touche, 1969; Van Reenen, 1973). Experiments with monkeys (Bowen, 1974) and rats (Russell and Jones, 1973) fed on carbohydrate-rich diets have shown that such diets promote the carriage and persistence of Candida species in the oral cavity. In clinical terms, Ritchie et al (1969) reported aggravation of symptoms in patients with oral candidal infections when their carbohydrate intake was raised above normal levels.

A unifying hypothesis encompassing the observations of several investigators is presented by Samaranayake and MacFarlane (1985) in an attempt to explain the role of a carbohydrate-rich diet in the pathogenesis of oral candidosis.

E. Types of Oral Candidosis

Candidosis is by far the most common mycotic infection of the oral cavity. Oral candidosis manifests itself in a number of forms and the classification of Lehner (1967) will be used in this thesis.

i. Acute Pseudomembraneous Candidosis (Oral thrush)

Acute pseudomembraneous candidosis is prevalent amongst children and the elderly. It has been reported as occurring in 5% of all newborn

infants (Soames et al, 1985) and in 10% of elderly, debilitated, hospitalized patients (Bodey, 1982).

The disease presents itself as a white patch (the pseudomembrane) on the affected mucosa which can be wiped away to leave a red, raw and often bleeding base. The pseudomembrane consists of desquamated epithelial cells, leukocytes, micro-organisms, keratin, necrotic tissue and food debris. Lesions may occur in any part of the mouth; the most prevalent locations being the sides and dorsum of the tongue, buccal mucosa, gingiva and oropharynx (Dreizen, 1984). Among adults, oral "thrush" may appear as a consequence of predisposition to infection in a number of clinical situations, eg. repeated oral candidosis has recently been reported as a common complication of acquired immuno-deficiency syndrome (AIDS) (Eversole et al, 1983).

ii. Chronic Atrophic Candidosis (Denture stomatitis, denture sore mouth)

Knowledge of this disease arises from studies published by Cawson (1963) and Budtz-Jörgensen (1971). It is probably the most common form of oral candidosis and has been found to occur in as high a proportion as 65% of elderly wearers of full dentures (Shepherd, 1982). The typical clinical presentation is a symptomless area of swelling and inflammation under the fitting surface of the upper denture. Candida can be recovered more readily from the fitting surface of the denture than from the surface of the mucosa, and evidence suggests that microscopic pores and irregularities on the fitting surface of a denture provide a suitable environment for growth and retention of the organism (Soames and Southam, 1985).

iii. Acute Atrophic Candidosis (Candida glossitis)

Acute atrophic candidosis may occur as a sequel to the shedding of fungal plaque in "thrush" or arise de novo during antibiotic therapy (Lehner, 1966). The disease is manifested by a painful erosion of the

oral mucosa and patchy depapillation of the dorsum of the tongue. The condition has been ascribed to an overgrowth of yeasts following antibiotic induced depression of the oral commensal flora. The symptoms of the disease usually subside spontaneously following withdrawal of the antibiotic (Odds, 1979).

iv. Angular Cheilitis (Perlèche)

Angular cheilitis is often associated with chronic atrophic candidosis and is characterized by soreness, erythema, and fissuring at the corners of the mouth. Deep folds of skin at the angles of the mouth which may be associated with loss of occlusal height in old age or result from incorrectly designed or old dentures, may be a contributory factor. Angular cheilitis has also been attributed to nutritional deficiencies (Russotto, 1980). In some patients with angular cheilitis there is no evidence of candida infection, and in such cases S. aureus, or less frequently, β-haemolytic streptococci are likely to be involved (MacFarlane and Helnarska, 1976).

v. Chronic Hyperplastic Candidosis (Candida leukoplakia)

This form of candidosis presents clinically as a persistent white patch on the oral mucosa which is indistinguishable from leukoplakia. Characteristically the lesions show dense, opaque white plaques with a rough surface, the thickness and density of the plaques usually being irregular. The most common sites are the buccal mucosa, tongue, lips and floor of the mouth. The disease occurs in the mouth as either the common homogeneous variety that is usually asymptomatic or as the speckled variety that is marked by stinging and burning during food intake (Dreizen, 1984).

It has not been conclusively shown as yet whether these lesions are primarily leukoplakia with a secondary candidal infection, or whether they are primarily chronic candidal infections which in time lead to epithelial hyperplasia.

III. Adherence of Micro-organisms

A. Importance of adherence

The ability of micro-organisms to adhere to a surface is essential for colonization by pathogenic as well as by commensal micro-organisms. Infection is considered to be a chain of events initiated in most cases by bacterial adherence to a tissue surface (Savage, 1972). Such adherence initially results in colonization with or without subsequent invasion and inflammation. From the point of view of the micro-organism, adherence to a surface is essential for at least three reasons: (1) to resist the cleansing action of secretions coating the mucosal surfaces, such as saliva, tears and urine; (2) to promote attachment to target tissues within the host distant from the point of entry, and (3) to allow delivery of toxin molecules in high concentration to receptors in host cell membranes (Shibl, 1985).

Marine microbiologists have known for many years that bacteria must stick to surfaces in order to avoid being swept away by moving streams of water (Zobell, 1943). However adherence has only relatively recently been recognized as an important ecological determinant in the colonization of specific sites in plants and animals, and in particular, an essential early event in the pathogenesis of many bacterial infections in animals and humans (Gibbons, 1977).

As early as 1908, Guyot reported studies on the adhesiveness of bacterial cells for blood erythrocytes. Then in 1965, Duguid and Old (1980) reported that the adherence of several enteric Gram-negative bacilli to erythrocytes and intestinal epithelial cells was mediated by mannose. However it was not until fourteen years ago when Gibbons and his colleagues began reporting on the selective nature of the adherence of bacteria to various human oral and dental surfaces that research in this field started

to flourish (Gibbons, 1977; Gibbons and van Houte, 1980).

B. Proposed mechanisms of adherence

A major problem that must be overcome for bacterial cells to associate with surfaces results from charge attraction and repulsion between the bacterial cell and surface. The surfaces of bacterial and eukaryotic cells have net negative charges so physical contact between the two surfaces is not favoured. These repulsive forces, however, may be overcome by the attractive forces between hydrophobic molecules present in varying numbers on the two cell surfaces. The two cell surfaces may also attract each other by long-range forces created by atomic and molecular vibrations that produce fluctuating dipoles of similar frequencies on each surface (the DLVO theory; Jones, 1977).

One of the earliest theories of adhesion was based on Zobell's (1943) observation that bacterial attachment was time-dependent. This idea was supported more recently by Marshall, Stout and Mitchell (1971), who described a two-stage attachment process, comprising reversible and irreversible adsorption. According to this theory, bacteria are initially attracted to a surface by a balancing of London-van der Waals attraction forces and electrostatic repulsion forces. This adsorption is reversible. Such weak adsorption may be followed by firm adhesion as the bacterium produces bridging polymers which can overcome the electrostatic repulsion barrier at the surface and thus become firmly adsorbed.

The selectivity with which bacteria attach to various cell surfaces suggests the existence of specific receptors on both the bacteria and the host cell that mediate adherence. A growing body of evidence indicates that bacteria adhere to surfaces by a specific lock-and-key mechanism (Beachey, 1981): ligand molecules (adhesins) on the surfaces of bacteria interact with complementary molecules (receptors) on the surface

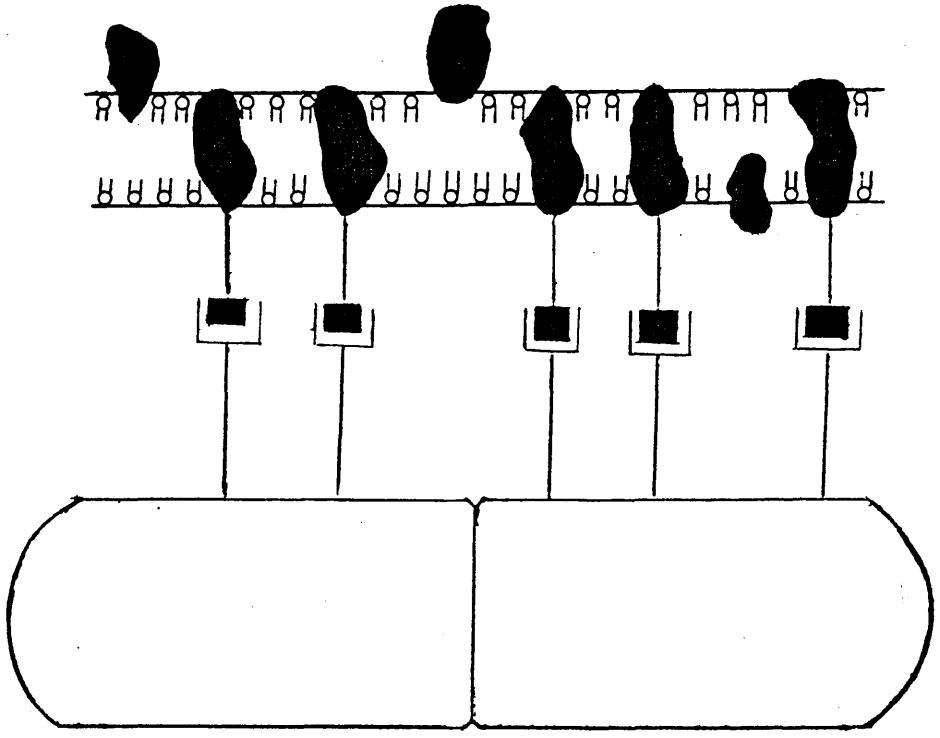
of tissue cells of the host (Fig. 1). The ability of micro-organisms to adhere to the host cell is dependent on their ability not only to synthesize the adhesin but also to express it in an accessible configuration on their surface.

The importance of adhesins in the adherence of bacteria to host cells has been shown in a number of studies. Competitive inhibition experiments have shown that bacterial adherence to cell surfaces can be competitively inhibited using either purified bacterial adhesin, host-cell receptor material, or their chemical analogues (Aronson et al, 1979). Experiments using anti-adhesive vaccines (Rutter and Jones, 1973) have demonstrated that vaccines evoke local immune responses which produce antibodies that mask bacterial adhesins, hence preventing bacterial attachment to the cell surface. Investigations of natural mucosal barriers have indicated that they may mask receptors on epithelial cells and prevent bacterial adhesion (Woods et al, 1980). Studies using antibiotics have resulted in the suppression of the synthesis and expression of bacterial surface adhesin, hence inhibiting adhesion of these organisms to cell surfaces (Shibl, 1985).

C. Host-cell receptors

The only bacterial receptors on host epithelial cells which have received much attention are various sugar moieties and fibronectin. It has been suggested that glycoproteins or glycolipids, especially those with blood group substance reactivity, serve as receptors for various organisms, such as streptococci (Gibbons et al, 1975). For example, pre-treatment of buccal epithelial cells with antisera to certain blood group substances, or the lectin, concanavalin A, masked the receptors required for the attachment of strains of Strep. mitior, Strep. sanguis and Strep. salivarius (Williams and Gibbons, 1975).

Figure 1: Proposed mechanism of bacterial adherence. Attachment of bacterial cells (bottom) via specific adhesins (fork-like structures) to complementary receptors (■) on the host-cell membrane (top) (From Ofek and Beachey, 1980).



Fibronectin is a high molecular weight glycoprotein which is found in various body fluids including plasma, on many host cell surfaces and in connective tissue matrices. The role of fibronectin as a lipoteichoic acid receptor for Group A Strep. pyogenes has been demonstrated by Simpson and Beachey (1983). It has also been shown to bind to other Gram-positive bacteria including S. aureus (Espersen and Clemmensen, 1982) and Lancefield groups C and G streptococci (Myhre and Kuusela, 1983). However, fibronectin does not bind to Gram-negative bacteria (Simpson et al, 1982).

There is also evidence that fibronectin may inhibit bacterial adherence since the removal of fibronectin from host cells resulted in increased attachment of Ps. aeruginosa (Woods et al, 1981). Moreover, Abraham et al (1983) have shown that buccal epithelial cells rich in fibronectin bind more streptococci and fewer mannose-sensitive E. coli as compared with fibronectin-poor cells where the opposite occurs.

The receptors on cell membranes for Gram-negative bacteria are, in general, carbohydrate moieties of glycoproteins or glycolipids. Mannose residues appear to be the sugar moieties involved for some strains of E. coli (Ofek and Beachey, 1978); fucose residues for Vibrio cholerae (Freter and Jones, 1976); sialic acid for mycoplasma species (Razin et al, 1981) and β-galactosyl residues for K88 positive E. coli (Gibbons et al, 1975). Adhesins of Ps. aeruginosa also appear to have D-mannosyl receptors (Gilboa-Garber et al, 1977).

D. Prevention of bacterial adherence

One of the goals in studies of bacterial adherence mechanisms is the eventual development of measures to prevent the adhesion of harmful bacteria to mucosal surfaces before the organisms can produce tissue damage. It should be borne in mind, however, that since the adherence of pathogens

to phagocytic cells is probably beneficial to the host the anti-adherence agents must not interfere with this interaction. Theoretically prevention may be achieved by topical application of the isolated and purified bacterial adhesin, membrane receptor or analogues of these substances as competitive inhibitors of bacterial adherence. Also, prevention could be achieved by the systemic administration of drugs that suppress the formation or expression of bacterial adhesins, for example, sub-lethal doses of antibiotics. In addition, adhesin vaccines could be developed that would induce the formation of local antibodies that could coat organisms and thereby prevent adhesion (Beachey, 1981).

IV. Adherence of streptococci

The axiom that bacteria must adhere in order to prevent removal from their niche is nowhere as evident, perhaps, as in the mouth. Whereas the mouth represents a hospitable bacterial growth chamber offering a wide variety of micro-niches, dislodgement results in the removal of bacteria to inhospitable surroundings; ie. the stomach. Very high numbers of bacteria may be found adhering to all oral surfaces, but in particular to the non-shedding tooth surface (Gibbons, 1980).

The members of the oral flora which have been most intensively studied are the oral streptococci. Gibbons and van Houte (1971) showed that there was a correlation between the relative adherence of streptococci species *in vitro* and their proportional distribution on oral surfaces *in vivo* They postulated that the ability of oral streptococci to adhere to oral surfaces was a determinant affecting their colonization.

A. Adherence to the tooth surface

The formation of dental plaque requires two types of adherent interactions (Gibbons and van Houte, 1973; 1980). Bacteria must first

adhere to the layer of salivary glycoprotein covering the enamel (pellicle) and then to each other while proliferating to avoid being removed by the mechanical washing actions of saliva. The adherence of Strep. mutans to tooth surfaces has been extensively studied because of its importance as an aetiological agent in dental caries (Gibbons and van Houte, 1975; Hamada and Slade, 1980). The exact mechanism by which Strep. mutans can adhere to and accumulate on the surface of teeth in vivo is unknown (Gibbons and van Houte, 1971). It has been suggested that the cell wall of Strep. mutans contains tightly- and possibly covalently-bound polypeptide molecules that may serve as a means of attachment to smooth surfaces (Nesbitt et al, 1980). Although glucans may promote accumulation of previously attached bacteria, they are not thought to act as adhesins (Gibbons and van Houte, 1975).

In vitro studies have shown that Strep. mutans can attach to saliva-coated hydroxyapatite independent of glucan synthesis. Under these conditions the attachment of Strep. mutans strains can be inhibited significantly by galactose and melibiose but not by lactose; inhibition occurs after pre-treatment of bacterial cells but not after pre-treatment of the saliva-coated apatite (Gibbons and Qureshi, 1979). Pre-treatment of salivary mucins of high molecular weight with α-galactosidase also inhibits their ability to aggregate Strep. mutans (Levine et al, 1978). These data suggest that Strep. mutans can bind to exposed α-galactoside residues of salivary glycoproteins present in the acquired pellicle. The adherence of Strep. mutans can also be inhibited by amines (Gibbons and Qureshi, 1979). Thus, the adherence of Strep. mutans to the acquired pellicle may involve lectin binding as well as basic pellicle residues.

Of all oral streptococcal species, Strep. sanguis has the highest affinity for enamel (Applebaum et al, 1979). However little is known about

the adherence mechanisms of this organism. Nesbitt et al (1982) have suggested that the adherence of Strep. sanguis to saliva-coated smooth surfaces is partially dependent on the formation of hydrophobic bonds between the cell and adsorbed salivary proteins. This is supported by the findings of Gibbons et al (1983) who showed that a non-hydrophobic mutant of Strep. sanguis had a diminished capacity for attaching to salivary pellicles on hydroxyapatite surfaces. This mutant also appeared to be defective in the synthesis of fimbriae, as judged by electron microscopy, a finding which has been confirmed by Fives-Taylor (1982). Recently, Fachon-Kalweit et al (1985) have shown that antibodies of Strep. sanguis that bind to fimbriae block the adhesion of this organism to saliva-coated hydroxyapatite.

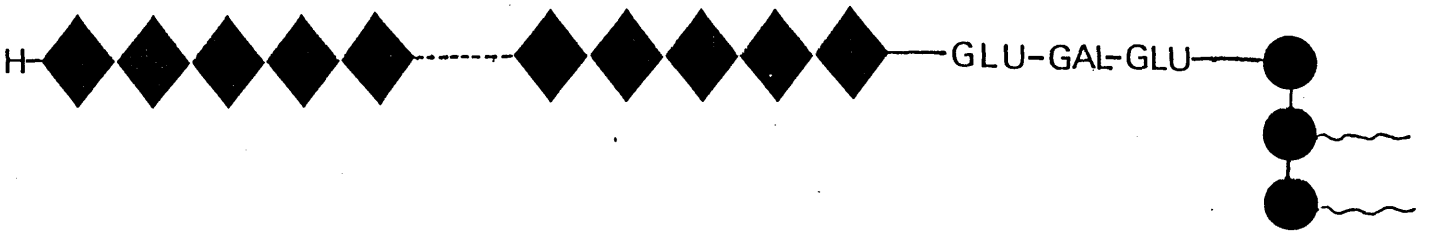
B. Adherence to mucosal surfaces

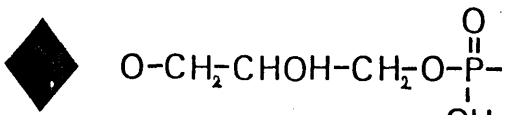
The mucosal surfaces of the healthy host are usually impervious to pathogenic organisms because of a number of cleansing mechanisms that operate on these surfaces. In health mucosal surfaces are constantly bathed by secretions which contain a wide range of constituents including antibacterial enzymes and antibodies. Successful pathogens are those capable not only of penetrating these local defenses and adhering to mucosal cells, but also of re-colonizing the fresh surfaces produced as bacteria-coated host cells are desquamated into saliva and swallowed. In time, the pathogen may penetrate the epithelial cell barrier and invade host tissues (Ofek and Beachey, 1980).

i. The role of lipoteichoic acid in the adherence and colonization of epithelial surfaces

Lipoteichoic acid (LTA), an amphipathic molecule found in most Gram-positive bacteria, consists of a chain of polyglycerol phosphate covalently linked to a glycolipid at one end (Fig. 2). Depending on the

Figure 2: Proposed structure of LTA from L. casei (Wicken, 1980).





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species of origin, the polyglycerol phosphate backbone is substituted to varying degrees with glycosyl and alanyl residues (Wicken and Knox, 1975). These polymers are normally bound to the cytoplasmic membrane but they usually extend through the wall to the cell surface and are often excreted into the surrounding environment (Alkan et al, 1977).

Lipoteichoic acid is believed to play an important role in several diseases by mediating the attachment of pathogenic organisms to host cells. It has been shown to bind to a variety of eukaryotic cells including oral mucosal cells (Beachey, 1975), human platelets (Beachey et al, 1977) and erythrocytes (Beachey et al, 1979a). Lipoteichoic acid has also been implicated in a number of inflammatory diseases in experimental animals, including arthritis (Ne'eman and Ginsburg, 1972), nephritis (Waltersdorff et al, 1977) and bone resorption (Hausmann et al, 1975). It also causes lymphocytes to undergo mitogenesis (Beachey et al, 1979) and stimulates the release of lysosomal enzymes from macrophages (Harrop et al, 1980).

The adhesion of group A streptococci to human skin epithelial cells and buccal mucosal cells has been the subject of a number of investigations. An early association of M-protein with fibrillar structures present on streptococci led to the idea that M-protein mediated the adhesion of group A streptococci to oral epithelial cells (Ellen and Gibbons, 1972). However, Alkan et al (1977) found that M-protein positive strains did not show significantly greater adherence than M-negative strains. Beachey and Ofek (1976) dissociated M-protein from group A streptococci without producing any change in the surface fibrillae or adherence activity to epithelial cells. They also examined various surface components of group A streptococci as possible candidates for the adhesin. Of the components tested, which included LTA, M-protein, carbohydrate and

a sonicate of peptidoglycan, only LTA was able to block adherence to any significant degree. In addition, they found that pre-treatment of streptococci with antiserum to LTA only, blocked subsequent binding of streptococci to epithelial cells.

The binding of LTA to host cells is mediated by the glycolipid end of the molecule. According to Beachey (1981) LTA and deacylated LTA form a complex network with the M-protein and other LTA-binding proteins on the surface of the Streptococcus (Fig. 3). These complexes allow the lipid ends of some LTA molecules to be intercalated into the cytoplasmic membranes of the bacterium, while others are free to interact with LTA-receptors on host cell membranes. It is thought that the LTA-protein complexes may be the main constituents of fibrillae on the surface of streptococci (Beachey et al, 1983).

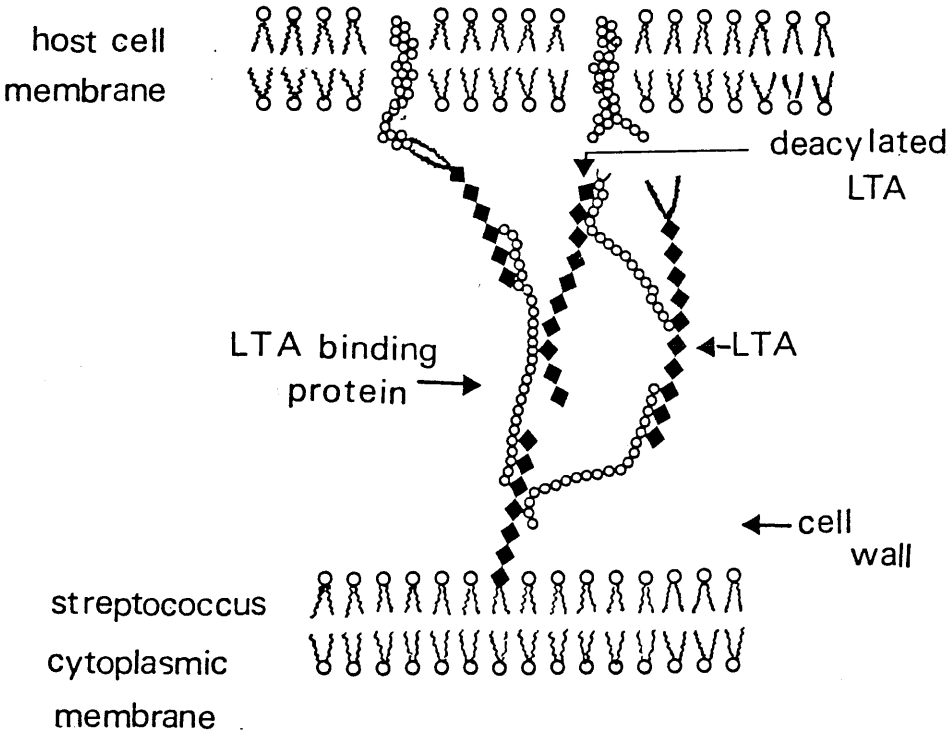
Lipoteichoic acid has also been shown to mediate the adherence of group B streptococci to human embryonic, foetal and adult buccal epithelial cells (Nealon and Mattingly, 1984). Similarly, Carruthers and Kabat (1983) have shown that pre-treatment of buccal cells with LTA in vitro markedly reduced the adherence of S. aureus.

Some studies have suggested that the hydrophobic character of bacteria plays a central role in their interaction with mammalian cells (Magnusson et al, 1977). Group A streptococci are known to have a strong tendency to hydrophobic interaction (Tylewska et al, 1979). A relationship between M-protein and the hydrophobic properties of these bacteria has previously been suggested (Mi̊rner et al, 1982; Tylewska et al, 1979). However, investigations carried out by Mi̊rner et al (1983) have shown that surface-associated LTA rather than M-protein contributes to the hydrophobic affinity of group A streptococci.

ii. The role of fibrils in mediating adherence

Electron microscopic studies have shown that oral streptococcal

Figure 3: Hypothetical model for the interaction between LTA and M-protein on the surface of Strep. pyogenes.
(From Beachey et al, 1983)


host cell
membrane
deacylated
LTA
LTA binding
protein
LTA
cell
wall
streptococcus
cytoplasmic
membrane

species possess a fibrillar coating. Liljemark and Gibbons (1972) demonstrated the presence of a fibrillar "fuzzy" coat on the surface of Strep. mitior which appeared to mediate their attachment to the epithelial cell membrane. The surface of Strep. mitior is now known to possess three types of fibrillae (Handley and Carter, 1979).

The presence of two different cell surface structures on Strep. salivarius has been shown by Handley et al (1984). Strains which reacted with Lancefield group K antiserum (K^+ strains) possessed fibrils and those organisms that were K^- possessed fimbriae. Fibrils were defined as being of consistent length but no consistent width, while fimbriae were categorized as much longer than fibrils and of consistent width (Handley et al, 1984). This subdivision of Strep. salivarius correlates with the observations of Weerkamp and McBride (1980), who divided Strep. salivarius strains into two groups on the basis of adherence characteristics. The K^+ strains were more adhesive than the K^- strains and were found to carry three categories of specific functional groups.

V. Adherence of Candida albicans

Adherence has been shown to play a central role in the pathogenesis of many bacterial infections (Gibbons and van Houte, 1975; Reed and Williams, 1978). Less emphasis, however, has been placed on the role that adherence may play in fungal infections.

A. Variability in the adherence of Candida species

Candida species have been shown to differ markedly in their ability to adhere to various surfaces. King et al (1980) examined the in vitro adherence capabilities of seven Candida species to mucosal cells. They found that C. albicans adhered to epithelial surfaces better than the

other species tested: C. tropicalis and C. stellatoidea exhibited moderate adherence while C. guilliermondii, C. pseudotropicalis and C. krusei did not adhere. Candida albicans has also been shown to adhere to epidermal corneocytes and buccal mucosal cells (Ray et al, 1984), vascular endothelium (Klotz et al, 1983) and fibrin-platelet matrices (Scheld et al, 1981) more avidly than any other species of Candida. This ability of C. albicans to adhere better than any other Candida species probably explains its greater potential for colonizing and infecting human hosts.

B. Adherence of C. albicans to epithelial surfaces

Although C. albicans has been shown to adhere to a variety of surfaces including acrylic (Samaranayake and MacFarlane, 1980), neutrophils (Diamond and Krzesicki, 1978) and plastic (Klotz et al, 1985), the vast majority of investigators have studied the adherence of C. albicans to epithelial cells.

Kimura and Pearsall (1978) demonstrated that adhesion of C. albicans to buccal epithelial cells was greater with organisms suspended in saliva than with organisms suspended in phosphate-buffered saline (PBS). They also found that germinated yeasts adhered better than non-germinated yeasts. In a study of C. albicans adhesion to mucosal epithelial cells, King et al (1980) found stationary-phase blastospores to be more adherent than exponential-phase yeasts, whereas Segal et al (1982) found the opposite to be true. This may have been due to the different experimental conditions used in these two studies. Although the optimal pH value for adherence to occur has been reported as 6.0, (Sobel et al, 1981), or 7.3 (King et al, 1980) adherence appears to occur over the pH range 4.0-10.0 (King et al, 1980).

King et al (1980) found adherence to vaginal epithelial cells to

be significantly higher than adherence to buccal epithelial cells, while Sobel et al (1981) found only a slight increase. Sobel et al (1981) showed that adherence of C. albicans to tissue culture cells of human vaginal stratified squamous epithelium compared well with that to exfoliated cells. Samaranayake and MacFarlane (1981) examined adherence of C. albicans to HeLa cells. They reported findings similar to those of Kimura and Pearsall (1978) who had examined adhesion to buccal cells. However they found that the HeLa cell system gave a significantly lower percentage co-efficient of variation between replicate experiments than did the buccal cell system.

C. Factors affecting adherence to epithelial cells

Several factors have been found to affect the adherence of C. albicans to epithelial cells. The adhesion of C. albicans to buccal epithelial cells and acrylic was substantially enhanced after growth of the yeast in defined media containing high concentrations of certain sugars, notably galactose and sucrose, as the carbon source (McCourtie and Douglas, 1981, 1984; Douglas et al, 1981). For example, organisms grown in media containing 500 mM galactose were up to eleven times more adherent than control yeasts grown in media with a relatively low concentration (50 mM) of glucose.

McCourtie and Douglas (1984) have shown that C. albicans strains grown to stationary phase in media containing high concentrations of certain sugars have an additional fibrillar surface layer which appears to enhance adherence.

Factors which enhance germination were also found to enhance adherence of C. albicans. Sobel et al (1981) showed that adherence was blocked by fucose, or by pre-incubation of germinated yeast or pre-incubation of epithelial cells with trypsin or chymotrypsin. Lee and King (1981) have

described a similar effect with papain. Treatment of blastospores with reducing agents, β-mercaptoethanol and dithiothreitol, have also been found to render C. albicans non-adherent (Lee and King, 1983). The same authors also discovered that cell-wall fragments prepared from C. albicans lost their adherence capability when treated with α-mannosidase or papain. Sandin et al (1982) reported that pre-treatment of C. albicans with concanavalin A inhibited yeast adherence to buccal epithelial cells as did methyl α-mannoside. These authors suggested that adhesion could be mediated by a mannose-containing component on the yeast surface. This is in agreement with work carried out by Maisch and Calderone (1981) who examined the role of surface mannan in the adherence of C. albicans to fibrin-platelet clots. They concluded that the cell-surface mannan may play a role in the adherence of C. albicans to fibrin-platelet matrices which form in vivo on the endocardium of heart valves.

Douglas and McCourtie (1983), using a different approach, also suggested the probable involvement of mannoprotein in mediating yeast adhesion. They treated C. albicans cells with tunicamycin, an antibiotic, which in yeasts, specifically inhibits the synthesis of mannoprotein. They found that tunicamycin-treated, galactose-grown yeast cells showed a decrease in adherence of 60% compared to untreated galactose-grown cells. The authors concluded that because of the established specificity of tunicamycin action, the fibrillar surface layer whose synthesis was shown to be inhibited by the antibiotic must consist largely, if not entirely, of mannoprotein. Furthermore, since the production of this layer enhanced yeast adherence, Douglas and McCourtie postulated that mannoprotein plays a key role in the interaction with buccal epithelial cells. McCourtie and Douglas (1985) also inhibited adhesion to buccal epithelial cells by pre-incubating the cells with a crude mannoprotein preparation obtained from

culture supernatants of C. albicans. However, Segal et al (1982) found that mannose, methyl-α-mannoside and mannan had no effect on the adherence of C. albicans to vaginal epithelial cells and only amino sugars and chitin acted as inhibitors. Lehrer et al (1983) blocked in vivo attachment of C. albicans to murine vaginal epithelial cells by pre-treating these cells with a soluble extract of chitin, or N-acetylglucosamine. Treatment of the mice after inoculation of the yeasts did not prevent infection thus indicating that chitin soluble-extract prevented infection by blocking attachment of yeasts to the host mucosal surfaces.

Vudhichamnong et al (1982) showed that secretory IgA isolated from human breast milk inhibited the adherence of C. albicans to human oral epithelial cells. This finding was supported by the investigations of Epstein et al (1982) who demonstrated an inverse correlation between salivary IgA anti-candida antibodies and the adherence of C. albicans to buccal epithelial cells, suggesting that IgA antibodies can inhibit the adherence of candida to the oral mucosa. This action of salivary IgA might help in limiting the oral population of the commensal yeast.

D. Effect of bacteria on yeast adherence

Although several investigators have demonstrated that C. albicans can colonize germ-free chicks and mice in higher numbers than conventional animals (Balish and Philips, 1966; Philips and Balish, 1966) there has been relatively little investigation into the types of bacteria which are capable of suppressing colonization of C. albicans or of the mechanisms involved in this microbial interaction.

Liljemark and Gibbons (1973) reported the suppression of C. albicans by Strep. salivarius and Strep. mitior, but not Strep. mutans, in gnotobiotic mice. They also reported that twice as many cells of C. albicans became attached to tongue and cheek epithelial cells from

germ-free rats as compared to cells from conventional animals. It was concluded that attachment inhibition by the indigenous flora may explain in part the suppression of candida colonization. However, little progress was made in this area of research and it is only in the past five years that any attempt has been made in the understanding of this microbial interaction.

