THE ANTIGENIC COMPOSITION OF *STREPTOCOCCUS FAECALIS* ASSOCIATED WITH INFECTIVE ENDOCARDITIS

submitted by

EILEEN JANE AITCHISON

for the degree of

Doctor of Philosophy

ASTON UNIVERSITY

September 1987

TO MY PARENTS, ALAN and JEAN

WITH LOVE

ASTON UNIVERSITY

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The composition of the cell wall of *Streptococcus faecalis* was examined for the presence of specialised structures or components responsible for attachment to host endocardial tissue in infective endocarditis (IE). Analysis of cells by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), crossed immunoelectrophoresis (CIE) and immunoblotting revealed that the protein and antigenic profiles of cells grown in complex laboratory medium were different to those of cells grown in conditions approximating to the in vivo environment. The antigenic pattern of *S. faecalis* was more complex after growth in brain-heart infusion than after growth in serum. Growth in serum resulted in expression of three major protein antigens, 73, 40 and 37K, which were characteristic of *S. faecalis* species and were absent from other streptococcal species and *Staphylococcus aureus*.

The three species-specific antigens were used in a trial for the serodiagnosis of *S. faecalis* endocarditis. The trial involved reaction of immunoblots prepared from electrophoretically separated streptococcal antigens with sera from patients with IE. This method of diagnosis was rapid and accurate, only 3/129 false-positive diagnoses arising.

S. faecalis antigens were located at the cell surface by immunofluorescence microscopy using cells labelled with monospecific antisera, and by radioiodination of surface proteins. Partial characterization of antigens was performed by proteolytic digestion; periodate oxidation; reacting antigens on immunoblots or CIE's with anti-group D streptococcal grouping serum; and by ligand blotting with lectins.

Preliminary isolation and purification of *S. faecalis*-specific antigens was carried out by precipitation using high concentrations of ammonium sulphate or by fast protein liquid chromatography.

Key words: *Streptococcus faecalis*; surface antigens; serodiagnosis; infective endocarditis

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ABBREVIATIONS

A	ampere
AMPS	ammonium persulphate
APL	asparagus pea lectin
ATCC	American Type Culture Collection
BDH	British Drug Houses
BHI	brain-heart infusion broth
Bis	N,N' methylene bisacrylamide
°C	degrees centigrade
c	centi-
CDM	chemically defined medium
CIA	clumping inducing agent
CIE	crossed immunoelectrophoresis
CIEWIG	crossed immunoelectrophoresis with intermediate gel
con A	concanavalin A
D-ala	D-alanine
D-glut	D-glutamic acid
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra-acetic acid
Fab	antigen-binding fragment of immunoglobulin
Fc	crystallizable fragment of immunoglobulin
FITC	fluorescein isothiocyanate
FN	fibronectin
FPLC	fast protein liquid chromatography
FUC	fucosyl residue

GLUC	glucosyl residue
GP	glycoprotein
HS	heat-inactivated horse serum
IE	infective endocarditis
Ig	immunoglobulin
I/M	intramuscularly
I/V	intravenously
K	thousand
k	kilo-
l	litre
L-ala	L-alanine
L-lys	L-lysine
LPS	lipopolysaccharide
LTA	lipoteichoic acid
M	moles per litre
m	metre
μ	micro-
Man	mannosyl residue
N-ac	N-acetylglucosamine
NBTE	non-bacterial thrombotic endocarditis
Ngal	N-acetyl galactosamine
Ngluc	N-acetylglucosamine
NHS	normal human serum
N-mur	N-acetylmuramic acid
OD	optical density
PG	peptidoglycan
PMSF	phenylmethylsulphonyl fluoride

PS	polysaccharide
Rf	retardation factor
RNA	ribonucleic acid
rpm	revolutions per minute
Sarkosyl	sodium lauroyl sarcosinate
SBA	soybean agglutinin
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TA	teichoic acid
TEMED	N,N,N,N' tetramethylene diamine
V	volts
v/v	volume per volume
WGA	wheatgerm agglutinin
w/w	weight per volume
/BHI	grown in BHI
/HS	grown in HS

1. INTRODUCTION

1.1.1 Streptococci: history and classification

The genus *Streptococcus* is a member of the family Streptococcaceae together with the genera *Pediococcus, Leuconostoc, Aerococcus* and *Gemella* (Deibel and Seeley, 1974). This genus contains characteristically spherical or ovoid microorganisms that have a Gram-positive cell wall. Cell division occurs in parallel vertical planes and after fission cells tend to cling together to form pairs or chains of varying lengths. Chain length is influenced by the growth medium (Davis et al, 1980). The poorer the medium, the greater the number of cocci per chain. Streptococci are "microaerophilic" - generally aerobic but usually grow better in an atmosphere of reduced oxygen (Facklam and Wilkinson, 1981). These organisms are non-motile, non-spore-forming and do not synthesize haem compounds. They have complex nutritional requirements and form lactic, acetic and formic acids, ethanol and CO_2 from carbohydrates (Jones, 1978).

In 1874 Billroth first described streptococci (which he termed "*streptococcos*" stemming from the Greek word "*streptos*", meaning twisted or winding) as globular microorganisms growing in chains in purulent exudates from erysipelas lesions and infected wounds of animals. The genus was renamed "*Streptococcus*" by Rosenbach in 1884, in which he included chain-forming cocci isolated from human suppurative lesions (designated *Streptococcus pyogenes*). This species was later discovered to be identical to the "*Streptococcos*" isolated from earlier erysipelas cases. In the following 20 years similar organisms were isolated from the blood in puerpureal fever and from the throat in scarlet fever. The emerging links between streptococci and many animal and human diseases led to attempts to identify and classify the various species in this genus.

The first definitive classification of the streptococci was produced by Andrewes and Horder in 1906. This was modified by Orla-Jensen in 1919 using a greater number of tests for classification criteria. He grouped the species according to fermentation characteristics; tolerance to heat and sodium chloride; temperature limits of growth; and cellular morphology (all under defined growth conditions). Thereby the following species of streptococci were recognised and grouped: *S. lactis*, *S. cremoris*, *S. mastitidis*, *S. agalactiae*, *S. thermophilus*, *S. bovis*, *S. inulinaceus*, *S. liquefaciens*, *S. pyogenes*, *S. equinus*, *S. mitis*, *S. salivarius*, *S. anginosus*, and *S. faecalis*. Not all streptococci could be satisfactorily grouped using Orla-Jensen's criteria, however (Orla-Jensen, 1919). In the same year Brown (1919) developed the use of blood agar in the study of streptococci. This grouped the species according to haemolytic properties. A zone of complete lysis surrounding the bacterial colonies on the blood agar plate was termed β-haemolysis. Incomplete lysis, appearing as a narrow green zone surrounding colonies, was known as α-haemolysis. If the bacteria had no noticeable effect on the blood agar, the streptococci were deemed non-haemolytic (or γ-haemolytic).

The following years saw refinement of established tests and development of new tests in an attempt to improve the classification of the streptococci. Several workers used bacteriophages to differentiate streptococci (Hadley and Dabney, 1926; Lancefield, 1932 and 1933; Evans, 1936) with varying degrees of success. Other laboratories used properties such as bacterial motility (Schieblich, 1932; Kloblmüller, 1935), bile tolerance (Houston, 1934) or fibrinolytic activity (Tillet and Garner, 1933) to distinguish certain species of streptococci. The most widely accepted streptococcal classification scheme of that time was introduced by Sherman in 1937. This was the first time an attempt had been made to segregate the genus into homogeneous divisions.

Sherman recognised four main physiological groups of streptococci. His divisions were based on such properties as temperature limits of growth; tolerance to salt and methylene blue; ability to grow at pH 9.6; reducing ability; production of ammonia from peptone and type of haemolysis produced on blood agar. Using these criteria, Sherman divided the streptococci into "pyogenic", "viridans", "lactic" and "enterococcus" groups. Initial assignation of species into one of these groups was followed by further tests to identify particular species within each group (see table 1).

The "pyogenic" group included most of the streptococci at that time recognised as being pathogenic to man (apart from *S. pneumoniae*; Deibel and Seeley, 1974). Generally, Sherman's pyogenic streptococci were β-haemolytic, sensitive to temperature extremes, non-tolerant to salt and produced ammonia from peptone. The "viridans" group were so named because of the tendency of some species to produce greening on blood agar (α-haemolysis). Species in this group grew at 45°C but not at 10°C, had weak reducing activity, were non-tolerant to methylene blue, salt and alkali, and did not produce ammonia from peptone. "Lactic" streptococci (so named because of their long association with dairy products) were non-haemolytic, grew at 10°C but not at 45°C, were non-tolerant to salt or alkali and did not produce ammonia from peptone. The "enterococcus" division included streptococci that were characteristically temperature-, salt-, alkali- and methylene blue-tolerant. Species in this group were also strongly reducing and had the ability to produce ammonia from peptone. All enterococci possessed the Lancefield Group D antigen (Lancefield, 1933; see section 1.1.2).

Nowadays these broad divisions are somewhat obsolete since many recently recognised species cannot be assigned to a particular group using Sherman's criteria (Deibel and Seeley, 1974). However, the tolerance tests

TABLE 1

PHYSIOLOGICAL AND BIOCHEMICAL TESTS FOR IDENTIFICATION OF STREPTOCOCCUS SPP.*†

Physiological Group	Serological Group	Sherman criteria: Hemolysis	Sherman criteria: Growth at 10C	Sherman criteria: Growth at 45C	Sherman criteria: Growth at pH 9.6	Sherman criteria: 0.1% Methylene blue	Sherman criteria: Survive 60C/30 min	Sherman criteria: NH_3 from arginine	Species Differentiation: β-hemolysis	Species Differentiation: Na hippurate hydrolysis	Species Differentiation: Lactose	Species Differentiation: Trehalose	Species Differentiation: Sorbitol	Species Differentiation: Gelatin liquefaction	Species Differentiation: 0.04% Tellurite tolerance	Species Differentiation: Starch hydrolysis	Species Differentiation: Glycerol (anaerobic)	Species Differentiation: Tetrazolium reduction	Species Differentiation: Growth at 50C	Species Differentiation: Mannitol	Species Differentiation: Arabinose	Species Differentiation: Melibiose	Species Differentiation: Melezitose	Species Differentiation: Maltose	Species Differentiation: Growth at 40C	Species Differentiation: Growth at pH 9.2	Species Differentiation: Growth in 4% NaCl	Species Differentiation: NH_3 from arginine	Species Differentiation: Acetoin production	Streptococcus spp.	Usual Habitat or Source	Mostly nonpathogenic	Occasional pathogens
Enterococcus	D	α, β, or γ	+	+	+	+	+	+	−	+	+	+	−	+	+	−	+	+	−	+	+	−	−	+	−	+	+	+	+	−	*faecalis*	Intestine of man and other warm-blooded animals		●
	D								−	+	+	+	±	+	+	−	+	+	−	+	+	−	−	+	−	+	+	+	+	−	*faecalis* var. *zymogenes*			
	D								−	+	+	+	−	+	+	−	+	+	−	+	+	−	−	+	−	+	+	+	+	−	*faecalis* var. *liquefaciens*			
	D								−	+	+	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	*faecium*			
	D								±	+	+	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	*faecium* var. *durans*			
Lactic	D	α or γ	−	+	−	−	−	−	−	−	±	−	−	±	−	±	−	−	±	−	−	±	+	−	−	−	−	−	−	−	*bovis*	Bovine intestine		●
	D								−	−	±	−	−	±	−	±	−	−	±	−	−	±	+	−	−	−	−	−	−	−	*equinus*	Equine intestine		
	N	γ	+	−	−	+	±	±			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	*lactis*	Dairy utensils, milk and milk products, vegetable material	●	
	N										+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*lactis* var. *diacetilactis*			
	N										−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	*cremoris*			
Viridans	Ungrouped	α or γ	−	+	−	−	−	±			±	±		−	−	+	+	S1													*mitis*	Human mouth and throat	●	
	Ungrouped										±	±		−	−	+	+	−													*salivarius*	Human mouth and throat		
	Ungrouped										+	−		+	−	−	−	−													*thermophilus*	Pasteurized milk and cheese		
Pyogenic	A	β	−	−	−	−	−	+	−	−																					*pyogenes*	Human – pathogen	●	
	B								+	−																					*agalactiae*	Cattle – pathogen		
	C								±	−																					*equi*	Horse – pathogen		
	C								−	−																					*equisimilis*	Human – parasite		
	C								+	−																					*dysgalactiae*	Cattle and sheep – pathogen		
	E								±	−																					*zooepidemicus*	Animal – pathogen		
	F								+	−																					sp.	Cattle – parasite		
	G								+	−																					*anginosus*	Human – pathogen		
	H1								±	−																					sp.	Human and animal – parasite		
																															sanguis	Human – parasite		

