THE ISOLATION AND CELL-SURFACE IMMUNOCHEMISTRY OF LANCEFIELD GROUP F AND RELATED STREPTOCOCCI

by

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Thesis presented for the degree of Doctor of Philosophy

University of Edinburgh

1989

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ABSTRACT

The literature was reviewed concerning the taxonomy, laboratory identification, clinical significance, cell-surface immunochemistry, and potential virulence factors of the *Streptococcus milleri* group, with special reference to those belonging to Lancefield group F.

A short series of biochemical tests was developed for the rapid and cheap presumptive identification of large numbers of *Streptococcus milleri* isolates from a range of sources. A small series of sugar fermentation tests, based on the presence of preformed enzymes was selected to allow not only the identification of those isolates with typical biochemical profiles, but also those displaying wide carbohydrate utilisation profiles.

Dental plaque was recovered from the mouths of twelve healthy adult volunteers by use of a simple sampling procedure, and plated onto a single selective medium. Over two-thirds of the isolates picked from the selective medium were identified by the short series of biochemical tests as *Streptococcus milleri*. *Streptococcus milleri* was recovered from the dental plaque of ten out of twelve subjects sampled, confirming the commensal role of this organism in the gingival crevice. Serological grouping allowed the identification of group F streptococci within this collection of oral *Streptococcus milleri* isolates, which together with organisms obtained from "clinical" material and reference strains, formed a suitably diverse collection of group F streptococci to form the basis of further studies.

Incubation of group F and related streptococci with EDTA was found to release both carbohydrates and complex arrays of proteins associated non-covalently with the cell-surface of these strains. Examination of the PAGE profiles of proteins extracted from cultures of reference strains grown under different atmospheric conditions indicated some stability of non-covalently bound cell-surface proteins in these strains. PAGE examination of a collection of wild-strain EDTA extracts showed considerable similarity in the protein profiles of many strains of diverse origin and haemolytic behaviour. However, differences in the PAGE profiles of some strains suggested that this technique may be of value for use in chemotaxonomic studies of the *Streptococcus milleri* group as a whole.

Application of Western blotting, with antisera raised in rabbits against the whole-cell vaccines of homologous reference strains, revealed

considerable serological diversity amongst the EDTA-extractable surface antigens of these strains. ELISA studies showed significant cross-reactivity amongst reference and wild strains, but was able to illustrate important differences in the balance of carbohydrate and protein antigens associated non-covalently with the cell-surface of different strains.

Immunoelectrophoretic examination of EDTA extracts revealed uncomplicated profiles of precipitating antigens, and showed that the major non-covalently linked precipitating antigens of many strains were carbohydrate in nature. The behaviour of these precipitating antigens in antibody-containing gel indicated that some carried a positive charge, whilst another carried a negative charge; comparison of a number of their characteristics with previous descriptions of the typing antigens found in group F and related streptococcal strains suggested that they had much in common.

Investigations were undertaken to isolate and analyse a number of carbohydrate polymers associated with the cell-membrane and cell-wall of Lancefield group F and related streptococcal reference strains. Antigens associated with the cell-membrane were found to be lipoteichoic acids or analogues thereof, and to behave in a similar manner to the carbohydrate antigens contained in EDTA extracts of these strains. Lipoteichoic acids were shown to be significant cell-surface antigens in many group F and related streptococci. Serologically similar carbohydrate polymers, which were not teichoic acids, were isolated from the purified cell-walls of a number of strains. The similarity of some of these antigens to previous reports of the typing antigens of group F and related streptococci was considered.

Suggestions were made for further immunochemical and biochemical studies of the antigens associated with the cell-surface of this group of microorganisms, whose recognition as pathogens of note is increasing.

ACKNOWLEDGEMENTS

It is difficult to contemplate that three brief years can bring about the changes necessary to transform an individual completely unacquainted with the art and science of laboratory-based bacteriological research into one preparing a PhD thesis for submission. A great many interactions have contributed to this metamorphosis, and it is my hope in the next few lines to communicate a measure of gratitude to a by no means exhaustive list of people who have been instrumental in laying this valuable foundation.

First of all, I wish to acknowledge the generous support of the Faculty of Medicine, University of Edinburgh in awarding financial backing in the form of Bonnar Research Fellowship E606627; without such support this research opportunity could never have arisen.

A special burden of gratitude is due to my supervisor Dr. Ian Poxton, who undertook to offer helpful guidance from the earliest days of this project, and who latterly has supported me through the final preparation of this thesis.

Cordial gratitude is also due to my other supervisors: Dr. Christopher Cumming for his valued input, particularly in the early stages of this project; and to Dr. Phillip Ross for his constant backing throughout the course of my studies.

Mr. Robert Brown and Mrs. Hannah Lough are thanked both for their friendship, and for their eagerness to share the knowledge and expertise honed by lengthy exposure to laboratory diagnostic and research bacteriology. To the research students and staff of MPRL, past and present, I extend my unreserved thanks, again for sharing considerable expertise, but also for the many moments of inspired hilarity which will gladly persist when the memory of the times of travail inevitably fade into oblivion.

To David Dirom, Ian, Susie, and Frances of the Medical Illustration Department, I offer my thanks in recompense for patient instruction in the rudiments of photographic printing, and for allowing me the insights gained from many frustrating hours in the dark room!

For a warm welcome, and an equally warm expression of interest and concern at all times throughout my stay in his Department, I should like to thank

Professor J. G. Collee, and communicate my gratitude for making my stay such a happy and valuable one.

Many others outside the Department of Bacteriology are no less worthy of a vote of thanks for their contribution to the learning process during the last three years. In this respect I should like to express thanks to the many friends at the Edinburgh City Fellowship who have shared it all, and on occasion restored a sense of proportion and assurance in God.

I honour my parents and my new parents through marriage for their unstinting support and for their confidence in me.

I finally wish to bless my wife Janet, who has honoured me by her longsuffering and steadfastness during the preparation of this thesis; my future with her deserves at the very least the same investment of time, effort, commitment, and attention to detail.

DECLARATION

All investigations described in this thesis were designed, performed and interpreted by the author unless indicated otherwise.

LIST OF ABBREVIATIONS

A_n	Absorbance at a given wavelength (n).
BSS	Buffer salt solution.
CIE	Crossed immunoelectrophoresis.
CFU	Colony forming unit.
DEAE-cellulose	Diethylaminoethylcellulose.
DNA	Deoxyribonucleic acid.
EDTA	Ethylenediaminetetra-acetic acid.
ELISA	Enzyme-linked immunosorbent assay.
FRIE	Fused rocket immunoelectrophoresis.
HRP	Horseradish peroxidase.
LTA	Lipoteichoic acid.
MPRL	Microbial Pathogenicity Research Laboratory.
NCTC	National Collection of Type Cultures.
PAGE	Polyacrylamide gel electrophoresis.
PBS	Phosphate buffered saline.
RCUT	Rapid carbohydrate utilisation test.
RIE	Rocket immunoelectrophoresis.
RNA	Ribonucleic acid.
RTF	Reduced transport fluid.
SDS	Sodium dodecyl sulphate.
TBS	Tris buffered saline.
TCA	Trichloroacetic acid.
TCIE	Tandem crossed immunoelectrophoresis.
THB	Todd Hewitt Broth.
TTBS	Tween 20 tris buffered saline.
u-v	Ultra-violet.
VP	Voges Proskauer reaction.

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CHAPTER 1

GENERAL INTRODUCTION

1. The group F and related streptococci.

Group F streptococci were first described by Lancefield and Hare (1935) in their pivotal studies on the serological classification of haemolytic streptococci. Their chief characteristics were slow and difficult growth and the formation of minute transparent colonies which produced a wide zone of haemolysis. No special pathological significance was attributed to group F strains, since the two strains examined had not given rise to infection.

1.1. Morphology.

Group F streptococci are Gram-positive, facultatively aerobic or capnophilic cocci, which form pairs or chains. Diverse colony morphology and haemolytic behaviour may be seen amongst strains belonging to this group (Rantz, 1942a). Colonies may range from very small and domed to large, flat and grey, and are capable of displaying alpha, beta or no haemolysis on blood agar. A distinctive but variable feature is the production of a caramel-like odour in culture.

1.2. Taxonomy.

According to current European taxonomy schemes, all group F streptococci belong to the broader *Streptococcus milleri* group (Hardie, J., 1988: personal communication).

In the following section, a historical review of the often confusing taxonomy of the *Streptococcus milleri* group is presented, in an attempt to clarify current views.

Organisms belonging to *Streptococcus milleri* have provided problems in

classification which have arisen out of their diversity of behaviour when standard methods of streptococcal identification have been applied. Traditional methods used in streptococcal classification have included the determination of physiological characteristics (Gordon, 1905; Andrewes and Horder, 1906a-e), haemolytic activity on blood agar (Schotmüller, 1903; Brown, 1919), and serological grouping (Lancefield, 1933).

In their list of five pathogenic strains of streptococci defined by characteristic fermentation reactions, Andrewes and Horder (1906) described a virulent, haemolytic streptococcus isolated from cases of suppurative peritonitis, appendicitis, endocarditis, meningitis and angina (sore throat). A variant of *Streptococcus pyogenes*, this organism was named *Streptococcus anginosus* because of its association with angina, and is believed to be the earliest reference to an organism of the *Streptococcus milleri* group. The association of this early isolate with the present day *Streptococcus milleri* group has been supported despite questionable results when properties of the current *Streptococcus anginosus* type strain (beta-haemolytic, group G) were compared with the results of Andrewes and Horder (Hardie, 1986). None of the original isolates is now available for examination.

Long and Bliss (1934b), described the recovery of a group of 'minute haemolytic streptococci' from the human throat. Isolates were characterised by slow and difficult growth aerobically on blood agar, and displayed marked zones of haemolysis. Serological examination by Lancefield and Hare (1935) showed them to belong to the hitherto undescribed group F. Bliss (1937) later showed that 91% of her 'minute haemolytic streptococci' were

group F, and the remainder group G. Serological typing was readily achieved, and Bliss's serotype I was found in representatives of group F and group G. Serotype II was found among strains that carried the group F antigen.

Similar organisms were isolated from human faeces by Smith and Sherman (1938), who retained the title 'minute haemolytic streptococci' to describe only their group F isolates. *Streptococcus anginosus* was preferred for their group G isolates which, "agreed entirely with the original description of Andrewes and Horder".

Niven (1957) on the other hand included haemolytic streptococci of group F and G under the heading *Streptococcus anginosus*.

Streptococcus MG, a non-haemolytic member of the current *Streptococcus milleri* group, was recovered by Mirick *et al.* (1944a) from the respiratory tract of patients suffering from primary atypical pneumonia. Serological examination (Mirick *et al.* 1944b; c) led to its classification as a separate genus related only to *Streptococcus salivarius* type 1 (Sherman *et al.*, 1943). Later examination of this serological relationship by Willers *et al.* (1964b) revealed it to be due to a cross-reaction with typing sera. *Streptococcus MG* was found to be a group F, Ottens type III (see later) strain, and cross-reactivity was explained by the similarity of the 'salivarius' antigen to the type III antigen contained in the *Streptococcus MG* strain (Kothari *et al.*, 1971).

The title "*Streptococcus milleri*" first appeared in 1956. Guthof (1956) used this name to describe a physiologically distinct group of non-haemolytic organisms recovered from dental abscesses and other suppurative

lesions around the mouth. Lancefield grouping was applied unsuccessfully to these non-haemolytic streptococci, but in their study of similar organisms recovered from dental root canal cultures, Winkler and van Amerongen (1959) reported that 23 of their 40 isolates belonged to group F, G, C, D or E. Ten were group F.

Differentiation of group F streptococci on the basis of their haemolytic activity on blood agar was brought into question by Ottens (1961), who noted only minor differences between the biochemical activities of indifferent and haemolytic strains (indifferent strains tended to ferment lactose). Ottens and Winkler (1962), were also able to demonstrate identity between the group F antigens of indifferent and haemolytic strains.

In addition to the grouping antigen, Ottens and Winkler (1962) observed the presence of five independent carbohydrate typing antigens (I-V) in group F strains. The type I and II antigens were as described by Bliss (1937). Distribution of the Ottens type antigens outside group F was subsequently observed as follows:

i. type I antigen was found in haemolytic and indifferent group G strains (Ottens and Winkler, 1962);

ii. type II was found in group T (de Moor, 1959) and group A strains (Jablon *et al.*, 1965);

iii. type III antigen was found in indifferent group C (Ottens and Winkler 1962), and in group L strains (Willers *et al.*, 1964b).

Typing antigens were also present in strains which lacked a recognised grouping antigen (Ottens and Winkler, 1962; Willers and Alderkamp, 1967; Michel *et al.*, 1967).

The group F streptococci thus presented problems of classification resulting from variables in colony morphology, haemolytic activity, and a series of typing antigens shared with other serological groups.

It was clear that there was much to be learned about the systematic relationships of these streptococci. The problems of classification within the non-haemolytic streptococci were addressed in a series of studies by Colman and Williams in the 1960's and early 1970's. These studies included examination of cell-wall composition (Colman and Williams, 1965), computer-aided numerical taxonomic investigation (Colman, 1968), and transformation experiments (Colman, 1969). The results of these investigations, in which a collection of 364 streptococcal strains was subjected to a wide range of physiological and serological tests, and analysis of cell-wall composition were summarised by Colman and Williams in 1972. Computer-aided 'cluster analysis', of the results allowed the relative similarities of strains to be established.

Six species were recognised among the viridans streptococci of man, namely: *Streptococcus pneumoniae*, *Streptococcus salivarius*, *Streptococcus mitior*, *Streptococcus milleri*, *Streptococcus sanguis*, and *Streptococcus mutans*. Each species was serologically heterogeneous, but possessed a distinctive combination of characteristics.

Within the *Streptococcus milleri* cluster were included the following organisms:

i. *Streptococcus milleri* (Guthof, 1956);

ii. *Streptococcus MG* (Mirick *et al.*, 1944a);

iii. all group F strains;

iv. certain non-haemolytic streptococci of groups A, C, and G, (comparison

being made with a variant group A strain described by Michel and Gooder, 1962);

v. strains possessing a type antigen, but no Lancefield group antigen and,

vi. the 'minute haemolytic streptococci' (Long and Bliss, 1934a, b; Deibel and Niven, 1955)). The inclusion of these strains was tentative at that stage, but was later supported by the work of Mejàre and Edwardsson (1975), Poole and Wilson (1976), and Lütticken *et al.* (1978).

The strains brought together as *Streptococcus milleri* (Colman and Williams, 1972), with few exceptions fermented lactose, sucrose, trehalose and salicin, formed acetoin from glucose, hydrolysed arginine and aesculin, and were resistant to bacitracin and nitrofurazone. The cell-walls contained rhamnose, and usually glucose, galactose, and galactosamine. Few grew at 45°C, or in 4% NaCl broth, fermented raffinose or inulin, or hydrolysed starch. None formed extracellular polysaccharide from sucrose, survived 60°C for 30 minutes, or hydrolysed hippurate.

Considerable clarity had therefore been gained by the Colman and Williams scheme. Acceptance of this scheme was not, however, universal, particularly in the United States, where preference was given to other titles to describe members of the *Streptococcus milleri* group.

Deibel and Seeley (1974) preferred the title *Streptococcus anginosus* for beta-haemolytic group F streptococci, and for strains previously designated *Streptococcus MG.*

Streptococcus constellatus (Prevot, 1924; 1933; Douglas, 1957; Holdeman and Moore, 1974), and *Streptococcus intermedius* (Prevot, 1925; Smith, 1957; Holdeman *et al.*, 1977) referred to two closely related organisms isolated

in anaerobic culture (sometimes referred to as *Peptostreptococcus constellatus* or *intermedius*). The former was variably haemolytic and did not ferment lactose, whilst the latter was non-haemolytic and did ferment lactose (Holdeman and Moore, 1974).

In his study of the physiological differentiation of viridans streptococci, Facklam (1977) was able to show identity between *Streptococcus MG* and *Streptococcus intermedius* strains. Identity was also demonstrated between *Streptococcus constellatus* and *Streptococcus anginosus*. In addition, considerable physiological and serological similarity was noted between the two clusters. Facklam agreed with the Colman and Williams description of *Streptococcus milleri*, and accepted the close relationship between these strains, but believed it was justified to differentiate between them for epidemiological purposes. Non-beta-haemolytic members of the group were divided on the basis of lactose fermentation into:

i. *Streptococcus MG-intermedius* (lactose fermenter) and,

ii. *Streptococcus anginosus-constellatus* (non-lactose fermenter).

Many of these strains belonged to Lancefield group F.

Beta-haemolytic group F streptococci were termed *Streptococcus anginosus*.

Streptococcus milleri was also not included in the 1980 Approved List of Bacterial Names (Skerman *et al.*, 1980), in which the nomenclature of Facklam was adopted. However, despite its absence from the Approved List, the name *Streptococcus milleri* remained in popular use in both the clinical (Ruoff and Kunz, 1982), and scientific literature (Ball and Parker, 1979; Bridge and Sneath, 1983).

The major differences in the American and British streptococcal taxonomy schemes, with special reference to *Streptococcus milleri* were further discussed by Facklam in 1984. The identification of streptococci was said to be necessarily dichotomous, Lancefield grouping applying to and conveying useful information only in the identification of beta-haemolytic streptococci. Serological grouping was of little value in the identification of non-beta-haemolytic streptococci, which were correctly speciated by the determination of physiological characteristics.
Table 1.1. summarises the proposed nomenclature for streptococci of the *milleri* group under the Facklam classification (Facklam, 1984). All of these organisms may be considered as *Streptococcus milleri* under the Colman and Williams (1972) scheme.

A single, universally accepted taxonomy scheme has therefore not emerged for the *Streptococcus milleri* group on the basis of traditional diagnostic measures. Lack of certainty regarding the degree of relatedness amongst members of the *milleri* group has consequently prompted a search for alternative, or additional parameters on which to assess the homogeneity of this group.

1.3. Alternative techniques applied to the classification of *milleri* group streptococci.

1.3.1. Fatty-acid composition. The whole-cell fatty-acid fingerprints of a number of streptococcal strains were investigated by Drucker and Lee (1981). Gas chromatographic analysis of the cellular fatty-acid profiles of *Streptococcus milleri* "shed light on the disparate nature of strains

Table 1.1: Summary of the proposed nomenclature for streptococci of the *Streptococcus milleri* group under the Facklam classification (Facklam, 1984).

DESCRIPTION	TITLE
1. Beta-haemolytic strains.	
Beta-haemolytic group F streptococci	= *Streptococcus anginosus* group F.
Minute beta-haemolytic group A	= *Streptococcus anginosus* group A.
Minute beta-haemolytic group C	= *Streptococcus anginosus* group C.
Minute beta-haemolytic group G	= *Streptococcus anginosus* group G.
Ungroupable beta-haemolytic	= *Streptococcus anginosus* no group.
2. Non-beta-haemolytic strains. (serological group not applicable)	
Streptococcus MG-intermedius	= *Streptococcus intermedius.*
Streptococcus anginosus-constellatus	= *Streptococcus constellatus.*

assigned to this species". Three separate groups were demonstrated:

i. strains of oral origin capable of inducing caries in gnotobiotes;

ii. strains of vaginal origin;

iii.'medical' strains from abscesses and wounds in humans.

However, in a similar study by Labbé *et al.* (1985), *Streptococcus milleri* strains representing all of Facklam's (1984) subdivisions were said to form a homogeneous group according to fatty acid composition. The small standard deviations observed suggested much homogeneity within the group, which was easily differentiated from other groups.

1.3.2. Pyrolysis gas chromatography. Gas chromatographic analysis of whole-cell pyrolysates was found by French *et al.* (1982; 1989b) to be of limited value for the identification of oral streptococci. Identification of *Streptococcus mutans* and *Streptococcus salivarius* was rapidly and accurately achieved, but differentiation between *Streptococcus milleri* and *Streptococcus sanguis* was not possible.

1.3.3. Cell-wall studies. Cummins and Harris (1956), and Colman and Williams (1965) investigated the use of cell-wall composition as an indicator of taxonomical status. Colman and Williams, as seen earlier, successfully used this technique as part of a numerical taxonomy scheme for the classification of streptococci. More recently, the whole-cell trimethylsilyl-sugar profiles of certain 'viridans' streptococci examined by gas chromatography were found to provide taxonomically useful information (Aluyi and Drucker, 1983). A good deal of uniformity in trimethylsilyl-sugar profile was noted within a collection of strains belonging to *Streptococcus milleri*.

More detailed consideration of the cell-wall composition of *Streptococcus milleri* with special reference to group F strains will be given later.

1.3.4. Sodium dodecyl sulphate polyacrylamide-gel-electrophoresis (SDS-PAGE).

Whole-cell protein composition was investigated as a possible chemotaxonomic marker for the oral streptococci by Whiley *et al.* (1982). The SDS-solubilised proteins of mechanically-disrupted cells were examined by SDS-PAGE, revealing characteristic profiles for accepted species of oral streptococci, including *Streptococcus milleri*.

Moore *et al.* (1982a) were also able to demonstrate close similarity between the soluble protein profiles of *Streptococcus anginosus* and *Streptococcus intermedius* strains in PAGE. Based on these, and on phenotypic reactions, Moore *et al.* (1982a) concluded that *Streptococcus anginosus* and *Streptococcus intermedius* were the same species.

1.3.5. DNA-DNA homology and base ratios. All of the methods described in the previous section are means of observing phenotypical qualities of organisms, which may be subject to modulation by agencies external to the bacterium. They are expressions of aspects of the bacterial genome. A more definitive method of assessing the relatedness of bacterial strains would therefore be to directly compare their genetic "blueprints". A commonly used method determines the cytosine-plus-guanine content of DNA. The value obtained indicates the quantities of these bases, rather than the precise sequence of nucleotides in the test DNA. Strains with similar C + G content may consequently be phenotypically different, and this technique, although of value, is not a definitive indicator of genetic relatedness. More

accurate assessment of genetic relatedness comes from DNA-DNA homology. Using this technique, the degree of similarity between one section of DNA and another is expressed as the percentage binding under given conditions and is highly dependent not only on the ratio of nucleotides present, but on their precise sequence.

Both of these powerful techniques have been applied in recent years to organisms of the *Streptococcus milleri* group.

Examination of the C + G content of *Streptococcus milleri* strains by Drucker and Lee, (1983) revealed that some degree of genetic heterogeneity may exist among members of this group.

However, DNA-DNA hybridisation studies by Welborn *et al.* (1983) indicated that certain strains of *Streptococcus intermedius*, *Streptococcus constellatus*, *Streptococcus mitis*, *Streptococcus MG-intermedius*, and group F strains were closely related to each other genetically. Relative binding ratios, the majority being over 85%, suggested that these strains should all be considered members of the same species, although physiological tests suggested that this was a relatively heterogeneous group of organisms. The division of strains on the basis of their ability to produce acid from lactose (Facklam, 1977) was considered to be unjustified on the basis of these results. Hybridisation results also indicated that the minute beta-haemolytic group F streptococci should be included in the *Streptococcus milleri* group, in agreement with Colman and Williams (1972). However, further hybridisation work was required to establish the position of minute beta-haemolytic group G strains.

Similar findings were reported by Farrow and Collins (1984) who demonstrated a close relationship between *Streptococcus anginosus* and *Streptococcus intermedius*, *Streptococcus constellatus*, and "*Streptococcus*

milleri". Hybridisations were performed under optimal conditions. Ezaki *et al.* (1986) also showed strong genetic relationships between the minute haemolytic streptococci and *Streptococcus anginosus*.

Using more stringent hybridisation conditions, Kilpper-Bälz *et al.* (1984) were able to demonstrate clear separation of the type strains of *Streptococcus constellatus*, and *Streptococcus anginosus*. *Streptococcus intermedius* was also shown to be clearly distinct although it was more closely related to *Streptococcus constellatus* than to *Streptococcus anginosus*. Two distinct groups were demonstrated within eight strains of "*Streptococcus milleri*" tested, one showing high homology with *Streptococcus constellatus*, and one with *Streptococcus anginosus*, suggesting their correct taxonomical designation to one of these groups. However, no reliable phenotypical markers were available to reflect the DNA homology findings.

In an extensive study by Coykendall *et al.* (1987), embracing "*Streptococcus milleri*" isolates of all biotypes, haemolytic types, and serotypes, in addition to the type strains *Streptococcus anginosus*, *constellatus*, *intermedius* and group F streptococcus, considerable genetic similarity was noted within the group. It was proposed on the basis of these results that this phenotypically diverse group of organisms should be unified under a single species name. Since, in his opinion *Streptococcus constellatus* and *Streptococcus milleri* were later synonyms of *Streptococcus anginosus*, and *Streptococcus intermedius* (Moore *et al.*, 1982b) was also a later synonym of *Streptococcus anginosus*, Coykendall recommended the adoption of *Streptococcus anginosus* as the unifying title.

The taxonomical relationships within this group of streptococci remain uncertain. More recently, Knight and Shlaes (1988), using highly stringent

hybridisation conditions found evidence of at least three genetically and phenotypically distinguishable groups within eighteen representatives of the *Streptococcus intermedius* taxon. No formal proposals were made on the basis of their limited data.

To date, it would seem that even the powerful techniques of DNA-DNA homology have been unable to reach unequivocal, definitive answers on the precise taxonomical position of a collection of organisms clustered within the *Streptococcus milleri* group by Colman and Williams (1972).

1.3.6. Final comments on the current status of *Streptococcus milleri* taxonomy, with a note on the nomenclature to be used in this thesis.

The protean character of *Streptococcus milleri* in culture may cause confusion, and difficulties of identification are compounded by persistent international differences in taxonomy. Despite ongoing academic discussions on the details of streptococcal taxonomy, organisms of clinical significance must have names which convey a clear meaning to those involved in the management of infected patients. *Streptococcus milleri* has become a meaningful title because of its association with deep-seated purulent lesions (See section 1.5.). Until clear evidence emerges to justify the use of more than one title to describe this group of related organisms, *Streptococcus milleri* will continue to be used by European taxonomists to describe a cluster of organisms listed in the 1986 edition of Bergey's Manual (Hardie, 1986) thus:

Streptococcus anginosus (Andrewes and Horder, 1906);

Streptococcus MG (Mirick *et al.*, 1944a;b;c);

Streptococcus constellatus (Prevot, 1924; Holdeman and Moore, 1974);

Streptococcus intermedius (Prevot, 1925; Holdeman *et al.*, 1977);

"*Streptococcus milleri*" (Guthof, 1956);

Minute beta-haemolytic streptococci of groups F and G (Long and Bliss, 1934b; Bliss, 1937).

Common worldwide usage of the name *Streptococcus milleri*, it was noted, would make it likely that admission to the Approved List would be sought in the near future.

Throughout this thesis, the European system of classification will be adhered to. Streptococci of Lancefield's group F will generally be referred to as *Streptococcus milleri* regardless of haemolytic activity or behaviour in biochemical tests. It should be noted, however, that in reviewing the published work of other researchers, the titles used in the original communication to describe members of the *Streptococcus milleri* group will be used.

1.4. Isolation and identification of *Streptococcus milleri* in the clinical laboratory.

1.4.1. Cultural requirements. Early isolates of group F streptococci were described as "minute" (Long and Bliss, 1934a), reflecting both their microscopic appearance (1/2-2/3 that of 'ordinary' beta-haemolytic streptococci), and their appearance on routine aerobic culture. Other strains have been considered as being anaerobic because of their improved recovery under anaerobic conditions. As with many other microorganisms, it was later found that growth could be enhanced by the addition of 5-10% CO_2 to the culture atmosphere (Rose, 1942; Niven *et al.*, 1946; Lui, 1954; Deibel and Niven, 1955; Sisson *et al.*, 1978). Facilities are now available in many service laboratories for the routine culture of clinical specimens under an increased CO_2 tension, which probably accounts for some of the

increased recognition of *Streptococcus milleri* from clinical specimens in recent years.

1.4.2. Selective media. No selective media have been formulated specifically for the recovery of group F streptococci, or for the *Streptococcus milleri* group. A selective medium which may be useful is the sulphonamide-containing "MC" agar of Carlsson (Carlsson, 1967a; Mejàre and Edwardsson, 1975; Yakushiji *et al.*, 1988a), originally designed for the recovery of *Streptococcus mutans* from the oral cavity.

1.4.3. Identification of *Streptococcus milleri* in the clinical laboratory.
Central to the identification of streptococci in the clinical laboratory is the colonial appearance on blood agar and the appearance in Gram-stained films. Haemolytic streptococci are usually serologically grouped, often using one of the commercially available latex-agglutination systems, which typically contain group A, B, C, D, F, and G specific latex-reagents.
Physiological and biochemical tests are necessary for the identification of the viridans streptococci and the enterococci.
Many studies on the physiological properties and biochemical activities of viridans streptococci in relation to taxonomy have used extensive series of tests in order to characterise strains as fully as possible (Carlsson, 1967b; 1968; Colman and Williams, 1972; Parker and Ball, 1976; Lütticken *et al.*, 1978; Facklam, 1977; Hardie *et al.*, 1982). The use of such exhaustive batteries of tests on a daily basis by service laboratories is impractical, and shortened schemes of identification have consequently evolved for the presumptive identification of streptococcal isolates (Hardie and Bowden, 1976; Waitkins *et al.*, 1980; Ruoff and Ferraro, 1986;

Manning and Hogg, 1987).

The advent of commercially-available, ready-made galleries of biochemical tests for the identification of streptococci has led to their widespread use in clinical diagnostic laboratories. The API-20 Strep system has been favourably reviewed for the identification of *Streptococcus milleri* isolates (Tillotson, 1982; Brown, 1982; Colman and Ball, 1984; French *et al.*, 1989a), accommodating the different sugar-fermentation patterns known to exist within this group (Colman and Ball, 1984).

Further consideration will be given to the physiological and serological identification of *Streptococcus milleri* isolates in chapter 3.

1.5. The clinical significance of *Streptococcus milleri.*

1.5.1. *Streptococcus milleri* as a commensal in man.

In common with other species of viridans streptococci, organisms of the *Streptococcus milleri* group have been considered as members of the complex resident microflora of a number of mucosal sites, and as pathogens of note only in infective endocarditis. Before discussing the current status of *Streptococcus milleri* as a pathogen in man, the literature will be reviewed concerning the sites in which this organism may be considered to be part of the resident flora.

1.5.2. The oral cavity.

Streptococcus sp. MG was regarded as being an inhabitant of the normal human oral cavity by Swift (1952), and by Kraus *et al.* (1953). *Streptococcus milleri* has been recovered from the healthy mouths of both children (Bowden *et al.* 1973, 1975; Edwardsson and Mejàre, 1978), and

adults (Mejàre and Edwardsson, 1975; Poole and Wilson, 1979; Theilade *et al.*, 1982; Crawford and Russel, 1983).

The pattern of distribution of *Streptococcus milleri* within the mouth was discussed by Mejàre and Edwardsson in 1975. Recovery of *Streptococcus milleri*, as a proportion of the total streptococcal flora, ranged from 14 to 56% in the gingival crevice, and from 4 to 25% in supragingival plaque. In swabbings from the cheek, the dorsum of the tongue and in saliva, recovery was less than 1%. The predilection of this organism for hard surfaces was further indicated by an increase in number relative to other streptococci as the number of deciduous teeth in the mouths of children increased (Edwardsson and Mejàre, 1978). *Streptococcus milleri* was also the commonest species recovered from the palatal surface of ten dentures associated with a healthy underlying oral mucosa (Theilade *et al.*, 1973), accounting for 11% of all isolates, and 26% of streptococcal isolates.

The regular isolation of *Streptococcus milleri* from the human oral cavity in health reinforces its position as a component of the normal oral flora of adults and dentate children. *Streptococcus milleri* appears to have a special association with dental plaque deposits found in relatively sheltered areas, notably the gingival crevice, and the fitting surface of dentures.

1.5.3. The upper respiratory tract.

The original descriptions of 'minute haemolytic streptococci' (Long and Bliss 1934a; b), group F streptococci (Lancefield and Hare, 1935; Hare, 1935), and *Streptococcus MG* (Mirick *et al.* 1944a), included isolates from the upper respiratory tract which were not associated with local inflammatory disease.

Organisms of the *Streptococcus milleri* group have been recovered in the absence of disease from the mixed microbial flora of the upper respiratory tract of healthy adults (Poole and Wilson, 1979; McBride *et al.*, 1980). Colonisation of children on the other hand appears to be slow (Poole and Wilson, 1979).

Alpha-haemolytic and microaerophilic streptococci were found to be components of the flora of normal maxillary sinuses by Brook (1981), and it is speculated that *Streptococcus milleri* may also contribute to the resident flora of this site.

1.5.4 The gastrointestinal tract.

A number of surveys of the complex faecal flora in health have included mention of members of the *Streptococcus milleri* group (Hare, 1935; Smith and Sherman, 1938; Attebery *et al.*, 1972; Finegold *et al.*, 1974; Holdeman *et al.*, 1976; Murray *et al.*, 1978).

Streptococcus milleri was recovered from 25.9% of uninflamed appendices obtained at necropsy, and 24.2% of histologically normal appendices removed at operation (Poole an Wilson, 1977). It was additionally found in 15% of normal faeces from humans of all ages. Significantly more isolates were made from the faeces of adults than children (Poole and Wilson, 1979; Wanatabe *et al.*, 1980).

In quantitative terms, *Streptococcus milleri* has been demonstrated in moderate numbers (10^3-10^6 per gram faeces) in the stools of healthy adults (Unsworth, 1980), suggesting that the gut is an important reservoir of this organism.

1.5.5. The urogenital system.

Group F streptococci have been isolated from the vagina and from urine samples, possibly as vaginal or perineal contaminants (Lancefield and Hare, 1935; Rantz, 1942a; Duma *et al.*, 1969; Wort, 1975; Bannatyne and Randall, 1977).

Reports of *Streptococcus milleri* in these sites (including further group F isolates) are equally numerous (Sisson *et al.*, 1978; Ball and Parker, 1979). Poole and Wilson (1979) recovered *Streptococcus milleri* from 18.2% of vaginal swabs, and Ruoff *et al.* (1983) found it to be a frequent isolate from both male and female urinary tracts. A pathogenic role was considered unlikely. Recently, Rabe *et al.* (1988), found *Streptococcus intermedius* to be the most frequently isolated species of viridans streptococcus from the healthy vagina.

1.5.6. *Streptococcus milleri* as a pathogen.

Virtually all infections due to viridans streptococci arise endogenously. It has been established in the preceeding section that *Streptococcus milleri* is frequently encountered, and often in high numbers, as a member of the complex bacterial flora of a number of mucosal surfaces in healthy human beings. Breakdown of normal skin or mucosal barriers allows normally commensal microorganisms access to deeper tissues. Provided that conditions are favourable to the invading organisms, this process may result in infection. Traditionally, the aerobic streptococci of the mouth have been regarded as a rather homogeneous group of low-grade pathogens. Today this belief is no longer valid (Brennan and Durack, 1984). *Streptococcus milleri* is now widely recognised as an important cause of pyogenic

infection, though it eluded suspicion for many years; its variable appearance and properties in culture were deceptive and attracted little attention until greater interest in the identification of "viridans" and other commensal streptococci established the organism as a distinct bacterial species. Recognition of *Streptococcus milleri* in the clinical laboratory may not always be easy, and less distinctive strains may be inconspicuous in mixed cultures (Lancet, 1985). Because nearly all the viridans streptococci are susceptible to penicillin, differentiating these strains into species has not been a pressing matter for the clinical microbiologist. Consequently, much of the literature concerning infection due to this group of organisms refers to "viridans" streptococci, or "*Streptococcus viridans*". In the following review of the occurrence of *Streptococcus milleri* in human infection, no attempt is made to change the title "*Streptococcus viridans*" into modern equivalents.

1.5.7. The mouth and perioral tissues.

1.5.7.a. Dental caries. *Streptococcus milleri*, unlike *Streptococcus mutans*, is not recognised as an agent of primary aetiological importance in dental caries. High levels of *Streptococcus milleri* were encountered in deep areas of carious dentine by Edwardsson (1974), but the isolates formed part of a mixed flora within which it was impossible to attribute specific roles.

In a longitudinal epidemiological study of dental plaque and the development of caries, Hardie *et al.* (1977) recovered *Streptococcus milleri* from 45% of approximal plaque samples from sites in which caries had not developed over a 2 year period.

Animal experiments have indicated varying degrees of cariogenicity for

organisms of the *Streptococcus milleri* group (Rosen and Kolstad, 1977; Drucker *and Green*, 1978; Yoshizaki, 1983; Horton *et al.*, 1985; Hosoi, 1985). All but one of these studies (Rosen and Kolstad (1977), who showed *Peptostreptococcus intermedius* to be more cariogenic than *Streptococcus mutans* 6715), demonstrated the capacity of *Streptococcus milleri* to produce caries in experimental animals, though less severely than *Streptococcus mutans* strains.

The involvement of *Streptococcus milleri* as a primary agent in the initiation and progression of dental caries is insubstantially documented, and would seem unlikely. Recent *in vitro* studies have shown that *Streptococcus intermedius* cannot reduce the pH at a tooth surface to the same extent as *Streptococcus mutans* (Bessho, 1985).

1.5.7.b. Periodontal disease. There are great difficulties associated with obtaining representative samples for the bacteriological investigation of diseased periodontal sites. Samples typically contain a plethora of bacterial species involved in complex interactions, both with each other and with the host, through whom much of the destruction seen in periodontal disease is wrought. Assignation of specific periodontopathogenicity in these circumstances is very difficult since the mere recovery of an organism from a diseased site does not necessarily implicate it in a primary pathogenic role.

Organisms of the *Streptococcus milleri* group have been recovered frequently, and in high numbers in association with periodontal lesions, including experimental gingivitis (Moore *et al.*, 1982a), early periodontitis (Darwish *et al.*, 1978), severe periodontitis (Moore *et al.*, 1982b), and severe generalised periodontitis (Moore *et al.*, 1983).

Recognising the association of *Streptococcus anginosus* with pyogenic infections in other body sites, it was speculated (Moore *et al.*, 1982b) that this organism was a potential agent in a model of episodic, destructive activity in periodontal disease caused by periods of aggressive pyogenic activity.

Crawford and Russell (1983), on the other hand, found no significant differences in the numbers of viridans streptococci recovered from sites described as "healthy", "gingivitis", "early or moderate periodontitis", or "severe periodontitis". *Streptococcus milleri* was identified in low numbers, and no pathological significance could be attributed to it.

It was observed by Haffajee *et al.* (1985) that the proportions of *Streptococcus intermedius* were elevated in both active and inactive sites in groups of patients who responded poorly to periodontal therapy, compared to a similar group who responded well. Such an observation, if confirmed, could be useful as a bacteriological prognostic indicator prior to the commencement of periodontal therapy.

Although associations are rarely clear-cut, it is possible that in certain circumstances, members of the *Streptococcus milleri* group, within a mixed flora, could contribute to the pathogenic processes of periodontal disease.

1.5.7.c. Dental root canals. Indifferent and haemolytic streptococci, many belonging to groups F, C, or G (Winkler and van Amerongen, 1959; Ottens and Winkler, 1962), and *Streptococcus milleri* (Mejàre, 1975) were found to be particularly numerous in 'infected' root canals. Winkler and van Amerongen (1959), described a higher recovery rate of indifferent streptococci from teeth with necrotic pulps than from those which had been vital. Haemolytic, indifferent, and indifferent anaerobic streptococci of groups

F, C, or G were thought to be the most likely pathogens in this site.

The culture of dental root canals is no longer considered a useful exercise in routine endodontic therapy. Failure to get any growth does not mean that the canal is sterile, nor does the growth of some microorganisms necessarily mean that the preparation has failed (Cawson, 1978). Just as *Streptococcus milleri* is a well recognised inhabitant of the gingival crevice, so it may be found in the protected environment of the open dental root canal. *Streptococcus milleri* from the gingival crevice, or as part of the mixed flora of an advancing carious lesion (Edwardsson, 1974) may gain access to the dental pulp, and become involved in pulpal and periapical pathology by virtue if its tolerance to a reduced redox potential.

1.5.7.d. Odontogenic abscesses. The normal flora surrounding the teeth, particularly those present in the densely populated gingival crevice, are the organisms usually isolated in soft-tissue infections of dental origin (Busch, 1984).

The association of *Streptococcus milleri* with purulent lesions of odontogenic origin was described by Guthof (1956). More recently, reports of *Streptococcus milleri* group isolates from dental abscesses, often in pure culture have confirmed a high recovery rate from such lesions (Wort, 1975; Bannatyne and Randall, 1977; Sisson *et al.*, 1978; Lütticken *et al.*, 1978; Poole and Wilson, 1979; Shlaes *et al.*, 1981; Libertin *et al.*, 1985; Kambal, 1987).

Recent improvements in anaerobe bacteriology have increasingly implicated organisms such as *Bacteroides* spp. as major pathogens in dental abscesses. *Streptococcus milleri* group organisms isolated anaerobically have been encountered within a mixed anaerobic flora (Sabiston, *et al.* 1976; Bartlett

and O'Keefe, 1979; Aderhold *et al.*, 1981; Brook *et al.*, 1981; Labriola *et al.*, 1983). They have also been shown to be the commonest facultative organisms isolated from dental abscesses (von Konow *et al.*, 1981; 1983). Their importance in abscess formation remains, however, unclear.

"With the viridans streptococci we are confronted with the enigma that the organisms most commonly isolated from purulent oral infections are non-pathogenic." (Sims, 1974).

The role of facultative streptococci in abscess formation was discussed by Aderhold *et al.* (1981), who suggested their involvement in a process of autogenic succession, in which facultative organisms reduce the Eh of a lesion prior to colonisation by anaerobes. This position was echoed by Heimdahl *et al.* (1985), and Lewis *et al.* (1986), who isolated *Streptococcus milleri* in pure culture from two patients presenting on the first day of clinical symptoms. Aspirates from dental abscesses symptomatic for 2-3 days, on the other hand, tended to yield a predominantly anaerobic flora. It has recently been shown (Lewis *et al.*, 1988) that *Streptococcus milleri* is capable of producing infection following subcutaneous injection into mice, and that severe local infection can be produced in combination with *Bacteriodes intermedius.* Such synergistic bacterial interactions with anaerobic Gram-negative bacilli are not limited to *Streptococcus milleri*, and are likely to be operational in purulent lesions containing a mixed flora.

1.5.8. Paranasal sinusitis. The close proximity of the root apices of the maxillary dentition to the floor of the maxillary antrum makes possible the direct spread of purulent material from maxillary periapical lesions to the sinus. The bacterial flora of the infected sinus in these circumstances is

likely to reflect that of the discharging dental lesion. *Streptococcus milleri* may be involved in a process of autogenic succession in sinusitis of non-dental origin, similar to its suggested role in dental abscesses. Closure of the sinus ostium by mucosal hyperaemia leads to a fall in oxygen tension within the sinus, which initially favours *Streptococcus pneumoniae* and *Haemophilus influenzae*. A continued fall in oxygen tension, coupled with a rise in CO_2 tension, encourages the growth of microaerophiles such as *Streptococcus milleri*, and ultimately strict anaerobes. Chronic sinusitis is commonly associated with anaerobic and microaerophilic streptococci (Brook *et al.*, 1980). Organisms of the *Streptococcus milleri* group have been recovered from cases of sinusitis, sometimes in pure culture, and from complicating lesions such as subdural empyema and periorbital cellulitis (Evans *et al.*, 1975; Wort, 1975; Bannatyne and Randall, 1977; Brook *et al.*, 1980; Blayney *et al.*, 1984; Van der Auwera, 1985). Other recognised complications include meningitis and intracranial abscess.

1.5.9. Intracranial and spinal infection. Appendix 1 documents 21 cases or series of cases of *Streptococcus milleri* group organisms in infections of the central nervous system and meninges.

Streptococci form the largest group of organisms isolated from intracranial pus, and of these *Streptococcus milleri* is the commonest (de Louvois, 1980). In one major series of intracranial abscesses (de Louvois *et al.*, 1977) all 20 of the *Streptococcus milleri* isolates encountered belonged to Lancefield group F, and possessed the Ottens type III antigen. Animal experiments (de Louvois *et al.*, 1974) have additionally shown that *Streptococcus milleri* has a well defined affinity for the central nervous

system of young mice.

Streptococcus milleri has been isolated from purulent lesions in a range of intracranial sites, but abscess of the frontal lobe secondary to sinusitis is most frequently observed (de Louvois *et al.*, 1977). Intracranial sepsis due to *Streptococcus milleri* may also arise following dental infection (Ingham *et al.*, 1978), traumatic injury (Puthucheary and Rapport, 1982), or metastatic spread from a distant purulent focus (Melo and Raff, 1978).

1.5.10. Infections of the cardiovascular system.

1.5.10.a. Bacteraemia. Transient bacteraemia is probably common and unsuspected in most cases. Viridans streptococci may enter the circulation following vigorous toothbrushing, and occasionally intestinal bacteria enter the portal circulation. More significant bacteraemia may follow trauma, surgical manipulation, or the development of a neoplastic or focal purulent lesion in a range of body sites.

Bacteraemia due to *Streptococcus milleri*, often following dental extraction, has been frequently documented (Gaudreau *et al.*, 1981; Crawford and Russel, 1983; Phillips *et al.*, 1976; Shanson *et al.*, 1978; Barnham, 1989; Nouri *et al.*, 1989). Bacteraemic infections due solely to *Streptococcus milleri* are so infrequent that the isolation of *Streptococcus milleri* from the blood of a febrile patient should always stimulate a search for pus in an internal organ (Parker and Ball, 1978; Moore-Gillon *et al.*, 1981; Libertin *et al.*, 1985). Recently, Minault *et al.* (1988) described a case of septic shock due to *Streptococcus milleri* following endoscopic sclerosis of oesophageal varices.

1.5.10.b. Infective endocarditis. *Streptococcus milleri* is not a common cause of endocarditis (Parker and Ball, 1976), even in elderly males, accounting for only 4-15% of alpha-haemolytic streptococcal endocarditis (Parker and Ball, 1976; Facklam, 1977; Roberts *et al.*, 1979). It has been suggested (Lancet, 1974), that dextran production may aid the establishment of streptococci on heart valves, by virtue of its stickiness. The inability of *Streptococcus milleri* to produce such extracellular polysaccharides was suggested by Parker and Ball (1976) as a possible reason for the low incidence of endocarditis due to this organism. Gossling (1988) suggested that the low incidence of *Streptococcus milleri* endocarditis simply reflected the relative numbers of this organism in dental plaque. This position assumes, however, that all endocarditis is due to organisms of oral origin, and fails to consider the gut and purulent foci as additional sources of *Streptococcus milleri* bacteraemia and endocarditis.

Although *Streptococcus milleri* is an infrequent cause of endocarditis, it was found by Murray *et al.* (1978) to be associated with an unusually high frequency of metastatic suppurative complications. Evidence for local cardiac tissue destruction could not however be found, despite the nature of extracardiac complications. Isolated reports have documented cases of myocardial abscess associated with endocarditis (Levandowski, 1985; Wallis *et al.*, 1986), or as a metastatic lesion secondary to a primary purulent focus (Gopalakrishna *et al.*, 1977), mitral valve aneurysm secondary to endocarditis (Akinjide-Obonyo *et al.*, 1983), and mycotic aneurysm of the aorta (Fox, 1980) due to *Streptococcus milleri*.

Although it is widely held that *Streptococcus milleri* endocarditis carries a high risk of purulent complications, Sussman *et al.* (1986) were firmly of the opinion that inaccuracies in speciation of viridans streptococci

rendered it meaningless, and that no well characterised species was associated with an outcome more serious than any other.

Streptococcus intermedius has been shown to be capable of producing infective endocarditis in experimental rats and rabbits whose heart-valves have been damaged (Glauser and Francioli, 1982; Héraïef *et al.*, 1982; Yersin *et al.*, 1982).

1.5.11. Infection of the respiratory tract. The possible role of *Streptococcus milleri* in paranasal sinusitis has been discussed (section 1.5.8.).

1.5.11.a. Pharyngitis. *Streptococcus anginosus* (Andrewes and Horder, 1906a-e), and group F streptococci (Lancefield and Hare, 1935; Duma *et al.*, 1969) have been recovered from infected throats.

More recent surveys have demonstrated extremely low (0.04-1%) recovery rates of beta-haemolytic group F streptococci in cases of acute pharyngitis (Wort, 1975; Schwartz *et al.*, 1986; Cimolai, *et al.*, 1988), although other reports have indicated higher pharyngeal recovery rates for *Streptococcus milleri* (Bannatyne and Randall, 1977; Ruoff *et al.*, 1985). Association with pharyngitis however, was not always clear.