Sobel et al (1981) have shown that lactobacilli inhibited the adherence of C. albicans to epithelial cells. Centeno et al (1983) found that the number of yeasts attaching to epithelial cells was enhanced by pre-incubation of epithelial cells with piliated strains of E. coli and Kl. aerogenes, whereas pre-incubation with non-piliated strains had no effect. This is in agreement with Makrides and MacFarlane (1983) who showed that pre-incubation of HeLa cell monolayers with E. coli cell suspensions resulted in a large, significant increase in candidal adherence. Other investigations by Makrides and MacFarlane (1982) have reported that some strains of oral streptococci inhibit adherence of C. albicans to HeLa cells. Recently Kennedy and Volz (1985) found that the indigenous microflora reduced adhesion of C. albicans to the gastro-intestinal mucosa by forming a dense layer in the mucous gel, out-competing yeast cells for adhesion sites, and by producing inhibitor substances.

VI. Microbial Interactions

The complex nature of the normal oral flora has aroused interest in the possible interactions, both stimulatory and inhibitory that may occur among micro-organisms in the mouth. These interactions influence the microbial composition, metabolic activity and consequently, pathogenicity of the microflora at specific sites in the mouth. Early workers considered using antagonistic members of the oral flora to prevent wounds in the oral cavity from being infected (Hugenschmidt, 1896), and in reducing the incidenc of dental caries (Rutter et al, 1961).

A. Interactions between oral streptococci and other organisms

Examples of the nature of the antagonistic agents produced *in vitro* by various streptococci have been described and include such agents as lactic and acetic acids and hydrogen peroxide (Donoghue and Tyler, 1975). A study by Allen (1985) showed that growth of several species of mycobacteria was inhibited by actively growing 'viridans' streptococci. He concluded that this effect was due to a peroxide-mediated antagonism. Another study by Allen and Swaffield (1982) showed that the growth of *M. tuberculosis* was inhibited by *Strep. mitior* and *Strep. sanguis* II. Other investigators have also reported streptococcal antagonism to mycobacteria. Gelbert *et al* (1974) isolated an α-haemolytic streptococcus that inhibited growth of *M. intracellulare*, *M. avium* and other closely related species.

The production of bacteriocins and bacteriocin-like substances have been detected *in vitro* in strains of *Strep. mutans*, *Strep. sanguis* (Donoghue and Tyler, 1975) and *Strep. mitior* (Dajani *et al*, 1976). These agents have activity against other streptococci commonly found in dental plaque, and some have wide ranges of activity against other oral bacteria. It is likely that the ability of a bacterial species to produce bacteriocins *in vivo* would assist the species to dominate a particular site when growing in competition with a wide range of different bacteria.

Co-aggregation between 'viridans' streptococci and other oral bacteria has been demonstrated by several investigators. Cisar *et al* (1979) reported co-aggregation between *Strep. sanguis* and *Actinomyces* species, an interaction that may play a part in the initial stages of plaque build-up. Co-aggregation reactions between *Strep. sanguis* and *Fusobacterium nucleatum* have also been demonstrated (Lancy *et al*, 1983; Handley *et al* 1985) as have those between *Strep. salivarius* and *Veillonella* (Weerkamp and McBride, 1980).

B. Interaction between lactobacilli and Candida albicans

As far back as 1956, Young et al demonstrated a marked decrease in the number of Candida cells when grown with lactobacilli whereas the lactobacilli showed an increase in numbers. These authors also found that lactobacilli which were unable to grow in vitamin-deficient culture media grew well in the same medium in mixed culture with C. albicans. Young et al suggested that in the normal mouth, a counterbalance exists between the two organisms, with Candida providing nutritional stimulation for lactobacilli and the latter producing lactic acid which prevents the excessive colonization by yeasts.

In studies of the vaginal flora, Saigh et al (1978) noted reduced counts of lactobacilli during and immediately after menstruation, during which times vaginal candidosis occurs with peak frequency. Savage (1969) demonstrated strong microbial interference between indigenous yeasts and lactobacilli in the gastro-intestinal tract of mice.

C. Interactions between C. albicans and other organisms

The interaction of bacteria and fungi on epithelial surfaces has aroused clinical interest for some time. Several investigators have observed the fact that the normal resident bacterial flora suppress fungal colonization, whereas alteration and reduction in surface bacteria, such as occur after antibiotic therapy, are associated with fungal colonization and often symptomatic disease.

Paine (1958) demonstrated the inhibition of Candida by bacteria representative of the usual human flora and, in 1960, Isenberg et al examined the inter-relationship between C. albicans and normal intestinal bacteria. Their results suggested that the bacterial residents of the gastro-intestinal tract may be categorized as inhibitory, stimulatory and indifferent with respect to the yeast's growth.

Several investigators have suggested a direct relationship between gastro-intestinal populations of other microbes and systemic dissemination of Candida (Steffen and Berg, 1983; van der Waaij et al, 1972). Recently Kennedy and Volz (1983) reported that several species of Candida could opportunistically colonize the gut and disseminate to visceral organs after intragastric challenge of antibiotic treated mice. The feeding of C. albicans to animals not given antibiotics did not lead to Candida dissemination from the gastro-intestinal tract.

Since antibiotic treatment predisposes animals to gastro-intestinal overgrowth and subsequent dissemination of Candida it has been suggested that members of the indigenous flora suppress growth of C. albicans within the gut thereby preventing systemic invasion (Myerowitz et al, 1977; Kennedy and Volz, 1985).

Several reports by Carlson (1982, 1983a, 1983) have shown a synergistic effect between toxic shock syndrome-associated S. aureus and C. albicans on the mortality of mice. She found that mice exhibited high resistance when inoculated intraperitoneally by either pathogen alone, but dual infection with the two organisms at doses which separately caused no animal deaths, resulted in 100% mortality. Furthermore, investigations by MacFarlane and Makrides (1982) have shown that the growth of C. albicans is inhibited by S. aureus and E. coli.

Although there have been a number of studies which have demonstrated inhibition of Candida growth by commensal bacteria, the precise nature of the inhibitory activity has not been studied in detail.

Object of Research

When this study was initiated the effect of some members of the commensal flora on yeast adherence had been demonstrated *in vivo* (Liljemark and Gibbons, 1973) and *in vitro* (Makrides and MacFarlene, 1982). In light of these results, the present investigation had the following main objectives:

1. To investigate the ability of fresh and type isolates of oral streptococci and lactobacilli to inhibit the adherence of *C. albicans* to epithelial cells.
2. To investigate whether the effect of the bacteria on yeast adhesion was modified by culture of the yeast or the bacteria in media containing different carbon sources, for example, 500 mM galactose, 500 mM sucrose or 50 mM glucose.
3. To investigate the mechanisms whereby bacteria inhibit yeast adherence to epithelial cells with specific reference to the involvement of lipoteichoic acid.

It was hoped that such studies might lead to a better understanding of the interactions between the commensal bacterial flora and *C. albicans* in the oral cavity.

MATERIALS AND METHODS

I. Micro-organisms Used

A. Origin of micro-organisms

Two strains of Candida albicans were used; strain MRL 3153 was obtained from the Mycological Reference Library, London and strain GDH 2346 was isolated at Glasgow Dental Hospital from a patient with denture stomatitis.

Nineteen strains of oral streptococci and four strains of lactobacilli were used, the origins of which are shown in (Table 1).

B. Isolation and identification of micro-organisms

In order to isolate fresh strains of streptococci and lactobacilli, oral swabs were collected from patients and inoculated onto 10% horse blood agar (Gibco, Paisley). Plates were incubated at 37°C for 24-48 h in an atmosphere of 95% oxygen and 5% CO_2. Colonies of interest, eg. those showing α-haemolysis, were examined by Gram stain and then inoculated onto 10% horse blood agar, and mitis-salivarius agar (Gibco, Europe) or man, rogosa and sharpe agar (MRS; Oxoid, Basingstoke) to obtain pure isolates for identification.

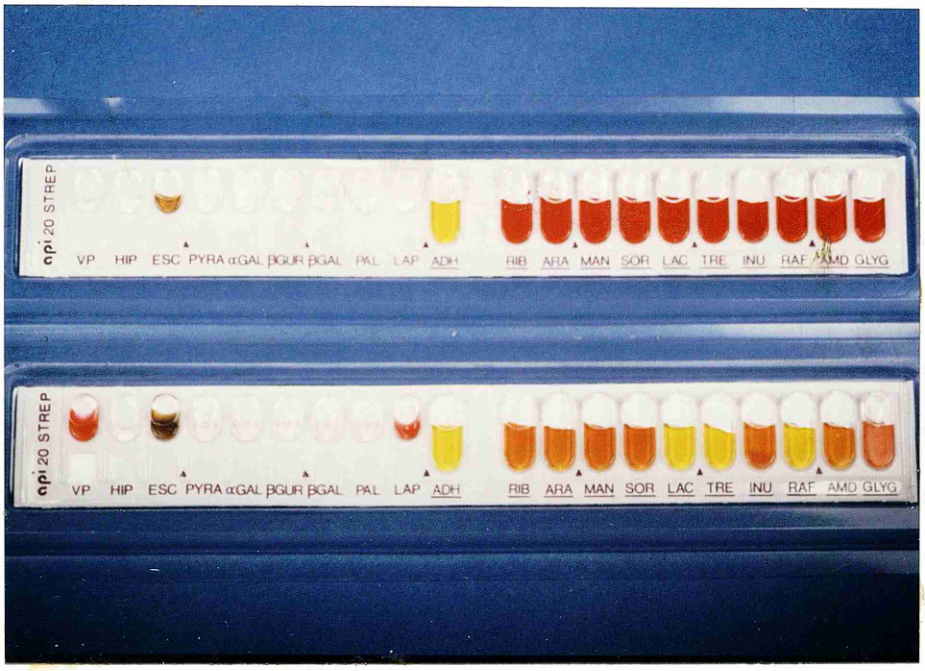
i. Identification of streptococcal strains

Presumptive streptococcal isolates were identified using the API 20 Strep system (API Lab. Products Ltd., Hampshire). This kit enables twenty biochemical tests, arranged in a gallery, to be carried out (Fig. 4). A dense suspension in distilled water (1 ml) was made from a pure culture and part of this suspension (0.5 ml) was used to inoculate the first half of the gallery. The remaining suspension (0.5 ml) was mixed with API Strep medium (2 ml) then pipetted into the remainder of the gallery. The strip was then incubated in an enclosed tray containing 5 ml of water and incubated at 37°C under aerobic conditions. Control strips were set up

Table 1 Origin and location of bacteria in the oral cavity

Organism	Origin of isolate	Predominant location in oral cavity
S. salivarius NCTC 7366	Type collection, Colindale	Dorsum of tongue
S. sanguis NCTC 7864	"	Tooth surface
S. mitior NCTC 10712	"	Buccal mucosa
L. casei NCTC 6375	"	Tooth surface
S. salivarius D66	Denture	Dorsum of tongue
S. salivarius P64	Palate	
S. salivarius M65	Buccal mucosa	
S. salivarius S32	Saliva	
S. salivarius P62	Palate	
S. salivarius C41	Buccal mucosa	
S. salivarius MU289	Manchester Dental Hospital	
S. mitior M65	Buccal mucosa	Buccal mucosa
S. mitior 863	Saliva	
S. mitior M62	Buccal mucosa	
S. mitior 841	Saliva	
S. mitior D32	Denture	
S. mitior P32	Palate	
S. mitior M65	Buccal mucosa	
S. sanguis 1762	Saliva	Tooth surface
S. sanguis 263	Saliva	
L. casei 0112	Saliva	Tooth
L. casei P64	Palate	
L. casei MU259	Manchester Dental Hospital	

Figure 4: API 20 Strep system showing identification of *Streptococcus salivarius*


api 20 STREP
VP HIP ESC PYRA αGAL βGUR βGAL PAL LAP ADH RIB ARA MAN SOR LAC TRE INU RAF AMD GLYG
api 20 STREP
VP HIP ESC PYRA αGAL βGUR βGAL PAL LAP ADH RIB ARA MAN SOR LAC TRE INU RAF AMD GLYG

using type strains. The gallery was examined after 4 h and then again after 24 h. Reactions, both positive and negative, were recorded on a results sheet and compared with the API 20 Strep index.

ii. Identification of lactobacillus strains

Presumptive lactobacillus strains, ie. Gram-positive rods, growth on MRS agar, were identified using API 50 carbohydrate lactobacillus kit (API 50CHL). To prepare the inoculum, the organisms were inoculated into 10 ml of MRS broth and incubated statically at 37°C under aerobic conditions. After 24 h the cells were harvested in sterile universal containers by centrifugation at 3000 g for 10 min on an MSE bench centrifuge. The pellet was washed twice with distilled water and centrifuged as before. A dense suspension of the organism was prepared in API 50CHL medium and used to inoculate the gallery. The gallery was incubated in an enclosed tray containing water and incubated aerobically at 37°C. Strips were examined after 3, 6, 24 and 48 hours of incubation. The positive reactions were recorded and a profile obtained which was compared with API 50CHL profiles for L. casei.

C. Maintenance of cultures

i. Bacteria

All bacterial cultures were freeze-dried by suspending the pellet in 10 ml of Todd-Hewitt broth (THB; Gibco, Paisley) supplemented with 1g sucrose and 2.5 ml rabbit serum (Gibco, Paisley). Ampoules were stored at 4°C. When required, cultures were obtained from the freeze-dried isolates by adding 1 ml of THB to the ampoule and inoculating the suspension onto blood agar. Organisms were subcultured once every month and maintained on 10% horse blood agar and stored at 4°C. A new freeze-dried culture was opened every two months.

ii. C. albicans

Cultures of C. albicans were freeze-dried by suspending the pellet in 2% skimmed milk. Ampoules were stored at 4°C. The yeast was removed from the ampoule by resuspending the pellet in 1 ml of yeast nitrogen base (YNB; Difco) containing 500 mM sucrose and spread onto a Sabouraud dextrose agar plate (Gibco, Paisley). Organisms were sub-cultured once every month and maintained on Sabouraud dextrose agar stored at 4°C. Cultures were replaced every two months from freeze-dried ampoules.

II. Growth of Micro-organisms

A. For adherence assays

Generally, bacterial isolates were cultured in THB. However in some of the adhesion assays to HeLa cells bacteria were cultured in THB which was prepared in the laboratory from individual constituents but containing different concentrations of glucose (50 mM), sucrose (500 mM) or galactose (500 mM). Batches of medium (50 ml in 250 ml Erlenmeyer flasks) were inoculated with overnight bacterial cultures (5 ml) and incubated statically at 37°C aerobically. Cells were harvested after 24 h in universal containers by centrifugation at 3000g for 10 min in an MSE bench centrifuge operating at room temperature. Organisms were washed twice in 0.15M phosphate-buffered saline, pH 7.2 (PBS).

Yeasts were grown in yeast nitrogen base containing 50 mM glucose or 500 mM sucrose or galactose as the carbon source. Batches of medium (50 ml in 250 ml Erlenmeyer flasks) were inoculated with overnight yeast cultures (5 ml) and incubated at 37°C in an orbital shaker operating at 100 rpm. Cells were harvested after 24 h in universal containers by

centrifugation at 1200g for 5 min in an MSE bench centrifuge operating at room temperature. Yeasts were washed twice in PBS before use in assays.

B. For lipoteichoic acid extraction

Bacteria were cultured in 5 x 2000 ml batches of THB. Overnight cultures (200 ml in 500 ml Erlenmeyer flasks) were inoculated with a starter culture (20 ml) and incubated statically at 37°C aerobically. Batches of medium (1800 ml in 2000 ml Erlenmeyer flasks) were inoculated with the overnight bacterial cultures (200 ml) and incubated statically at 37°C for 24 h. Cells were harvested in 500 ml containers by centrifugation at 10000g for 15 min in a Sorval RC-5 centrifuge operating at 4°C. Organisms were washed twice in PBS.

C. For agar disc assays

Bacteria were cultured in THB. Batches of medium (20 ml in sterile universal containers) were inoculated with a loopful of bacteria and incubated statically at 37°C for 24 h aerobically. Cells were harvested by centrifugation at 3000g for 10 min. Organisms were washed twice in PBS before use in assays.

Yeasts were grown in YNB supplemented with 50 mM glucose. Batches of medium (50 ml in 250 ml Erlenmeyer flasks) were inoculated with an overnight culture (5 ml) and incubated at 37°C on an orbital shaker operating at 100 rpm. Cells were harvested after 24 h in universal containers by centrifugation at 1200g for 5 min. Organisms were washed twice in PBS before use.

D. For electron microscopy

Bacteria were grown in Brain Heart Infusion broth (BHI; Gibco). Batches of medium (30 ml in 100 ml flasks) were inoculated with a loopful

of culture from a blood agar plate and incubated statically and aerobically at 37°C overnight. Cells were harvested in universal containers by centrifugation at 3000g for 10 min in an MSE bench centrifuge. Organisms were washed three times in distilled water.

E. For rocket immunoelectrophoresis

Bacteria were grown in Todd-Hewitt broth. Batches of medium (100 ml in 250 ml flasks) were inoculated with an overnight culture (10 ml) and incubated statically and aerobically at 37°C for 24 h. Cultures were centrifuged at 10,000g in a Sorval RC-5 centrifuge operating at 4°C. The cell pellets were discarded and the supernatants retained.

The culture supernatants (100 ml) were passed through a filter (0.22 μm; Sartorius, Calif.) to remove any residual bacteria and dialysed against eight 3 litre changes of distilled water over a period of 3 days. The dialysed culture supernatants were concentrated 20 fold by freeze-drying.

III. Isolation and Purification of Lipoteichoic Acid

A. For Adherence Assays

i. Extraction of LTA

The method used was that of Fischer et al (1983). Bacteria were harvested by centrifugation and suspended in sodium citrate buffer (0.1M; pH 3.0), to give a concentration of 40g wet cells/100 ml. An equal volume of Ballotini beads was added and the mixture shaken for 3 min in a Braun disintegrator (No.2 setting) fitted with a CO_2 cooling device. The beads were removed from the disrupted cell suspension by filtration through a sintered glass funnel (coarse grade 0) and the beads washed with sodium citrate buffer. The pH of the broken cell suspension was adjusted to 4.7

with $NaHCO_3$ (1M). An equal volume of 80% (v/v) aqueous phenol was added, and the mixture shaken at 65°C for 1 h in a water bath. After cooling, the emulsion was centrifuged (1200g for 30 min; MSE bench centrifuge) and the aqueous layer collected. The phenol layer and the insoluble residue were stirred with an equal volume of sodium acetate (0.1M; pH 4.7), centrifuged as before and the aqueous layer collected. The aqueous layers were combined and dialysed against four 5 litre changes of sodium acetate buffer (0.1M; pH 5.0) over a period of 24 h.

ii. Digestion with nucleases

After dialysis of the aqueous extracts, insoluble material was removed by centrifugation (17,000g, 20 min). The mixture was concentrated by ultrafiltration using an Amicon Stirred Ultrafiltration Cell (Amicon Corp., Danvers, Mass.) containing a Diaflo PM10 membrane to give a phosphorus concentration of approximately 20 mM. Deoxyribonuclease II (30 units/ml), ribonuclease (9 units/ml) and ribonuclease T_1 (150 units/ml) were added, together with EDTA (disodium salt; 15 mg/ml), sodium azide (0.2 mg/ml) and chloramphenicol (0.1 mg/ml). The mixture was incubated under toluene at 20°C for 24 h. Subsequently the enzymes were removed by phenol/water extraction at ambient temperature (as described above). The extract was dialysed against distilled water for 48 h and then freeze-dried to give a crude extract of LTA.

iii. Preparation of LTA for adherence assay

LTA was dissolved in saline to give concentrations of 100 μg/ml and 1 mg/ml. Solutions of LTA (1 ml) were then prepared in the range 0-100 μg/ml or 200-1000 μg/ml by diluting aliquots of the stock solutions in saline.

B. Preparation of LTA for antiserum production

For the preparation of antiserum to LTA, the polymer was extracted

by the method of Wicken et al (1973). The preparation yielded by this method is more immunogenic than the phenol extracted material due to the high concentration of contaminating protein present in the crude extract. The organism used was L. casei NCTC 6375 since the antiserum raised against the LTA from this organism will cross-react with LTA from other strains because it has an unsubstituted polyglycerol phosphate backbone.

Cells were extracted with chloroform:methanol (2:1, v/v) in the proportion 1:20 (w/v), by stirring at 18°C for 2.5 h. Organisms were recovered by filtration through a sintered glass filter (No.5), and the extraction procedure was repeated twice. Residual solvent was removed in vacuo and the dry residue of organisms suspended in distilled water (1:10, w/v) at 100°C for 30 min. This procedure was repeated twice, and the combined aqueous extracts were dialysed against six 5 litre changes of distilled water over a period of 48 h. The retentate was clarified by centrifugation (17,000g for 30 min) and freeze-dried to give a crude extract of LTA.

C. Purification of LTA by column chromatography

Crude phenol extracted LTA was purified by column chromatography.

i. Anion-exchange chromatography

The method used was that of Fischer et al (1983). Columns of DEAE-Sephacel (2.5 x 40 cm) were equilibrated in sodium acetate buffer (0.1M; pH 4.7) containing Triton X-100 (0.05%, v/v) by running several litres of this buffer through the gel. The void volume was determined by applying 2 ml of Blue Dextran 2000 (Pharmacia, Uppsala, Sweden) at a concentration of 2 mg/ml in acetate buffer (pH 4.7). This material was eluted with acetate buffer; the volume of buffer collected before the elution of Blue Dextran was equivalent to the void volume.

Lipoteichoic acid (80-100 mg in 8 ml of acetate buffer) was

applied to the column and eluted with sodium acetate buffer (0.1M; pH 4.7) containing 0.05% (v/v) Triton X-100 and a linear gradient (0.05M-1.5M) of sodium chloride (400 ml) which was formed by a gradient mixer (BRL, Bethesda). Flow was downwards at a rate of 25 ml/h. Fractions of 6 ml were collected with an LKB Ultrarac 7000 fraction collector and their absorbance at 280 nm was monitored with a Unicam SP8-100 spectrophotometer. Protein, phosphorus and carbohydrate estimations were carried out on representative samples.

To remove Triton X-100, the combined LTA-containing fractions were diluted with an equal volume of sodium acetate buffer (0.1M; pH 4.7) and concentrated to 40 ml by ultrafiltration with an Amicon stirred ultrafiltration cell containing a Diaflo PM10 membrane. The extract was washed by passing 5 litres of acetate buffer through the ultrafiltration cell. The extract was dialysed against four 5 litre changes of distilled water over a period of 48 h and was then freeze-dried to give a purified preparation of LTA.

ii. Hydrophobic interaction chromatography

A second method of column chromatography was used to purify the phenol extracted LTA. The method used was that of Fischer et al (1983). Columns of Octyl-sepharose (1.5 cm x 30 cm) were equilibrated in sodium acetate buffer (0.1M; pH 4.7) containing 15% (v/v) propan-1-ol by running several litres of this buffer through the column. The void volume was determined as before (i).

Lipoteichoic acid (60-100 mg in 5 ml of the above buffer) was applied to the column and elution carried out by a two-step procedure:

Step 1 - the column was first eluted with 15% (v/v) propan-1-ol in 0.1M acetate buffer. Flow was upwards at a rate of 5 ml/h and fractions of 4 ml were collected.

Step 2 - the column was eluted with sodium acetate buffer (0.1M;

pH 4.7) containing a linear gradient (15-80%, v/v) of propan-1-ol (250 ml) formed by a glass gradient maker (LKB). Flow was upwards at a rate of 25 ml/h. Fractions of 4 ml were collected and their absorbance measured at 280 nm using a Unicam SP8-100 spectrophotometer. Analyses of samples were carried out as for ion-exchange samples. Lipoteichoic acid-containing fractions were combined and dialysed against four 5 litre changes of distilled water over a period of 48 h then freeze-dried to give a purified preparation.

D. Deacylation of Lipoteichoic Acid

This was carried out by the method of Ofek et al (1975). Purified LTA (10 mg) was dissolved in distilled water (1 ml). A small volume (0.5 ml) of 30% ammonium hydroxide was added to 0.5 ml of the LTA solution and incubated at ambient temperature overnight. The mixture was dried under a stream of nitrogen gas and the residue resuspended in distilled water (1 ml). To this was added 1 ml of chloroform:methanol (2:1) and the mixture left at room temperature for 1 h. This extraction procedure was repeated three times and the aqueous layers were pooled, dialysed against distilled water and freeze-dried.

IV. Chemical and Chromatographic Analyses

Chemical and chromatographic analyses were carried out on column fractions, crude LTA preparations and concentrated culture supernatants.

A. Chemical analysis

Phosphorus determination: phosphorus was estimated by the method of Chen et al (1956) using KH_2PO_4 as a standard (Appendix I).

Carbohydrate determination: carbohydrate was estimated by the method of Dubois et al (1956) using glucose as a standard (Appendix I).

Protein determination: protein was estimated by the method of Lowry et al (1951) using bovine serum albumin as a standard (Appendix I).

B. Paper chromatography

i. Acid hydrolysis

A sample of LTA (5 mg) was placed in an ampoule and 1 ml of 2N HCl was added. The ampoule was sealed and heated at 105°C for 3 h in an oven; it was then opened and the hydrolysate transferred to a bijou and evaporated to dryness over KOH in a dessicator. The hydrolysate was re-dissolved in 4 drops of distilled water.

ii. Chromatography of hydrolysates

Descending chromatography on Whatman No.1 paper (23 cm x 56 cm) was done at room temperature in butan-1-ol:pyridine:water (6:4:3; v/v) for 24 h (Jeanes et al, 1957). Standard solutions of glucose, galactose, rhamnose, glucosamine (10 mg/ml) and glycerol (10% v/v) were co-chromatographed with each LTA hydrolysate. After 24 h the chromatogram was removed, dried and developed for a further 24 h. Hydrolysis products were visualised using the alkaline silver nitrate dip reagent for sugars (Trevelyan et al, 1950; Appendix II).

V. Adherence Assays

A. HeLa Cell Assay

i. Preparation of HeLa cell monolayers on glass coverslips

HeLa cells obtained from the Virology Department, Glasgow University, were grown as monolayers in 81 sq.cm. plastic tissue culture flasks (Gibco, Europe) in Eagle's Minimal Essential Medium (MEM: Gibco) supplemented with 10% newborn calf serum, penicillin/streptomycin (100 μg/ml) and glutamine (292 μg/ml). The flasks were incubated for three days at 37°C in an atmosphere of 95% air and 5% CO_2. Once confluent monolayers

were obtained, the growth medium was discarded and the monolayers washed for 15 s with sterile PBS. Trypsin solution (0.25%:1 ml) was added to the monolayers and left at room temperature for 20 s. Excess trypsin was discarded and the flasks placed in an incubator at 37°C until cell detachment was observed. The cells were then suspended in the growth medium described above to an approximate concentration of 10^5 cells/ml as determined by counting in an improved Neubauer haemocytometer chamber (Hawksley, England).

Glass coverslips (22 x 22 mm) were washed, dried, wrapped individually in aluminium foil and sterilized in a hot air oven prior to monolayering with HeLa cells. The coverslips and cell suspension (3.5 ml) were placed in a sterile multiwell tissue culture tray (Sterilin, Teddington, Middlesex) and incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. Over a period of 2-3 d confluent monolayers were obtained, and prior to carrying out the adherence assay, the monolayers were washed briefly in PBS.

ii. Preparation of bacterial suspensions

Bacteria were harvested by centrifugation at 3000g for 10 min (MSE minor bench centrifuge) and washed twice in PBS. Organisms were resuspended in PBS to a concentration of 10^8/ml, determined as before.

iii. Preparation of yeast suspensions

Yeasts were harvested by centrifugation at 1200g for 5 min (MSE minor bench centrifuge), washed twice and then resuspended in PBS to give a concentration of 10^8/ml, determined as before.

iv) Adherence assay

The method used was that of Samaranayake and MacFarlane (1981). Washed HeLa cell monolayers on glass coverslips were incubated with 2 ml of a bacterial cell suspension. Incubation was carried out at 37°C on an orbital shaker at 60 rpm for 30 min. The bacterial cell suspension

was sucked out using a pasteur pipette and the monolayers washed for 20 s with PBS. The monolayers were then incubated with a suspension of C. albicans (2 ml) at 37°C for 30 min on an orbital shaker operating at 60 rpm. The yeast suspension was removed with a pasteur pipette and the monolayers washed for 20 s with PBS. The monolayers were then fixed in cold acetone for 3-4 min, dried in air and then stained using Grams method. Finally, the coverslips were mounted on glass microscope slides using Harlecco synthetic resin (Kodak, Liverpool). Control monolayers were incubated with PBS instead of a bacterial suspension prior to incubation with the yeast suspension.

Each assay was carried out in duplicate and repeated four times.

The number of adherent yeasts were counted using the Optomax III System (Fig. 5) which is an image analyzer. The stained monolayer is placed on a microscope to which there is a camera attached. The monolayer with adherent yeasts is projected onto the screen of the central processing unit (Fig. 6). The size of field to be counted is set using the adjustable circular frame. The number of yeasts attaching to the monolayer are detected by virtue of their contrast as compared with the surrounding background. A count pulse is generated and the machine simply counts the number of 'count pulses' within the measuring frame, for example, in this situation the number of yeasts in the field. This information is then passed into the computer for statistical analysis. Thirty fields were counted at random for each slide and the mean calculated.

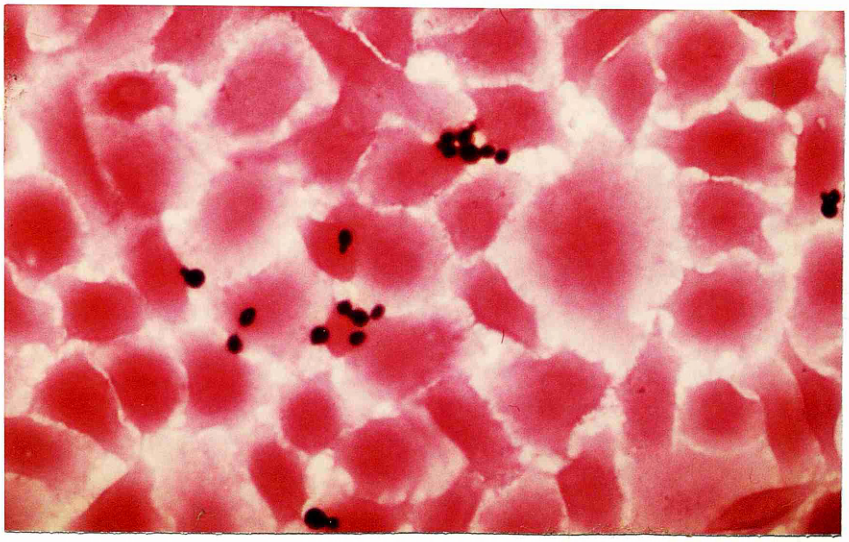
v. Statistical analysis

The Student t test was used to evaluate the differences in inhibition of yeast adhesion produced by the bacteria. A P value of < 0.05 was considered significant.

B. Adherence to human buccal epithelial cells


SYSTEMS
Nikon

Figure 6: HeLa cell monolayer with adherent yeasts (X40).



i. Collection and preparation of epithelial cells

Buccal epithelial cells were obtained from a healthy adult by gently rubbing the inside of the cheeks with sterile swabs which were then agitated in PBS. Epithelial cells were collected by centrifugation at 1200g for 5 min (MSE bench centrifuge), washed twice in PBS to remove any unattached micro-organisms and resuspended in PBS to a concentration of 10^5 cells/ml as determined by counting in an improved Neubauer haemocytometer chamber (Hawksley, England).

ii. Preparation of lipoteichoic acid suspensions

Lipoteichoic acid (LTA) was resuspended in PBS to give a concentration of 100 μg/ml. In some assays the concentration was increased to 1 mg/ml.

iii. Preparation of yeast suspension

Yeast suspensions were prepared as described for the HeLa cell assay (page 73).

iv. Adherence assay

The method of Kimura and Pearsall (1978) was used with minor modifications. Buccal epithelial cells were centrifuged for 1 min (Beckman microfuge). LTA solution of the appropriate concentration (1 ml) was added to the pelleted epithelial cells and the mixture incubated in small screw-capped bottles at 37°C for 30 min in an orbital shaker operating at 100 rpm. After 30 min the epithelial cells were pelleted (Beckman microfuge), washed twice with PBS and resuspended in 1 ml of PBS. Standardized suspensions of epithelial cells (0.1 ml) and yeasts (0.1 ml) were mixed in small screw-capped bottles and incubated at 37°C with gentle shaking for 45 min. The epithelial cells with attached yeasts were collected on polycarbonate filters (12 μm pore size; 25 mm diameter; Nucleopore Corp., Pleasanton, USA) and washed with 30 ml of PBS to remove unattached yeasts. The washed epithelial cells on the filters were air-

dried, fixed with absolute methanol and stained by the Gram procedure. In the control assay the epithelial cells were incubated with PBS (1 ml) instead of LTA solution prior to incubation with the yeast suspension.