*Adapted from Sharpe, Fryer, and Smith in Gibbs and Skinner (Eds.): Identification Methods for Microbiologists, Part A, Academic Press–London, 1966.

†Symbols: + = positive; − = negative; Sl = slight; ± = majority positive; ∓ = majority negative.

‡Serological groups K-T have also been designated.

used by Sherman (1937) to subdivide species within his divisions are still used today in the routine identification of streptococci.

Twenty-one species of streptococci are now officially recognised and listed in the eighth edition of *Bergey's Manual of Determinative Bacteriology (1974)*:-

S. pyogenes, S. equisimilis, S. zooepidemicus, S. equi, S. dysgalactiae, S. sanguis, S. pneumoniae, S. anginosus, S. agalactiae, S. acidominimus, S. salivarius, S. mitis, S. bovis, S. equinus, S. thermophilus, S. faecalis, S. faecium, S. avium, S. uberis, S. lactis and *S. cremoris* (Deibel and Seeley, 1974). Identification of the individual species is based on serological and physiological tests, arising from the knowledge of streptococcal classification gained over the past century (listed extensively by Facklam and Wilkinson, 1981). Species lacking a group antigen are identified by physiological tests only and nominally placed in a serological group on the basis of physiological similarities to species possessing a group antigen.

Several anaerobic species have been excluded from the genus *Streptococcus* in *Bergey's Manual* because they are not facultative anaerobes. Such species include *S. intermedius, S. constellatus, S. morbillorum, S. hansenni* and *S. pleomorphus*. The placement of these species in the genus *Streptococcus* is questionable and more work needs to be performed before a common agreement is reached on their taxonomic status (Jones, 1978). Several other unlisted (in *Bergey's Manual*) species include *S. rattus, S. sorbrinus, S. cricetus* and *S. ferus*, which are all varieties of *S. mutans* (Jones, 1978).

Knowledge accumulated from almost a century of study has not yet led to the development of a universally accepted classification scheme for the genus *Streptococcus*. Most of the existing systems are less than ideal in that they concentrate on too narrow a spectrum of characteristics to be applied to such a large group of diverse species (Jones, 1978). In order to

produce a satisfactory classification scheme for the streptococci, exhaustive broad biological and chemical studies of species from all sources must be carried out under defined growth conditions and taking into account the effect of plasmid-carriage upon the organism. Only thus may a reliable identification and classification system may emerge which can be applied to all streptococcal species and their many varieties.

1.1.2 Serological grouping

In 1924 Hitchcock observed the soluble specific substance upon which the serological grouping of streptococci came to be based (Hitchcock, 1924a). This "residue antigen" was initially believed to be common to almost all haemolytic streptococci but in 1933, Lancefield discovered the serological specificity of this substance. This led to the development of her grouping system for streptococci which divided the genus into approximately 20 groups, each assigned a letter of the alphabet (Lancefield, 1933). Members of each Lancefield group are generally biochemically uniform (except for group C which contains recognisably different biotypes) and cause similar diseases in a characteristic host range (Parker, 1975)

The group-specific antigen (or "C substance") was detected serologically by precipitin techniques using antisera prepared by injecting whole cells into rabbits (Hitchcock, 1924a and 1924b; Lancefield, 1928a and 1933). This led to a better understanding of the antigenic structure of streptococci (Lancefield 1928a, b, c, d and e).

In common with most classification systems the Lancefield serological grouping scheme has limitations, in that it is inapplicable to certain species. In some cases this is because the organism possesses an incomplete group antigen, an antigen with dual group specificity, or lacks a group antigen altogether. Such non-typable species include *S. acidominimus*, *S. anginosus-constellatus*, *S. mitis*, *S. morbillorium*, *S. mutans*, *S. salivarius*,

S. sanguis and *S. uberis* (Lerner, 1975; Parker and Ball, 1976; Facklam, 1977; Facklam and Wilkinson, 1981). Cross-reaction may occur between certain species of streptococci. Recently, strains of *S. faecalis* have been reported that react with group G serum as well as group D (Birch et al, 1984; Harvey and McIllmurray, 1984). This gives rise to the likelihood of misidentification if the Lancefield grouping technique is used as the sole identification criterion. Ideally the serological grouping should be carried out in conjunction with biochemical tests when classifying or identifying streptococci. However, the Lancefield grouping technique is widely accepted as providing an excellent means of initial "sorting out" of streptococcal species prior to more detailed identification.

The grouping procedure is relatively simple to perform. Lancefield's original method involves extraction of the group-specific antigen by incubating streptococcal cells in 0.2N HCl at 100°C for 10 minutes. The acid-extract is then neutralized and separated from residual whole cells by centrifugation (Lancefield, 1933). The precipitin test is carried out on the extracted antigen as follows: a sterile capillary tube is dipped into Lancefield grouping serum and a column of approximately 1cm is drawn up. An equivalent amount of antigen extract is drawn up in the same way. The lower end of the capillary tube is sealed with a plasticine plug without mixing the reactants. The tube is then inverted and the open end sealed with plasticine. Within five minutes a ring of white precipitate should form at the interface between the extract and its homologous grouping serum (weaker reactions may need up to 30 minutes to develop).

The group-specific antigen may be obtained from streptococci by other methods including enzymatic extraction using lysozyme (Watson et al, 1975), pronase B (Ederer et al, 1972) or an enzyme from *Streptomyces albus* (Maxted, 1948a); extraction with nitrous acid (El Kholy et al, 1974) or by autoclaving (Rantz and Randall, 1955). The Lancefield, Fuller, Rantz-Randall

and Watson methods are suitable for extraction of groups A, B, C, D, F and G streptococci but the El Kholy and Maxted extraction methods are not suitable for group D streptococci. Antigen extracts obtained by the pronase B method (Ederer et al, 1972) must be grouped using high quality sera in the precipitin test in order to gain a satisfactory result (Facklam and Wilkinson, 1981).

Other serological methods of grouping streptococci have been developed in addition to the Lancefield's original capillary tube technique. The double-diffusion method of Rotta et al (1971) dispelled the need for the use of absorbed antisera. However this test had its main application in typing of group A streptococci according to M protein serotypes (see section 1.2). Dajani (1973) developed a grouping method which involved counter-current immunoelectrophoresis. This method was rapid and inexpensive, with reputedly no cross-reactions or false positive results occurring. A method involving immunofluorescence with labelled $F(ab')_2$ fragments of anti-group IgG was introduced by Cars et al (1975). This method had the advantage that cross-reaction with staphylococcal protein A did not occur and excluded non-specific reactions between normal rabbit IgG and streptococci of groups A, C and G. Improved specificity and strength of the grouping reactions has been made possible with the advent of monoclonal antibody technology (Nahm et al, 1980).

The group-specific antigens of streptococci are either polysaccharides (as in groups A, B, C, E, F, G, H, K, L, P and U) or teichoic acids (as in groups D and N). Polysaccharide group-specific antigens are located in the cell wall (Krause,1963), attached to N-acetylmuramic acid residues in the peptidoglycan network via a phosphodiester linkage. For some streptococcal groups the chemical nature of the antigen has now been established. The group antigen in group A and B streptococci is composed of L-rhamnose and N-acetylglucosamine; in group C streptococci the antigen is rhamnose and

N-acetylgalactosamine (Hammond et al, 1984). The amino sugar is responsible for the antigenic specificity of the polysaccharide. In group G streptococci the rhamnose backbone of the group antigen is thought to be the specific antigenic determinant (Krause, 1963).

Group D and group N streptococci possess a teichoic acid group-specific antigen (Elliott, 1960; Jones and Shattock, 1960; Wicken et al, 1963). Detailed studies have led to the conclusion that the group antigen is a membrane- or lipoteichoic acid (Archibald and Baddiley, 1966: Wicken and Knox, 1975a). Although not strictly associated with the cell wall, the membrane teichoic acid group antigen is thought to traverse the cell wall and extend into the external environment from the surface of the streptococcal cell (Knox and Wicken, 1973; Beachey, et al, 1983; Orefici et al, 1986). The immunodeterminant in the teichoic group antigen of group D streptococci is glucose-α-1→2-glucose, whereas in group N streptococci it is α-D-galactose (Wicken and Knox, 1975a and b). The cellular location and chemical characteristics of some streptococcal group antigens are shown in table 2.

The group-specific antigens of some streptococcal species have been implicated in the process of pathogenicity or virulence (see section 1.2.2 for teichoic acid antigens and section 1.2.3 for carbohydrate antigens).