Poole and Wilson (1976) showed a trend towards heavier growths of *Streptococcus milleri* from the throats of patients with symptoms than from asymptomatic carriers. Of 25 patients complaining of sore throat or tonsillitis, 21 (84%) yielded the organism in heavy growth compared with 5 (33%) of 16 symptomless carriers.

Streptococcus milleri is not generally regarded as an important pathogen in the throat, and recent studies have indicated the importance of differentiating between *Streptococcus milleri* isolates and their "large-

colony-forming" counterparts in throat cultures (Bucher and von Graevenitz, 1984; Ruoff *et al.*, 1985). *Streptococcus milleri* is not associated with the serious "post streptococcal" complications seen in group A streptococcal infection.

1.5.11.b. Pleuropulmonary disease. In recent years, *Streptococcus milleri* has become recognised as a cause of purulent pleuropulmonary disease, notably pleural empyema (Foley, 1947; Parker and Ball, 1976; Plump and Haponik, 1983; Frankish and Kolbe, 1984; Hocken and Dussek,1985; Ferber *et al.*, 1987). The mouth and upper respiratory tract have frequently been regarded as the source of *Streptococcus milleri*, and infections preceded by aspiration (Shlaes *et al.*, 1981; Plump and Haponik, 1983; Waitkins *et al.*, 1985). Cases have also followed pneumonia (Hocken and Dussek, 1985), and hepatic abscess (Koshi and John, 1971). The recovery of *Streptococcus milleri* in pure culture from pulmonary abscesses and empyema is not uncommon, particularly when sepsis is confined to the pulmonary cavity, whilst the presence of gastrointestinal fistulae often gives rise to a mixed flora (Waitkins *et al.*, 1985).

A preponderance in the distribution of empyemas caused by *Streptococcus milleri* in males was noted by Shlaes *et al.* (1981) and Hocken and Dussek (1985).

Streptococcus milleri has also been isolated in cases of purulent pericarditis (Braunstein *et al.*, 1971; Shlaes *et al.*, 1981; Reder, *et al.*, 1984; Akashi *et al.*, 1988).

1.5.12. Abdominal infection.

1.5.12.a. Surgical sepsis. The presence of *Streptococcus milleri* within the normal flora of the gut, including the appendix was discussed earlier. In addition to its commensal role, *Streptococcus milleri* has been implicated in abdominal sepsis, including acute appendicitis, peritonitis, pelvic abscess, and purulent wound discharge (Rogers, 1957; Wort, 1975; Poole and Wilson, 1976; Bannatyne and Randall, 1977; Sisson *et al.*, 1978; Murray *et al.*, 1978; Parker and Ball, 1978; Tresadern *et al.*, 1983; Madden and Hart, 1985; Admon *et al.*, 1986; 1987; Kambal, 1987).

Poole and Wilson (1977) recognised a highly significant increase in the isolation rate in the presence of inflammation, and a pronounced correlation between the presence of *Streptococcus milleri* in the appendix and the purulent manifestations of appendicitis. Swabs from other abdominal sites were received five times more often from patients harbouring *Streptococcus milleri* in the appendix than from patients who were not, and three-quarters of these swabs yielded *Streptococcus milleri*.

It was suggested by Tresadern *et al.* (1983) that the prophylactic use of antibiotic combinations such as gentamicin and metronidazole (active against coliforms and *Bacteroides*) in patients undergoing colorectal surgery may be important in promoting the emergence of *Streptococcus milleri* as a significant pathogen. Tresadern *et al.* (1983), and Madden and Hart (1985) proposed that *Streptococcus milleri* be considered in the formulation of prophylactic and therapeutic antibiotic regimens for use with patients undergoing abdominal surgery.

1.5.12.b. Hepatic abscess. Anaerobic and microaerophilic streptococci are the commonest isolates from pyogenic liver abscesses (Finegold, 1977).
Cases of hepatic abscess due to *Streptococcus milleri*, often carrying the group F antigen, and often in pure culture have appeared frequently in the literature of this rare condition (Patterson *et al.*, 1967; Bateman *et al.*, 1975; Reid and Davidson, 1976; Parker and Ball, 1976; Sisson *et al.*, 1978; Murray *et al.*, 1978; Shlaes *et al.*, 1981; Brasitus *et al.*, 1983; Gleeson *et al.*, 1983; Hatoff, 1983; Allison *et al.*, 1984; Chua *et al.*, 1989).
Sixteen cases of pyogenic liver abscess encountered over a ten year period were described by Moore-Gillon *et al.* (1981). In 10 instances, *Streptococcus milleri* was isolated in pure culture, and in mixed culture in a further 3. Of the 13 *Streptococcus milleri* isolates, 12 belonged to Lancefield group F. Hatoff (1983) also recognised *Streptococcus milleri* as the most frequent isolate from liver abscesses, and as in surgical sepsis (Tresadern *et al.*, 1983), suggested overgrowth of this organism in metronidazole therapy as a possible factor in the development of liver abscesses.
In the majority of cases the source of *Streptococcus milleri* was probably abdominal via the portal circulation, though a dental origin was suggested in one case (Reid and Davidson, 1976).

1.5.13. Other infections.

1.5.13.a. Neonatal infection. *Streptococcus milleri* has infrequently been associated with intrauterine pneumonia (Wells and Keeney, 1980; MacGowan and Terry, 1987), fulminant sepsis (Spencer *et al.*, 1982; Cox *et al.*, 1987), and septicaemia of the newborn (Nyhan and Fousek, 1958). In most

cases, infection was associated with premature rupture of the membranes, with consequent ascending infection.

1.5.13.b. Infection of bone and joints. There have been sporadic reports of the association of *Streptococcus milleri* with septic arthritis (Houston *et al.*, 1980; Lever *et al.*, 1982; Hynd *et al.*, 1984; Libertin *et al.*, 1985), and osteomyelitis (Gopalakrishna *et al.*, 1977; Shlaes *et al.*, 1981). Such infections have usually been associated with an immunocompromised host suffering from alcoholism, diabetes, or receiving immunosuppressive treatment.

1.5.13.c. Infection of skin and subcutaneous tissue. Miller *et al.* (1983) reported a very high incidence of subcutaneous sepsis caused by *Streptococcus milleri* following human bites. A similarly high incidence of small distal extremity abscesses secondary to trauma was reported by Libertin *et al.* (1985). Rarer conditions have included necrotising fasciitis (Giuliano *et al.*, 1977; Shlaes *et al.*, 1981), and hydradenitis suppurativa (Highet *et al.*, 1980).

1.5.14. Summary of the clinical significance of *Streptococcus milleri.*

Streptococcus milleri has in recent years been increasingly associated with serious purulent infection in a variety of body sites. Infection is usually endogenous, and preceded by disease or trauma to a mucosal surface. Often, a systemic condition which compromises the immune system predisposes the host to endogenous infection. However, unlike many opportunistic infections, no significant increase in incidence is noted in the elderly (Gossling, 1988). A notable feature is the preponderance of reported cases

of infection in men, compared with women after the first decade of life (see table 1.2.).

1.6. Management of infection caused by *Streptococcus milleri.*

The antibiotic of choice for infections caused by *Streptococcus milleri* is penicillin, to which all but a few strains are very sensitive (Phillips et al., 1976; de Louvous, 1978; Shlaes *et al.*, 1981; Tillotson *et al.*, 1984). Surgical drainage remains central to the management of abscesses, and is often augmented by antibiotics. In the case of liver abscesses, and a small number of patients with persistent surgical sepsis, courses of 28 days or longer have been recommended (Bateman *et al.*, 1975; Reid and Davidson, 1976; Tresadern *et al.*, 1983). Suitable alternatives to penicillin include erythromycin, clindamycin, and cephaloridine. Sensitivity to tetracyclines is variable (Bateman *et al.*, 1975; Shlaes *et al.*, 1981; Tillotson *et al.*, 1984). Sulphonamides are inactive against *Streptococcus milleri* and have been used as a selective agent for the recovery of this organism from the oral cavity (Mejàre and Edwardsson, 1975; Yakushiji *et al.*, 1988a). The consideration of *Streptococcus milleri* in prophylactic and therapeutic regimens for patients undergoing colorectal surgery has already been discussed.

Table 1.2: Distribution of patients with suppurative infections due to *Streptococcus milleri*, by age and sex.

	No. of patients of indicated sex.	
AGE (yrs.).	MALE.	FEMALE.
0-9	4	6
10-19	20	4
20-29	11	0
30-39	6	4
40-49	9	0
50-59	8	3
60-69	13	2
70-79	2	1
80-89	1	0
TOTAL	74	20

(From Gossling, 1988).

1.7. The cell-surface and potential virulence factors of group F and related streptococci.

The capacity of a microorganism to produce disease is dependent upon a balance between the pathogenic properties of the organism in question and its interactions with specific and non-specific defence mechanisms present in the host.

Streptococcus milleri is not considered to be an aggressively invasive organism. Most infection is of endogenous origin and results from the breakdown of protective skin or mucosal barriers, allowing access of organisms to the deeper tissues. Once established in deeper tissues, invading organisms must be capable of surviving host defences, whilst tissue damage is brought about by bacterial and host factors.

It is clear from the literature cited that *Streptococcus milleri* is capable of producing infection in a range of sites, and is unusual amongst the viridans streptococci in its capacity to produce purulent disease. The literature relating to work carried out on the cell-surface characteristics and potential virulence factors of *Streptococcus milleri*, with special reference to those belonging to Lancefield group F, will now be considered.

1.7.1. The bacterial cell-surface and its relationship with pathogenicity.

The structural components of the bacterial cell envelope are often fundamental to pathogenicity, and structural components may be primary factors in disease production. A diagram illustrating the basic components of the Gram-positive cell envelope is shown in figure 1.1.

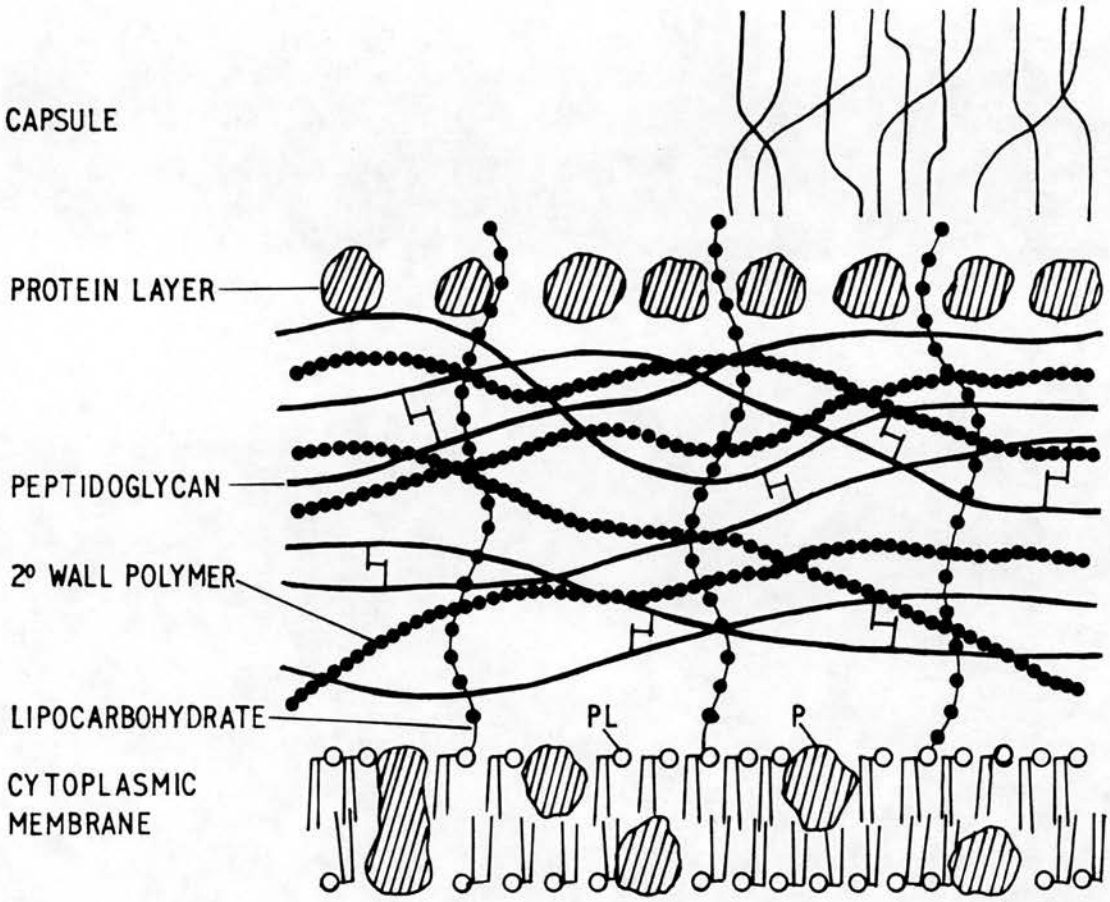


Figure 1.1: The Gram-positive cell envelope.

P = protein.

PL = phospholipid.

⊢ = cross-linking peptide chain.

(courtesy of Dr. I.R. Poxton).

1.7.2. Capsule. Capsules, when present, represent the outer layer of the Gram-positive bacterial cell, and are mainly polysaccharide in nature. Encapsulation is a well-established bacterial virulence factor, important in conferring various degrees of resistance to phagocytosis by inflammatory cells. Classical examples of antiphagocytic capsules include the *Streptococcus pyogenes* hyaluronic acid capsule, and the *Streptococcus pneumoniae* polysaccharide capsule.

Some organisms of the *Streptococcus milleri* group have been shown by Brook and Walker (1985) to possess a polysaccharide capsule, demonstrated by staining with Hiss stain or ruthenium red. Only strains possessing a capsule were able to produce subcutaneous abscesses when injected alone into mice. Strains without a demonstrable capsule were unable to produce subcutaneous abscesses when injected alone into mice, but when passaged with other encapsulated organisms the capsule was often restored, and so too was pathogenicity. Lewis *et al.* (1988), also demonstrated the capacity of *Streptococcus milleri* to produce abscesses in pure culture, and although they did not investigate its presence in their strains, considered encapsulation to be a possible virulence factor. The presence of capsules in *Streptococcus milleri* strains has rarely been reported. Mirick *et al.* (1944a) observed capsular swelling in strains of *Streptococcus MG* when mixed with homologous antiserum, though encapsulation was not obvious until this was done. Their strains were almost completely non-pathogenic in animal experiments.

Repeated laboratory subculture of organisms may lead to the loss of capsule and pathogenicity (Ward and Berkeley, 1981). Against this background, *Streptococcus milleri* strains isolated from infection may rarely possess

capsules, may rarely be investigated for the presence of capsules, or may rapidly transform to non-capsulation on laboratory media. This probably explains the scarcity of information on encapsulation within this group of organisms.

The typing antigens present in group F and related streptococci have often been regarded as microcapsular structures, capable of preventing phagocytosis (Huis in't Veld and Willers, 1973) and will be discussed later.

1.7.3. Wall-associated proteins. The preparation of bacterial cell-walls for analytical work usually involves certain procedures to free them from cytoplasmic contaminants. Popular techniques have included treatment with proteolytic enzymes (Krause and McCarty, 1961), with hot phenol, or with hot detergents (Huis in't Veld and Willers, 1973). The inevitable loss or denaturation of wall-associated proteins has resulted in little protein being found in association with peptidoglycan, and a general disregard for their importance in the cell-wall.

A variety of proteins are now recognised as wall components. Some wall-associated proteins are structural, and others are enzymes involved in wall assembly or modification. Examples include staphylococcal protein A, and autolytic enzymes. Covalently bound proteins were demonstrated in the cell-wall of a z_3III strain by Huis in't Veld and Willers (1973).

Some streptococci contain proteins associated with the cell-wall which may confer antiphagocytic properties, for example, group A streptococcal M protein. Lütticken *et al.* (1978) demonstrated the presence of one or two protein antigens ("sm" antigens) in HCl, but not formamide extracts of many *Streptococcus milleri* strains. It was suggested that these so-called "sm"

proteins were present at or near the cell-surface, and possibly played a part in virulence analogous to that produced by M protein. At least three serologically distinct protein antigens were isolated from group F reference strain O'Mahoney (Colindale) by Nakayama and Maekawa (1979).

Proteins non-covalently associated with the cell-wall may be released by the action of detergents or chaotropes.

1.7.4. Surface appendages. The term 'fibrillae' has been used to describe fine wisps of M protein on the surface of *Streptococcus pyogenes* (Fischetti *et al.*, 1988). Fine fimbrial structures have been demonstrated on the surface of oral streptococci, including *Streptococcus milleri* (Handley *et al.*, 1978; 1985; personal communication, 1986). The presence of surface appendages may have an important role in adherence to surfaces, and in the inter-species interactions common in the mixed flora of mucosal surfaces, dental plaque, and purulent lesions.

1.7.5. The Gram-positive cell-wall. The Gram-positive cell-wall is a rigid structure located external to the cytoplasmic membrane, providing physical strength, maintaining cell shape, and possibly having involvement in molecular sieving.

1.7.5.a. Peptidoglycan. The 'backbone' polymer of the cell-wall is peptidoglycan which, as the name implies, contains both polysaccharide and peptide moieties. Repeating units of the disaccharide (N-acetylglucosamine β1-4 N-acetylmuramic acid) are polymerised by β1-4 glycosidic linkages to form parallel polysaccharide chains. Short peptide chains covalently cross-

link the longer carbohydrate chains to form a single, cell-shaped macromolecule, which typically represents between 50 and 80% of the dry weight of the cell-wall.

It has been suggested that both the peptide, and the hexosamine components of streptococcal peptidoglycan may be antigenic (Karakawa *et al.*, 1966, 1967). From a taxonomical point of view, the analysis of peptidoglycan components may be of limited value since despite identical amino-acid composition between different organisms, the sequence or even the mode of cross-linkage may be quite different (Schleifer and Kandler, 1972).

The remainder of the wall is made up of 'accessory polymers', including teichoic acids, teichuronic acids, polysaccharides, and proteins. Accessory polymers are covalently linked to the structural component, peptidoglycan, throughout the thickness of the wall.

The principal accessory polymers of the Gram-positive cell-wall are usually polysaccharides, or the related phosphate-containing teichoic acids. These polymers are frequently the dominant antigenic determinants at the cell-surface, and may represent species-specific antigens.

1.7.5.b. Teichoic acids. Teichoic acids were discovered by Armstrong *et al.* (1958), and defined as linear polymers of glycerol or ribitol phosphate, in which the repeating units were joined by phosphodiester linkages. Poly (ribitol phosphate) or (glycerol phosphate) chains were substituted to various extents by ester-linked D-alanine, and often also glycosyl residues. The definition has since been broadened to include a range of polymers possessing phosphodiester groups, polyols and/or sugar residues, and usually but not always, D-alanine ester residues (Baddiley, 1970). The nature of these substituent groups (eg. D-alanyl groups) may have an

important influence on the molecular properties of teichoic acid chains. The high phosphate content means that teichoic acids are acidic, they carry a net negative charge, and are largely responsible for the negative surface charge carried by cells in which they occur.

This definition encompasses a group of polymers which includes both the wall teichoic acids, and the membrane-associated lipoteichoic acids which will be considered later. One important function of the teichoic acids is to bind cations (eg. Mg^{2+}) and maintain an adequate supply of them to the cell membrane.

1.7.5.c. Teichuronic acids. The teichuronic acids are linear polysaccharide polymers containing uronic acid and usually amino-sugar residues, and which share similar properties to the teichoic acids. Phosphate is not contained within the repeating units, but attachment to the peptidoglycan probably involves a single phosphodiester linkage. In general, the polymers do not contain alanyl-ester substituents. The negative charge of teichuronic acids comes from the carboxyl groups of the uronic acid residues.

The synthesis of teichuronic acids by some organisms is critically dependent upon the conditions under which they are grown. Conditions where phosphate is limited may cause cells to cease the production of teichoic acids, and to incorporate teichuronic acids into their walls instead.

1.7.5.d. Neutral polysaccharides. In addition to the more widely distributed teichoic and teichuronic acids, the walls of some Gram-positive bacteria contain uncharged polysaccharides. This is so for the walls of some streptococci, in which they represent the C antigens used in the serological grouping of streptococci (Lancefield, 1933). Recognition of the

antigenicity of these carbohydrate accessory wall polymers was made long before their composition was determined, and it would appear that the chemical basis of the difference between groups resides in the amino-sugar present. Examples include N-acetylglucosamine, which is the antigenic determinant for the group A streptococcus (Krause, 1963), and N-acetylgalactosamine for the group C streptococcus. Carbohydrate typing antigens may also be found amongst the streptococci, and are believed to be located in the cell-wall, forming a surface layer, or even a superficial capsule (Parker, 1983).

1.7.5.e. Attachment of accessory polymers to peptidoglycan. Most carbohydrate-related secondary wall polymers are attached via phosphodiester bonds formed between C1 of a terminal sugar in the accessory polymer, and a 6-hydroxyl of a muramic acid residue on the peptidoglycan (Ward, 1981). In the case of teichoic acids and some teichuronic acids, a specialised 'linkage unit' containing a tri-(glycerophosphoryl)-N-acetylhexosamine group intervenes between the terminal residue of the polymer and the muramic acid residue.

1.7.6. Isolation of accessory carbohydrate polymers from the Gram-positive cell-wall. The sugar 1-phosphate group involved in the attachment of accessory polymers is exceptionally labile to the action of dilute acids, which may facilitate removal of these polymers from the cell-wall.
Similarly, the ease with which teichoic acids are extracted from cell-wall preparations by dilute alkali (Archibald *et al.*, 1969) may be explained by the high vulnerability to alkali-attack of the glycerophosphoryl-N-acetylhexosamine group of the specialised 'linkage unit'. Choice of

extraction agents and conditions is of importance, since degradation and consequent loss of antigenicity may result from the application of inappropriate methods.

Trichloroacetic acid has been used most frequently but may lead to some hydrolysis of phosphodiester bonds, and therefore some chain fragmentation of teichoic acids. Dilute HCl (Hughes and Tanner, 1968), and hot acidic buffers (Pavlik and Rogers, 1973; Kojima *et al.*, 1983) have also been recommended, the latter giving some selectivity in polymer extraction.

Alkali extraction results in less teichoic acid degradation than does acid (Archibald *et al.*, 1969), and may selectively extract teichoic acids from walls containing teichuronic acids or polysaccharides in addition (Hughes, 1970).

A great many techniques have been successfully used for the extraction of group and type specific material from streptococci, and include:

Hot HCl (Lancefield, 1933), hot formamide (Fuller, 1938), *Streptomyces albus* enzyme (Maxted, 1948), *Streptomyces albus* enzyme with lysosyme (Watson *et al.*, 1975), *Streptomyces griseus* enzyme (pronase B) (Ederer *et al.*, 1972), autoclaving in saline (Rantz and Randall, 1955), hot trichloroacetic acid (Slade, 1965), nitrous acid (El Kholy *et al.*, 1974; 1978), and achromopeptidase (Slifkin and Cumbie, 1987). Most of these techniques have been applied to whole cells, and the crude antigenic extracts obtained used in diagnostic serological tests. Some have in addition been used to obtain material for use in the characterisation of cell-wall polymers. Clearly, the material from which extracts are made (ie: whole cells, trypsin-treated cell-walls, sodium dodecyl sulphate treated cell - walls), as well as the extraction technique and subsequent

purification procedures, can have a bearing on the chemical nature of the material finally analysed.

1.7.7. Accessory carbohydrate polymers of the group F streptococci.

Accessory wall polymers in the form of grouping and typing antigens have been described in group F strains. The group F 'C' substance was described by Lancefield and Hare (1935), and was believed to be carbohydrate, in common with the grouping antigens of other streptococcal strains. Ottens and Winkler (1962) described 5 independent carbohydrate typing antigens in group F and related strains. Both the group and type antigens were regarded as cell-wall components located in or on the cell-wall, though the presence of some antigenic material within the cell could not be excluded (Ottens and Winkler, 1962).

Cummins and Harris (1956) showed that the wall of a group F streptococcus (NCTC 5389) contained large amounts of rhamnose and glucose, and smaller amounts of galactose, glucosamine, galactosamine, and 'unknown hexosamine'. Similar paper chromatographic cell-wall analysis of 19 serologically diverse *Streptococcus milleri* strains by Colman and Williams (1965) showed them all to possess rhamnose and glucose. Sixteen contained galactosamine, and 15 galactose. None contained glycerol, anhydroribitol or fucosamine.

Gas-liquid chromatographic examination of the whole-cell trimethylsilyl-sugar profiles *Streptococcus milleri* strains also showed that the major neutral sugars present were rhamnose, glucose and galactose. Smaller amounts of xylose were also detected (Aluyi and Drucker, 1983).

The group-specific oligosaccharide for group F strains was isolated from formamide extracts of trypsinised cell-walls by Willers *et al.* (1964a), and characterised more fully by Michel and Willers (1964). Quantitative

inhibition tests (McCarty and Lancefield, 1955) indicated that the most probable structure of the group F antigenic determinant was 3-ß-D-glucopyranosyl-N-acetyl-D-galactosamine. This finding was later verified by Michel and Krause (1967).

1.7.7.a. Typing antigens of group F streptococci.

Type I: Type I antigen is probably identical to the type I antigen of Bliss, (1937). It has been found in group F strains, and in indifferent and haemolytic strains of group G (Ottens and Winkler, 1962). It may also be found in strains lacking a recognised grouping antigen. Examination of this antigen revealed the presence of galactose, glucose, rhamnose and galactosamine in the approximate ratio 4:2:2:1. The determinant group probably contained N-acetyl-galactosamine (Willers *et al.*, 1964a; Heidelberger *et al.*, 1969).

Type II: Type II antigen is again probably identical to the type II antigen of Bliss, (1937) and has been found in group F and non-groupable strains as well as occasional group A (Jablon *et al.*, 1965) and group T strains (Ottens and Winkler, 1962). Michel and Krause (1967) showed the type II antigen to contain rhamnose, glucose, galactose, and galactosamine. Later work by Michel *et al.* (1969) confirmed that the wall-derived type II antigen contained galactosamine, galactose, rhamnose, and glucose in the molar ratios 1:1:2:2. The antigenic determinant consisted of ß-(N-acetyl-D-galactosaminyl)-(1-2)-α-D-galactosyl-(1-2)-α-L-rhamnosyl-(1-4)-ß-D-glucosyl-(1-4)D-glucose. N-acetyl-galactosamine was the terminal sugar.

Type III: Type III antigen has been found in group F and indifferent strains of group C, as well as strains lacking a recognised grouping antigen. Preliminary studies on the Ottens type III antigen suggested the

presence of a ß-glucosidic and galactosidic group as possible antigenic determinants (Willers *et al.*, 1964a; 1964b). Willers and Alderkamp (1967) showed that the type III antigen contained glucose, galactose and rhamnose in the ratio 5:3:1. Rhamnose was not serologically active and was believed to be the structural backbone of the molecule. That a lower than expected proportion of 'structural' rhamnose was found was attributed to destruction of rhamnose during formamide extraction. Later studies by Willers *et al.* (1973a) showed that the type III antigen cleaved from cell-walls was a linear polymer containing mainly rhamnose, glucose, and galactose in the approximate ratio 1:2:3. The most probable structure of the type III determinant group was suggested as ß-glucosyl-(1-6)-galactosyl-(1-6)-galactosyl-(1-3)-rhamnose. A second determinant group was also considered possible.

Type IV: Only a few strains possess a type IV antigen. These strains usually belong to group F or possess no recognised grouping antigen, and are non-haemolytic (Willers *et al.*, 1973b). Type IV antigen was shown by Willers *et al.* (1973b) to contain rhamnose, glucose, galactose and N-acetyl-glucosamine in the molar ratio 4:4:4:1. Inhibition reactions indicated that both of the trisaccharides ß-galactosyl-glucosyl-galactose and ß-galactosyl-glucosyl-rhamnose were determinant groups of the type IV antigen. No clear information was provided on the linkages within these trisaccharides, or on the role of N-acetylglucosamine in the antigen molecule.

Type V: Although the type V antigen was described by Ottens and Winkler (1962) and partially characterised by Willers *et al.* (1964a), it was later found not to be a distinct type on further examination (Willers, personal communication, cited by Lütticken *et al.*, 1978).

1.7.7.b. Distribution of group F and type-antigens within the bacterial envelope.

Group and type-antigens were both believed to be cell-wall components, and could be extracted from cell-walls by a variety of methods. A superficial position in the wall was proposed for typing antigens, based on the following evidence:

i. the release of type-antigen into the culture medium during growth, and

ii. the production of antibodies directed only against type-antigen when strains containing group and type-antigen were used to raise antisera in rabbits.

Further evidence was provided by Willers *et al.*, (1968). Whole bacterial cells containing both group and type-antigens were probed with fluorescein-labelled anti-group and anti-type antibodies. The intensity of fluorescence indicated that the typing antigen had a more superficial location than the grouping antigen. Weaker fluorescence was observed when the group antigen was probed, suggesting a sub-surface location. A surface, microcapsular location was proposed for the type-antigen, though this 'capsule' was not demonstrable by negative staining with India ink. However, electron micrographic analysis of cell-sections treated with ferritin-labelled anti-type sera by Huis in't Veld and Linssen (1973) showed that type-antigen was present in abundance throughout the thickness of the cell envelope. Very dense localisation of ferritin particles was additionally seen in the cytoplasm, close to the cytoplasmic membrane. This was suggested as a site of antigen synthesis or storage. Negative staining with India ink again did not demonstrate the presence of a capsule.

1.7.7.c. Examination of type-antigen released into the culture medium.

Willers *et al.* (1964a) observed that various group F and related streptococci released material which was serologically identical to wall-extracted type-antigen into the culture medium during growth. This material was believed to represent type-antigen in a more native form than that violently extracted from cells with formamide, and attempts were consequently made to characterise it. Distinct differences were observed between material from the two different sources. Material released into the culture medium contained less rhamnose, and contained mannose which was almost absent from wall-derived material. It also possessed high levels of glucosamine. High levels of glucosamine were also found in the wall antigen, though this was thought to be partly derived from the 'mucopeptide complex' (peptidoglycan).

Michel and Krause (1967) demonstrated differences between type II antigen released into the culture medium and that extracted with formamide (see table 1.3). Serological identity was demonstrated in Ouchterlony gel, and the differences in composition were tentatively attributed to incomplete purification of the antigen obtained from the culture medium.

Type III antigen isolated from the culture medium was characterised by a lower glucose content, and a fucose content of 20%. In some cases, the purified polysaccharide contained considerable amounts of glycogen-like material (first observed by Heidelberger *et al.*, 1969). Pyrolysis gas chromatography illustrated again the differences in sugar composition between type III antigen derived from the culture medium and type III antigen extracted from cells (Huis in't Veld *et al.*, 1973). Serological identity could not necessarily be correlated with chemical or structural identity.

Table 1.3: Chemical composition of wall and medium-derived type II antigen. (Michel and Krause, 1967).

Constituent.	Medium-extracted type II antigen.	Wall-extracted type II antigen.
Rhamnose	4.6%	24.5%
Glucose	4.7%	25.9%
Galactose	15.2%	12.2%
Mannose	1.4%	0
Glucosamine	15.8%	0.1%
Galactosamine	11.5%	15.8%

Recently, Yakushiji *et al.*(1988b) were able to demonstrate at least ten distinct serological types within oral *Streptococcus milleri* strains. This new typing system was based upon carbohydrate antigens derived from whole cells by the Rantz and Randall (1955) method. Although this typing system depends on antigens other than the Lancefield grouping antigen, strains belonging to serotypes a, c, and f corresponded strictly with those of Lancefield groups A, C, and F respectively. To date, only the type b antigen has been investigated in any detail (Yakushiji *et al.*, 1988c). Analysis of type b antigen purified from a trichloroacetic acid extract of the trypsin-treated cell-walls of NCTC 10708 has shown that it contains rhamnose and glucose in the molar ratio 1.7:1.0, with a trace of galactosamine (0.1). No glycerol or ribitol was detected, and the amounts of protein and phosphorus were also negligible. Quantitative inhibition tests suggested that rhamnose was structurally involved in the immunodeterminant.

1.7.8. The cytoplasmic membrane. All Gram-positive bacteria with teichoic acids as accessory polymers in their cell-walls also possess a membrane-associated or lipoteichoic acid. These are thought to be exclusively glycerol phosphate polymers, terminating not in a covalent link to peptidoglycan, but in a diglyceride, or glycolipid moiety, whose structure may be species-specific (Iwasaki *et al.*, 1986). Originating from their glycolipid anchorage in the outer-leaflet of the cytoplasmic membrane, lipoteichoic acid chains extend through the cell-wall, with which they are loosely associated (Chui *et al.*, 1974), and may present as a cell-surface antigen (Lambert *et al.*, 1977; Wicken and Knox, 1975). Lipoteichoic acids, often in deacylated form, may be found in association with the cell-wall,

or as extracellular products, since they are released into the culture medium during bacterial growth and division (Chiu *et al.*, 1974; Kessler and Shockman, 1979). Functions of the lipoteichoic acids are believed to include the binding of magnesium ions, the non-specific inhibition of autolytic enzymes, and the adhesion of bacteria to surfaces.

The isolation of lipoteichoic acid has not been described for the group F streptococcus, although a hexosamine-free component with the properties of a lipoteichoic acid was demonstrated in a z_3III strain by Huis in't Veld and Linssen,(1973).

Plackett and Shaw (1967) recovered immunologically active diglucosyl diglycerides from *Streptococcus MG* (NCTC 8037) which cross-reacted with antisera to *Mycoplasma laidlawii* (*pneumoniae*). Similar glycolipids were found in the cell-walls of z_3III and F III strains by Huis in't Veld and Willers (1973). The complexing of glycolipids with teichoic acids in the walls of Gram-positive organisms had been described earlier by Wicken and Knox (1970), and could not be excluded from occurring in the cell-walls of streptococci (Huis in't Veld and Willers, 1973).

1.7.9. Extracellular products of *Streptococcus milleri.*

1.7.9.a. Enzymes.

i. Hyaluronidase. Hyaluronidase production has been described for some strains of group F streptococcus and *Streptococcus milleri*, especially beta-haemolytic representatives (Colman and Williams, 1972; Poole and Wilson, 1979). The hyaluronidases produced by some strains of *Streptococcus milleri* were examined by Unsworth *et al.* (1980) who were able to demonstrate four distinct serotypes of hyaluronidase; these generally correlated with the source of the strain. For example, isolates from dental

plaque, and from purulent lesions predominantly produced type IV hyaluronidase, suggesting an oral origin for "abscess-strains". High titres of antibodies in blood-donor sera to type III and type IV hyaluronidases additionally suggested a greater invasiveness for strains producing these serotypes of enzyme. The results of further studies on hyaluronidase production in relation to infection were published recently by Unsworth (1989). Hyaluronidase production was again found to be commonest amongst beta-haemolytic isolates, many of which were group F, although it was also found in alpha and non-haemolytic isolates. A strong correlation was observed between hyaluronidase production and isolation of the organism from internal abscesses. This was not so in isolates recovered from non-abscess sites. It was consequently suggested that production of hyaluronidase may be important in the pathogenicity of some *Streptococcus milleri* isolates, and could be helpful in predicting the likelihood of deep sepsis in isolates from blood culture.

ii. Ribo- and deoxyribonuclease. Production of RNase and DNase has been observed in some strains of *Streptococcus intermedius* (Marshall and Kaufman, 1981), and *Streptococcus milleri* (Drucker and Lee, 1983). No correlation has been shown between pathogenicity and production of nucleases by these strains.

iii. Proteolytic enzymes. The release of extracellular proteins, including potentially damaging proteolytic enzymes, was demonstrated in an endocarditis strain of *Streptococcus MG-intermedius* by Straus *et al.* (1977). When grown in conditions of essential amino-acid deprivation to mimic the conditions in a fibrotic heart lesion, the release of proteins as a proportion of the dry weight of bacteria increased 4-8 fold. It was therefore speculated that in infected sites where nutritional conditions

may not be optimal, certain bacterial strains may still be able to bring about damage to host tissues by the continued release of destructive enzymes.

iv. Rutinase. Strains of *Streptococcus milleri* (though not strains of *Streptococcus salivarius*, or *Streptococcus mutans*) from the mouths of healthy individuals were found by Parisis and Pritchard (1983) to be capable of releasing carcinogenic substances by the hydrolysis of common foodstuffs. Hydrolysis of rutin, a common component of foods and beverages, liberated quercetin, a genotoxic substance which may be involved in the production of epithelial carcinoma. It was speculated that certain oral populations may be instrumental in carcinogenesis in the mouth, and a possible correlation between poor oral hygiene and oral cancer was suggested.

1.7.9.b. Bacteriocins. The production of substances that are antagonistic to other microorganisms has important implications in terms of colonisation in a mixed flora.

Bacteriocin-like activity was demonstrated in 78% of alpha-haemolytic streptococci by Dajani *et al.* (1976). Antagonistic activities of alpha-haemolytic streptococci in the urogenital tract have also been recognised by McBride *et al.* (1980). Drucker and McKillop (1982) described the widespread production of antagonistic substances in the form of H_2O_2 production and bacteriocin-like activity amongst *Streptococcus milleri* strains. It was also observed that all *Streptococcus milleri* isolates were sensitive to the bacteriocin-like activity of *Streptococcus mutans* (NCTC 10832). However, recent work by Dajani (1989) has suggested a low rate of

bacteriocin-like activity among alpha-haemolytic *Streptococcus milleri* strains.

1.7.9.c. Interaction with the host immune system. Higherd *et al.* (1978) found that the crude extracellular products liberated by gentle washing of *Streptococcus intermedius* cells (CEP-Si) supressed fibroblast proliferation and altered lymphocytic immunological responses *in vitro*. Subsequent investigation revealed a strongly immunosuppressive, non-cytotoxic substance in this crude extract (Arala-Chaves *et al.*, 1979). Purification showed that the immunosuppressive factor was a 90 kilodalton protein, designated f3'EP-Si, which was further shown to induce T-suppressor lymphocytes, and to have B cell mitogenic activity (Arala-Chaves *et al.*, 1981; 1983; 1986). The extent of production of this immunosuppressive and fibroblast-inhibiting substance in *Streptococcus milleri* strains has not been established, but it is possible that it may emerge as an important virulence factor for this species.

The possibility that the occurrence of recurrent aphthous ulceration represents a hypersensitivity reaction to oral streptococci is well established. Shore and Shelley (1974) described a case of recurrent aphthous stomatitis, in which the lesions were associated with a heavy intralesional growth of alpha-haemolytic group F streptococci. A hypersensitivity state was believed to have developed to this organism, and clinical improvement followed the use of appropriate antimicrobial chemotherapy. There have been no other reports implicating group F streptococci or *Streptococcus milleri* in the aetiology of auto-immune states.

1.7.10. Final comments on the cell-surface and virulence factors of *Streptococcus milleri.*

There can be little doubt that certain organisms belonging to the *Streptococcus milleri* group are capable of causing infection in man. It is unclear from the literature whether the capacity to produce infection is, in the right circumstances, a quality possessed uniformly by all members of this diverse group, or whether it is a property of certain members only.

The work done hitherto on the potential virulence mechanisms involved in *Streptococcus milleri* disease has revealed a number of potentially important agencies including: the capacity to tolerate conditions of low Eh; possession of antiphagocytic capsular material; possession of antiphagocytic surface-protein structures; the release of material which interacts with the host immune system; and the release of hyaluronidase. The relative importance of these and other factors and their distribution within the *Streptococcus milleri* group as a whole have yet to be clearly established. Pathogenic mechanisms within the *Streptococcus milleri* group remain largely unclear.

Although much work on the chemical nature of the carbohydrate grouping and typing antigens of the group F and related streptococci was carried out by Willers and co-workers in the 1960's and early 1970's, it would appear from the literature that there is room for further study of the cell-surface antigens of group F and related streptococci.

Further work is required to re-examine the composition of covalently-linked carbohydrate antigenic material derived from cell-walls which have been more thoroughly freed from cytoplasmic contaminants. In addition, the membrane-associated or lipoteichoic acids appear to have much in common

with the "excreted" type-antigen material described previously. Studies on the lipoteichoic acids of group F and related streptococci, and their importance as cell-surface antigens have not previously been reported.

The results of some investigations into the nature of covalent, and non-covalently bound antigenic material associated with the cell-surface of group F and related streptococci will be presented in the following chapters of this thesis.

CHAPTER 2

MATERIALS AND METHODS

2.1. Culture of streptococcal isolates.

2.1.1. Solid media.

a. Blood agar. Streptococcal isolates were routinely cultured on Columbia Agar Base (Oxoid, Basingstoke, Hampshire) containing 5% (v/v) horse blood.

b. Selective medium. The only selective medium employed in these studies was a modification of Carlsson's (1967a) MC agar, used in the recovery of *Streptococcus milleri* isolates from the oral cavity (see chapter 3). In this medium, 1g sulphadimetine (Ciba), which has no product licence, and is not available in the United Kingdom (Ciba Laboratories, Horsham, U.K. - personal communication), was replaced by 1g sulphadimidine (3ml ampoule Sulphamethazine, ICI). In all other respects, the medium was as described by Carlsson (1967a).

2.1.2. Liquid media.

a. Culture medium. Unless otherwise stated, the liquid medium used for broth-culture was Todd-Hewitt Broth (Oxoid), reconstituted according to the manufacturer's instructions. Personal observations indicated superior growth of *Streptococcus milleri* isolates in filter-sterilised, as opposed to autoclaved broths. Membrane filtration was consequently employed for all THB used in these studies.

b. Transport medium. A reduced transport fluid (RTF) described by Syed and Loesche (1972) was used in the isolation of *Streptococcus milleri* from the oral cavity (see chapter 3).

2.1.3. Culture conditions.

Unless otherwise stated, all plate-cultures were incubated in anaerobic jars from which the catalysts had been removed in an atmosphere of: 10% CO_2, and 90% H_2, for 24-48 hours at 37°C.

Broth cultures were routinely incubated statically in air, at 37°C.

2.1.4. Storage of organisms.

Streptococcal isolates were stored lyophilised in a medium comprising: Oxoid Nutrient Broth number 2 (100ml), inactivated horse serum (10ml), 20% (w/v) aqueous glucose solution (5ml).

Organisms in current use were stored on blood-agar plates at 4°C, and subcultured at least fortnightly to maintain viability, and to prevent mutation.

2.2. Isolation of *Streptococcus milleri* from the oral cavity.

Chapter 3 describes the results of a study on the recovery of *Streptococcus milleri* from the oral cavity. Details of the materials and methods used are given here.

2.2.1. Sampling procedure.

Dental plaque and debris was recovered from two sites in each mouth: lingual to a lower first permanent molar, and disto-labial to an upper canine tooth. Potential sample-sites were examined for the presence of active periodontal disease, as evidenced by bleeding on gentle probing of the gingival crevice (Löe, 1967). Only sites where no bleeding was evident were sampled. Where the designated teeth were absent, samples were taken from convenient adjacent teeth.

Samples were collected using sterile McCall's curettes, and dispersed immediately into 1ml RTF (Syed and Loesche, 1972).

Plaque-suspensions were transported to the laboratory within one hour for processing.

2.2.2. Laboratory procedures.

Plaque was more thoroughly dispersed in RTF by gentle vortex-mixing, before its serial dilution in RTF. Dilutions (0.2ml of the 10^{-3}, 10^{-4}, and 10^{-5}) were spread onto the surface of MC agar plates (modified from Carlsson, 1967a), using sterile glass-spreaders. Plates were incubated in anaerobic jars from which the catalysts had been removed, in an atmosphere of 10% CO_2, 90% H_2, for 48 hours at 37°C.

With the aid of a hand-lens, colonies morphologically resembling *Streptococcus milleri* (Mejàre and Edwardsson, 1975) were picked from MC agar plates, and subcultured onto a quarter of a blood agar plate. Blood agar cultures were incubated as before for 24-36 hours.

Following incubation, cultures were assessed visually for purity, and were examined microscopically by Gram-staining. Sometimes, one or more subcultures was required before pure cultures were obtained.

Ten colonies of pure cultures giving the appearance of Gram-positive cocci forming chains were finally subcultured onto blood agar for further incubation (24 hours) as before. Gram-films were repeated to check microscopic morphology.

Isolates were either lyophilised, or retained on blood agar prior to biochemical and serological characterisation.

Fresh, overnight cultures of isolates on full blood agar plates were used in biochemical and serological identification procedures.

2.3. Identification of streptococcal isolates.

2.3.1. Biochemical characterisation: the rapid carbohydrate utilisation test (RCUT).

This technique was modified from Young *et al.* (1976). In the RCUT, preformed enzymes are measured by adding a suspension of the overnight growth of the test organism to a buffered (non-nutrient) solution containing the sugar to be tested and a pH indicator (phenol red). Acid production resulting from sugar utilisation is detected by a colour change of the pH indicator from red to yellow (occasionally orange).

Buffer-salt solution (BSS). This was prepared by mixing the following solutions: 40ml 0.1 mol/litre K_2HPO_4; 12ml 0.1mol/litre KH_2PO_4; 100ml 8% (w/v) KCl; 10ml 1% (w/v) aqueous phenol red; and 838ml sterile distilled water. The pH was checked and if necessary adjusted to 7.10-7.15. It was then dispensed in screw-cap bottles and stored at -20°C.

Sugar solutions. These were prepared as 100ml volumes of 10% (w/v) solutions in distilled water of mannitol, raffinose, sorbitol, melibiose, and trehalose. They were filter sterilized, dispensed in 4ml amounts, and stored at -20°C.

Method.

The RCUT was conveniently performed in the wells of microtitre plates.

1. 100*ul* of BSS was added to 6 microtitre wells, and 250*ul* to a sterile Wassermann tube for each organism to be examined. An additional row of 5 wells which would receive no organism, but which would receive each of the test sugars respectively was also included in the microtitre plate. (See

figure 2.1).

2. 25*ul* of mannitol, raffinose, sorbitol, melibiose, and trehalose solution were added respectively to one of 5 wells for each test organism, and to the row to which no organism was to be added. The remaining well received no sugar solution.

3. Growth from a fresh, overnight blood-agar culture was harvested with a cotton-tipped swab, and used to make a dense suspension in the 250*ul* of BSS contained in a Wasserman tube. This suspension (25*ul*) was added to each of the six wells for each test organism. (See figure 2.1 for a schematic representation of the layout of a RCUT test plate set up to identify three test isolates).

4. Plates were incubated at 37°C in a waterbath. A definitive reading was made within 4 hours.

Each day, a fresh culture of NCTC 10707 was used as a positive *Streptococcus milleri* control.

2.3.2. Voges-Proskauer (VP) reaction.

The method employed for determination of the VP reaction was the rapid method of Bucher and von Graevenitz (1984). NCTC 10707 was used daily as a positive *Streptococcus milleri* control.

Method. A loopful of culture from the plates used to inoculate the RCUT was emulsified in 200*ul* MR-VP broth (Difco Laboratories, Detroit, MI.) in a Wasserman tube, and incubated in a waterbath at 37°C for 5 hours with occasional shaking. One drop of 0.5% creatine, one drop of 5% (w/v) alpha-naphthol in absolute ethanol, and one drop of 40% (w/v) KOH were added and

the tubes thoroughly shaken. Tubes were subsequently left at room temperature, and angled to allow the maximum surface area of their contents to be in contact with air. A positive reaction was read within 15-20 minutes by the development of a pink to red colour.

2.3.3. Arginine hydrolysis.

Hydrolysis of arginine was determined by the method of Niven *et al.* (1942), as described by Cowan and Steel (1965).

A laboratory stock-strain of *Streptococcus faecalis* was used as a positive control.

2.3.4. Aesculin hydrolysis.

The routine method for the determination of aesculin hydrolysis was the aesculin agar method described by Cowan and Steel (1965).