The number of adherent yeasts on each of 100 epithelial cells were counted on each filter under X400 magnification using a Vickers microscope. Each slide was counted 'blind' to avoid prejudice. Filters were prepared in triplicate for each assay. Adherence values quoted represent mean figures derived from three independent assays.

v. Statistical analyses

The Student t test was used to evaluate differences in inhibition of yeast adhesion produced by LTA. A P value of < 0.05 was considered significant.

VI. Immunological Studies

A. Preparation of anti-lipoteichoic acid antiserum

Crude LTA (10 mg) was dissolved in saline (1 ml) and mixed thoroughly with Bayol F (1.2 ml: Esso; kindly provided by Dr. D.E.S. Stewart-Tull) and 0.6 ml Arlacel A (Honeywell-Atlas Ltd., Carshalton, Surrey). A 3 month old New Zealand white rabbit was injected intramuscularly into each hind limb with 0.5 ml of this mixture of LTA in incomplete Freund's adjuvant (Freund and Bonanto, 1944). The rabbit was bled from the ear before injection to test for the presence of antibodies to LTA. After 4 weeks a booster injection was administered intraperitone (1 ml of LTA/adjuvant mixture). After a further 2 weeks the rabbit was again bled and tested for antibody production. The rabbit was given a booster injection every 8 weeks for a further six months and then finally bled by cardiac puncture.

B. Double-diffusion precipitin test

Serum was tested for the presence of antibodies to LTA by the slide method of Ouchterlony (1958). A microscope slide was covered in agarose (Appendix III), which was allowed to set before wells (2 mm diameter and 4 mm apart) were cut out with a punch. Wells were filled with antiserum or antigen (5 mg/ml). Slides were incubated in a moist atmosphere (a petri dish containing dampened filter paper) for 24 h at 37°. Slides were washed in saline over a period of 24 h at 4°C and then given a final wash in distilled water. The gel was covered with a piece of moist filter paper and dried with a hot air dryer. The slide was stained with 0.5% Coomassie brilliant blue R (Appendix III), destained and finally dried.

C. Quantitative precipitin test

The method used was that of Dean and Webb (1928). Increasing amounts of PBS (0.1 ml-0.9 ml) were pipetted into test tubes with decreasi amounts of LTA (100 μg/ml) to give a final volume in each tube of 1 ml. Serum (0.1 ml) was added to each tube and the contents mixed by gentle shaking. The tubes were incubated at 37°C in a water bath and observed at regular intervals for the development of turbidity. After 1 h the tubes were removed and placed at 4°C for 3 days to allow complete precipitation to occur.

The contents were centrifuged for 15 min at 1200g in an MSE minor bench centrifuge. The pellet was resuspended in cold PBS and centrifugation repeated. The pellet was washed three times. The antibo content of the precipitate was quantitated by the method of Lowry et al (1951, Appendix I).

D. Rocket immunoelectrophoresis

Culture supernatants were examined for the presence of extracellular LTA by rocket immunoelectrophoresis using the method of Jacques et al (1979). Glass plates (5 cm x 5 cm) were coated with 1% agarose in phosphate buffer (0.01M; pH 7.2), containing 0.01% sodium azide and 200 µl of antiserum (3.5 ml of gel per plate). Antigen samples (5 µl) were pipetted into wells cut in the agarose (2 mm diameter) and the electrophoresis was run in phosphate buffer at 120V for 2 h. The temperature was controlled at 10-12°C by circulating cold water. When the electrophoresis was completed, the plates were covered with filter paper presoaked in 0.85% NaCl and dried at 37°C for 24 h. The plates were then washed over a period of 24 h with three changes of 0.85% NaCl, covered with filter paper presoaked in distilled water and dried at 37°C for 24 h. The precipitin lines were stained by immersion in 0.5% Coomassie brilliant blue R for 10 min. The gel was then destained in 96% ethanol:glacial acetic acid:water (9:2:9; v/v) and dried.

VII. Agar Disc Assay

The method used was that of MacFarlane and Makrides (1982) with slight modification in that the discs were stained before examination. In this assay the test micro-organisms (C. albicans) are incorporated int agar discs and the effector organisms (bacteria) are suspended in fluid growth media which is pipetted on top of the agar discs.

A. Preparation of agar discs

Purified agar (1%) was prepared in deionized water, dispensed in 7 ml volumes in universal containers and autoclaved at 121°C for 20 mi The final pH of the agar was 6.0.

Yeasts were resuspended in PBS to a concentration of 10^7/ml

determined as before, and 20 µl of the standardized suspension added to 7 ml of molten agar and poured into a sterile petri dish (9 cm diam: Sterilin, Middlesex). The plate was stored at 4°C for 1 h. Small discs of agar, approximately 1 mm thick and 4 mm in diameter, were cut out using a No.2 cork borer, sterilized by heating in a bunsen flame.

B. Preparation of bacterial suspensions

Washed cell pellets were resuspended to a concentration of 10^6/m in fresh Todd-Hewitt broth, determined as before.

C. Growth-inhibition assay

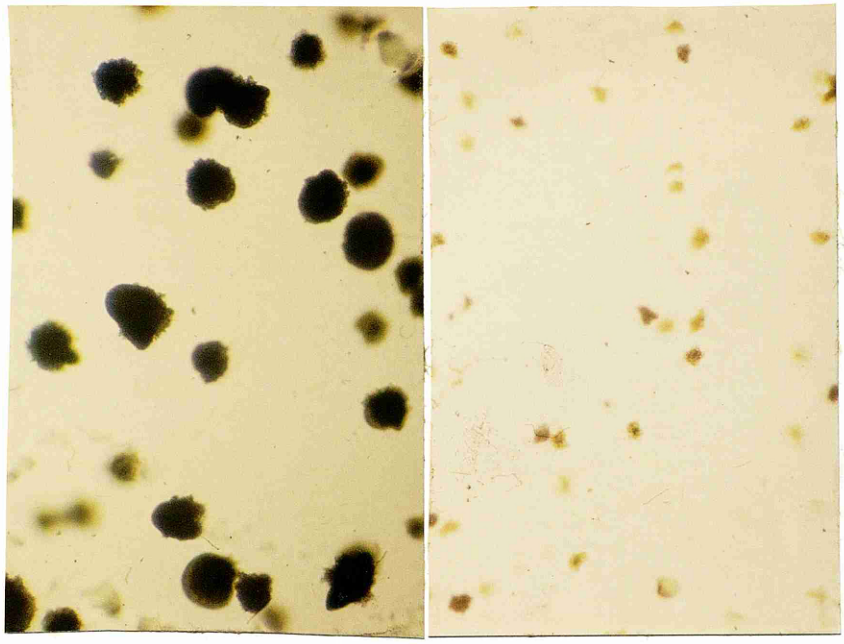
The appropriate bacterial suspension (0.3 ml) was added to the wells of a sterile microtitre tray (Nunc, Denmark). The agar discs containing the yeasts were then added to the wells using a loop and the tray incubated at 37°C for 24 h. Control discs were incubated in THB (0.3 ml) under the same conditions. The assays were carried out in triplicate and repeated three times. After 24 h, discs were stained with 1% crystal violet, placed on glass microscope slides, air dried and examined at X10 magnification.

Microbial growth in the test and control discs was compared microscopically and results were expressed as total inhibition (TI), partial inhibition (PI) or no inhibition (NI). In PI, the colonies in test discs were smaller in size than those in control discs (Fig. 7).

VIII. Electron Microscopy

Mesh grids were coated with formvar (0.25%, w/v) and carbon evaporated onto the surface. For negative staining, one drop of 1% ammonium molybdate was added to one drop of turbid suspension on the grid

Figure 7: Agar disc assay showing partial inhibition of yeast growth.



Excess liquid was removed from the grid using filter paper. The preparations were examined and photographed using a Philips EM300 electron microscope operating at 60 kV.

RESULTS

I. Identification of isolates

Streptococcal strains which were isolated from various sites of the oral cavity (Table 1, Materials and Methods) were identified by the API 20 Strep system.

Streptococcus salivarius strains produced large mucoid colonies when grown on mitis-salivarius agar and when inoculated into the API strips gave positive reactions for acetoin production, β-glucosidase activity, alkaline phosphatase activity, leucine arylamidase activity, and fermented trehalose, raffinose and starch (Table 2).

Streptococcus mitior strains showed two different reaction patterns, both of which are acceptable in the identification of this organism. Four of the isolates, C32, 863, P32 and D32 gave positive reactions for leucine arylamidase activity and lactose fermentation (Table 3). The other two Strep. mitior strains (M65 and P62) gave positive reactions for β-galactosidase activity and leucine arylamidase activity and fermented lactose (Table 4).

Only two strains of Streptococcus sanguis were identified. Strain 1762 gave positive reactions for β-glucosidase activity, alkaline phosphatase, leucine arylamidase activity, arginine dehydrolase activity and fermented lactose, trehalose and inulin (Table 5). Strain 263 produced a different reaction pattern. In addition to being positive for all the reactions shown by strain 1762, it was also positive for α-galactosidase activity (Table 6). Again both profiles are acceptable for the identification of Strep. sanguis.

Lactobacillus strains were identified using the API 50CHL system. Two strains of Lactobacillus casei (0112 and P64) were identified, both giving the same biochemical profile (Tables 7 and 8).

II. The effect of bacteria on the adherence of Candida albicans to HeLa cells

Table 2 Identification of Streptococcus salivarius isolates

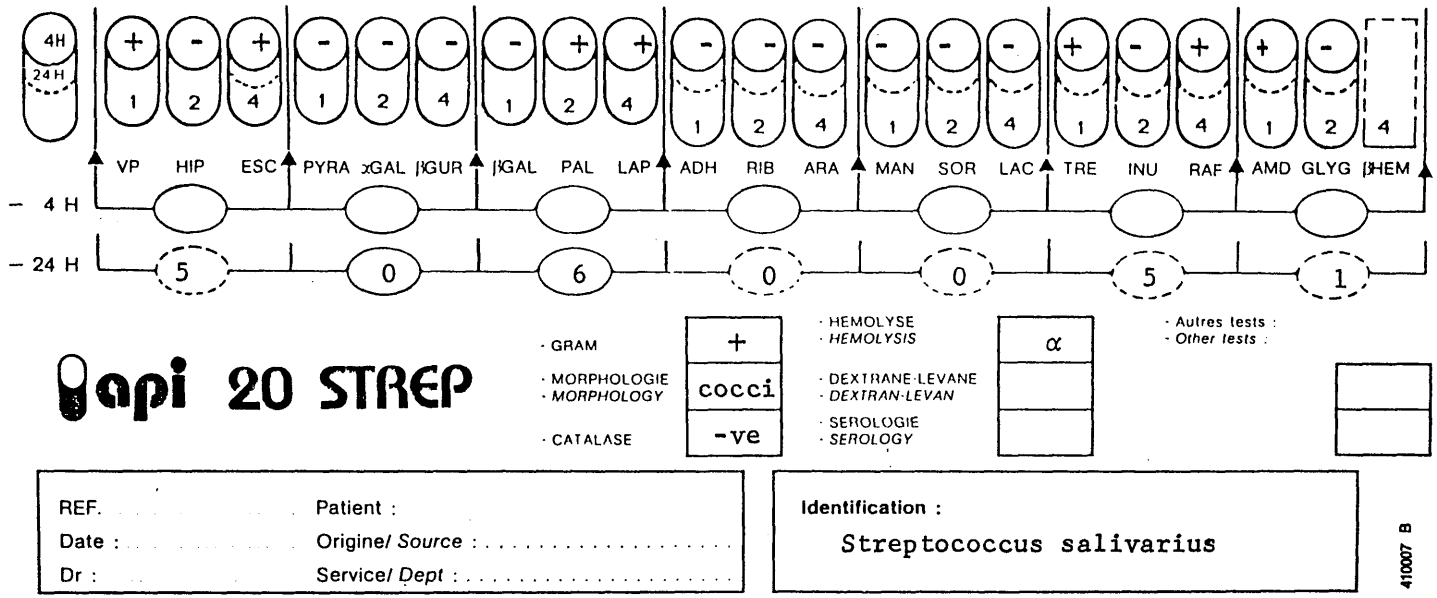


Table 3 Identification of Streptococcus mitior isolates

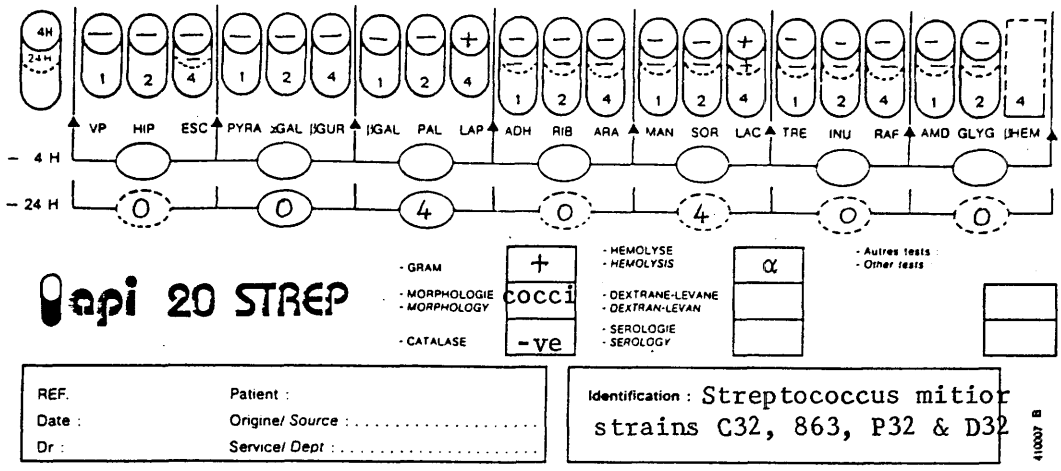


Table 4 Identification of Streptococcus mitior isolates

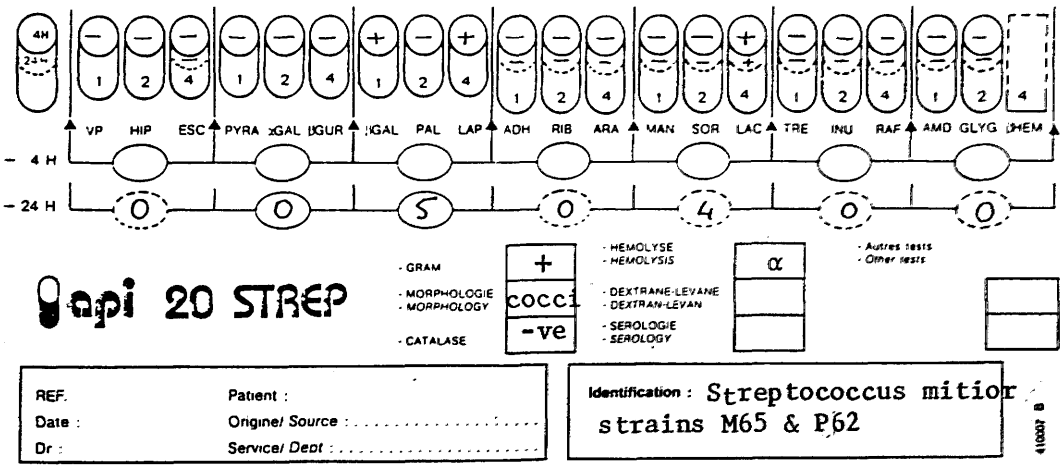


Table 5 Identification of Streptococcus sanguis isolate

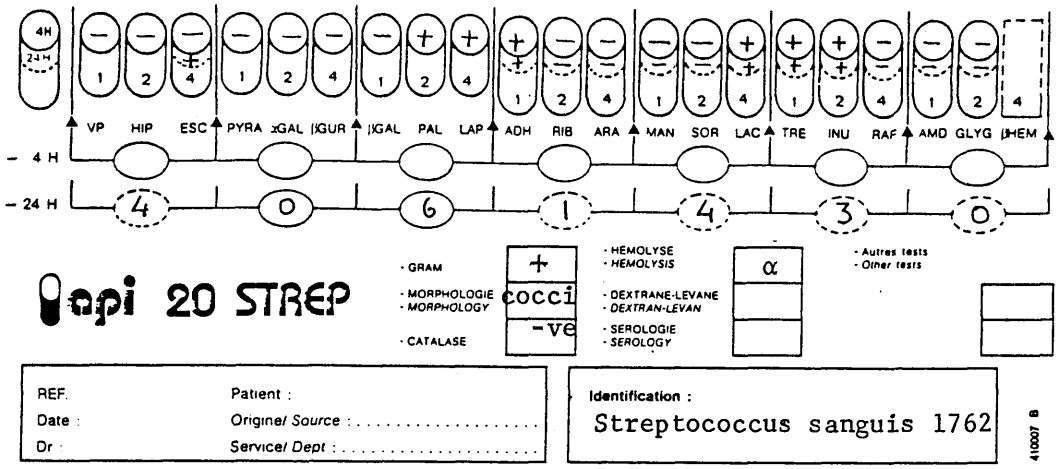


Table 6 Identification of Streptococcus sanguis isolate

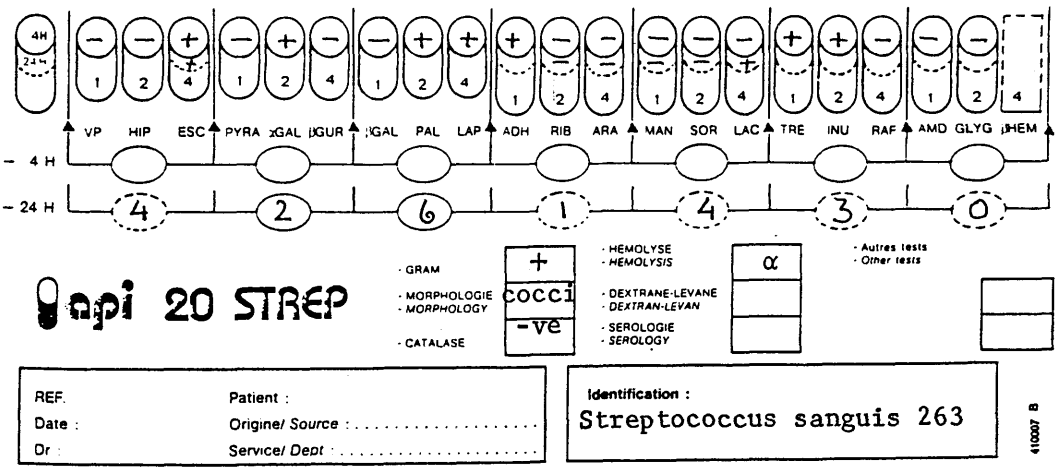


Table 7 Identification of Lactobacillus isolates

api 50 CH

Reference _____ Date _____

Origine/Source Saliva (0112) Dept./Service _____

AUTRES/OTHER TESTS AUTRES/OTHER TESTS

code API 20			

IDENTIFICATION

L. casei (0112)

Température d'incubation / Incubation temperature : _____

Milieu d'inoculation / Inoculation medium : _____

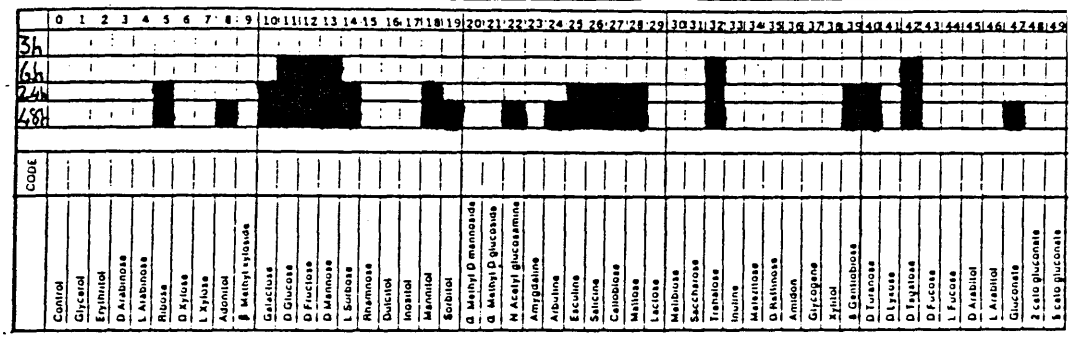


Table 8 Identification of Lactobacillus isolates

api 50 CH

Reference _____ Date _____

Origine/Source Palate Dept./Service _____

AUTRES/OTHER TESTS AUTRES/OTHER TESTS

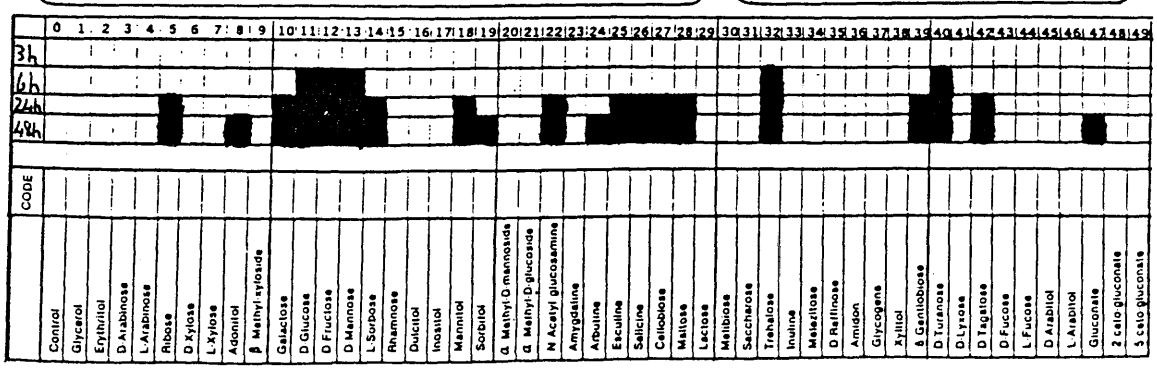
code API 20			

IDENTIFICATION

L. casei P64

Température d'incubation / Incubation temperature : _____

Milieu d'inoculation / Inoculation medium : _____



A. Adherence of Candida albicans MRL 3153 to HeLa cells as affected by pre-incubation of monolayers with a bacterial suspension for different time periods

To determine whether the length of pre-incubation of HeLa monolayers with bacterial suspension affected the subsequent adherence of C. albicans, pre-incubation of monolayers with Strep. salivarius NCTC 7366 was varied at 5-min intervals for a period of 1 h prior to performing adherence assays with Candida.

For this experiment the yeast was grown in yeast nitrogen base (YNB) supplemented with 50 mM glucose with the streptococci being grown in Todd-Hewitt broth (THB).

As can be seen from Table 9, adherence of C. albicans was only affected when the monolayers were pre-incubated with the bacterial suspension for 25 min or longer. Since a pre-incubation period of 30-35 min gave optimal inhibition of yeast adhesion to HeLa cells this time was chosen for all further experiments.

B. Effect of bacteria on the adherence of Candida albicans MRL 3153 to HeLa cells after growth of the yeast in defined medium supplemented with different carbon sources

In the following experiments Candida albicans was grown in YNB supplemented with either 50 mM glucose, 500 mM sucrose or 500 mM galactose; the bacteria were grown in THB.

i. Effect of eight strains of Streptococcus salivarius on the adherence of Candida albicans MRL 3153 to HeLa cells

Eight strains of Strep. salivarius were examined for their effect on the adherence of C. albicans to HeLa cells. The effect of the eight strains on the adherence of C. albicans cultured in 50 mM glucose is shown

Table 9 Adherence of 50 mM glucose-grown Candida albicans MRL 3153 to HeLa cells as affected by the period of pre-incubation, with Streptococcus salivarius NCTC 7366

Pre-incubation time (min)	Mean number of adherent yeasts/field ± SE[a]	Relative adherence[b]	P[c]
5	40.5 ± 2.6	1.05	NS
Control	38.7 ± 3.0		
10	36.2 ± 3.1	0.92	NS
Control	39.4 ± 3.6		
15	32.8 ± 4.0	0.89	NS
Control	36.9 ± 3.4		
20	30.9 ± 3.6	0.91	NS
Control	34.1 ± 2.8		
25	34.3 ± 2.6	0.75	< 0.01
Control	45.6 ± 3.1		
30	29.7 ± 2.8	0.68	< 0.001
Control	43.8 ± 3.0		
35	36.1 ± 2.7	0.62	< 0.0001
Control	58.2 ± 2.1		
40	27.7 ± 2.0	0.67	< 0.001
Control	41.5 ± 3.6		
60	34.9 ± 2.8	0.68	< 0.001
Control	51.2 ± 3.9		

[a]The mean of four different assays carried out in duplicate

[b]Adherence relative to that in control assays in which bacteria were replaced by buffer

[c]Probability values comparing test and control assays for each pre-incubation period

NS - not significant

in Table 10. The adherence of C. albicans to HeLa cells was inhibited by all eight strains of Strep. salivarius, with the degree of inhibition ranging from 28% for Strep. salivarius M65 to 53% for Strep. salivarius D66.

When C. albicans was cultured in YNB + 500 mM sucrose or 500 mM galactose and experiments with the same Strep. salivarius isolates were performed similar results were found.

The adherence of C. albicans cultured in 500 mM sucrose was inhibited by all eight strains of Strep. salivarius, with the degree of inhibition ranging from 31% for Strep. salivarius S32 to 48% for Strep. salivarius P62 (Table 11).

When C. albicans was cultured in 500 mM galactose adhesion of the yeast was once more inhibited by all strains of Strep. salivarius, this time with the degree of inhibition ranging from 33% for Strep. salivarius C41 to 57% for Strep. salivarius MU 289 (Table 12).

The adherence of C. albicans in both control and test assays increased according to the nature and concentration of the carbon source, ie. maximal adherence of C. albicans in both tests and controls was recorded for galactose-grown cells. However the increased ability of the yeast to adhere had little effect on the ability of Strep. salivarius to inhibit adhesion, eg. Strep. salivarius NCTC 7366 inhibited adherence of C. albicans to HeLa cells by approximately 40% irrespective of the carbon source used in the growth of the yeast (Table 13).

ii. Effect of eight strains of Streptococcus mitior on the adherence of Candida albicans MRL 3153 to HeLa cells

Seven strains of Strep. mitior were isolated from various areas of the oral cavity (Table 1, Materials and Methods) and, along with a type strain, were examined for their effect on the adherence of C. albicans to HeLa cells.

Table 10 Effect of eight Streptococcus salivarius isolates on the adhesion of 50 mM glucose-grown Candida albicans MRL 3153 to HeLa cells

Strain of Strep. salivarius	Mean number of adherent yeasts/field ± SE[a]	Relative adherence[b]	P[c]
NCTC 7366	23.0 ± 2.9	0.58	< 0.005
Control	39.5 ± 4.2		
D66	38.0 ± 3.9	0.47	< 0.001
Control	80.4 ± 9.0		
P64	36.6 ± 6.0	0.57	< 0.005
Control	65.0 ± 6.6		
M65	44.3 ± 3.8	0.72	< 0.025
Control	61.4 ± 6.4		
S32	46.2 ± 3.4	0.71	< 0.001
Control	65.4 ± 4.3		
P62	35.8 ± 4.1	0.52	< 0.001
Control	69.4 ± 5.0		
C41	46.1 ± 5.3	0.70	< 0.01
Control	65.8 ± 4.5		
MU289	36.6 ± 6.0	0.57	< 0.01
Control	65.0 ± 8.6		

[a] Mean of four different assays carried out in duplicate

[b] Adherence relative to that in control assays in which bacteria were replaced by buffer

[c] Probability values comparing test and control assays for each bacterial strain.

GRAY, Richard
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Table 11 Effect of eight Streptococcus salivarius isolates on the adhesion of 500 mM sucrose-grown Candida albicans MRL 3153 to HeLa cells

Strain of Strep. salivarius	Mean number of adherent yeasts/field ± SE[a]	Relative adherence[b]	P[c]
NCTC 7366	41.2 ± 4.2	0.56	< 0.001
Control	73.7 ± 6.7		
D66	76.6 ± 5.3	0.55	< 0.001
Control	138.0 ± 8.1		
P64	64.6 ± 7.6	0.58	< 0.001
Control	110.9 ± 6.1		
M65	65.3 ± 4.9	0.61	< 0.001
Control	106.8 ± 7.2		
S32	79.1 ± 9.2	0.69	< 0.005
Control	113.3 ± 6.0		
P62	51.0 ± 3.0	0.52	< 0.001
Control	98.7 ± 7.1		
C41	70.1 ± 6.2	0.67	< 0.001
Control	104.4 ± 4.3		
MU289	64.6 ± 6.6	0.58	< 0.001
Control	110.9 ± 5.8		

[a] Mean of four different assays carried out in duplicate

[b] Adherence relative to that in control assays in which bacteria were replaced by buffer

[c] Probability values comparing test and control assays for each bacterial strain.

Table 12 Effect of eight Streptococcus salivarius isolates on the adhesion of 500 mM galactose-grown Candida albicans MRL 3153 to HeLa cells

Strain of Strep. salivarius	Mean number of adherent yeasts/field ± SE[a]	Relative adherence[b]	P[c]
NCTC 7366	70.1 ± 4.2	0.61	< 0.001
Control	114.8 ± 8.1		
D66	99.9 ± 8.3	0.47	< 0.0001
Control	213.1 ± 10.1		
P64	87.8 ± 7.0	0.51	< 0.0001
Control	172.4 ± 8.3		
M65	90.9 ± 8.6	0.55	< 0.0001
Control	166.0 ± 9.1		
S32	104.1 ± 8.3	0.66	< 0.0001
Control	158.8 ± 4.1		
P62	76.6 ± 4.3	0.61	< 0.0001
Control	125.6 ± 6.1		
C41	88.0 ± 4.0	0.67	< 0.0001
Control	132.3 ± 7.1		
MU289	67.8 ± 4.2	0.43	< 0.0001
Control	157.3 ± 7.1		

[a]Mean of four different assays carried out in duplicate

[b]Adherence relative to that in control assays in which bacteria were replaced by buffer

[c]Probability values comparing test and control assays for each bacterial strain.

Table 13 Summary of the effect of *Streptococcus salivarius* isolates on adherence of *Candida albicans* MRL 3153 to HeLa cells

Strain of *Strep. salivarius*	Relative adherence after growth of yeast in YNB containing: 50 mM glucose	500 mM sucrose	500 mM galactose
NCTC 7366	0.58	0.56	0.61
D66	0.47	0.55	0.47
P64	0.57	0.58	0.51
M65	0.72	0.61	0.55
S32	0.71	0.69	0.66
P62	0.52	0.52	0.61
C41	0.70	0.67	0.67
MU 289	0.57	0.58	0.43

The effect of the eight strains of Strep. mitior on the adherence of C. albicans cultured in 50 mM glucose is shown in Table 14. All strains of Strep. mitior inhibited the adherence of C. albicans with the degree of inhibition ranging from 30% for Strep. mitior M65 to 47% for Strep. mitior D32.

When C. albicans was cultured in YNB + 500 mM sucrose or 500 mM galactose and experiments with the same Strep. mitior isolates were performed similar results were found.

The adherence of C. albicans cultured in 500 mM sucrose was inhibited by 27% with Strep. mitior M65 to 49% with Strep. mitior (D32 and P32)(Table 15).

When C. albicans was cultured in 500 mM galactose yeast adhesion was inhibited by all strains of Strep. mitior in the range of 30-57% (Table 16).

As was the case with Strep. salivarius isolates, all eight strains of Strep. mitior inhibited adherence of C. albicans to HeLa cells to a similar extent irrespective of the carbon source used in the growth of the yeast, eg. Strep. mitior 863 inhibited adherence of C. albicans by 45-50% whether the yeast was cultured in 50 mM glucose, 500 mM sucrose or 500 mM galactose (Table 17).

iii. Effect of three strains of Streptococcus sanguis on the adherence of Candida albicans MRL 3153 to HeLa cells

Only two strains of Strep. sanguis were isolated from the oral cavity: strain 1762 was isolated from saliva and strain 263 from the palate. Both isolates were examined, together with a type strain, for their effect on the adherence of C. albicans MRL 3153 to HeLa cells. As with the other Streptococcus species all strains of Strep. sanguis inhibited adherence of C. albicans whether the yeast was cultured in 50 mM glucose, 500 mM sucrose or 500 mM galactose.