TABLE 2

Some chemical characteristics and cellular location of streptococcal antigens

Group antigen (hapten)			Type antigen (hapten)			Species
Designation	Chemical nature	Cellular location	Designation	Chemical nature	Cellular location	
A	Rhamnose-*N*-acetyl-glucosamine polysaccharide	Wall	M R T	Protein Protein Protein	Envelope Envelope Envelope	*S. pyogenes*
B	Rhamnose-glucosamine polysaccharide	Wall	S (3 types)	Glucose-galactose-*N*-acetyl-glucosamine polysaccharide	Envelope	*S. agalactiae*
C	Rhamnose-*N*-acetyl-galactosamine polysaccharide	Wall	(2 types described)	Protein		*S. equisimilis*
			(3 types)	Protein		*S. zooepidemicus*
			(Only 1 type described)	Protein	Envelope	*S. equi*
			(3 types)	Protein		*S. dysgalactiae*
D	Glycerol teichoic acid containing D-alanine and glucose	"Intracellular" between wall and membrane	(11 types established)	Rhamnose-glucosamine-glucose polysaccharide	Cell wall	*S. faecalis*
D			(19 types described; many more exist)			*S. faecium*
D, Q						*S. avium*
D			(Many types exist)	Carbohydrate?	Capsule	*S. bovis*
D						*S. equinus*
E	Rhamnose polysaccharide	Wall	I to V	Polysaccharide		*Streptococcus sp.*
F	Rhamnose and a glucopyranosyl-*N*-acetyl-galactosamine tetrasaccharide	Wall	I to V	Carbohydrates—some types contain glucose, galactose and rhamnose		*S. anginosus*
G	Rhamnose-galactosamine polysaccharide	Wall	(3 types described)			*Streptococcus sp.* (large colony)
H	Rhamnose polysaccharide	Wall	(5 types described)			*S. sanguis*
K	Rhamnose polysaccharide	Wall	I and II, and I-II	Galactose, glucose rhamnose Type I—*O*-β-D galactopyranosyl-(1->6)-D-galactose	Cell wall	*Streptococcus sp., S. salivarius*
N	Glycerol teichoic acid containing D-alanine and galactose phosphate	"Intracellular" between wall and membrane	Many types exist			*S. lactis* *S. cremoris*
D			(Only 1 type)	Contains glucose, glucosamine, galactosamine	Capsule	*S. suis*
None	Ribitol teichoic acid with choline phosphate	Wall	(90 types)	Carbohydrate	Capsule	*S. pneumoniae*
			M	Protein	Envelope	

1.2 Structure and composition of the streptococcal cell wall and its relation to pathogenicity

The structure of the cell wall of Gram-positive bacteria (figure 1a) is much less complex than that of Gram-negative bacteria (figure 1b). In the Gram-staining procedure the Gram-positive cell wall retains the dye-iodine complex whereas Gram-negative bacteria are decolourized by alcohol. This is thought to be due to differences in cell-wall permeability (Salton, 1964). The structure and composition of major components of the streptococcal cell wall will be discussed with reference to the virulence of streptococci.

1.2.1 Peptidoglycan

Peptidoglycan (PG) is the only cell wall polymer common to both Gram-negative and Gram-positive bacterial cells. Although only a minor component of Gram-negative walls (≈10%), it is the major component of Gram-positive cell walls, comprising ≈50% of the total dry weight of the wall. The remainder of the cell wall consists of teichoic acids (section 1.2.2), polysaccharides (section 1.2.3) and proteins (section 1.2.4), a proportion of which are covalently bound to the PG (Schleifer and Kandler, 1972).

PG, alternatively known as mucopeptide, glycopeptide, basal structure, murein or glycosaminopeptide (Ghuysen, 1968; Schleifer and Kandler, 1972), forms a thick layer (approximately 20-50nm) around the cell outside the cytoplasmic membrane. The PG forms a three-dimensional network which gives strength and rigidity to the bacterial cell (Baddiley, 1972; Wicken and Knox, 1980; Rogers, 1970; Shockman and Barrett, 1983). This network is composed of glycan strands cross-linked by peptide subunits, which are themselves joined by interpeptide bridges. The glycan strands are made up

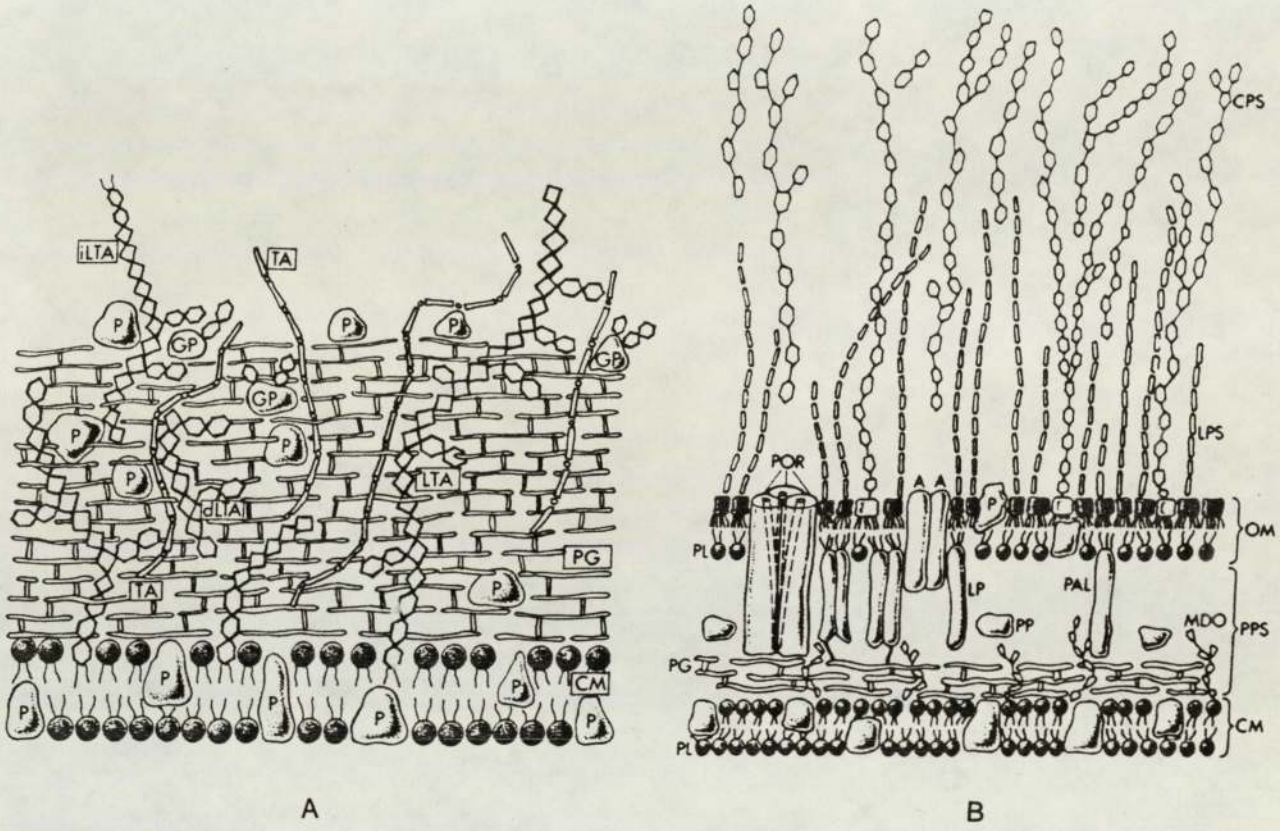


Figure 1a Representation of Gram-positive bacterial cell wall.
Key:

CM	cytoplasmic membrane
GP	glycoprotein
LTA	lipoteichoic acid
dLTA	deacylated LTA
iLTA	inverted LTA
P	protein
PG	peptidoglycan
TA	teichoic acid

Figure 1b Representation of Gram-negative bacterial cell envelope.
Key:

A	outer membrane protein A
CM	cytoplasmic membrane
CPS	capsular polysaccharide
LP	Braun lipoprotein
LPS	lipopolysaccharide
MDO	membrane-derived oligosaccharide
OM	outer membrane
P	protein
PAL	peptidoglycan-associated lipoprotein
PG	peptidoglycan
PL	phospholipid
POR	porin protein
PPS	periplasmic space (periplasm)

of alternating molecules of N-acetylglucosamine and N-acetylmuramic acid joined through β-1→4 links (Ghuysen, 1968; Schleifer and Kandler, 1972). The structure of the glycan strands shows little variation but may occassionally show O-acetyl substitution on the C_6 of some N-acetylmuramic acid residues (Rogers, 1970). The short peptide subunits which link the glycan strands are usually composed of L-alanine (joined to the N-acetylmuramic acid residue) linked to D-glutamic acid which is itself linked by its γ-carboxyl group to an L-diamino acid (this is L-lysine in the case of *S. faecalis*), followed by D-alanine (Schleifer and Kandler, 1972). The distal amino group of the L-diamino acid is involved in formation of an interpeptide bridge with D-alanine on an adjacent peptide subunit (Ghuysen, 1968; Schleifer and Seidl, 1977). In a few Gram-positive organisms the link between the L-diamino acid and D-alanyl residues on adjacent peptide chains is direct and not via an interpeptide bridge. The amino acid composition and sequence of the interpeptide bridge varies greatly among Gram-positive species. In *S. faecalis* PG the composition is L-lysine-L-alanine-L-alanine-L-alanine (Deibel and Seeley, 1974). A representation of the structure of *S. faecalis* PG is shown in figure 2.

The variation in the composition of interpeptide bridges in bacterial PG has been used as a means of classifying species. Kandler et al (1968) noted two different characteristic sequences of amino acids in the PG interpeptide bridges of enterococcal strains. Kandler's group differentiated *S. faecalis* (interpeptide chain type: L-lysine-D-alanine-L-alanine-L-alanine-L-alanine) from *S. faecium* (interpeptide chain type: L-lysine-D-alanine-D-asparagine). Also by this method *S. liquefaciens* and *S. durans* were identified as being varieties of the former and latter species, respectively. This method of bacterial classification showed good

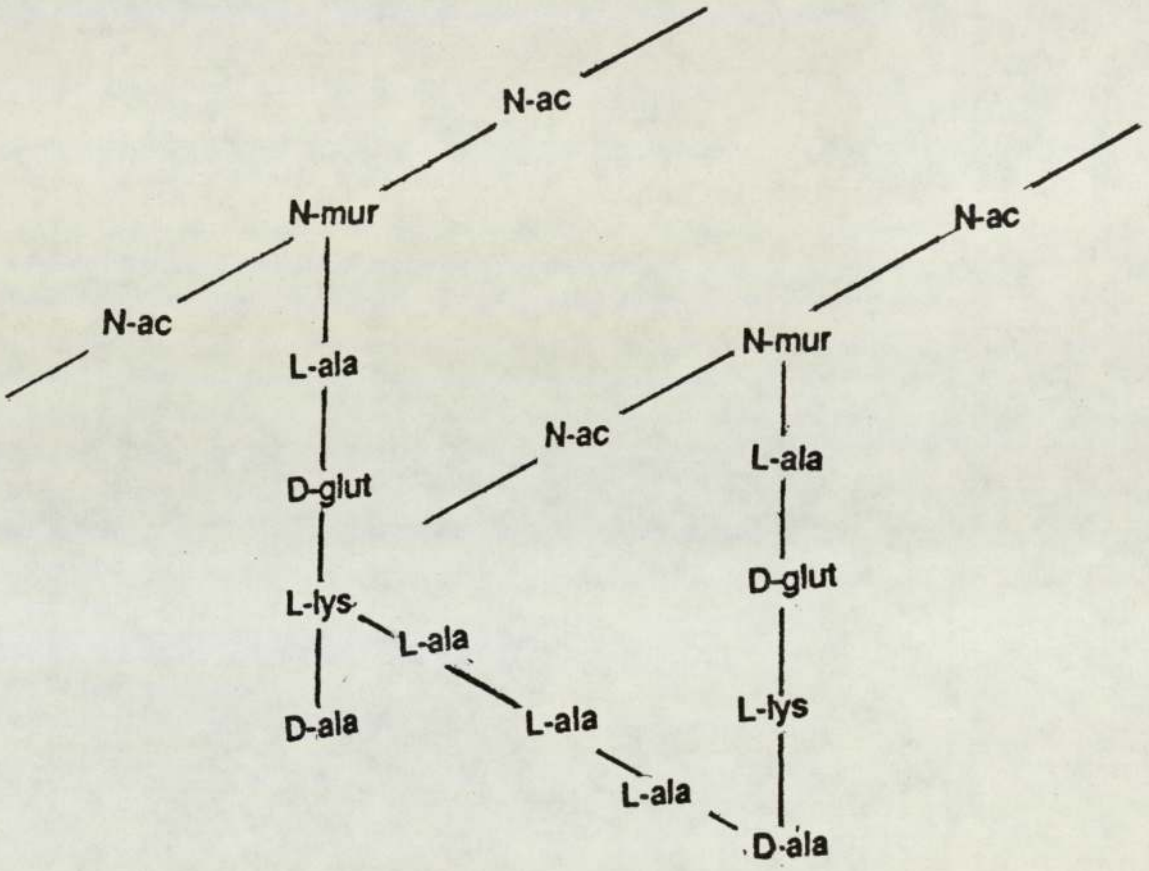


Figure 2 Representation of peptidoglycan structure of *Streptococcus faecalis* cell wall.