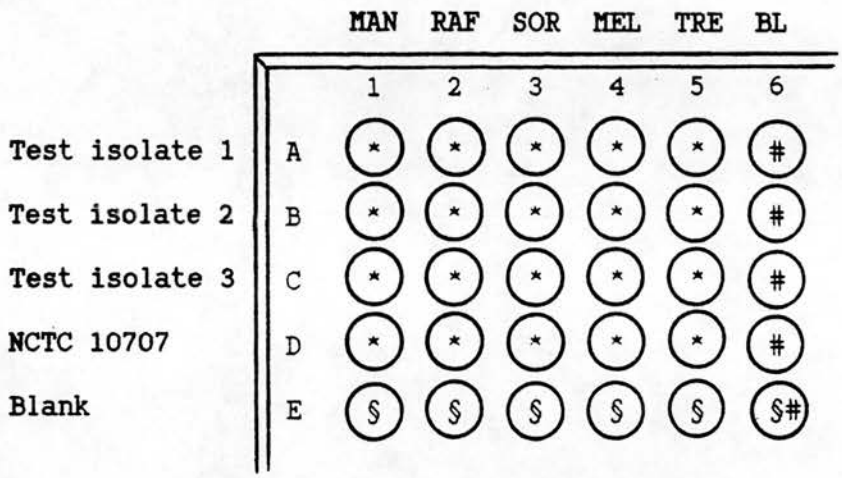
An alternative method was modified from that of Brown *et al.* (1989). THB (1ml) containing 0.2% aesculin was inoculated with a loopful of culture, and incubated at 37°C for 24-48 hours. A positive result was given by the formation of a black precipitate on the addition of 100*u*l of 1% aqueous ammonium ferric citrate solution. A stock laboratory strain of *Streptococcus faecalis* was again used as a positive control.

2.3.5. Serological identification.

Serological identification was performed by gel-diffusion in Ouchterlony gels.

i. Preparation of gel-slides. Gel-slides were prepared by casting 4ml 1% agarose (w/v) in distilled water onto the acetone-cleaned surface of a

Figure 2.1. Schematic representation of the layout of a RCUT test prepared for the identification of 3 test isolates.



Key.

Numbers 1-6, and letters A-E represent row numbers of a microtitre plate.

MAN = Mannitol
RAF = Raffinose
SOR = Sorbitol
MEL = Melibiose
TRE = Trehalose
BL = Blank

Wells marked * received both indicated organism and sugar.
Wells marked # received organism, but no sugar.
Wells marked § received sugar, but no organism.
The well marked §# received neither sugar, nor organism.

microscope slide. Wells, one central and 6 peripheral, were cut with a Feinberg cutter.

ii. Extraction of C antigen. Carbohydrate grouping antigen was extracted from the cells contained in a heavy sweep of fresh blood agar culture by the method of El Kholy *et al.* (1974; 1978). Each day, stock laboratory cultures of beta-haemolytic group A, C, and G streptococci, and NCTC 10707 (group F) were used as positive controls.

iii. Determination of Lancefield group. Antigenic extracts (10*ul*) were placed in peripheral wells, and antisera (undiluted) placed in the central wells of Ouchterlony gels. Each extract was reacted with commercial group A, C, F, and G antisera (Wellcome), and with antisera raised in rabbits against NCTC 10707 (see section 2.4.). Slides were stored at room temperature in a humid atmosphere, and lines of immunoprecipitation read within 4 hours.

2.4. Preparation of antisera for diagnostic serology, and immunochemical studies.

Antisera were raised against ultra-violet (u-v) - killed bacterial cells by the method of Poxton (1979). The following reference strains were used for the production of sera: NCTC 5389, NCTC 8037, NCTC 10707, NCTC 10714, NCTC 11065, NCTC 11063 (see table 3.1. for details of these strains).

Bacteria were grown up in 100ml volumes of THB (Oxoid), harvested by centrifugation (4,000*g*, 15min), and washed three times in sterile 0.85% saline. Broth cultures were checked for purity by subculture on blood agar. Following the final wash, cells were resuspended in 5ml saline, and after counting under phase-contrast microscopy, adjusted to a concentration of 10^9 CFU per ml. Suspensions were irradiated as a thin film in glass petri-

dishes with a bactericidal u-v unit (Hanovia Lamps, Slough, England) positioned 20cm from the cells for 5 minutes. The irradiated cell-suspensions were stored frozen at -70°C in aliquots of 1.5ml until used. Aliquots of cells were thawed immediately prior to use and 1ml volumes given intravenously to the marginal ear-vein of six (one for each bacterial strain) Dutch rabbits according to the following schedule: weeks one and two, three consecutive daily injections; weeks three and four, one injection only; week five, test bleeds (5ml). Doubling dilutions of test sera were reacted against hot HCl (Lancefield, 1933) extracts of homologous strains in Ouchterlony-gel (Freimer, 1963) to indicate their activity. Rabbits were subsequently exsanguinated by cardiac puncture and their sera collected.

2.5. Storage of sera.

Sera were stored frozen at -20°C in aliquots of 5ml. Aliquots in current use were stored at 4°C with the addition of merthiolate (1:10,000 w/v) as a preservative.

2.6. Enzyme-linked immunosorbent assay (ELISA).

The method was adapted from Poxton (1979). One hundred microlitres of antigen diluted in 50mM carbonate buffer (pH 9.6, containing 0.02% sodium azide) to give a final concentration of 25ug/ml (protein), were added to each well of a microtitre plate (Nunc immuno module polysorb F8, Gibco). Plates were incubated at 37°C for 4 hours, then held at 4°C overnight. Plates were washed three times with 0.15M NaCl containing 0.05% Tween 20, and shaken dry. Antiserum, as doubling dilutions (beginning at 1 in 100) in 50mM phosphate buffered saline, pH 7.4 (PBS) containing 0.05% Tween 20, and

0.02% sodium azide (antibody/conjugate buffer) was added to each well (100*ul*) and incubated at room temperature for 4 hours. The plates were washed as before, and anti-rabbit IgG-conjugated alkaline phosphatase (Sigma, Poole, Dorset), diluted 1 in 1000 in antibody/conjugate buffer, was added to each well (100*ul*) and incubated overnight at room temperature. After washing the plates as before, 100*ul* volumes of substrate (a 1mg/ml solution of *p*-nitrophenylphosphate (Sigma, Poole, Dorset) in 50mM carbonate buffer, pH 9.8, containing 1mM $MgCl_2$) were added to each well. Following a 1 hour incubation at room temperature, the colour change was read on a Titertek Multiscan Spectrophotometer (Organon Teknika, St. Neots, Cambridgeshire) at a wavelength of 405nm. End points of titrations were taken as the highest dilution of antisera to give an A_{405} of 1.0.

2.7.1. ELISA inhibition reactions. (Adapted from Cousland and Poxton, 1984).

Inhibition of ELISA was performed by pre-incubating dilutions of potential inhibitors with equal volumes of antiserum diluted to twice the titre for 45 minutes at 37°C.

Potential inhibitors were:

i. Untreated EDTA extract as positive control (see section 2.17. for the production of EDTA extracts from streptococcal isolates);

ii. Heat-treated EDTA extract: EDTA extracts were heated to 121°C for 15 minutes;

iii. Periodate-treated EDTA extract: EDTA extracts were treated with 0.01M sodium periodate in sodium acetate buffer, pH 5.0 (Poxton *et al.*, 1989) for 16 hours at room temperature. Reaction was stopped by the addition of ethanediol (BDH, Analar).

2.7.2. Alternative method for ELISA inhibition reactions.

An alternative method was developed for the ELISA inhibition reaction. 100*ul* of antigen diluted to 25*ug*/ml (protein) was bound to the microtitre plate as described above. Each antigen was used to inoculate 7 wells. Following washing of the plate, 100*ul* of potential inhibitor diluted in PBS (pH 7.8) was added to each of the wells respectively. Potential inhibitors were:

i. 0.01M sodium periodate solution,

ii. 0.02M sodium periodate solution,

iii. 40u/ml trypsin (Sigma),

iv. 400u/ml trypsin,

v. 10 PUK units/ml pronase (BDH),

vi. 0.2u/ml proteinase K (Protease-Sigma).

The final well received 100*ul* PBS (as uninhibited control).

Plates were incubated for 2 hours at room temperature, washed, and shaken dry before the addition of 100*ul* antiserum diluted to twice titre. From this point, the procedure was as described for conventional ELISA.

2.8. Protein assay.

Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin (2mg/ml) as standard.

2.9. Carbohydrate assay.

Carbohydrates, as glucose equivalents, were assayed by the phenol sulphuric acid method (Dubois *et al.* 1956).

2.10. Phosphate assay.

Organic and inorganic phosphate was estimated by the method of Chen *et al.* (1956).

2.11. Choline assay.

Choline was determined by the method of Appleton *et al.* (1953).

2.12. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

The method was modified from that of Laemmli (1970), as described by Poxton and Brown (1979). Vertical slab gels (170mm x 140mm x 1mm), consisting of a stacking gel (10mm of 4% acrylamide gel) above a 10% acrylamide separating gel were used to run up to 20 samples in a BioRad Slab Gel Apparatus (BioRad Laboratories Ltd., Watford). Samples, after boiling in a waterbath for 3 minutes, were loaded to wells in the stacking gel and electrophoresed at 60V until they had entered the separating gel as a sharply focussed line (1-2 hours). The voltage was subsequently increased to 150V until the sample buffer front (indicated by bromophenol blue) had travelled 7.5cm from the top of the separating gel (2-3 hours).

Details of the buffers are given in appendix 4.

2.13. SDS-free-PAGE.

A modification of the above method used gels prepared from buffers containing no SDS. In every other respect, the method was identical to that described above.

2.14. Staining of polyacrylamide gels.

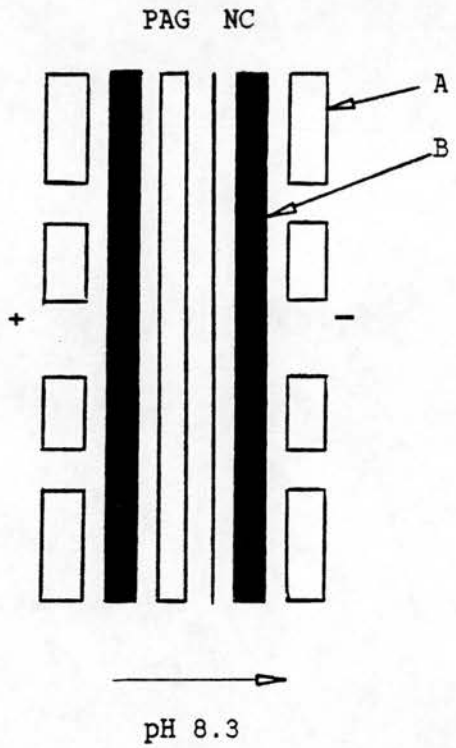
Gels containing proteins were silver-stained by the method of Tsai and Frasch (1982).

Gels containing carbohydrates were silver-stained by a method adapted from that of Tsai and Frasch (1982). Gels were fixed in 40% ethanol (v/v) and 5% acetic acid (v/v) overnight. Oxidisation was with 100ml of freshly prepared 0.7% (w/v) periodic acid in 40% ethanol and 5% acetic acid for 45-60 min. This was followed by washing for 3 x 30 minutes in distilled water. Fresh ammoniacal silver nitrate solution was added (1.4ml ammonia solution plus 21ml 0.36% NaOH with 4ml of 19.4% $AgNO_3$, made up to 100ml with distilled water) for 15 minutes, after which gels were washed in distilled water (2 x 10 minutes). Gels were transferred to fresh 0.005% (w/v) citric acid in 0.19% formaldehyde, and allowed to develop until the required staining intensity was reached. The staining reaction was stopped by repeated washing in large volumes of distilled water.

2.15. Immunoblot transfer.

The method of Towbin *et al.* (1979), as described by Poxton *et al.* (1984) was followed. Extracts separated by PAGE (SDS-free) were transferred to nitrocellulose membrane (Schleicher and Schuell, Dassel, West Germany), in an immunoblotting tank (See figure 2.2). Electrophoresis was at 12V (40mA) for 18 hours at 4°C. The membrane was washed (10 minutes) in Tris-buffered saline (TBS: 20mM Tris, 500mM NaCl, pH 7.5), and the unbound sites blocked with a solution of 3% gelatin in TBS for 45 minutes. The membrane was then transferred to antiserum (1st antibody) diluted in TBS containing 1% gelatin (antibody buffer) and incubated at room temperature for 3 hours. Following a brief wash in distilled water, and two 10 minute washes in

Figure 2.2. Diagram illustrating immunoblot transfer from polyacrylamide gel to nitrocellulose membrane.



Key:

A = Plastic cassette.

B = Scotchbrite™ pad which supports the PAG and NC within the cassette.

PAG = Polyacrylamide gel.

NC = Nitrocellulose membrane.

0.025% Tween 20 TBS (TTBS), the membrane was placed into goat-anti-rabbit IgG horseradish peroxidase (HRP) conjugate (2nd antibody; ICN Biomedicals, High Wycombe, Bucks.) diluted 1 in 3,000 in antibody buffer. After 1 hour, the membrane was washed as before, and placed into a solution consisting of: 30mg HRP colour development reagent (BioRad), 10ml ice-cold methanol, 30*u*l H_2O_2 and 50ml of TBS. Colour development was allowed to proceed until the desired intensity was reached, and the reaction stopped by repeated washing in distilled water. All steps were performed with gentle shaking.

2.16. Dot-Blotting.

Nitrocellulose membrane (Schleicher and Schuell, Dassel, West Germany) was washed for 10 minutes in TBS, and allowed to dry on a sheet of filter paper in air at room temperature (10-15 minutes). Samples (2*u*l) were applied using a micropipette, and allowed to dry. When the dots were dry, the membrane was blocked in 3% gelatin and treated exactly as for immunoblotting (see section 2.15).

2.17. Extraction of whole bacterial cells with ethylenediaminetetra-acetic acid (EDTA). (Standard method).

Following overnight culture in 500ml THB, cells were harvested by centrifugation (20,000*g*, 10 minutes, 4°C), and washed three times in 150ml PBS. The washed pellet was resuspended in 5ml 10mM EDTA (BDH Chemicals, Thornliebank, Glasgow) in PBS by vortexing (10 seconds), and placed in a 45°C waterbath for 1 hour. Bacterial suspensions were re-vortexed for 15 seconds at 30 minutes. Supernatants were collected after two cycles of centrifugation at 10,000*g* (2.5 minutes, room temperature). This material was used undialysed as antigen.

2.18. Preparation of cell-walls.

Cell-walls were prepared essentially as described by Hancock and Poxton (1988).

2.18.1. Preparation of crude cell-walls.

Starter cultures were prepared by inoculation of 100ml THB with >10 colonies of organism from a fresh blood agar culture, and incubated overnight at 37°C.

Starter culture was used to inoculate 8-10l THB which was then incubated for 18 hours at 37°C. Cells were harvested by centrifugation at 19,000*g* for 10 minutes at 4°C and washed twice in 0.85% NaCl, following which the pellet was frozen at -20°C overnight. Purity of all broth cultures was checked by Gram-filming, and plating-out on blood agar.

The thawed bacteria were resuspended in 40ml 0.05M-Tris-HCl, pH 8.0, containing 1mM $MgCl_2$, 0.2mM dithiothreitol (disruption buffer) and 50µg each of RNAse A and DNAse 1 (Sigma) per ml. The cell-suspension was disrupted by passage through a pre-chilled French Pressure Cell (Aminco, Silver Springs, Maryland, USA) at 10^7 Pa. Three passages were required to provide adequate disruption (as assessed by examination of wet films under phase-contrast microscopy). Unbroken cells were removed by centrifugation at 6,000*g* for 30 minutes at 4°C. Removal of unbroken cells was checked by examination of wet-films, and centrifugation repeated if necessary. Crude cell-walls were harvested by centrifugation at 44,500*g* for 30 minutes at 4°C, and washed in disruption buffer, with the addition of KCl to 0.2M. The supernate (containing the membranes) was frozen (-20°C) and lyophilised.

2.18.2. Purification of crude wall preparations.

Crude walls were resuspended in the minimum volume of ice-cold water, and

added dropwise to boiling 4% SDS (40ml). The suspension was kept at boiling-point for 15 minutes, and then stirred overnight at room temperature. SDS-treated walls were harvested by centrifugation at 44,500*g* for 30 minutes at 15°C (to prevent crystallisation of SDS), and washed with four changes of 0.85% NaCl, and two changes of distilled water to remove all traces of SDS. Material was lyophilised and stored at room temperature.

2.19. Extraction of secondary wall polymers from purified cell-walls.

2.19.1. Extraction with dilute HCl.

Lyophilised, purified cell-walls were resuspended to an approximate concentration of 0.8% (w/v)in 0.1M HCl, and incubated for 90 minutes at 37°C. Samples were placed on ice and neutralised with 1M aqueous ammonia. Neutralised suspensions were centrifuged at 40,000*g* for two periods of 1 hour at 4°C to sediment the extracted walls. Supernates were retained, and desalted by dialysis against distilled water for two periods of 24 hours. Desalted material was lyophilised, and stored at room temperature.

2.19.2. Extraction with dilute NaOH.

The method was adapted from that of Archibald *et al.* (1969). Purified cell-walls were extracted with 0.5M NaOH at an approximate concentration of 0.8% (w/v), by stirring for 90 minutes at room temperature. Samples were placed on ice and neutralised with 0.5M HCl. Removal of wall fragments and desalting was performed as described in section 2.19.1.

2.19.3. Extraction with trichloroacetic acid (TCA).

Purified walls were resuspended in ice-cold distilled water to an approximate concentration of 0.8% (w/v). An equal volume of 10% (w/v) TCA

was then added, and the resultant suspension stirred for 48 hours at 4°C. Extracted walls were again removed as in section 2.19.1. TCA was removed by six successive partitions against an equal volume of diethyl ether, and the remaining ether allowed to evaporate off before storage of the extract.

2.19.4. Extraction by autoclaving in saline.

Extraction was performed essentially as described by Rantz and Randall (1955).

Purified walls were resuspended to approximately 0.8% (w/v) in sterile 0.85% saline (pH 7.2). Wall-suspensions were then autoclaved at 121°C for 20 minutes. Following cooling, extracted walls and salts were removed as in section 2.19.1.

Extracts were stored either in aqueous solution at -20°C, or lyophilised at room temperature before further purification.

2.20. Purification of crude secondary wall polymers.

Purification of cell-wall extracts was achieved by anion-exchange chromatography on a column (18cm x 15mm) of DEAE-cellulose (DE-52, Whatman). Samples, re-suspended in distilled water, were washed on with a downward flow of distilled water (100ml), and 50 fractions of 2ml collected. Subsequently, the column was eluted with a downward flow of 0-1M pyridinium acetate buffer, pH 5.3 from a gradient mixer. Pyridinium acetate buffer was prepared by the addition of pyridine (BDH, Analar) to a 1M aqueous solution of acetic acid until the pH was 5.3. Fifty 2ml fractions were again collected. All fractions were assayed for carbohydrate. Column

fractions were additionally examined for immunological activity with homologous antiserum by immunoelectrophoresis.

2.21. Preparation of membrane antigen.

Membrane fraction was prepared from mechanically disrupted cells harvested from overnight broth cultures (see section 2.18.1.). The lyophilised supernate was finely divided and defatted with two successive overnight extractions in 200ml of chloroform/methanol (2:1 v/v). Following filtration through a Whatman No. 1 filter paper, the residue was dried in a 37°C incubator overnight, ground to a fine powder, and weighed. Defatted membranes were extracted with phenol by a method adapted from that of Coley *et al.* (1975). The membranes were mixed with distilled water to give a 10% (w/v) suspension. An equal volume of 80% (w/v) aqueous phenol was added, and the mixture stirred for 30 minutes at room temperature. This was centrifuged at 2,500*g* for 20 minutes at 4°C. The upper, aqueous phase was carefully removed and transferred to a clean centrifuge tube at 4°C. An additional 2ml distilled water was added to the phenol phase, which was vortexed, and centrifuged again. The aqueous phase was again removed and pooled with the previous fraction before centrifugation at 10,000*g* for 10 minutes at 4°C. The supernate was dialysed against running water overnight, after which an equal volume of 0.2M sodium acetate/acetic acid buffer, pH 5.0, containing 0.02M magnesium chloride was added. DNAse 1 and RNAse A (Sigma) were mixed with the extract, a drop of toluene placed on the surface of the fluid to prevent bacterial growth, and the flask covered with a foil lid for overnight incubation at 37°C. The extracts were subsequently re-extracted with an equal volume of phenol as before. After overnight dialysis against running water, material was dialysed against

distilled water for a further 2 hours, lyophilised, weighed, and stored in a sealed container at room temperature.

Crude membrane antigen was purified by gel filtration on a Sepharose 6-B column (58cm x 15mm - Pharmacia, Uppsala, Sweden), with 0.1M sodium acetate/acetic acid buffer, pH 4.7 (Fischer *et al.*, 1983), by upward pumped flow. Fractions (60 x 2.3ml) were collected, and assayed for carbohydrate, phosphate and nucleic acids (A_{260}). Column fractions were additionally screened for immunological activity with homologous antiserum by immunoelectrophoresis, or by dot-blotting.

Peak fractions were pooled and desalted on a 10ml Sephadex G25 column with distilled water. Desalted fractions were lyophilised and stored at room temperature.

2.22. Immunoelectrophoresis.

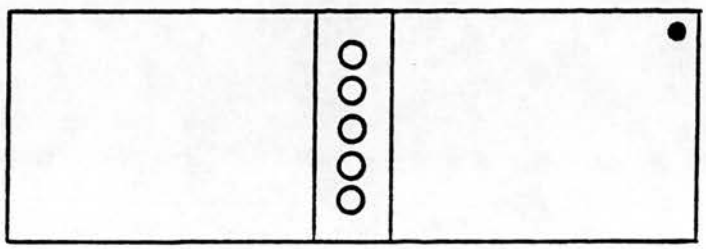
Immunoelectrophoresis was performed with Shandon Southern electrophoresis apparatus (Camberley, Surrey). The barbital/glycine electrophoresis buffer (pH 8.8) described by Svendsen (cited by Weeke, 1973a) was used both in the wells of the electrophoresis tank, and in the agarose used to cast gels. Details of the preparation of buffers and agarose gels for use in immunoelectrophoresis are given in appendix 4.

Antibody-free agarose gel (15ml) was initially cast onto the surface of a clean, acetone-wiped glass slide (8cm x 8cm). Antibody-containing gels were cast onto appropriately sized pieces of GelBond film (Marine Colloids).

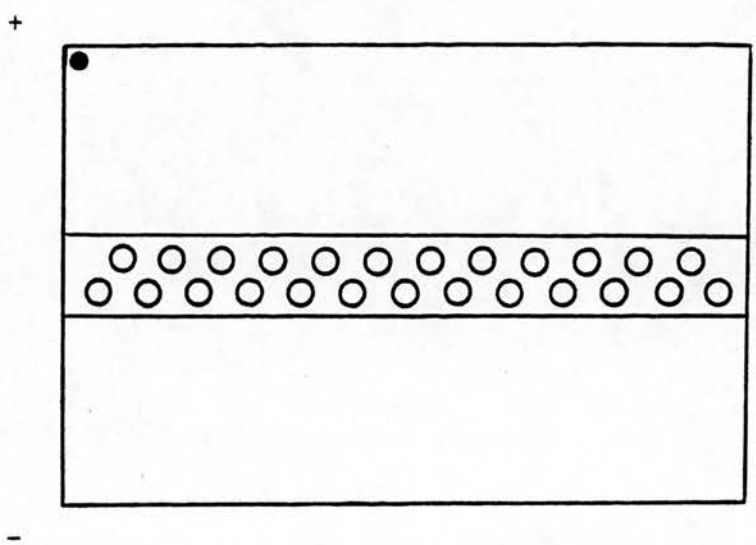
A variety of templates was employed, some of which are illustrated in figures 2.3. and 2.4.

Figure 2.3. Some of the templates used in the preparation of gel-slides for RIE (a) and FRIE (b).

a.



b.



● indicates the marker used to allow correct orientation of gels.

2.22.1. Rocket immunoelectrophoresis [RIE] (Weeke, 1973b) and fused-rocket immunoelectrophoresis [FRIE] (Svendsen, 1973).

RIE: Wells were cut in a block of antibody-free gel placed centrally on a piece of GelBond film measuring 100mm x 30mm (see figure 2.3.a.). Antiserum (250μl) was added to molten agarose gel (1.5ml, 50°C), and a total volume of 1.75ml antibody-containing gel cast onto each half of the gel-slide. A sample of antigen (5-10μl) was added to each well, and the slide electrophoresed at 12V/cm (120V) overnight at 4°C.

FRIE: Wells were cut in a block of agarose placed centrally on a piece of GelBond film measuring 900mm x 600mm (see figure 2.3.b.). A sample (5-10μl) was added to each well, and the slide held in a moist-chamber at 4°C for 30 minutes to allow diffusion of samples. Antibody (250μl) was added to 2.8ml molten agarose (50°C) and 3.05ml antibody-containing gel cast onto each half of the gel-slide. Electrophoresis was again at 12V/cm (72V), overnight at 4°C.

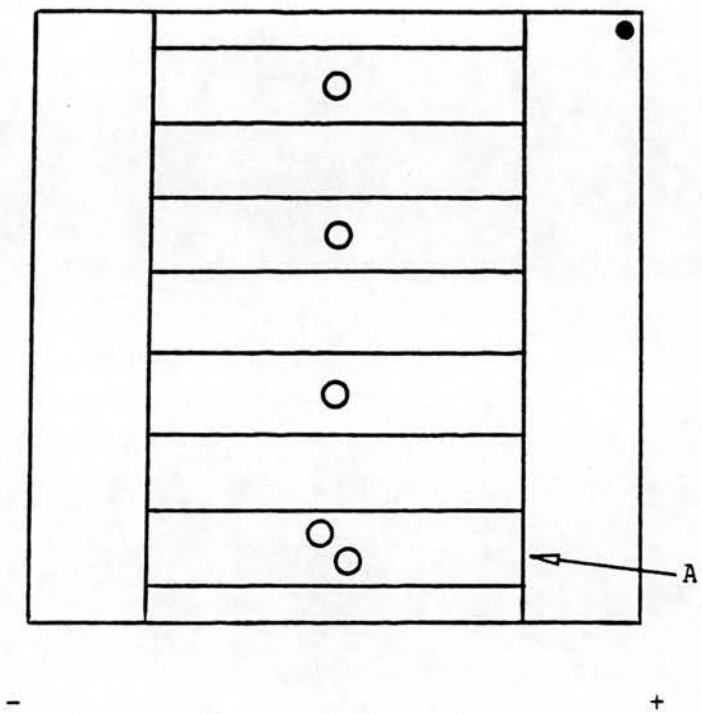
2.22.2. Crossed-immunoelectrophoresis [CIE]. (Weeke, 1973c).

First dimension: Agarose (15ml) was cast onto a glass slide (8cm x 8cm), and wells punched as illustrated in figure 2.4.a. Wells were loaded with sample (2.5-10μl), and the slide electrophoresed at 12.5V/cm (100V) for 1.5-2 hours at 4°C.

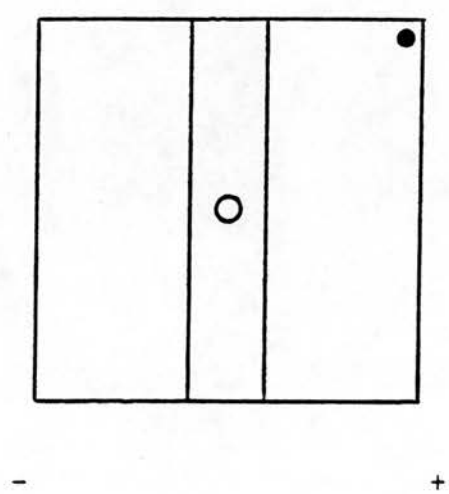
Second dimension: Blocks of agarose were cut from the first-dimension gel in a plane parallel to the line of first-dimension electrophoresis (see figure 2.4.a.), and placed centrally on a piece of GelBond film measuring 50mm x 50mm (see figure 2.4.b.). Antibody (62.5-500μl) was added to molten agarose (3ml, 50°C), and an equal volume cast on each side of the gel-

Figure 2.4. Some templates used in the preparation of gel-slides for first (a) and second (b) dimension CIE.

a.



b.



● indicates the marker used to allow correct orientation of gels.

A represents the placement of wells in first-dimension TCIE.

slide. Electrophoresis was at 12V/cm (60V) overnight at 4°C in a plane normal to that of the first dimension (see figure 2.4.b.).

2.22.3. Tandem-crossed-immunoelectrophoresis (TCIE). (Modified from Kroll, 1973).

In TCIE, two wells were prepared in each track of the first-dimension gel (see figure 2.4.a.(A)), and loaded with antigen. In every other respect, the procedure was performed in exactly the same manner as CIE.

2.22.4. Staining of agarose gels. (Weeke, 1973a).

Following electrophoresis, gels were covered with Whatman No. 1 filter paper cut to the same size, and pressed under 2-3cm of weighted blotting paper for 15 minutes. Paper was removed, and the pressed gels washed for two periods of 15 minutes in 0.1M NaCl, and once in distilled water. Following a further period of pressing for 15 minutes, gel-slides were dried to a thin film using a hairdryer. Dried gels were stained for at least 30 minutes in a solution containing: 5% (w/v) Coomassie Brilliant Blue R-250; 45% (v/v) ethanol; 10% (v/v) glacial acetic acid, and 45% (v/v) distilled water.

Destaining was achieved in a solution comprising: 45% (v/v) ethanol; 45% (v/v) distilled water, and 10% (v/v) glacial acetic acid. Following destaining, gel-slides were again dried to a thin film using a hairdryer, and stored indefinitely without deterioration.

2.23. Preparation of purified secondary wall polymers, and membrane-associated antigens for chemical analysis.

2.23.1. Hydrolysis of carbohydrate polymers.

Cell-wall and membrane carbohydrate antigens were hydrolysed in 2M HCl at 100°C for 2 hours, and dried several times *in vacuo* over NaOH pellets and phosphorus pentoxide. The hydrolysate was dissolved in 1.5ml distilled water and 0.75ml stored at 4°C before examination by paper-chromatography. The remaining 0.75ml of carbohydrate solution was converted to alditol acetates by a method modified from that of Lindberg *et al.* (1978), (see section 2.23.3.).

2.23.2. Hydrolysis of material for amino-acid analysis.

Samples were hydrolysed in 6M HCl at 100°C, for 6 hours. Hydrolysed material was dried and freed from acid *in vacuo* over NaOH and phosphorus pentoxide as described in section 2.23.1. The hydrolysate was subsequently taken up in 0.75ml distilled water and stored at 4°C until used.

2.23.3. Preparation of alditol acetates.

Dry hydrolysates of carbohydrates (see section 2.23.1.) were dissolved in 0.75ml distilled water, and made alkali by the addition of 100*ul* 18M aqueous ammonia solution. Alditols were prepared by the addition of 10mg potassium borohydride to each sample, and incubating at 20°C overnight. Excess borohydride was destroyed by the dropwise addition of glacial acetic acid, and removed as methyl borate by three distillations with methanol (3ml). Dry alditols were acetylated in 5ml pyridine/acetic anhydride (1:1, v/v) at 100°C for 60 minutes. Excess pyridine/acetic anhydride/acetic acid was removed by several co-distillations with toluene in a rotary

evaporator, and the alditol acetates taken to dryness. Alditol acetates were taken up in 1ml chloroform, washed three times with an equal volume of distilled water, dried by rotary evaporation, and finally dissolved in 100μl chloroform. Samples were subsequently examined by gas chromatography.

2.24. Gas chromatography.

Samples (1μl) were examined using a Perkin Elmer 8410 gas chromatograph fitted with a single flame ionisation detector, split injector, and data handling facility.

2.24.1. Detection of pentoses, and hexoses.

For pentose and hexose derivatives, a fused silica capillary column (25m x 0.22mm internal diameter) coated with a 0.2μm film of CP-Sil 84 liquid phase (Chrompak) was used. The temperature was controlled as follows: 220°C for two minutes, followed by a 1°C/minute rise in temperature to 240°C at which the temperature was held for 3 minutes. The carrier gas was helium, at a pressure of 17.5 psi (2.54 x 10^{-3}Pa), giving a flow-rate of 0.6ml/minute.

2.24.2. Detection of amino-sugars.

Amino-sugars were detected using a fused silica capillary column (25m x 0.22mm internal diameter) coated with a 0.25μm film of BP20 bonded liquid phase (SGE). The temperature was controlled as follows: 220°C for 4 minutes, followed by a 2°C/minute rise in temperature to 240°C at which the column was held for 4 minutes. The temperature was then increased again by 2°C/minute to 250°C where it was held for 27 minutes. The carrier gas was again helium, at 15 psi (2.175 x 10^{-3}Pa), giving a flow-rate of 0.55ml/minute.

2.25. Paper chromatography.

Hydrolysates were examined by descending chromatography on Whatman number 1 paper, using Shandon Southern paper chromatography apparatus. Chromatographs were conveniently run overnight.

2.25.1. Detection of polyols.

Samples were separated by a solvent comprising: propan-1-ol/aqueous ammonia (S.G. 0.88)/ water (6:3:1, by volume). Polyols were stained with the periodate-Schiff reagents (Baddiley *et al.*, 1956).

2.25.2. Detection of reducing sugars.

Sugars were separated by a solvent comprising: butan-1-ol/pyridine/water (6:4:3, by volume). Reducing sugars were stained with the alkaline silver nitrate reagents (Trevelyan *et al.*, 1950).

2.25.3. Detection of amino-acids.

Amino-acids were separated by a two-phase solvent comprising: butan-1-ol/acetic acid/water (4:1:5) (Dawson *et al.*, 1969). Amino-acids were stained with 0.5% indane-trione hydrate in butan-1-ol (Ninhydrin, BDH).

CHAPTER 3

THE ISOLATION AND CHARACTERISATION OF *STREPTOCOCCUS MILLERI* FROM THE HUMAN ORAL CAVITY AND OTHER SOURCES

3.1. Introduction and aims.

The distribution of *Streptococcus milleri* amongst the complex mixed flora of the human oral cavity was considered in section 1.5.2. In this chapter, a study is described in which a collection of *Streptococcus milleri* isolates recovered mainly from the mouth, but also from "clinical" material, in addition to reference strains, was investigated by means of biochemical and serological tests. "Clinical" isolates were received from the Clinical Bacteriology Laboratory, University of Edinburgh, the identification being either: group F streptococcus, or *Streptococcus milleri*. A list of the "clinical" and reference strains included in this study is given in table 3.1.

The major aims of this study were as follows:

1. To design a simple and reliable protocol for the recovery of *Streptococcus milleri* isolates from defined sites in the human oral cavity.
2. To develop a simple, rapid, and cheap battery of biochemical and serological tests for the presumptive identification of *Streptococcus milleri* isolates, and to apply it to a collection of streptococcal isolates from a range of sources.
3. To establish a culture collection of Lancefield group F streptococci which would form the basis of further studies on this group of organisms.

Full details of the experimental procedures are included in sections 2.2. and 2.3.

TABLE 3.1. Reference and "clinical" strains used in this study, and their source.

1. List of reference strains:

NUMBER.	SPECIES.	GROUP.*	TYPE.$	SOURCE.
NCTC 10707	*Streptococcus milleri*	F	None	NCTC, London.
NCTC 10714	*Streptococcus milleri*	F	I	NCTC, London.
NCTC 5389	*Streptococcus milleri*	F	II	NCTC, London.
NCTC 8037	*Streptococcus milleri*	F	III	NCTC, London.
NCTC 11065	*Streptococcus milleri*	None	IV	NCTC, London.
NCTC 11063	*Streptococcus milleri*	None	II	NCTC, London.
NCTC 11169	*Streptococcus milleri*	None		Dr.T.W. MacFarlane, Glasgow Dental School.
NCTC 10708	*Streptococcus milleri*	None		Dr. T.W. MacFarlane, Glasgow Dental School.
NCTC 7864	*Streptococcus sanguis*			M.P.R.L. stock culture collection.
NCTC 8606	*Streptococcus salivarius*			M.P.R.L. stock culture collection.
NCTC 10449	*Streptococcus mutans*			M.P.R.L. stock culture collection.
NCTC 10712	*Streptococcus mitior*			M.P.R.L. stock culture collection.

2. List of "clinical" isolates:

NUMBER.	DIAGNOSIS.	HAEMOLYSIS.	SOURCE.
JW1	*Streptococcus milleri*	None	High-vaginal swab.
JW2	*Streptococcus milleri*	None	High vaginal swab.
JW3	Group F streptococcus	Beta	Pilonidal sinus.
JW5	Group F streptococcus	Beta	Intra-oral swab: infected dental cyst.
JW6	Group F streptococcus (API: *Streptococcus faecium*)	Alpha	Skin swab: special care baby unit.
JW8	Group F streptococcus	None	Throat swab.
JW9	*Streptococcus milleri*	None	Arm abscess.
JW10	Group F streptococcus	Alpha	Denture swab.
H957	Group F streptococcus	Beta	Chin abscess.

* = Applies to *Streptococcus milleri* isolates only.

$ = Ottens and Winkler type - applies to *Streptococcus milleri* isolates only.

Consideration is also conveniently given here to the production of antisera in rabbits against *Streptococcus milleri* reference strains (see sections 2.4. and 2.5 for details of the inoculation schedule, and the storage of sera respectively). Antiserum raised against a group F reference strain (NCTC 10707) was used in diagnostic serology in the current study.

Some of the results of an earlier pilot study on the recovery of group F streptococci from the oral cavity have been presented elsewhere (Whitworth *et al.*, 1987).

Significant differences exist between the experimental procedures used in the previously reported study and those presented in this thesis. Differences exist in sampling protocol, in the use of selective media, and also in both the range and means of performing biochemical and serological tests for the presumptive identification of isolates. The results of this pilot study will consequently not be presented in the following chapter of this thesis, other than to discuss some of the problems previously encountered in the light of the system finally used.

3.2. Results.

3.2.1. Characterisation of a collection of streptococcal reference strains using a short series of tests.

A collection of reference strains was first examined using the short series of biochemical tests described in section 2.3. to establish the ability to differentiate *Streptococcus milleri* from other oral streptococci, and to examine the reproducibility of results.

Table 3.2. shows the results obtained for a number of *Streptococcus milleri*, and other oral streptococcal reference strains when subjected to the short series of biochemical tests. All tests were repeated in triplicate, and gave absolutely reproducible results for all strains on three consecutive days.

RCUT tests were clearly read after four hours, and VP tests after five hours. Determination of arginine hydrolysis required incubation for 24 hours, and determination of aesculin hydrolysis often required incubation for 48 hours before a definitive reading could be made.

Eight *Streptococcus milleri* reference strains formed a relatively homogeneous group on the basis of the short scheme of tests. No *Streptococcus milleri* isolates displaying a wide carbohydrate-utilisation pattern (Ball and Parker, 1979; Ruoff and Kunz, 1982) were represented amongst this reference collection. Two beta-haemolytic *Streptococcus milleri* reference strains (NCTC 10714 and NCTC 11063) were unable to hydrolyse aesculin either by the tube (Brown *et al.*, 1989), or by the plate method (Cowan and Steel, 1965). Each of these methods was employed on a further two occasions, with incubation for 48 hours. In all cases, negative results were recorded for NCTC 10714 and NCTC 11063.

Although differentiating *Streptococcus milleri* strains quite clearly from

Table 3.2. Biochemical characterisation of some streptococcal reference strains.

Strain	MAN[1]	RAF[1]	SOR[1]	MEL[1]	TRE[1]	ARG[2]	AESC[2]	VP[3]
NCTC 7864	-	+	-	+	+	-	-	-
NCTC 8606	-	+	-	+	+	-	+	-
NCTC 10449	+	+	+	+	+	-	+	+
NCTC 10712	-	+	-	+	+	-	-	-
NCTC 10707	-	-	-	-	+	+	+	+
NCTC 10714	-	-	-	-	+	+	-	+
NCTC 5389	-	-	-	-	+	+	+	+
NCTC 8037	-	-	-	-	+	+	+	+
NCTC 11065	-	-	-	-	+	+	+	+
NCTC 11063	-	-	-	-	+	+	-	+
NCTC 10708	-	-	-	-	+	+	+	+
NCTC 11169	-	-	-	-	+	+	+	+

Key.
1. Rapid carbohydrate utilisation test:
MAN = Mannitol. RAF = Raffinose.
SOR = Sorbitol. MEL = Melibiose.
TRE = Trehalose.
2. Hydrolysis reactions:
ARG = Arginine. AESC = Aesculin.
3. Production of acetoin from glucose:
VP = Voges-Proskauer reaction.

\+ = Positive test reaction.
\- = Negative test reaction.

the single representatives of *Streptococcus sanguis*, *Streptococcus salivarius*, and *Streptococcus mitior*, differentiation amongst the latter three strains was not clear. The single *Streptococcus mutans* strain (NCTC 10449) was identified clearly by its wide carbohydrate utilisation profile, positive VP reaction, and failure to hydrolyse arginine.

Reference strains were also subjected to serological identification as described in section 2.3.5. Results were readable within four hours.
In all cases, agreement was obtained between antiserum raised against NCTC 10707 (see section 3.2.3.), and commercial group F antiserum in the serological identification of group F streptococci. NCTC 10707, NCTC 10714, NCTC 5389, and NCTC 8037 were correctly identified as group F streptococci. None of the other reference strains reacted with the range of antisera used.

3.2.2. Oral sampling.

The use of a simple sampling procedure, in combination with a single selective medium allowed the isolation of 177 streptococcal isolates which were examined further. Within this group of organisms, it was possible to make the presumptive diagnosis of *Streptococcus milleri* on the basis of the short series of biochemical and serological tests described. In all, 127 *Streptococcus milleri* isolates were identified from the mouths of ten of the twelve subjects sampled.

The biochemical and serological results obtained for 127 oral *Streptococcus milleri* isolates recovered in this study are shown in table 3.3. Biochemical and serological tests were performed twice (on consecutive

Table 3.3. Summary of the characteristics of 127 *Streptococcus milleri* isolates recovered from the mouths of ten volunteers.

		Number of isolates positive	(%)
a. Biochemical tests			
Mannitol		6	4.7
Raffinose		6	4.7
Sorbitol		6	4.7
Melibiose		2	1.6
Trehalose		119	93.7
Arginine		118	92.9
Aesculin		103	81.1
VP		119	93.7
b. Serological group	A	0	0
	C	1	0.8
	F	46	36.2
	G	7	5.5
	No group	73	57.5
c. Haemolysis	Alpha	33	26.0
	Beta	5	3.9
	None	89	70.1

days) for each isolate, and were entirely reproducible. The execution of biochemical and serological tests was very simple, and it was possible to set up all diagnostic tests from a single blood-agar culture plate inoculated to give a large well covering over 2/3 of the plate.

Streptococcus milleri formed a relatively homogeneous group of organisms, with most isolates fermenting trehalose, hydrolysing arginine and aesculin, and giving a positive VP reaction. A small number of isolates was found to be able to utilise mannitol, raffinose, sorbitol or melibiose (see table 3.3.). The majority (70.1%) of isolates were non-haemolytic, and of the five (3.9%) beta-haemolytic isolates, three were group G, and two were ungroupable within the range of antisera used. No beta-haemolytic group A, C, or F isolates were encountered.

In total, 42.5% of oral *Streptococcus milleri* isolates recovered were groupable within the range of antisera used. No isolates carrying the group A antigen were recovered.

Within the collection of 127 *Streptococcus milleri* isolates, 46 (36.2%) were shown to possess the Lancefield group F antigen (see table 3.3.). In all instances, the results obtained with antiserum raised against NCTC 10707 agreed with the commercial group F antiserum. Including four NCTC reference strains, and nine "clinical" isolates (all of which proved to be group F, see table 3.1.), a total of 59 group F streptococcal isolates was examined. No attempt was made to type group F isolates serologically.

The range of biochemical profiles encountered within this collection of group F streptococci is shown in table 3.4. Thirty eight (64.4%) were non-haemolytic, sixteen (27.1%) alpha-haemolytic, and five (8.5%) beta-haemolytic.

Although the majority (74.6%) of group F isolates formed a biochemically

Table 3.4. Biochemical characterisation of 59 group F streptococci, including oral, "clinical", and NCTC isolates.

No	MAN[1]	RAF[1]	SOR[1]	MEL[1]	TRE[1]	ARG[2]	AESC[2]	VP[3]
44	-	-	-	-	+	+	+	+
4	-	-	-	-	+	+	+	-
2	-	-	-	-	+	+	-	+
2	-	+	-	+	-	+	+	+
2	-	+	-	-	+	-	+	+
1	-	-	-	-	+	-	-	+
1	+	-	+	-	+	+	+	+
1	-	-	-	-	+	+	-	-
1	-	-	-	-	+	-	+	+
1	-	-	-	+	-	-	-	+
Total: 59	1	4	1	3	56	54	54	54
(%) (100)	(1.7)	(6.8)	(1.7)	(5.1)	(94.9)	(91.5)	(91.5)	(91.5)

Key.
No = Number of isolates with given biochemical profile.

1. Rapid carbohydrate utilisation test:
MAN = Mannitol RAF = Raffinose
SOR = Sorbitol MEL = Melibiose
TRE = Trehalose
2. Hydrolysis reactions:
ARG = Arginine AESC = Aesculin
3. Production of acetoin from glucose:
VP = Voges-Proskauer reaction

\+ = Positive test reaction.
\- = Negative test reaction.

homogeneous group, a number of isolates displayed differing biochemical profiles. A single mannitol positive isolate was encountered (JW6, see table 3.1.). This was a highly unusual isolate identified initially by the API-20 Strep system as *Streptococcus faecium*, but found to possess Lancefield group F antigen, both by Streptex (Wellcome) and by the current serological scheme.

No differentiation could be made between group F and non-group F *Streptococcus milleri* isolates on the basis of biochemical tests. Neither could differentiation be made between haemolytic types on the basis of biochemical tests.

This collection of organisms derived from a range of sources, displaying a range of haemolytic activity, and a degree of biochemical heterogeneity was considered to represent a suitably diverse culture collection on which to base further studies of the group F streptococci.

3.2.3. Production of antisera against reference strains.

Details of the immunisation schedule and the storage of sera are given in sections 2.4. and 2.5. respectively.

Test-bleeds (5ml) were obtained in week five, and doubling dilutions of serum in PBS reacted in Ouchterlony gel against hot HCl extracts (Lancefield, 1933) of homologous strains to give an indication of activity. Test-bleed sera from all five rabbits gave clear lines of precipitation against homologous antigenic extracts at dilutions of 1 in 8. This was considered satisfactory, and all five animals were exsanguinated. Volumes of sera obtained from single Dutch rabbits ranged from 30ml to 62ml.

The use of antiserum raised against NCTC 10707 in diagnostic serology was considered in sections 3.2.1. and 3.2.2.

3.3. Discussion.

The streptococci have long been established as organisms of medical importance in man and other animals. Classically, only beta-haemolytic isolates were considered to be noteworthy pathogens, although "viridans" streptococci, often of oral origin were recognised as important agents in infective endocarditis. The development of a rapid and accurate serological means of classifying the majority of haemolytic streptococci (Lancefield, 1933) led to its widespread use by medical microbiologists in identifying the classical purulent varieties. The viridans streptococci cannot be reliably classified within the Lancefield scheme, and despite numerous attempts to design a separate serological grouping scheme (Lancefield, 1925a; b; Solowey, 1942; Selbie *et al.*, 1949; Williamson, 1964; Austrian *et al.*, 1972; Karakawa *et al.*, 1973a; b; c), a satisfactory system has not emerged. Viridans streptococci are necessarily classified by physiological and biochemical tests.

Streptococcus milleri represents a special case, in that its members traverse traditional haemolytic boundaries, and are known to be capable of possessing "C" antigens A, C, F, or G which are believed to be identical to those of their large-colony counterparts. Isolates may be classified as *Streptococcus milleri* on the basis of characteristic biochemical tests, or as with the group F streptococci, on the basis of Lancefield grouping (see section 1.2.).

Streptococcus milleri has been described by many workers as a relatively homogeneous group in terms of behaviour in biochemical and physiological tests (Colman and Williams, 1972; Mejàre and Edwardsson, 1975; Hardie and Bowden, 1976; Hardie *et al.*,1982; Parker and Ball, 1976; Lütticken *et al.*, 1978). Behaviour is not, however, entirely uniform. Although their comments

were not confined to *Streptococcus milleri*, Parker and Ball (1976) noted that "the cultural and biochemical characters of individual strains in each recognised (streptococcal) species or group were far from uniform, and few tests were free from occasional anomalous results". Referring specifically to *Streptococcus milleri* isolates from a range of sources, Ball and Parker (1979) reported "considerable variation in single and in small groups of characters". In addition to a central group of *Streptococcus milleri* isolates displaying typical biochemical reactions (Colman and Williams, 1972: see section 1.2., p 28), two main types of deviation from this pattern were noted:

1. Those displaying "loss" of one or more reactions, and
2. those "gaining" the ability to acidify additional sugars, notably raffinose, melibiose, or mannitol.

Characteristically, though not exclusively, isolates displaying wide carbohydrate utilisation profiles have been associated with the female genital tract (Ball and Parker, 1979; Ruoff and Kunz, 1982).