Table 14 Effect of eight Streptococcus mitior isolates on the adhesion of 50 mM glucose-grown Candida albicans MRL 3153 to HeLa cells

Strain of Strep. mitior	Mean number of adherent yeasts/field ± SE[a]	Relative adherence[b]	P[c]
NCTC 10712	44.5 ± 4.2	0.60	< 0.0001
Control	74.0 ± 5.1		
D32	39.3 ± 5.0	0.53	< 0.0001
Control	74.6 ± 6.1		
P32	43.5 ± 5.0	0.65	< 0.025
Control	66.6 ± 8.2		
M65	55.8 ± 8.6	0.70	< 0.025
Control	79.6 ± 5.1		
863	36.7 ± 4.8	0.55	< 0.005
Control	66.6 ± 8.2		
C32	40.2 ± 5.5	0.65	< 0.01
Control	61.4 ± 6.4		
M62	51.2 ± 3.1	0.65	< 0.0001
Control	78.3 ± 5.2		
841	45.6 ± 8.2	0.66	< 0.005
Control	69.1 ± 4.4		

[a] Mean of four different assays carried out in duplicate

[b] Adherence relative to that in control assays in which bacteria were replaced by buffer

[c] Probability values comparing test and control assays for each bacterial strain.

Table 15 Effect of eight Streptococcus mitior isolates on the adhesion of 500 mM sucrose-grown Candida albicans MRL 3153 to HeLa cells

Strain of Strep. mitior	Mean number of adherent yeasts/field ± SE[a]	Relative adherence[b]	P[c]
NCTC 10712	78.6 ± 5.1	0.63	< 0.0001
Control	124.5 ± 4.3		
D32	63.4 ± 5.8	0.51	< 0.0001
Control	123.1 ± 6.2		
P32	58.6 ± 4.4	0.51	< 0.0001
Control	113.8 ± 6.8		
M65	106.7 ± 8.4	0.73	< 0.0001
Control	147.0 ± 4.3		
863	60.3 ± 2.2	0.53	< 0.0001
Control	113.8 ± 6.8		
C32	60.6 ± 2.8	0.57	< 0.0001
Control	106.8 ± 8.8		
M62	65.3 ± 4.7	0.63	< 0.0001
Control	103.5 ± 6.4		
841	70.4 ± 5.6	0.68	< 0.001
Control	103.5 ± 6.4		

[a]Mean of four different assays carried out in duplicate

[b]Adherence relative to that in control assays in which bacteria were replaced by buffer

[c]Probability values comparing test and control assays for each bacterial strain.

Table 16 Effect of eight *Streptococcus mitior* isolates on the adhesion of 500 mM galactose-grown *Candida albicans* MRL 3153 to HeLa cells

Strain of *Strep. mitior*	Mean number of adherent yeasts/field ± SE[a]	Relative adherence[b]	p[c]
NCTC 10712	102.6 ± 8.5	0.56	< 0.0001
Control	184.7 ± 6.5		
D32	82.9 ± 5.3	0.43	< 0.0001
Control	192.7 ± 8.8		
P32	95.4 ± 8.1	0.58	< 0.0001
Control	164.2 ± 6.1		
M65	123.3 ± 9.1	0.63	< 0.0001
Control	196.4 ± 6.0		
863	70.2 ± 4.3	0.49	< 0.0001
Control	144.2 ± 5.9		
C32	85.9 ± 4.2	0.52	< 0.0001
Control	166.0 ± 7.1		
M62	110.2 ± 8.3	0.67	< 0.0001
Control	164.3 ± 6.6		
841	75.9 ± 6.3	0.70	< 0.005
Control	108.2 ± 8.5		

[a] Mean of four different assays carried out in duplicate

[b] Adherence relative to that in control assays in which bacteria were replaced by buffer

[c] Probability values comparing test and control assays for each bacterial strain.

Table 17 Summary of the effect of *Streptococcus mitior* isolates on adherence of *Candida albicans* MRL 3153 to HeLa cells

	Relative adherence after growth of yeast in YNB containing:		
Strain of *Strep. mitior*	50 mM glucose	500 mM sucrose	500 mM galactose
NCTC 10712	0.60	0.63	0.56
D32	0.53	0.51	0.43
P32	0.65	0.51	0.58
M65	0.70	0.73	0.63
863	0.55	0.53	0.49
C32	0.65	0.57	0.52
M62	0.65	0.63	0.67
841	0.66	0.68	0.70

With the 50 mM glucose-grown C. albicans, inhibition of yeast inhibition varied from 47% for Strep. sanguis NCTC 7864 to 23% for Strep. sanguis 1762 (Table 18).

When C. albicans was cultured in 500 mM sucrose inhibition of yeast adhesion ranged from 45% for Strep. sanguis NCTC 7864 to 33% for the two oral isolates (Table 19).

When C. albicans was cultured in 500 mM galactose yeast adhesion was inhibited by 40% with Strep. sanguis NCTC 7864, 35% with Strep. sanguis 1762 and 37% with Strep. sanguis 263 (Table 20).

If the results obtained for the effect of all strains of Strep. sanguis on the adherence of C. albicans cultured on the three carbon sources are compared (Table 21) we can see that the degree of inhibition of yeast adhesion was similar for the two oral isolates. Both strains inhibite yeast adhesion by approximately 25 to 35% irrespective of the carbon source. The type strain, Strep. sanguis NCTC 7864, inhibited adherence of C. albicans to a greater extent than the two isolated strains. It inhibited yeast adherence by 40-55%.

iv. Effect of four strains of Lactobacillus casei on the adherence of Candida albicans MRL 3153 to HeLa cells

Four strains of L. casei, two of which were fresh isolates, were examined for their effect on the adherence of C. albicans to HeLa cells.

When C. albicans was cultured in YNB + 50 mM glucose inhibition of yeast adhesion ranged from 63% for L. casei NCTC 6375 to only 26% for L. casei 0112 (Table 22). Similar effects on yeast adhesion were found when C. albicans was cultured in 500 mM sucrose or 500 mM galactose. With the 500 mM sucrose-grown yeast, inhibition of adhesion ranged from 54% for L. casei NCTC 6375 to 29% for L. casei 0112 (Table 23). With the 500 mM galactose-grown yeast, inhibition of adhesion ranged from 59% for L. casei NCTC 6375 to 30% for L. casei 0112 (Table 24).

Table 18 Effect of three Streptococcus sanguis isolates on the adhesion of 50 mM glucose-grown Candida albicans MRL 3153 to HeLa cells

Strain of Strep. sanguis	Mean number of adherent yeasts/field ± SE[a]	Relative adherence[b]	P[c]
NCTC 7864	20.9 ± 2.4	0.53	< 0.001
Control	39.5 ± 4.2		
1762	34.4 ± 2.5	0.77	< 0.025
Control	44.6 ± 4.0		
263	35.8 ± 3.1	0.74	< 0.01
Control	48.2 ± 3.9		

[a]Mean of four different assays carried out in duplicate

[b]Adherence relative to that in control assays in which bacteria were replaced by buffer

[c]Probability values comparing test and control assays for each bacterial strain.

Table 19 Effect of three Streptococcus sanguis isolates on the adhesion of 500 mM sucrose-grown Candida albicans MRL 3153 to HeLa cells

Strain of Strep. sanguis	Mean number of adherent yeasts/field ± SE[a]	Relative adherence[b]	P[c]
NCTC 7864	40.6 ± 5.6	0.55	< 0.001
Control	73.7 ± 6.3		
1762	50.3 ± 2.5	0.67	< 0.001
Control	75.2 ± 3.7		
263	45.8 ± 4.3	0.67	< 0.001
Control	68.4 ± 5.0		

[a]Mean of four different assays carried out in duplicate

[b]Adherence relative to that in control assays in which bacteria were replaced by buffer

[c]Probability values comparing test and control assays for each bacterial strain.

Table 20 Effect of three Streptococcus sanguis isolates on the adhesion of 500 mM galactose-grown Candida albicans MRL 3153 to HeLa cells

Strain of Strep. sanguis	Mean number of adherent yeasts/field ± SE[a]	Relative adherence[b]	P[c]
NCTC 7864	68.8 ± 6.3	0.60	< 0.001
Control	114.8 ± 8.1		
1762	74.2 ± 2.9	0.65	< 0.001
Control	113.3 ± 3.6		
263	77.6 ± 4.1	0.63	< 0.001
Control	122.5 ± 6.7		

[a]Mean of four different assays carried out in duplicate

[b]Adherence relative to that in control assays in which bacteria were replaced by buffer

[c]Probability values comparing test and control assays for each bacterial strain.

Table 21 Summary of the effect of *Streptococcus sanguis* isolates on adherence of *Candida albicans* MRL 3153 to HeLa cells

	Relative adherence after growth of yeast in medium containing:		
Strain of *Strep. sanguis*	50 mM glucose	500 mM sucrose	500 mM galactose
NCTC 7864	0.53	0.55	0.60
1762	0.77	0.67	0.65
263	0.74	0.67	0.63

Of the 4 strains of L. casei that were examined, the type strain L. casei NCTC 6375, had the greatest effect on yeast adhesion; inhibiting it by 54 to 63%. The poorest inhibitor of yeast adhesion was L. casei 0112. This strain was isolated from the saliva of a patient with oral candidosis.

If the results obtained for the effect of all strains of L. casei on the adherence of C. albicans cultured on the three carbon sources are again compared (Table 25) we can see that the degree of inhibition of yeast adhesion was similar for a given bacterial strain irrespective of the carbon source used in the growth of C. albicans, eg. L. casei 0112 inhibited yeast adhesion to HeLa cells by around 30% under all cultural conditions.

If the effect of all bacterial strains on yeast adhesion is examined no one species shows up as being the most inhibitory. Strains of Strep. salivarius and Strep. mitior inhibited yeast adhesion by approximately 40% on average. Since only half the number of strains of L. casei and Strep. sanguis were examined it is more difficult to compare their effect on yeast adhesion with that of Strep. salivarius and Strep. mitior. Although L. casei NCTC 6375 showed the strongest effect on yeast adhesion of all the bacteria examined, the other strains of L. casei did not have such a pronounced effect, and were on average slightly poorer inhibitors of yeast adhesion compared with Strep. salivarius or Strep. mitior.

C. Effect of bacteria on the adherence of Candida albicans MRL 3153 to HeLa cells after growth of the bacteria in medium containing different carbon sources

In the previous experiments the effect of bacteria on yeasts grown on different carbon sources was examined. In the following experiments the bacteria were grown in Todd-Hewitt broth (THB) containing one of 3 different carbon sources: 50 mM glucose, 500 mM sucrose or 500 mM galactose. Candida albicans was cultured in YNB containing 50 mM glucose for all these experiments.

Table 22 Effect of four Lactobacillus casei isolates on the adhesion of 50 mM glucose-grown Candida albicans MRL 3153 to HeLa cells

Strain of L. casei	Mean number of adherent yeasts/field ± SE[a]	Relative adherence[b]	P[c]
NCTC 6375	27.2 ± 8.6	0.37	< 0.001
Control	74.0 ± 5.1		
0112	43.8 ± 4.3	0.74	< 0.025
Control	58.8 ± 5.2		
MU259	27.2 ± 3.0	0.62	< 0.025
Control	43.9 ± 4.2		
P64	33.3 ± 4.7	0.70	< 0.025
Control	47.6 ± 4.0		

[a] Mean of four different assays carried out in duplicate

[b] Adherence relative to that in control assays in which bacteria were replaced by buffer

[c] Probability values comparing test and control assays for each bacterial strain.

Table 23 Effect of four Lactobacillus casei isolates on the adhesion of 500 mM sucrose-grown Candida albicans MRL 3153 to HeLa cells

Strain of *L. casei*	Mean number of adherent yeasts/field ± SE[a]	Relative adherence[b]	P[c]
NCTC 6375	57.1 ± 5.0	0.46	< 0.001
Control	124.5 ± 4.3		
0112	74.4 ± 4.2	0.71	< 0.001
Control	105.4 ± 6.1		
MU259	49.2 ± 4.1	0.59	< 0.001
Control	83.9 ± 5.8		
P64	58.2 ± 6.3	0.67	< 0.001
Control	87.5 ± 4.4		

[a] Mean of four different assays carried out in duplicate

[b] Adherence relative to that in control assays in which bacteria were replaced by buffer

[c] Probability values comparing test and control assays for each bacterial strain.

Table 24 Effect of four Lactobacillus casei isolates on the adhesion of 500 mM galactose-grown Candida albicans MRL 3153 to HeLa cells

Strain of L. casei	Mean number of adherent yeasts/field ± SE[a]	Relative adherence[b]	P[c]
NCTC 6375	75.3 ± 6.3	0.41	< 0.001
Control	184.7 ± 6.5		
0112	112.0 ± 5.4	0.70	< 0.001
Control	159.0 ± 4.2		
MU259	67.8 ± 6.6	0.56	< 0.001
Control	122.1 ± 7.3		
P64	80.2 ± 7.0	0.63	< 0.001
Control	126.8 ± 4.1		

[a]Mean of four different assays carried out in duplicate

[b]Adherence relative to that in control assays in which bacteria were replaced by buffer

[c]Probability values comparing test and control assays for each bacterial strain.

Table 25 Summary of the effect of *Lactobacillus casei* isolates on adherence of *Candida albicans* MRL 3153 to HeLa cells

Strain of *L. casei*	Relative adherence after growth of yeast in medium containing: 50 mM glucose	500 mM sucrose	500 mM galactose
NCTC 6375	0.37	0.46	0.41
0112	0.74	0.71	0.70
MU259	0.62	0.59	0.56
P64	0.70	0.67	0.63

i. Effect of 50 mM glucose-grown strains of Streptococcus salivarius and Streptococcus sanguis on the adherence of Candida albicans to HeLa cells

Since all bacterial strains inhibited yeast adhesion in the previous experiments, the number of isolates examined in these experiments was reduced. Three strains of Strep. salivarius were tested (2 fresh isolates and the type strain) and two strains of Strep. sanguis (1 fresh isolate and one type strain).

Of the 4 strains examined, Strep. salivarius NCTC 7366 inhibited yeast adherence to the greatest extent (by more than 50%; Table 26), while the other strains of Strep. salivarius (D66 and M65) inhibited adherence by 37% and 31% respectively. The type strain of Strep. sanguis inhibited yeast adhesion by 32% and the fresh isolate inhibited it by 28%.

ii. Effect of 500 mM sucrose-grown strains of Streptococcus salivarius and Streptococcus sanguis on the adherence of Candida albicans to HeLa cells

The effect of pretreating cells with Strep. salivarius and Strep. sanguis grown in sucrose on the adherence of yeast are shown in Table 27. Of the five strains tested, Strep. salivarius D66 gave the strongest inhibition of yeast adhesion (> 50%). The other two strains of Strep. salivarius (NCTC 7366 and M65) inhibited adhesion by 22% and 27% respectively. Streptococcus sanguis NCTC 7864 had no effect on the adherence of C. albicans, and the fresh isolate (Strep. sanguis 1762) only reduced adhesion by less than 20%.

iii. Effect of 500 mM galactose-grown strains of Streptococcus salivarius and Streptococcus sanguis on the adherence of Candida albicans MRL 3153 to HeLa cells

The effect of pretreating cells with Strep. salivarius and Strep. sanguis grown in galactose on the adherence of yeast are shown in Table 28. Of the five strains of bacteria tested, only three strains had any significant

Table 26 Effect of 50 mM glucose-grown strains of *Streptococcus salivarius* and *Streptococcus sanguis* on the adherence of 50 mM glucose-grown *Candida albicans* MRL 3153 to HeLa cells

Strain	Mean number of adherent yeasts/field ± SE[a]	Relative adherence[b]	P[c]
S. salivarius NCTC 7366	31.7 ± 2.4	0.47	< 0.0001
Control	67.1 ± 3.2		
S. salivarius D66	43.5 ± 3.6	0.63	< 0.0001
Control	69.0 ± 1.8		
S. salivarius M65	49.3 ± 4.0	0.69	< 0.0001
Control	71.2 ± 3.4		
S. sanguis NCTC 7864	40.3 ± 2.9	0.68	< 0.0001
Control	59.5 ± 3.3		
S. sanguis 1762	45.6 ± 2.7	0.72	< 0.001
Control	63.3 ± 4.5		

[a]Mean of four different assays carried out in duplicate

[b]Adherence relative to that in control assays in which bacteria were replaced by buffer

[c]Probability values comparing test and control assays for each bacterial strain.

Table 27 Effect of 500 mM sucrose-grown strains of Streptococcus salivarius and Streptococcus sanguis on the adherence of 50 mM glucose-grown Candida albicans MRL 3153 to HeLa cells

Strain	Mean number of adherent yeasts/field ± SE[a]	Relative adherence[b]	P[c]
S. salivarius NCTC7366	52.7 ± 2.5	0.78	< 0.001
Control	67.1 ± 3.2		
S. salivarius D66	31.7 ± 2.3	0.46	< 0.0001
Control	69.0 ± 1.8		
S. salivarius M65	52.3 ± 2.9	0.73	< 0.0001
Control	71.2 ± 3.4		
S. sanguis NCTC 7864	61.4 ± 4.0	1.00	NS
Control	59.5 ± 3.3		
S. sanguis 1762	51.7 ± 2.1	0.82	< 0.025
Control	63.3 ± 4.5		

[a]Mean of four different assays carried out in duplicate

[b]Adherence relative to that in control assays in which bacteria were replaced by buffer

[c]Probability values comparing test and control assay for each bacterial strain

NS - not significant.

Table 28 Effect of 500 mM galactose-grown strains of Streptococcus salivarius and Streptococcus sanguis on the adherence of 50 mM glucose-grown Candida albicans MRL 3153 to HeLa cells

Strain	Mean number of adherent yeasts/field ± SE[a]	Relative adherence[b]	P[c]
S.salivarius NCTC7366	53.4 ± 3.7	0.80	< 0.01
Control	67.1 ± 3.2		
S.salivarius D66	46.7 ± 4.4	0.68	< 0.0001
Control	69.0 ± 1.8		
S.salivarius M65	56.6 ± 2.0	0.79	< 0.001
Control	71.2 ± 3.4		
S. sanguis NCTC 7864	68.1 ± 4.4	1.14	NS
Control	59.5 ± 3.3		
S. sanguis 1762	54.8 ± 3.7	0.87	NS
Control	63.3 ± 4.5		

[a]Mean of four different assays carried out in duplicate

[b]Adherence relative to that in control assays in which bacteria were replaced by buffer

[c]Probability values comparing test and control assays for each bacterial strain

NS - not significant.

effect on the adherence of C. albicans. Streptococcus salivarius NCTC 7366 reduced yeast adhesion by 20% while the other two strains of Strep. salivarius (D66 and M65) inhibited adherence by 32% and 21% respectively. Neither strain of Strep. sanguis had any effect on the adherence of the yeast.

If the effect of the bacteria on yeast adhesion after growth in 500 mM sucrose is compared with that of the bacteria cultured in 50 mM glucose we can see that most strains had a reduced effect on the adherence of C. albicans when cultured in sucrose (Table 29). Streptococcus salivarius NCTC 7366 inhibited adherence by only 22% when grown in sucrose compared with 53% inhibition when grown in glucose. Streptococcus salivarius M65 also had a reduced effect on adhesion but not to as great an extent as Strep. salivarius NCTC 7366. Only Strep. salivarius D66 showed a greater effect when grown in sucrose as compared with glucose.

The effect of the two Strep. sanguis strains was also different when grown in sucrose. Streptococcus sanguis NCTC 7864, when grown in glucose, inhibited yeast adhesion by 32%; but when grown in sucrose it had no effect on the adherence of C. albicans to HeLa cells. The effect of the other Strep. sanguis strains (1762) on yeast adhesion was reduced from 28% to only 18% inhibition.

Growth of the bacteria in galactose also altered their effect on yeast adhesion (Table 29). All strains of bacteria had a reduced effect on yeast adhesion when grown in galactose compared with glucose-grown organisms. Streptococcus salivarius D66, which had a greater effect on yeast adherence when grown in sucrose compared with glucose-grown strains, had a reduced effect when grown in galactose. The other two strains of Strep. salivarius had a similar effect on the adherence of C. albicans when grown in galactose as compared with sucrose-grown strains. The effect on yeast adhesion of the two Strep. sanguis strains grown in

Table 29 Summary of the effect of *Streptococcus salivarius* and *Streptococcus sanguis* isolates on the adherence of *Candida albicans* after growth of bacteria on different carbon sources

	Relative yeast adherence in assays with bacteria grown in medium containing:		
Bacterium used in assay	50 mM glucose	500 mM sucrose	500 mM galactose
Strep.salivarius NCTC7366	0.47	0.78	0.80
Strep.salivarius D66	0.63	0.46	0.68
Strep.salivarius M65	0.69	0.73	0.79
Strep. sanguis NCTC7864	0.68	1.00	1.14
Strep. sanguis 1762	0.72	0.82	0.87

galactose was also similar to the effect produced by the sucrose-grown bacteria.

iv. Effect of 50 mM glucose-grown strains of Streptococcus mitior and Lactobacillus casei on the adherence of Candida albicans MRL 3153 to HeLa cells

The effect of pretreating HeLa cells with Strep. mitior and L. casei grown in glucose on the subsequent adherence of candida are shown in Table 30. All six strains tested inhibited the adhesion of C. albicans. Of the three strains of Strep. mitior tested, C32 inhibited yeast adhesion by only 16% while the other two strains, 863 and M65, reduced adhesion by 36% and 32% respectively. Of all the strains tested, including the lactobacilli, Strep. mitior 863 had the greatest effect on yeast adhesion. When the three Lactobacillus strains were tested, L. casei NCTC 6375 was found to produce the best inhibition of adhesion (34%) with the other two lactobacillus isolates (0112 and MU 259) inhibiting adhesion by 27% and 21% respectively.

v. Effect of 500 mM sucrose-grown strains of Streptococcus mitior and Lactobacillus casei on the adherence of Candida albicans MRL 3153 to HeLa cells

The effect of pretreating HeLa cells with Strep. mitior and L. casei grown in sucrose on the subsequent adherence of C. albicans is shown in Table 31. Of the three strains of Strep. mitior tested, two reduced the adherence of the yeast (863 and M65) while Strep. mitior C32 had no effect on yeast adhesion. All three strains of L. casei reduced yeast adhesion, with L. casei NCTC 6375 again being the strongest inhibitor of the three.

vi. Effect of 500 mM galactose-grown strains of Streptococcus mitior and Lactobacillus casei on the adherence of Candida albicans MRL 3153 to HeLa cells

The effect of pretreating HeLa cells with Strep. mitior and L. casei

Table 30 Effect of 50 mM glucose-grown strains of Streptococcus mitior and Lactobacillus casei on the adherence of 50 mM glucose-grown Candida albicans MRL 3153 to HeLa cells

Strain	Mean number of adherent yeasts/field ± SE[a]	Relative adherence[b]	P[c]
S. mitior C32	51.2 ± 2.0	0.84	< 0.001
Control	62.0 ± 2.6		
S. mitior 863	45.8 ± 3.2	0.64	< 0.0001
Control	72.0 ± 4.7		
S. mitior M65	48.6 ± 2.7	0.68	< 0.0001
Control	70.8 ± 4.3		
L. casei NCTC6375	49.0 ± 3.6	0.66	< 0.0001
Control	74.1 ± 2.5		
L. casei 0112	50.1 ± 2.6	0.73	< 0.0001
Control	68.3 ± 3.0		
L. casei MU259	49.7 ± 3.7	0.79	< 0.025
Control	63.1 ± 2.2		

[a]Mean of four different assays carried out in duplicate

[b]Adherence relative to that in control assays in which bacteria were replaced by buffer

[c]Probability values comparing test and control assays for each bacterial strain.

Table 31 Effect of 500 mM sucrose-grown strains of *Streptococcus mitior* and *Lactobacillus casei* on the adherence of 50 mM glucose-grown *Candida albicans* MRL 3153 to HeLa cells

Strain	Mean number of adherent yeasts/field ± SE[a]	Relative adherence[b]	P[c]
S. mitior C32	57.2 ± 3.2	0.92	NS
Control	62.0 ± 2.6		
S. mitior 863	27.8 ± 1.8	0.39	< 0.0001
Control	72.0 ± 4.7		
S. mitior M65	53.3 ± 3.6	0.75	< 0.005
Control	70.8 ± 4.3		
L. casei NCTC 6375	56.7 ± 2.5	0.76	< 0.0001
Control	74.1 ± 2.5		
L. casei 0112	54.9 ± 2.9	0.80	< 0.005
Control	68.3 ± 3.0		
L. casei MU259	55.0 ± 2.8	0.87	< 0.005
Control	63.1 ± 2.2		

[a]Mean of four different experiments carried out in duplicate

[b]Adherence relative to that in control assays in which bacteria were replaced by buffer

[c]Probability values comparing test and control values for each bacterial strain

NS - not significant.

grown in galactose on the subsequent adherence of C. albicans is shown in Table 32. Of the six strains tested only two had any effect on yeast adhesion. Streptococcus mitior 863 inhibited adhesion by 41%, with Strep. mitior M65 giving a 15% reduction. None of the lactobacillus strains had any effect on adhesion.

If the effect on yeast adhesion produced by the sucrose-grown bacteria is compared with that of the glucose-grown organisms (Table 33) we can see that all the strains, apart from S. mitior 863, produced less of an effect when grown in sucrose as compared with glucose. When Strep. mitior C32 was grown in glucose it inhibited yeast adhesion by 16%, but when grown in sucrose it had no significant effect on candidal adherence. All three lactobacillus strains had a reduced effect on yeast adhesion when grown in sucrose as compared with glucose.

Growth of the bacteria in galactose once again affected their ability to inhibit adherence of C. albicans to HeLa cells (Table 33). Only Strep. mitior 863 had an increased effect on yeast adhesion when grown in galactose as compared with glucose. However, in comparison to sucrose-grown cells, the effect of Strep. mitior 863 on yeast adhesion was markedly reduced when cultured in galactose. The effect of the three galactose-grown lactobacillus strains on yeast adhesion was markedly affected compared with glucose or sucrose-grown strains, with none of the three strains having any effect on yeast adhesion when grown in galactose.

The majority of the strains tested in these assays had a reduced effect on the adherence of C. albicans when grown in either sucrose or galactose as compared with glucose.

As a result of these experiments, ten isolates were chosen for lipoteichoic acid experiments. The strains chosen represent a cross-section based on the effect they had on yeast adhesion, ie. from the most inhibitory to the least inhibitory strain.

Table 32 Effect of 500 mM galactose-grown strains of Streptococcus mitior and Lactobacillus casei on the adherence of 50 mM glucose-grown Candida albicans MRL 3153 to HeLa cells

Strain	Mean number of adherent yeasts/field ± SE[a]	Relative adherence[b]	P[c]
S. mitior C32	58.1 ± 3.2	0.98	NS
Control	62.0 ± 2.6		
S. mitior 863	42.7 ± 4.1	0.59	< 0.0001
Control	72.0 ± 4.7		
S. mitior M65	60.3 ± 2.8	0.85	< 0.05
Control	70.8 ± 4.3		
L. casei NCTC 6375	81.1 ± 2.6	1.10	NS
Control	74.1 ± 2.5		
L. casei 0112	60.2 ± 3.2	0.88	NS
Control	68.3 ± 3.0		
L. casei MU 259	61.7 ± 2.7	0.98	NS
Control	63.1 ± 2.2		

[a]Mean of four different assays carried out in duplicate

[b]Adherence relative to that in control assays in which bacteria were replaced by buffer

[c]Probability values comparing test and control assays for each bacterial strain

NS - Not significant.

Table 33 Summary of the effect of *Streptococcus mitior* and *Lactobacillus casei* isolates on adherence of *Candida albicans* after growth of bacteria on different carbon sources

	Relative yeast adherence in assays with bacteria grown in medium containing:		
Bacterium used in assay	50 mM glucose	500 mM sucrose	500 mM galactose
Strep. mitior C32	0.84	0.92	0.98
Strep. mitior 863	0.64	0.39	0.59
Strep. mitior M65	0.68	0.75	0.85
L. casei NCTC 6375	0.66	0.76	1.10
L. casei 0112	0.73	0.80	0.88
L. casei MU 259	0.79	0.87	0.98

III. Analyses of Lipoteichoic Acid (LTA)

Since lipoteichoic acid has been implicated as mediating adhesion of some streptococci, it was decided to extract this polymer from a selection of the bacteria used in the previous studies to see if it was responsible for the inhibition of yeast adhesion. The method for extracting LTA was the hot phenol/water procedure (Materials and Methods, page 67) since this gives a crude material which has very little contaminating protein present.

A. Chemical analysis of LTA

The carbohydrate, protein and phosphorus content of the crude LTA preparation extracted from the ten selected bacteria are shown in Table 34.

In most strains the protein content of the LTA preparation was very low, accounting for only about 2.4% of the LTA. However, LTA extracted from L. casei NCTC 6375 by chloroform:methanol:water (Materials and Methods, page 68) had a much higher percentage of contaminating protein (15%). This LTA preparation was used to raise anti-LTA antiserum in rabbits because the relatively high percentage of contaminating protein makes it more immunogenic than the hot phenol/water extract.

Carbohydrate and phosphorus accounted for around 55 to 70% of the dry weight of the crude LTA preparations. The remaining 30-45% could probably be accounted for by glycerol, which was not determined and is a major constituent of LTA, nucleic acid and fatty acids.

B. Examination of lipoteichoic acid by paper chromatography

Crude LTA preparations extracted from all ten strains of bacteria were examined after acid hydrolysis, by paper chromatography (Materials and Methods, page 72). The hydrolysis products that were detected are shown in Table 35. For two strains, Strep. mitior C32 and L. casei 0112 only trace amounts of glycerol could be detected. Glycerol was found in hydro-

Table 34 Biochemical analysis of crude lipoteichoic acid extracts

LTA extracted from:	Percent composition		
	Carbohydrate	Phosphorus	Protein
S. salivarius NCTC 7366	34.2	28.1	2.3
S. salivarius M65	36.9	32.3	2.4
S. salivarius D66	29.8	25.1	2.2
S. salivarius MU 289	37.1	34.9	2.1
S. sanguis NCTC 7864	35.4	18.7	2.4
S. mitior C32	32.2	24.1	2.3
S. mitior 863	30.2	26.4	2.4
L. casei NCTC 6375	33.5	31.5	2.4
L. casei 0112	31.7	28.7	2.6
L. casei MU 259	29.4	24.6	2.4
L. casei NCTC 6375 (Chloroform:methanol)	33.6	28.0	14.7

Table 35 Products obtained after acid hydrolysis of crude lipoteichoic acid preparations as detected by paper chromatography

LTA from:	Glycerol	Glucose	Galactose	Glucosamine
S. salivarius NCTC 7366	+	-	+	-
S. salivarius M65	+	+	-	+
S. salivarius D66	+	+	+	-
S. salivarius MU 289	+	+	-	-
S. sanguis NCTC 7864	+	+	+	-
S. mitior C32	+/-	-	-	-
S. mitior 863	+	+	-	+
L. casei NCTC 6375	+	+	+/-	-
L. casei 0112	+/-	-	-	-
L. casei MU 259	+	+	+/-	-

\+ = product present

\- = no product

+/- = trace amounts

lysates from all other strains along with a few other sugars, of which glucose was the most common.

Lipoteichoic acid from L. casei is known to contain glucose and galactose substituents both of which were found in the hydrolysates of L. casei NCTC 6375 and L. casei MU 259. The LTA composition of the other organisms tested has not been described previously.

C. Immunological analysis

Antiserum for use in detecting LTA was prepared against lipoteichoic acid from L. casei NCTC 6375 since it is known that such serum cross-reacts with different LTAs on the basis of the common glycerol phosphate backbone (Wicken and Knox, 1971).

The reactions given by the ten crude LTA preparations to anti-LTA antiserum are shown in Table 36. All LTA preparations gave a positive reaction with the antiserum apart from the material extracted from Strep. sanguis NCTC 7864, which did not react. From this result it would appear that the material extracted from Strep. sanguis NCTC 7864 was not LTA but some other phosphorylated polysaccharide.

D. Purification of lipoteichoic acid

Lipoteichoic acid from four strains of bacteria was purified either by anion-exchange or hydrophobic interaction chromatography as described in Materials and Methods (page 69).

i. Anion-exchange chromatography of crude lipoteichoic acid extracts from L. casei NCTC 6375 and Strep. salivarius NCTC 7366

Crude preparations of lipoteichoic acid from L. casei NCTC 6375 and Strep. salivarius NCTC 7366 were purified by anion-exchange chromatography using DEAE-Sephacel.