Key:

D-ala	D-alanine
D-glut	D-glutamic acid
L-ala	L-alanine
L-lys	L-lysine
N-ac	N-acetylglucosamine
N-mur	N-acetylmuramic acid

correlation with the classification systems of Andrewes and Horder (1906) and Orla-Jensen (1919). Other such studies have successfully distinguished organisms on the basis of their PG composition and structure (Schleifer and Kandler, 1967; Ghuysen, 1968; Slade and Slamp, 1972; Schleifer and Kandler, 1972). These studies have shown that PG structure and composition can be used to divide members of an immunological group into subgroups and also to establish a relationship between members of the same genus not related immunologically (Slade and Slamp, 1972).

The composition of PG is relatively stable but may be affected in exceptional circumstances by the medium in which the bacterium is grown (Shockman and Barrett, 1983). Hydroxylysine may be incorporated into the PG polymer of *S. faecalis* in place of lysine. However this can only happen if a high concentration of hydroxylysine is added to the growth medium and the lysine content is limited (Smith and Henderson, 1964).

In unencapsulated streptococci the PG has a surface location in the bacterial cell. Surface-exposed PG is protected from the action of lysozyme and other lytic enzymes by O-acetylation of the glycan strands and by covalently bound teichoic acids and polysaccharides (Schwab, 1979; Rosenthal et al, 1982).

Various biological activities have been attributed to bacterial PG:- adjuvant activity, endotoxin-like properties, arthrogeneity and immunogenicity (Schwab, 1979; Seidl et al, 1983). Schleifer and Seidl (1977) have shown there to be at least three antigenic determinants in PG. These antigenic sites are contained in the glycan strands, the peptide subunits and the interpeptide bridges. N-acetylglucosamine is the immunodominant group on the glycan strand rather than the N-acetylmuramic acid (Seidl et al, 1983). In the peptide subunits it is the carboxyl terminal

D-alanine-D-alanine group which is immunologically active (but only on peptide subunits which are not cross-linked with interpeptide bridges). The interpeptide bridge stimulates production of antisera acting specifically against each type of interpeptide bridge (Seidl et al, 1983). Antibodies to PG are present in normal human and animal sera (Heymer at al, 1973) - with raised titres in Gram-positive infections (Zeiger et al, 1981; Wergeland et al, 1984) - and a strong antibody response can be elicited by injection of PG preparations (Heymer and Rietschel, 1977; Rotta et al, 1982). In addition to being immunogenic independently, PG can act as an immunoadjuvant. When administered as a water-in-oil emulsion with unrelated antigens, PG stimulates antibody production and induces delayed hypersensitivity. A muramyldipeptide is the active subunit of PG in its capacity as an immunoadjuvant (Heymer and Reitschel, 1977; Rotta et al, 1983).

Other virulent actions of PG are summarized as follows:- Group A streptococcal cell wall fragments cause a chronic inflammatory response when injected intradermally into rabbits (Schwab, 1979). In particular it is a PG-polysaccharide complex which is responsible for this effect. Group D cell wall fragments only produce a short-lived reaction. The PG-polysaccharide complex of group A streptococci is resistant to biodegredation because the PG is protected by the group-specific polysaccharide. Such protection is not afforded group D PG because of the absence of a group-specific polysaccharide, thus the PG is quickly eliminated (Stimpson et al, 1986). The alternate complement pathway is activated by PG (Greenblatt et al, 1978; Schwab, 1979). PG is known to be pyrogenic (Heymer and Reitschel, 1977). This endotoxin-like activity is most pronounced with a muramyldipeptide PG fragment (Rotta et al, 1982),

pyrogenicity lessening with increasing peptide chain length. PG may inhibit phagocytosis of bacteria by rabbit and human polymorphonuclear leukocytes, due to a direct toxic action on phagocytes (Jones and Schwab, 1970; Leong and Cohen, 1984). PG can also cause B-cell mitogenicity (Damais et al, 1975); cytotoxic activation of macrophages(Schwab, 1979); cardiac necrosis (Heymer and Reitschel, 1977; Parker, 1975; Davis et al, 1980); and lysis of red blood cells and platelets (Ryc and Rotta, 1975).

1.2.2 Teichoic Acids

Teichoic acids (TA) are water-soluble, phosphate-containing polymers associated with the cell wall or cytoplasmic membrane of Gram-positive bacteria. These polymers are amphipathic - having both hydrophilic and hydrophobic regions (Wicken and Knox, 1980). The TA's associated with the cytoplasmic membrane, initially known as "intracellular TA's" were thought to be located in the cell membrane or between the membrane and the PG network (Shattock and Smith, 1963; Wicken et al, 1963). The intracellular TA was renamed "membrane TA" (also known as lipoteichoic acid (LTA)) by Archibald and Baddiley (1966) because of their linkage to the cytoplasmic membrane (Shockman and Slade, 1964).

Wall-associated TA is not common to all Gram-positive species and its presence in a particular bacterial cell may depend upon composition of the growth medium, or upon the rate of growth (Ellwood and Tempest, 1972; Knox and Wicken, 1984). Under phosphate limitation wall TA may be replaced by an acidic polysaccharide, teichuronic acid (Ellwood and Tempest, 1972). The structure of wall TA's varies widely between bacterial species. Basically they are polymers of glycerol- or ribitol phosphate, covalently linked through a specialized linkage unit via a phosphodiester bond to the

6-position of N-acetylmuramic acid residues in the glycan strands of PG (Ghuysen et al, 1965: Button et al, 1966; Ghuysen, 1968; Coley et al, 1978). Many TA's possess glycosyl and D-alanyl substituents on hydroxyl groups of the glycerol or ribitol residues (Baddiley, 1972; Beachey, 1980), thus allowing many variations on the basic TA structure.

Membrane TA (LTA) is found in all Gram-positive bacteria apart from some *Actinomyces* and *Micrococcus* species (Archibald and Baddiley, 1966; Wicken and Knox, 1975a). This polymer acts as the group-specific antigen in group D and group N streptococci (Archibald and Baddiley, 1966; Shockman and Slade, 1964; Shattock and Smith, 1963; Wicken et al, 1963; Toon et al, 1972). The structure and composition of LTA is relatively stable (Knox and Wicken, 1973; Wicken and Knox, 1977), being little affected by growth rate or medium. LTA is composed of repeating units of glycerol phosphate joined through a 1→3 phosphodiester linkage (Coley et al, 1978). The LTA is covalently bound to a glycolipid fraction in the cytoplasmic membrane through a phosphodiester bond (Toon et al, 1972), only about 12% of membrane glycolipid being involved in binding to LTA. In the case of *S. faecalis* the membrane anchorage of LTA is a phosphatidyl kojibiosyl [α-D-glucopyranosyl-(1→2)-D-glucopyranosyl] diglyceride (Toon et al, 1972; Ganfield and Pieringer, 1975; see figure 3). The non-polar tail of this molecule consists of four long-chain fatty acids which form a broad hydrophobic region embedded in the cell membrane. The backbone of *S. faecalis* LTA is heavily glycosylated (Beachey, 1980). The hydroxyl groups of the polyglycerol phosphate chain are substituted with kojibiosyl groups together with D-alanine residues (Toon et al, 1972).

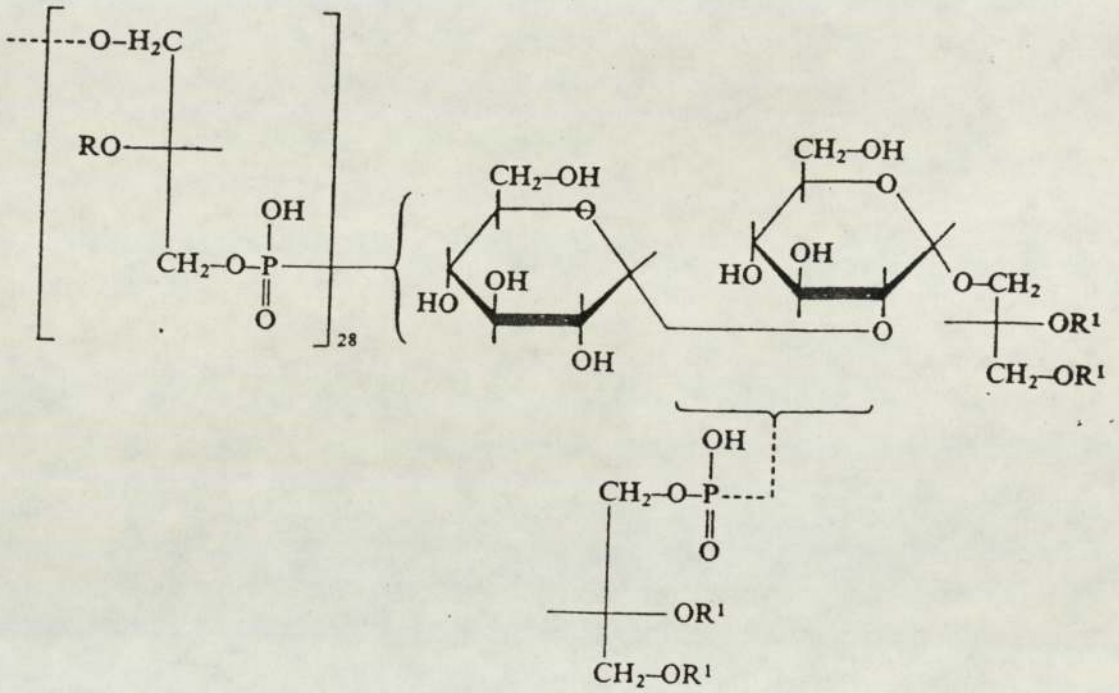


Figure 3 Linkage of LTA to cytoplasmic membrane in *Streptococcus faecalis*. The fatty acid tails of the phosphatidyl kojibiosyl diglyceride unit are anchored in the cytoplasmic membrane; the LTA chain extends through the cell wall to the cell surface.

As a result of several studies the position of LTA within the bacterial cell has been clarified. The LTA molecule extends from its cytoplasmic membrane glycolipid anchor, through the cell wall to the surface of the bacterial cell. In 1973, van Driel et al demonstrated that the LTA of *Lactobacillus fermenti* was immunogenic. When whole-cells were injected into rabbits, anti-LTA antibodies were raised. Also anti-LTA antisera agglutinated whole cells of *L. fermenti* (van Driel et al, 1973). These workers proposed a model of the Gram-positive bacterial cell in which LTA, bound by its lipid end to the cytoplasmic membrane, showed various depths of penetration into the PG-polysaccharide network of the cell wall, some molecules reaching the cell surface. Further serological studies together with electron microscopy using ferritin linked to anti-LTA (or goat anti-rabbit γ-globulin) antibodies showed that part of the LTA polyglycerol phosphate chain extends from the cell wall into the external environment (Hewett et al, 1970; van Driel et al, 1973; Orefici et al, 1986).