The current study reported in the preceeding pages utilised a dichotomous (physiological and serological) system for the identification of *Streptococcus milleri* isolates, and within them Lancefield group F streptococci.

3.3.1. Development of a protocol for the presumptive identification of *Streptococcus milleri*.

Earlier studies on the identification of group F streptococci (Whitworth *et al.*, 1987) employed a series of biochemical tests executed in the

traditional way, by observing the production of acid in peptone water culture containing 1% (w/v) sugar and 1% (v/v) Andrades indicator. The use of such conventional biochemical tests in the identification of streptococci has been described as slow, tedious and time-consuming (Tillotson, 1982). Tests were dependent upon bacterial growth, and incubation for up to 72 hours was often required before results could be read, particularly with slow-growing isolates. The VP test was not included in the range of diagnostic tests employed.

This system was found to be fraught with problems in the hands of an inexperienced single-handed operator dealing with the identification of a large number of clinical isolates. Not only were tests time-consuming, but results were often found to be unclear, even after prolonged incubation, and the attendant problem of test contamination was a real consideration. Considerable variation in biochemical profile was noted amongst *Streptococcus milleri* isolates using this system.

Serological grouping was routinely performed in Ouchterlony gel using hot HCl extracts (Lancefield, 1933) of test isolates harvested from overnight broth-cultures in THB (100ml) against a range of antisera. The use of HCl extracts was found to be satisfactory in terms of accuracy, but less so both in terms of the time required to grow-up broth cultures, harvest and wash cells, and then perform extraction, and in terms of the cost of consumables. An attempt was also made to type group F streptococcal isolates serologically using appropriately absorbed sera raised against NCTC reference strains (see section 2.4. and table 3.1). The results of typing procedures were most unsatisfactory, because of multiple cross-reactions appearing between typing sera. Whilst failing to yield useful diagnostic information, and prompting its discontinuation in the current

research, the observation of many cross-reactions within the group suggested that further work was required to investigate the antigenic determinants of these organisms. Greater consideration will be given to some immunochemical aspects of this group of organisms in the following chapters of this thesis.

A system for the determination of carbohydrate utilisation profiles by detecting the presence of preformed enzymes was shown to be a more reliable means of identifying isolates than previously described methods. Inoculation of tests from a pure plate culture, in addition to short incubation times in non-nutrient medium reduced to a minimum the potential problems of contamination. The small volumes of reagents used, the absence of need for broth starter-culture, and the use of non-sterile microtitre plates kept the cost of media and consumables low.

The range of biochemical tests was based on the short identification schemes of Hardie and Bowden (1976), and Manning and Hogg (1987). A minimum number of tests was selected for the presumptive identification of *Streptococcus milleri* isolates, but allowing the recognition of isolates with wide carbohydrate utilisation profiles described previously (Ball and Parker, 1979; Ruoff and Kunz, 1982).

For serological identification, the nitrous acid (El Kholy *et al.*, 1978) method of extraction was used in the current study for the following reasons:

1. This technique has been shown to be highly efficient in extracting C antigen from streptococci (Hodgins and Raybould, 1988),
2. It has been shown (El Kholy *et al.*, 1974) that amplification of lines of immunoprecipitation in gel occurs when this method of extraction is

used. Salting-out of sodium acetate along lines of immunoprecipitation was shown by El Kholy *et al.* (1974) to produce a 20-fold increase in the density of bands.

3. As a consequence of 1. and 2., small amounts of test organism taken from the pure culture used to inoculate biochemical tests could be used for extraction, avoiding the need for broth-culture of isolates for diagnostic serology. Another benefit of this was the consequent reduction in time required to obtain a serological result.

4. Since reagents were inexpensive, the El Kholy *et al.* (1978) technique was a cheap method to apply to a large collection of isolates.

The successful use of this technique in the serological grouping of *Streptococcus milleri* isolates was described previously by Lütticken *et al.*, (1978).

3.3.2. Application of a short series of biochemical and serological tests to a collection of streptococcal reference strains.

The short series of biochemical tests was found to be satisfactory for the presumptive identification of *Streptococcus milleri* reference strains. Differentiation of these strains from other oral streptococcal reference strains was easily achieved. Identification of the single *Streptococcus mutans* reference strain was also satisfactory. The capacity of this short series of tests (unmodified) to differentiate all of the common oral streptococci must, however, be questioned. Whilst beyond the scope of the current research, which was aimed at the presumptive identification of *Streptococcus milleri* isolates, a fuller evaluation of this scheme with a far larger collection of oral streptococci of known identity would be

required in order to evaluate the system for this purpose. It would seem reasonable to assume that the results of such studies might suggest the use of supplementary tests, for example: the production of H_2O_2 in culture (Hardie and Bowden, 1976; French *et al.*, 1989a), the production of urease (French *et al.*, 1989a), and the production of extracellular polysaccharide from sucrose (Hardie and Bowden, 1976; Colman and Ball, 1984) to allow more certain identification of all of the common oral streptococci. It was considered highly advantageous in the current study to be able to perform all diagnostic tests (including a sub-culture for repeats on the following day) from growth on a single blood-agar culture plate. The inclusion of a number of supplementary tests may necessitate the inoculation of two blood-agar plates per isolate as the source of culture for a broader range of tests.

Serological identification within this small reference collection was simply and rapidly achieved using the El Kholy *et al.*(1978) technique. The results corroborated with Lütticken *et al.* (1978), and El Kholy *et al.* (1974), who found that the technique could reliably identify group F streptococci.

3.3.3. The recovery of *Streptococcus milleri* isolates from dental plaque.

Streptococcus milleri is a prominent commensal in the human oral cavity, appearing to have a special association with dental plaque deposits in relatively sheltered areas, notably the gingival crevice (Mejàre and Edwardsson, 1975; Yakushiji *et al.*, 1988a). This finding was borne out in the present study in which *Streptococcus milleri,* identified by a short

series of tests was isolated from ten out of twelve mouths sampled. No attempt was made to isolate and identify other members of the complex plaque flora in order to assess the contribution made by *Streptococcus milleri* to whole plaque in the sites sampled. The modification of Carlsson's (1967a) MC agar used in this study proved to be an effective selective medium for the recovery of *Streptococcus milleri*, colony forms being selected with the aid of a hand-lens. These findings are again in agreement with the work of Mejàre and Edwardsson, (1975) and Yakushiji *et al.*, (1988a). Over two-thirds of the streptococcal isolates picked from the surface of MC agar plates in this study were identified as *Streptococcus milleri*. This finding may reflect a high level of *Streptococcus milleri* in the mouths of the subjects sampled, or it may be that the incorporation of sulphadimidine instead of sulphadimetine makes this medium more selective for *Streptococcus milleri*. Studies on the relative resistance of oral streptococci to different sulphonamide preparations may provide an answer to the latter question.

3.3.4. The application of a short series of tests to the presumptive identification of *Streptococcus milleri* isolates from the human oral cavity.

The application of a short series of tests to the identification of streptococcal isolates recovered from the oral cavity allowed rapid, reproducible and cheap presumptive identification of *Streptococcus milleri* isolates.

The results of biochemical tests were generally quite predictable. In common with a large number of other workers (Colman and Williams, 1972; Mejàre and Edwardsson, 1975; Hardie and Bowden, 1976; Hardie *et al.*, 1982;

Lütticken *et al.*, 1978; Yakushiji *et al.*, 1988a) *Streptococcus milleri* isolates from the oral cavity represented a relatively homogeneous group on the basis of the short series of tests used. With few exceptions, isolates fermented trehalose, hydrolysed arginine and aesculin, and produced acetoin from glucose. A small number of isolates fermented mannitol, raffinose, sorbitol, or melibiose. Whilst isolates with wide carbohydrate utilisation profiles are most commonly recovered from the female genital tract (Ball and Parker, 1979; Ruoff and Kunz, 1982), Ball and Parker (1979) reported that they formed 12.5% of isolations from the mouth and respiratory tract. In the current study, only 9.4% organisms presumptively identified as *Streptococcus milleri* possessed wide carbohydrate utilisation profiles (ie: the ability to ferment mannitol, raffinose, sorbitol, or melibiose).

Mejàre and Edwardsson (1975), in their study which included 91 oral *Streptococcus milleri* isolates, found 40 (44%) of them to be alpha-haemolytic, 5 (5%) beta-haemolytic, and 46 (50.5%) non-haemolytic. Yakushiji *et al.* (1988a) on the other hand , found 90% of their isolates to be non-haemolytic, and 10% alpha-haemolytic, with no beta-haemolytic isolates being recovered. The results of the current study are intermediate to these groups with 26% being alpha, 3.9% beta, and 70.1% non-haemolytic. These findings are contrary to the statement of Ball and Parker (1979) who stated that *Streptococcus milleri* strains from the teeth and gums were nearly always non-haemolytic. It is widely accepted that the haemolytic behaviour of streptococci may vary according to the composition, and the type of blood incorporated into blood-agar plates. Haemolytic behaviour must therefore be viewed with a degree of caution in the classification of isolates, and in the comparison of results from different centres.

Ball and Parker (1979) also noted that oral strains of *Streptococcus*

milleri were rarely groupable. Lütticken *et al.* (1978), however, showed that amongst a collection of 86 *Streptococcus milleri* isolates, mainly of oral or circumoral origin, 52% were non-groupable, and 26% possessed group antigen F. Similar results were reported by Yakushiji *et al.*, (1988a), who found 56% of their oral *Streptococcus milleri* isolates to be non-groupable, and 32% group F. Group C and G isolates comprised 8% and 3% respectively, whilst no group A isolates were reported. These results are again in agreement with the current study in which 57.5% of isolates were ungroupable, 36.2% group F, 5.5% group G, and 0.8% group C. It seems clear from these studies that a large proportion of oral *Streptococcus milleri* isolates are groupable, and that of the groupable organisms, group F is by far the most common.

Multiple recoveries of *Streptococcus milleri* were made from the dental plaque of ten subjects in whose mouths it was detected. Whether or not multiple isolates represented clones of the same parent cell is impossible to deduce, but commonly, a number of isolates from the same mouth could be differentiated on the basis of serological, haemolytic, or biochemical reactions.

3.3.5. Establishment of a culture collection of group F streptococci.

A collection of group F streptococci from a number of sources was found to be indistinguishable from a collection of oral *Streptococcus milleri* isolates. Based upon this small collection of isolates, and the small range of diagnostic tests employed, support is once again given to the synonymity of group F streptococcus with *Streptococcus milleri*. This is in aggreement with the work of Colman and Williams, (1972), Parker and Ball (1976) and Ball and Parker (1979). All of the group F isolates were classified, for

the purposes of this study as *Streptococcus milleri* including the mannitol-positive isolate (JW6), which had been identified by the API-20 Strep system as *Streptococcus faecium*. It is possible that this organism had colonised the neonate from the birth-canal during parturition. The shortcomings of the current presumptive identification scheme are consequently highlighted in the identification of this isolate.

In almost universal use in clinical diagnostic laboratories is a more comprehensive system of 20 biochemical tests (API-20 Strep), encompassing sugar fermentation tests, hydrolysis reactions, and enzyme detection tests. Based again on the presence of pre-formed enzymes, and delivering results within hours, the presentation of these kits is in a pre-dispensed form in which substrates are lyophilised. This system has developed into an immensely powerful diagnostic tool, with biochemical test behaviour being translated into a coded number which is in turn translated into an appropriate identification. In circumstances where the diagnosis is indefinite, supplementary tests are often suggested by the manufacturer which will allow clearer identification. This system is backed up by a constantly updated database built up from the profiles demonstrated by an ever-increasing number of clinical isolates. The result is a sharing of diagnostic information from a large number of centres, and a capacity within the system for correct identification even if isolates demonstrate unusual biochemical profiles. At present, three biotypes are identified within *Streptococcus milleri* by this system. Such a situation can seldom arise in a centre using its own series of diagnostic tests, where only the experience of the operator, or the assistance of the Streptococcal Reference Laboratory may be of help in difficult cases. Whilst accepting the clear benefits of this system, and the quality of results obtained, the

major drawback in terms of its use in the present study was its cost. Application of tests costing several pounds per isolate could not reasonably be applied to the screening of large numbers of clinical isolates, and this is reason enough to support the value of cheap presumptive tests.

3.3.6. Summary.

Streptococcus milleri was consistently recovered from dental plaque associated with the gingival crevice by the use of a simple sampling technique.

A rapid, cheap and simple battery of diagnostic tests was developed and satisfactorily used in the identification of *Streptococcus milleri* isolates from dental plaque and other sources. This system would appear to be versatile, and capable of adaptation for the identification of a wider range of oral streptococci if required.

Serological identification of isolates allowed the establishment of a culture collection of group F streptococci of suitably diverse nature to form the basis of further studies on this group of organisms.

CHAPTER 4

STUDIES ON THE NATURE OF ANTIGENIC MATERIAL ASSOCIATED NON-COVALENTLY WITH THE CELL-SURFACE OF GROUP F AND RELATED STREPTOCOCCI

4.1. Introduction.

Review of the literature (see chapter 1) revealed that considerable work was undertaken by Willers and co-workers in Utrecht during the 1960's and early 1970's on carbohydrate antigens associated covalently with the cell-walls of group F and related streptococci. However, little information was encountered regarding the nature of surface antigens associated in a non-covalent manner.

In this chapter, the results are reported of some investigations into the nature of antigenic material associated non-covalently with the cell-surface of a collection of streptococcal strains, the majority of which belonged to Lancefield group F.

The major aims of this study were as follows:

1. To examine the ability of EDTA to extract non-covalently bound material from the cell-surface of a collection of streptococci belonging mainly to Lancefield group F, and to examine the composition of the material extracted;

2. To examine in PAGE the protein profiles of EDTA extracts derived from reference strains cultured under differing atmospheric conditions, in order to establish the stability of profiles for given strains;

3. To apply a number of immunochemical techniques, including ELISA, Western blotting, and crossed and rocket immunoelectrophoresis using antisera raised against *Streptococcus milleri* reference strains, in an attempt to recognise antigenic material within EDTA extracts;

4. To investigate the general biochemical nature of important non-covalently bound cell-surface antigens in this group of organisms.

A list of strains included in the current study is given in table 4.1. Wild strains derived from a range of sources, and displaying a range of biochemical and haemolytic behaviour were selected from a collection of group F streptococci described in chapter 3.

Details of the materials and methods employed in this study are given in chapter 2. Some additional experimental details are included in the narrative of this chapter.

Table 4.1. List of strains included in the current study:

1. List of reference strains:

NUMBER.	GROUP.	TYPE.	HAEMOLYSIS.	SOURCE.
NCTC 10707	F	None	None	NCTC, London.
NCTC 10714	F	I	Beta	NCTC, London.
NCTC 5389	F	II	Beta	NCTC, London.
NCTC 8037	F	III	None	NCTC, London.
NCTC 11065	None	IV	None	NCTC, London.

2. List of wild strains:

NUMBER.	GROUP.	HAEMOLYSIS.	SOURCE.
JW1	F	None	High vaginal swab.
JW2	F	None	High vaginal swab.
JW3	F	Beta	Pilonidal sinus.
JW5	F	Beta	Intra-oral swab: infected dental cyst.
WJ8	F	None	Throat swab.
WJ9	F	None	Arm abscess.
WJ10	F	Alpha	Denture swab.
WJ12	F	None	Dental plaque.
WJ15	F	Alpha	Dental plaque.
WJ49	F	Alpha	Dental plaque.
WJ55	F	Alpha	Dental plaque.
OS25a	F	Alpha	Dental plaque.
OS25n	F	None	Dental plaque.
H957	F	Beta	Chin abscess.

Single laboratory stock strains of beta-haemolytic group A, C, and G streptococci were also used in this study.

4.2. Results.

4.2.1. Preparation of THB for broth-culture of *Streptococcus milleri* strains.

Four reference strains were examined in a pilot study to compare the relative growth of *Streptococcus milleri* strains in THB sterilised by membrane filtration, or by autoclaving. Broths of identical volume (500ml) received identical (10ml) inocula from a common broth starter culture, and were incubated in parallel, aerobically at 37°C. Following overnight incubation (17 hours), the density of growth in broth-cultures was assessed by reading the A_{600} against an uninoculated broth incubated in parallel. This investigation was performed twice for each strain on consecutive days, and the mean A_{600} readings recorded (see table 4.2.). Growth of all strains was consistently superior in filter-sterilised broths, although considerable strain-to-strain variation in maximum growth (A_{600}) was noted. Membrane filtration was consequently employed as the standard method for preparation of THB throughout the investigations described in chapters 4 and 5.

4.2.2. Establishment of standard conditions for the extraction of cells with EDTA.

A pilot study was undertaken to compare the protein yields obtained by incubating equal amounts of freshly harvested cells with 10mM EDTA at 45°C:

1. For 30 minutes, or
2. For 60 minutes, with a period of vortex-mixing (15 seconds) at 30

Table 4.2. The effect of THB preparation on the growth of *Streptococcus milleri* reference strains as evidenced by A_{600} of overnight cultures.

	A_{600} AFTER OVERNIGHT INCUBATION		PERCENTAGE INCREASE IN A_{600} WITH FILTERED BROTH
STRAIN	AUTOCLAVED BROTH	FILTERED BROTH	
NCTC 8037	1.26	1.45	15
NCTC 11065	1.035	1.3	26
NCTC 10707	0.755	1.075	42
NCTC 10714	0.636	0.85	33.6

minutes.

Extension of the incubation time to 60 minutes led to increases in protein yield ranging from 10 to 60%, depending upon the strain. Comparison of extracts in SDS-PAGE failed to demonstrate the presence of additional bands in the 60 minute extracts, indicating that the lengthier extraction procedure had not led to large-scale cell lysis. This point was additionally confirmed by the examination of cell-suspensions by phase contrast microscopy. The latter method was consequently adopted as the standard method for EDTA extraction of streptococcal isolates (see section 2.15.).

Considerable variation in protein yield was observed for different strains cultured and extracted under conditions as uniform as could be achieved. Yields ranged from 300 to 950ug/ml.

4.2.3. PAGE.

Figure 4.1. shows the appearance in SDS-PAGE of proteins contained in the EDTA extracts of five *Streptococcus milleri* reference strains. Three 'large colony' beta-haemolytic streptococci were included for comparison. Considerable similarity, though not identity, was noted amongst the five *Streptococcus milleri* strains in SDS-PAGE (tracks 1-5). A comparable degree of uniformity was noted amongst the three 'large-colony' beta-haemolytic strains (tracks 6-8), but distinction between them and the *Streptococcus milleri* strains was clear.

Pilot studies showed that transfer of EDTA extracted material to nitrocellulose membranes for the purpose of immunoblotting was superior from SDS-free polyacrylamide gels than from gels in which SDS was present. As it was planned at a later stage to examine EDTA extracts separated in PAGE by Western blotting, all further gels run in this study were prepared without SDS to allow some consistency of results.

The EDTA extracts of *Streptococcus milleri* reference strains, and the group A streptococcus shown in figure 4.1. were subsequently examined in SDS-free PAGE (figure 4.2). Far less similarity in PAGE profile was noted amongst the *Streptococcus milleri* reference strains in SDS-free PAGE, and distinction from the group A strain on visual examination was more difficult. Despite the application of standard quantities of protein to each track, some tracks stained more heavily than others.

4.2.4. Growth of *Streptococcus milleri* reference strains under different atmospheric conditions.

In order to assess the degree of stability of EDTA-extractable protein profiles obtained from *Streptococcus milleri* reference strains, each of the strains included in figure 4.2. was grown-up in identical volumes of THB as follows:

i. Aerobically, static;

ii. In THB which had been held in an atmosphere containing 10% CO_2, 90% H_2 for 48 hours prior to incubation with culture in the same atmosphere;

iii. In THB pre-reduced by anaerobic storage for 48 hours prior to incubation with culture in an anaerobic atmosphere.

Figure 4.1. SDS-PAGE of EDTA extracts from five *Streptococcus milleri* reference strains, and three 'large colony' beta-haemolytic streptococci.

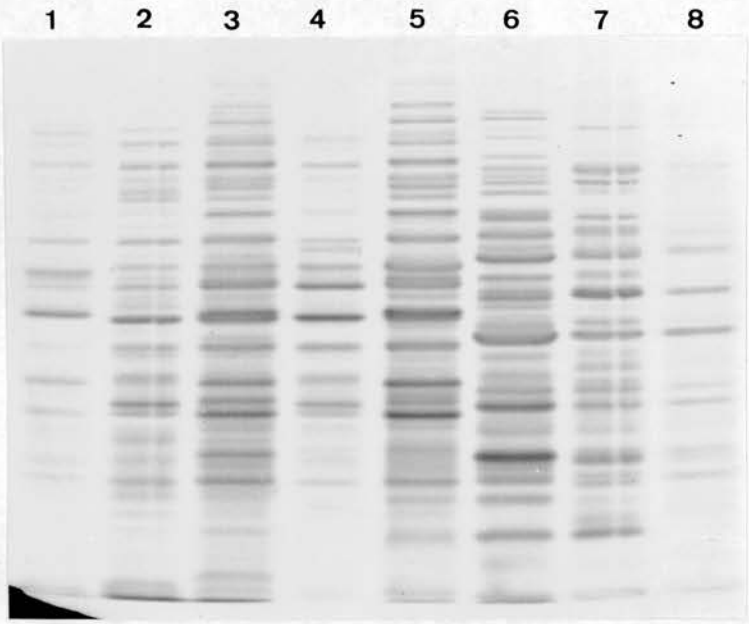
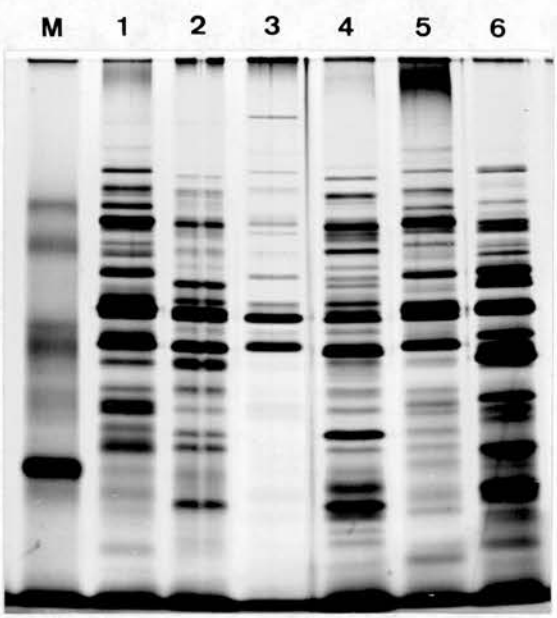


Figure 4.2. SDS-free PAGE of EDTA extracts from five *Streptococcus milleri* reference strains, and a beta-haemolytic group A streptococcus.



KEY: Applies to figures 4.1. and 4.2. Both gels silver stained for protein. Track 1 = NCTC 8037; 2 = NCTC 5389; 3 = NCTC 10707; 4 = NCTC 10714; 5 = NCTC 11065; 6 = Beta-haemolytic group A streptococcus; 7 = Beta-haemolytic group C streptococcus; 8 = Beta-haemolytic group G streptococcus. M = molecular mass markers (BDH). Approximately 40μg protein loaded to each track.

The variations in conditions described represented those under which *Streptococcus milleri* isolates might be routinely cultured in the laboratory. It was beyond the scope of the current study to investigate the action of highly variable conditions on the expression of EDTA-extractable proteins.

All three flasks for each strain received identical inocula from a common starter culture, and were incubated for 17 hours at 37°C.

EDTA extracts were prepared from each of the fifteen cultures, and were assayed for protein and carbohydrate as described in sections 2.8. and 2.9. respectively. The ratio of carbohydrate to protein (mg/ml) contained in each extract is given in table 4.3.(A). An appreciable amount of carbohydrate, in addition to protein, was contained in extracts of these strains. The ratio of carbohydrate to protein for individual strains was relatively uniform under the conditions investigated. Comparison of the carbohydrate to protein ratio for each strain allowed comparison independent of finite growth level and indicated considerable differences between some strains.

EDTA extracts from NCTC 10707 and NCTC 11065 contained high levels of protein relative to carbohydrate, compared to NCTC 8037, NCTC 5389, and NCTC 10714, in which the predominance of protein was less marked. Extracts from NCTC 8037 contained the highest amount of carbohydrate relative to protein. The meaning of these observations in terms of the nature of antigenic material contained in the EDTA extracts of *Streptococcus milleri* reference strains was, however, unclear.

Table 4.3. Examination of EDTA extracts from 5 *Streptococcus milleri* reference strains cultured under three conditions: A = Ratio of carbohydrate (CHO) to protein for each extract (mg/ml); B = Antibody titre against homologous antiserum, determined by ELISA.

STRAIN	CULTURE CONDITIONS	A CHO:PROTEIN RATIO	B ANTIBODY TITRE
NCTC 8037	Aerobic	1 : 2.37	12,800
NCTC 8037	CO_2 enriched	1 : 1.22	12,800
NCTC 8037	Anaerobic	1 : 1.25	12,800
NCTC 5389	Aerobic	1 : 3.91	3,200
NCTC 5389	CO_2 enriched	1 : 3.56	1,600
NCTC 5389	Anaerobic	1 : 3.98	1,600
NCTC 10707	Aerobic	1 : 12.8	12,800
NCTC 10707	CO_2 enriched	1 : 11.3	12,800
NCTC 10707	Anaerobic	1 : 12.38	12,800
NCTC 10714	Aerobic	1 : 3.37	1,600
NCTC 10714	CO_2 enriched	1 : 3.12	3,200
NCTC 10714	Anaerobic	1 : 3.65	12,800
NCTC 11065	Aerobic	1 : 7.47	12,800
NCTC 11065	CO_2 enriched	1 : 5.48	12,800
NCTC 11065	Anaerobic	1 : 5.58	12,800

4.2.5. Antibody titres.

Prior to their examination in PAGE, EDTA extracts described in section 4.2.4. were examined in ELISA against doubling dilutions of homologous antiserum (see sections 2.4. and 2.6.). The results of titrations, repeated in triplicate, were entirely reproducible and are given in table 4.3.(B).

Three EDTA extracts from NCTC 10707, NCTC 11065, and NCTC 8037 gave uniform, high titres with homologous antiserum. The titres obtained for NCTC 5389 EDTA extracts were lower than those obtained for NCTC 10707, NCTC 11065, and NCTC 8037, and showed a degree of non-uniformity. A higher titre was obtained for the EDTA extract of aerobically cultured NCTC 5389 than for the CO_2 enriched, or anaerobic cultures. The titres for NCTC 10714 extracts were again generally lower and, like NCTC 5389, showed some variation. A markedly higher titre was recorded for the EDTA extract of anaerobically cultured NCTC 10714.

ELISA titration results demonstrated that EDTA extracts of *Streptococcus milleri* reference strains contained antigenic material. Antibody titres indicated that the antisera were adequately potent and were sensitive in the detection of non-covalently bound cell-surface antigens extracted from their homologous strain.

ELISA titration results also indicated possible quantitative or qualitative differences in the antigens contained in extracts derived from cultures grown under different conditions. This finding was particularly marked for NCTC 10714, although the minor variations of carbohydrate to protein ratio

for the three extracts of this strain did not appear to correlate with variations in antibody titre (see table 4.3.).

4.2.6. Examination of reference strain EDTA extracts by PAGE and Western blotting.

Following the demonstration of antigenic material in EDTA extracts, they were examined by PAGE and Western blotting. Material immobilised to nitrocellulose membranes was probed with homologous antiserum at a dilution of 1 in 50 in antibody diluent.

Figures 4.3.a., 4.4.a., 4.5.a., 4.6.a., and 4.7.a. show the PAGE profiles of EDTA soluble proteins from three cultures of: NCTC 11065, NCTC 5389, NCTC 10707, NCTC 8037, and NCTC 10714 respectively. The results in all cases indicated a stability of PAGE protein profiles from strains cultured under differing atmospheric conditions. Slight variation in staining intensity was observed for tracks within individual gels despite efforts to load standard quantities of protein to each track. Uncontrollable variations in running conditions, and variations in the staining intensities of individual silver-stained gels made comparison of protein profiles seen in different gels difficult.

Figures 4.3.b., 4.4.b., 4.5.b., 4.6.b., and 4.7.b. show the corresponding immunoblot profiles for EDTA extracts of NCTC 11065, NCTC 5389, NCTC 10707, NCTC 8037, and NCTC 10714 respectively.

Figure 4.3.b. shows consistent antigenic profiles from three EDTA extracts of NCTC 11065, consisting of: a series of approximately ten closely spaced

bands of high molecular mass (A), and a fainter band of lower molecular mass (B).

Figure 4.4.b. shows a similar pattern of bands for NCTC 5389, although not as sharp as for NCTC 11065; again, antigenic profiles were constant and did not appear to reflect the variations in antibody titre for different extracts detected by ELISA.

Figure 4.5.b. shows immunoblot profiles for culture extracts of NCTC 10707 and demonstrates a highly contrasting series of bands compared to figures 4.3.b. and 4.4.b. The antigenic profiles again remained constant, and were dominated by two well-separated bands (A and B). Additional, very weakly reacting material was observed corresponding to material of both high molecular mass, and associated with the gel-front.

Figure 4.6.b. shows another highly contrasting series of antigenic bands obtained consistently for three EDTA extracts of NCTC 8037. In addition to three major antigenic bands in the body of the immunoblot, a heavily-staining smear of antigenic material was observed in a position corresponding to the gel-front.

Figure 4.7.b. illustrates once again a different series of antigenic bands for NCTC 10714 EDTA extracts. However, in contrast to the previous immunoblots, additional bands were clearly demonstrated in the EDTA extract derived from an anaerobic culture of this strain (A and B). The presence of additional antigenic bands did not reflect obvious variations in the corresponding PAGE gel, but did reflect the significantly higher antibody titre obtained in ELISA for this extract (see table 4.3.B.).

It was therefore shown that the PAGE profiles of EDTA-soluble proteins contained in extracts from *Streptococcus milleri* reference strains remained

constant under the range of culture conditions investigated. Immunoblotting demonstrated considerable inter-strain differences in the profiles of antigenic material derived from reference strains, and for NCTC 10714 showed that antigenic variation may occur despite seemingly identical PAGE protein profiles for the various extracts of this strain.

Results were purely qualitative, with no attempt being made to calculate the molecular masses of the various antigenic bands visualised, which may or may not have been protein in nature. Some difficulty was encountered in matching bands on immunoblots to the corresponding bands on silver-stained gels. Part of the explanation for this is that the initial fixing process involved in silver staining results in shrinkage of the gel, a phenomenon which is not encountered during the development of the corresponding immunoblot on nitrocellulose membrane.

It was of interest following these results to attempt to investigate the general biochemical nature of important antigenic material involved in the reactions of EDTA extracts with homologous antisera.

Figure 4.3. PAGE and immunoblot profiles of NCTC 11065 EDTA extracts.

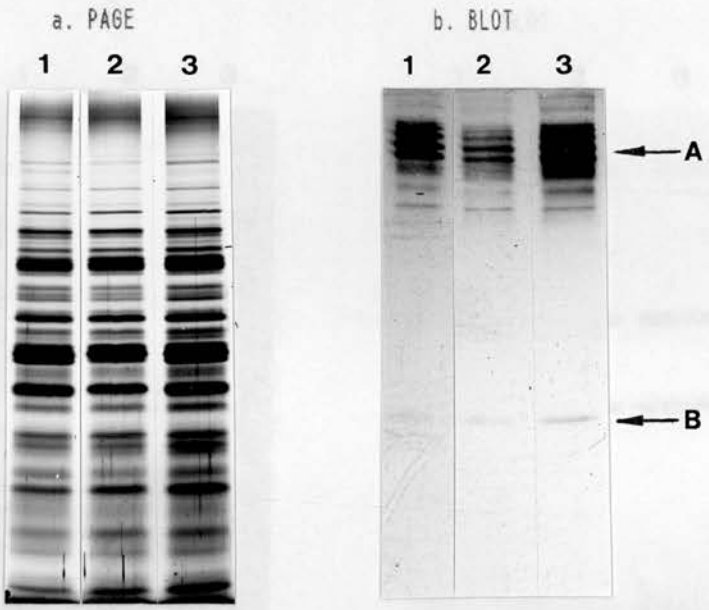


Figure 4.4. PAGE and immunoblot profiles of NCTC 5389 EDTA extracts.

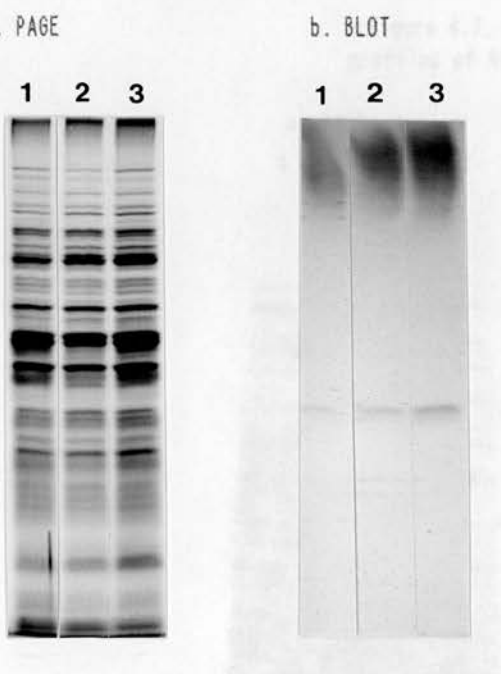
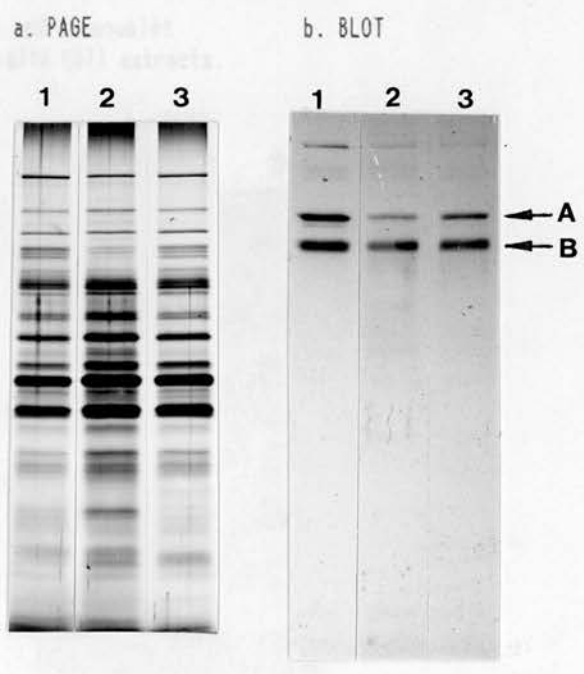


Figure 4.5. PAGE and immunoblot profiles of NCTC 10707 EDTA extracts.



Figures 4.3. to 4.7.: Tracks 1 = EDTA extract of aerobic culture; 2 = EDTA extract of CO_2 enriched culture; 3 = EDTA extract of anaerobic culture. PAGE gels silver stained for protein, approximately 30μg protein loaded to each track.

Figure 4.6. PAGE and immunoblot
profiles of NCTC 8037 EDTA extracts.

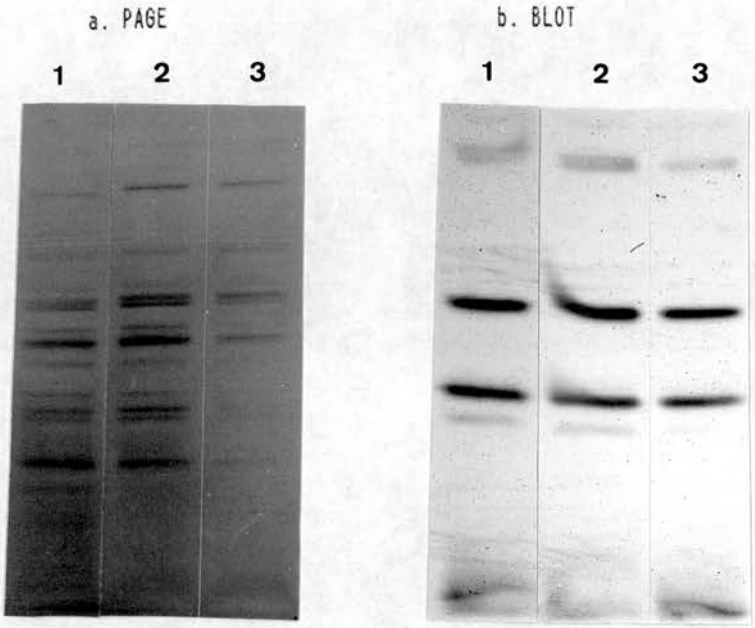
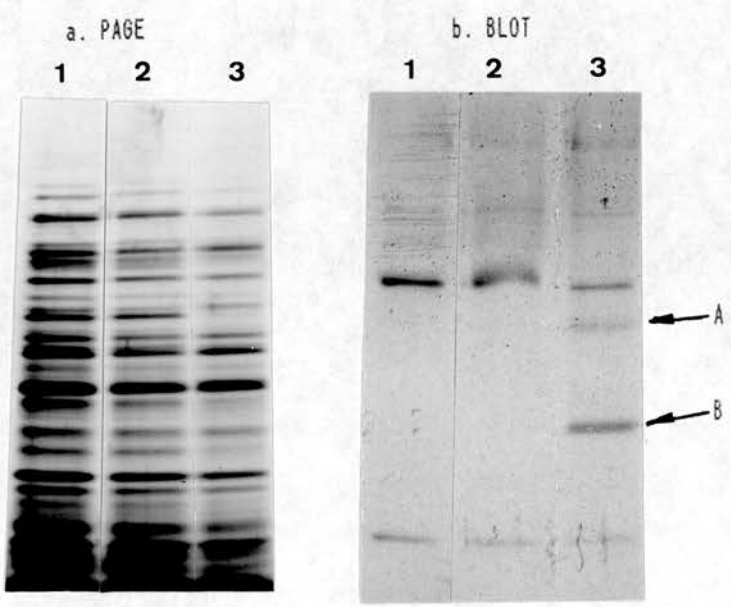


Figure 4.7. PAGE and immunoblot
profiles of NCTC 10714 EDTA extracts.



See previous page for legend.

4.2.7. ELISA inhibition reactions.

In order to get some impression of the nature of antigenic material contained in EDTA extracts and reacting with homologous antiserum, the system for ELISA inhibition reactions described in section 2.7.1. was applied. An ELISA system was adopted in this study in order to obtain a general picture of the antigenicity of the cell-surface of these strains, and not simply to demonstrate precipitating antigens.

Results for the aerobic culture extracts of NCTC 11065 and NCTC 8037 were representative of all extracts of these strains and are shown in figure 4.8.a. and b. respectively.

Figure 4.8.a. shows the reduced inhibitory action following heat treatment of the NCTC 11065 EDTA extract on the NCTC 11065 EDTA extract/NCTC 11065 antiserum ELISA reaction. Sodium periodate (0.01M) treatment, on the other hand, was markedly less active in this respect. This result indicated that the predominant antigenic material contained in EDTA extracts from NCTC 11065 and detected by homologous antiserum in the current model was protein in nature.

Figure 4.8.b. shows the converse situation for NCTC 8037, where the denaturing action of periodate had more effect than heat treatment. This result indicated that the predominant antigenic material contained in EDTA extracts of NCTC 8037, and detected by homologous antiserum in the current model was carbohydrate in nature.

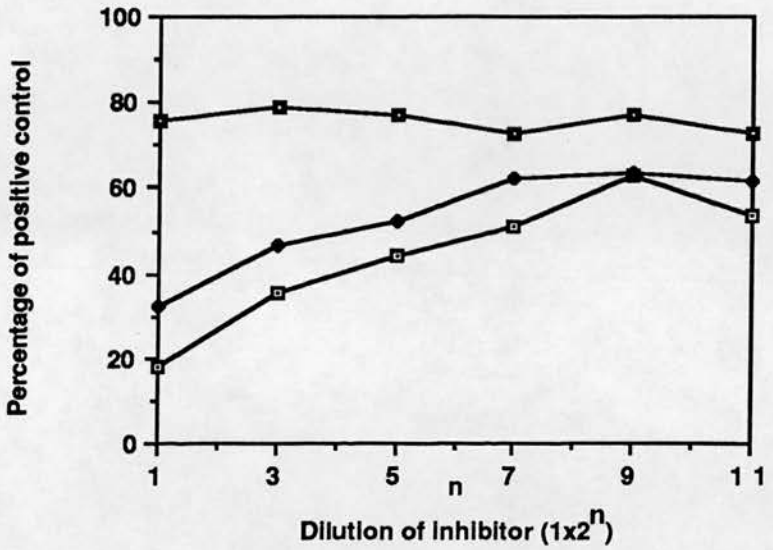
The results for other strains were generally far less clear, and it was indeterminate whether carbohydrate or protein predominated.

Extensive use of the technique described for ELISA inhibition reactions revealed that it was laborious and time-consuming. As a consequence of this observation, a simpler method was developed (see section 2.7.2.).
Some results obtained by this simplified method are given in figures 4.9. to 4.13., and represent the mean of triplicate reactions for each test. Variation of results amongst the repeat reactions was very slight.
In some of the reactions, one of the proteolytic enzymes (proteinase K) was omitted, since it was expensive, and appeared to offer no advantage over trypsin and pronase in proteolytic activity under the conditions studied.

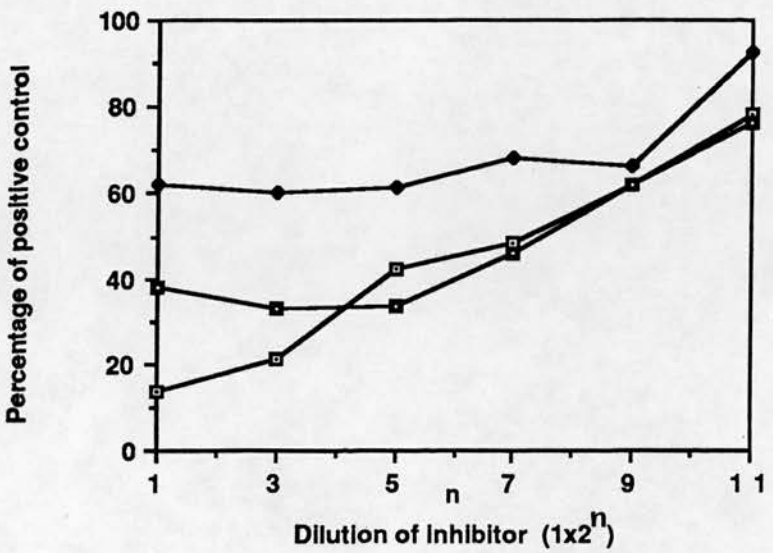
Figure 4.9. shows ELISA inhibition reactions for EDTA extracts of NCTC 11065. The results corroborate those in figure 4.8.a. In all cases, proteolytic enzymes reduced the antigenicity of EDTA extracts to a significantly greater level than sodium periodate was able to. It was consequently confirmed that the predominant antigenic material contained in EDTA extracts of NCTC 11065, and detected in ELISA by homologous antiserum was protein in nature.
Figure 4.10. shows ELISA inhibition reactions for EDTA extracts of NCTC 8037. Again, the results corroborated those of figure 4.8.b. In contrast to the results for NCTC 11065, sodium periodate was able to significantly diminish the antigenicity of NCTC 8037 EDTA extracts, whilst the action of proteolytic enzymes was limited. It was confirmed by this result that the predominant antigenic material contained in EDTA extracts of NCTC 8037 and detected in ELISA by homologous antiserum was carbohydrate in nature.

Figure 4.8. ELISA inhibition reactions to determine the general biochemical nature of non-covalently bound cell-surface antigens of two *Streptococcus milleri* reference strains.

a. NCTC 11065.



b. NCTC 8037.



Inhibition of ELISA was by preincubating doubling dilutions of potential inhibitors with equal volumes of antiserum diluted to twice the titre for 30 minutes at 37°C. Antigen was homologous EDTA extract at a dilution of 25*ug*/ml protein.
Inhibitors were: untreated homologous EDTA extract (-■-) as positive control; homologous EDTA extract heated to 121°C for 15 minutes (-■-); and homologous EDTA extract treated with 0.01M sodium periodate (pH 5.0) for 16 hours at 20°C (-◆-).

Inclusion of NCTC 11065 and NCTC 8037 in the same investigation provided reciprocal internal controls that the denaturing agents applied were having the stated effects.

Figure 4.11. shows a less clear-cut result for NCTC 5389 than was observed for the previous two strains. This was not surprising in the light of results obtained by the earlier system. The action of proteolytic enzymes and sodium periodate both substantially decreased the antigenic activity of the EDTA extract as a whole, though it could be tentatively reported that proteolytic enzymes had slightly greater activity in this respect, illustrated most clearly in figure 4.11.a.

Figures 4.12. and 4.13. illustrate the results for NCTC 10707, and NCTC 10714 respectively. Unlike the results obtained for NCTC 11065 and NCTC 8037, no clear predominance of protein or carbohydrate material was observed for these strains. The results indicated that both protein and carbohydrate antigens were equally important as non-covalently bound antigens in these strains. Figure 4.13.c. gave some evidence to suggest a greater importance of carbohydrate in the extract derived from an anaerobic culture of NCTC 10714, compared to the other two extracts. It would be unreasonable to assume that any definite conclusion could be made from this observation with respect to the increased antibody titre to this extract demonstrated in table 4.3.(B), and the additional bands observed in the immunoblot of this extract.

Figures 4.9. to 4.13.: ELISA inhibition reactions to determine the general biochemical nature of non-covalently bound cell-surface antigens reacting in ELISA with homologous whole-cell antiserum.

Antigen applied to the plate was homologous EDTA extract, diluted to 25µg/ml protein in antigen diluent (50mM carbonate buffer containing 0.02% sodium azide).

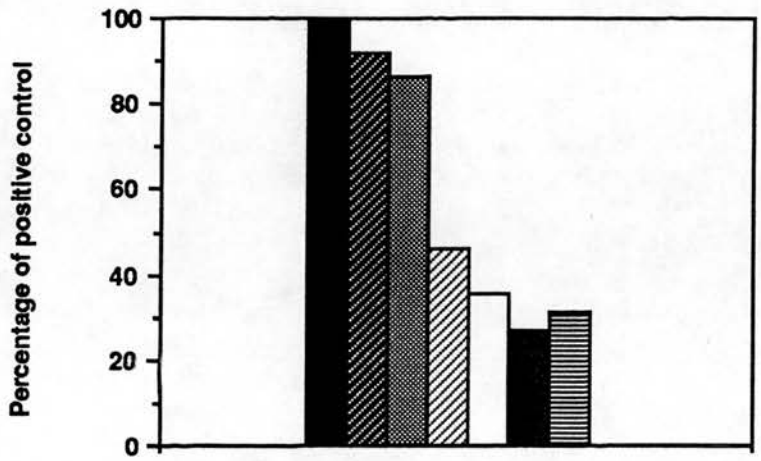
Potential inhibitors were:

PBS (pH 7.8)	(■)	as positive control
Sodium periodate (0.01M) in PBS	(▨)	
Sodium periodate (0.02M) in PBS	(▩)	
Trypsin (40u/ml) in PBS	(▨)	
Trypsin (400u/ml) in PBS	(□)	
Pronase (10 PUK units/ml) in PBS	(■)	
Proteinase K (0.2u/ml) in PBS	(▤)	

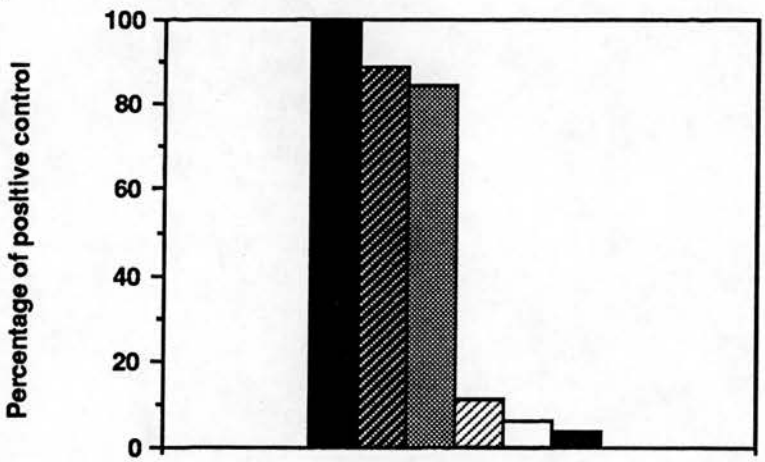
Antigen was incubated with potential inhibitors for 2 hours at room temperature. Homologous antiserum was subsequently applied at twice titre. Following addition of conjugate, and development of the reaction in the usual manner, the A_{405} was read, and the results recorded.