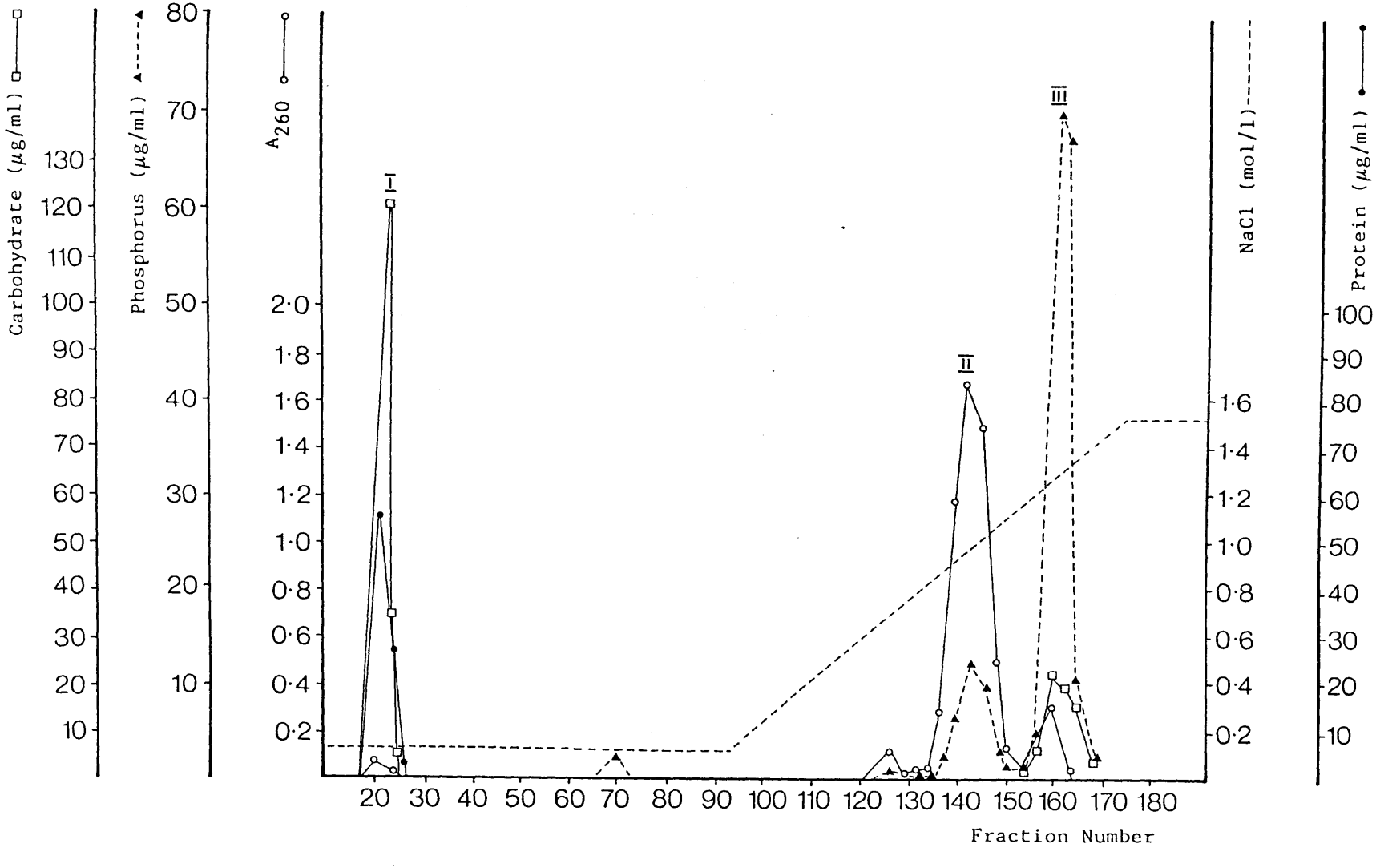
The elution profile obtained for L. casei NCTC 6375 (Fig. 8) was

Table 36 Reaction of LTA preparations with anti-serum raised against LTA from *L. casei* NCTC 6375

LTA from:	Reaction with anti-serum[a]
S. salivarius NCTC7366	+
S. salivarius M65	+
S. salivarius D66	+
S. salivarius MU 289	+
S. sanguis NCTC 7864	-
S. mitior C32	+
S. mitior 863	+
L. casei NCTC 6375	+
L. casei 0112	+
L. casei MU 259	+

[a]Determined by the slide Ouchterlony test described in Materials and Methods, page 79

Figure 8: Purification of lipoteichoic acid from L. casei NCTC 6375 by anion-exchange chromatography.


Carbohydrate (μg/ml)
Phosphorus (μg/ml)
A260
NaCl (mol/l)
Protein (μg/ml)
I
II
III
Fraction Number

similar to that shown by Fischer et al (1983) for Micrococcus varians in that the LTA fraction was the last to be eluted. Lipoteichoic acid from L. casei was bound very strongly to the column shown by the fact that the NaCl gradient had to be increased to greater than 1M to remove bound LTA. This avid binding of LTA from L. casei may be due to the fact that the polyglycerol phosphate backbone is unsubstituted. The material in peak II was confirmed as LTA by using anti-LTA antiserum which had been prepared against LTA from L. casei NCTC 6375. The material from peak III gave a single precipitin band when tested with the antiserum. The material in peaks I and II did not react with the antiserum.

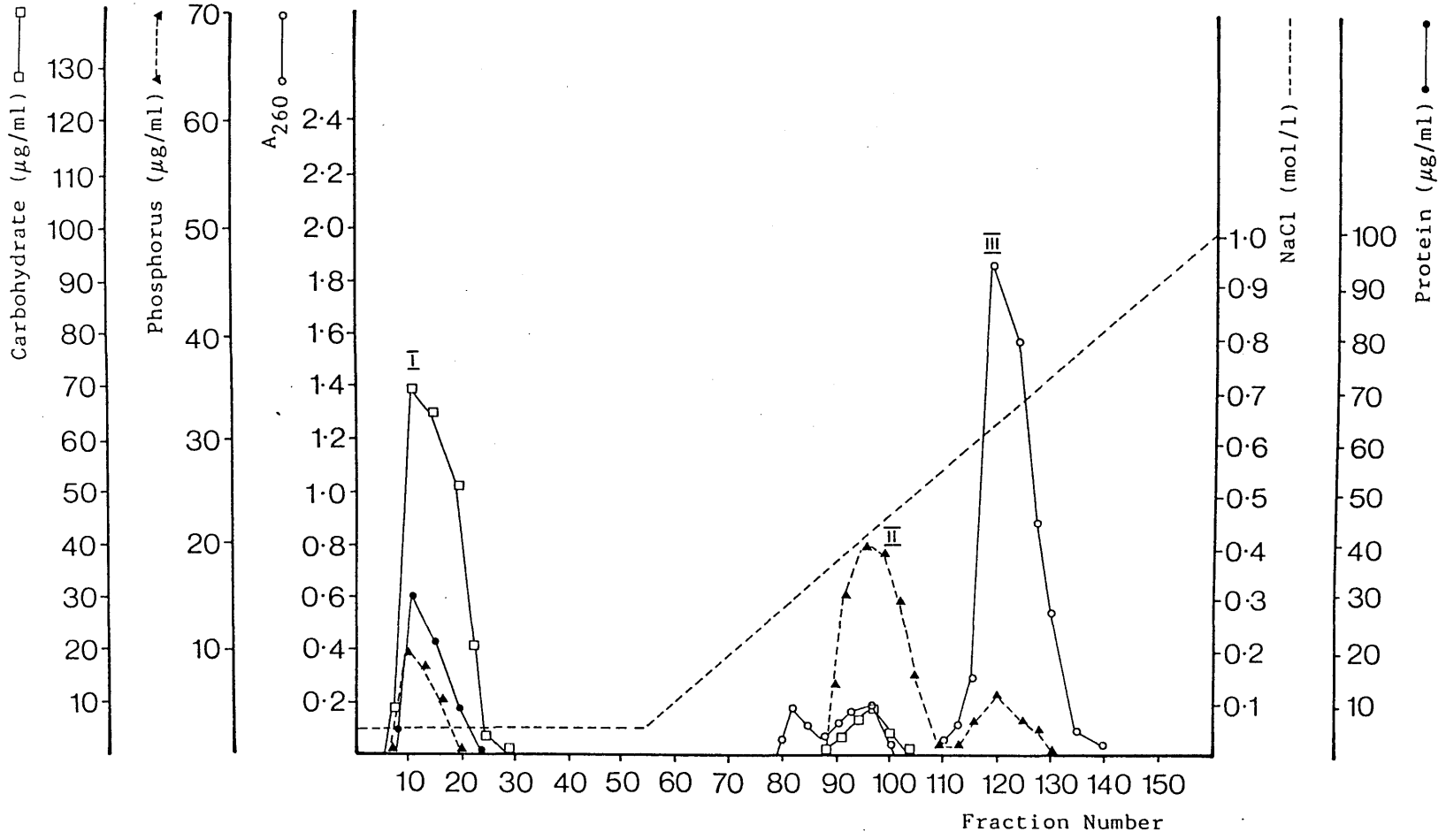
Separation of Strep. salivarius NCTC 7366 lipoteichoic acid (II) from carbohydrate, protein (I) and nucleic acid (III) is shown in Fig. 9. With this preparation, the LTA containing peak was eluted before the nucleic acid peak and is similar to the elution profile for the separation of Strep. faecalis LTA shown by Fischer et al (1983). Lipoteichoic acid from Strep. salivarius NCTC 7366 did not bind as avidly to the column as that of L. casei NCTC 6375. This may be due to a higher carbohydrate substitution of the polyglycerol phosphate backbone. Peak II was confirmed as the LTA containing peak by its positive reaction with the antiserum. Peaks I and III gave a completely negative reaction with antiserum.

ii. Hydrophobic-interaction chromatography of crude lipoteichoic acid extracts from Streptococcus salivarius D66 and Streptococcus mitior C3

Crude preparations of lipoteichoic acid from Strep. salivarius D66 and Strep. mitior C32 were purified by hydrophobic interaction chromatography.

According to Fischer et al (1983) hydrophobic interaction chromatography is more effective at separating polyanionic contaminants and LTA than anion-exchange chromatography. With both Strep. mitior C32 and Strep. salivarius D66, the polymeric contaminants, including nucleic acid, were

Figure 9: Purification of lipoteichoic acid from Strep. salivarius NCTC 7366 by anion-exchange chromatography.


Carbohydrate (μg/ml)
Phosphorus (μg/ml)
A260
NaCl (mol/l)
Protein (μg/ml)
I
II
III
Fraction Number

eluted at step one (Figs. 10 & 11), whereas with anion-exchange chromatography nucleic acid was not eluted until after the gradient had been applied.

Separation of S. mitior C32 LTA (III) from polysaccharide, protein (I) and nucleic acids (I, II) is shown in Fig. 10. Peak III was confirmed as the LTA fraction by its positive reaction with antiserum. The LTA containing peak was split, probably indicating molecular species with different numbers of fatty acid esters. If the flow rate was reduced to 5 ml/h during gradient elution, LTA would separate into molecular species according to the number of their acyl groups.

Separation of Strep. salivarius D66 LTA (II), from polysaccharide, protein and nucleic acid (I) is shown in Fig. 11. The nucleic acid fraction eluted completely in peak I. Again, LTA was confirmed as peak II by its positive reaction with antiserum.

Apart from the more effective separation of polyanionic contaminants and LTA, the other advantage of hydrophobic interaction chromatography over anion-exchange chromatography is the ready removal of propanol as compared to Triton X-100.

IV. Effect of lipoteichoic acid on the adherence of Candida albicans to buccal epithelial cells

In the assays previously described the system chosen to study the effect of bacteria on yeast adhesion involved the use of HeLa cells because such cells are free of any indigenous flora. However, in the following experiments buccal epithelial cells were used in adhesion studies since they represent more closely the in vivo situation.

Buccal epithelial cells were pretreated with the appropriate dilution of LTA (as described in Materials and Methods, page 77) before the addition of the yeast suspension.

Figure 10: Purification of lipoteichoic acid from Strep. mitior C32 by hydrophobic interaction chromatography.

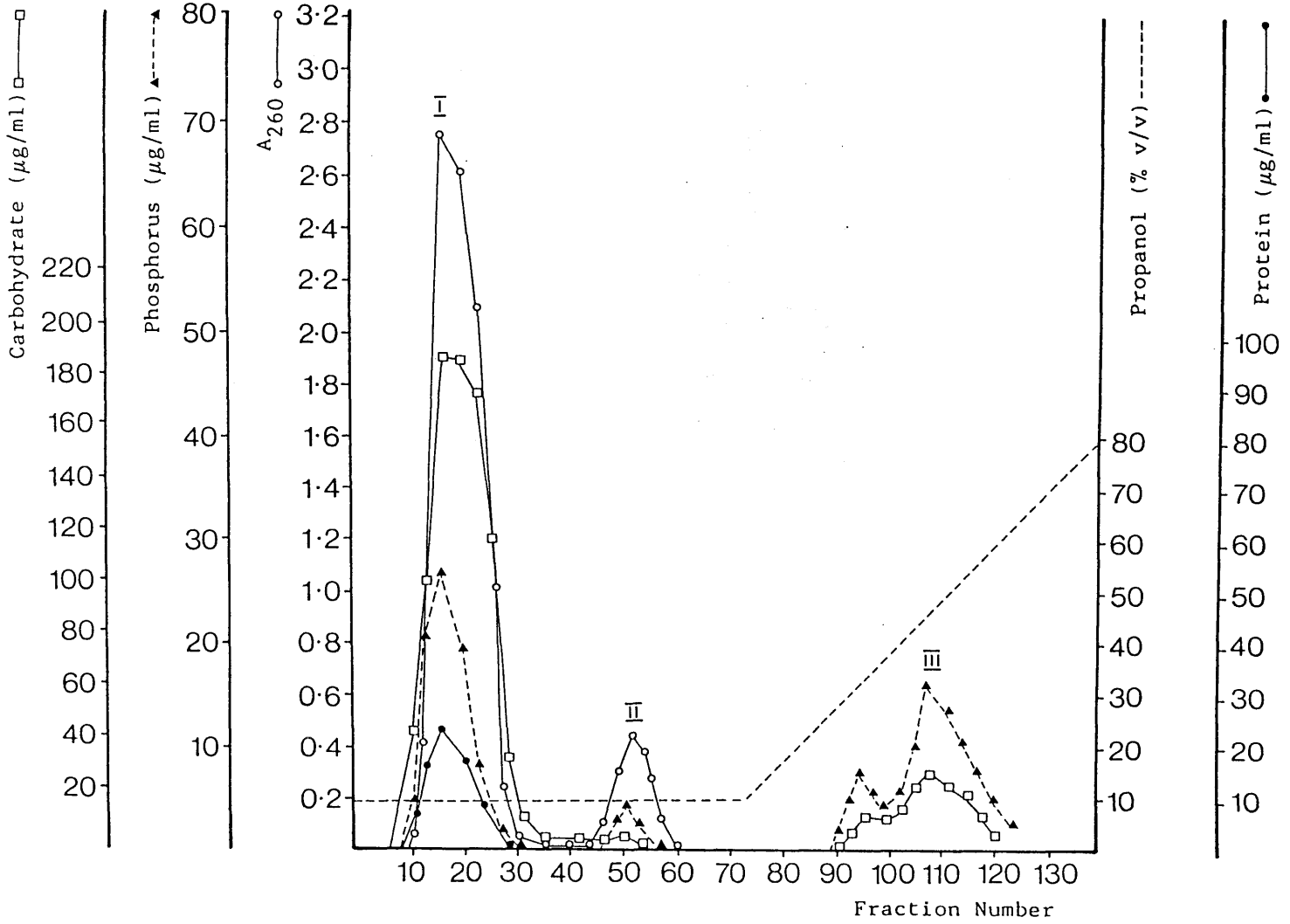
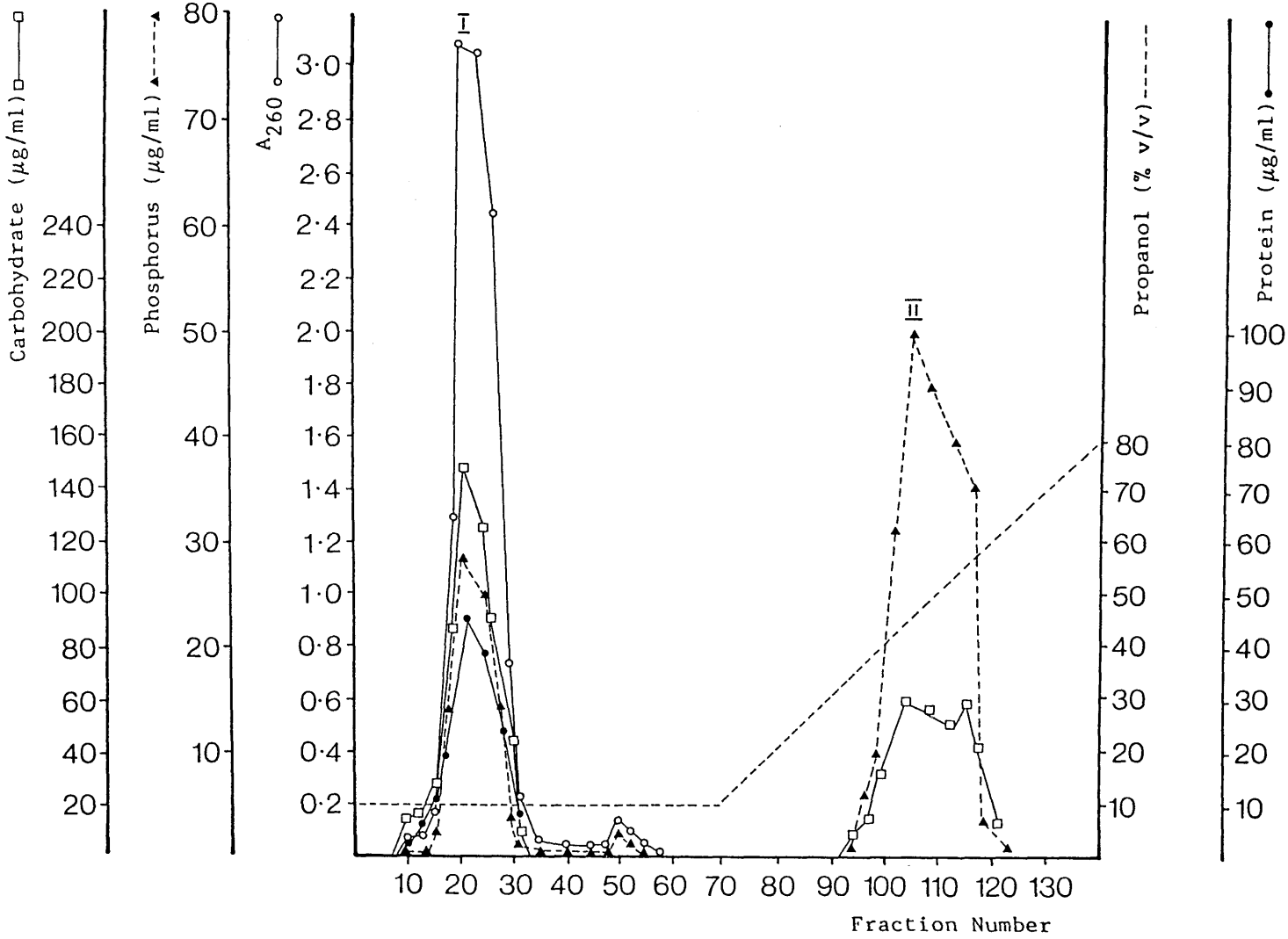

Carbohydrate (μg/ml)
Phosphorus (μg/ml)
A260
Propanol (% v/v)
Protein (μg/ml)
I
II
III
Fraction Number

Figure 11: Purification of lipoteichoic acid from Strep. salivarius D66 by hydrophobic interaction chromatography.


Carbohydrate (μg/ml)
Phosphorus (μg/ml)
A260
Propanol (% v/v)
Protein (μg/ml)
I
II
Fraction Number

A.i. Effect of crude LTA from Lactobacillus casei NCTC 6375 on the adherence of 500 mM galactose-grown Candida albicans MRL 3153 to buccal epithelial cells

Crude LTA extracted from Lactobacillus casei NCTC 6375 was examined for its effect on yeast adhesion to buccal epithelial cells. The concentration of LTA examined initially was in the range 0 to 100 μg/ml (Table 37A). Even at concentrations as low as 2 μg/ml inhibition of yeast adhesion was almost 20%. As the concentration of LTA was increased the inhibition of adhesion increased up to a maximum of 35% at an LTA concentration of 50 μg/ml. At higher concentrations of LTA (50-100 μg/ml) inhibition remained constant at this figure.

Subsequently the effect of LTA at concentrations greater than 100 μg/ml on yeast adherence was studied. The results showed that by increasing the concentration of LTA from 0.1 mg/ml to 1 mg/ml had no additional effect on the adherence of C. albicans to epithelial cells (Table 37B).

ii. Effect of purified LTA from Lactobacillus casei NCTC 6375 on the adherence of Candida albicans MRL 3153 to buccal epithelial cells

Lipoteichoic acid, which had been purified by anion-exchange chromatography, was examined for its effect on adherence of both 50 mM glucose-grown and 500 mM galactose-grown C. albicans MRL 3153 to epithelial cells.

The effect of purified LTA on the adherence of 50 mM glucose-grown C. albicans was examined in the range 0 to 1000 μg/ml (Table 38). The adherence of C. albicans was inhibited by 42% at an LTA concentration of 10 μg/ml and reached 55% at a concentration of 80 μg/ml. At concentrations of LTA >80 μg/ml the inhibitory effect was reduced. At concentrations of 1 mg/ml inhibition was only 22% (Table 38).

Table 37 Effect of crude lipoteichoic acid from Lactobacillus casei NCTC 6375 on adherence of 500 mM galactose-grown Candida albicans MRL 3153 to buccal epithelial cells

	Concentration of LTA (μg/ml)	Mean number of adherent yeasts/ 100 epithelial cells ± SE[a]	Relative adherence[b]	P[c]
A.	0	1362 ± 106	1.00	-
	2	1114 ± 90	0.82	< 0.05
	5	986 ± 85	0.72	< 0.01
	10	943 ± 87	0.69	< 0.005
	20	905 ± 83	0.66	< 0.001
	50	896 ± 81	0.65	< 0.001
	80	954 ± 82	0.70	< 0.005
	100	886 ± 84	0.65	< 0.001
B.	0	1430 ± 88	1.00	-
	200	903 ± 81	0.63	< 0.001
	400	928 ± 76	0.65	< 0.001
	600	920 ± 78	0.64	< 0.001
	800	931 ± 74	0.65	< 0.001
	1000	919 ± 79	0.64	< 0.001

[a] Mean of three different assays carried out in triplicate

[b] Adherence relative to that in control assays in which LTA was replaced by saline

[c] Probability values comparing control and test assays for each concentration of LTA.

Table 38 Effect of purified lipoteichoic acid from Lactobacillus casei NCTC 6375 on adherence of 50 mM glucose-grown Candida albicans MRL 3153 to buccal epithelial cells

	Concentration of LTA (μg/ml)	Mean number of adherent yeasts/ 100 epithelial cells ± SE[a]	Relative adherence[b]	P[c]
A.	0	221 ± 21	1.00	-
	10	128 ± 16	0.58	< 0.001
	20	123 ± 22	0.56	< 0.005
	40	115 ± 24	0.52	< 0.005
	60	109 ± 19	0.49	< 0.001
	80	99 ± 15	0.45	< 0.001
	100	123 ± 18	0.56	< 0.001
B.	0	212 ± 9.5	1.00	-
	200	134 ± 12	0.63	< 0.001
	400	126 ± 5	0.59	< 0.001
	600	150 ± 14	0.71	< 0.001
	800	159 ± 14	0.75	< 0.005
	1000	165 ± 14	0.78	< 0.01

[a] Mean of three different assays carried out in triplicate

[b] Adherence relative to that in control assays in which LTA was replaced by saline

[c] Probability values comparing test and control assays for each concentration of LTA.

The effect of purified LTA on galactose-grown C. albicans was examined in the range 0 to 100 μg/ml; even at 2 μg/ml there was approximately 20% inhibition of yeast adhesion (Table 39). As the concentration of LTA was increased the extent of inhibition increased but levelled off to around 56% inhibition at LTA concentrations of 80-100 μg/ml. Thus, the effect of the purified polymer on the adherence of C. albicans to epithelial cells was greater than that of the crude preparation which only inhibited adherence by 35% at an LTA concentration of 100 μg/ml (Table 37A).

iii. Effect of crude LTA from Lactobacillus casei NCTC 6375 on the adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

The effect of LTA on the adherence of a second strain of Candida albicans was examined.

The effect of LTA on the adhesion of C. albicans GDH 2346 previously cultured in 500 mM galactose, was examined in the range 0 to 100 μg/ml (Table 40). At 2 μg/ml LTA there was around 30% inhibition of yeast adhesion. At higher concentrations of LTA, inhibition of yeast adhesion increased slightly to around 35 to 40%.

The results showed that LTA had a similar effect on both yeast strains, inhibiting the adherence of both by around 35-40% at LTA concentrations of 20-100 μg/ml. However, at the lower concentrations of LTA (2-10 μg/ml), the adherence of C. albicans GDH 2346 was inhibited to a greater extent than the adherence of C. albicans MRL 3153 (30-35% as compared with 18-30%).

iv. Effect of purified LTA from Lactobacillus casei NCTC 6375 on the adherence of Candida albicans GDH 2346 to buccal epithelial cells

The effect of purified LTA on the adherence of 50 mM glucose-grown C. albicans GDH 2346 was examined in the range 0 to 100 μg/ml

Table 39 Effect of purified LTA from L. casei NCTC 6375 on the adherence of 500 mM galactose-grown Candida albicans MRL 3153 to buccal epithelial cells

Concentration of LTA (μg/ml)	Mean number of adherent yeasts/ 100 epithelial cells ± SE[a]	Relative adherence[b]	P[c]
0	1532 ± 82	1.00	-
2	1208 ± 62	0.79	< 0.01
5	1082 ± 71	0.71	< 0.001
10	981 ± 63	0.64	< 0.001
20	841 ± 41	0.55	< 0.001
50	679 ± 53	0.44	< 0.001
80	668 ± 50	0.44	< 0.001
100	652 ± 52	0.43	< 0.001

[a]Mean of three different assays carried out in triplicate

[b]Adherence relative to that in control assays in which LTA was replaced by saline

[c]Probability values comparing test and control assays for each concentration of LTA.

Table 40 Effect of crude LTA from Lactobacillus casei NCTC 6375 on the adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

Concentration of LTA (μg/ml)	Mean number of adherent yeasts/ 100 epithelial cells ± SE[a]	Relative adherence[b]	P[c]
0	1408 ± 71	1.00	-
2	984 ± 51	0.69	< 0.001
5	944 ± 79	0.67	< 0.001
10	910 ± 64	0.65	< 0.001
20	859 ± 63	0.61	< 0.001
50	902 ± 97	0.64	< 0.001
80	901 ± 61	0.64	< 0.001
100	881 ± 85	0.63	< 0.001

[a]Mean of three different assays carried out in triplicate

[b]Adherence relative to that in control assays in which LTA was replaced by saline

[c]Probability values comparing test and control assays for each LTA concentration.

(Table 41). Again, even at a low concentration of LTA (2 μg/ml) inhibition of yeast adhesion was around 30% and increased to almost 60% at an LTA concentration of 20 μg/ml. At the higher concentrations of LTA (50 - 100 μg/ml) inhibition of yeast adhesion remained fairly stable at 50-53%.

With 500 mM galactose-grown yeasts there was 35% inhibition of adhesion at an LTA concentration of 2 μg/ml which increased to around 55% inhibition at higher concentrations of LTA (50-80 μg/ml) (Table 42).

If the results for the relative effect of crude and purified LTA on the adherence of C. albicans GDH 2346 are compared we can see that inhibition of yeast adhesion was greater with purified LTA than with the crude polymer. With purified LTA (Table 42) inhibition was 35% at 2 μg/ml and reached 55% at the higher concentrations (50-80 μg/ml) compared with around 40% inhibition of adhesion with crude LTA (Table 40).

The effect of purified LTA on the adherence of C. albicans GDH 2346 was similar to that observed with C. albicans MRL 3153 (Table 39), the adhesion of both yeast strains being inhibited by around 55% at an LTA concentration of 80 μg/ml.

v. Effect of deacylated lipoteichoic acid (dLTA) on the adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

In order to determine which part of the LTA molecule was responsible for inhibiting yeast adhesion some purified LTA from L. casei NCTC 6375 was treated with ammonium hydroxide to remove the fatty acid portion of the molecule. The fatty acids were extracted with chloroform: methanol:water and the remainder of the LTA molecule which is found in the aqueous phase was used in adhesion assays.

Results obtained for the effect of dLTA on the adherence of 500 mM galactose-grown C. albicans GDH 2346 to epithelial cells are shown in Table 43. At the concentrations of LTA examined (20 and 50 μg/ml) the

Table 41 Effect of purified LTA from L. casei NCTC 6375 on the adherence of 50 mM glucose-grown Candida albicans GDH 2346 to buccal epithelial cells

Concentration of LTA (μg/ml)	Mean number of adherent yeasts/ 100 epithelial cells ± SE[a]	Relative adherence[b]	P[c]
0	61.7 ± 3.3	1.00	-
2	42.7 ± 4.6	0.69	< 0.001
5	34.7 ± 4.8	0.56	< 0.001
10	29.7 ± 5.1	0.48	< 0.001
20	25.3 ± 4.2	0.41	< 0.001
50	31.6 ± 4.7	0.51	< 0.001
80	31.6 ± 3.6	0.51	< 0.001
100	28.9 ± 4.9	0.47	< 0.001

[a]Mean of three different assays carried out in triplicate

[b]Adherence relative to that in control assays in which LTA was replaced by saline

[c]Probability values comparing test and control assays for each concentration of LTA.

Table 42 Effect of purified LTA from *L. casei* NCTC 6375 on the adherence of 500 mM galactose-grown *Candida albicans* GDH 2346 to buccal epithelial cells

Concentration of LTA (μg/ml)	Mean number of adherent yeasts/ 100 epithelial cells ± SE[a]	Relative adherence[b]	P[c]
0	1113 ± 102	1.00	-
2	720 ± 58	0.65	<0.001
5	658 ± 56	0.59	<0.001
10	609 ± 48	0.55	<0.001
20	527 ± 60	0.47	<0.001
50	499 ± 53	0.45	<0.001
80	506 ± 63	0.45	<0.001
100	596 ± 62	0.54	<0.001

[a]Mean of three different assays carried out in triplicate

[b]Adherence relative to that in control assays in which LTA was replaced by saline

[c]Probability values comparing test and control assays for each concentration of LTA.

Table 43 Effect of deacylated LTA on the adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

Nature and concentration of LTA (μg/ml)		Mean number of adherent yeasts/ 100 epithelial cells ± SE[a]	Relative adherence[b]	P[c]
0		1256 ± 84	1.00	-
20	deacylated	1346 ± 63	1.07	NS
50	deacylated	1276 ± 51	1.02	NS
20	untreated	621 ± 23	0.49	< 0.0001
50	untreated	602 ± 24	0.48	< 0.0001

[a]Mean of three different assays carried out in triplicate

[b]Adherence relative to that in control assays in which LTA was replaced by saline

[c]Probability values comparing test and control assays for each concentration of LTA

NS - Not significant.

untreated polymer inhibited yeast adhesion by almost 50%, but dLTA had no effect on adherence of C. albicans. From these results it would appear that removal of the fatty acid portion of the molecule resulted in the loss of its ability to inhibit yeast adherence to epithelial cells.

vi. Effect of crude LTA from Lactobacillus casei MU 259 on the adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

Lipoteichoic acid was extracted from L. casei MU 259 and examined for its effect on the adherence of 500 mM galactose-grown C. albicans GDH 2346 to epithelial cells over the range 0 to 1000 µg/ml (Table 44).

At LTA concentrations of 2 and 5 µg/ml there was no significant inhibition of yeast adhesion. At concentrations of 10-80 µg/ml, inhibition of adhesion was fairly level at around 30-35%, but then increased sharply to more than 40% inhibition at 100 µg/ml. Because of this sudden increase at 100 µg/ml, the concentration of LTA was increased and examined over the range 200-1000 µg/ml. At a concentration of 200 µg/ml there was an increase in inhibition to 47% but this levelled off at around 40-42% over the higher LTA concentrations (400-1000 µg/ml).