In 1975, Wicken and Knox proposed a new model for the cellular location of LTA (Wicken and Knox, 1975a). This followed the discovery, by Joseph and Shockman, (1975) that LTA was found outside the bacterial cell. This extracellular LTA is either actively secreted or lost naturally from the cell as a result of cell wall turnover during growth and division. Extracellular LTA may exist either in micellar form or in a deacylated monomeric form (Wicken and Knox, 1977). During the transportation of LTA to the external environment the polymer becomes detatched from the cytoplasmic membrane, and there is a transient stage at which the LTA molecule exists solely in the cell wall (Wicken and Knox, 1975a). The transient LTA either retains its fatty acid tail or is deacylated (see figure 4). Further to these observations Beachey et al, (1983) reported that during transit through the cell wall (of group A streptococci) some

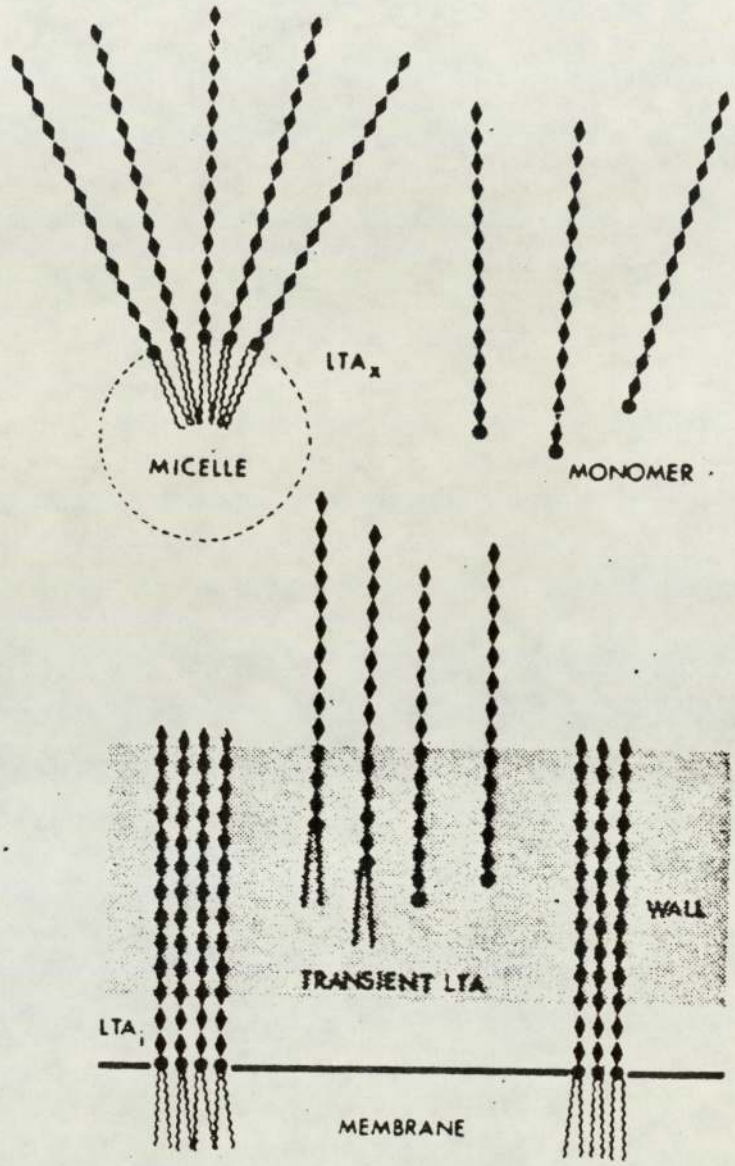


Figure 4 Representation of relationship of extracellular LTA and Intracellular LTA to the streptococcal cell wall and cytoplasmic membrane.
Key:
LTA_x extracellular LTA
LTA_i intracellular LTA

LTA_x is represented in an acylated micellar form as well as a deacylated monomeric form. Transient LTA may be acylated or deacylated, and is shown in the process of being excreted from the cell.
(from Wicken and Knox, 1977a).

molecules of LTA become reorientated to expose their lipid end at the surface of the bacterial cell (shown in figure 1a as iLTA - "inverted LTA"). The hydrophilic region of the LTA polymer interacts with charged areas of protein molecules in the bacterial cell wall, resulting in the formation of a stable complex, with reorientation of the LTA (Ofek et al, 1982; Beachey et al, 1983). The surface exposure of the lipid fraction of LTA in accordance with Ofek and Beachey's cell wall model would explain the findings of Tylewska et al (1979) who demonstrated that group A streptococci had very hydrophobic surfaces. The reorientation of LTA may possibly occur in other Gram-positive species as well as group A streptococci (Wicken et al, 1973). Group D streptococci may be the exception since their cell surface is hydrophilic (Simpson et al, 1980).

Although changes in growth conditions primarily affect surface polymers of bacteria rather than those covalently attached to the wall (Knox et al, 1979), production of LTA can be influenced in this way. Growth in the presence of sucrose or fructose; under nutrient limitations; or in a medium having a high pH increased the amount of cellular and extracellular LTA produced by bacteria (Jacques et al, 1979a; Jacques et al, 1979b; Rolla et al, 1980).

The main function of LTA and wall TA within the bacterial cell is related to their ability to bind bivalent cations required to maintain the correct physical functions and enzymatic activities of the cytoplasmic membrane (Toon et al, 1972). The amphipathic LTA and wall TA molecules interact to form a route from the external environment, across the cell wall, to the membrane, so facilitating passage of cations (especially Mg^{2+}) to the membrane (Lambert et al, 1977; Wicken and Knox, 1977).

The location of LTA on the surface of bacterial cells and excretion of LTA into the external environment suggests that LTA has the potential to interact with host cells. Several comprehensive reviews have been published

concerning the biological properties of TA/LTA, principally those by Wicken and Knox (Knox and Wicken, 1973; Wicken and Knox, 1975a; Knox and Wicken, 1977; Wicken and Knox, 1977; Beachey, 1980; Wicken and Knox, 1980). There are three major immunodominant areas on the LTA molecule:- the poly glycerol phosphate backbone; the carbohydrate and D-alanine substituents; and the glycolipid region (Wicken and Knox, 1975a; Knox and Wicken, 1977). The carbohydrate substituents povide the basis for the group specificity of those species possessing TA group antigens (Knox and Wicken 1977). These substituents are usually glucose and/or galactose in mono-, di- or tri-saccharide form (Wicken and Knox, 1975). For example, the immunodeterminant of the group D antigen in *S. faecalis* is a [α-D-glucopyranosyl-(1→2)-D-glucopyranosyl] residue (Wicken et al, 1963; Toon et al, 1972; Kessler et al, 1984). Antibodies to the polyglycerol phosphate backbone of LTA are found in normal human serum (Wicken and Knox, 1975a) and, in raised titres, in patients with infections due to Gram-positive bacteria (Crowder and White, 1972; Wergeland et al, 1984). Cross-reaction between different bacterial species may occur with anti-polyglycerol phosphate antibodies - if one particular organism is responsible for the primary stimulation of anti-"LTA backbone" antibodies, their production may subsequently be stimulated by LTA from other Gram-positive species (Wicken and Knox, 1975a). The D-alanyl substituents of LTA are relatively weakly antigenic, possibly due to susceptibility to biodegredation of the alanine-ester linkage (Knox and Wicken, 1973).

LTA has the property of sensitizing sheep or human red blood cells (Knox and Wicken, 1973). The glycolipid fraction of LTA is thought to play a key part in this activity, probably by forming a hydrophobic association with lipid in the erythrocyte membrane (Hewett et al, 1970; Knox and Wicken, 1973; Ofek et al, 1975). However. Chorpenning, Cooper and colleagues hold the opinion that deacylated LTA can also absorb to erythrocytes

(Cooper et al, 1978; Chorpenning et al, 1979). These workers argue that the procedures that other researchers have used to obtain lipid-free LTA could partially destroy the polyglycerol phosphate backbone of LTA, and this might account for the failure of deacylated LTA to interact with the red blood cells. Whilst acknowledging this argument, the main body of opinion lies with the lipid component of LTA being responsible for its membrane-binding properties. Indeed, most biological activities of LTA are lost if the glycolipid "membrane anchor" is removed. These activities include:- immunogenicity; production of a Schwartzman reaction; bilateral kidney necrosis; hypersensitivity; stimulation of bone resorption; mitogenic stimulation of T- and B-lymphocytes; and activation of complement by the classical and/or alternative pathway (Hewett et al, 1970; van Driel et al, 1973; Wicken and Knox, 1977; Beachey et al, 1980; Courtney et al, 1981; Hamada et al, 1985). All these actions are implicated in disease processes. Although the presence of the glycolipid fraction is a prerequisite to the biological properties of LTA, the length of the polyglycerol phosphate chain may also influence pathogenicity. Nealon and Mattingly (1985) observed that virulent strains of group B streptococci possessed a backbone of 30-35 glycerol phosphate units whereas non-pathogenic strains of the same species had an average LTA chain length of 10-12 glycerol phosphate units. Wall TA's from a wide range of Gram-positive species are also immunogenic (Crowder and White, 1972; Knox and Wicken, 1973); immunogenicity decreasing with decreasing molecular weight of TA.

The hydrophobic interaction between erythrocytes and the LTA lipid moiety stimulated interest in the potential of LTA to mediate bacterial adherence to other host tissues. Ofek et al (1982) showed that the LTA-M protein complex in group A streptococci remained stable at neutral pH and acylated LTA-M protein bound ten times more albumin than deacylated LTA-M protein. This confirmed the theory that a proportion of LTA has its lipid

end free to interact with other molecules (Beachey et al, 1983). LTA binds to human oral mucosal cells (Ofek et al, 1975; Beachey et al, 1983), platelets (Beachey et al, 1977), polymorphonuclear leukocytes (Courtney et al, 1981) and a variety of other mammalian cells (Stewart and Martin, 1962). Only fully acylated LTA is active is these respects (Beachey, 1980). Colonization of the buccal cavity by oral streptococci has been extensively studied. The antiphagocytic M protein of group A streptococci is involved in attachment to human epithelial cells (Ellen and Gibbons, 1972 and 1974; Gibbons and van Houte, 1975). Beachey and Ofek (1976) demonstrated that as well as M protein, LTA was a component of group A streptococcal fimbriae. These workers found that cells with intact fimbriae that had been selectively denuded of M protein still retained the ability to adhere to human buccal epithelial cells (Beachey and Ofek, 1976). This suggested that the LTA component of the fimbriae acted as an adhesin. Further studies in this vein by Beachey and colleagues have led to an accumulation of evidence that LTA does mediate adherence of bacteria to human tissues possessing LTA receptors (Beachey et al, 1980; Beachey, 1981; Beachey et al, 1983), possibly albumin-like proteins (Simpson et al, 1980). Also emerging from these studies was the fact that LTA may also be involved indirectly in adherence of bacteria to mammalian cells, through binding to fibronectin.