Figure 4.9. ELISA inhibition reactions for EDTA extracts of NCTC 11065.

a. AEROBIC CULTURE



b. CO_2 ENRICHED CULTURE



c. ANAEROBIC CULTURE

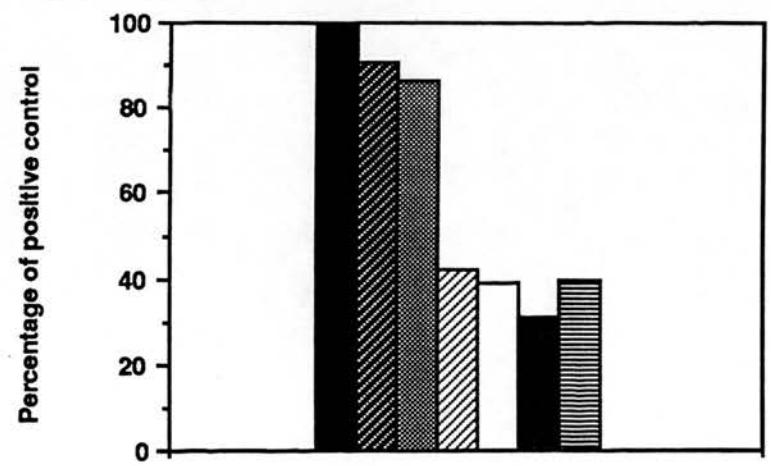
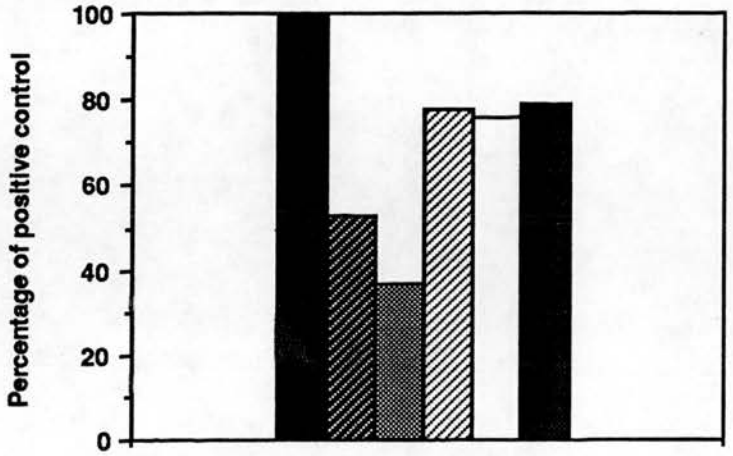
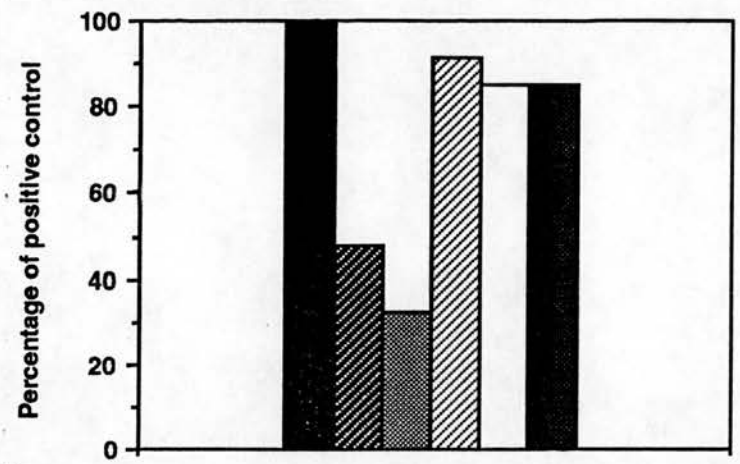


Figure 4.10. ELISA inhibition reactions for EDTA extracts of NCTC 8037.

a. AEROBIC CULTURE



b. CO_2 ENRICHED CULTURE



c. ANAEROBIC CULTURE

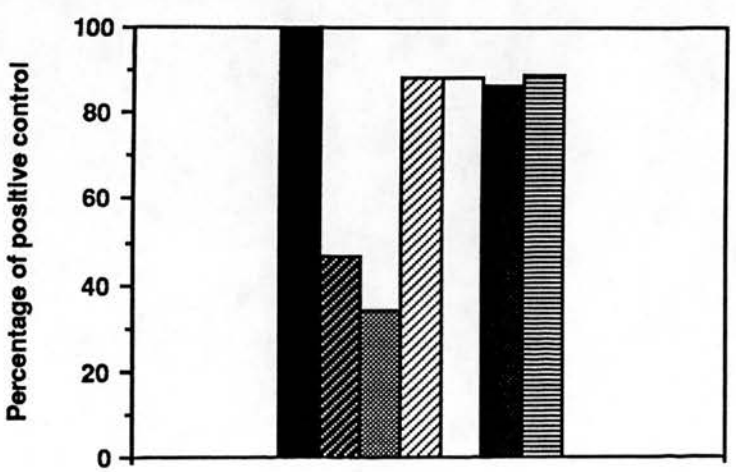
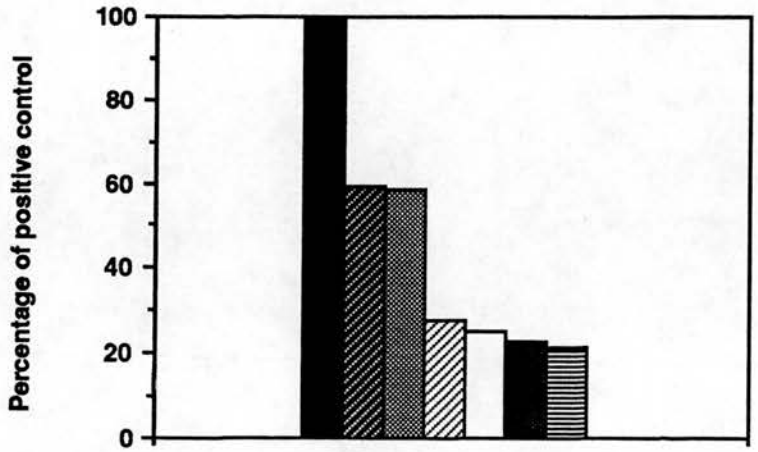
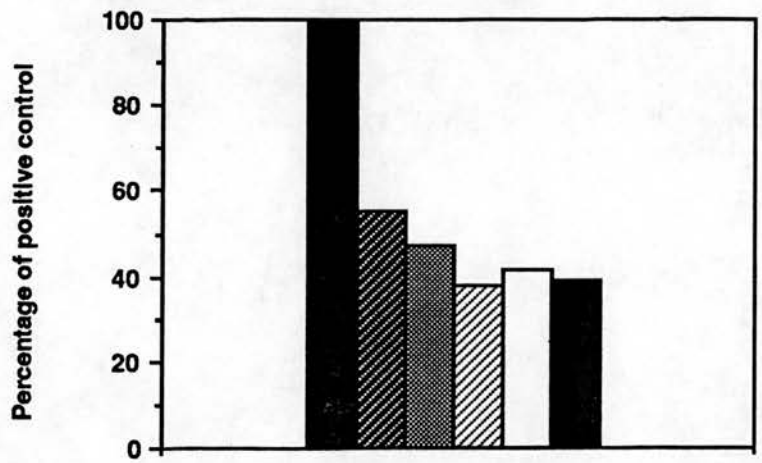


Figure 4.11. ELISA inhibition reactions for EDTA extracts of NCTC 5389.

a. AEROBIC CULTURE



b. CO_2 ENRICHED CULTURE



c. ANAEROBIC CULTURE

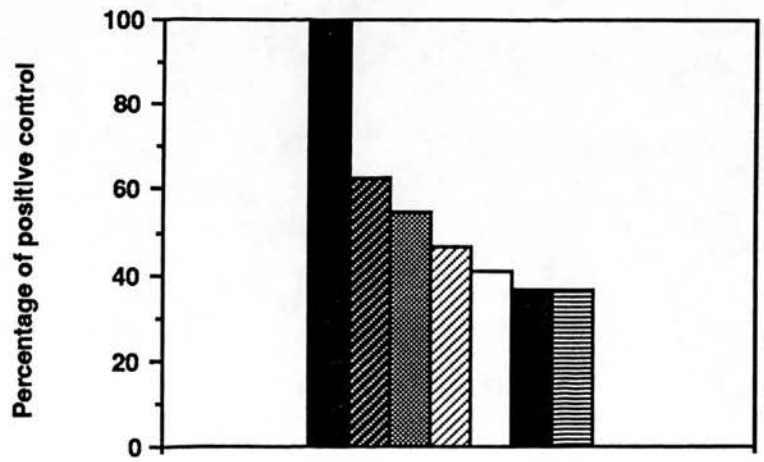
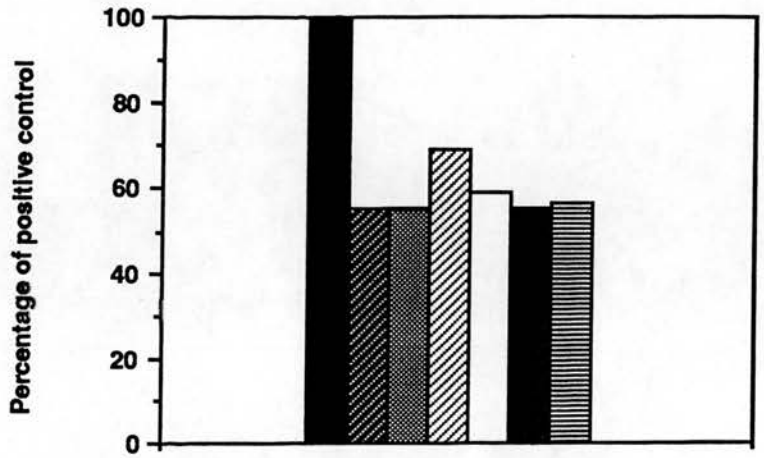
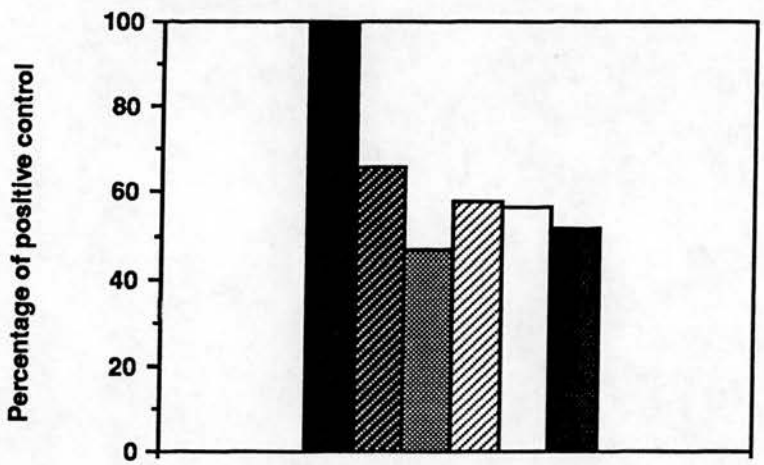


Figure 4.12. ELISA inhibition reactions for EDTA extracts of NCTC 10707.

a. AEROBIC CULTURE



b. CO_2 ENRICHED CULTURE



c. ANAEROBIC CULTURE

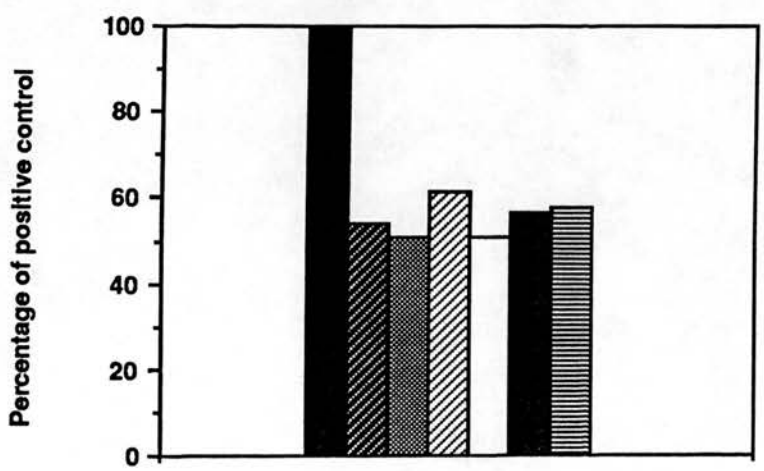
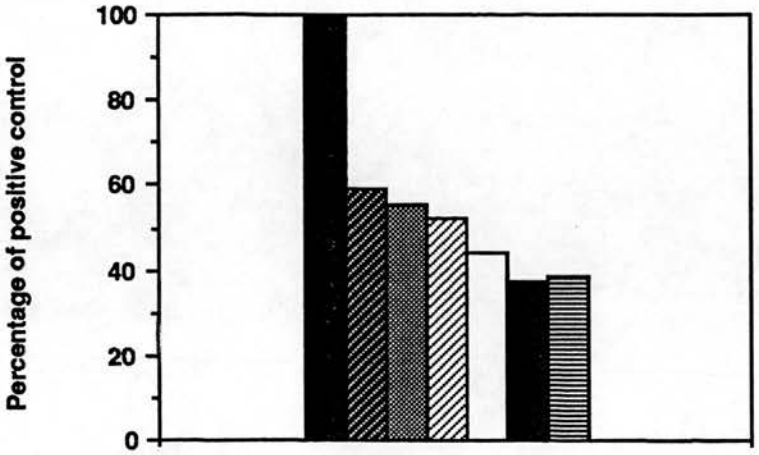
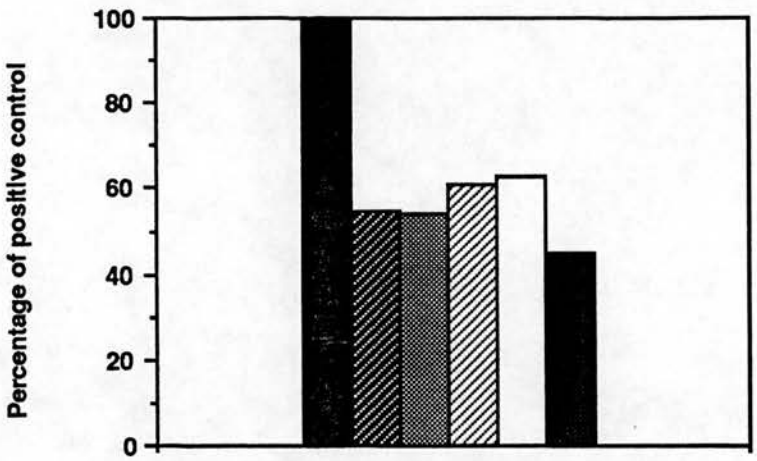


Figure 4.13. ELISA inhibition reactions for EDTA extracts of NCTC 10714.

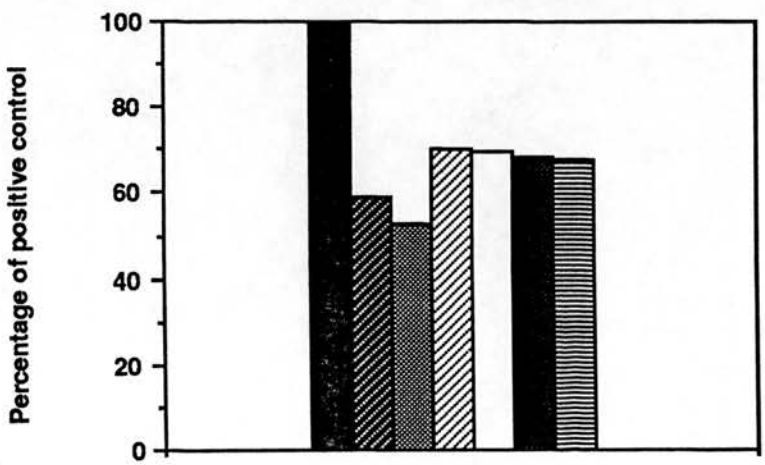
a. AEROBIC CULTURE



b. CO_2 ENRICHED CULTURE



c. ANAEROBIC CULTURE



ELISA inhibition reactions demonstrated diversity in the predominant non-covalently linked surface antigens of a collection of *Streptococcus milleri* reference strains, and echoed the results obtained by immunoblotting. Although general patterns of predominant antigenic material emerged for each strain, differences in the balance of surface antigens for strains cultured under different conditions were suggested which were not apparent from PAGE examination of proteins, or in most circumstances by examination of immunoblots.

4.2.8. Examination of the cross-reactivity between reference strains and their antisera in ELISA.

As a means of investigating the overall degree of cross-reactivity amongst reference strains, an ELISA system was again adopted to establish the antibody titres of EDTA extracts against heterologous antisera. Only the EDTA extracts derived from anaerobic cultures were considered in this study.

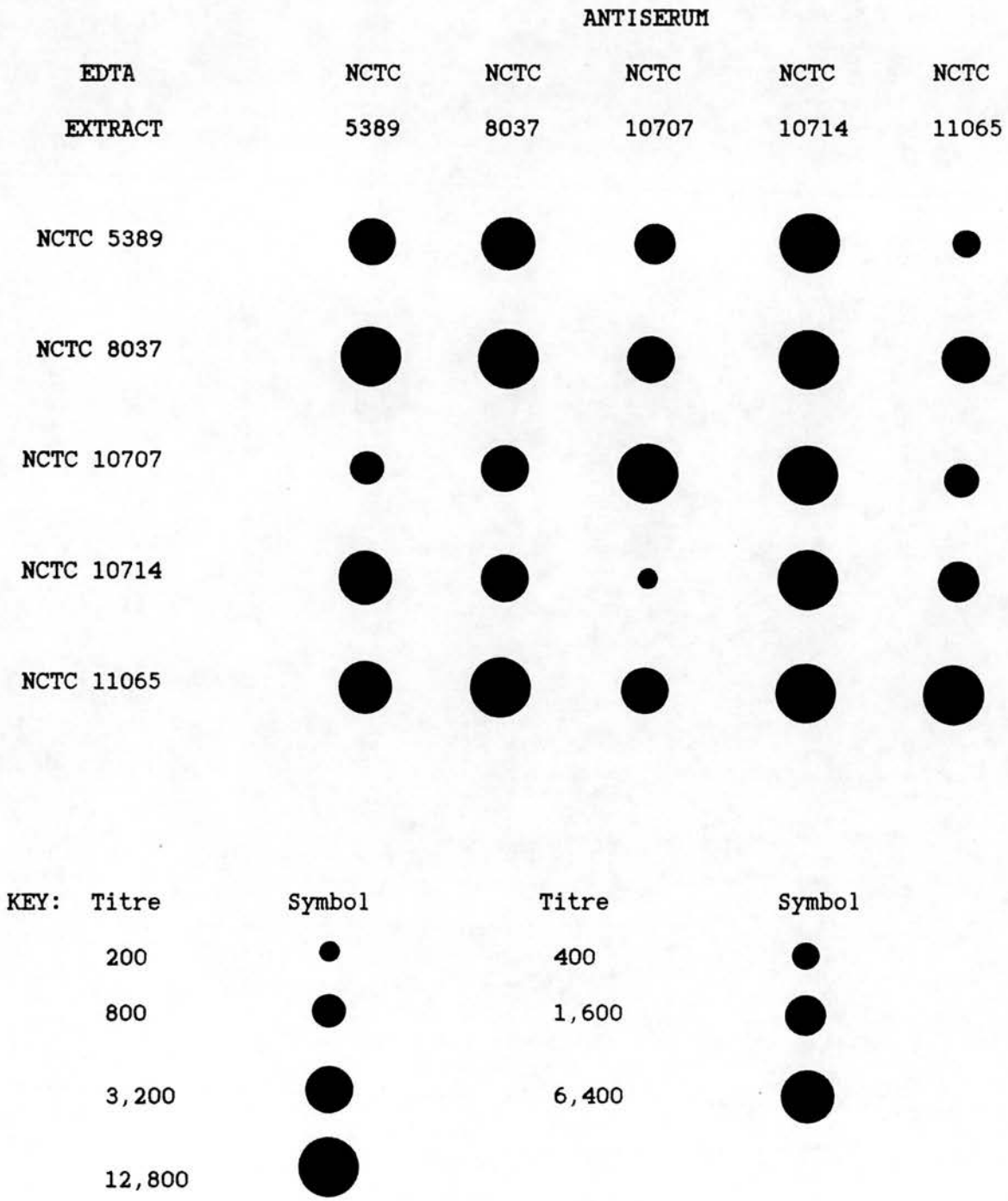
Actual figures for the end-points of titrations are given in appendix 2.(i).

Figure 4.14. shows the results in diagramatic form and indicates a generally high degree of cross-reactivity amongst five *Streptococcus milleri* EDTA extracts and their corresponding antisera. The reaction of NCTC 5389 EDTA extract was stronger with NCTC 8037 and NCTC 10714 antisera than with homologous antiserum. Generally, cross-reactions with NCTC 8037 and NCTC 10714 antisera were the strongest, whilst those with NCTC 11065 antiserum were the weakest.

As an extension to this study, an attempt was made to elucidate the general biochemical nature of predominant antigenic material involved in cross-reactions between strains by ELISA inhibition. In this study, PBS was again used as the positive control, whilst sodium periodate (0.02M in PBS), and pronase (10 PUK units/ml in PBS) were used as potential inhibitors. Reactions were again performed in triplicate and varied little on repeat. The mean results are represented in table 4.4. Actual figures for the action of the two potential inhibitors, expressed as a percentage of the positive control reaction are given in appendix 2.(ii).

In figure 4.14., antiserum raised against NCTC 10714 was generally the most reactive with heterologous strains. ELISA inhibition reactions (table 4.4.) showed that the predominant material involved in cross-reactions between all heterologous strains and this antiserum was carbohydrate in nature.
A clear predominance of carbohydrate antigens was indicated in the cross-reactions between NCTC 8037 antiserum and the EDTA extracts of NCTC 10707 and NCTC 5389, whilst the reactions with NCTC 10714 and NCTC 11065 EDTA extracts showed no clear predominance of protein or carbohydrate.
Figure 4.14. also indicated that NCTC 11065 antiserum was generally the least reactive with heterologous strains. The two strongest cross-reactions (with NCTC 8037, and NCTC 10714 EDTA extracts) involved protein antigens predominantly. Cross-reactions with NCTC 5389, and NCTC 10707 EDTA extracts were unclear, with neither carbohydrate nor protein clearly predominating. Cross-reactions with NCTC 5389 antiserum were again variable from strain to strain. With NCTC 10707 EDTA extract (the weakest cross-reaction) a clear predominance of carbohydrate antigens was demonstrated.

Figure 4.14. Titration of EDTA extracts from five *Streptococcus milleri* reference strains against heterologous *Streptococcus milleri* antisera in ELISA.



Cross-reactions with NCTC 10714, and NCTC 11065 EDTA extracts, on the other hand, were clearly protein in nature. The reaction with NCTC 8037 EDTA extract showed no clear predominance of carbohydrate or protein.

Cross-reactions between NCTC 10707 antiserum and NCTC 5389, NCTC 8037, and NCTC 11065 EDTA extracts were mediated predominantly by carbohydrate antigens. The weakest reaction of NCTC 10707 antiserum shown in figure 4.14. was with NCTC 10714 EDTA extract, the nature of which predominated neither carbohydrate, nor protein.

A complex patchwork of cross-reactions thus emerged from this ELISA study of interrelationships amongst *Streptococcus milleri* reference strains. Further attempts were unsuccessfully made to investigate cross-reactivity between strains by immunoblotting with heterologous antisera. The results of this investigation were confused, and inconsistent when repeated. In the author's hands, it was not possible to demonstrate any simplified patterns of single or groups of common bands amongst these strains by the application of immunoblotting.

4.2.9. Immunoelectrophoresis.

i. Crossed immunoelectrophoresis (CIE).

In a further attempt to investigate the antigens contained in EDTA extracts of *Streptococcus milleri* reference strains, extracts were run in CIE against homologous antiserum in order to visualise major precipitating antigens.

Table 4.4. Schematic representation of the predominant biochemical nature of material involved in cross-reactions between NCTC strains and heterologous antisera.

EDTA extract	ANTISERUM NCTC 5389	NCTC 8037	NCTC 10707	NCTC 10714	NCTC 11065
NCTC 5389	PC	C	C	C	PC
NCTC 8037	PC	C	C	C	P
NCTC 10707	C	C	PC	C	PC
NCTC 10714	P	PC	PC	PC	P
NCTC 11065	P	PC	C	C	P

KEY:

P = clear predominance of protein in reaction.

C = clear predominance of carbohydrate in reaction.

PC = no clear predominance of carbohydrate or protein in reaction.

In a pilot examination, whole *Streptococcus milleri* cells were extracted with cold TCA, and the extracts examined with little success in CIE against homologous antiserum. However, an unintentional reversal of the polarity of the power pack on one occasion led to the visualisation of antigenic peaks, and resulted in modification of the templates employed in immunoelectrophoresis to allow the detection of precipitin arcs migrating towards both the anode and the cathode.

Figures 4.15.a; 4.16.a; 4.17.a; and 4.18.a. show the appearance in CIE of the EDTA extracts derived from anaerobic cultures of NCTC 11065, NCTC 5389, NCTC 8037, and NCTC 10714 respectively. Each was run against homologous antiserum. No precipitating antigens were demonstrated in CIE for the EDTA extract of NCTC 10707. NCTC 11065 gave two peaks (1 and 2) which both migrated towards the anode, peak 1 being markedly heavier than peak 2. The remaining three strains: NCTC 5389, NCTC 8037, and NCTC 10714 gave strong peaks of immunoprecipitation toward the cathode, a rather unexpected result as most bacterial cell-surface antigens are believed to carry a negative charge. NCTC 5389, like NCTC 11065, gave an additional very weak peak towards the anode (peak 2).

In order to assess the biochemical nature of precipitating antigens demonstrated in CIE, EDTA extracts were subjected to treatments designed to denature carbohydrate, or protein antigens respectively:

1. A sample of EDTA extract was incubated for four hours with an equal volume of sodium periodate solution (0.02M in PBS) at room temperature. Excess periodate was inactivated by the addition of 2μl ethanediol after 4 hours.

2. A further sample of EDTA extract was heat-treated at 121°C for 15 minutes to denature protein material. Heat-treated extracts were then diluted to the same concentration as the periodate-treated extracts with PBS (pH 7.8).

Figure 4.15 b. shows the effect of periodate treatment on the EDTA extract of NCTC 11065; peak 1 was completely eradicated by periodate, leaving peak 2 intact. The converse was true for heat-treatment (figure 4.15.c.).

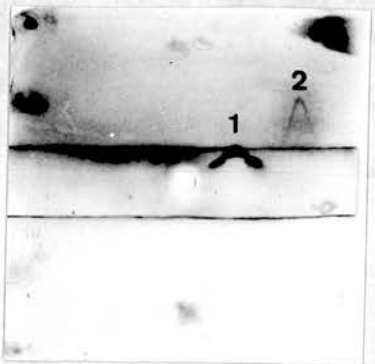
The single positively-charged peaks of NCTC 8037 and NCTC 10714 were completely eradicated by periodate treatment (figures 4.17.b. and 4.18.b. respectively), whilst being unaffected by heat-treatment (figures 4.17.c., and 4.18.c. respectively). A similar finding was also noted for peak 1 of the NCTC 5389 EDTA extract (figure 4.16.b and c). Peak 2 of the NCTC 5389 extract was not demonstrable following either treatment, and it was suspected that dilution of the samples had led to its loss. However, repeated attempts to visualise this peak following treatment without dilution were unsuccessful, perhaps indicating the presence of a glycoprotein antigen.

It was therefore shown that the major precipitating antigens associated non-covalently with the cell-surface of *Streptococcus milleri* reference strains were carbohydrate in nature. Only NCTC 11065 and NCTC 5389 gave more than one peak in CIE. In both cases, the additional peak was a faint line of immunoprecipitation towards the anode. Treatment of the EDTA extract of NCTC 11065 indicated that this second peak was protein. There was, however, no clear evidence that this was the case for the second peak contained in NCTC 5389 EDTA extract.

Figure 4.15. CIE of NCTC 11065 EDTA extract against homologous antiserum.

a. NCTC 11065, untreated
EDTA extract.

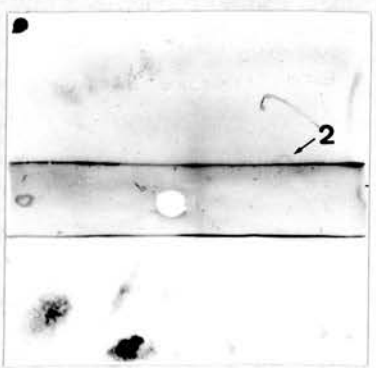
+



-

b. NCTC 11065, periodate-treated
EDTA extract.

+



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c. NCTC 11065, heat-treated
EDTA extract.

+

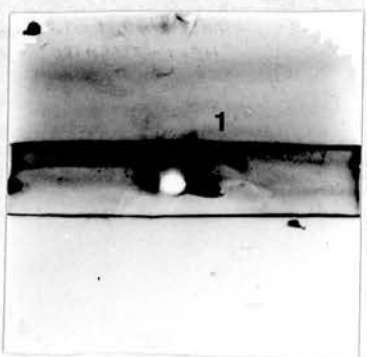

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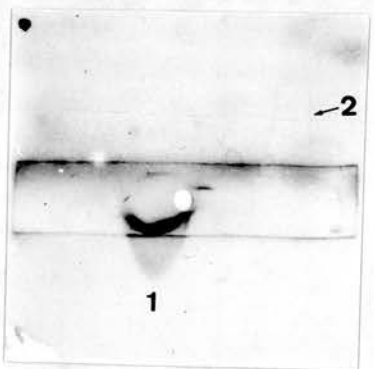
Figure 4.16. CIE of NCTC 5389 EDTA extract against homologous antiserum.

a. NCTC 5389 untreated
EDTA extract.

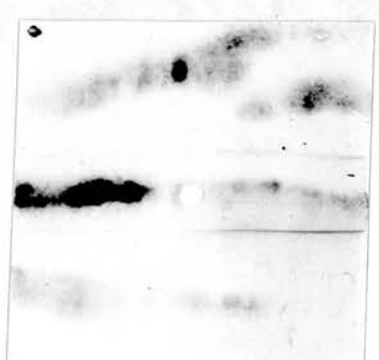
+



-

b. NCTC 5389 periodate-treated
EDTA extract.

+



-

c. NCTC 5389 heat-treated
EDTA extract.

+

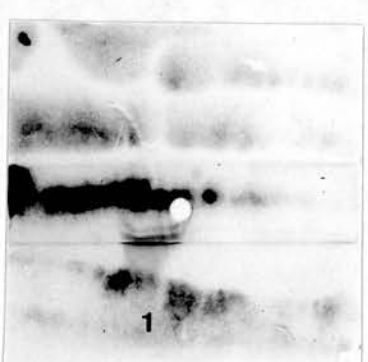


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Figure 4.17. CIE of NCTC 8037 EDTA extract against homologous antiserum.

a. NCTC 8037 untreated
EDTA extract.

+

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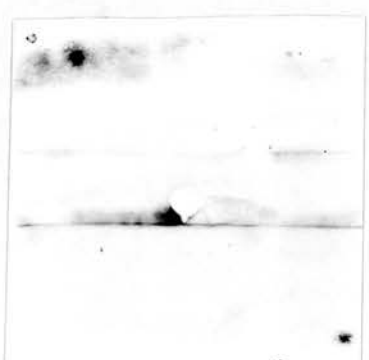
b. NCTC 8037 periodate-treated
EDTA extract.

+



-

c. NCTC 8037 heat-treated
EDTA extract.

+

-

Figure 4.18. CIE of NCTC 10714 EDTA extract against homologous antiserum.

a. NCTC 10714 untreated

EDTA extract.

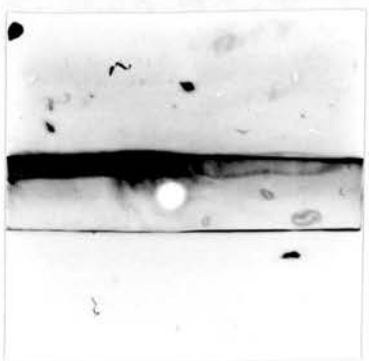
+

-

b. NCTC 10714 periodate-treated

EDTA extract.

+

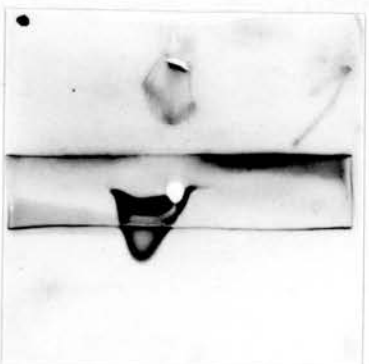


-

c. NCTC 10714 heat-treated

EDTA extract.

+



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ii. Fused rocket immunoelectrophoresis (FRIE).

In an extension to the study of precipitating antigens by immunoelectrophoresis, reference-strain EDTA extracts were run in FRIE against heterologous antisera to investigate cross-reactivity between strains.

No peaks of immunoprecipitation could be demonstrated between heterologous reference strains and antisera raised against NCTC 10707, or NCTC 8037.

Figure 4.19. shows the peaks of immunoprecipitation observed with antiserum raised against NCTC 11065. Despite the results of ELISA which showed generally the weakest cross-reactions with this antiserum, faint peaks were obtained with all heterologous strains, and migrated towards the anode.

Examination of the general biochemical nature of precipitating antigens was again performed, but without sample dilution. Heat-treatment of samples led to the abolition of all reacting peaks. Periodate-treatment, on the other hand abolished all of the peaks except that for NCTC 10714, which was reduced in height but not eradicated.

This result suggests that cross-reactions with NCTC 11065 antiserum involved glycoprotein antigens which bore serological similarity. Reference to appendix 2.(ii). shows that the reaction of NCTC 11065 antiserum with NCTC 10714 EDTA extract was most clearly protein in nature, whilst for the other strains, a more significant part was additionally played by carbohydrate. This may explain the reduced but not abolished antigenicity of NCTC 10714 EDTA extract by the action of periodate.

Cross reactions with NCTC 5389 antiserum were too faint to be satisfactorily photographed, but showed a similar appearance of negatively charged peaks as shown with NCTC 11065 antiserum. An additional, faint,

positively charged peak was demonstrated for the EDTA extract of NCTC 8037. As had been the case with peak 2 of the homologous reaction with this antiserum, the negatively charged peaks could not be visualised following heat or periodate treatment, and it was suggested that these peaks could again represent glycoprotein antigens. The second peak for NCTC 8037 EDTA extract was also eradicated by both heat and periodate treatment.

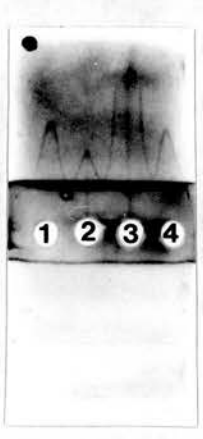
Cross-reactions with NCTC 10714 antiserum were again very weak. Visible peaks of immunoprecipitation were observed with EDTA extracts of NCTC 5389, NCTC 8037 and NCTC 11065. All of these negatively charged peaks were eradicated by heat-treatment, and all but that with NCTC 5389 EDTA extract were also eradicated by periodate treatment.

On no occasion during this study of cross-reactions between reference strains were heavy peaks created by carbohydrate antigens similar to those encountered in homologous reactions observed, indicating some strain specificity of these antigens. All cross-reactions were weak, but precipitating, cross-reactive antigens were encountered between many strains and heterologous sera. The majority of these cross-reactions appeared to involve antigens labile to the action of both heat and periodate. Only very limited correlation was possible with the results of ELISA.

4.10.2. Examination of EDTA extracts from wild group F strains.

Limitation of time allowed only brief examination of EDTA extracts derived from a small number of wild group F streptococcal strains. These strains were cultured aerobically prior to extraction.

Figure 4.19. EDTA extracts of NCTC 10707, NCTC 10714, NCTC 5389, and NCTC 8037 reacted with antiserum raised against NCTC 11065 in FRIE.



Well 1 = NCTC 10707 EDTA extract; 2 = NCTC 10714 EDTA extract; 3 = NCTC 5389 EDTA extract; 4 = NCTC 8037 EDTA extract.

Examination of EDTA extracts for the presence of carbohydrate and protein indicated less diversity amongst these strains than observed for reference strains (table 4.3.A.). Carbohydrate to protein ratios ranged from 1:2 to 1:5, and averaged 1:4.

4.2.11. Titration of EDTA extracts from group F wild strains in ELISA.

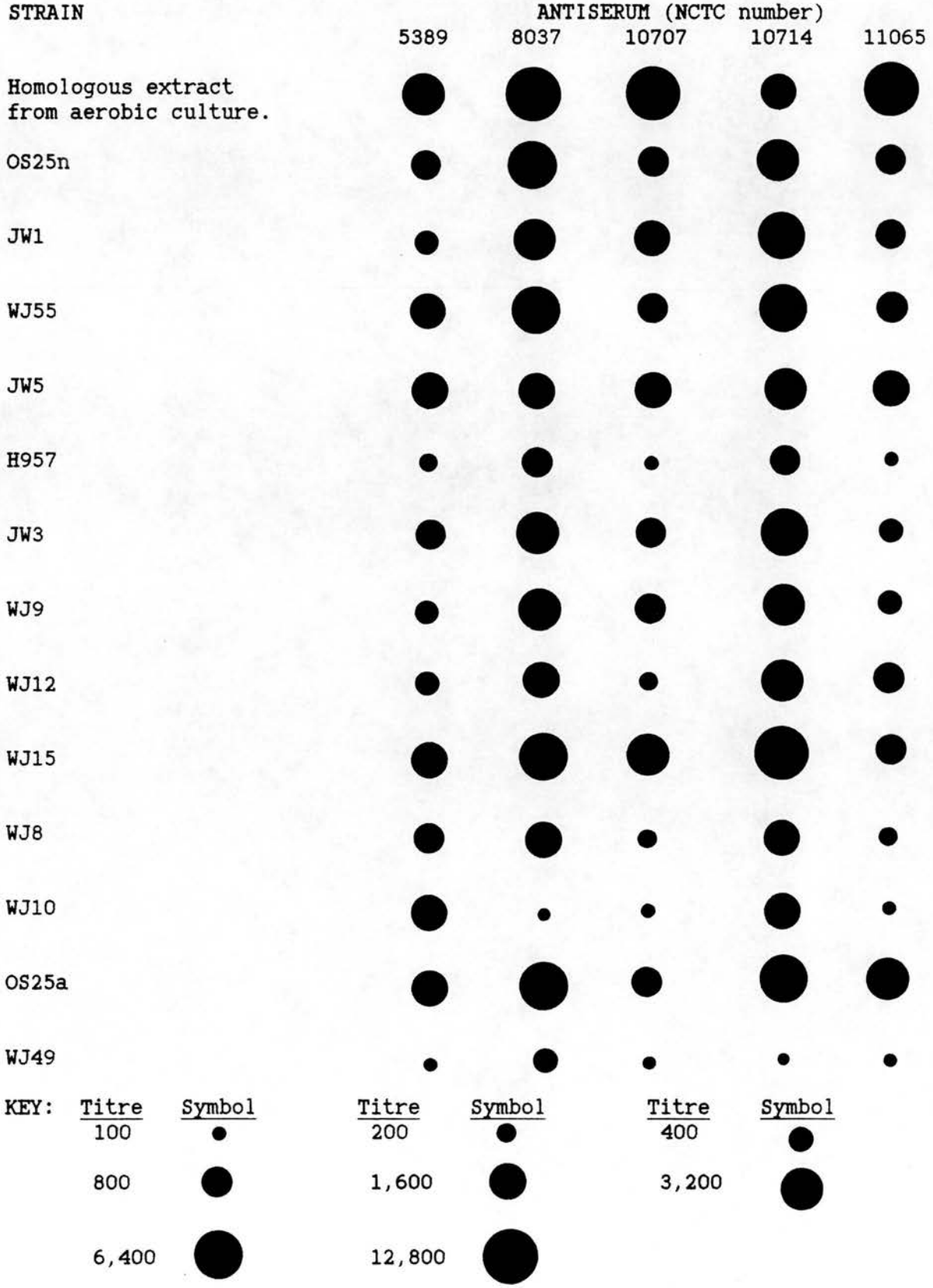
The results of titrations between EDTA extracts and doubling dilutions of five *Streptococcus milleri* antisera are shown diagramatically in figure 4.20. Actual figures for the end-points of titrations may again be found in appendix 2.(iii).

A high general degree of cross-reactivity was noted amongst wild strains and the five test antisera. As encountered with reference strains, antiserum raised against NCTC 10714 was most cross-reactive, whilst that raised against NCTC 11065 was least reactive in this respect.
No attempt was made to investigate the biochemical nature of antigenic material involved in these reactions.

4.2.12. Examination of EDTA extracts from group F wild strains in SDS-free PAGE.

Extracts were additionally examined in PAGE in order to assess the degree of similarity of protein profiles from a varied collection of wild strains. Figure 4.21. shows the PAGE profiles of seven group F streptococcal strains derived from a range of sources, and displaying either non-haemolysis (tracks 2-5), or beta-haemolysis (tracks 6-8). This collection of strains

Figure 4.20. Titration of EDTA extracts from wild group F strains against five *Streptococcus milleri* antisera.



formed a relatively homologous group in terms of biochemical behaviour, all behaving typically (see chapter 3) except for JW5 (track 6) and JW3 (track 8) which failed to hydrolyse aesculin.

A marked degree of general similarity was noted amongst the PAGE profiles of this small collection of strains, although minor differences between non-haemolytic and beta-haemolytic strains were suggested.

Figure 4.22. shows the PAGE profiles of a further collection of non-haemolytic (tracks 2-3) and alpha-haemolytic (tracks 4-8) group F strains.

Some physiological diversity was noted amongst these strains:

i. Typical biochemical profiles were observed for: WJ9 (track 2), WJ12 (track 3), WJ15 (track 4), and OS25a (track 7); whilst

ii. WJ8 (track 5) failed to hydrolyse arginine;

iii. WJ10 (track 6) gave positive reactions in the melibiose fermentation test, and the VP test only; and

iv. WJ49 (track 8) failed to hydrolyse aesculin and gave a negative VP reaction.

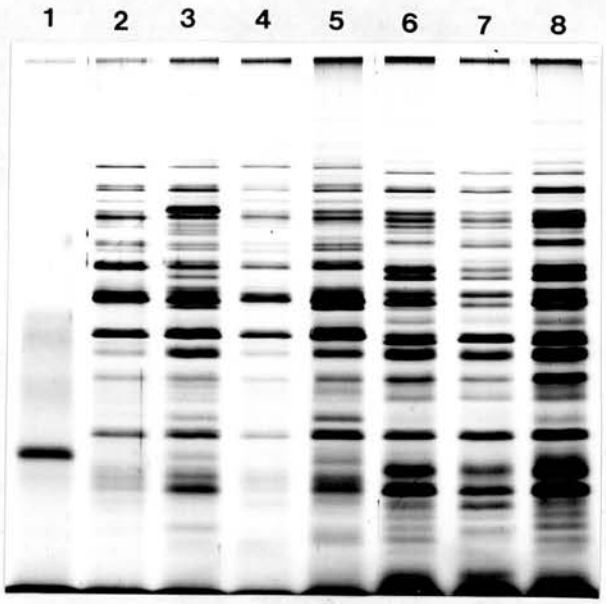
The diversity of biochemical behaviour was reflected in the PAGE profiles of the latter three strains. However, despite giving a typical biochemical profile, OS25a (track 7) presented a different PAGE profile.

Figure 4.23. confirmed the marked similarity of profiles from strains of all haemolytic types included in figures 4.21. and 4.22.

Differences observed between non-haemolytic and beta-haemolytic strains in figure 4.21. were not apparent in this gel.

Differences in PAGE protein profile for some of the alpha-haemolytic

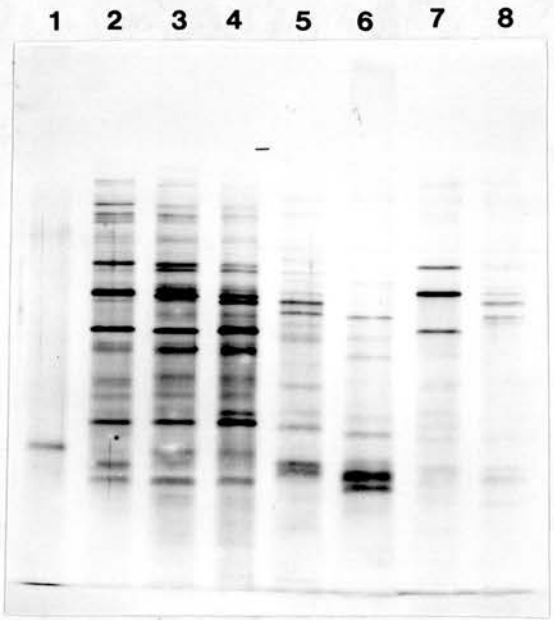
Figure 4.21. PAGE of EDTA extracts from wild non-haemolytic, and beta-haemolytic group F streptococci.



Gel silver stained for protein.

KEY: Track 1 = Molecular mass markers (BDH); 2 = OS25n; 3 = JW1; 4 = JW2; 5 = WJ55; 6 = JW5; 7 = H957; 8 = JW3. Approximately 15µg protein per track.

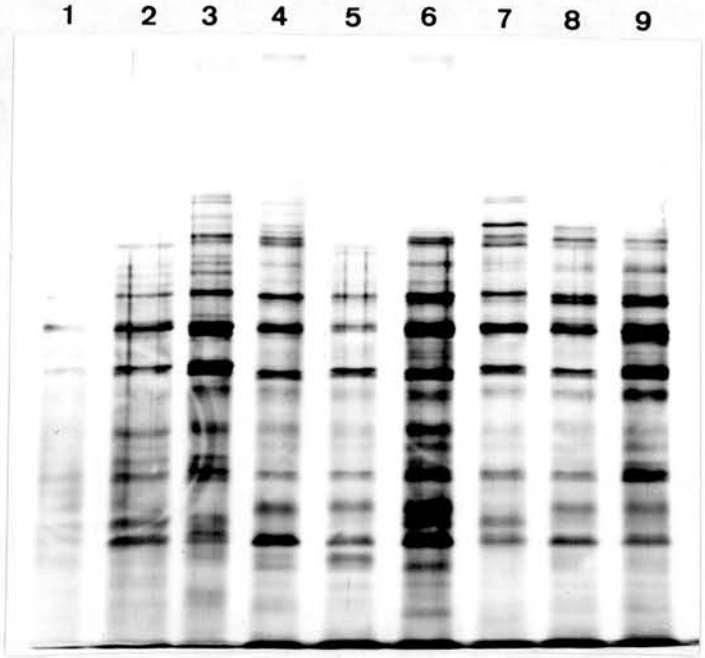
Figure 4.22. PAGE of EDTA extracts of wild non-haemolytic and alpha-haemolytic group F streptococci.



Gel silver stained for protein.

KEY: Track 1 = Molecular mass markers (BDH); 2 = WJ9; 3 = WJ12; 4 = WJ15; 5 = WJ8; 6 = WJ10; 7 = OS25a; 8 = WJ49. Approximately 15μg protein per track.

Figure 4.23. PAGE of EDTA extracts of a collection of alpha, beta, and non-haemolytic group F streptococci.



KEY: Track 1 = OS25n; 2 = JW1; 3 = WJ55; 4 = JW5; 5 = H957; 6 = JW3; 7 = WJ9; 8 = WJ12; 9 = WJ15. Approximately 15μg protein per track.

strains did not reliably reflect low antibody titres against *Streptococcus milleri* reference strains, with the exception of WJ49.

Attempts were made to visualise common groups of antigens in wild strains by immunoblotting with *Streptococcus milleri* antisera. Again, this investigation was unsuccessful, with highly confusing and variable arrays of antigenic bands being inconsistently recognised by different antisera.

4.2.13. Examination of wild-strain EDTA extracts in fused rocket immunoelectrophoresis.

EDTA extracts derived from a number of wild strains were examined in FRIE against *Streptococcus milleri* antisera. Reactions with different antisera varied considerably. Again, no visible peaks were encountered with NCTC 10707 antiserum. Faint, negatively-charged peaks were again observed between wild strains and antiserum raised against NCTC 11065, despite low ELISA antibody titres with this antiserum. Figure 4.24. shows the peaks observed between wild strains and antiserum raised against NCTC 8037. Unlike the investigation of cross-reactions between reference strains, some wild strains gave the same sort of heavy peaks as encountered in reactions between reference strain EDTA extracts and their homologous antiserum. For three oral strains, the peaks migrated towards the cathode. Heavy peaks of immunoprecipitation migrating towards the anode were also encountered for two wild strains (JW1 and JW9) reacted with NCTC 8037 antiserum. These peaks also occurred between the latter two strains and NCTC 5389 antiserum, and between JW1 and NCTC 10714 antiserum.