If these results are compared with those obtained with crude LTA extracted from L. casei NCTC 6375 (Table 40) we can see that the latter had a greater effect on yeast adhesion at the lower LTA concentrations (2-20 µg/ml) inhibiting it by around 30-40% compared with 30% inhibition produced by crude LTA from L. casei MU 259.

vii. Effect of crude LTA from Lactobacillus casei 0112 on the adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

Lipoteichoic acid was extracted from L. casei 0112 and examined for its effect on yeast adhesion to epithelial cells (Table 45). Adherence

Table 44 Effect of crude LTA from Lactobacillus casei MU 259 on adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

Concentration of LTA (µg/ml)		Mean number of adherent yeasts/ 100 epithelial cells ± SE[a]	Relative adherence[b]	P[c]
A.	0	1300 ± 66	1.00	-
	2	1137 ± 98	0.87	NS
	5	1113 ± 97	0.86	NS
	10	919 ± 82	0.71	< 0.005
	20	909 ± 64	0.70	< 0.001
	50	844 ± 54	0.65	< 0.001
	80	938 ± 67	0.72	< 0.005
	100	747 ± 53	0.57	< 0.001
B.	0	1231 ± 52	1.00	-
	200	653 ± 43	0.53	< 0.001
	400	720 ± 57	0.58	< 0.001
	600	698 ± 60	0.57	< 0.001
	800	754 ± 53	0.61	< 0.001
	1000	705 ± 61	0.57	< 0.001

[a] Mean of three different assays carried out in triplicate

[b] Adherence relative to that in control assays in which LTA was replaced by saline

[c] Probability values comparing test and control assays for each concentration of LTA

NS - Not significant.

Table 45 Effect of crude LTA from Lactobacillus casei 0112 on the adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

Concentration of LTA (μg/ml)	Mean number of adherent yeasts/ 100 epithelial cells ± SE[a]	Relative adherence[b]	P[c]
0	1216 ± 82	1.00	-
2	1066 ± 60	0.87	NS
5	1020 ± 57	0.84	NS
10	1038 ± 51	0.85	NS
20	1015 ± 46	0.83	< 0.05
50	984 ± 49	0.81	< 0.05
80	887 ± 53	0.73	< 0.005
100	900 ± 48	0.74	< 0.005

[a]Mean of three different assays carried out in triplicate

[b]Adherence relative to that in control assays in which LTA was replaced by saline

[c]Probability values comparing test and control assays for each concentration of LTA

NS - Not significant.

of Candida albicans was not significantly affected by the lower concentrations of LTA (2-10 μg/ml). Even at 20-50 μg/ml LTA, inhibition of yeast adhesion was only around 20% and increased only to around 25% at 80-100 μg/ml LTA.

This inhibition of yeast adhesion was much lower than that obtained with LTA from the type strain or the other isolate, L. casei MU 259. Even at 2 μg/ml, L. casei NCTC 6375 inhibited yeast adhesion by 30% increasing to around 40% at 20 μg/ml (Table 40). And, although LTA from L. casei MU 259 had no effect on yeast adhesion at 2 and 5 μg/ml it did inhibit by 40-45% at the higher concentrations (Table 44).

Lactobacillus casei 0112 was the strain that was isolated from the saliva of a patient with oral candidosis and was the strain which produced the poorest inhibition of yeast adhesion to HeLa cells.

B.i. Effect of crude lipoteichoic acid from Streptococcus salivarius NCTC 7366 on the adherence of 50 mM glucose-grown Candida albicans MRL 3153 to buccal epithelial cells

Lipoteichoic acid extracted from Strep. salivarius NCTC 7366 was examined for its effect on the adherence of C. albicans MRL 3153 to epithelial cells over the range 0 to 1000 μg/ml (Table 46). At an LTA concentration of 10 μg/ml there was no significant effect on yeast adhesion. At 20 μg/ml inhibition of adhesion was around 30% and increased to around 50% at 60-80 μg/ml LTA. At 100 μg/ml there was a decrease in the effect of LTA on yeast adhesion to 40% inhibition. Subsequently the effect of LTA at concentrations greater than 100 μg/ml on yeast adherence was studied. At the higher concentrations of LTA, inhibition of yeast adhesion varied a little around 40-50%.

ii. Effect of crude LTA from Streptococcus salivarius NCTC 7366 on the adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

Table 46 Effect of crude LTA from Streptococcus salivarius NCTC 7366 on adherence of 50 mM glucose-grown Candida albicans MRL 3153 to buccal epithelial cells

Concentration of LTA (μg/ml)	Mean number of adherent yeasts/ 100 epithelial cells ± SE[a]	Relative adherence[b]	P[c]
A. 0	282.9 ± 26.8	1.00	-
10	254.6 ± 23.1	0.90	NS
20	189.5 ± 18.2	0.67	<0.01
40	159.6 ± 18.3	0.56	<0.001
60	150.0 ± 13.4	0.53	<0.001
80	151.3 ± 21.6	0.54	<0.001
100	170.8 ± 20.8	0.60	<0.005
B. 0	275.8 ± 24.3	1.00	-
200	170.1 ± 17.4	0.62	<0.001
400	155.1 ± 18.2	0.56	<0.001
600	154.1 ± 12.6	0.56	<0.001
800	170.5 ± 19.9	0.62	<0.001
1000	147.0 ± 23.2	0.53	<0.001

[a]Mean of three different assays carried out in triplicate

[b]Adherence relative to that in control assays in which LTA was replaced by saline

[c]Probability values comparing test and control assays for each concentration of LTA

NS - not significant.

The effect of LTA from Strep. salivarius NCTC 7366 on the adherence of C. albicans GDH 2346 was examined in the concentration range 0-100 μg/ml (Table 47). No effect was observed at low concentrations (2-5 μg/ml) LTA. However, at 10 μg/ml there was almost 30% inhibition and this increased as the concentration of LTA was increased, reaching almost 50% inhibition of yeast adhesion at 100 μg/ml.

iii. Effect of purified lipoteichoic acid from Streptococcus salivarius NCTC 7366 on the adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

Lipoteichoic acid from Strep. salivarius NCTC 7366 was purified by anion-exchange chromatography and examined for its effect on adherence of 500 mM galactose-grown C. albicans GDH 2346 to epithelial cells (Table 48). Purified LTA inhibited yeast adhesion by 16% at a concentration of 5 μg/ml but at 2 μg/ml had no effect on adherence. There was an increase in inhibition with increasing LTA concentration to a maximum of 37% at 50 μg/ml. At LTA concentrations of 80-100 μg/ml the degree of inhibition levelled out to around 34%, compared to 47% inhibition obtained with the crude polymer (Table 47).

In contrast to results with L. casei NCTC 6375 (Table 42) purified LTA from Strep. salivarius NCTC 7366 did not inhibit yeast adhesion as strongly as the crude polymer (Table 47).

iv. Effect of crude lipoteichoic acid from Streptococcus salivarius D66 on the adherence of 500 mM galactose-grown Candida albicans to to buccal epithelial cells

Lipoteichoic acid extracted from Strep. salivarius D66 was examined for its effect on adherence of both C. albicans MRL 3153 and C. albicans GDH 2346, grown on 500 mM galactose, to epithelial cells.

With C. albicans MRL 3153 there was 25% inhibition of adherence

Table 47 Effect of crude LTA from Streptococcus salivarius NCTC 7366 on the adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

Concentration of LTA (μg/ml)	Mean number of adherent yeasts/ 100 epithelial cells ± SE[a]	Relative adherence[b]	P[c]
0	1100 ± 61	1.00	-
2	884 ± 94	0.80	NS
5	894 ± 89	0.81	NS
10	795 ± 78	0.72	< 0.05
20	679 ± 81	0.62	< 0.001
50	648 ± 91	0.58	< 0.001
80	612 ± 74	0.56	< 0.001
100	584 ± 53	0.53	< 0.001

[a]Mean of three different assays carried out in triplicate

[b]Adherence relative to that in control assays in which LTA was replaced by saline

[c]Probability values comparing test and control assays for each concentration of LTA

NS - Not significant.

Table 48 Effect of purified LTA from Streptococcus salivarius NCTC 7366 on the adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

Concentration of LTA (μg/ml)	Mean number of adherent yeasts/ 100 epithelial cells ± SE[a]	Relative adherence[b]	P[c]
0	1273 ± 75	1.00	-
2	1192 ± 61	0.94	NS
5	1064 ± 62	0.84	< 0.05
10	941 ± 51	0.74	< 0.005
20	907 ± 54	0.71	< 0.005
50	802 ± 46	0.63	< 0.001
80	843 ± 61	0.66	< 0.001
100	826 ± 68	0.65	< 0.001

[a]Mean of three different assays carried out in triplicate

[b]Adherence relative to that in control assays in which LTA was replaced by saline

[c]Probability values comparing test and control assays for each concentration of LTA

NS - Not significant.

at an LTA concentration of 2 μg/ml (Table 49). However, the degree of inhibition remained fairly constant over the concentration range, before reaching its maximum at 100 μg/ml where yeast adhesion was inhibited by 35%.

With C. albicans GDH 2346 the extent of inhibition produced by LTA was similar to that observed with strain MRL 3153. There was 24% inhibition of adhesion at 2 μg/ml LTA and this remained constant at around 25 to 32% for concentrations of LTA from 2-80 μg/ml (Table 50A). At 100 μg/ml LTA the level of inhibition increased to 42%, and therefore higher concentrations of LTA (200-1000 μg/ml) were also used (Table 50B). However, increasing the concentration of LTA did not increase the inhibitory effect on yeast adhesion; in fact, the degree of inhibition levelled out at around 40% over the range 200-1000 μg/ml.

v. Effect of purified lipoteichoic acid from Streptococcus salivarius D66 on the adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

Lipoteichoic acid from Strep. salivarius D66 was purified by hydrophobic interaction chromatography and then examined for its effect on yeast adherence to epithelial cells (Table 51).

With purified LTA there was no significant inhibition of yeast adhesion at low concentrations (2-5 μg/ml) of LTA. At 10 μg/ml there was 18% inhibition which increased to around 30% at an LTA concentration of 80-100 μg/ml.

As with Strep. salivarius NCTC 7366, the effect of purified LTA on yeast adhesion was not as good as the effect of the crude preparation. With crude LTA inhibition of yeast adhesion was around 42% (Table 50) compared with only 30% inhibition with the purified polymer (Table 51).

From these results it would appear that LTA combined with some other component, possibly protein, is more effective in inhibiting yeast

Table 49 Effect of crude LTA from Streptococcus salivarius D66 on the adherence of 500 mM galactose-grown Candida albicans MRL 3153 to buccal epithelial cells

Concentration of LTA (μg/ml)	Mean number of adherent yeasts/ 100 epithelial cells ± SE[a]	Relative adherence[b]	P[c]
0	1945 ± 68	1.00	-
2	1468 ± 77	0.75	< 0.001
5	1480 ± 87	0.76	< 0.001
10	1482 ± 79	0.76	< 0.001
20	1370 ± 95	0.70	< 0.001
50	1352 ± 51	0.69	< 0.001
80	1418 ± 84	0.73	< 0.001
100	1256 ± 68	0.65	< 0.001

[a]Mean of three different assays carried out in triplicate

[b]Adherence relative to that in control assays in which LTA was replaced by saline

[c]Probability values comparing test and control assays for each concentration of LTA.

Table 50 Effect of crude LTA from Streptococcus salivarius D66 on adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

Concentration of LTA (μg/ml)		Mean number of adherent yeasts/ 100 epithelial cells ± SE[a]	Relative adherence[b]	P[c]
A.	0	1234 ± 94	1.00	-
	2	942 ± 91	0.76	< 0.025
	5	849 ± 68	0.69	< 0.001
	10	906 ± 83	0.73	< 0.001
	20	839 ± 77	0.68	< 0.001
	50	929 ± 64	0.75	< 0.01
	80	920 ± 64	0.74	< 0.01
	100	760 ± 61	0.58	< 0.001
B.	0	1332 ± 98	1.00	-
	200	781 ± 73	0.59	< 0.001
	400	803 ± 76	0.60	< 0.001
	600	832 ± 85	0.62	< 0.005
	800	810 ± 71	0.61	< 0.001
	1000	800 ± 70	0.60	< 0.001

[a] Mean of three different experiments carried out in triplicate

[b] Adherence relative to that in control assays in which LTA was replaced by saline

[c] Probability values comparing test and control assays for each concentration of LTA.

Table 51 Effect of purified LTA from Streptococcus salivarius D66 on the adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

Concentration of LTA (μg/ml)	Mean number of adherent yeasts/ 100 epithelial cells ± SE[a]	Relative adherence[b]	P[c]
0	1172 ± 81	1.00	-
2	1008 ± 46	0.86	NS
5	1005 ± 51	0.86	NS
10	960 ± 47	0.82	< 0.05
20	891 ± 84	0.76	< 0.05
50	846 ± 81	0.72	< 0.025
80	807 ± 60	0.69	< 0.001
100	805 ± 42	0.69	< 0.001

[a]Mean of three different experiments carried out in triplicate

[b]Adherence relative to that of control assays in which LTA was replaced by saline

[c]Probability values comparing test and control assays for each concentration of LTA

NS - Not significant.

adhesion than LTA alone, suggesting that LTA is not the sole adhesin on the surface of Strep. salivarius.

vi. Effect of crude LTA from Streptococcus salivarius M65 and Streptococcus salivarius MU 289 on the adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

Crude lipoteichoic acid preparations from two further strains of Strep. salivarius were examined for their effect on the adherence of 500 mM galactose-grown C. albicans GDH 2346 to epithelial cells.

Lipoteichoic acid from Strep. salivarius M65 inhibited yeast adhesion by 24% at a concentration of 5 μg/ml (Table 52). Inhibition of yeast adherence increased with increasing concentrations of LTA reaching its maximum of 46% at 100 μg/ml.

With Strep. salivarius MU 289, LTA did not significantly inhibit yeast adhesion at low concentrations of 2-5 μg/ml (Table 53). However, from 10-100 μg/ml, inhibition of adherence increased steadily to a maximum of 48% at an LTA concentration of 100 μg/ml.

Crude LTA extracted from the four strains of Strep. salivarius had a similar effect on yeast adhesion, with all four preparations producing a maximum of 42-48% inhibition (Tables 47, 50, 52 and 53).

C. Effect of crude lipoteichoic acid from Streptococcus sanguis NCTC 7864 on the adherence of 500 mM galactose-grown Candida albicans to buccal epithelial cells

Lipoteichoic acid was extracted from Strep. sanguis NCTC 7864 and examined for its effect on the adherence of both Candida albicans MRL 3153 and C. albicans GDH 2346 to buccal cells.

With C. albicans GDH 2346 there was almost 30% inhibition of adhesion at 2 μg/ml LTA (Table 54). Over the concentration range 5-100 μg/ml, the extent of inhibition remained fairly constant at around 40%.

Table 52 Effect of crude LTA from Streptococcus salivarius M65 on the adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

Concentration of LTA (μg/ml)	Mean number of adherent yeasts/ 100 epithelial cells ± SE[a]	Relative adherence[b]	P[c]
0	1133 ± 89	1.00	-
2	937 ± 63	0.83	NS
5	860 ± 91	0.76	< 0.05
10	840 ± 58	0.74	< 0.01
20	787 ± 54	0.69	< 0.005
50	732 ± 68	0.65	< 0.005
80	761 ± 70	0.67	< 0.005
100	615 ± 30	0.54	< 0.001

[a]Mean of three different assays carried out in triplicate

[b]Adherence relative to that of the control in which LTA was replaced by saline

[c]Probability values comparing test and control assays for each concentration of LTA

NS - Not significant.

Table 53 Effect of crude LTA from Streptococcus salivarius MU 289 on the adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

Concentration of LTA (μg/ml)	Mean number of adherent yeasts/ 100 epithelial cells ± SE[a]	Relative adherence[b]	P[c]
0	1141 ± 83	1.00	-
2	1016 ± 82	0.89	NS
5	995 ± 62	0.87	NS
10	904 ± 53	0.79	< 0.05
20	787 ± 55	0.69	< 0.005
50	710 ± 58	0.62	< 0.001
80	644 ± 61	0.56	< 0.001
100	599 ± 51	0.52	< 0.001

[a]Mean of three different experiments carried out in triplicate

[b]Adherence relative to that in control assays in which LTA was replaced by saline

[c]Probability values comparing test and control assays for each concentration of LTA

NS - Not significant.

Table 54 Effect of crude LTA from Streptococcus sanguis 7864 on the adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

Concentration of LTA (μg/ml)	Mean number of adherent yeasts/ 100 epithelial cells ± SE[a]	Relative adherence[b]	P[c]
0	1428 ± 79.3	1.00	-
2	1036 ± 66.0	0.73	< 0.005
5	875 ± 52.3	0.61	< 0.001
10	913 ± 57.0	0.64	< 0.001
20	889 ± 57.3	0.62	< 0.001
50	877 ± 53.0	0.62	< 0.001
80	815 ± 43.6	0.57	< 0.001
100	885 ± 53.0	0.62	< 0.001

[a]Mean of three different assays carried out in triplicate

[b]Adherence relative to that in control assays in which LTA was replaced by saline

[c]Probability values comparing test and control assays for each concentration of LTA.

With C. albicans MRL 3153 there was 20% inhibition of adherence with an LTA concentration of 2 μg/ml. The level of inhibition increased over the concentration range 5-80 μg/ml LTA, reaching a maximum of around 40% (Table 55).

Although the preparation from Strep. sanguis NCTC 7864 has been referred to as LTA, it must be remembered that it did not react with anti-LTA antiserum and thus, may be another polymer and not LTA. The effect that this preparation had on yeast adherence appears to be similar to the effect of LTA extracted from the other strains.

D. Effect of crude lipoteichoic acid from Streptococcus mitior on the adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

The final two organisms from which LTA was extracted were Strep. mitior (C32 and 863). Both of these strains were freshly isolated from the oral cavity: strain C32 was isolated from the buccal mucosa and strain 863 from saliva.

i. Effect of crude LTA from Streptococcus mitior 863 and Streptococcus mitior C32 on the adherence of Candida albicans GDH 2346 to epithelial cells

Crude LTA from Strep. mitior 863 inhibited adherence of C. albicans by 17% at a concentration of 2 μg/ml (Table 56). As the concentration of LTA was increased the degree of inhibition of yeast adhesion increased only slightly, reaching a maximum of around 30% at 50-80 μg/ml LTA. This was significantly less than the 50% inhibition of yeast adhesion to HeLa cells caused by the intact bacteria of Strep. mitior 863 (Table 16).

From these results it would appear unlikely that LTA is the sole adhesin on the surface of Strep. mitior 863.

Table 55 Effect of crude LTA from Streptococcus sanguis NCTC 7864 on the adherence of 500 mM galactose-grown Candida albicans MRL 3153 to buccal epithelial cells

Concentration of LTA (μg/ml)	Mean number of adherent yeasts/ 100 epithelial cells ± SE[a]	Relative adherence[b]	P[c]
0	1634 ± 81.6	1.00	-
2	1308 ± 76.4	0.80	< 0.025
5	1253 ± 61.8	0.77	< 0.005
10	1108 ± 60.7	0.68	< 0.001
20	1006 ± 54.2	0.62	< 0.001
50	992 ± 56.1	0.61	< 0.001
80	1004 ± 58.4	0.61	< 0.001
100	1086 ± 55.5	0.66	< 0.001

[a]Mean of three different assays carried out in triplicate

[b]Adherence relative to that in the control assays in which LTA was replaced by saline

[c]Probability values comparing test and control assays for each concentration of LTA.

Table 56 Effect of crude LTA from Streptococcus mitior 863 on the adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

Concentration of LTA (µg/ml)	Mean number of adherent yeasts/ 100 epithelial cells ± SE[a]	Relative adherence[b]	P[c]
0	1156 ± 50	1.00	-
2	958 ± 57	0.83	< 0.025
5	939 ± 36	0.81	< 0.005
10	951 ± 50	0.82	< 0.025
20	929 ± 51	0.80	< 0.01
50	819 ± 67	0.71	< 0.005
80	853 ± 58	0.74	< 0.005
100	959 ± 61	0.83	< 0.025

[a]Mean of three different assays carried out in triplicate

[b]Adherence relative to that in control assays in which LTA was replaced by saline

[c]Probability values comparing test and control assays for each concentration of LTA.

Crude LTA from Strep. mitior C32 produced a slightly greater effect on yeast adhesion to epithelial cells than LTA from Strep. mitior 863 (Table 57). At 2 μg/ml LTA, there was 25% inhibition of adhesion and this remained fairly constant from 10-100 μg/ml, reaching a maximum of 36% at 50 μg/ml. Again, this was in contrast to almost 50% inhibition of yeast adhesion to HeLa cells shown by the intact bacteria of Strep. mitior C32 (Table 16).

ii. Effect of purified lipoteichoic acid from Streptococcus mitior C32 on the adherence of Candida albicans GDH 2346 to buccal epithelial cells

Lipoteichoic acid from Strep. mitior C32 was purified by hydrophobic interaction chromatography to see whether its effect on yeast adhesion could be enhanced.

At concentrations of 2-5 μg/ml LTA there was no significant effect on yeast adhesion (Table 58). At 10 μg/ml there was only 17% inhibition, and even when the concentration of LTA was increased the inhibitory effect was only marginally higher, reaching 24% at 50-100 μg/ml LTA. This inhibition of yeast adherence to epithelial cells was greater with the crude polymer than with the purified preparation. With the crude polymer there was 35% inhibition of adhesion at an LTA concentration of 50 μg/ml (Table 57) whereas at the same concentration of purified LTA, inhibition of adhesion was only 24% (Table 58).

These results would appear to suggest that LTA may play a minor role in mediating adherence of Strep. mitior, but is not the main adhesin on the surface of these bacteria.

V. Surface structures of streptococci and lactobacilli as revealed by electron microscopy

In some oral streptococci, lipoteichoic acid is contained within -

Table 57 Effect of crude LTA from Streptococcus mitior C32 on the adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

Concentration of LTA (μg/ml)	Mean number of adherent yeasts/ 100 epithelial cells ± SE[a]	Relative adherence[b]	P[c]
0	1310 ± 65	1.00	-
2	979 ± 59	0.75	< 0.005
5	1131 ± 57	0.86	NS
10	996 ± 48	0.76	< 0.005
20	933 ± 52	0.76	< 0.001
50	843 ± 61	0.64	< 0.001
80	952 ± 63	0.73	< 0.005
100	937 ± 68	0.72	< 0.005

[a]Mean of three different assays carried out in triplicate

[b]Adherence relative to that in control assays in which LTA was replaced by saline

[c]Probability values comparing control and test assays for each concentration of LTA

NS - Not significant.

Table 58 Effect of purified LTA from Streptococcus mitior C32 on the adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

Concentration of LTA (μg/ml)	Mean number of adherent yeasts/ 100 epithelial cells ± SE[a]	Relative adherence[b]	P[c]
0	1342 ± 86	1.00	-
2	1271 ± 71	0.95	NS
5	1206 ± 60	0.90	NS
10	1115 ± 62	0.83	< 0.05
20	1106 ± 51	0.82	< 0.05
50	1023 ± 54	0.76	< 0.01
80	1039 ± 50	0.77	< 0.01
100	1016 ± 56	0.76	< 0.01

[a]Mean of three different assays carried out in triplicate

[b]Adherence relative to that in control assays in which LTA was replaced by saline

[c]Probability values comparing test and control assays for each concentration of LTA

NS - Not significant.

or complexed with - surface fibrils following its partial excretion from the cell (Beachey et al, 1983). These fibrils appear to be involved in streptococcal attachment to mucosal surfaces. The ten bacterial strains which were used in the lipoteichoic acid studies on yeast adhesion were examined under a transmission electron microscope after negative staining with ammonium molybdate to see whether they possessed any fibrillar structures.

All strains of streptococci, apart from Strep. salivarius NCTC 7366 showed the presence of fibrils (Figs. 12-15) (Table 59). No such structures were found on the surface of the lactobacillus strains. The distribution and length of fibrils varied between strains, eg. Strep. salivarius M65 had a quite dense arrangement of fibrils about 0.04 μm - 0.08 μm in length (Fig. 12a) compared to Strep. sanguis NCTC 7864 (Fig. 12b) where the fibrils were sparsely arranged on the cell surface and were only 0.01 μm to 0.02 μm in length.

VI. Biochemical and Immunological Examination of Bacterial Culture Supernatants

A. Immunological examination of culture supernatants

Markham et al (1975) have shown that a number of oral streptococci and lactobacilli excrete LTA into the surrounding environment. Culture supernatants were concentrated twenty fold and examined by rocket immuno-electrophoresis (RIE) to see whether any of the strains excreted LTA. Before the assay could be carried out the amount of antibody in each batch of serum had to be calculated since the height of the rocket obtained by RIE is influenced by the amount of antibody in the serum. This was done by the quantitative precipitin test (Materials and Methods, page 79).

Table 59 Surface structures of streptococci and lactobacilli as revealed by electron microscopy

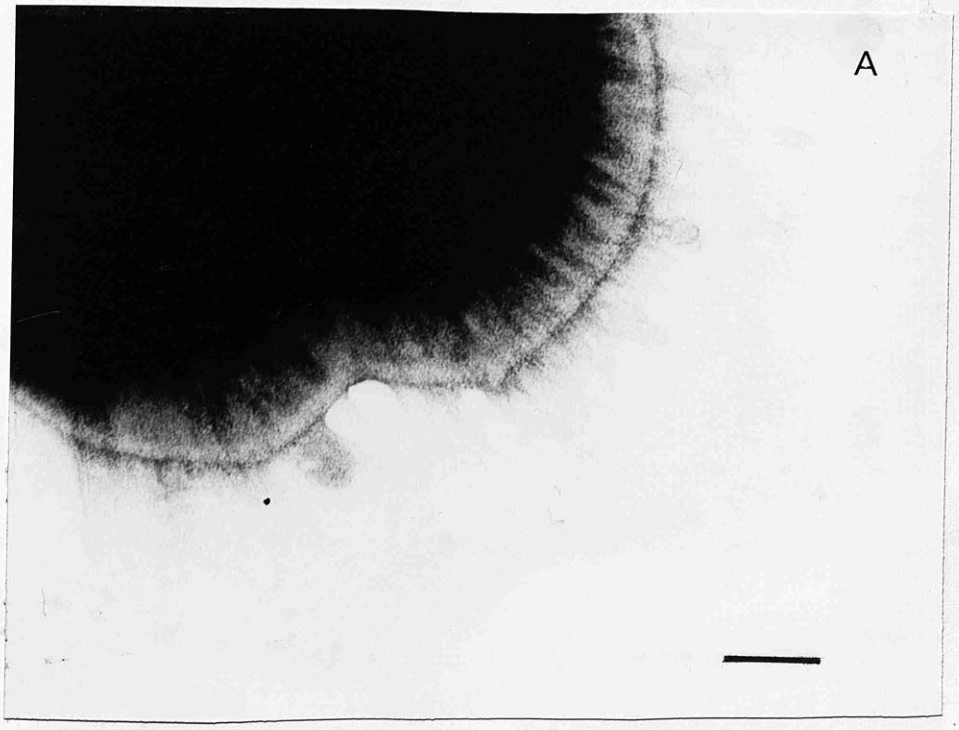
Organism	Presence of fibrils	Length
S. salivarius NCTC 7366	-	-
S. salivarius M65	+	0.04 - 0.08 µm
S. salivarius D66	+	≤ 0.04 µm
S. salivarius MU 289	+	0.02 - 0.06 µm
S. sanguis NCTC 7864	+	≤ 0.02 µm
S. mitior C32	+	≤ 0.04 µm
S. mitior 863	+	≤ 0.05 µm
L. casei NCTC 6375	-	-
L. casei 0112	-	-
L. casei MU 259	-	-

Figure 12: Electron microscopy of cell-surface structures of

a - Streptococcus salivarius M65

b - Streptococcus sanguis NCTC 7864

Bar represents 0.1 μm.


A

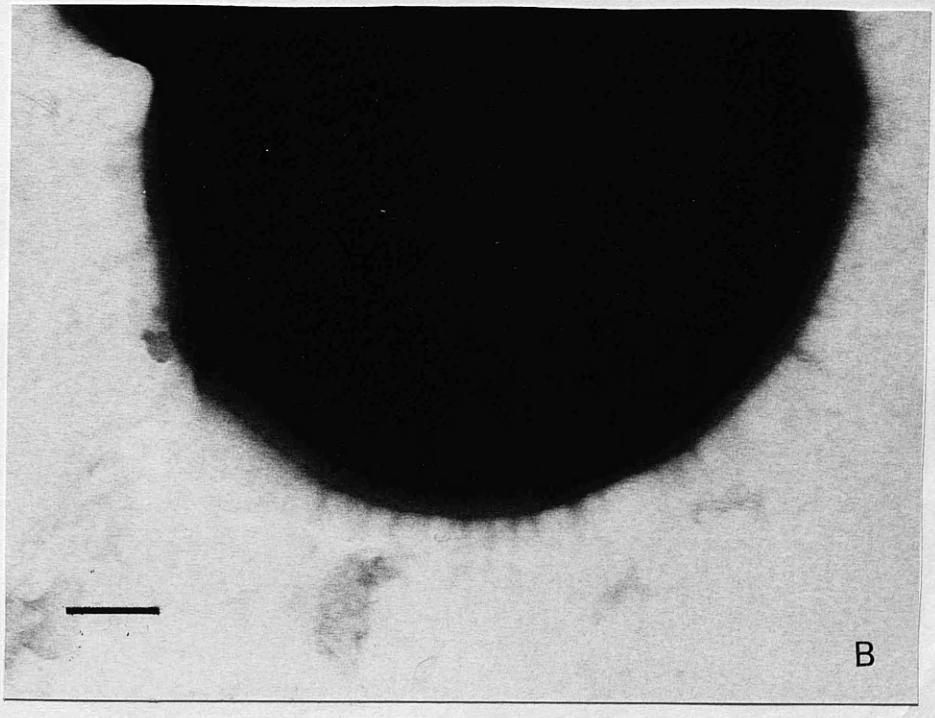
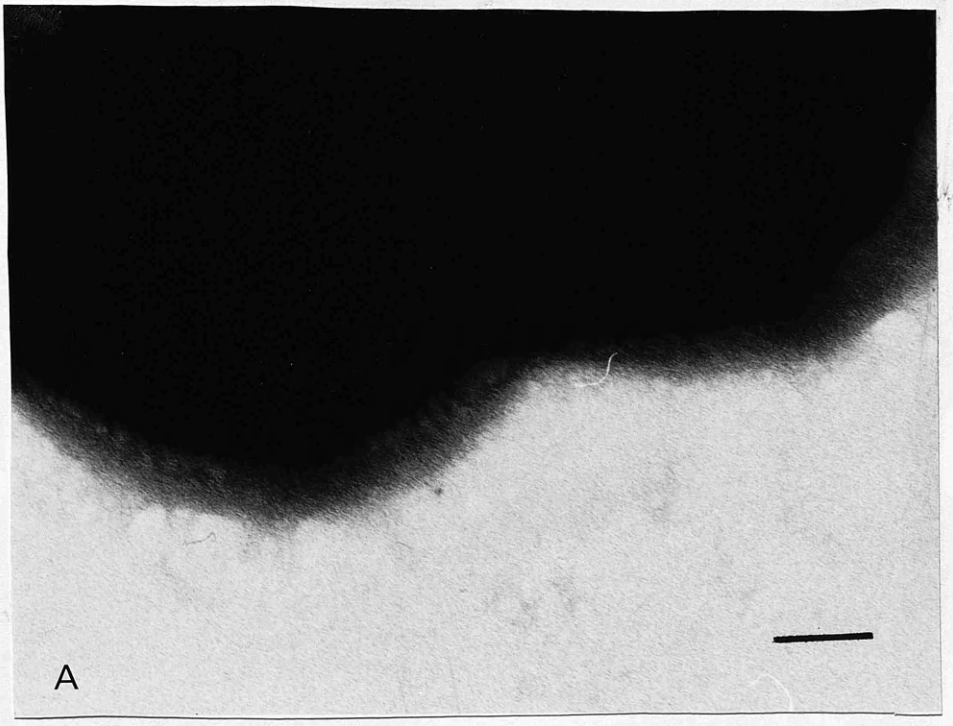

B

Figure 13: Electron microscopy of surface structures of

a - Streptococcus salivarius D66

b - Streptococcus salivarius MU 289

Bar represents 0.1 µm.