Fibronectin (FN) is a large glycoprotein (approximately 440K) found in a soluble form in many body fluids and in an insoluble form in basement membranes, the extracellular matrix of connective tissue and as a component of cell surfaces (Yamada and Olde, 1978; Pearlstein et al, 1980; Mosher, 1984). This large dimeric molecule possesses several discrete domains which have specific binding activity. These include binding sites for staphylococci and streptococci (Kuusela, 1978: Mosher, 1984; Kuusela et al, 1984; Hörmann, 1985; Yamada et al, 1985). FN has been shown to mediate the attachment of these bacteria to human buccal epithelial cells (Beachey and

Simpson, 1982; Abraham et al, 1983; Stanislawski et al, 1985); human umbilical vein endothelial cells (Vercellotti et al, 1984); fibrin thrombi (Toy et al, 1985) and non-bacterial thrombotic endocarditis (Scheld et al, 1985). In some cases LTA has been identified as the bacterial agent acting as a receptor for FN (Beachey et al, 1983; Courtney et al, 1983; Courtney et al, 1985; Nealon et al, 1986). However it should be noted that several other workers have demonstrated the presence of a protein receptor for FN on some bacterial species (Espersen and Clemmensen, 1982; Ryden et al, 1983; Vercellotti et al, 1984; Speziale et al, 1984; Ouaissi et al, 1986).

Whether or not FN is involved, there is a wealth of evidence that suggests LTA contributes to the pathogenic process of attachment of streptococci to host cells. There is an inherent dificutly in gaining conclusive proof that LTA acts as an independent bacterial adhesin, or that all the other biological activities attributed to LTA are indeed solely due to LTA. This stems from the difficutly of extracting pure LTA without contaminating proteins or polysaccharides (Beachey, 1980). Wicken et al (1973b) demonstrated that the immunogenicity of LTA directly correlated with the amount of protein associated with the LTA (even though the antibodies raised were specific for LTA and not for the protein contaminant).

Realistically it seems likely that the virtually irreversible binding of a bacterium to a host cell surface is a multifactorial process, several mechanisms acting independently or in conjunction to form numerous bonds between the bacterium and host cell (Gibbons, 1977; Bell, 1978; Beachey, 1981).

1.2.3 Polysaccharides

Polysaccharides (PSs) exist in the streptococcal cell wall either as group-specific antigens covalently attached to PG (Krause, 1963; Krause and McCarty, 1961 and 1962); as substituents on the hydroxyl groups of TA's (Baddiley, 1972; Toon et al, 1972); or they may be in the form of exopolysaccharides associated with the outer surface of the bacterial cell (Costerton et al, 1978; Gibbons and Banghart, 1967; Costerton et al, 1981).

Streptococcal groups A, B, C, E, F, G, H, K, L, O, P and U possess PG-bound group-specific PS antigens. Although the group-specific antigens of streptococcal groups D and N are TA's, the immunodeterminants of the antigens are PSs (Wicken and Knox, 1975a; Knox and Wicken, 1977). Much work has been undertaken in the characterization of the PS group-specific antigens of streptococci (Krause and McCarty, 1961 and 1962; Wicken et al, 1963; Michel and Willers, 1964; Heidelberger et al, 1967; Kane and Karakawa, 1977; Carey et al, 1980; Pritchard et al, 1984). Lancefield groups B, D, E, F, H and K can be subdivided serologically by virtue of type-specific PS antigens. Slight differences in the carbohydrate immunodeterminants enables distinction between serotypes (Elliott, 1960; Sharpe, 1964; Munoz et al, 1967; Chiongole and Hayashi, 1969; Hewett et al, 1970; Knox and Wicken, 1973; Wicken and Knox, 1975a). For a brief summary of the immunodeterminants of streptococcal group- and type-specific antigens see table 2.

The type-specific PS antigens of group B streptococci have been studied in detail (Lancefield, 1972). There are several serotypes, designated I, II, I/II, III and IV, distinguished according to specificity for typing sera raised against PS antigens (Ross, 1984). The type-specific antigens may be extracted from group B streptococci using a number of methods including:- sonication in cold trichloracetic acid (Wilkinson, 1975); stirring in EDTA

(Baker et al, 1976); boiling in neutral buffer (Kane and Karakawa, 1977); digestion with lytic enzymes (Tai et al, 1979); and concentration of culture supernatants (Anthony et al, 1982; Carey et al, 1980). These PS antigens are located on the surface of the cell wall (Wagner et al, 1980). Immunoelectron microscopy shows that the group specific PS antigen of these organisms may also be surface-associated (in addition to being wall-bound) but is often masked by the type-specific PS surface antigens (Wagner et al, 1980). The type-specific antigens of group B streptococci are accessible to binding with lectins. Lectins have proved very useful in the purification and characterization of bacterial PSs (Sharon, 1977). Wheatgerm agglutinin interacts with the terminal N-acetylneuraminic acid residue, a component which is thought to be immunologically important to group B streptococci (Gray et al, 1984). A lectin with specificity for rhamnose binds to the group-specific PS of group B streptococci (which is composed of rhamnose, glucitol and phosphate with a side chain of rhamnose (1→3), galactose (1→3)N-acetylglucosamine linked to the 4 position of rhamnose in the PS backbone; Pritchard et al, 1984) and also to group G streptococcal PS (composed of rhamnose and galactosamine). Both of these group-specific antigens contain rhamnose at the non-reducing terminal of side chains, and this possibly accounts for cross-reactions with certain grouping sera (Heidelberger et al, 1967).

The type-specific PS antigens of group D streptococci are associated with the cell wall (in contrast with the group-specific antigen which is cytoplasmic membrane-associated). Sharpe and Shattock (1952) identified 24 distinct types by precipitin tests. The group D type-specific PS may be extracted from cells by similar methods as those of group B streptococci - hot hydrochloric acid extraction (Lancefield, 1928a), or by enzymic extraction using *Streptomyces albus* enzyme or an autolytic enzyme (Bleiweis and Krause, 1965). Little is known about the type PSs of group D

streptococci except that the immunodeterminant of the serotype I antigen is composed of D-glucose and N-acetylglucosamine (Krause, 1972) borne on a rhamnose polymer backbone (Elliott et al, 1971).

The accessibility of group- and type-specific streptococcal antigens to antibodies in grouping/typing sera indicates that these PSs may also be accessible to host molecules. These cell-wall or cell-surface PSs may act as receptors and participate in pathogenic processes (Linzer et al, 1984). The group-specific antigens of groups A and B streptococci have been implicated in arthritis (Cromartie et al, 1977; Schwab, 1979). PG-PS complexes are the active agents of arthrogeneity. The PS moiety has a protective function, reducing the rate of PG biodegredation, so prolonging the presence of PG in the host and inducing a chronic inflammation (Schwab, 1979). The PS immunodeterminant of group D streptococci does not have this protective capacity since it is associated with LTA and not PG (Elliott, 1960; Wicken and Knox, 1975a; Stimpson et al, 1986). In *S. sanguis*, the serotype I PS antigen (composed of glucose, rhamnose and N-acetylglucosamine, with a terminal α-glucosidic linkage as the immunodeterminant) may participate in attachment of *S. sanguis* to tooth surfaces (Okahashi et al, 1983).

PSs associated with TAs of gram-positive bacteria vary widely between species (Baddiley, 1972; Beachey, 1980). This variation may arise from the type of PS substituent or from the degree of substitution of the hydroxyl groups on the TA polyglycerol phosphate chain. For example *S. lactis* TA is substituted with galactosyl residues; *Lactobacillus casei* TA is rarely glycosylated and *S. faecalis* TA is highly substituted with kojibiosyl residues (Wicken and Knox, 1975a). The degree of glycosylation of the LTA backbone affects the immunogenicity of LTA PSs (Wicken and Knox, 1975a). The PS substituents of TA may be responsible for some cases of cross-reaction between grouping sera (Knox and Wicken, 1973). Antibodies to wall ribitol TA of *L. planitarum* cross-react with LTA of other species because

of common α-D-glucosyl substituents (Knox and Wicken, 1972). PS substituents of LTA have not been directly linked to bacterial pathogenesis except for a possible contribution to the dermal toxicity produced by *Staphylococcus aureus*. Kowalski and Bergman (1971) reported that TA-PG fragments of *Staphylococcus aureus* evoked hypersensitivity reactions in guinea pigs sensitized with whole cells. PG alone did not have this effect. PG which was α-linked to PS through N-acetylglucosamine had a greater sensitizing capacity than did PG-PS with a β-linkage (Kowalski and Bergman, 1971).

Some species of bacteria possess the ability to synthesize high molecular weight exopolysaccharides ("glycocalyx") from simple sugars (Costerton et al, 1978; Cheng et al, 1981; Costerton et al, 1981a and 1981b). Oral viridans streptococci synthesize dextrans from sucrose (Gibbons and van Houte, 1975). The exopolysaccharide synthesized by *S. mutans* is made up of glucose units polymerised mainly through α-(1→6) linkages (Gibbons and Banghart, 1967). It is similar to soluble dextrans and to insoluble mutans containing more than 50% α-(1→3) linkages (Guggenheim, 1970). Most *S. mutans* strains also synthesize β-(2→6) linked fructans which is identical to levans (Gibbons and van Houte, 1975). The enzymes glucosyl- and fructosyltransferase assemble the polymers extracellularly (Robrish et al, 1972). Essentially the exopolysaccharide glycocalyx forms a highly hydrated anionic "ion exchange" matrix surrounding the bacteria which might aid entrapment and transport of nutrients (Mills et al, 1984; Gristina et al, 1985).

The high molecular weight exopolysaccharides synthesized by some Gram-positive bacteria have been linked to adherence to host surfaces in the process of colonization (Costerton et al, 1978). Dextrans, levans, glucans and fructans synthesized by oral streptococci mediate aggregation of these bacteria (Gibbons and Fitzgerald, 1969; Gibbons and van Houte, 1975), and

aid their colonization of the tooth surface, possibly via lectin-PS interactions (Gibbons, 1977; Gibbons and van Houte, 1975). Bacteria which exist in glycocalyces in their natural or pathogenic environment are protected from the action of phagocytes (Caputy and Costerton, 1982; Zimmerli et al, 1982), opsonins (Wilkinson et al, 1979) and antibiotics (Govan and Fyfe, 1978; Costerton and Marrie, 1983; Pulliam et al, 1985). The production of exopolysaccharides by certain strains of bacteria in infective endocarditis has been demonstrated *in vitro* and *in vivo* (Scheld et al, 1978; Ramirez-Ronda, 1978 and 1980; Mills et al, 1984). The exopolysaccharide surrounding bacteria in cardiac vegetations is different to the dextrans produced by bacteria in the oral cavity since sucrose is not normally available in the bloodstream (Mills et al, 1984). Dextran-producing bacteria form larger cardiac vegetations than do nondextran-forming bacteria (Mills et al, 1984), either because of an increase in the number of adherent organisms or because of increased stimulation of fibrin and platelet deposition (Hook and Sande, 1974; Sullam et al, 1985a). The dextran-positive organisms in these larger vegetations are more resistant to antibiotic treatment than are dextran-negative strains in smaller vegetations (Yersin et al, 1982; Pulliam et al, 1985). This is due to the relative metabolic inactivity of these organisms rather than failure of the antibiotics to penetrate the vegetation (McColm and Ryan, 1985). Only those bacterial cells growing at or near the surface of vegetations are metabolically active (Durack and Beeson, 1972b), so therefore large vegetations contain a greater proportion of metabolically inactive organisms which remain relatively unaffected by antibiotics. Scheld et al (1978) demonstrated that the adherence of *S. sanguis* to artificial fibrin-platelet vegetations was increased when the organisms were grown in the presence of sucrose and that there was a direct correlation between the amount of dextran produced and the ability of a bacterial strain to adhere

to fibrin or a fibrin-platelet matrix. This indicates that production of dextran is important in the adherence of oral streptococci to nonbacterial thrombotic endocarditis and that this exopolysaccharide may be a contributory factor in the pathogenesis of infective endocarditis. There is no evidence that dextran production promotes streptococcal adherence to undamaged endocardium, however (Sullam et al, 1985). The molecular weight of the exopolysaccharide influences the adherence capacity. High molecular weight, water-insoluble dextrans promote adherence to surfaces (Mukasa and Slade, 1973; Ramirez-Ronda, 1980) whilst dextrans with a molecular weight less than 70K may interfere with the adherence mechanisms mediated by high molecular weight dextrans (Ramirez-Ronda, 1980).