Figure 4.24. Examination in FRIE of EDTA extracts from group F wild strains against NCTC 8037 antiserum.

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Well 1 = JW3 EDTA extract; 2 = JW1 EDTA extract; 3 = JW9 EDTA extract; 4 = WJ12 EDTA extract; 5 = OS25n EDTA extract; 6 = OS25a EDTA extract; 7 = WJ55 EDTA extract; 8 = WJ49 EDTA extract.

The limitations of time prevented a fuller examination of the extracts of wild strains, but the inclusion of these results indicates that there is still much work to be done on the non-covalently bound cell-surface antigens of group F and related streptococci.

4.3. Discussion.

The great sensitivity of modern analytical techniques, particularly immunochemical methods has led to the detection of cell-surface components on whole bacteria which are not observed during gross wall analysis. These substances include proteins and amphiphilic carbohydrates such as lipoteichoic acids. Although lost during the course of cell-wall purification, these wall components that are not tightly bound may present at the cell-surface of intact cells as important antigenic determinants.

Studies reported in the current chapter were designed to investigate some aspects of cell-surface components associated in a non-covalently linked manner with a collection of streptococci, the majority of which belonged to Lancefield group F.

In the first part of this study, EDTA, a chelating agent used in biochemical systems *in vitro* for the chelation of divalent cations, was investigated for its ability to release material from the surface of whole streptococcal cells. Previous reports of Poxton and Byrne, (1981), Sharp and Poxton (1985) and Smith *et al.*, (1986) demonstrated the ability of EDTA to extract surface antigens from *Clostridium* spp., *Clostridium difficile* and Gram positive anaerobic cocci respectively, by destabilisation of the cell-wall and membrane.

Incubation of *Streptococcus milleri* strains with 10mM EDTA led to the release of both proteins and carbohydrates from the cell-surface of whole cells. In all cases, EDTA extracts contained higher levels of protein than

carbohydrate. Incubation for 60 minutes at 45°C greatly improved protein yields compared to 30 minute incubation, and gave no evidence of large-scale cell lysis.

Protein yields varied considerably between strains, but were similar in range to those reported for cultures of Gram-positive anaerobic cocci, whose yields varied between 100ug/ml and 1,000ug/ml (Smith, 1985). Both the results of the current study, and those of Smith (1985) represented low protein levels compared to those derived from some strains of Gram-positive anaerobes treated in a similar manner. For example, EDTA extracts prepared from cultures of *Clostridium* spp. may yield 1.5 - 2.0mg/ml protein (Mr. R. Brown, Chief MLSO: personal communication). No details of typical protein yields from other Gram-positive aerobes were available for comparison.

Some of the variation in protein yield inevitably reflected variation in growth level for individual strains, and it is likely that differences would have been less marked if extraction had been performed on equal weights of freshly harvested cells for each strain.

4.3.1. PAGE.

Silver staining of extracts separated in PAGE demonstrated the presence of complex arrays of proteins released into solution by the action of EDTA.

Initial examination of reference strains in SDS-PAGE illustrated similarity of a biochemically quite homologous collection of reference strains of varying haemolytic types. Profiles obtained in SDS-free gels were, however, less convincingly similar, and strains could not be said to bear more than a general likeness in terms of PAGE appearance.

Examination of the influence of minor differences in growth conditions

indicated that the profiles of EDTA-extractable proteins displayed some stability for individual strains.

It was beyond the scope of the current research to monitor the influence of more severe alterations in culture conditions on the expression of cell-surface proteins. Such studies would ideally be carried out with the aid of a chemostat, in which very strict and reproducible control of the culture environment could be achieved. Sophisticated culture apparatus of this sort was not available for the current investigations, but every effort was made to ensure that culture and extraction procedures varied only in the ways indicated.

It is consequently suggested on the basis of these results that provided reasonable care is taken in experimental procedures, EDTA extractable protein profiles in PAGE may be regarded as relatively stable features for individual strains.

Although the current studies were primarily concerned with antigenic aspects of the cell-surface of this group of organisms, PAGE examination of EDTA extracted protein profiles from a small number of wild group F strains was undertaken. PAGE profiles showed a high general degree of similarity amongst strains representative of all haemolytic types. The strains examined did not, however, represent an entirely homologous group, particularly with respect to a number of alpha-haemolytic strains in which differences in biochemical behaviour were reflected by differences in protein profile. In the case of strain OS25a, the different protein profile did not reflect an atypical biochemical profile. Observations from this small collection of strains suggested that examination of EDTA extracts in PAGE may potentially offer an additional method of value in

chemotaxonomical studies of the *Streptococcus milleri* group. Examination of a large collection of strains spanning organisms of all representative Lancefield groups, haemolytic types, biochemical activities, sources, and suspected pathological involvement may allow the recognition of natural sub-groups within this taxon. Such investigations may be of assistance in establishing the typical associations of pathological members of this diverse group of organisms.

It is suggested that such an investigation would ideally be accomplished with the aid of densitometry and numerical analysis facilities, to aid the processing of protein profiles. Surface protein examination may be more sensitive in this respect than the examination of whole-cell proteins (Whiley *et al.*, 1982) in which potentially significant variations in cell-surface proteins associated with certain sub-groups may be lost in a sea of common intracellular proteins.

Differences in the appearance of EDTA extracts run in different gels (see figures 4.21. and 4.23.) were encountered in the current investigations, highlighting the problems of reproducing running conditions accurately. Another problem encountered was in obtaining uniform staining density between gels. This problem arises out of the fact that the point at which development of a silver-stained gel is arrested is a matter of judgement based upon the knowledge that development will continue for some minutes following rinsing in distilled water. An additional problem which was occasionally encountered was the observation of differences in staining intensity of individual tracks when standard quantities of protein were loaded (see figure 4.2.). An explanation for this finding was offered by Davies *et al.* (1973), who suggested that the presence of phenolic compounds

in cell extracts may compromise the accuracy of protein assays. Any such discrepancies are likely to have a marked effect on the silver stained appearance of gels owing to the low amounts of protein loaded, and the extreme sensitivity of the stain.

It is suggested that should the examination of EDTA-extractable proteins be applied in taxonomic studies, the Coomassie blue stain would offer a more reproducible alternative in staining. An additional point in favour of the Coomassie blue stain in such studies is its lower sensitivity, which allows a more general appraisal of the likeness of profiles than is possible by the application of a highly sensitive silver stain.

4.3.2. Immunoblotting.

Demonstration of antigenic material in EDTA extracts of *Streptococcus milleri* reference strains by ELISA prompted the use of immunoblotting to visualise antigenic bands. It was hoped that the application of immunoblotting would allow simplification of the complex arrays of protein bands visualised in PAGE, and demonstrate either single, or groups of antigenic bands common to many strains. Probing of material transferred from PAGE gels of reference strains to nitrocellulose with homologous antiserum demonstrated the presence of numerous antigenic bands in each strain. No general uniformity of immunoblot appearance was noted amongst the reference strains studied, although NCTC 11065 and NCTC 5389 appeared to bear some similarity. The demonstration of additional antigenic bands in the EDTA extract of anaerobically cultured NCTC 10714 suggested that whilst PAGE protein profiles had apparently remained constant under the range of

culture conditions considered, differences in the quantity, or means of expression of certain surface antigens may have occurred, allowing detection of additional bands by immunoblotting. This initial use of immunoblotting in the examination of reactions with homologous sera gave reproducible results and suggested considerable diversity amongst the non-covalently bound antigenic determinants of this collection of *Streptococcus milleri* reference strains.

However, the probing of gels with heterologous antisera added greatly to the complexity of the picture, with different bands being inconsistently detected by different antisera, and no picture of common antigens emerging. Similar results were obtained when wild strains were probed with the various *Streptococcus milleri* antisera, and despite every effort being made to keep conditions uniform, no clear, reproducible, or meaningful information was obtained by the use of this technique in the hands of the author. A confusing picture of cross-reactions was observed.

Whether the bands observed were proteins or not could not be stated with certainty, as the matching of bands to equivalent bands on the acrylamide gel was not always easy. A measure of shrinkage occurs during the fixing process which forms the first part of silver-staining, adding to the difficulties of comparison between bands visible on gel and blot. Jackson and Thompson (1984), however, described a modification of the immunoblotting procedure which seeks to avoid problems of accurate superimposition of gel and blot in the following manner: polyacrylamide gels are run, and stained with Coomassie blue in the customary manner. Following staining, the bands of the gel are transferred (with stain) to nitrocellulose membrane, which is then appropriately probed with antisera and developed as an immunoblot. Photographs obtained with the aid of

filters allow the production of two identical images, one showing the blue Coomassie-stained bands of the gel, and the other, the red-stained antigenic bands of the blot. Despite the potential advantages of this technique, it is not in routine use in our laboratory since the facilities for photography under the conditions of filtration suggested are not currently available.

The appearance of non-covalently bound carbohydrate antigens, notably lipoteichoic acids, in PAGE and immunoblot has not to my knowledge been reported for the group F and related streptococci, and therefore comparisons could not be made with the gels and blots observed in this study. Further consideration will be given to this issue in chapter 5.

4.3.3. ELISA studies.

Since its development by Engvall and Perlmann in 1972, ELISA has developed into a highly versatile technique, with a wide range of applications in modern biology. One of the advantages of ELISA over a number of other immunochemical techniques (including immunoblotting and immunoelectrophoresis) is its ability to detect a wide range of antigens, many of which are too small to bind to nitrocellulose membranes, or to form precipitating complexes in gel. That, on the other hand, is not to say that all antigens may successfully be detected by ELISA, as all antigens do not satisfactorily bind to the wells of microtitre plates to allow detection.

ELISA was first used with success in this study for the titration of antisera raised against uv irradiated whole-cell vaccines of *Streptococcus*

milleri reference strains against the EDTA extracts of homologous strains. Despite the reportedly low virulence of *Streptococcus milleri* isolates for laboratory animals, limited resources made the potential loss of an animal due to infection from live streptococcal vaccines an unacceptable risk. Killing by uv irradiation was consequently employed in the current study as it was believed to be less capable of damaging cell-surface antigenic structures than such alternative methods as formalinisation or heat-treatment.

Antisera prepared in the manner described were capable at high dilution of detecting non-covalently linked cell-surface antigens of *Streptococcus milleri* reference strains. Even the lowest titres obtained (1 in 1,600 dilution) indicated a high sensitivity of antiserum for EDTA extracted antigens of homologous strains. Variations in ELISA titration end-points for different EDTA extracts of NCTC 5389 and NCTC 10714 suggested antigenic differences between extracts from organisms grown under differing atmospheric conditions. None but the major increase in antibody titre for anaerobically cultured NCTC 10714 with homologous antiserum were reflected by differences in immunoblot profiles against homologous antisera. These differences in titre may simply have reflected minor discrepancies in the protein concentration of samples, or may have reflected alterations in the quantity or expression of antigens undetectable by less sensitive means than ELISA.

ELISA inhibition reactions reaffirmed the diversity of immunoblot profiles observed for reference strains, and reflected a great deal of diversity in the overall nature of antigenic material presenting at the cell-surface of different strains. Results obtained by two different methods for ELISA

inhibition reactions corroborated one another and reaffirmed the impression of antigenic variation indicated by immunoblotting. The predominant cell-surface antigens detected by this technique varied from predominantly protein for NCTC 11065 to predominantly carbohydrate for NCTC 8037. Referring back to the ratios of carbohydrate to protein for these strains (table 4.3.A.) NCTC 11065 EDTA extracts did possess high levels of protein relative to carbohydrate, whilst the extracts of NCTC 8037 contained the highest levels of carbohydrate compared to protein of all the strains examined. This does not, however, explain the result of ELISA inhibition reactions for NCTC 10707, where the level of protein relative to carbohydrate was by far the highest of any of the strains studied. Although it would have been pleasing to find a simple correlation between the levels of carbohydrate and protein in extracts and the nature of predominant cell-surface antigens, this was not reliably found to be the case.

A further aspect of antigenic differences was suggested by the ELISA inhibition results obtained for extracts derived from different culture conditions. Although general trends remained constant for given strains, variations in the balance of antigenic material at the cell-surface of strains cultured under different conditions was suggested. Such variations passed undetected in most circumstances by immunoblotting with homologous antiserum, but indicated that even minor fluctuations in culture conditions could influence the balance of non-covalently bound cell-surface antigens.

It is possible that greater correlation between ELISA and immunoblotting results might have been observed if the antisera used to probe immunoblots had been diluted further than the level at which they were used in the current studies (1 in 50 dilution). ELISA results indicated that this dilution represented an excess of serum, which may perhaps have

compromised the fine sensitivity to detect minor changes which were detected in ELISA by immunoblotting.

Very minor alterations in culture atmosphere and pH have been shown to be capable of causing significant qualitative changes in the expression of cell-surface proteins in coagulase negative staphylococci (Dr. D.G.E. Smith: personal communication). Such alterations in labile surface antigens may be of significance in terms of the virulence of organisms under different conditions. It is conceivable that organisms living as commensals in one body site may undergo antigenic modification on introduction to deeper tissue following trauma, and adopt altered cell-surface characteristics which may be of importance in terms of their interactions with the host immune response and pathogenicity.

The unsuccessful application of immunoblotting to the study of cross-reactions between reference strains and their corresponding antisera led to the return to an ELISA system for this purpose. The results of ELISA investigations were at least reproducible, and therefore presented a major advantage over the results of immunoblotting with heterologous antisera.

Titration of EDTA extracts against heterologous antisera showed that reference strains did possess considerable antigenic similarity, illustrated by the generally high antibody titres obtained. The results of ELISA inhibition reactions applied to cross-reactions again demonstrated the diversity of reacting antigenic determinants for this collection of reference strains.

ELISA is undoubtedly a highly sensitive and versatile assay system for use in the investigation of antibody/antigen reactions. In the current studies, extensive use of ELISA led to an appreciation of the complexity of the many carbohydrate, protein and glycoprotein antigens non-covalently linked and presenting as cell-surface antigens within strains of *Streptococcus milleri*. However, ELISA has not been able to produce more than a general overview of the cell-surface of these strains within the current system. Probing complex mixtures of antigens contained in EDTA extracts with polyvalent antisera results in the complexity of observations reported. Whilst for example, it is possible to say that protein antigens are important at the cell-surface of NCTC 11065, it is not possible within the current framework to define the size or likely function of such material, or the proportion made up by particular functional units of interest. Whilst the demonstration of protein antigens in NCTC 11065 is an interesting finding, the value of it in practical terms of deepening the understanding of important cell-surface proteins for this strain is limited.

More meaningful information would perhaps come from probing complex antigenic mixtures with monoclonal antibodies directed against certain proteins, or carbohydrates of specific functional interest, or of suspected importance in virulence.

Few conclusions may be drawn from the brief examination of wild strains in ELISA. The general degree of cross-reactivity demonstrated between these strains and the five *Streptococcus milleri* antisera suggested the presence of much common antigenic material in EDTA extracts. Simple correlations were not consistently found between antibody titres against *Streptococcus*

milleri antisera in ELISA, and the similarity of PAGE protein profiles compared to other strains.

It may be possible to couple the sort of PAGE examination suggested in section 4.3.1. with an ELISA examination of EDTA extracts as a means of diagnosing different sub-groups on the basis of non-covalently linked antigens. However, the application of such polyvalent sera as described here would appear to be of little value, and sera raised against purified components of EDTA extracts may be of more use in screening samples for this sort of study.

4.3.4. Immunoelectrophoresis.

i. CIE.

Perhaps the most interesting observations to emerge from the series of investigations presented in this chapter came from the examination of EDTA extracts in CIE.

Immunoelectrophoretic examination of extracts revealed surprisingly uncomplicated patterns of peaks for each strain, all of which were entirely reproducible. Despite the demonstration by other immunochemical techniques of antigenic material in EDTA extracts of NCTC 10707, no peaks of immunoprecipitation were observed for this strain in CIE. The lack of peaks of immunoprecipitation noted for this strain may reflect the low level of carbohydrate relative to protein in its extracts. Strong peaks of immunoprecipitation, which are believed to represent carbohydrate antigens, were observed as the only visible peaks for NCTC 10714 and NCTC 8037, and as the strongest peak for NCTC 5389. The fortuitous chance encounter with

these apparently positively charged antigens was described in the narrative of this chapter. The major carbohydrate peak visualised in the EDTA extract of NCTC 11065 was unusual compared to the other strains in that it migrated towards the anode, and therefore carried a negative charge.

Additional, weaker peaks of immunoprecipitation were demonstrated in the extracts of NCTC 11065 and NCTC 5389, both migrating towards the anode. The second peak for NCTC 11065 was shown to be protein, but the second peak for NCTC 5389 appeared to contain both carbohydrate and protein. Failure to detect this peak following heat or periodate treatment may suggest that this is a glycoprotein antigen. Previous reports of Lütticken *et al.* (1978), and Nakamaya and Maekawa (1979) have described the presence of precipitating protein antigens associated with the cell-surface of *Streptococcus milleri* strains, the latter describing them in strain O'Mahoney (NCTC 5389). ELISA studies highlighted the prominence of surface protein antigens in both of these strains, especially for NCTC 11065. These strains also shared similar profiles in immunoblotting with homologous antiserum. It is likely that a wide range of protein antigens present at the surface of streptococcal cells, many of which may be too small to form precipitating complexes, despite being detected by techniques such as ELISA. The proportion of the whole represented by the precipitating protein antigen demonstrated in NCTC 11065 EDTA extract, its importance, and function remain unknown.

ii. FRIE.

Examination of reference strains in FRIE failed to demonstrate cross-reactions mediated by the carbohydrate antigens illustrated in homologous

reactions. However, certain wild strains were found to give strongly reacting, positively charged peaks with NCTC 8037 antiserum. This finding suggested that these non-covalently bound carbohydrate antigens are quite strain-specific. Additional, weakly reacting peaks were demonstrated amongst many reference and wild strains, suggesting the presence of common cell-surface protein or glycoprotein antigens for many of these strains. Further work is required to investigate more fully the antigens contained in wild strains. It would, on the other hand, seem sensible to pursue more fully the investigation of certain antigens contained in reference strains before extending the study to include wild strains.

Reports in the literature have described the typing antigens of group F and related streptococci as carbohydrate polymers associated with the cell-wall (see section 1.7.7.a.). These polymers were said to be "excreted" into the culture medium during bacterial growth (Huis in't Veld and Willers, 1973) a property now known to be true for the lipoteichoic acids of Gram-positive organisms. As to the distribution of these polymers, Huis in't Veld and Linssen (1973) showed type-antigen to be present in abundance throughout the thickness of the cell envelope, with very dense localisation in the cytoplasm, close to the cytoplasmic membrane. This observation suggested also that the typing antigens for this group of organisms may in fact be lipoteichoic acids or their analogues: polymers capable of extraction from whole cells by the action of EDTA. It was therefore thought possible that the carbohydrate antigens demonstrated in CIE were lipoteichoic acid typing antigens for these strains.

Further support was given to this possibility by the facts that:

i. No precipitating carbohydrate antigen was noted for NCTC 10707, which is

believed to contain no typing antigen, and

ii. studies by Willers *et al.* (1973b) revealed that the elution behaviour of type IV antigen from DEAE-cellulose columns differed from those of type I-III antigens. This may reflect in the difference observed in the charge of the NCTC 11065 carbohydrate antigen in CIE compared to those of NCTC 5389, NCTC 8037, and NCTC 10714.

All of these interesting observations regarding non-covalently bound carbohydrate antigens of group F and related streptococci appeared worthy of further investigation, and reinforced still further the need to examine purified lipocarbohydrate material from this group of organisms. Consideration will be given in chapter 5 to the isolation, purification and analysis of membrane associated lipocarbohydrate antigens from group F and related streptococci.

CHAPTER 5

THE ISOLATION AND ANALYSIS OF MEMBRANE LIPOCARBOHYDRATE AND SECONDARY CELL-WALL POLYMERS FROM GROUP F AND RELATED STREPTOCOCCI

5.1.1. Introduction.

As awareness of the association of *Streptococcus milleri* with purulent disease in man increases, so does the need for greater understanding of the particular features of this group of organisms which result in the observed pathogenic behaviour. Perhaps of fundamental importance in this respect is an understanding of cell-surface antigens which may interact with the host immune system and provoke a protective immune response. This information is consequently of value not only in terms of enhancing the understanding of complex virulence mechanisms at work in bacterial disease, but is potentially of value in serological diagnostic and classification schemes. Information currently available on the cell-surface structure and antigenicity of group F and related streptococci has been reviewed in chapter 1.

One of the most interesting findings to emerge from the series of investigations described in chapter 4 was that certain *Streptococcus milleri* strains contained precipitating, non-covalently linked carbohydrate antigens, which appeared to be strain-specific. These antigens seemed to have much in common with descriptions of the carbohydrate typing antigens which are believed to be contained by some group F and related streptococci (Ottens and Winkler, 1962; Huis in't Veld and Linsson, 1973; Huis in't Veld and Willers, 1973). Many of the characteristics of these non-covalently linked carbohydrate antigens also suggested that they may be membrane-associated lipocarbohydrate antigens, whose distribution is believed to be widespread among Gram-positive bacteria (Lambert *et al.*, 1977).

Much of the analysis previously undertaken on the carbohydrate polymers associated with the cell-surface of group F and related streptococci was carried out on formamide extracts of whole cells (Michel and Willers, 1964; Willers *et al.*, 1964b; Willers *et al.*, 1973a), or of bacterial cell-walls purified by incubation with proteolytic enzymes (Willers *et al.*, 1964a). This method of purification is now believed to be capable of leaving considerable amounts of cytoplasmic material in association with cell-walls (Huis in't Veld and Willers, 1973).

The possibility that such preparations contained mixtures of cytoplasmic and truly wall-linked carbohydrate polymers cannot be excluded, and suggested that a fresh look at them would be justified.

No reports in the literature have described the isolation and analysis of membrane-associated lipocarbohydrate antigens from the group F and related streptococci.

Aims.

The major aims of the current study were:

1. To isolate and purify membrane lipocarbohydrate material from *Streptococcus milleri* reference strains and to examine it immunochemically for the presence of antigenic material associated with this cellular location;

2. To isolate and purify carbohydrate polymers derived from cell-walls freed from cytoplasmic contaminants by treatment with hot SDS and to examine them immunochemically for the presence of antigenic material;

3. To undertake some chemical analysis of the antigenic material identified from these sources;

4. To visualise membrane and wall associated antigens in immunoelectrophoresis and by immunoblotting to investigate the serological relationship of wall and membrane associated polymers.

5.1.2. List of strains investigated in the current study:

The following *Streptococcus milleri* reference strains were included in the current study: NCTC 11065, NCTC 10714, NCTC 5389, NCTC 8037, and NCTC 10707. Details of these strains may be found in table 3.1.

Details of the experimental procedures are given in chapter 2. Some additional points are included in the narrative of this chapter.

5.2. Results.

5.2.1. Extraction of membrane-associated lipocarbohydrates from *Streptococcus milleri* reference strains.

Experimental details are given in section 2.21. Membrane lipocarbohydrates were isolated from cultures of: NCTC 11065, NCTC 10707, NCTC 10714, and NCTC 5389.

The dry weights of defatted membrane fraction and crude phenol extract derived from 10l broth cultures of each strain are given in table 5.1. Some strain to strain variation was noted in both respects.

5.2.2. Purification of crude lipocarbohydrate material.

In addition to lipocarbohydrate material, phenol extracts of Gram-positive bacterial cell-membranes are believed to contain variable quantities of nucleic acids, proteins and cell-wall carbohydrates (Fischer *et al.*, 1983). Lipocarbohydrate extracts were purified by gel-filtration on a Sepharose 6-B column, and the fractions screened for carbohydrate, phosphate and nucleic acid; serological activity was detected by immunoelectrophoresis, or by dot-blotting against homologous antiserum. The void volume (V_0) of the column was at fraction 20 (approximately 46ml).

Figure 5.1. shows the elution profile of the crude phenol extract of NCTC 11065 cell-membranes from a Sepharose 6-B column. The first peak was observed just after the void volume.

Table 5.1. Dry weights of defatted membrane fraction and crude phenol extract derived from 10l broth cultures of four *Streptococcus milleri* reference strains.

STRAIN	DRY WEIGHT OF DEFATTED MEMBRANES (g)	DRY WEIGHT OF CRUDE PHENOL EXTRACT (mg)	% YIELD FROM MEMBRANES
NCTC 11065	0.816	56.1	6.9
NCTC 10714	0.65	26.5	4.1
NCTC 10707	1.056	53.8	5.1
NCTC 5389	0.61	60	9.8

Figure 5.2. shows the odd numbered NCTC 11065 Sepharose 6-B column fractions (21 to 59 inclusive) in FRIE against homologous antiserum. Strongly antigenic material migrating towards the anode was observed in the purified lipocarbohydrate material (peak 1) of this strain. Column fractions corresponding to peak 1 (fractions 22 to 36 inclusive), and peak 2 (fractions 39 to 46 inclusive) were pooled, desalted and lyophilised.

Figure 5.4. shows the elution profile of the crude phenol extract of NCTC 10707 cell-membranes from a Sepharose 6-B column. Again, the first peak was observed just after the void volume.

Examination in FRIE against homologous antiserum failed to demonstrate precipitating antigens in any of the column fractions. To exclude the possibility that antibody/antigen concentrations were resulting in the formation of non-precipitating complexes, further attempts were made to visualise immunoprecipitation by altering the relative concentrations of antigen and antiserum. All attempts were unsuccessful, and it was concluded that precipitating antigens could not be demonstrated for this strain. As an alternative means of demonstrating antigenic activity, column fractions were examined by dot-blotting against homologous antiserum. Fractions corresponding to peak 1 (fractions 21 to 29 inclusive) were strongly antigenic, and were pooled. Significantly less activity was noted in fractions corresponding to peak 2 (fractions 38 to 46 inclusive), and again in those corresponding to peak 3 (fractions 47 to 54 inclusive). Each collection of fractions was separately pooled, desalted and lyophilised. A repeat preparation for NCTC 10707 confirmed the result that the lipocarbohydrate material extracted from the cell-membranes of this strain contained no precipitating antigens, but that antigenicity could be

detected by dot-blotting. This antigenic activity was again mainly confined to column fractions corresponding to peak 1.

Figure 5.5. shows the elution profile of the crude phenol extract of NCTC 10714 cell-membranes from a Sepharose 6-B column. No peaks were observed until after the void volume. The profile shown in figure 5.5. indicated that the carbohydrate peak (peak 1) did not coincide with the phosphate or nucleic acid (A_{260}) peaks.

Figure 5.3. shows the even numbered NCTC 10714 Sepharose 6-B column fractions (20 to 60 inclusive) in FRIE against homologous antiserum. Strong peaks of immunoprecipitation were observed for this strain, corresponding to the carbohydrate peak observed in figure 5.5., and migrating towards the cathode. Column fractions corresponding to peak 1 (fractions 28 to 38 inclusive), and peak 2 (fractions 41 to 48 inclusive) were separately pooled, desalted, and lyophilised.

Figure 5.6. shows the elution profile of the crude phenol extract of NCTC 5389 cell-membranes from a Sepharose 6-B column. Again, no peaks were observed until after the void volume. In a similar manner to that observed for NCTC 10714 (figure 5.5.), the carbohydrate peak (peak 1) appeared not to coincide with the phosphate and nucleic acid peaks for this strain. The meaning of this observation in terms of the purity of lipocarbohydrate antigens prepared by gel-filtration on this column was, however, unclear. Figure 5.7. shows the even numbered column fractions (16 to 60 inclusive) in FRIE against homologous antiserum. Definite peaks of immunoprecipitation migrating towards the cathode were observed in column fractions

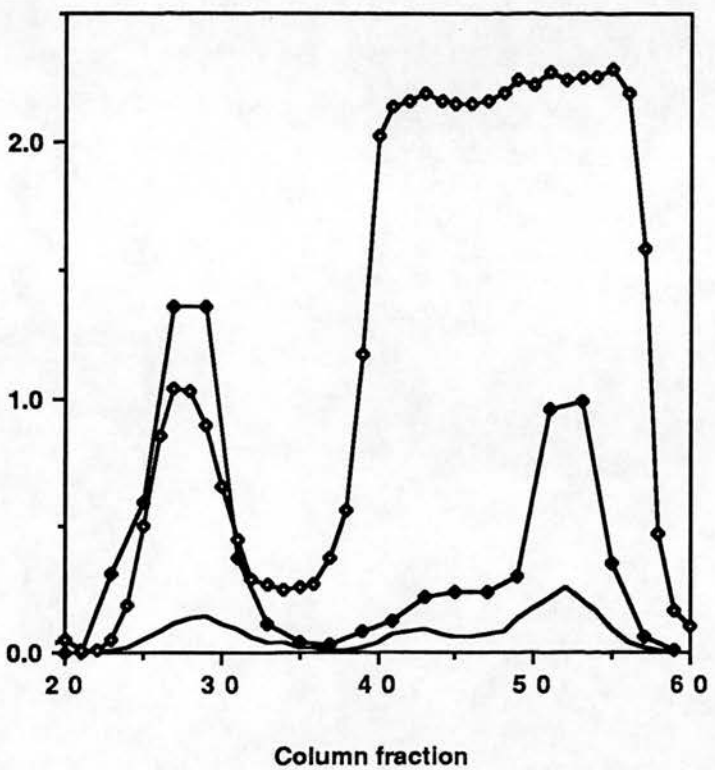


Figure 5.1. Separation of NCTC 11065 lipocarbohydrate from membrane contaminants by gel-filtration on a Sepharose 6-B column. Column eluted with 0.1M sodium acetate buffer, pH 4.7.
Carbohydrate content of fractions (mg/ml) = —
Phosphate content of fractions (ug/ml) = —●—
Nucleic acid content of fractions (A_{260}) = —○—
Peak 1 = fractions 22 to 36 (inclusive), peak 2 = fractions 39 to 46 (inclusive).

Figure 5.2. FRIE against homologous antiserum of odd numbered fractions (21 to 59 inclusive) eluted from the NCTC 11065 Sepharose 6-B column shown in figure 5.1.

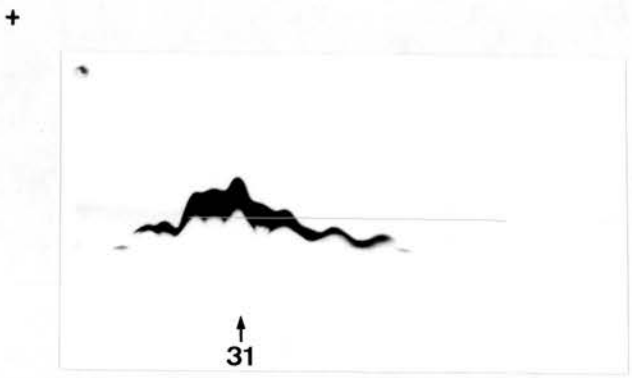
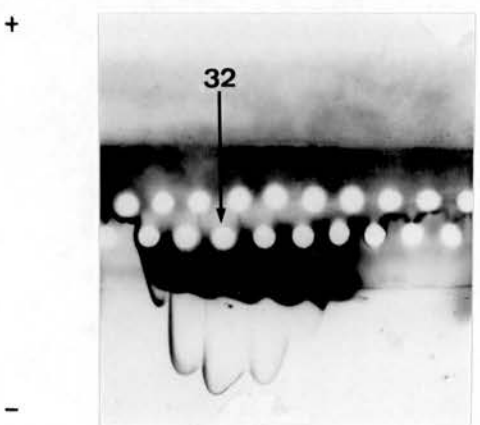


Figure 5.3. FRIE against homologous antiserum of even numbered fractions (20 to 60 inclusive) eluted from the NCTC 10714 Sepharose 6-B column shown in figure 5.5.



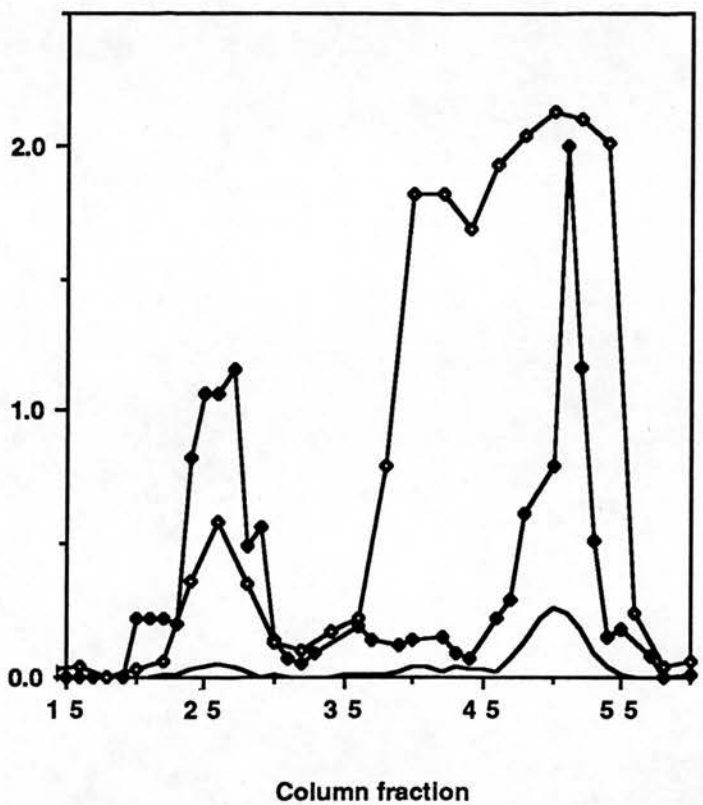


Figure 5.4. Separation of NCTC 10707 lipocarbohydrate from membrane contaminants by gel-filtration on a Sepharose 6-B column. Column eluted with 0.1M sodium acetate buffer, pH 4.7.
Carbohydrate content of fractions (mg/ml) = —
Phosphate content of fractions (μg/ml) = —◆—
Nucleic acid content of fractions (A_{260}) = —○—
Peak 1 = fractions 21 to 29 (inclusive), peak 2 = fractions 38 to 46 (inclusive), peak 3 = fractions 47 to 54 (inclusive).

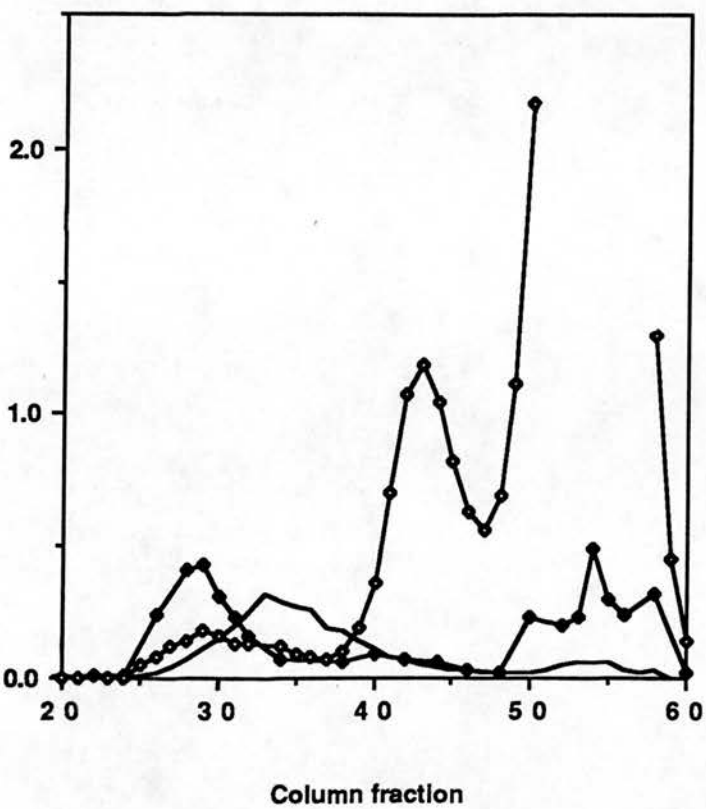


Figure 5.5. Separation of NCTC 10714 lipocarbohydrate from membrane contaminants by gel-filtration on a Sepharose 6-B column. Column eluted with 0.1M sodium acetate buffer, pH 4.7.
Carbohydrate content of fractions (mg/ml) = —
Phosphate content of fractions (ug/ml) = -◆-
Nucleic acid content of fractions (A_{260}) = -◆-
Peak 1 = fractions 28 to 38 (inclusive), peak 2 = fractions 41 to 48 (inclusive).

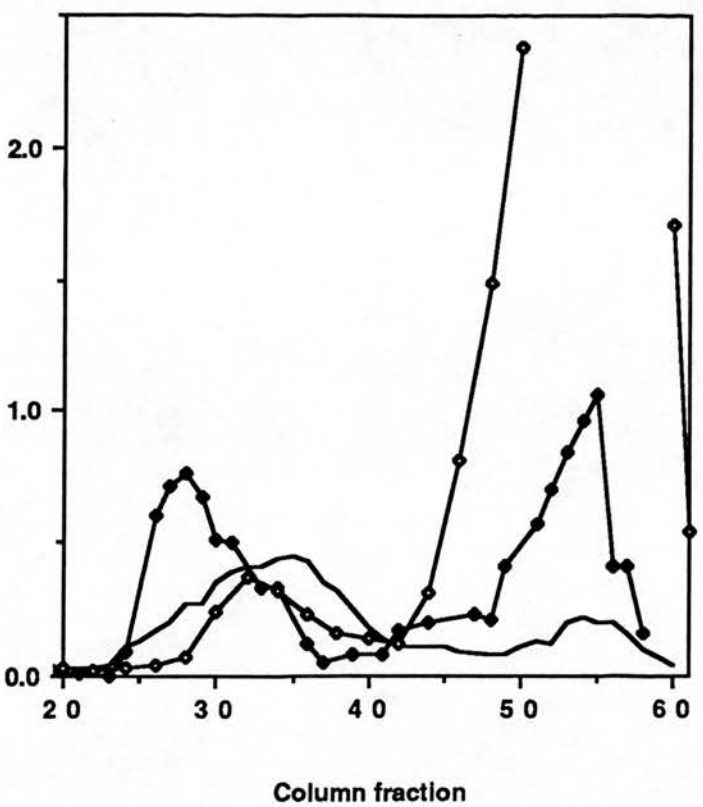
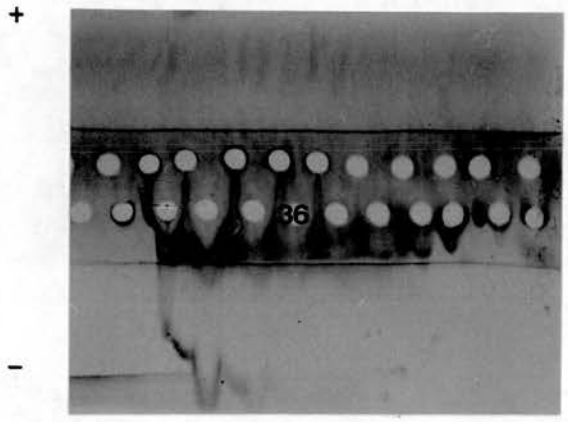


Figure 5.6. Separation of NCTC 5389 lipocarbohydrate from membrane contaminants by gel-filtration on a Sepharose 6-B column. Column eluted with 0.1M sodium acetate buffer, pH 4.7.
Carbohydrate content of fractions (mg/ml) = —
Phosphate content of fractions (ug/ml) = —●—
Nucleic acid content of fractions (A_{260}) = —○—
Peak 1 = fractions 24 to 38 (inclusive).

Figure 5.7. FRIE against homologous antiserum of even numbered fractions (16 to 60 inclusive) eluted from the NCTC 5389 Sepharose 6-B column shown in figure 5.6.



corresponding to the carbohydrate peak 1. Fractions corresponding to this peak (fractions 24 to 38 inclusive) were pooled, desalted and lyophilised. In all cases, purified lipocarbohydrate material (peak 1 for each strain) eluted close to the exclusion volume of the Sepharose 6-B column. All purified lipocarbohydrates were shown to be antigenic and behaved in a similar manner in CIE to the non-covalently linked carbohydrate antigens contained in EDTA extracts of their homologous strain (see chapter 4). The extract of NCTC 10707 failed to give immunoprecipitation in gel against homologous antiserum. For the other strains examined, single peaks of immunoprecipitation were observed against homologous antiserum. NCTC 11065 lipocarbohydrate antigen migrated towards the anode, but those of NCTC 10714 and NCTC 5389 migrated towards the cathode. No membrane lipocarbohydrate antigen was prepared from NCTC 8037.

No cross-reactivity of purified lipocarbohydrate antigens was demonstrated in immunoelectrophoresis. No attempt was made to investigate possible cross-reactivity amongst purified lipocarbohydrate antigens in an ELISA system.

5.2.3. Analysis of purified lipocarbohydrate antigens.

No additional purification steps were undertaken prior to carrying out chemical analysis to determine the general composition of these membrane-associated antigens.

All of the purified lipocarbohydrate antigens were found to contain glycerol phosphate, by staining of paper chromatographs with the periodate Schiff reagents (Baddiley *et al.*, 1956).

Purified NCTC 11065 lipocarbohydrate antigen contained 0.079 ug of phosphate for every 1 ug total carbohydrate, or in molar proportions 0.5 : 1. This represented the highest level of phosphate in relation to carbohydrate of all of the membrane-associated antigens prepared.
Gas chromatographic analysis showed that this antigen contained: glycerol, glucose, rhamnose, galactose and xylose in the approximate molar ratios of 4 : 4 : 1 : 1 : 0.3. A trace amount of deoxyribose was also present.

Purified NCTC 10707 lipocarbohydrate antigen contained 0.007 ug of phosphate for every 1 ug total carbohydrate, or in molar proportions 0.044 : 1.
Gas chromatographic analysis showed that this antigen was the simplest of those examined in the current study, and contained: glycerol, glucose and xylose in the approximate molar ratio of 3 : 2 : 1. No rhamnose was detected in this antigen, but a trace amount of deoxyribose was present.

Purified NCTC 10714 lipocarbohydrate antigen contained 0.0097 ug of phosphate for every 1 ug total carbohydrate, or in molar proportions 0.0 6 : 1.
Gas chromatographic examination revealed the presence of: glycerol, glucose, rhamnose, galactose and galactosamine in the approximate molar ratio of 2 : 5 : 3 : 5 : 1. Trace amounts of arabinose and deoxyribose were also found.

Purified NCTC 5389 lipocarbohydrate antigen contained 0.014 ug of phosphate for every 1 ug total carbohydrate, or in molar proportions 0.09 : 1.
Gas chromatographic examination showed that this antigen contained: glycerol, glucose, rhamnose and galactose in the approximate molar ratio of

1 : 4 : 2 : 1. Trace amounts of deoxyribose, galactosamine, arabinose and xylose were also detected.

No choline could be detected in any of these purified antigens by the method of Appleton *et al.* (1953).

A summary of the results of chemical analysis is given in tabulated form in appendix 3.

5.2.4. Isolation and analysis of covalently linked cell-wall antigens.

5.2.5. Preparation of cell-walls.

Crude cell-walls were routinely prepared by explosive decompression of cells in a French Pressure Cell (see section 2.18.1.). A pilot study showed that ultrasonic irradiation of *Streptococcus milleri* cell suspensions resulted in barely detectable levels of disruption when examined by phase contrast microscopy.

Repeated passaging (three passages) through the French Press at high pressure was required to obtain approximately 70% cell breakage. No appreciable improvement in the degree of cell breakage was obtained by further passaging. Crude cell-walls were freed from cytoplasmic contaminants by treatment with hot SDS. The yields of purified, lyophilised cell-walls obtained from 81 broth cultures of five reference strains harvested after 17 hours incubation are given in table 5.2.

5.2.6. Extraction of secondary wall polymers.

Samples of lyophilised cell-walls were subjected to a range of extraction procedures designed to release carbohydrate secondary wall polymers from their covalent linkages (see section 2.19). Wherever possible, more than one extraction technique was applied to the cell-walls of each strain, as it was felt the material extracted may vary quantitatively or qualitatively depending upon the method used. The total carbohydrate extracted by each technique is shown in table 5.3.

All of the extraction procedures employed were able to release carbohydrate from the purified cell-walls of *Streptococcus milleri* reference strains. Considerable variation was noted in the amount of carbohydrate released by different extraction techniques, but autoclaving cell-walls in saline (Rantz and Randall, 1955) resulted in the highest carbohydrate yield per 100mg cell-walls for all of the strains studied.

The small quantity of cell-walls obtained from NCTC 10714 was extracted by only one method. Autoclaving in saline was the obvious choice in the light of the results with other strains.

Carbohydrate yields were generally quite low; the mean carbohydrate yield being approximately 2.5% of the initial weight of lyophilised cell-walls.

5.2.7. Examination of crude cell-wall extracts by immunoelectrophoresis.

Crude cell-wall extracts were examined by immunoelectrophoresis to detect the presence of precipitating antigens.

Table 5.2. Dry weights of purified cell-walls obtained from overnight broth cultures (81) of five *Streptococcus milleri* reference strains.

STRAIN	DRY WEIGHT OF PURIFIED WALLS (mg)
NCTC 11065	245
NCTC 10714	70
NCTC 8037	117
NCTC 5389	170
NCTC 10707	170

Figure 5.8. shows four cell-wall extracts of NCTC 11065 reacted in CIE against homologous antiserum. Single, strong peaks of immunoprecipitation were observed for all extracts, migrating towards the anode. FRIE demonstrated serological identity of the four extracts, and confirmed that no additional peaks migrating towards the cathode had been missed.

Figure 5.9.a. shows the autoclave prepared cell-wall extract of NCTC 10714 in CIE against homologous antiserum. A single peak of immunoprecipitation was again observed, which migrated towards the cathode.

Figure 5.9.b. shows the NaOH extract of NCTC 8037 cell-walls in CIE against homologous antiserum. The single, positively charged peak was representative of all extracts of this strain, which showed serological identity in FRIE.

Cell-wall extracts of NCTC 5389 again behaved in a similar manner to those of NCTC 10714 and NCTC 8037 in CIE against homologous antiserum. Only single peaks of immunoprecipitation were again observed, migrating towards the cathode.

Figure 5.10. shows the NaOH (a) and autoclave (b) prepared extracts of NCTC 10707 in CIE against homologous antiserum. Very unusual peaks were reproducibly observed for both of these extracts, suggesting that they may have contained more than one antigen.

In the extracts of all other strains, however, it had been possible to visualise only one peak of immunoprecipitation against homologous antiserum. On no occasion were two peaks corresponding to a grouping and

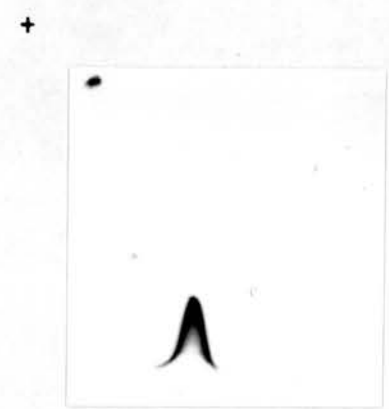
Table 5.3. Summary of the carbohydrate yields (mg/ml) obtained from samples of streptococcal cell-walls extracted by a number of agents.

STRAIN	EXT[1]	WT. WALLS (mg)[2]	CHO (mg)[3]	CHO (mg/100mg WALLS)[4]
NCTC 11065	NaOH	97.4	0.7	0.72
	HCl	31	0.29	0.93
	TCA	53.4	0.87	1.62
	Autoclave	59.6	1.26	2.12
NCTC 10707	NaOH	47.9	0.34	0.71
	TCA	62.3	0.33	0.53
	Autoclave	58.1	0.96	1.65
NCTC 8037	NaOH	40	0.45	1.13
	TCA	30	0.6	2.0
	Autoclave	47	3.2	6.81
NCTC 10714	Autoclave	68.3	5.8	8.49
NCTC 5389	NaOH	66	1.2	1.82
	TCA	52	0.26	0.5
	Autoclave	52	3.3	6.35

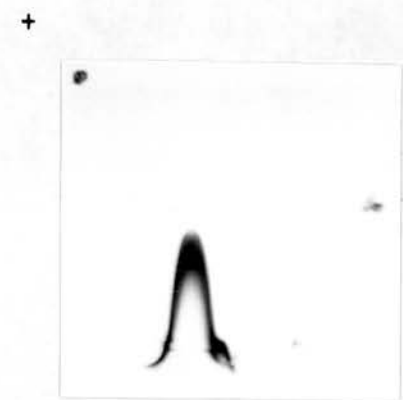
KEY:
1: EXT = extraction technique (see section 2.19. for details).
2: WT. WALLS = weight of lyophilised cell-walls extracted.
3: CHO = quantity of carbohydrate extracted by the given technique.
4: CHO = quantity of carbohydrate extracted by the given technique, expressed as the yield per 100mg of cell-walls (percentage yield).