A

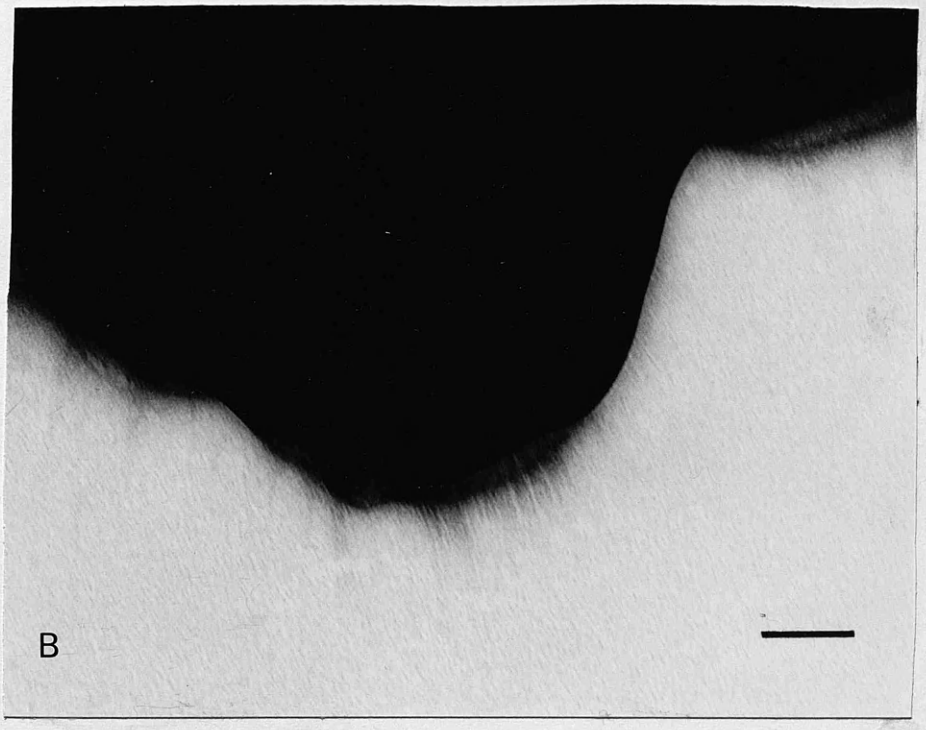

B

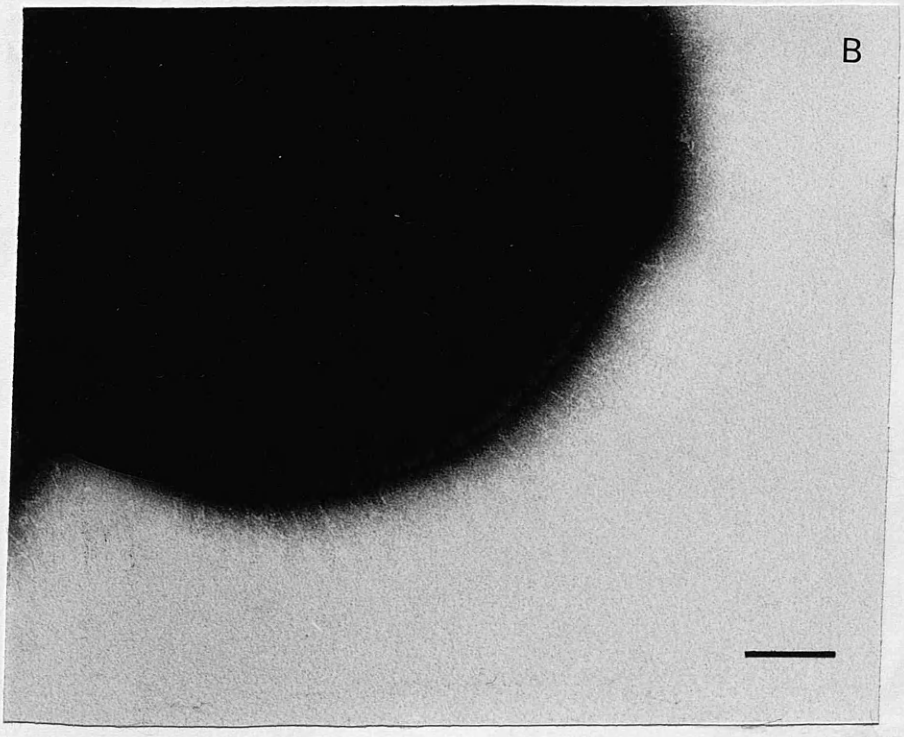
Figure 14: Electron microscopy of cell-surface structures of

a - Streptococcus mitior C32

b - Streptococcus mitior 863

Bar represents 0.1 μm.


A


B

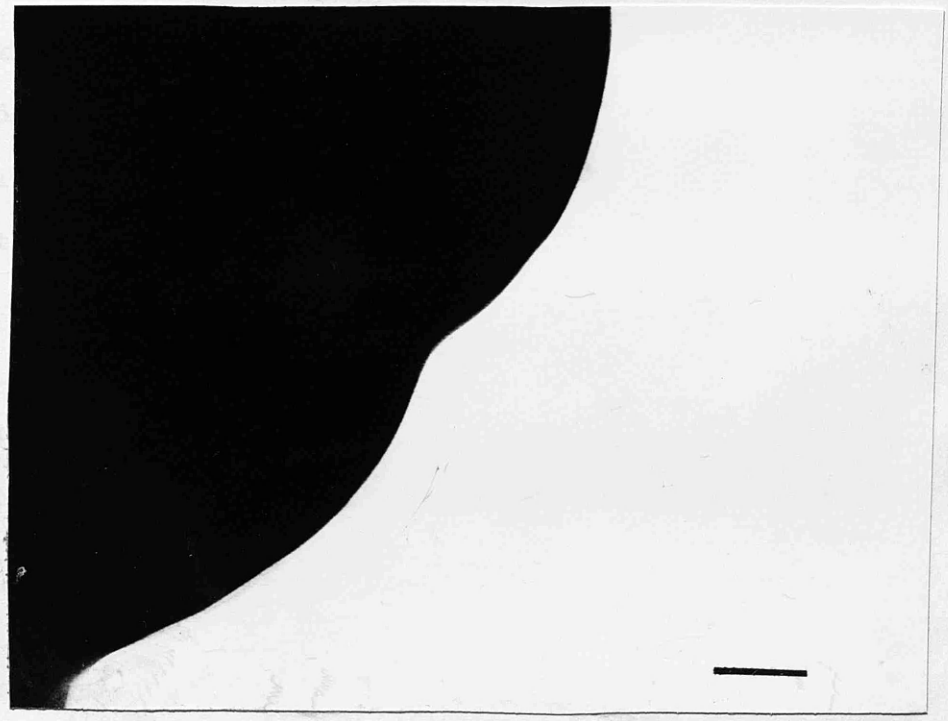


Figure 15: Electron microscopy of cell-surface structures of *Streptococcus salivarius* NCTC 7366

Bar represents 0.1 μm.

i. Quantitative precipitin test

A series of test tubes were set up containing varying amounts of antigen (LTA) and a constant amount of antibody (0.1 ml). The protein content of the precipitate formed in these tubes was estimated by the method of Lowry et al (1951).

The amount of protein in the precipitate was plotted against the amount of antigen added to form a quantitative precipitin curve. The antibody content for two batches of serum was determined in this way. With the first batch of serum, maximum precipitation of antibody occurred with 50 μg of LTA (Fig. 16a). The precipitate formed in this tube contained 0.11 mg protein. Since only 0.1 ml of serum was used the actual amount of reacting antibody present in batch 1 (15 ml) was 1.10 mg/ml.

With the second batch of serum, maximum precipitation of antibody again occurred with 50 μg of LTA (Fig. 16b). The precipitate formed in this tube contained 0.14 mg protein, therefore, the amount of reacting antibody present in batch 2 (15 ml) was 1.40 mg/ml.

ii. Rocket immunoelectrophoresis of culture supernatants

Concentrated bacterial culture supernatants were examined for the presence of extracellular LTA by RIE as described in Materials and Methods, page 80, using purified LTA from L. casei NCTC 6375 as the standard. Only two organisms, Strep. salivarius M65 and Strep. salivarius MU 289 were found to excrete LTA under the growth conditions employed (Table 60). In the RIE assay, culture supernatant from Strep. salivarius M65 produced a rocket height of 1.4 cm (Fig. 17a) while that from Strep. salivarius MU 289 produced a rocket height of 0.9 cm (Fig. 17b). The height of the rocket for the standard LTA was 1.8 cm.

B. Biochemical analysis of culture supernatants

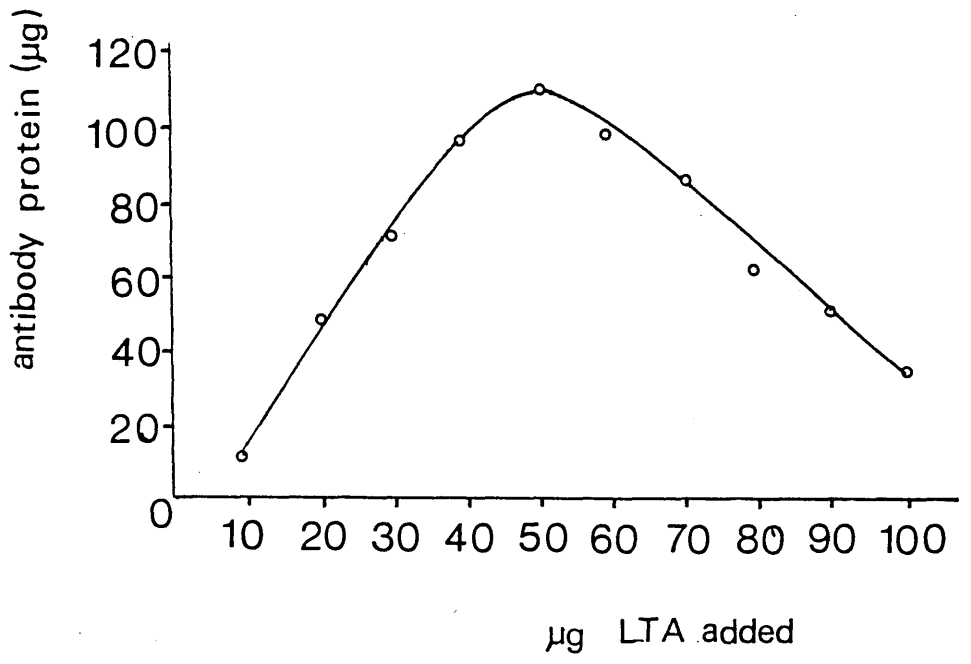
Concentrated culture supernatants were analysed biochemically for

Figure 16: Estimation of antibody content of serum by the quantitative precipitin test -

a - batch 1

b - batch 2

A


antibody protein (µg)
120
100
80
60
40
20
0
10 20 30 40 50 60 70 80 90 100
µg LTA added

B

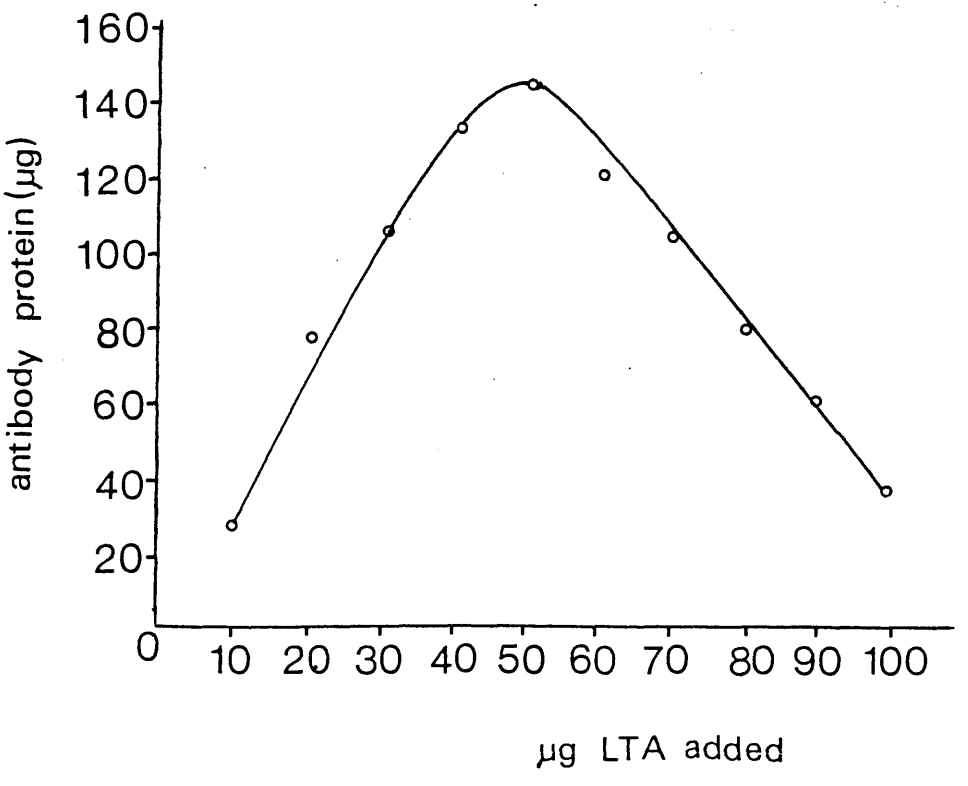

antibody protein (µg)
160
140
120
100
80
60
40
20
0
10 20 30 40 50 60 70 80 90 100
µg LTA added

Table 60 Production of extracellular LTA as measured by rocket immunoelectrophoresis

Organism	Production of extracellular LTA	Rocket Height (cm)
S. salivarius NCTC 7366	-	0
S. salivarius M65	+	1.4 cm
S. salivarius D66	-	0
S. salivarius MU 289	+	0.9 cm
S. sanguis NCTC 7864	-	0
S. mitior C32	-	0
S. mitior 863	-	0
L. casei NCTC 6375	-	0
L. casei 0112	-	0
L. casei MU 259	-	0
Standard (Purified LTA from *L. casei* 6375)		1.8 cm

Figure 17: Examination of bacterial culture supernatants by rocket immunoelectrophoresis.

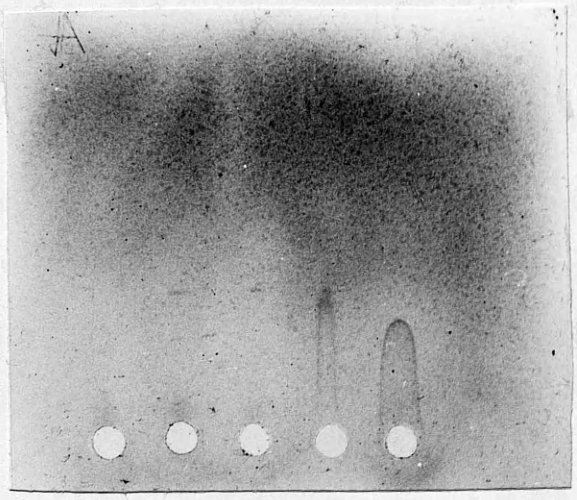
A.

Well 1 - Strep. salivarius NCTC 7366.
2 - Strep. sanguis NCTC 7864
3 - L. casei NCTC 6375
4 - Standard
5 - Strep. salivarius M65

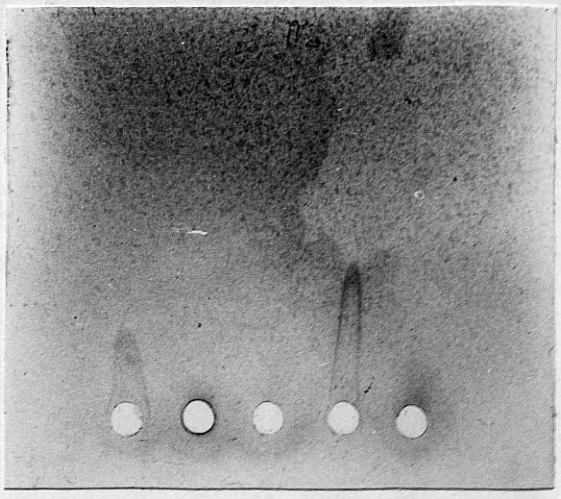
B.

Well 1 - Strep. salivarius MU 289
2 - Strep. mitior C32
3 - L. casei 0112
4 - Standard
5 - L. casei MU 259

a



b



carbohydrate, phosphorus and protein (Table 61). The most abundant component detected in the supernatants was protein. With Strep. sanguis NCTC 7864 the protein content was almost 3 mg/ml. The next most abundant component detected was carbohydrate. In most cases the phosphorus content was quite low. Two strains of Strep. salivarius (M65 and MU 289) were found to have the highest content of phosphorus in their supernatants (60.3 and 51.8 μg/ml, respectively). This was double the phosphorus content of the supernatants from most of the other strains. These organisms were the two Strep. salivarius strains shown to excrete LTA (Table 60).

VII. Effect of bacterial culture supernatants on the adherence of 500 mM galactose-grown Candida albicans GDH 2346 to buccal epithelial cells

After immunological and biochemical examination, the concentrated culture supernatants were examined for their effect on adherence of 500 mM galactose-grown C. albicans GDH 2346 to epithelial cells (Table 62). The supernatants from only three strains were found to significantly reduce yeast adhesion: Strep. salivarius NCTC 7366, Strep. salivarius M65 and Strep. salivarius MU 289. All reduced adherence of C. albicans by around 20%. None of the supernatants from the other strains had any effect on yeast adherence to epithelial cells.

The amount of protein excreted by the three strains was less than that excreted by the majority of the other strains which had no effect on adherence and, therefore, it seems unlikely that the inhibitor of yeast adhesion was protein in nature. Two of the strains (Strep. salivarius M65 and Strep. salivarius MU 289) excreted LTA into the supernatant (Table 60). It is possible that in the case of these two streptococci soluble extracellular LTA inhibited adherence of C. albicans to epithelial cells.

Table 61 Biochemical analysis of concentrated bacterial culture supernatants

Culture supernatant from[a]	Content of		
	Carbohydrate (mg/ml)	Phosphorus (mg/ml)	Protein (mg/ml)
S. salivarius NCTC 7366	0.10	0.02	0.62
S. salivarius M65	0.07	0.06	0.80
S. salivarius D66	0.14	0.02	1.18
S. salivarius MU 289	0.18	0.05	1.70
S. sanguis NCTC 7864	0.18	0.01	2.92
S. mitior C32	0.17	0.03	1.35
S. mitior 863	0.19	0.03	1.76
L. casei NCTC 6375	0.19	0.03	1.43
L. casei 0112	0.05	0.01	0.88
L. casei MU 259	0.14	0.01	0.53

[a] All organisms were grown in Todd-Hewitt broth. All culture supernatants were concentrated 20-fold by freeze drying.

Table 62 Effect of bacterial culture supernatants on the adherence of 500 mM galactose-grown *Candida albicans* GDH 2346 to buccal epithelial cells

Culture supernatant from	Mean number of adherent yeasts/100 BEC ± SE[a]	Relative adherence[b]	P[c]
S. salivarius NCTC 7366	1021.0 ± 58.3	0.81	< 0.025
S. sanguis NCTC 7864	1139.4 ± 66.1	0.90	NS
L. casei NCTC 6375	1076.6 ± 88.2	0.85	NS
S. salivarius D66	1409.2 ± 83.2	1.12	NS
S. salivarius M65	996.6 ± 62.5	0.79	< 0.01
S. salivarius MU 289	1009.0 ± 77.4	0.80	< 0.025
S. mitior C32	1299.7 ± 80.5	1.03	NS
S. mitior 863	1463.7 ± 99.9	1.16	NS
L. casei 0112	1151.2 ± 79.1	0.91	NS
L. casei MU 259	1080.1 ± 85.1	0.86	NS
Control	1260.9 ± 81.8	-	-

[a] Mean of three different experiments which were carried out in triplicate

[b] Adherence was measured relative to that of the control in which the supernatant was replaced with buffer

[c] Probability values comparing test and control assays for different culture supernatants

NS - Not significant.

VIII. Effect of bacterial cell suspensions on growth of Candida albicans

The effect of bacterial suspensions on the growth of both C. albicans MRL 3153 and C. albicans GDH 2346 was investigated by the agar disc method (Table 63). Partial inhibition of yeast growth was judged to have taken place when the colony size in the test system was smaller than the colony size in the control system (Fig. 6, Materials and Methods).

All strains of Strep. salivarius partially inhibited the growth of both yeast strains as did suspensions of Strep. sanguis NCTC 7864 and L. casei NCTC 6375. The greatest inhibition of yeast growth was caused by Strep. salivarius M65. Colony size was reduced by approximately 90% compared to the control, as judged by eye, when the yeasts were incubated with a suspension of Strep. salivarius M65. Colony size was also greatly reduced (70-80%) by Strep. salivarius MU 289. The other two strains of L. casei (0112 and MU 259) had no effect on the growth of either yeast strain, as was the case with Strep. mitior C32. However, Strep. mitior 863 partially inhibited C. albicans MRL 3153 but had no effect on the growth of C. albicans GDH 2346.

The pH value of the assay mixture was monitored to see whether pH could be responsible for inhibition of yeast growth (Table 63). The pH value of all assay mixtures before incubation was 7.8. After 24 h incubation the greatest decrease in pH was produced by Strep. salivarius M65 and the two lactobacillus isolates (0112 and MU 259). Since neither of the lactobacillus strains had any effect on yeast growth, it is unlikely that the reduction in pH produced by Strep. salivarius M65 is responsible for the partial inhibition of yeast growth shown by this organism.

Table 63 Effect of bacterial suspensions on the growth of Candida albicans

Organism	Strain MRL 3153		Strain GDH 2346	
	Type of inhibition[a]	Final pH[b]	Type of Inhibition[a]	Final pH[b]
S. salivarius NCTC 7366	Partial (143)	7.1	Partial (125)	7.1
S. sanguis NCTC 7864	Partial (138)	7.3	Partial (147)	7.3
L. casei NCTC 6375	Partial (124)	7.4	Partial (117)	7.2
S. salivarius D66	Partial (181)	7.0	Partial (146)	7.1
S. salivarius M65	Partial (143)	6.8	Partial (125)	6.6
S. salivarius MU 289	Partial (198)	7.0	Partial (142)	6.9
S. mitior C32	None (200)	7.3	None (159)	7.0
S. mitior 863	Partial (178)	7.2	None (161)	7.1
L. casei 0112	None (204)	6.9	None (167)	6.8
L. casei MU 259	None (209)	6.8	None (160)	6.8
Control	None (211)	7.5	None (165)	7.6

[a]Partial inhibition is measured as a reduction in colony size compared with that of the control. Number of colonies is given in parentheses.

[b]The pH value of the assay mixture after 24 h incubation.

DISCUSSION

I. Selection and Isolation of Microorganisms

Since it is known that repeated sub-culture of micro-organisms can alter their ability to adhere to surfaces (Westergren and Emilso, 1982) it was decided to use both freshly isolated and type cultures in this study. The fresh isolates were obtained by sampling various areas of the oral cavity and identifying a selection of streptococci and lactobacilli as described in Materials and Methods.

It was not surprising that many of the streptococcal isolates were identified as either Strep. salivarius or Strep. mitior since both these organisms comprise a large percentage of the oral streptococci cultivable from the mucosal surfaces of the oral cavity and represent 30-40% of the streptococci found in saliva (van Houte et al, 1971; Gibbons and van Houte, 1975). More difficulty was experienced in isolating L. casei and Strep. sanguis. Since, in health, L. casei represents a very small proportion of the oral microbial flora this is not surprising. In contrast, however, one might have expected a higher percentage of the organisms isolated to have been Strep. sanguis as this organism is a common inhabitant of the oral microflora. The small number that were isolated is probably a reflection of the areas of the oral cavity that were sampled. If, for example, the tooth surface had been swabbed then it is likely that more strains of Strep. sanguis would have been isolated since this is the preferred habitat of this organism (Gibbons and van Houte, 1975).

II. Effect of bacteria on the adherence of C. albicans to HeLa cells

The ability of an organism to adhere to a host surface is an essential pre-requisite for successful colonization and infection (Gibbons and van Houte, 1975). Although the adherence of both bacterial and candidal species has been studied, the role of commensal bacteria in

promoting or inhibiting yeast adhesion has received very little attention.

Perhaps the best indication that commensal bacteria may be involved in regulating the numbers of C. albicans is broad spectrum antibiotic treatment. Reports by Seelig (1966), Shastry et al (1969) and Kennedy and Volz (1985) have shown that antibiotic therapy can contribute to the increasing incidence and severity of clinical candidosis since the alteration of the normal flora by antibiotics can lead to the proliferation of candida in the absence of competing micro-organisms. Investigations carried out twenty years ago showed that C. albicans colonized the gut of germ-free chicks and mice in higher numbers than conventional animals, again suggesting that the indigenous bacterial flora play a role in suppressing colonization of C. albicans (Balish and Philips, 1966; Philips and Balish, 1966).

The effect of bacteria on yeast adhesion was investigated using the HeLa cell system of Samaranayake and MacFarlane (1981). In most studies of yeast adhesion, investigators have employed an in vitro microscopical method to quantify the number of yeasts attached to desquamated human epithelial cells of buccal and vaginal origin (Kimura and Pearsall, 1978; King et al, 1980). A different technique for assessing yeast adhesion was described by Samaranayake and MacFarlane (1981) who used epithelial monolayers of human origin grown in tissue culture. This method appears to have a number of advantages over previous techniques, especially when studying the role of the commensal microflora. Buccal epithelial cells are inevitably contaminated with commensal organisms which are often difficult to dislodge even after repeated washings (Sklavanou and Germaine, 1980; Varian and Cooke, 1980). Moreover, buccal cells are continuously bathed in saliva and perhaps contaminated with serum and food debris to varying degrees prior to collection. Indeed,

King et al (1980) showed that the numbers of adherent yeasts varied considerably with vaginal epithelial cells from different donors. They also noticed a considerable day-to-day fluctuation in adherence values when the cells were tested on a daily basis.

Samaranayake and MacFarlane (1981) found that the eukaryotic cell line exhibited more uniform characteristics making them more amenable to a wider range of experiments. By using this system to study the effect of bacteria on yeast adhesion the conditions can be more easily controlled due to the uncontaminated nature of the epithelial monolayers.

A. Adherence of C. albicans to HeLa cells as affected by the time of pre-incubation of monolayers with a bacterial cell suspension

In previous studies carried out by Makrides and MacFarlane (1982), HeLa cell monolayers were pre-incubated with bacterial cell suspensions for periods of 30 and 60 minutes. Both periods of pre-incubation were found to produce similar results. However, to determine whether a pre-incubation period of less than 30 minutes had any significant effect on yeast adhesion, assays were performed in which HeLa cell monolayers were incubated with a cell suspension of Strep. salivarius NCTC 7366 for 5 to 30 minutes.

It was found that a pre-incubation period of at least 25 minutes was required for the bacteria to have any effect on yeast adhesion. This length of time may be necessary either for the bacteria themselves to attach to the epithelial cells or for the release of products that interfere with yeast adhesion. Since optimum inhibition of candidal adhesion was achieved after 30-35 minutes pre-incubation of monolayers with bacteria, this period was chosen for all further adhesion studies.

B. Effect of bacteria on adherence of C. albicans MRL 3153 to HeLa cells after growth of the yeast in YNB containing a carbon source

It has been shown that the commensal microflora of the mouth (Gibbons, 1980), the gut (Fuller and Brooker, 1980) and the vagina (Saigh et al, 1978) can affect the adherence and colonization of micro-organisms on mucosal surfaces. The results of this study on the effect of twenty three strains of bacteria on the adherence of C. albicans to HeLa cells substantiate these observations as all organisms were found to significantly reduce yeast adhesion.

The finding that Strep. salivarius and Strep. mitior significantly reduced candidal adhesion to epithelial cells in vitro is in agreement with the in vivo experiments of Liljemark and Gibbons (1973) who demonstrated that secondary inoculation of Strep. salivarius and Strep. mitior inhibited intra-oral candidal colonization in germ-free rodents. These authors also obtained similar results in vitro when they incubated buccal epithelial cells from gnotobiotic mice with streptococci and candida.

More recent investigations by Samaranayake and MacFarlane (1982) have shown that Strep. salivarius and Strep. mitior can reduce the adherence of C. albicans to HeLa cells.

It has been suggested that the indigenous bacterial flora may suppress C. albicans by secreting antifungal substances or by competing for nutrients (Isenberg et al, 1960; Koser et al, 1960). However, Liljemark and Gibbons (1973) observed no zones of inhibition indicative of the production of growth-inhibiting materials when cultures of Strep. salivarius and Strep. mitior were cross-streaked with C. albicans.

A more likely cause of C. albicans suppression is bacterial interference with the adherent interactions of the yeast. Strains of Strep. salivarius and Strep. mitior have been shown to adhere well to oral

mucosal surfaces (Gibbons and van Houte, 1971; Liljemark and Gibbons, 1972) and may well compete with C. albicans for epithelial cell receptors. This hypothesis is further supported by the finding that Strep. mutans has no effect on yeast adhesion (Liljemark and Gibbons, 1973). This organism has been shown to attach poorly to oral epithelial cells (Gibbons and van Houte, 1971) and so would not be expected to compete with C. albicans for the same epithelial cell receptor.

Of all the organisms examined in this study, L. casei NCTC 6375 inhibited the adherence of Candida albicans by the greatest amount, with values of up to 50-65%. In contrast to this, L. casei 0112 could only inhibit yeast adhesion by 30% or less. The fact that this organism was isolated from the saliva of a patient with oral candidosis may be related to its poor effect on yeast adhesion. However, since no other strain examined in this study was isolated from an active yeast infection one can only speculate that this was the reason that L. casei 0112 was a poorer inhibitor of yeast adhesion than L. casei NCTC 6375.

Lactobacilli have, in fact, been found by a number of investigators to affect yeast adhesion. Sobel et al (1981) showed that there was a significant decrease in the adherence of C. albicans when vaginal epithelial cells were coated with lactobacilli. This is in agreement with the investigations of Savage (1969) who, using an in vivo model, showed strong microbial interference between indigenous yeasts and lactobacilli. Lactobacilli normally are layered on the surface of the keratinized gastric epithelium, and yeasts are layered on the non-keratinized portion of gastric epithelium. When mice were given a penicillin solution in place of drinking water, the lactobacilli disappeared and were replaced by yeasts. When the penicillin was discontinued, the lactobacilli displaced the yeasts from the keratinized epithelium. The yeasts never populated the keratinized

epithelium when lactobacilli were present suggesting that displacement of the yeast from the epithelium was due either to interference with yeast multiplication or adherence by the lactobacilli.

Investigations by Makrides and MacFarlane (1982) have also shown inhibition of yeast adhesion to HeLa cells by L. casei. In the present study, which is an extension of their work, L. casei inhibited adherence of C. albicans to HeLa cells by a similar extent irrespective of the carbon source used in the growth of the yeast. This finding was true for all the bacteria examined. Since growth of the yeast in medium supplemented with 500 mM galactose or 500 mM sucrose enhances adhesion (Douglas et al, 1981), reduced inhibition of yeast adhesion might have been expected when C. albicans was cultured using these carbon sources. The finding that bacterial inhibition of C. albicans adhesion remained fairly similar may be related to the adhesin(s) expressed on the surface of the yeast in response to environmental factors. It has been suggested that there is more than one type of adhesin present on the yeast cell surface since glycosides containing L-fucose, N-acetyl-D-glucosamine or D-mannose may all function as epithelial cell receptors for the yeast (Douglas, 1985). The subsequent expression of these adhesins is probably dependent upon environmental conditions. Galactose and sucrose-grown yeasts may possess more than one type of adhesin on their surface. However, if the bacteria block one group of adhesins, the percentage inhibition relative to the control will remain similar whether the yeasts are cultured in glucose, sucrose or galactose. A further explanation of these results is that the high concentration of bacteria used to pre-treat the HeLa cells blocks many of the available attachment sites for candidal adherence irrespective of any variation in fibrillar adhesins.

C. Adherence of C. albicans to HeLa cells as affected by growth of the bacteria in THB containing different carbon sources

When bacteria were cultured in THB containing different carbon sources, the degree of inhibition of yeast adhesion varied depending on the carbon source used in the growth of the bacteria. When the bacteria were cultured in 50 mM glucose there was 30-40% inhibition of yeast adhesion. However, when the carbon source was changed to 500 mM sucrose most of the bacteria had a diminished inhibitory effect on candidal adhesion. Some of the organisms, particularly Strep. salivarius NCTC 7366 and Strep. mitior C32, produced large amounts of extracellular polysaccharide when grown on sucrose; this may have affected their ability to inhibit yeast adhesion possibly by masking the bacterial adhesin. When the bacteria were grown in media containing 500 mM galactose the effect they had on candidal adhesion was further affected. All strains, apart from Strep. mitior 863, were found to be less inhibitory than those organisms grown on 50 mM glucose. Furthermore six strains lost their ability to inhibit the adherence of C. albicans to HeLa cells when they were grown in media containing 500 mM galactose.

It would appear from these results that the carbon source used in the growth of the bacteria is either affecting their surface properties or is affecting the synthesis and possibly secretion of extracellular products which are involved in the inhibition of yeast adhesion. Hardy et al (1981), investigated the effect of different carbohydrates on the surface properties, lipoteichoic acid production and extracellular proteins of Strep. mutans and found that both cell-bound and extracellular lipoteichoic acid production was affected by the carbon source available for bacterial growth. These authors found also that the growth conditions affected the amounts of a number of individual extracellular proteins.