1.2.4 Cell wall proteins

Various proteins are found in association with the streptococcal cell wall: the type-specific M, T, and R proteins of group A, C and G streptococci are the best documented. M protein was first noted in group A streptococci (Lancefield, 1928a and 1943; Swanson et al, 1969). The M protein of group A streptococci is a fibrous, coiled-coil dimer protruding from the surface of the cell (Phillips et al, 1981) which forms fine "hair-like" fimbriae together with LTA (Beachey and Ofek, 1976). Over 60 different M protein types of *S. pyogenes* have been recorded (Fox, 1974). This protein or an M-like protein is also found on streptococci of groups B (Maxted, 1948a), C (Maxted, 1948b; Woolcock, 1974), E (Daynes and Armstrong, 1973) and G (Maxted, 1948b; Maxted and Potter, 1967). The M protein of group G streptococci has recently been shown to be closely related to that of group A streptococci (Bisno et al, 1987; Jones and Fischetti, 1987). The relationship of group A M protein to the M-like proteins of other streptococcal groups has yet to be established. M protein may be extracted from group A streptococcal whole cells by numerous means including the use of:- hot acid (Lancefield, 1928a); sonic oscillation (Ofek et al, 1969); alkali (Fox and Wittner, 1969); pepsin (Cunningham and Beachey, 1974); guanidine hydrochloride (Russell and Facklam, 1975); other enzymes (Schmidt, 1965) and extraction with nonionic detergent (Fischetti et al, 1976). The extracted antigens may then be typed using typing sera (as in the precipitin grouping technique). Certain strains of *S. pyogenes* are untypeable in this way because of a lack of M protein and/or slight alterations in serological specificity and/or emergence of noncross-reacting serotypes due to mutation of typeable strains (Fox, 1974).

M protein has a direct role in streptococcal virulence (Davis et al, 1980). The protrusion of this protein from the surface of the

bacterial cell makes it easily accessible to antibodies. Anti-M protein antibodies act as opsonins and are protective (Fox, 1974; Jones and Fischetti, 1987). Evidence has arisen that shows antibodies to some M protein epitopes cross react with sarcolemma and subsarcolemma of mammalian cardiac myofibres (Kaplan, 1965; Dale and Beachey, 1985; Poirier et al, 1985). This presents problems in developing a streptococcal vaccine. M protein protects group A streptococci from phagocytosis (Lancefield, 1962; Fox, 1974). The type-specific determinant of the M protein is uninvolved in this antiphagocytic action (Fischetti et al, 1976). M protein has a further virulence-related activity in mediating attachment of streptococci to host tissues (Ellen and Gibbons, 1972 and 1974; Gibbons and van Houte, 1975). Wadström et al (1984), suggested that M protein contributed to the hydrophobicity of group A streptococcal cell surfaces (Tylewska et al, 1979), thus enhancing aggregation of streptococci and promoting hydrophobic interactions between streptococcal and host cells. M protein also increases cell-surface hydrophobicity/mediates hydrophobic interactions in an indirect way by complexing with LTA and causing reorientation of this polymer to expose its glycolipid end at the surface of the bacterial cell (Ofek et al, 1982; Beachey et al, 1983).

The T protein antigens are also concerned in the type-specificity of group A streptococci and are found in streptococci of groups C, G and L (Parker, 1984). T antigens may give rise to cross-reactions when using M proteins to type streptococci. These antigens are not associated with virulence and non-soluble in alcohol, in contrast to M proteins (Lancefield and Dole, 1946; Lancefield, 1962). The amounts of M and T proteins produced by streptococci depends upon the growth temperature (Parker, 1984). Generally the distribution of M and T antigens vary between streptococcal types but occasionally may be matched (Davis, 1980).

The R protein antigens are found in cell walls of groups A, B, C, G and L streptococci (Parker, 1984). R antigens are not generally exploited in typing systems and are not associated with virulence.

Many other proteins are located in the cell wall or are associated with the surface of streptococcal cells, which until recently, have remained largely unstudied. Some may act as receptors involved in bacteria-bacteria or bacteria-host interactions and some function as enzymes. Groups A, B, C and G streptococci possess surface proteins similar to the protein A of *Staphylococcus aureus* (Forsgren and Sjönquist, 1966), which act as receptors for the Fc portion of immunoglobulins (Kronvall, 1973; Chhatwal and Blobel, 1987). Streptococcal Fc receptors can bind all four human immunoglobulin G subclasses and group A Fc receptors possess the capacity to bind to IgA (Myhre and Kronvall, 1977; Björck and Kronvall, 1984). Such Fc receptors are often lost upon repeated subculturing of organisms in the laboratory (Christensen and Oxelius, 1974). Burova et al (1982) suggested that possibly IgG Fc receptors inhibit phagocytosis of streptococci by interfering with the classical pathway of complement activation. However, it has not yet been determined conclusively whether the Fc receptor proteins contribute to streptococcal pathogenicity.

The most extensively studied group of streptococcal cell-wall/surface proteins are those implicated in the adhesion of oral streptococci to salivary components, buccal epithelial tissue or to tooth surfaces. Proteins tightly associated to the cell wall of *S. mutans* may act as attachment factors to salivary components and host epithelial cells (Russell, 1979; Nesbitt et al, 1980; Ogier et al, 1984). Ogier et al (1984) showed that four surface proteins of *S. mutans* contained carbohydrate fractions indicating that the saliva-interacting agents were likely to be glycoproteins. Furthermore, Russell (1979) identified a cell-surface, M-like protein in *S. mutans* that was antigenically related to human heart tissue. *S. mutans*

has now been reclassified, and subdivided into several species according to serotypes:- *S. mutans* (serotypes c, e, and f); *S. sorbrinus* (serotypes d and g); *S. rattus* (serotype b); and *S. cricetus* (serotype a) (Hardy et al, 1986). The cell-wall protein profiles of these organisms have been used to distinguish between serotypes (Coykendall, 1971). Four major *S. mutans* proteins have been recognised and characterized - I, II, I/II, and III (Russell and Lehner, 1978; Zanders and Lehner, 1981). Antigen I/II has been shown by ^{125}I-lactoperoxidase labelling to have a surface location in the *S. mutans* cell (Zanders and Lehner, 1981) and is highly immunogenic. Expression of proteins by *S. mutans* is influenced by the growth medium (Hardy et al, 1986). Some *S. mutans* surface-bound proteins have been recognised as glucosyltransferases. These act as receptors for exopolysaccharides, in addition to other non-enzymic dextran receptors (Kuramitsu and Ingersoll, 1978; Robrish et al, 1972; Gibbons and van Houte, 1975; Ramirez-Ronda, 1980). Similar receptors are also present in *S. bovis* but are absent from dextran-producing strains of *S. sanguis* (Spinell and Gibbons, 1974). Those streptococci possessing cell-bound glucosyltransferase dextran receptors show an increased tendency to aggregate, thus these receptors may participate in dental plaque formation and tooth colonization (Spinell and Gibbons, 1974)

A link has emerged between the ability of oral streptococci to adhere to saliva-coated surfaces and the surface hydrophobicity of these organisms (Nesbitt et al, 1982; Olsson and Westergren, 1982). LTA and M protein have both been held responsible for bacterial surface hydrophobicity (Tylewska et al, 1979; Ofek et al, 1983). However, Oakley et al (1985) demonstrated that other cell-wall proteins also contibute to the hydrophobic properties of *S. sanguis* cell surfaces. Several other workers have shown that cell-surface proteins are involved in the adherence of *S. sanguis* to saliva-coated hydroxyapatite (Liljemark and Bloomquist, 1981; McBride et al, 1984).

Applebaum and Rosan (1984) used SDS-PAGE to identify a number of surface proteins of *S. sanguis* and suggested that this method was a suitable tool for taxonomic studies of oral streptococci.

The surface-associated proteins of streptococci may be carried on hair-like fibrillar structures in a similar manner to M protein in *S. pyogenes* (Weerkamp et al, 1976; Handley et al, 1985; Morris et al, 1985; Weerkamp et al, 1987). Weerkamp et al (1986) reports that antigen C, a glycoprotein involved in the attachment of *S. salivarius* to host surfaces (Weerkamp and Jacobs, 1982) is mainly located in a fibrillar layer outside the bacterial cell wall. In contrast, antigen B, a protein responsible for bacterial aggregation (Weerkamp and McBride, 1981), is located within the cell wall. LTA is not specifically associated with the fibrillar antigens of *S. salivarius*, makes no contribution to cell hydrophobicity and is in no way involved in adherence mechanisms associated with the cell wall protein antigens of *S. salivarius* (Weerkamp et al, 1987).

Wyatt et al (1987) have shown that there is a lack of correlation between fibrils, hydrophobicity and adhesion to saliva-coated hydroxyapatite for strains of *S. sanguis*. These workers found that non-fibrillar strains adhered better than did strains bearing peritrichous fibrils. Unlike Westegren and Olsson (1982), Wyatt et al found that mutants of a *S. sanguis* strain that were low in adhesion had a high surface hydrophobicity. This might indicate that the role of surface proteins in bacterial adherence in certain instance, may be chiefly as receptors for host tissue components with less emphasis on involvement in hydrophobic interaction.

In addition to enzymes involved in exopolysaccharide production, other enzymes are associated with the streptococcal cell wall. Several species of streptococci possess autolytic enzymes which hydrolyze PG linkages during cell growth and normal turnover of the cell wall (Daneo-Moore and Shockman, 1977). In *S. faecalis* this enzyme is a β-1,4,N-acetylmuramide

glycanhydrolase (Cornett et al, 1979; Joseph and Shockman, 1974) which is bound tightly to the cell wall. This enzyme is located in discrete areas of the cell wall, at the point of initiation of cell division and new wall elongation (Shockman et al, 1967; Higgins et al, 1970). Control over the activity of autolysins is exerted in part by interactions of TA and/or teichuronic acids and LTA. These polymers are thought to bind to the enzyme (Hammond et al, 1984). Autolytic enzymes are not thought to be involved in streptococcal pathogenicity - other than providing means of bacterial cell proliferation!