Figure 5.8. CIE of NCTC 11065 cell-wall extracts against homologous antiserum.

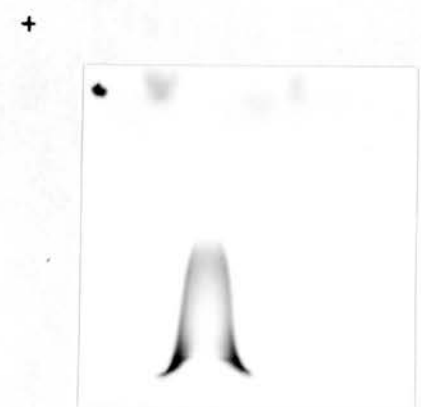
a. HCl extract



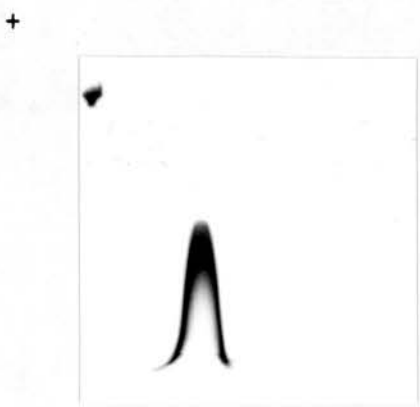
b. NaOH extract.



c. TCA extract



d. Autoclaved extract.



5*ul* of crude cell-wall antigen (1mg/ml carbohydrate) reacted against NCTC 11065 antiserum (125*ul* in 3ml agarose) in CIE. First dimension electrophoresed at 100V for 90 minutes; second dimension electrophoresed at 60V for 17 hours. Both dimensions run at 4°C.

a typing antigen visualised. The antiserum raised against NCTC 10707 was reacted in RIE against crude extracts of NCTC 8037, NCTC 10714 and NCTC 5389, and revealed that reaction of all group F strain extracts with this antiserum could be observed. Immunoprecipitation was rather diffuse in all cases, migrating towards the cathode. No sharp or clear peaks were observed on any occasion.

5.2.8. Purification of cell-wall extracts.

Prior to chemical analysis, cell-wall extracts were purified by application to an anion-exchange column (see section 2.20.). Despite the inability to demonstrate the presence of more than one peak of immunoprecipitation in the cell-wall extracts of these strains, it was hoped that ion-exchange chromatography would reveal the presence of two distinct carbohydrate peaks within extracts, which would be serologically distinct.

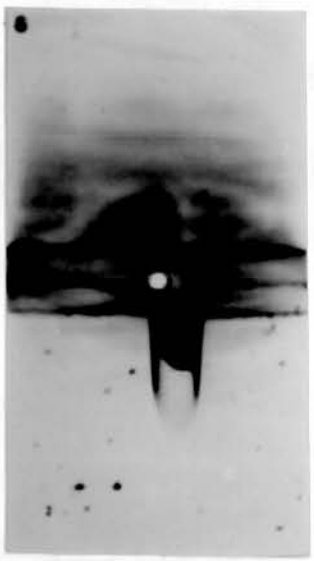
Figure 5.11. shows the elution profile for the HCl prepared cell-wall extract of NCTC 11065 from a DEAE-cellulose column. This profile is representative of all cell-wall extracts of NCTC 11065 from the anion-exchange column. A single carbohydrate peak eluted between 0.4 and 0.5M pyridinium acetate. Figure 5.13.a. shows column fractions 69 to 79 (inclusive) in RIE against homologous antiserum. No antigenic material was observed in the wash-on fractions (fractions 1-50).

Figure 5.13.b. shows odd numbered column fractions 51 to 99 (inclusive) from the NaOH prepared extract of NCTC 11065 in FRIE against homologous antiserum. Fractions corresponding to antigenic peaks were pooled and lyophilised in each case.

Figure 5.9. CIE of crude cell-wall extracts of NCTC 10714 and NCTC 8037 reacted against homologous antiserum.

a. NCTC 10714 autoclave prepared cell-wall extract.

+



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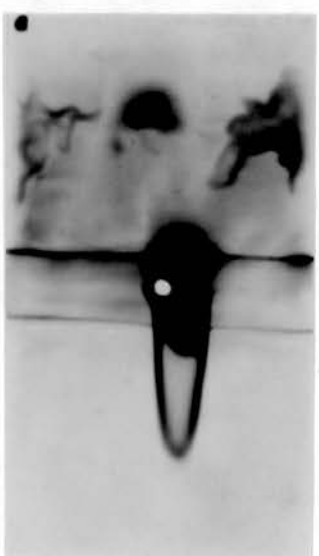
b. NCTC 8037 NaOH prepared cell-wall extract.

+



-

10μl of crude cell-wall antigen (1mg/ml carbohydrate) reacted against homologous antiserum (250μl in 3ml agarose on each side of antibody free gel) in CIE. First dimension electrophoresed at 100V for 90 minutes; second dimension electrophoresed at 84V for 17 hours. Both dimensions run at 4°C.

Figure 5.10. CIE of crude NCTC 10707 cell-wall extracts against homologous antiserum.

a. NaOH extract.

+

-

b. Autoclaved extract.

+

-

10*ul* of crude cell-wall antigen (1mg/ml carbohydrate) reacted against homologous antiserum (250*ul* in 3ml agarose on each side of antibody free gel) in CIE. First dimension electrophoresed at 100V for 90 minutes; second dimension electrophoresed at 84V for 17 hours. Both dimensions run at 4°C.

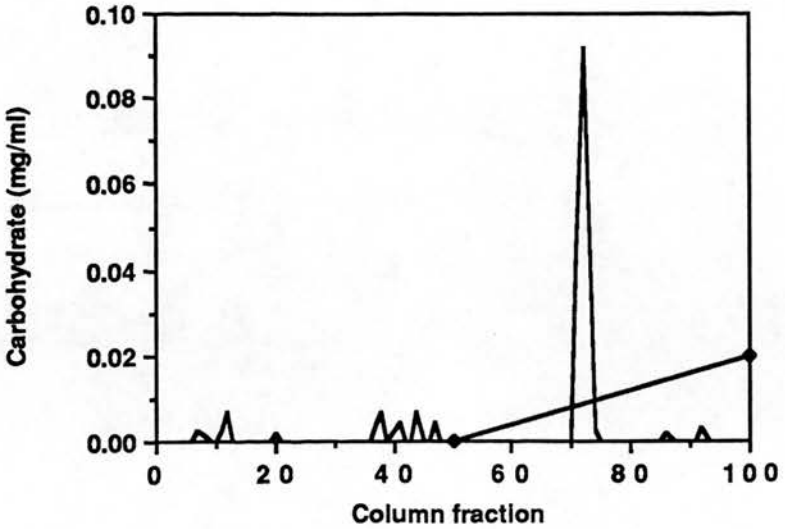


Figure 5.11. Purification of NCTC 11065 HCl prepared cell-wall extract by anion-exchange chromatography on a DEAE-cellulose column. Column eluted with a linear gradient of 0-1M pyridinium acetate buffer, pH 5.3.
Carbohydrate content of fractions (mg/ml) = —
Pyridinium acetate gradient = -●-

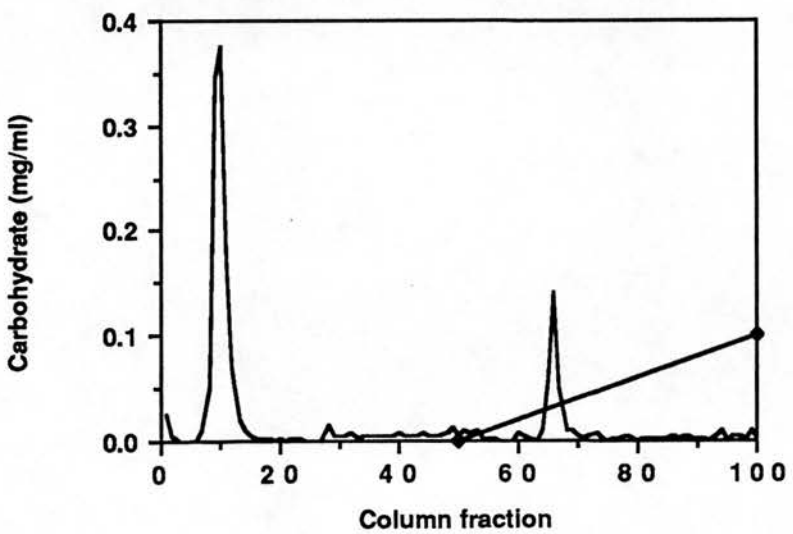


Figure 5.12. Purification of NCTC 10714 autoclave prepared cell-wall extract by anion-exchange chromatography on a DEAE-cellulose column. Column eluted with a linear gradient of 0-1M pyridinium acetate buffer, pH 5.3.
Carbohydrate content of fractions (mg/ml) = —
Pyridinium acetate gradient = -●-

Figure 5.13. RIE and FRIE against homologous antiserum of NCTC 11065 cell-wall extracts eluted from a DEAE-cellulose column.

a. NCTC 11065 HCl extract; column fractions 69-79 (inclusive) in RIE.

b. NCTC 11065 NaOH extract; column fractions 51-99 (inclusive) in FRIE.

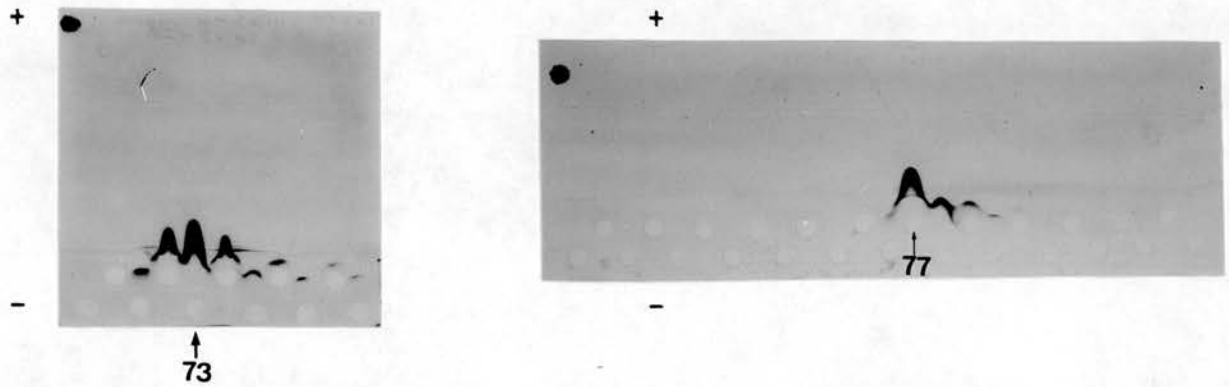


Figure 5.14. CIE against homologous antiserum of peak fractions eluted from a DEAE-cellulose column during purification of NCTC 10714 autoclave prepared cell-wall extract.

a. Wash-on peak (fraction 10).

b. Gradient peak (fraction 66).

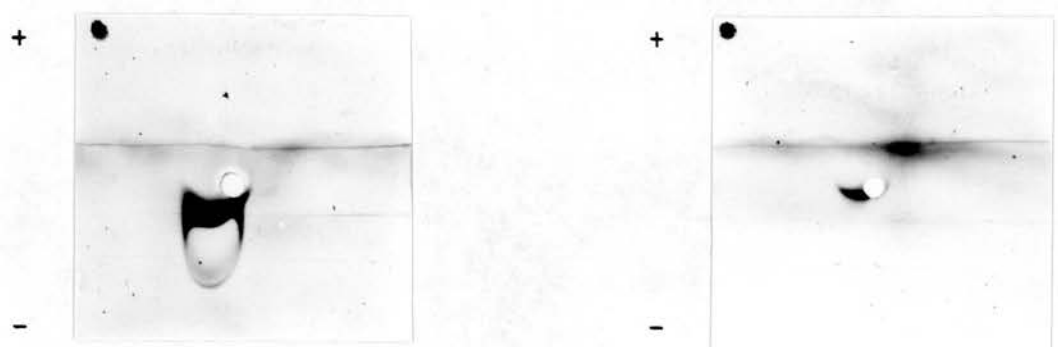


Figure 5.12. shows the elution profile of the autoclave prepared cell-wall extract of NCTC 10714 from a DEAE-cellulose column. The majority of carbohydrate material passed through the column without binding. However, a smaller carbohydrate peak eluted from the column between 0.26 and 0.4M pyridinium acetate buffer.

Figure 5.14. shows fractions corresponding to the wash-on (a) and gradient (b) peaks of NCTC 10714 autoclave prepared cell-wall extract in CIE against homologous antiserum.
Both samples contained antigenic material which formed single peaks of immunoprecipitation towards the cathode. TCIE did not allow any discrimination between the material contained in the two peaks. Fractions corresponding to the two peaks were pooled and lyophilised.

Figure 5.15. shows the elution profile of the autoclave prepared cell-wall extract of NCTC 8037 from a DEAE-cellulose column, and is illustrative of all extracts from this strain. The majority of carbohydrate material contained in these extracts passed through the column without binding. A small peak of carbohydrate was again demonstrated eluting between 0.28 and 0.44M pyridinium acetate.
Figure 5.17. shows fractions corresponding to the wash-on (a) and gradient (b) peaks of NCTC 8037 autoclave prepared cell-wall extract in CIE against homologous antiserum. Both samples contained material which gave single peaks of immunoprecipitation towards the cathode. TCIE again failed to demonstrate serological differences between the material contained in the two peaks. Peak fractions were again pooled and lyophilised in each case.

Figure 5.16. shows the elution profile of the autoclave prepared cell-wall extract of NCTC 5389 from a DEAE-cellulose column. In common with the extracts of NCTC 10714 and NCTC 8037, the majority of carbohydrate material contained in extracts of this strain passed through the column without binding. However, no additional carbohydrate peak was eluted by the gradient of pyridinium acetate buffer for extracts of this strain.
Figure 5.18. shows column fractions 4 to 14 (inclusive) in FRIE against homologous antiserum, with peaks of immunoprecipitation migrating to the cathode.

Figure 5.19. shows the elution profile of the autoclave prepared extract of NCTC 10707 from a DEAE-cellulose column. Two definite carbohydrate peaks were again visualised, one corresponding to material which passed through the column without binding, and a larger peak eluting between 0.26 and 0.52M pyridinium acetate. Examination of the fractions corresponding to the wash-on peak in FRIE revealed very faint peaks of immunoprecipitation towards the anode. FRIE examination of the fractions corresponding to the gradient peak showed unclear peaks of immunoprecipitation in both directions, and migrating only fractionally from the wells. The small amounts of carbohydrate material contained in these extracts presented problems of visualising clear peaks of immunoprecipitation, although it was possible to identify antigenicity without the need to resort to dot-blotting.
Fractions corresponding to the peaks were pooled and lyophilised as before.

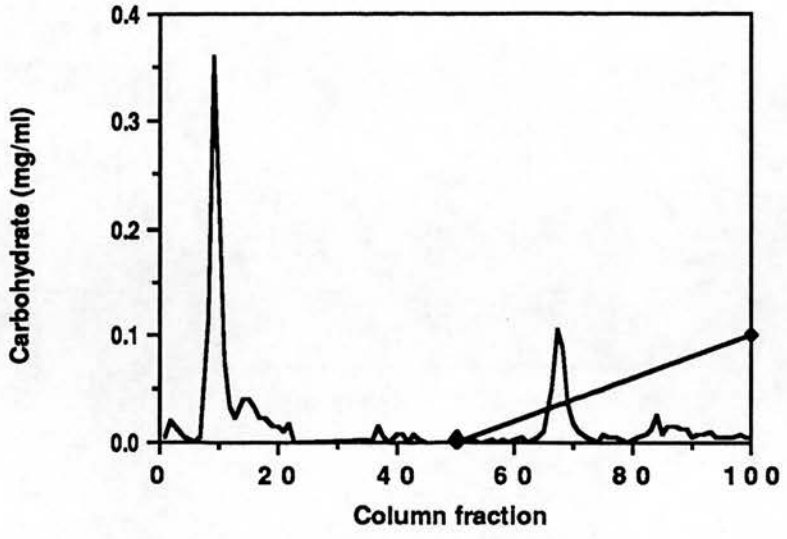


Figure 5.15. Purification of NCTC 8037 autoclave prepared cell-wall extract by anion-exchange chromatography on a DEAE-cellulose column. Column eluted with a linear gradient of 0-1M pyridinium acetate buffer, pH 5.3.
Carbohydrate content of fractions (mg/ml) = —
Pyridinium acetate gradient = -◆-

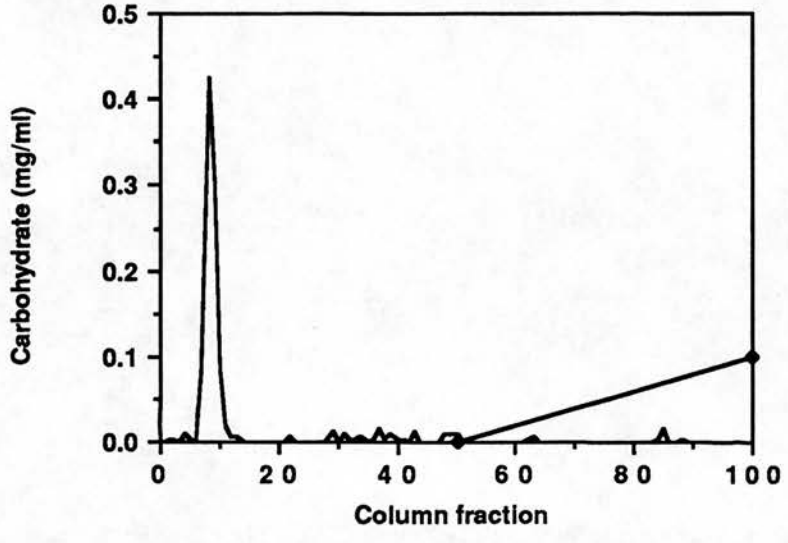


Figure 5.16. Purification of NCTC 5389 autoclave prepared cell-wall extract by anion-exchange chromatography on a DEAE-cellulose column. Column eluted with a linear gradient of 0-1M pyridinium acetate buffer, pH 5.3.
Carbohydrate content of fractions (mg/ml) = —
Pyridinium acetate gradient = -◆-

Figure 5.17. CIE against homologous antiserum of fractions corresponding to carbohydrate peaks eluted from a DEAE-cellulose column in the purification of NCTC 8037 autoclave prepared cell-wall extract.

a. Wash-on peak (fraction 9). b. Gradient peak (fraction 67).

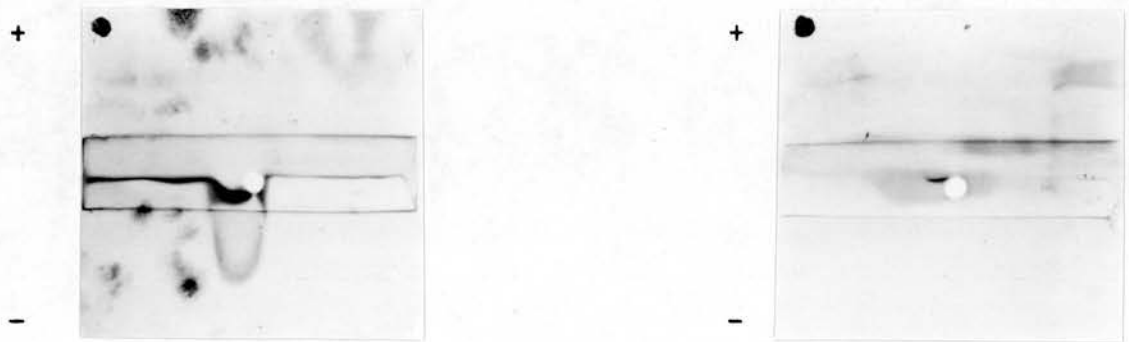
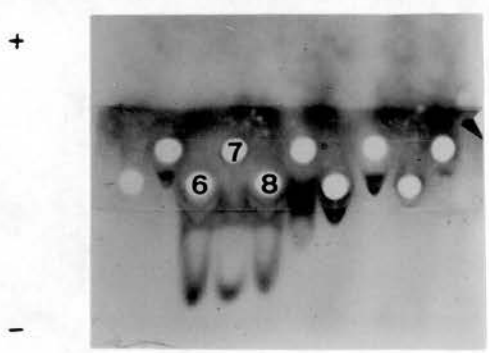


Figure 5.18. FRIE against homologous antiserum of column fractions 4-14 (inclusive) eluted from a DEAE-cellulose column in the purification of NCTC 5389 autoclave prepared cell-wall extract.



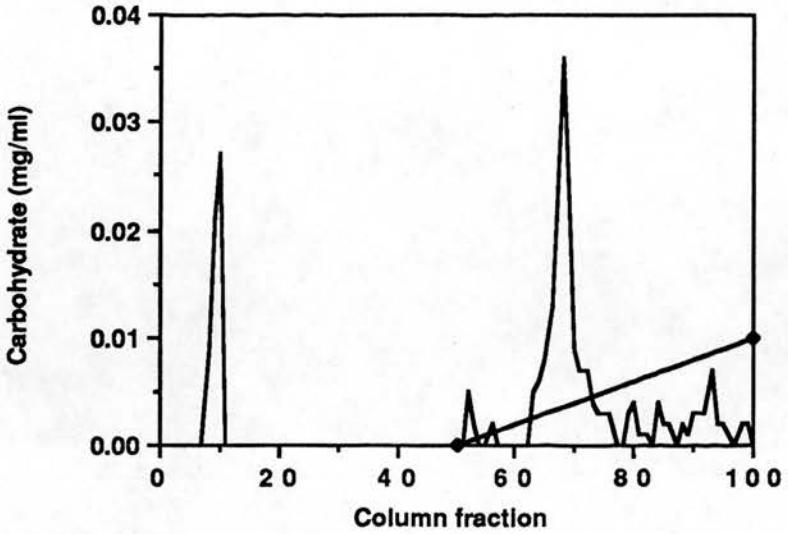


Figure 5.19. purification of NCTC 10707 autoclave prepared cell-wall extract by anion-exchange chromatography on a DEAE-cellulose column. Column eluted with a linear gradient of 0-1M pyridinium acetate buffer, pH 5.3.
Carbohydrate content of fractions (mg/ml) = —
Pyridinium acetate gradient = -●-

5.2.9. Analysis of some purified cell-wall antigens.

Attempts were made to undertake some chemical analysis of the antigenic cell-wall polymers purified by anion-exchange chromatography.

Generally, all of the cell-wall antigens examined by gas chromatography contained varying quantities of rhamnose, glucose, galactose, and xylose. The presence of these sugars was confirmed in some cases by staining of paper chromatographs with the alkaline silver nitrate reagents (Trevelyan *et al.*, 1950). No glycerol or ribitol phosphate was detected in any of the samples by staining of paper chromatographs with the periodate-Schiff reagents (Baddiley *et al.*, 1956). Amino-sugars could not be detected by the ninhydrin stain. No choline, and only very small traces of phosphate were detected in purified antigens by the methods of Appleton *et al.*, (1953) and Chen *et al.* (1956) respectively.

i. NCTC 11065 cell-wall antigen.

All of the antigenic material contained in NCTC 11065 cell-wall extracts was eluted from the column in a single peak by the action of pyridinium acetate buffer. Gas chromatographic examination showed that the HCl prepared antigen contained: glucose, rhamnose, galactose and xylose in the approximate ratio of 4 : 3 : 3 : 1.

The serologically identical TCA prepared antigen contained glucose, rhamnose, galactose and xylose in the approximate molar ratio of 2 : 2 : 2 : 1.

The NaOH prepared antigen also contained rhamnose, glucose, galactose and xylose.

ii. NCTC 10714 cell-wall antigen.

The majority of antigenic material contained in this sample passed through the ion-exchange column without binding. This peak of antigenic material contained: glucose, rhamnose, galactose and xylose in the approximate molar ratio of 4 : 6 : 4 : 1. The small amount of material contained in the peak eluted by the pyridinium acetate gradient presented difficulties in analysis but also contained rhamnose, glucose, galactose, and xylose. The levels of galactose and glucose, however, appeared to be reduced compared to the levels found in the major antigenic peak.

iii. NCTC 5389 cell-wall antigen.

All of the antigenic material contained in these samples was contained in a single peak which passed through the column without binding.

The autoclave prepared antigen contained glucose, rhamnose, galactose and xylose in the approximate molar ratio of 3 : 3 : 1 : 1.

The NaOH prepared antigen contained: glucose, rhamnose and galactose in the approximate molar ratio of 3 : 3 : 1, with a trace amount of xylose.

iv. NCTC 8037 cell-wall antigen.

The majority of antigenic material contained in the extracts of this strain also passed through the column without binding. The antigenic material contained in the autoclave prepared extract contained: glucose, rhamnose, galactose and xylose in the approximate molar ratio of 6 : 6 : 6 : 1. The TCA prepared antigen also contained glucose, rhamnose, galactose and xylose.

The small amount of material contained in the fractions eluted by the pyridinium acetate gradient could not be satisfactorily analysed.

v. NCTC 10707 cell-wall antigen.

The very small amounts of material contained in purified extracts of NCTC 10707 again presented great problems for analysis. Only the peak of material contained in the autoclave prepared extract, and eluted from the column by the pyridinium acetate gradient was analysed with any success, and contained rhamnose, with trace amounts of glucose, galactose and xylose.

Some of the results of chemical analysis are summarised in tabulated form in appendix 3.

Investigations into the chemical composition of all samples was compromised by the small amounts of purified antigenic material available for analysis.

5.2.10. Examination of the serological relationship of cell-wall and cell-membrane antigens.

A number of investigations were undertaken to compare antigens isolated from the cell-wall and cell-membrane of group F and related streptococci.

i. Tandem crossed immunoelectrophoresis (TCIE).

Cell-wall and cell-membrane antigens were run in TCIE against homologous antiserum to examine the serological relationship of wall and membrane derived antigens for each strain.

Figure 5.20. shows the crude NaOH prepared cell-wall extract of NCTC 11065 in TCIE with NCTC 11065 membrane lipocarbohydrate against homologous antiserum. The appearance in TCIE suggested partial, though not complete

serological identity of cell-wall and membrane derived antigens.

Figure 5.21. shows the crude autoclave prepared cell-wall extract of NCTC 10714 in TCIE with NCTC 10714 membrane lipocarbohydrate against homologous antiserum. Serological identity was suggested by the complete superimposition of antigenic peaks.

Figure 5.22. shows the crude NaOH prepared cell-wall extract of NCTC 5389 in TCIE with NCTC 5389 membrane lipocarbohydrate against homologous antiserum. The possibility of minor serological differences between the wall and membrane derived antigens was suggested by the slight asymmetry of the single peak visualised in this gel.

It was not possible to make any comparison of NCTC 8037 wall and membrane antigens as no lipocarbohydrate antigen had been prepared for this strain. The NCTC 10707 membrane antigen failed to give immunoprecipitation in gel, preventing its comparison with the cell-wall derived antigens by this method.

ii. SDS-free PAGE and Western blotting.

A further investigation was performed to examine and compare the appearance of membrane lipocarbohydrate antigens and some cell-wall derived antigens by SDS-free PAGE and immunoblotting. The sort of appearance to expect for lipocarbohydrate antigens in PAGE and Western blotting was unknown, and it was of special interest to see how these appearances correlated with those of the EDTA-extracts illustrated in chapter 4.

Figure 5.20. NCTC 11065 NaOH prepared cell-wall antigen, and purified lipocarbohydrate antigen in TCIE against homologous antiserum.

+

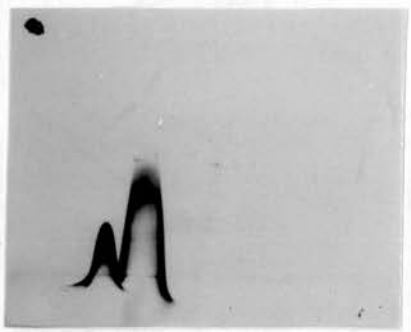


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Figure 5.21. NCTC 10714 autoclave prepared cell-wall antigen, and purified lipocarbohydrate antigen in TCIE against homologous antiserum.

+

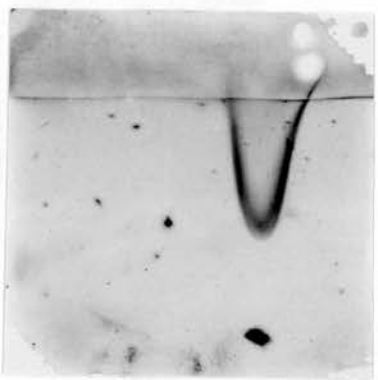

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Figure 5.22. NCTC 5389 NaOH prepared cell-wall antigen, and purified lipocarbohydrate antigen in TCIE against homologous antiserum.

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Figure 5.23.a. shows material corresponding to peaks 1 (track 1) and 2 (track 2) pooled during the preparation of NCTC 11065 lipocarbohydrate antigen, along with the NaOH (track 3) and autoclave (track 4) prepared cell-wall extracts in PAGE.

The purified lipocarbohydrate antigen of this strain (track 1), contained only a small negatively staining area corresponding to the gel-front. Track 2 contained a far less distinct area of negative staining at the gel-front, in addition to faint bands beyond it.

Both the NaOH (track 3) and autoclave (track 4) prepared cell-wall extracts gave evidence in PAGE of carbohydrate material throughout the gel, with some definite areas of banding visible within the NaOH extract.

Immunoblotting (figure 5.23.b.) showed strongly reacting antigenic material throughout the length of the track containing the autoclave prepared cell-wall extract (track 4). Only a faint band of antigenic material was detected at the gel-front for the NaOH extract of this strain. No reaction was observed with material contained in track 2 (peak 2 fractions from the NCTC 11065 Sepharose 6-B column), but a very faint reaction (arrowed) corresponding to the negatively-staining area seen in PAGE was detected in the purified lipocarbohydrate material from this strain (track 1). This very faint band did not photograph well.

Figure 5.24.a. shows material corresponding to peaks 1 (track 1), 2 (track 2) and 3 (track 3) pooled during the preparation of NCTC 10707 lipocarbohydrate antigen, and crude autoclave prepared cell-wall antigen (track 4) in PAGE. Purified lipocarbohydrate antigen (track 1) showed a very marked area of negative staining associated with the gel-front. Peak 2 (track 2) lipocarbohydrate material contained a less marked area of

negative staining associated with the gel-front, whilst peak 3 (track 3) material gave evidence of numerous bands, possibly reflecting the high levels of cytoplasmic debris in this sample. Little staining was seen in track 4 which contained the autoclave prepared cell-wall extract.

Immunoblotting again revealed the presence of antigenic material in the cell-wall antigen (track 4), but also demonstrated more clearly the antigenicity of the negatively staining area of the purified membrane antigen (track 1, arrowed). Similar results were obtained for the purified membrane antigens of NCTC 10714 (figure 5.25.a and b. track 1) and NCTC 5389 (figure 5.26.a. and b.). In all cases, the purified membrane antigen appeared as an area of negative staining at the gel-front and was detected as a rather indistinct smear on the corresponding immunoblot.

A similar appearance of indistinct antigenic material associated with the gel-front was observed faintly in the immunoblots of NCTC 10707 EDTA extracts (figure 4.5.b.), and more clearly in the immunoblots of NCTC 8037 EDTA extracts (figure 4.6.b.). This observation will be discussed later.

Crude cell-wall extracts appeared to contain antigenic material across a broad range of molecular mass, which may have represented fragmented cell-wall antigen contained in these preparations. Whether or not whole, unfragmented cell-wall antigen would have been able to enter the polyacrylamide gel is uncertain.

a. PAGE

b. Immunoblot

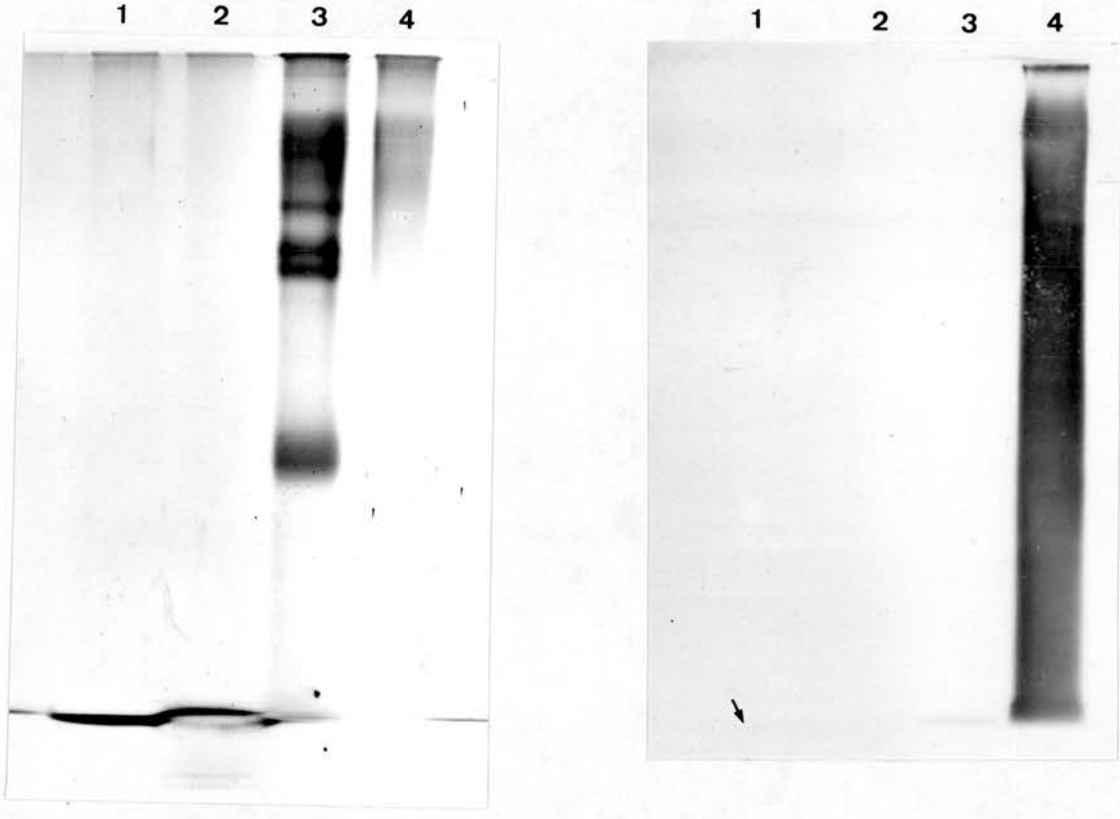


Figure 5.23. PAGE and immunoblot of NCTC 11065 membrane and crude cell-wall antigens. Track 1 contains purified lipocarbohydrate antigen (Sepharose 6-B column fractions corresponding to peak 1; 2 contains Sepharose 6-B fractions corresponding to peak 2; 3 contains NaOH prepared cell-wall antigen; 4 contains autoclave prepared cell-wall antigen. PAGE silver stained for carbohydrate. Immunoblot probed with NCTC 11065 antiserum at a dilution of 1 in 50 in antibody diluent.

a. PAGE

b. Immunoblot

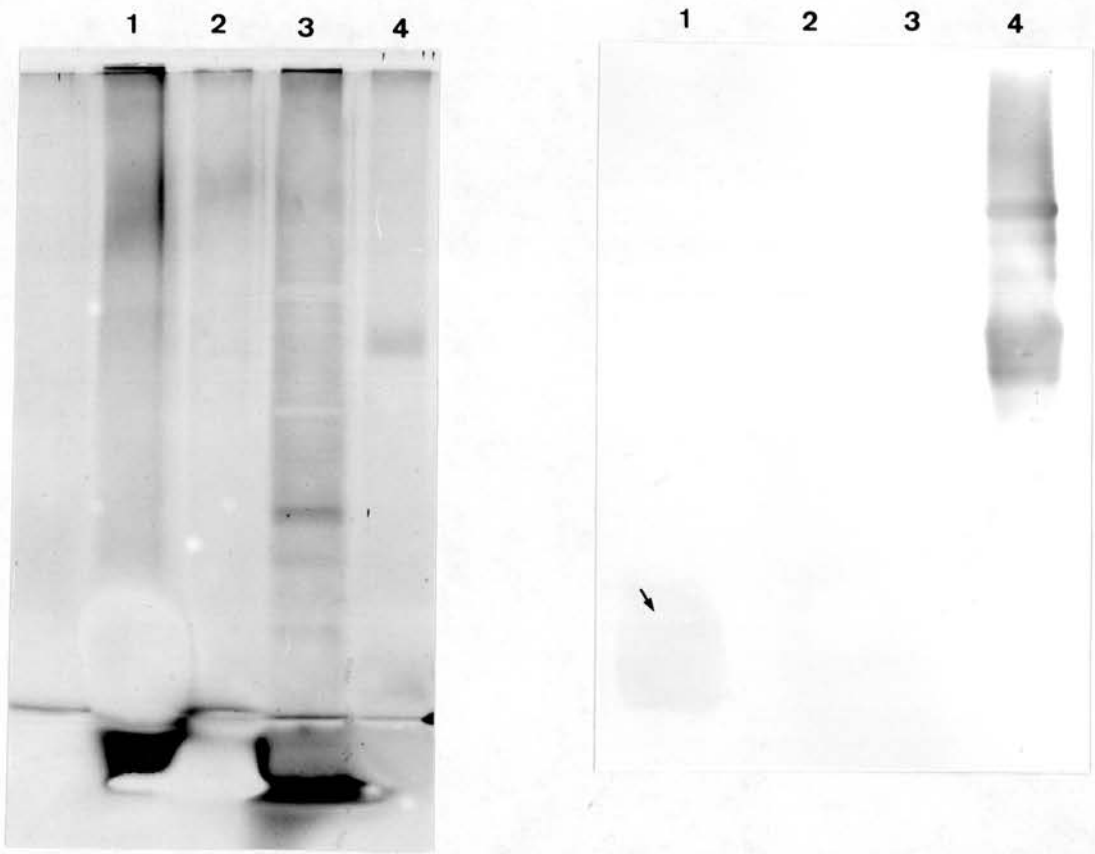


Figure 5.24. PAGE and immunoblot of NCTC 10707 membrane and crude cell-wall antigens. Track 1 contains purified lipocarbohydrate antigen (Sepharose 6-B column fractions corresponding to peak 1; 2 contains fractions corresponding to Sepharose 6-B column peak 2; 3 contains fractions corresponding to Sepharose 6-B column peak 3; 4 contains crude autoclave prepared cell-wall antigen. PAGE silver stained for carbohydrate. Immunoblot probed with NCTC 10707 antiserum at a dilution of 1 in 50 in antibody diluent.

a. PAGE

b. Immunoblot

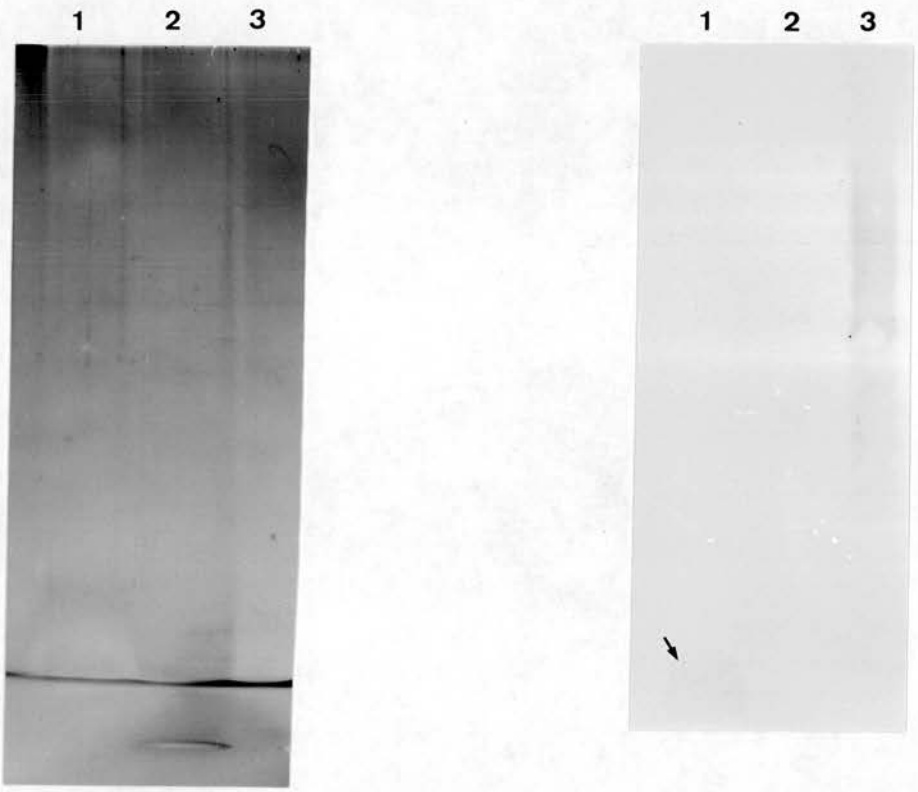


Figure 5.25. PAGE and immunoblot of NCTC 10714 membrane and crude cell-wall antigens. Track 1 contains purified lipocarbohydrate antigen (Sepharose 6-B column fractions corresponding to peak 1; 2 contains Sepharose 6-B column fractions corresponding to peak 2; 3 contains autoclave prepared cell-wall antigen. PAGE silver stained for carbohydrate. Immunoblot probed with NCTC 10714 antiserum at a dilution of 1 in 50 in antibody diluent.

a. PAGE b. Immunoblot

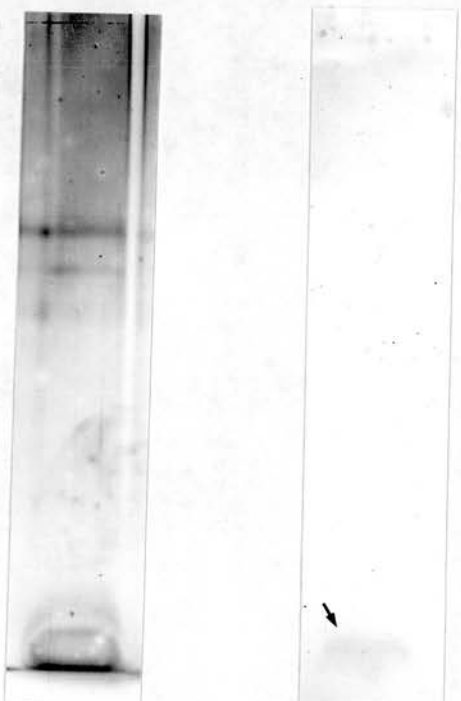


Figure 5.26. PAGE and immunoblot of NCTC 5389 purified lipocarbohydrate antigen (fractions corresponding to Sepharose 6-B column peak 1).
Gel silver stained for carbohydrate. Immunoblot probed with NCTC 5389 antiserum at a dilution of 1 in 50 in antibody diluent.

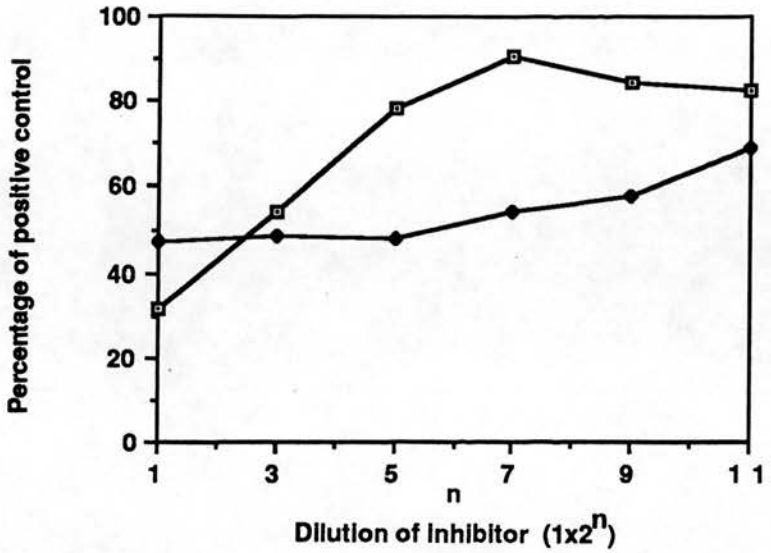
5.2.11. ELISA inhibition reactions.

A final investigation was carried out in the current studies to investigate the ability of purified lipocarbohydrate antigens from two reference strains to inhibit the homologous EDTA extract/antiserum ELISA reaction. Two strains whose EDTA extracts had been shown in chapter 4 to contain different balances of protein and carbohydrate antigens were selected for this study. NCTC 11065, whose EDTA extracts contained predominantly protein antigens was selected as one strain, whilst in the absence of a lipocarbohydrate antigen from NCTC 8037, NCTC 10714 was selected as a contrasting strain whose extracts contained proportionately more carbohydrate antigen. Inhibition of ELISA was performed essentially as described in section 2.7.1. Briefly, antiserum at twice titre was preincubated with an equal volume of dilutions of: i. homologous EDTA extract at an initial concentration of 50µg/ml protein in antibody diluent, or ii. purified lipocarbohydrate at an initial concentration of 1mg/ml (w/v) in antibody diluent. The antigen coated onto the plates was homologous EDTA extract at a concentration of 25µg/ml protein.

Figure 5.27.a. shows the activity of NCTC 10714 purified lipocarbohydrate antigen in inhibiting the NCTC 10714 EDTA extract/antiserum ELISA reaction. Figure 5.27.b. shows that the converse was true for NCTC 11065, where considerably less inhibitory action was provided by preincubation of antiserum with lipocarbohydrate antigen.

ELISA inhibition reactions were not undertaken for other strains, but it was felt that the investigation of NCTC 11065 whose predominant surface antigens were shown in chapter 4 to be protein, and NCTC 10714, where a

a. NCTC 10714 ELISA inhibition reaction.



b. NCTC 11065 ELISA inhibition reaction.

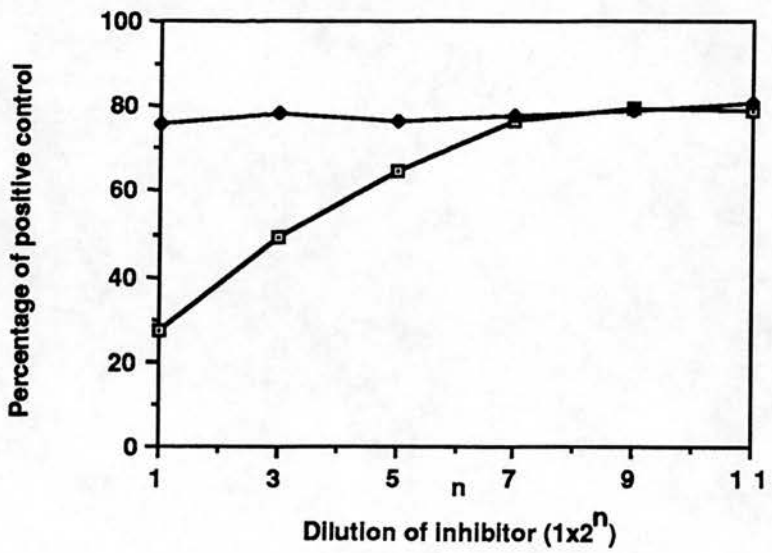


Figure 5.27. ELISA inhibition reactions to determine the inhibitory effect of purified lipocarbohydrate antigen on the homologous EDTA extract/antiserum ELISA reaction of a. NCTC 10714, and b. NCTC 11065.
Antiserum pre-incubated with:
Homologous EDTA extract = -□-
Purified lipocarbohydrate antigen = -◆-

greater proportion was made up by carbohydrate antigens provided illustration of the importance of lipocarbohydrate antigens at the cell-surface of some *Streptococcus milleri* reference strains.

No investigations were made to examine the ability of the serologically similar cell-wall antigens of these strains to inhibit the ELISA reactions of EDTA extracts and their homologous antiserum.

5.3. Discussion.

The studies presented in the current chapter have sought to explore some aspects of the cell-surface antigens of a small collection of group F and related streptococci. Investigations have largely been confined to the isolation and analysis of carbohydrate-containing polymers associated with two cellular locations, namely the cell-membrane and the cell-wall.