It is known that the adherence properties of bacteria may be affected by such changes and hence their ability to inhibit yeast adhesion may also be diminished.

A study on the effect of indigenous intestinal bacteria on yeast colonization in the gut of hamsters by Kennedy and Volz (1985) reported that the bacteria reduced colonization of the mucosa by C. albicans possibly by competition for adhesion sites or by a physical barrier formed by dense populations of micro-organisms. These authors also found that the bacteria produced inhibitor substances which may reduce mucosal colonization by the yeast. They suggested, therefore, that the indigenous intestinal microflora suppresses C. albicans colonization and dissemination from the gut by inhibiting yeast adhesion and reducing C. albicans population levels in the gut.

These findings by Kennedy and Volz (1985) may be similar to the events that occur in the oral cavity. In the present study it has been shown that the oral commensal flora can inhibit adherence of C. albicans to epithelial cells in vitro. Therefore, it is possible that population levels of C. albicans in the oral cavity are reduced by competition for adhesion sites or by a physical barrier formed by dense populations of micro-organisms.

III. Effect of lipoteichoic acid on adherence of C. albicans to buccal epithelial cells

There is now considerable evidence that lipoteichoic acid (LTA) mediates the attachment of some streptococci, most notably Strep. pyogenes, to mucosal surfaces (Beachey, 1981). Although LTA has been shown to be associated with the cytoplasmic membrane of Gram-positive bacteria (Knox and Wicken, 1973), a number of investigators have suggested that some LTA

molecules are exposed on the surface of the organisms (Chiu et al, 1974; Beachey and Ofek, 1976), and mediate their attachment to mucosal surfaces.

Ten strains of bacteria used in the previous adhesion studies were chosen for further examination. The strains were selected because they inhibited the adherence of C. albicans to HeLa cells to different extents covering a range from the most inhibitory to the least inhibitory.

Buccal epithelial cells were used instead of HeLa cells to study the effect of LTA on yeast adhesion as they resemble more closely the in vivo situation. A preliminary investigation using HeLa cells yielded results similar to those of the buccal cell assays. Pretreatment of HeLa cells with crude LTA from L. casei NCTC 6375 resulted in 32% inhibition of yeast adhesion. A similar result was obtained when buccal cells were pre-treated with crude LTA from L. casei NCTC 6375: yeast adhesion was inhibited by 37%. However, since the HeLa cell assay was only repeated once the results have not been included. Although buccal cells and HeLa cells are not closely related, candidal adhesion to both cell types shows a number of similarities, for example, in both systems adherence of C. albicans was enhanced to a similar extent by growth in media containing 500 mM galactose or 500 mM sucrose (Samaranayake and MacFarlane, 1982). Also, in both systems adhesion was dependent upon yeast cell concentration and was detectable above 10^4 yeast cells/ml of incubation mixture, and formol-killed non-viable yeasts adhered significantly less than the viable yeasts (Kimura and Pearsall, 1978; Samaranayake and MacFarlane, 1981).

A. Effect of LTA from L. casei on yeast adhesion

Lipoteichoic acid samples from three strains of L. casei were examined for possible effects on yeast adhesion to buccal epithelial cells. With L. casei NCTC 6375 low concentrations of LTA caused inhibition of

y east adhesion to buccal cells. As the concentration of LTA was increased inhibition of yeast adhesion increased. However, if the effect of isolated LTA on yeast adhesion is compared with that of the whole cell suspension (Table 64), then we can see that LTA was not as effective as the bacterial cells in inhibiting yeast adhesion.

To see if the effectiveness of LTA could be enhanced, the crude polymer from L. casei NCTC 6375 was purified by anion-exchange chromatography. This treatment enhanced the effect of LTA on yeast adhesion; increasing the degree of inhibition to a level much closer to that produced by the whole cell suspension (Table 64) suggesting that LTA may play a role in mediating adherence of L. casei NCTC 6375 to epithelial cells.

The effect of both crude and purified LTA from L. casei NCTC 6375 on a second strain of C. albicans was investigated. Candida albicans GDH 2346 was originally isolated at Glasgow Dental Hospital from a patient with denture stomatitis. In a study carried out by McCourtie and Douglas (1984) on the adherence of nine different strains of C. albicans, strain GDH 2346 was found to be one of the most adherent.

The effect of both crude and purified LTA on the adherence of C. albicans GDH 2346 was similar to the effect on C. albicans MRL 3153 (Table 65) with the adherence of both strains being inhibited by 35-40% by crude LTA and 55% with the purified polymer. The similar effect of LTA on the two yeast strains might be expected if one looks at the findings of McCourtie and Douglas (1985). These authors showed that incubation of suspensions of C. albicans MRL 3153 with antiserum raised against extracellular polymeric material (EP) from C. albicans GDH 2346 produced a pattern of titres similar to that obtained with the homologous yeast cells, indicating a similarity between the EP produced by these two strains. Since this EP is derived from a fibrillar layer which promotes yeast adhesion

Table 64 Comparison between the effect of whole cells and isolated LTA on the adherence of 500 mM galactose-grown *Candida albicans* to epithelial cells

Organism	Relative adherence of *C. albicans* as affected by:	
	LTA[a]	Whole Cell suspension
S. salivarius NCTC 7366	0.53 (0.63)[b]	0.61
S. salivarius D66	0.58 (0.69)[b]	0.47
S. salivarius M65	0.54	0.55
S. salivarius MU 289	0.52	0.43
S. sanguis NCTC 7864	0.57	0.60
S. mitior 863	0.71	0.49
S. mitior C32	0.64 (0.76)[b]	0.52
L. casei NCTC 6375	0.63 (0.45)[b]	0.41
L. casei 0112	0.73	0.70
L. casei MU 259	0.53	0.56

[a]Maximum inhibition achieved with crude LTA

[b]Maximum inhibition given by purified LTA.

Table 65 Comparison of the effect of isolated crude LTA on the adherence of *C. albicans* MRL 3153 and GDH 2346 to epithelial cells

Crude LTA from	Relative adherence of *C. albicans*[a]	
	MRL 3153	GDH 2346
L. casei NCTC 6375	0.63	0.63
Strep. salivarius NCTC 7366	0.53	0.53
Strep. salivarius D66	0.65	0.59
Strep. sanguis NCTC 7864	0.61	0.57

[a]Maximum inhibition achieved with crude LTA.

(McCourtie and Douglas, 1981), it is likely that both these yeast strains attach to the same host cell receptor. If LTA inhibits yeast adhesion by competing for the host-cell receptor then the effect it has on these two yeast strains will be the same.

Lipoteichoic acid extracted from the other two strains of L. casei (MU 259 and 0112) inhibited the adherence of C. albicans GDH 2346 to buccal cells. The degree of inhibition produced by crude LTA from both strains was similar to that produced by the whole cell suspensions (Table 64). Of the three strains of L. casei examined, the LTA extracted from strain 0112 was less inhibitory than the LTA from the other two strains. L. casei 0112 was isolated from the saliva of a patient with oral candidosis and was the strain of L. casei which had the least effect on adherence of C. albicans to HeLa cells.

The studies of Chan et al (1985) suggest that LTA is responsible for the adherence of lactobacillus cells to uroepithelial cells. These authors examined the effect of the indigenous flora of the cervix, vagina and urethra on the adherence of uropathogenic bacteria to uroepithelial cells and found that blockage with whole cells and LTA-peptidoglycan complexes was more effective than blockage by LTA alone. This suggests that steric hindrance of receptor sites is more important in the blockage of uropathogen adherence than direct competition for receptor sites. However in this present study, the results obtained with the isolated LTA from the three strains of L. casei were similar to those produced by the whole cells (Table 64), suggesting that inhibition of yeast adhesion by L. casei is a result of direct competition for the same epithelial cell receptor site. This is supported by the finding that purified LTA from L. casei NCTC 6375 was more effective at inhibiting yeast adhesion than the crude polymer.

B. Effect of deacylated LTA on yeast adherence to buccal cells

Purified LTA from L. casei NCTC 6375 was treated with ammonium hydroxide as described in Materials and Methods, page 71. This treatment effectively removes the fatty acid portion of the molecule (Nealon and Mattingly, 1984). When deacylated LTA was examined for its effect on yeast adhesion it was found to have lost its ability to inhibit candidal adherence to buccal cells. The untreated polymer inhibited yeast adherence to buccal cells by 50% whereas the deacylated polymer had no effect on adhesion indicating that the fatty acid portion of the molecule is necessary for inhibition. This is in agreement with the findings of previous investigators; for example, Beachey (1981, 1975) demonstrated that the binding of LTA from Strep. pyogenes to epithelial cells is mediated by the glycolipid region of the molecule. The lipid moiety of the hydrolyzed molecule inhibited the binding of whole streptococci, whereas the deacylated LTA did not. Carruthers and Kabat (1983) also demonstrated a loss of inhibition of adherence of S. aureus to buccal cells by staphylococcal LTA after deacylation of the preparation, suggesting that the fatty acids on the molecule are essential to binding.

C. Effect of LTA from Strep. salivarius on yeast adhesion

Streptococcus salivarius is a common inhabitant of the mucosal surfaces of the oral cavity. Investigations on the effect of whole cell suspensions on yeast adherence to HeLa cells demonstrated 40% inhibition of candidal adhesion. A crude preparation of LTA from Strep. salivarius NCTC 7366 inhibited the adherence of both C. albicans MRL 3153 and GDH 2346 by a similar amount (50%) (Table 65), suggesting that both yeast strains attach to the same epithelial cell receptor.

Crude LTA from Strep. salivarius NCTC 7366 was purified to see if

its effect on yeast adhesion could be enhanced. However, unlike L. casei NCTC 6375, purification of isolated LTA from Strep. salivarius NCTC 7366 did not increase the inhibitory effect of the polymer, but rather, decreased its effect on yeast adherence to epithelial cells.

Lipoteichoic acid was extracted from a further three strains of Strep. salivarius and examined for its effect on yeast adherence to buccal cells. With Strep. salivarius D66, the effect of crude LTA on both C. albicans MRL 3153 and GDH 2346 was similar, with approximately 35-40% inhibition of both yeast strains. Once more purification of the polymer resulted in a decrease in its effect on yeast adhesion.

With the remaining two strains of Strep. salivarius, M65 and MU 289, crude preparations of LTA inhibited adhesion of C. albicans. In the case of Strep. salivarius M65 the degree of inhibition produced by isolated LTA was comparable to that of the whole cells (Table 64) suggesting that LTA may be competing with the yeast for the same epithelial cell receptor. Lipoteichoic acid extracts from strains MU 289 and D66 were slightly less inhibitory than the whole cell suspensions, but the results still suggest competition between the bacteria and the yeasts for the same epithelial cell receptor. However, when crude LTA was purified its effect on yeast adhesion decreased suggesting that another component in addition to LTA may be involved in mediating adherence of the bacteria, or that the crude preparation may block yeast adhesion by steric hindrance. It is also possible that there is more than one epithelial cell receptor for LTA that may not be common to C. albicans.

Unlike Strep. pyogenes, which has been the subject of a number of investigations by Beachey and co-workers, little is known about the adherence mechanisms of Strep. salivarius. Streptococcus pyogenes has been shown to attach to buccal epithelial cells via LTA complexed with M-protein

(Beachey et al, 1983). According to Beachey, the binding of LTA to a variety of host cells is mediated by the glycolipid end of the LTA molecule, which can form ionic complexes with streptococcal surface proteins, permitting the re-orientation of the LTA to expose some of its lipid ends toward the surface of the organism (Fig. 3). These LTA-protein complexes are now thought to compose the fibrillae on the surface of Strep. pyogenes. The fibrillae, as seen by electron microscopy, appear to be involved in the binding of the organism to epithelial cells (Beachey et al, 1983).

The streptococcal strains used in the present study were examined for the presence of fibrils. Of the four strains of Strep. salivarius examined, only one - Strep. salivarius NCTC 7366 - did not show the presence of such structures. This proved to be an unusual finding as reports by other investigators suggest that all strains of Strep. salivarius possess some kind of fibrillar structure (Handley et al, 1984). It is possible that only a few cells in a culture possess these structures and this may explain the apparent lack of fibrils on Strep. salivarius NCTC 7366.

In their studies of Strep. salivarius strains, Handley et al (1984) found that there were two structural subgroups, carrying either fibrils or fimbriae. They described fibrils as being of consistent length but not of consistent width, while fimbriae were longer and were consistent in width. Lancefield Group K strains possessed fibrils whilst strains which did not possess the Group K antigen carried fimbriae. Their studies on surface structure correlated well with the observations of Weerkamp and McBride (1980) who divided Strep. salivarius strains into two groups on the basis of adherence characteristics. The joint implication of these two separate studies is that fibrillar strains carry the adhesins necessary for host-associated adherence properties and that

fimbriated strains lack them. It is likely that the fibrils are the structural components upon which a number of separate adhesins are carried. Three types of adhesin on the surface of Strep. salivarius HB have been discovered by Weerkamp and McBride (1980). The first two adhesins were isolated and partially characterized as a glycoprotein and a protein respectively, the third has not yet been characterized (Weerkamp and Jacobs, 1982). Since Handley et al (1984) demonstrated only two distinct structural components on Strep. salivarius HB, ie. fibrils and fimbriae, it seems likely that the fibrils carry more than one kind of adhesin.

There is a great structural and functional diversity within the biochemically homogeneous species of Strep. salivarius, so what applies to one particular strain may not apply to others. In the present study, LTA inhibited yeast adhesion to buccal cells, but it is unlikely to be the sole component of the bacterial adhesin since the purified preparation had less effect on yeast adhesion than did the crude preparation. Like Strep. pyogenes, it may be necessary for LTA from Strep. salivarius to form complexes with cell surface proteins.

D. Effect of LTA from Strep. sanguis on yeast adhesion

A crude LTA extract from Strep. sanguis NCTC 7864 inhibited adherence of both C. albicans MRL 3153 and GDH 2346 by the same margin (Table 65). The inhibition caused by this extract was consistent with that produced by the whole cells (Table 64), thus implying that LTA or LTA complexes block yeast adhesion to epithelial cells by competing with the yeast for the same epithelial cell receptor.

As with most strains of streptococci, the possession of fibrils has been related to the ability of the organism to adhere to various surfaces. Electron microscopy of Strep. sanguis NCTC 7864 did in fact reveal the presence of surface structures. The fibrils were short in

length and sparsely distributed (Fig. 12b).

Streptococcus sanguis strains have been shown to possess a number of different fibrillar structures. Handley et al (1985) demonstrated the presence of two main structural sub-groups for each of the two Strep. sanguis biotypes. They showed that the fibrillar strains co-aggregated well with strains of Actinomyces and Fusobacterium. However, they did not present evidence that the fibrils were responsible for co-aggregation. A large proportion of cells that did not carry fibrils still co-aggregated, so it is probable that in some strains co-aggregation adhesins are located in the cell wall.

The evidence so far produced for the composition of possible adhesin components in Strep. sanguis is somewhat confusing. The three major surface antigens detected so far on Strep. sanguis strains are polysaccharide, protein and lipoteichoic acid. Rosan and Argenbright (1982) claim that LTA is the group specific determinant ($\equiv$ antigen a) whereas Okahashi et al (1983) described the characterization of a serotype 1 group specific carbohydrate composed of glucose, rhamnose and N-acetylglucosamine. So far the polysaccharide has not been implicated in adhesion. Liljemark and Bloomquist (1982) have partially characterized a protein-containing cell surface component from Strep. sanguis isolated by sonication, which inhibited adherence to saliva-coated hydroxyapatite. However, since LTA has been found to bind strongly to hydroxyapatite (Ciardi et al, 1977) and Strep. sanguis strains are known to produce both cellular and extracellular LTA (Chiu et al, 1974; Horne and Tomasz, 1979) there is good evidence for its role in adherence. Bolton (1980) showed that adherence of Strep. sanguis and Strep. mutans to uncoated hydroxyapatite was significantly reduced by antibody to LTA. Furthermore, Bolton demonstrated that incubation of the antiserum with purified soluble

lipoteichoic acid reduced its inhibitory effect. When saliva-coated hydroxyapatite was used in adherence assays the inhibitory effect of antibody to lipoteichoic acid was still apparent but at a lower level than that observed with untreated hydroxyapatite. This would suggest that mechanisms additional to LTA-mediated binding are involved in the adherence of bacteria to hydroxyapatite. A report by Hogg and Embery (1982) suggests that LTA is the bacterial cell receptor for blood group-reactive glycoproteins. They showed that LTA inhibited the interaction between Strep. sanguis NCTC 7864, and a high molecular weight blood group-reactive glycoprotein isolated from human saliva. A study carried out by Hogg et al (1981) demonstrated a correlation between blood group-reactive glycoprotein binding and the presence of surface fibrils on Strep. sanguis NCTC 7864. Together, the results of these two studies suggest that LTA may be located on the surface fibrils of Strep. sanguis. This strain of Strep. sanguis used by Hogg et al is the same strain as was examined in this present study. The results of this investigation along with those of Hogg et al (1982, 1981) suggest a role for LTA in mediating adherence of Strep. sanguis NCTC 7864. Since isolated LTA inhibited adherence of C. albicans, it is likely that Strep. sanguis competes with the yeast for the same epithelial cell receptor.

However, to complicate matters still further, there is contradictory evidence relating not only to the role of LTA, but to its actual presence in some strains of Strep. sanguis. A recent report by Yamamoto et al (1985) suggests that Strep. sanguis NCTC 7864 does not possess lipoteichoic acid, but has in its place a unique amphipathic antigen which they termed a lipoheterosaccharide. This polymer was extracted from whole cells of Strep. sanguis by the phenol/water method, ie. the same extraction procedure that is used for obtaining LTA. The major portion of

the purified amphiphile was composed of a heterosaccharide of mannose, galactose, glucose and glycerol. Very low contents of phosphorus and glycerol indicated that the composition was different from the LTA of Strep. sanguis 7863. The latter contained no galactose, mannose or rhamnose. A study by Hamada et al (1980) found that 47 strains of Strep. sanguis biotype B lacked LTA. Subsequent work by Mizuno et al (1983) revealed that several strains of biotype B Strep. sanguis possessed a unique amphipathic antigen.

This finding by Yamamoto et al (1985) may help to explain why the crude extract of LTA from Strep. sanguis NCTC 7864, used in the present study, did not react with antiserum prepared against LTA whilst all other extracts did. However, since the preparation did still inhibit yeast adhesion it is possible that this polymer, if it is not LTA, does participate in the adherence of Strep. sanguis to host cell surfaces.

E. Effect of LTA from Strep. mitior on yeast adhesion

Streptococcus mitior is the predominant streptococcal species found adhering to the vestibular mucosa (Liljemark and Gibbons, 1972). Like Strep. sanguis there are conflicting reports as to whether LTA is present on all strains of Strep. mitior or not. Rosan (1978) has shown that some strains of this organism lacked LTA whilst the studies of Markham et al (1975) demonstrated the excretion of this polymer into the culture supernatant.

Unlike the material that was extracted from Strep. sanguis NCTC 7864, the crude preparations from Strep. mitior C32 and 863 both gave positive reactions with antiserum prepared against LTA from L. casei NCTC 6375 suggesting that both extracts were lipoteichoic acid.

Inhibition of yeast adhesion by the crude LTA preparations was

lower than the inhibitory effect produced by the whole cell suspensions of Strep. mitior (Table 64). Purification of the crude extract from Strep. mitior C32 resulted in a further decrease in its effect on yeast adhesion. These results suggest that adhesion of Strep. mitior is not mediated by LTA alone but by some other component possibly in conjunction with LTA. Investigations by Beachey et al (1981) have demonstrated the formation of complexes between LTA and protein from strains of Strep. mitior and Strep. pyogenes. Since LTA has been shown to bind to host cells only via its glycolipid moiety (Beachey and Ofek, 1976) some of the molecules need to be oriented and anchored in such a way as to permit exposure of the glycolipid moieties on the surfaces of the organisms. Complex formation between the polyanionic backbone of LTA and cell wall proteins would leave the glycolipid moieties free to interact with receptor sites on host cells (Beachey et al, 1983).

Since whole cell suspensions of Strep. mitior blocked yeast adhesion by 50% it would appear that both bacteria and yeasts compete for the same epithelial cell receptor. However, since isolated crude LTA was less inhibitory than the whole cell suspensions it appears that this polymer alone is not responsible for mediating adherence of the bacteria. This is further supported by the finding that purified LTA from Strep. mitior C32 was less inhibitory than the crude preparation. It therefore seems likely that either there is more than one adhesin on the streptococcal surface or that some kind of complex formation between LTA and another cell surface component, eg. protein, is necessary in order to mediate adhesion of the bacteria.

Electron microscopy of the two Strep. mitior isolates revealed the presence of a "fuzzy-coat" similar to that shown by Liljemark and Gibbons (1972). A study by Handley and Carter (1979) demonstrated the

presence of three types of fibrils on the surface of Strep. mitior. The short fibrils were probably equivalent to the "fuzzy-coat" described by Liljemark et al (1972) and in this study. As is the case with Strep. salivarius and Strep. sanguis it would appear likely that the components which mediate attachment of Strep. mitior are located on the surface fibrils since the fibrillar surface coating appears to bind them directly to the epithelial cell membrane, Liljemark and Gibbons (1972). These authors have also shown that the "fuzzy-coat" is trypsin-sensitive implying that a protein component may be involved in adhesion. However, this interpretation must be viewed with caution since trypsin has been shown to completely remove surface appendages on Strep. pyogenes and so may remove other components such as lipoteichoic acid (Beachey and Ofek, 1976).

F. Conclusions

For all organisms examined in this study it would appear that there is more than one type of adhesin present on their surfaces and so they probably attach to a number of receptors on the host cell surface.

Already, several components which may act as epithelial cell receptors have been shown to bind C. albicans (Douglas, 1985), suggesting that there is more than one type of adhesin present on the yeast cell surface. If there are several host cell receptors for both bacteria and yeasts then it is possible that they share one particular group and so the bacteria could affect colonization by the yeasts by interfering with adhesion. If bacteria and yeasts both adhered to only one specific group of receptors then one would have expected inhibition levels to have been closer to 100% especially when whole cell suspensions were examined for their effect on yeast adhesion. Instead, the highest level of inhibition achieved was less than 65%.

In the majority of species examined, inhibition of yeast adhesion by crude LTA was comparable to that produced by whole cell suspensions. It therefore appears likely that LTA is involved in mediating bacterial adhesion of these species to epithelial cells. However, the finding that purified LTA was not as effective as the crude polymer in inhibiting yeast adhesion, suggests that complex formation between LTA and some other cell surface component is necessary to mediate bacterial adhesion.

As yet, no investigator has demonstrated a common host cell receptor for C. albicans or the bacterial strains used in this study, although it has been suggested that fibronectin may serve this function. It has been shown that Strep. pyogenes attaches to fibronectin via LTA (Simpson and Beachey, 1983). However, this association has not been demonstrated for other streptococci, although fibronectin has been shown to bind Strep. salivarius, Strep. sanguis and Strep. mitior but to a much lesser degree than Strep. pyogenes (Babu et al, 1983). It is also unlikely that fibronectin serves as the receptor for L. casei LTA as Imai et al (1984) have shown that LTA from L. casei bound plasma fibronectin very weakly. There has been one report of C. albicans binding to fibronectin (Skerl et al, 1984); however, this has not been confirmed by any other investigators. Indeed, recent work indicates that fibronectin does not act as a receptor for C. albicans or any other Candida species, but rather serves as a blocking agent for yeast attachment (Lancaster and Douglas, unpublished results).

IV. Effect of bacterial supernatants on yeast adhesion to epithelial cells

Culture supernatants from ten bacterial strains were examined for their effect on the adherence of C. albicans GDH 2346 to buccal epithelial cells. The three supernatants which inhibited yeast adhesion by approxi-

mately 20% were from cultures of Strep. salivarius NCTC 7366, M65 and MU 289.

Biochemical analysis of all culture supernatants revealed a higher phosphorus content in the supernatants of Strep. salivarius M65 and MU 289 compared with the supernatants from the other organisms. Both these strains were found to produce extracellular LTA when their supernatants were examined by rocket immunoelectrophoresis.

The degree of inhibition of yeast adhesion was low and this may be related to the low amount of LTA excreted. With Strep. salivarius NCTC 7366, no LTA was detected in its supernatant although this strain did inhibit yeast adhesion. It is possible that the amount of LTA produced was too low to be detected, or that some other component was involved in the inhibition of candidal adherence.

The formation of LTA by cultures of streptococci has been investigated in detail by Shockman and co-workers. Culture fluid of Strep. mutans FA-1 was found to contain both LTA and deacylated LTA, whereas only the latter component was detected in the culture fluid of Strep. faecium (Joseph and Shockman, 1975). A study by Markham et al (1975) demonstrated the presence of LTA in the culture fluid for strains of Strep. mutans, Strep. sanguis, Strep. salivarius and Strep. mitior, with Strep. mutans producing relatively high concentrations. Strains of the other three species produced much smaller amounts.

The carbohydrate source is known to affect the production of extracellular LTA and Jacques et al (1979) has shown that fructose enhances the production of extracellular LTA. There also appears to be considerable variation within a species (Wicken et al, 1982). These authors found that fructose-grown Strep. sanguis M5 produced five times more extracellular LTA than Strep. sanguis Blackburn and almost twice as much as Strep. sanguis Challis. With the glucose-grown strains, Strep. sanguis 10558 produced

the largest amounts, 3-4 times that produced by other strains of Strep. sanguis. Such variation has also been found with other strains of streptococci and lactobacilli (Markham et al, 1975). Such variation in the production of extracellular LTA by different strains of the same species may explain why only two strains (Strep. salivarius M65 and MU 289) examined in the present study, were found to excrete LTA into the culture supernatant.

In addition to finding that growth conditions can affect the amount of LTA excreted, Hardy et al (1981) also reported that the secretion of extracellular proteins could be affected by the carbohydrate used in the culture of bacteria. In addition they found that some of the protein components had a low isoelectric point and suggested that this may be due to an association with LTA.

Although these studies were performed in vitro it is interesting to speculate on the in vivo situation. It is possible that the oral commensal flora suppress colonization of the oral cavity by C. albicans by competing for the same epithelial cell receptor.

V. Effect of bacterial cell suspensions on growth of C. albicans

The ten bacterial strains that were used in the LTA adhesion studies were examined for their effect on the growth of both C. albicans MRL 3153 and GDH 2346 by the method described earlier. No organism showed total inhibition of yeast growth. All strains of Strep. salivarius partially inhibited the growth of the yeast strains as did cell suspensions of Strep. sanguis NCTC 7864 and L. casei NCTC 6375. The greatest inhibition of yeast adhesion was produced by two strains of Strep. salivarius - M65 and MU 289.

The pH value of the assay mixture was monitored to see if it was

responsible for inhibition of yeast growth. However, there was no significant difference in pH values between the tests and controls. It therefore seems more likely that the bacteria are secreting a substance which is inhibitory to growth of C. albicans.

The production of bacteriocins and bacteriocin-like substances have been detected in vitro in strains of Strep. sanguis (Donoghue and Tyler, 1975) and Strep. mitior (Dajani et al, 1976). However, even if such products are secreted in this system it is unlikely that they would affect growth of C. albicans since they normally only affect organisms closely related to the strain that produces the bacteriocin.

Lactobacilli have been shown to inhibit the growth of C. albicans (Young et al, 1956). It was suggested that this inhibition of yeast growth was caused by the production of lactic acid. However, this would seem unlikely to be the reason why L. casei NCTC 6375 inhibited growth of both yeast strains since there was only a small decrease in the pH value of the assay mixture. Also, the other two strains of L. casei used in this study had no effect on yeast growth even although the pH of the assay mixture was lower than that of L. casei NCTC 6375. It is also unlikely that depletion of nutrients was responsible for inhibition of yeast growth as one would have expected all strains of the same species to have had a similar effect on the growth of both yeast strains.

The two strains which caused the greatest inhibition of yeast growth, Strep. salivarius M65 and MU 289, excreted LTA into their culture supernatant. It is possible that LTA may be involved in the inhibition of yeast growth; however there have been no reports as to the possibility of this polymer acting as a growth inhibiting substance.

There have been several studies in which in vitro inhibition by α-haemolytic streptococci was shown to be due to the production of inhibitory

concentrations of hydrogen peroxide. Holmberg and Hallander (1973) and Le Bien and Bromel (1975) detected an accumulation of hydrogen peroxide in cultures of Strep. sanguis and Strep. mitior. Allen (1985) demonstrated the in vitro inhibition of mycobacterial growth by several strains of viridans streptococci. Although it is catalase-positive it is possible that growth of C. albicans may be inhibited by peroxide. Allen (1985) demonstrated that catalase-positive M. kansasi was sensitive to 35% of the streptococci tested. Inhibition was due to a peroxide-mediated antagonism. However, there are no reports in the literature as to the possibility of hydrogen peroxide inhibiting yeast growth.

As well as competing with C. albicans for the same epithelial cell receptor, it is possible that the oral commensal flora also suppress colonization of the oral cavity by C. albicans by producing substances which are inhibitory to the growth of the yeast.

I. COLORIMETRIC ASSAYS

(A) Estimation of protein using the Lowry method (Lowry et al, 1951)

Reagents:
(A) 2% Na_2CO_3 in 0.1N NaOH
(B) 0.5% $CuSO_4.5H_2O$ in 1% sodium tartrate (separate double strength solutions mixed 1:1 before use)
(C) Folin-Ciocalteau reagent diluted 1:1 with distilled water
(D) 50 volumes of reagent (A) mixed with 1.0 volume of reagent (B), renewed daily.

Procedure: Protein samples containing 25 to 100 µg protein in 1.0 ml were used. Solution D (5 ml) was added to each tube and the contents mixed and allowed to stand at room temperature for at least 10 min. Solution C (0.5 ml) was added rapidly with immediate mixing. All solutions were allowed to stand for 30 min at room temperature before their absorbance at 750 nm was recorded.

(B) Estimation of phosphorus (Chen et al, 1956)

Reagent:

1 vol 6N sulphuric acid 2 vol distilled water 1 vol 2.5% (w/v) ammonium molybdate 1 vol 10% (w/v) ascorbic acid	reagent should be prepared fresh before use

Procedure: To digestion mixture 3.9 ml of water was added followed by 4.0 ml colour reagent, and samples mixed by inversion. Tubes were incubated at 37°C for 1.5-2 h, and absorbance at 820 nm recorded.

(C) Estimation of carbohydrate by the phenol-sulphuric acid method (Dubois et al, 1956)

Reagents: (A) Concentrated sulphuric acid

(B) 80% (w/v) phenol

Procedure: Carbohydrate samples containing 10-70 μg of sugar in 2 ml were used, 0.05 ml of the phenol reagent was added to each solution, followed by the rapid addition of 5.0 ml of concentrated sulphuric acid. Samples were left at room temperature for at least 30 min and the absorbance at 485 nm recorded.

II. REAGENTS FOR PAPER CHROMATOGRAPHY

(A) Alkaline silver nitrate dip reagent for sugars (Trevelyan et al, 1950)

Solution A: 0.1 ml of saturated aqueous silver nitrate solution was diluted to 20 ml with acetone; water was added dropwise with shaking until the white precipitate formed dissolved.

Solution B: 1 ml of 40% sodium hydroxide was added to 20 ml ethanol.

Solution C: 5% sodium thiosulphate solution.

Procedure: Chromatograms were passed through (A) and dried; this procedure was repeated once. The papers were then passed through (B) and left until reducing compounds showed up as black or silver against a brown background of silver dioxide. Excess silver oxide was removed and chromatograms fixed by passing through (C). Papers were finally washed well with water.

III. Slide Ouchterlony

A. Preparation of gel.

1. Dissolve 2 g agarose in 100 ml distilled water by steaming for 20 min. Dispense into universal containers (2 ml) and sterilize.
2. Prepare a solution of 0.02M phosphate buffer (pH 7.2) containing 0.01% sodium azide.
3. Mix an equal volume of 1 and 2 then pour onto a clean microscope slide and allow to set.
4. Punch out wells of 2 mm diameter and 4 mm apart.
5. Fill wells with antiserum or antigen (5 µl).
6. Incubate in a moist atmosphere for 24 h at 37°C.
7. Wash with saline over a period of 24 h at 4°C.
8. Wash with distilled water for 1 h.
9. Place a piece of moist filter paper on top and dry with a hot air dryer.
10. Stain by immersing in 0.5% Coomassie Brilliant blue R for 5 min.
11. Destain and dry.

B. Preparation of stain

Dissolve 5 g of Coomassie Brilliant blue R in 450 ml of 96% ethanol, 100 ml glacial acetic acid and 450 ml distilled water.

C. Preparation of destain

450 ml of 96% ethanol

100 ml glacial acetic acid

450 ml distilled water.

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