5.3.1. Isolation of lipocarbohydrate antigens.

Lipoteichoic acids are cell-membrane associated polymers which may traverse the Gram-positive bacterial cell-wall and present as antigens at the surface of intact cells (see section 1.7.8.). Unlike secondary cell-wall polymers, they are believed to loosely interdigitate with the bacterial cell-wall and not to form strong covalent linkages with it. It is this property which allows the release of these membrane-associated antigens into solution by the action of EDTA on whole cells, whilst leaving covalently bound secondary cell-wall polymers firmly attached.

Immunoelectrophoretic examination of the EDTA extracts of certain group F and related streptococcal strains (see chapter 4) revealed the presence of precipitating carbohydrate antigens which appeared to have some strain specificity. It was suspected that the antigens visualised were the membrane-associated lipocarbohydrate antigens (lipoteichoic acids or their analogues) of these strains, antigens which to my knowledge had not previously been studied for this group of organisms. The isolation of a hexosamine-free component with the properties of lipoteichoic acid from a

z_3III strain was described by Huis in't Veld and Linssen (1973), but no detailed examination of this polymer was reported.

Following these observations, it was of interest to attempt to isolate and purify membrane-associated lipocarbohydrate antigens from *Streptococcus milleri* reference strains in large enough quantities to allow some immunochemical and biochemical analysis. No attempt was made to isolate and purify carbohydrate antigens from the EDTA extracts of these strains.

The cold aqueous phenol procedure of Coley *et al.* (1975) was shown to be capable of extracting antigenic carbohydrate-containing material from the cell-membranes of *Streptococcus milleri* reference strains. Coley *et al.* (1975), working on preparations of *Staphylococcus*, *Micrococcus*, and *Bacillus* spp. reported that the dry weight of crude phenol extract typically represented between 8 and 10% of the initial weight of defatted membrane material. The dry weight of crude phenol extracts obtained in the current study ranged from 4.1 to 9.8% of the initial weight of defatted membrane fraction. Comparable results were obtained by Smith (1985), who recovered 56mg of crude antigen (4.3% of the dry weight of defatted membranes) from a 10l broth culture of *Peptostreptococcus anaerobius*.
No comment may be made in absolute terms regarding the efficiency of this extraction technique, as there was no way of measuring the quantity of lipocarbohydrate antigen contained in whole cells prior to extraction. In addition, no investigations were undertaken to determine the amount, or relative purity of material extracted under different conditions (eg: by varying the temperature, or the time of extraction). Such investigations were considered beyond the scope of the current research.

Reports for other organisms have suggested that virtually complete extraction of lipoteichoic acids can be achieved by the treatment of unfractioned disrupted cell suspensions with aqueous phenol at 65°C for one hour (Fischer *et al.*, 1983). This method was not adopted in the current study for the following reasons:

i. It was considered wasteful in terms of the bulk cultures required to subject mixtures of cell-walls and membranes to phenol extraction, if satisfactory lipocarbohydrate preparations could be made from the cell-membrane fraction alone. The cell-walls were then free to use for other purposes, and

ii. One of the initial aims of this study was to obtain cell-membrane and cell-wall antigens free from contamination by the other. In this respect, the cold extraction of cell-membrane fraction was regarded as the method of choice in keeping contamination by cell-wall components to a minimum. On the other hand, it was speculated that the extraction of whole, disrupted cells with the application of heat, would carry an increased risk of significant contamination by cell-wall polymers.

5.3.2. Purification of lipocarbohydrate antigen.

Purification of crude phenol extracts was confined in this study to gel-filtration on Sepharose 6-B. The micellar nature of the antigenic lipocarbohydrate material was reflected in each case by its elution close to the exclusion volume of the column. It was interesting to observe from the elution profiles that the carbohydrate peaks corresponding to the lipocarbohydrate antigens of NCTC 10714 and NCTC 5389 did not appear to coincide with the phosphate or A_{260} peaks as had been the case for NCTC

11065 and NCTC 10707. It is possible that the purification of NCTC 11065 and NCTC 10707 lipocarbohydrates on Sepharose 6-B gave less than ideal separation from a small peak of phosphate-containing nuclease-resistant nucleic acids, accounting for the superimposition of the three peaks. It cannot therefore be assured that the lipocarbohydrate antigens prepared in this study were entirely free from all vestiges of the cytoplasmic contaminants with which they were co-extracted. The level of purity was, however, considered to be satisfactory for the purposes of the investigations to be performed in the current research.

The application of additional purification steps may become necessary prior to analysis of lipocarbohydrate antigens by more exacting means than those undertaken in the present study.

Possibilities for further purification include:

i. Application to an immunoabsorbent column (Poxton and Cartmill, 1982). This process is of value in removing non-antigenic contaminants from crude membrane preparations, particularly where antigens are heavily contaminated with nucleic acids. In the context of the current research, it was felt that application of material eluted from the Sepharose 6-B column to an immunoabsorbent column would offer little further purification as the level of nucleic acid contamination was probably quite small.

ii. Ion-exchange chromatography, which will be considered in greater detail with respect to purification of cell-wall derived antigens later, or

iii. Hydrophobic interaction chromatography. Fischer *et al.* (1983) described the use of hydrophobic interaction chromatography on octyl-Sepharose following ion-exchange chromatography as the final step in purifying lipoteichoic acids for analysis. Purified preparations were described as being essentially free of polymeric contaminants. Hydrophobic

interaction chromatography also made it possible to recognise contamination of lipoteichoic acid with its deacylated and lyso-form, and to discriminate molecular species containing two and three, or two and four acyl groups. Such detailed observations on the composition and contamination of lipocarbohydrate antigens were beyond the limitations of the techniques employed in the current study.

5.3.3. Examination of lipocarbohydrate antigens by immunoelectrophoresis.

Immunoelectrophoretically, each purified lipocarbohydrate antigen behaved in a manner similar to that observed for the homologous carbohydrate containing antigen present in whole cell EDTA extracts (see chapter 4). No attempt was made to compare these antigens directly in TCIE.

The purified lipocarbohydrate antigen of NCTC 10707 did not form precipitating complexes in antibody containing gel. Numerous attempts were unsuccessfully made to demonstrate immunoprecipitation following the adjustment of antigen and antibody concentrations. Antigenicity could, however, be demonstrated by dot-blotting and by Western blotting, the results of which will be discussed later. Why this antigen did not form precipitating complexes in gel is unclear, but some possibilities will be considered in section 5.3.4.

The purified lipocarbohydrate antigen of NCTC 11065 gave a single, sharply focussed peak of immunoprecipitation in gel, which migrated towards the anode. This behaviour in immunoelectrophoresis was regarded as being consistent with the typical nature of lipoteichoic acids, which contain high levels of negatively charged phosphate.

The lipocarbohydrate antigens of NCTC 10714 and NCTC 5389 again produced

single peaks of immunoprecipitation in gel, but as already reported for their EDTA-extractable carbohydrate antigens, migrated unexpectedly towards the cathode.

These membrane-associated antigens again appeared to be strain specific from the absence of cross-reactions observed in immunoelectrophoresis. No attempt was made to investigate the cross-reactivity of lipocarbohydrates in a sensitive ELISA system, but it is not beyond question that certain cross-reactivity may have been demonstrable, especially if small amounts of cell-wall or cytoplasmic contaminants persisted.

5.3.4. Analysis of purified lipocarbohydrate antigens.

Apart from providing information on the chemical composition of the lipocarbohydrate antigens contained in group F and related streptococci, it was hoped that the analysis undertaken might throw some light on the reasons for the observed behaviour of some of these polymers in immunoelectrophoresis.

As regards the general classification of these membrane associated antigens, the demonstration of glycerol phosphate by staining of paper chromatographs with the periodate-Schiff reagents (Baddiley *et al.*, 1956) confirmed that they were lipoteichoic acids or analogues thereof. No analysis of the lipid component of these polymers was undertaken in the current studies.

Gas chromatographic analysis revealed that the major neutral sugars contained in these antigens were: glycerol, rhamnose, glucose, galactose and xylose. These were also the major neutral sugars detected by Aluyi and

Drucker, (1983) in the whole cell trimethylsilyl-sugar profiles of 39 *Streptococcus milleri* strains. In common with the lipoteichoic acid-like component detected in a z_3III strain by Huis in't Veld and Linssen (1973), hexosamines did not appear to be prominent components of these antigens; galactosamine was detected in a quantifiable amount only in the antigen prepared from NCTC 10714.

Only a trace amount of deoxyribose was detected in each preparation, which supported the belief that an acceptable degree of antigen purification had been achieved by gel-filtration on Sepharose 6-B.

Rhamnose was absent from the antigen of NCTC 10707, and it was considered possible that this lack of rhamnose may have been of significance with respect to the behaviour of this antigen in antibody containing gel. Rhamnose is believed to be of importance in many streptococcal carbohydrate antigens, either as a structural component with no direct antigenic activity of its own, or as an important antigenic determinant in its own right (Krause, 1963; Willers *et al.*, 1964a; Parker, 1983). Its absence from the NCTC 10707 lipocarbohydrate antigen may suggest that the antigenic determinant is composed of a carbohydrate unit whose conformation is such that it does not form precipitating complexes in gel. Another possibility was that the level of carbohydrate antigen contained in this preparation was too low to allow satisfactory visualisation of immunoprecipitation in gel. This was considered highly unlikely as repeated attempts to visualise immunoprecipitation following adjustment of antigen and antibody concentrations were unsuccessful. A second preparation of this antigen also gave the same result. Perhaps the most likely explanation for this behaviour is that the lipocarbohydrate antigen may be only weakly expressed as a surface antigen in this strain, resulting in a proportionately weaker

antibody response to it than in some other strains. The analysis undertaken in the current study can offer no information on the length of lipocarbohydrate polymer chains anchored to the cell-membrane of these strains, and consequently the degree of projection and presentation of these antigenic polymers at the cell-surface. Such structural investigations would be interesting to pursue, but were beyond the scope of the current research.

If, as it is speculated, these lipocarbohydrate polymers represent the "excreted" typing antigens of the group F and related streptococci, the absence of immunoprecipitation in gel would perhaps explain the belief that NCTC 10707 does not contain an Ottens and Winkler (1962) typing antigen. Any serologically identical covalently linked cell-wall antigen in this strain would presumably also go undetected by immunoprecipitation in gel techniques.

The purified lipocarbohydrate antigen of NCTC 11065 contained the highest level of phosphate in relation to carbohydrate. This was in keeping with the migration of this antigen towards the anode in antibody containing gel. Observations on the elution behaviour of this antigen from a Sepharose 6-B column have already been considered, but in the light of the low level of deoxyribose detected, it is suspected that the contribution made to this high phosphate level by contaminating nucleic acids was probably quite small.

The levels of phosphate in the purified lipocarbohydrate antigens of NCTC 10714 and NCTC 5389 were considerably lower than that detected in the NCTC 11065 antigen. However, the level of phosphate was still considered quite

high, and certainly no reasonable suggestion could be made that this reduced level of phosphate was alone responsible for the behaviour of these antigens in immunoelectrophoresis. It was felt that the most likely reason for the observed behaviour of these antigens was that they contained high levels of a positively-charged substituent such as choline, which is found in the pneumococcus (Briles and Tomasz, 1973), or a positively-charged amino-acid. Unfortunately, no choline was detectable in these samples by the method of Appleton *et al.* (1953). Amino-acid analysis of these antigens was not undertaken in the current research, as it was felt that contamination by residual cytoplasmic polypeptides may create difficulties in meaningful amino-acid analysis. However, it is suggested that examination of more thoroughly purified antigens for the presence of associated positively-charged amino-acids may allow some valuable insight, and be worthy of pursuit in future studies. The reason for the positive charge, however, remains at present a mystery. Examination of the fatty-acid content may also provide useful information with regard to understanding the properties of these antigenic polymers more thoroughly.

It is difficult to draw clear correlations between findings presented in the current study, and the previous work of Willers's group on the carbohydrate antigens associated with the cell-surface of group F and related streptococci. The outstanding difference observed in the current studies is that the polymers described here are lipoteichoic acids or closely related polymers, and consequently contain glycerol phosphate which was not a prominent component of previously described antigens. Other notable differences from the work of Willers's group were that these antigens contained high levels of xylose, not previously reported, and that

in general, amino-sugars were not prominent components. No investigations were performed in the current studies to elucidate the composition of the specific determinant groups of these antigens.

Direct isolation of these antigens from the cell-membranes of group F and related streptococci has not previously been reported, and against this background it is perhaps not surprising that important differences in composition were noted between the current research and the observations of previous workers.

5.3.5 Examination of purified lipocarbohydrate antigens by SDS-free PAGE and Western blotting.

There have been very few reports of the examination of lipoteichoic acids or their analogues in PAGE. In the current study, purified lipocarbohydrate antigens were visualised in PAGE gels silver-stained for carbohydrate. In each case the purified antigen presented as a well-circumscribed area of negative staining associated with the gel-front. This appearance was regarded as being consistent with the lipid content of these polymers. The application of Western blotting in each case demonstrated the antigenicity of material contained in this negatively-staining area, which gave the appearance of a smear of antigenic material corresponding to the gel-front. It was interesting to note that very similar results were obtained when membrane lipocarbohydrate antigens extracted by the same method from *Listeria monocytogenes* strains were examined by PAGE and in immunoblots probed with homologous antiserum (Mr. C. Lowe, 1989: personal communication).

Comparison was made between the current results and the appearance of EDTA extracts in PAGE and Western blotting shown in chapter 4. Special attention was drawn to the appearance of NCTC 8037 EDTA extracts (figure 4.6.), which were shown to contain the highest levels of non-covalently linked carbohydrate antigen. Examination of the PAGE gel for this strain (figure 4.6.a.) showed pale, negatively-staining areas at the gel-front, whilst examination of the immunoblot (figure 4.6.b.) showed corresponding smears of antigenic material. No lipocarbohydrate antigen was prepared for this strain in the current study, but it is probable that the results obtained for other strains provide sufficiently strong circumstantial evidence that this was the nature of the antigenic material visualised.

A similar smear associated with the gel-front was also noted for the immunoblotted lipocarbohydrate antigen of *Peptostreptococcus anaerobius* by Smith (1985). However, very different appearances were noted for the lipocarbohydrate antigens of *Clostridium difficile* strains extracted and purified in a similar manner (Sharp and Poxton, 1986). Extracts of this species were found to form regular ladder patterns, highly reminiscent of the appearances in silver-stained PAGE and immunoblots of smooth lipopolysaccharide extracted from Gram-negative bacteria.

A note of caution must be expressed in interpreting the results of the current study. It is possible that the appearance of the lipocarbohydrate antigens in immunoblots may have represented non-specific affinity of IgM antibodies contained in polyvalent antisera to the lipids contained in these extracts. No investigations were undertaken to explore this possibility, neither was any attempt made to ascertain the level of different classes of immunoglobulins contained in the various *Streptococcus milleri* antisera used to probe the transferred material.

5.3.6. ELISA inhibition reactions.

Two strains were selected to investigate the ability of purified lipocarbohydrate antigen to inhibit the homologous EDTA extract/antiserum ELISA reaction. NCTC 11065, whose EDTA extract was shown in chapter 4 to contain predominantly protein antigens was selected, along with NCTC 10714 whose EDTA extract contained proportionately greater amounts of carbohydrate antigen. A large excess of lipocarbohydrate antigen was used as a potential inhibitor in the current study in order to give clear results.

The results of this investigation corroborated the findings of chapter 4. The purified lipocarbohydrate antigen of NCTC 11065 had limited ability to inhibit the homologous EDTA extract/antiserum ELISA reaction, whilst a far higher inhibitory action was noted for the NCTC 10714 reaction. It is suspected that an even more marked result would have been observed had a similar investigation been performed for NCTC 8037.

From all of these findings, it is considered highly likely that the precipitating carbohydrate antigens contained in EDTA extracts of group F and related streptococcal strains were lipoteichoic acids or their analogues. Lipoteichoic acids have been shown in the current studies to present as significant cell-surface antigens in at least some of the group F and related streptococci. It is also considered highly likely that these antigens represent the previously referred to typing antigens which have been recovered from the culture medium following bacterial growth.

5.3.7. Isolation and examination of covalently bound antigens associated with the cell-wall of group F and related streptococci.

Studies were undertaken to isolate and examine antigenic material associated covalently with the purified cell-walls of group F and related streptococcal strains. Willers *et al.* (1964a) reported that certain strains of these organisms possessed typing antigens in their cell-walls, which were serologically indistinguishable from antigens released into the culture medium during bacterial growth. It was consequently of interest to compare the cell-membrane antigens described in the previous section with antigenic material derived from the cell-walls of these strains.

Despite the undoubted sensitivity of currently available analytical techniques, there is still no satisfactory alternative to having sufficient material available for analysis, and repeat analysis, allowing for inevitable losses during purification procedures. The single most limiting feature of the current studies was the small amount of cell-wall antigens available for analysis. This was due partly to the small yields of purified cell-walls derived from these strains, and partly to the small amounts of carbohydrate material subsequently extracted from them. These difficulties were compounded by the decision to apply several extraction procedures to each strain, a decision which was recognised to be increasingly misguided as the studies progressed.

5.3.8. Cell disruption.

Pilot studies had investigated the efficacy of sonication in the breakage of *Streptococcus milleri* cell-suspensions. The poor results echoed the findings of Cooper *et al.* (1975) for *Streptococcus mutans* strains. Cooper *et al.* showed that vigorous sonication of *Streptococcus mutans* cell-suspensions resulted in approximately 8.5% cell disruption, whilst application of the same technique to *Streptococcus pyogenes* strains resulted in over 95% breakage. Satisfactory results were obtained by use of the French press, but cell-wall yields were lower than those reported for other related organisms in this laboratory. Cumming *et al.* (1983) reported a yield of 950mg lyophilised cell-walls from a 10l broth culture of group B streptococcus type II. Yields for *Streptococcus milleri* strains in the current study were, at best, only one third of this, and frequently far lower. These results corroborated those of Michel and Willers (1964) who recovered 300mg dry cell-walls from a 10l broth culture of a group F strain. A comparable yield was reported by Smith (1985), who recovered 285mg of purified cell-walls from a 10l broth culture of *Peptostreptococcus anaerobius*. Slightly improved yields may have followed cell-disruption in a Braun homogeniser or similar device, but such equipment was not available for use in the current studies.

The low levels of cell-wall production may also have reflected low growth levels for *Streptococcus milleri* strains in broth culture compared to some other streptococcal species. Improved growth levels in THB sterilised by filtration as opposed to autoclaving was described in chapter 4. Extension of the incubation time for broth cultures beyond 17 hours was not shown in pilot studies to improve the yields of cells, but rather to carry an

increased risk of autolysis, and possible damage to cell-surface antigens. It is suggested that future studies should use far larger volumes of broth than the 8 to 10l batch-cultures used in the current study. Michel and Willers (1964), for example, described the preparation of group F antigen from an 80l broth culture of organism. More recently, Yakushiji *et al.* (1988c) reported the extraction of wall-associated polymers from 1.4g of *Streptococcus milleri* cell-walls. Given the yields of cell-walls reported in the current study, the production of 1.4g of cell-walls would represent a broth culture of at least 50l. The logistics of handling large volumes of culture prohibited batches in excess of 10l in the current study.

Ideally, culture for exacting analytical work should be undertaken in a fermenter, allowing the strictly controlled, and reproducible culture of very large amounts of organism, and avoiding the uncontrollable variables arising out of multiple batch-cultures in flasks.

5.3.9. Extraction of secondary cell-wall polymers.

All of the extraction procedures applied to group F and related streptococcal cell-walls were able to release carbohydrate material from its covalent linkages within the cell-wall. Carbohydrate yields showed considerable variation, ranging from 0.5 to 8.49mg per 100mg cell-walls. Yields were consistently the highest when the Rantz and Randall (1955) method of extraction was employed.

Cumming *et al.* (1983) reported that the total carbohydrate extracted from group B type II cell-walls represented approximately 1% of the total quantity of cell-walls, whilst the carbohydrate extracted from *Peptostreptococcus anaerobius* by Smith (1985) represented approximately 10%

of the dry weight of cell-walls. Carbohydrate yields in the current study, were generally intermediate between these two results, and erred on the lower side. Knox *et al.* (1979) showed that the autoclaving method of Rantz and Randall (1955) probably releases carbohydrate material from the walls of oral streptococci by the hydrolysis of phosphodiester linkages. It is suggested that this technique is suitable for application in future immunochemical studies on larger quantities of purified cell-walls, in order to ensure high carbohydrate yields for analysis. It must, however, be borne in mind that important differences may be noted in the composition and structure of antigenic material extracted by different techniques, and that following examination of extracts prepared by the method of Rantz and Randall, studies should be extended to include antigens prepared by other methods. As far as the current studies are concerned, given the small amounts of purified cell-walls obtained, it would probably have been preferable to apply one extraction technique only to the cell-walls of each strain, before investigating numerous other extraction procedures. The Rantz and Randall method would have been the single extraction procedure of choice for use in this study.

5.3.10. Examination of crude cell-wall extracts by immunoelectrophoresis.

Immunoelectrophoretic examination of secondary wall polymers derived from NCTC 11065, NCTC 8037, NCTC 10714 and NCTC 5389 revealed the presence of only one precipitating antigen in each of the extracts. For each strain, all of the cell-wall extracts were serologically indistinguishable. The demonstration of only single precipitating antigens in these strains was somewhat disappointing, as it was hoped that it would be possible to

demonstrate two antigens in the extracts of NCTC 8037, NCTC 10714 and NCTC 5389; one representing the Lancefield grouping antigen, and one the typing antigen. It was also hoped to demonstrate a group-like (z) antigen in addition to a typing antigen in the cell-wall extract of NCTC 11065. Several reports have described the formation of antibodies directed against only the type antigen when streptococci possessing both a group-specific and type-specific antigen are used as vaccines for the production of antisera (Bliss, 1937; Willers *et al.*, 1973a). There is also no precedence for the occurrence of more than one secondary wall polymer in the cell-walls of streptococci. In the light of these observations, it is less surprising that only single peaks of immunoprecipitation were observed when extracts of these strains were reacted with homologous antisera in immunoelectrophoresis.

The only cell-wall extracts which gave any evidence of perhaps containing more than one precipitating antigen were those of NCTC 10707, which is believed to contain only a group-specific antigen. Although the peaks observed for extracts of this strain against homologous antiserum were somewhat strange, the group-specific activity of this antiserum could not, in the light of the results in chapter 3, be doubted. In addition, all of the crude cell-wall extracts prepared from group F strains in the current study (NCTC 8037, NCTC 10714 and NCTC 5389) did form immunoprecipitation in gel containing NCTC 10707 antiserum, but peaks were not sharp and were characterised by diffuse areas of staining, which migrated towards the cathode.

Crude cell-wall extracts were additionally run in TCIE with the homologous membrane-associated antigen. No comparison could be made between the non-

precipitating membrane antigen of NCTC 10707, and the cell-wall derived antigens of this strain. The cell-wall derived antigen of NCTC 10714 showed serological identity with the membrane-derived antigen, and those of NCTC 5389 were also very similar, if not identical. The cell-wall and cell-membrane antigens of NCTC 11065 again showed some serological similarity in TCIE, but were probably not identical.

Numerous reports from Willers's group have reported serologically identical antigens contained in the cell-wall and recovered from the culture medium of group F and related streptococci (Willers *et al.*, 1964a; 1973a; Michel *et al.*, 1967). It was initially thought that this observation may simply have represented the isolation of lipocarbohydrate antigen from the culture medium and from inadequately purified cell-walls. This is now not believed to be the case, as the cell-walls prepared in this study were treated with hot SDS and should therefore have been free from all cytoplasmic contaminants. As a note of caution, this was not confirmed by electron microscopy. Similarly, it is believed that the purified lipocarbohydrate antigens prepared in the previous section were free from cell-wall contamination. It is therefore suggested that serologically similar antigens may be found in association with the cell-wall and the cell-membrane of a number of group F and related streptococcal strains. Commenting upon the analysis of type III antigen isolated from the cell-walls and culture medium of streptococcal strains, Huis in't Veld *et al.*, (1973) observed that serological identity of antigens was not necessarily correlated with chemical or structural identity. A similar finding was reported by Poxton and Cartmill (1982) for the cell-wall and cell-membrane antigens of *Clostridium difficile* which showed partial serological identity in CIE, but were chemically quite distinct. Removal of the lipid moiety

from the membrane-associated antigen by treatment with NaOH made the membrane antigen serologically indistinguishable from the wall antigen, despite the fact that these polymers were chemically distinct.

Despite their appearances in immunoelectrophoresis, comparison of the current antigens by PAGE and Western-blotting revealed very distinct appearances of the cell-wall and cell-membrane material. How much may be interpreted from the results of this investigation is unclear. It is suspected that the antigenic material observed in the immunoblot of the crude autoclave-prepared extracts, and spanning a wide range of molecular mass may have simply represented fragmented cell-wall material. Whether unfragmented, native cell-wall antigens would have satisfactorily entered the polyacrylamide gel is unclear.

5.3.11. Purification of cell-wall antigens.

Purification of secondary cell-wall polymers was confined in the current study to anion-exchange chromatography. Attempts were initially made to purify samples on a DEAE-Sephadex A-25 column, equilibrated with ammonium carbonate solution (0.05M), pH 7.8 (Yakushiji *et al.*, 1988c). The unexplained formation of bubbles in the column buffer as it traversed the peristaltic pump led to breakdown of the column, and was found to be quite unsatisfactory in my hands. No such problems were encountered with the DEAE-cellulose/pyridinium acetate buffer system finally employed.

The antigenic material contained in extracts from NCTC 11065 cell-walls, which had migrated towards the anode in antibody containing gels bound well to the anion exchange column and were eluted predictably from the column by pyridinium acetate buffer. Single peaks of carbohydrate were eluted from

the column, confirming that extracts of the cell-wall of this strain contained only one carbohydrate antigen.

The antigenic material contained in cell-wall extracts of NCTC 8037, NCTC 10714 and NCTC 5389 showed far less affinity for the column, which might have been suspected from the positive charge which they displayed by their migration in immunoelectrophoresis. The majority of antigenic material contained in these cell-wall extracts passed through the column without binding, although a small amount of antigenic material did sometimes bind, and was later eluted by the increasing ionic strength of pyridinium acetate. The degree of purity of these wall-derived antigens could not be assured, but it was believed that most of the possible contaminants would have carried a negative charge, and therefore bound to the column, whilst the positively charged antigenic material passed through without binding. Serologically, no differences could be demonstrated between the components of these extracts which had affinity for the anion-exchanger, and those which did not. On no occasion was it possible to infer that two antigens, analogous to a grouping and a typing antigen were contained within the cell-wall extracts of these strains.

The purification of typing antigens from group F and related streptococci by anion-exchange chromatography on a DEAE-cellulose column was described by Willers *et al.* (1964a; 1973a, b). The majority of antigenic material believed to correspond to the type I, II and III antigens was found to be eluted from the anion-exchange column by distilled water, indicating that this material had little or no affinity for the ion-exchanger. The type IV antigen, on the other hand, was not eluted by distilled water, but by the application of phosphate buffer, indicating greater affinity of this antigen for the column, and suggesting the presence of a negative charge.

There is therefore a good deal of similarity between the findings of the current study, and those of Willers's group, lending more weight to the belief that the antigens visualised for NCTC 10714, NCTC 5389, NCTC 8037, and NCTC 11065 were type I to IV antigens respectively, and that type I, II, and III antigens carry a net positive charge. These charges were also reflected in the current study by the serologically similar cell-membrane antigens of these strains. Some possibilities for further purification of cell-membrane antigens were considered in section 5.3.2. The behaviour of these lipocarbohydrate antigens in ion-exchange chromatography is not known, but it is suspected that they would behave in a similar manner to their homologous cell-wall antigens on such columns.

Because of their passage through the ion-exchange column without binding, the purity of the positively charged antigens must be held in some question, and it would be advantageous in future to attempt the purification of these polymers on cation exchange columns. No attempt was made in the current study to investigate the behaviour of cell-wall extracts on cation exchange columns. The purity of the negatively charged carbohydrate antigens of NCTC 11065 following anion-exchange chromatography may be accepted with greater certainty.

The failure to demonstrate material corresponding to the group antigen of some of these strains cannot be explained, but it is suggested that fuller investigation would be allowed by the analysis of antigenic material derived from larger quantities of cell-walls than those available in the current studies.

5.3.12. Analysis of cell-wall derived antigens.

Analysis of acid hydrolysates of cell-wall derived polymers showed that they all contained glucose, galactose, rhamnose and xylose in varying amounts. Unlike the cell-membrane associated antigens of these strains, glycerol was not present, as revealed by gas chromatographic and paper chromatographic analysis. No anhydroribitol was detected in these samples, and only small traces of phosphate. These findings indicated that the cell-wall derived antigens were not teichoic acids. Again the question arose as to why some of these polymers migrated towards the cathode in immunoelectrophoresis.

Samples were again examined for the presence of choline, but in this case it was felt unlikely that high levels of choline would be present in the absence of large amounts of phosphate. This was confirmed by the inability to detect choline in any of the cell-wall antigens examined.

Another possibility was that associated in some way with the carbohydrate polymers were amino-acids which carried a highly positive charge. Examples of common positively charged amino-acids include lysine, arginine and histidine. Amino-acid analysis was carried out on crude cell-wall extracts of NCTC 5389 and NCTC 8037 in a Locarte amino-acid analyser. This analysis failed to reveal any useful information. A range of amino-acids were present in each of the unpurified samples, and there was no evidence to suggest the presence of high levels of positively charged amino-acids in them. It is, however, suggested that following careful purification, preferably by cation exchange chromatography the examination of these polymers for positively charged amino-acids would be worthy of further attention.

It is suggested that all of the strongly precipitating antigens visualised in the cell-wall extracts of NCTC 11065, NCTC 5389, NCTC 8037, and NCTC 10714 represented the carbohydrate typing antigens of these strains. Serologically similar lipocarbohydrate antigens were also recovered from three of these strains, corroborating the results of earlier workers. Limited correlation could be made with the results of previous workers in terms of the chemical analysis of these antigens. No experimentation was undertaken in the current research to identify the composition of specific determinant groups present in purified antigenic extracts. It must, however, be inferred that the antigenic material contained in the autoclave prepared cell-wall extract of NCTC 10707 represented the group F antigen derived from this strain. Detailed analysis of this antigen was restricted by the small amount of antigen available, but some correlation was possible with the findings of Willers *et al.*, (1964a). Willers *et al.* noted that this antigen contained mainly rhamnose, galactosamine and glucose, with a small amount of galactose. In the current studies, the major neutral sugars were also rhamnose, glucose and galactose, but no galactosamine was, however, detected. Again, no investigations were undertaken to determine the nature of the determinant groups present in the various antigens isolated.

It is not suggested that the findings presented in this chapter represent the final word in the isolation and analysis of cell-wall and cell-membrane associated antigens for the group F and related streptococci. Much further work is required to elucidate the composition, structure and functional aspects of these antigens more fully. The preparation and stringent purification of large amounts of antigen is prerequisite to detailed

analysis by techniques including high pressure liquid chromatography (HPLC), mass-spectrometry, and structural elucidation by nuclear magnetic resonance spectroscopy (NMR). On the other hand, it is hoped that the current studies may offer some guidance for further work on the cell-surface immunochemistry of this group of microorganisms.

CHAPTER 6

CONCLUDING REMARKS

In the preceeding chapters, a number of investigations were reported concerning the isolation and cell-surface immunochemistry of a collection of streptococci, the majority of which belonged to Lancefield group F.

Review of the current literature revealed that taxonomically, the group F streptococci are believed to form part of the broader *Streptococcus milleri* group, organisms which are increasingly implicated in serious purulent disease in man. It emerged from the literature, however, that the taxonomy of the *Streptococcus milleri* group as a whole remains unclear. Despite the recent implementation of powerful techniques of genetic analysis, it appears that debate over the taxonomy of this group of organisms is far from complete, and that much further work is required to bring universally accepted clarity to this area.

Improvements in the methods of isolation and culture of anaerobic and capnophilic microorganisms, in addition to an increased awareness of the potential importance of non-beta-haemolytic streptococci in serious disease, have doubtless been instrumental in the increased recognition of *Streptococcus milleri* in infection in recent years. No virulence factors have been established with any certainty for the *Streptococcus milleri* group, and it would appear that here too there is much scope for further investigation of the complex processes instrumental in the inception and progression of disease caused by *Streptococcus milleri*.

Review of the literature regarding the cell-surface antigenic structures of the Lancefield group F streptococci suggested that further work was required to aid understanding of the antigens presenting at the cell-surface of this group of organisms, which may have a bearing on interactions with host defences in disease.

The investigations undertaken in this thesis fell into three main areas:

i. Studies on the isolation and biochemical characterisation of *Streptococcus milleri* isolates from the oral cavity and other sources,

ii. Studies on the non-covalently bound cell-surface antigens of group F and related streptococci, and

iii. Studies on carbohydrate-containing antigens associated with the cell-wall and cell-membrane of group F and related streptococci.

In the first part of these studies, it was found possible to develop a series of biochemical and serological tests for the rapid and cheap presumptive identification of *Streptococcus milleri* isolates, and within them, group F streptococci.

Implementation of these tests, in combination with a simple sampling procedure, allowed the recovery of *Streptococcus milleri* from the human oral cavity, confirming the commensal role of this organism in the gingival crevice. Of the groupable oral isolates, Lancefield group F antigen was by far the most common. Within the series of tests employed in the current studies, it was not possible to define any consistent correlations between particular biotypes and Lancefield group status, haemolytic types, sources, or believed pathogenic behaviour amongst *Streptococcus milleri* isolates. Little contribution was made towards deepening the understanding of taxonomical designations within the *Streptococcus milleri* group as a whole, but it was possible to support the view that group F streptococcus appears to be synonymous with *Streptococcus milleri* on the basis of the tests performed.

The series of tests developed fulfilled the requirements made of it in being rapid, cheap, accurate and reproducible for the presumptive

identification of *Streptococcus milleri* isolates, but was limited in its capacity to identify other common oral streptococci. It is suggested, however, that the system is suitably versatile to be modified for the identification of other organisms by the incorporation of supplementary tests.

The establishment of a culture collection of group F streptococci, diverse in terms of biochemical activity, haemolytic activity, source and pathogenic association was readily achieved using the system developed.

Studies were undertaken to investigate material liberated from whole cells of group F and related streptococci by the action of EDTA.

Examination of EDTA extracts illustrated that both carbohydrates and complex arrays of proteins are released from their non-covalent associations with the cell-surface by this agent. Studies undertaken suggested that the PAGE profiles of proteins liberated from *Streptococcus milleri* strains by EDTA remained stable under a variety of culture conditions. Although the information obtained from the small number of strains examined in the current study was inconclusive, it was suggested that the examination of non-covalently linked cell-surface proteins in PAGE may be of value in chemotaxonomical studies of the *Streptococcus milleri* group as a whole.

ELISA studies suggested the presence of considerable shared antigenic material amongst reference and wild group F streptococcal strains. The application of immunoblotting was not, however, found useful in identifying common antigenic bands contained in EDTA extracts of these strains. The complexity of antigenic material presenting at the surface of whole cells was illustrated in ELISA investigations, and considerable

strain to strain variation in the balance of carbohydrate and protein cell-surface antigens was demonstrated. Minor alterations of the culture atmosphere were also shown to be capable of creating alterations in the balance of cell-surface antigens, as detected in an ELISA inhibition system. It was suggested that such processes may be of importance in diseased sites, where alterations in the environment within, for example, a developing pyogenic lesion may allow organisms to evade host defences by undergoing cell-surface antigenic modification. No attempts were made to isolate and purify individual components contained in EDTA extracts, and the specific functional importance of the various antigenic components encountered is unclear. Further investigation is required to identify cell-surface components in a less general manner, with a view to identifying specific carbohydrate or protein structures which may be of importance in pathogenic processes. Isolation and purification of specific components may then allow the production of more specific antisera, or monoclonal antibodies which may be used to screen large collections of *Streptococcus milleri* isolates for the presence or absence of certain cell-surface antigenic structures implicated with pathogenicity. Such studies may allow the establishment of certain sub-groups within the *Streptococcus milleri* group which have a greater association with certain pathogenic processes than others.

Immunoelectrophoretic examination of EDTA extracts demonstrated the presence of precipitating carbohydrate antigens in the extracts of many strains which were believed to represent the carbohydrate typing antigens of these strains.

The final component of these studies focussed on the isolation and analysis of carbohydrate containing antigenic polymers associated with the cell-wall and cell-membrane of group F and related streptococci. As studies in this area progressed, it became increasingly apparent that an entire research programme, and thesis could be devoted to the extraction, purification and exhaustive analysis of a single carbohydrate antigenic polymer.

Lipoteichoic acids, or their analogues, were found to be capable of presenting at the cell-surface of *Streptococcus milleri* strains as significant antigenic structures. Serologically similar cell-wall derived antigens, which were not teichoic acids and chemically quite distinct, were also demonstrated in preparations of these strains. These polymers were believed to represent the typing antigens described previously for the group F and related streptococci. Limited chemical analysis allowed certain insight into the nature of these polymers, but it was not possible to ascertain the reason why many of these polymers migrated towards the cathode in immunoelectrophoresis, and appeared to carry a positive charge. Consideration was previously given to possible investigations of value in understanding these antigenic polymers more fully.

Perhaps the most intriguing aspect of these studies, and one which would seem worthy of pursuit is the question of the location of the group F antigen contained in these strains. Whether the grouping antigen is cell-wall or cell-membrane associated is not clear, but it is suggested that the group F antigen is probably a cell-wall associated polymer. Cross-reactions of whole-cell EDTA extracts did not suggest that the group F antigen was associated non-covalently with the cell-surface of these strains. Neither was cross-reactivity noted amongst the lipocarbohydrate antigen preparations of these strains.

Limitation of time has unfortunately not allowed a fuller exploration of this interesting aspect of the current research, and it is suggested that a single-minded approach to cell-surface immunochemistry would be required in order to take the current work further. As discussed previously, the inability to define the grouping antigen may have been due in part to the small amounts of antigenic material available for analysis, and larger scale antigen production may be useful in that case. Alternatively, as it is known from the studies reported in chapter 3 that whole-cell HCl (Lancefield, 1933) and nitrous acid (El Kholy *et al.*, 1974; 1978) extracts contain antigenic material which reacts well with commercial and "home-produced" group F antiserum, it may be found useful to undertake preliminary examination of crude whole-cell extracts in an attempt to identify the grouping antigen. Again, if sufficient crude extract were obtained, it is not inconceivable that grouping antigen could be identified, isolated and purified to some extent. Comparison may then be possible with antigens extracted from cell-wall and cell-membrane preparations. Questions remain as to whether the grouping and typing antigens co-exist as separate secondary polymers residing in the streptococcal cell-wall, or whether the grouping and typing antigens have distinct locations, one in the wall, and one in the cell-membrane. Another possibility is that the grouping and typing antigens may not in fact be distinct entities, but that some antigenic complex is present with constant components which may represent a grouping antigen, and more variable areas representing typing antigens (when present), or absence of typing antigen (when absent).

How much bearing the findings of these studies have on the understanding of virulence mechanisms of the group F and related streptococci is uncertain.

Simple statement that the teichoic acids of some streptococci have immunomodulatory effects offers little indication as to the role of the polymers isolated in this research in *Streptococcus milleri* disease, but further research may bring clarity in this regard.

As the studies undertaken in this thesis have developed, it has emerged that there are innumerable avenues of research which are worthy of pursuit regarding organisms belonging to the *Streptococcus milleri* group. It is hoped that this thesis has generated some interest in this group of microorganisms, and that at least some of the very many questions which it has brought to light will be answered by further research initiatives in this area.

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APPENDICES

APPENDIX 1: Intracranial sepsis due to *Streptococcus milleri*.

AUTHOR	DATE	DESCRIPTION	ORGANISM	COMMENTS
1: Thomas	1939	Purulent meningitis	Haemolytic streptococcus (Long and Bliss)	
2: Rantz	1942(b)	1/4 cases meningitis	alpha haemolytic streptococcus group F	
3: Koepke	1965	1 case meningitis	*Streptococcus anginosus* (group F)	Multiple loculated abscesses over cerebral cortex. History of trauma
4: Lerner	1975	2/10 cases meningitis	Group F streptococcus	1 following ruptured brain abscess; 1 with spinal osteomyelitis/epidural abscess
5: Parker and Ball	1976	13/16 cases streptococcal brain abscess, 8/65 cases streptococcal meningitis	*Streptococcus milleri*	
6: Facklam	1977	20/28 cases viridans streptococcal brain abscess 3/28 cases viridans streptococcal brain abscess	*Streptococcus MG-intermedius* *Streptococcus anginosus-constellatus*	
7: Holland *et al.*	1977	2 cases brain abscess	*Streptococcus intermedius*	Pure cultures
8: de Louvois *et al.*	1977	14/35 streptococcal brain abscesses 6/8 cases subdural empyema	*Streptococcus milleri* (all group F type III)	*Streptococcus milleri* predominant in all but temporal lobe abscesses
9: Murray *et al.*	1978	1 case brain abscess	*Streptococcus milleri*	Post traumatic
10: Melo and Raff	1978	3 cases brain abscess	*Streptococcus MG-intermedius*	Pure cultures. 2 associated with liver abscesses
11: Ingham *et al.*	1978	2 cases brain abscess	*Streptococcus milleri*	Mixed cultures typical of dental abscesses, secondary to dental sepsis
12: Brook *et al.*	1980	3/10 cases subdural empyema 1/9 cases brain abscess	1 beta-haemolytic, group F; 2 *Peptostreptococcus intermedius; 1 Peptostreptococcus constellatus*	
13: Plotkin	1982	1 case subdural empyema 1 C.S.F. culture	*Streptococcus anginosus-constellatus*	Following pneumonia Following radical neck dissection and sinusitis
14: Hendrickx *et al.*	1982	8/10 cases brain abscess.	*Streptococcus milleri*	3/8 pure cultures; 5/8 with anaerobes
15: Puthucheary and Rapport	1982	7 cases brain abscess.	*Streptococcus milleri*	4/7 pure cultures; 3/7 with anaerobes
16: Tecson Tumang *et al.*	1982	1 case meningitis.	*Streptococcus MG-intermedius*	
17: Wu and Tsung	1983	1 case meningitis	*Streptococcus milleri*	Following dental extraction in diabetic
18: Blayney *et al.*	1984	3 cases subdural empyema	*Streptococcus milleri*	Complications of sinusitis
19: de Moor and Beatty	1985	2 cases brain abscess	*Streptococcus milleri*	
20: Admon *et al.*	1987	1 case brain abscess	*Streptococcus milleri*	
21: Ghosh *et al.*	1988	1 case acute spinal epidural abscess	*Streptococcus milleri*	

APPENDIX 2

i. Numerical values for the end-points of ELISA titrations between *Streptococcus milleri* reference strain EDTA extracts, and their corresponding antisera (values depicted diagramatically in figure 4.22).

EDTA	ANTISERUM				
EXTRACT	NCTC 5389	NCTC 8037	NCTC 10707	NCTC 10714	NCTC 11065
NCTC 5389	3,200	6,400	1,600	12,800	400
NCTC 8037	12,800	12,800	3,200	12,800	3,200
NCTC 10707	800	3,200	12,800	12,800	800
NCTC 10714	6,400	3,200	200	12,800	1,600
NCTC 11065	6,400	12,800	3,200	12,800	12,800

ii. Numerical values for ELISA inhibition reactions to investigate cross-reactions between EDTA extracts of reference strains, and their corresponding antisera. Results expressed as the percentage of the positive control reaction following application of sodium periodate (PER), or Pronase (PRO). These results were depicted schematically in table 4.3.

EDTA	ANTISERUM									
EXTRACT	NCTC 5389		NCTC 8037		NCTC 10707		NCTC 10714		NCTC 11065	
	PER	PRO	PER	PRO	PER	PRO	PER	PRO	PER	PRO
NCTC 5389	---	---	15	66	12	68	20	62	53	66
NCTC 8037	65	55	---	---	14	70	19	97	79	46
NCTC 10707	41	105	19	91	---	---	13	79	66	64
NCTC 10714	98	22	71	59	12	68	---	---	93	15
NCTC 11065	88	36	42	54	23	71	26	74	---	---

iii. Numerical values for the end-points of ELISA titrations between wild group F streptococcal strains and doubling dilutions of antisera raised against five *Streptococcus milleri* reference strains (values depicted diagramatically in figure 4.23).

EDTA	ANTISERUM				
EXTRACT	NCTC 5389	NCTC 8037	NCTC 10707	NCTC 10714	NCTC 11065
OS25n	800	6,400	800	3,200	800
JW1	400	3,200	1,600	6,400	800
WJ55	1,600	6,400	800	6,400	800
JW5	1,600	1,600	1,600	3,200	1,600
H957	200	800	100	800	100
JW3	800	3,200	800	6,400	400
WJ9	400	3,200	800	3,200	400
WJ12	400	1,600	200	3,200	800
WJ15	1,600	6,400	3,200	12,800	800
WJ8	800	1,600	200	1,600	200
WJ10	1,600	100	100	1,600	100
OS25a	1,600	6,400	800	6,400	3,200
WJ49	100	400	100	100	100

APPENDIX 3

a. Summary of the results obtained from the chemical analysis of purified membrane lipocarbohydrate antigens. Quantities of components expressed as approximate molar ratios.

	COMPONENT					
STRAIN	GLYCEROL	GLUCOSE	RHAMNOSE	GALACTOSE	XYLOSE	PHOSPHATE
NCTC 11065	4	4	1	1	0.3	5.15
NCTC 10707	3	2	1			0.264
NCTC 10714	2	5	3	5	(1 galactosamine)	0.96
NCTC 5389	1	4	2	1		0.72

b. Summary of some of the results obtained from the chemical analysis of secondary cell-wall polymers. Quantities of components expressed as approximate molar ratios.

		COMPONENT			
STRAIN	EXTRACT	GLUCOSE	RHAMNOSE	GALACTOSE	XYLOSE
NCTC 11065	HCl	4	3	3	1
	TCA	2	2	2	1
NCTC 10714	AUTOCLAVE	4	6	4	1
NCTC 5389	AUTOCLAVE	3	3	1	1
	NaOH	3	3	1	TRACE
NCTC 8037	AUTOCLAVE	6	6	6	1

APPENDIX 4

Buffers for SDS-PAGE.

Electrode buffer.

0.025M Tris
0.192M glycine
0.1% SDS

Method:

Weigh out 6.057g Tris (hydroxymethyl) methylamine (BDH, Analar), 28.827g glycine (BDH, chromatographically homogeneous) and 2.0g SDS (BDH, specially pure) into separate containers.

Dissolve Tris and glycine in approximately 1,000ml of distilled water, adjust the pH to 8.3 with 1M NaOH (BDH, Analar).

Add SDS. After it dissolves, make up the volume to 2,000ml with distilled water. Store at room temperature.

Stacking gel buffer.

0.25M Tris-HCl, pH 6.8
0.2% SDS

Method:

Weigh out 15.143g Tris (hydroxymethyl) methylamine (BDH, Analar) and 1.0g SDS (BDH, specially pure) into separate containers.

Dissolve the Tris in approximately 250ml of distilled water; adjust to pH 6.8 with 1M HCl (BDH, Analar).
Add SDS. After it dissolves, make up to 500ml with distilled water.

Store at room temperature.

Separating gel buffer. (Double strength).

0.75M Tris-HCl, pH 8.8
0.2% SDS

Method:

Weigh out 90.855g Tris (hydroxymethyl) methylamine (BDH, Analar) and dissolve it in approximately 500ml of distilled water; adjust the pH to 8.8 with 1M HCl (BDH, Analar).

Add 2.0g SDS (BDH, specially pure), dissolve and make up the volume to 1,000ml with distilled water. Filter through Whatman No. 1 paper.

Store at room temperature.

Buffers for immunoelectrophoresis.

Electrode buffer.

Solution 1:	barbital sodium	26.0g
	barbital (barbitone)	4.24g
	distilled water	2,000ml
Solution 2:	glycine	112.4g
	Tris (not Analar grade)	90.4g
	distilled water	2,000ml

Method:

Mix equal volumes of solutions 1 and 2 (final molarity is 0.187M).

Check the pH is 8.8.

Store at 4°C.

1% agarose for immunoelectrophoresis.

25ml electrode buffer (see above)
75ml distilled water
1.0g agarose (BDH)

Method:

Mix ingredients and dissolve by brief boiling, stirring continuously. Add Triton X-100 (scintillation grade) to 1% (v/v).

Dispense in 15ml volumes for first dimension CIE, and into 3ml volumes for second dimension CIE.

Store at 4°C.