There is very little information about non-enzymic cell wall proteins of group D streptococci. Perhaps the best documented enterococcal proteins are the pheromone-induced surface antigens of *S. faecalis*. The multiple antibiotic resistances of *S. faecalis* are plasmid-mediated (Clewell et al, 1974; van Embden et al, 1977; Dunny et al, 1978). There is evidence to suggest that bacterial conjugation (and subsequent plasmid transfer) is mediated by production of a peptide sex pheromone (also known as clumping-inducing-agent, or CIA) by recipient cells of *S. faecalis*, in response to the plasmid(s) carried by donor cells (Dunny et al, 1978; Franke and Clewell, 1981; Kessler and Yagi, 1983; Tortorello and Dunny, 1985). In response to the CIAs, changes occur on the surface of donor cells which mediate bacterial aggregation (Dunny et al, 1978), thus faciliting plasmid transfer. These changes involve expression of multiple protein antigens on the surface of *S. faecalis* donor cells which act as adhesins to bind recipient cells (Kessler and Yagi, 1983; Tortorello and Dunny, 1985; Tortorello et al, 1986). Each plasmid type contained in donor cells induces (through stimulation by CIAs) expression of particular surface proteins. For example resistance plasmid pCF10 caused CIA-induction of two proteinaceous antigens 73 and 130K, on the surface of *S. faecalis* donor cells (Tortorello and Dunny, 1985; Tortorello et al, 1986), whereas the plasmid pAD1, via CIAs,

causes expression of *S. faecalis* surface protein antigens of 157, 153, 130 and 74K (Ehrenfeld et al, 1986). These surface proteins produced in response to the plasmid-induced CIAs have their principal pathogenic activity in the spread of multiple antibiotic resistances among strains of *S. faecalis* (Franke and Clewell, 1981).

1.3 Group D enterococcus: *Streptococcus faecalis*

In the streptococcal classification scheme of Andrewes and Horder (1906), the organism predominantly isolated from human faeces was termed *Streptococcus faecalis*. In 1919 Orla-Jensen divided heat-resistant faecal streptococci into two distinct groups:- (i) *S. faecium* and (ii) *S. glycerinaceus* and *S. liquefaciens* (the latter two differing only in the ability of *S. liquefaciens* to liquefy gelatin). *S. equinus*, isolated from horse faeces and *S. bovis*, isolated from cow faeces were given separate status since they required higher growth temperatures and had distinct physiological characteristics. *S. faecalis* was distinguished from *S. faecium* by its ability to ferment melezitose, sorbitol, glycerol (anaerobically), citrate and gluconate; lack of ability to ferment arabinose and melibiose; strong reducing capacity; growth on tellurite-supplemented agar; absence of folic acid requirement and lack of α-haemolysis on blood agar (Shattock, 1955; Deibel et al, 1963). The species *S. zymogenes* and *S. liquefaciens* differed from *S. faecalis* only in proteolytic capacity and thus were awarded status as varieties of *S. faecalis* (Sherman, 1938). Similarly, *S. durans* was designated a variety of *S. faecium* since the only means of distinguishing the two species was the ability of *S. durans* to ferment arabinose and mannitol and inability to ferment sucrose (Deibel, 1963).

S. faecalis, *S. faecalis* var. *zymogenes*, *S. faecalis* var *liquefaciens* and *S. durans* were included in the enterococcus division of Sherman (1937). Sherman equated Andrewes and Horders' *S. faecalis* with S. glycerinaceus (Smith and Sherman, 1938). The term "enterococcus" had first been used by Thiercelin in 1899 to describe a potentially pathogenic *Streptococcus* isolated from faeces. It is now accepted that this term is only applied to *S. faecalis* and *S. faecium* and their varieties (Deibel, 1963). Although *S. durans* was reluctantly classed as an enterococcus by Sherman (1938),

S. faecium was not recognised as such (Smith and Sherman, 1938). This species was included in this division by subsequent workers (Deibel, 1964) because of characteristic nutritional patterns, metabolic activities and cell structure. The enterococci (*S. faecalis*, *S. faecalis* var. *zymogenes*, *S. faecalis* var. *liquefaciens*, *S. faecium* and *S. faecium* var. *durans*) and the non-enterococcal faecal streptococci (*S. equinus* and *S. bovis*) possess the Lancefield group D antigen (Lancefield, 1933). Atypical organisms or variants are assigned to the species or variety with the most closely matching physiological and metabolic properties (Deibel, 1964; Facklam and Wilkinson, 1981).

A number of classification systems have been used for taxonomic purposes or for identification of the enterococci. Raj and Colewell (1966) developed a numerical taxonomy scheme involving computer analysis of a variety of biochemical tests. This method showed good correlation with previous schemes in which *S. bovis* was clearly distinguishable from the enterococci (Raj and Colewell, 1966). Other classification schemes have used the structure of cell wall PG to successfully speciate the enterococci (Schleifer and Kandler, 1967; Ghuysen, 1968; Kandler et al, 1968; Schleifer and Kandler, 1972; Slade and Slamp, 1972). Genetic properties and plasmid-transfer characteristics have also been used as a means of subdividing *S. faecalis* species (Clewell and Franke, 1974; Dunny and Clewell, 1975).

The natural habitat of *S. faecalis* is the intestinal tract of healthy humans and animals. Diet, geographical location, age and species of host may account for variation in faecal streptococci (Deibel, 1964; Mead, 1978). In Great Britain, *S. faecalis* is the predominating enterococcus in human faeces, but in other European countries and the USA *S. faecium* has been found to preside over *S. faecalis* (Deibel, 1964).

Once outside the gut, *S. faecalis* may act as a pathogen. This was observed as early as 1899 when MacCallum and Hastings isolated an organism

which they named "*Micrococcus zymogenes*" (equivalent to *S. faecalis* var. *zymogenes*) as the causative agent from a case of infective endocarditis. Since then *S. faecalis* has been linked to cases of infective endocarditis, urinary tract infection, bacteraemia, septicaemia and, occasionally, has been reported to cause suppurative conditions including peritonitis and meningitis (Deibel, 1964; Krause, 1972; Parker, 1978; Kaye, 1982; Malone et al, 1986). In virtually all cases infection with *S. faecalis* is self-originating and arises from trauma to the gastro-intestinal tract, resulting in "escape" of the faecal flora to other body sites (Mandell et al, 1970; Krause, 1972; Le Frock et al, 1973; Kaye, 1982).

Most streptococci are susceptible to antibiotic treatment (Parker, 1978). But *S. faecalis* is resistant to a wide range of antibiotics (Moellering Jr., 1975; Parker, 1975 and 1978) including tetracycline, benzylpenicillin, aminoglycosides, methicillin, sulphonamides, trimethoprim and the newer cephalosporins (Basker et al, 1977; Krogstad and Parquette, 1980; Cherubin et al, 1981; Mederski-Samoraj and Murray, 1983; Goodhart, 1984; Fernandez-Guerrero et al, 1987). Indeed, the increased use of cephalosporins has been implicated in enterococcal bacteraemia (Yu, 1981; Moellering Jr., 1982; Dougherty et al, 1983). The multiple antibiotic resistances of *S. faecalis* are plasmid-borne and are transferred by conjugation between *S. faecalis* cells (Dunny and Clewell, 1975; Dunny et al, 1981; Mederski-Samoraj and Murray, 1983; Murray et al, 1986). Such transfer can take place at high frequencies (Franke et al, 1978). The lack of sensitivity of *S. faecalis* to penicillins is largely overcome by using a synergistic combination of antibiotics. This usually involves treatment with penicillin plus an aminoglycoside antibiotic (Watanakunakorn, 1971; Calderwood et al, 1977; Watt, 1978; Indrelie et al, 1984).

1.4 Infective endocarditis

1.4.1 Natural history

Infective endocarditis (IE) is an inflammation of the endocardium as a result of infection. The disease may involve the heart valves and/or the wall of the chambers of the heart. The condition has in the past been inaccurately termed "bacterial endocarditis". "Infective" is a more precise description of the endocarditis since microorganisms other than bacteria may cause the disease (Scheld and Sande, 1985). Thayer (1931) and Lerner and Weinstein (1966) were chiefly involved in adoption of the more correct terminology. Traditionally IE has been divided into an acute form and a subacute form. Acute IE affects previously undamaged heart valves and is usually a secondary manifestation of an infection sited elsewhere in the body, and tends to be caused by a wide range of virulent microorganisms (Netter, 1978). Typical symptoms of acute IE are high fever, systemic toxicity, leukocytosis and if untreated, the patient dies in less than 6 weeeks - possibly even within several days (Scheld and Sande, 1985). In subacute IE the primary infection site is the endocardium and tends to involve organisms originating from the natural flora of the body (Parker, 1984b). A patient with subacute IE may present with low grade fever, night sweats, weight loss and various vague systemic complaints. If untreated, death may occur within 6 months. Patients vary in their symptoms of IE and this lack of definitive clinical presentation often leads to delay in diagnosis (von Reyn et al, 1981; King and Harkness, 1986a). The division of endocarditis into acute and subacute forms is somewhat outdated since it is based upon the course followed by the untreated disease. Now IE is classified according to the aetiological agent causing the disease (King and Harkness, 1986a).

The mortality rate for IE dropped from virtually 100% to ≈ 30% with the introduction of antibiotic treatment and has remained more or less at that level ever since (Dormer, 1958; Hayward, 1973a; Wilson and Geraci, 1983; Newsom, 1984). The incidence of IE has changed little since the pre-antibiotic era, affecting approximately 1 in 40,000 people in the UK and 1 in 1,000,000 worldwide (Oakley, 1980; Freedman, 1982). Other changes in the characteristics of IE have become evident over these years. Since the 1940s there has been a gradual increase in the mean age of patients with IE from under 30 years to over 50 years (Kaye et al, 1961; Garvey and Neu, 1978), with an accompanying shift in emphasis from adolescent females to elderly males (Hayward, 1973a; Parker and Ball, 1976; Welsby, 1977; Whitby and Fenech, 1985). Patients with IE caused by group D enterococci have an even higher mean age of over 60 years (Welsby, 1978; Come, 1982). The reduction in the number of adolescents with IE has been due in part to:-

(i) the reduction in the number of young patients with rheumatic heart disease and rheumatic fever,

(ii) the more widespread use of antibiotics to control streptococcal throat infections and

(iii) the earlier surgical correction of congenital heart defects (Hayward, 1973a; Parker, 1984b).

The increase in mean age of IE patients may be attributed to the increased longevity of the general population that has accompanied progress in medical techniques (Scheld and Sande, 1985).

Another noticeable change in the pattern of IE is in the type of organism responsible for the disease. In the years before antibiotics, viridans streptococci accounted for the vast majority of IE cases, and although today this group of organisms are still the most common cause of the disease, the viridans streptococci cause less than 40% of IE - with a rise in the frequency with which non-haemolytic streptocci, enterococci

and staphylococci are isolated as the causative agents (Geraci and Martin, 1954; Hayward, 1973a; Welsby, 1977 and 1978; Whitby and Fenech, 1985; King and Harkness, 1986a). Currently the most common aetiological agents of IE are still the oral viridans streptococci which account for approximately 30-40% of cases, followed in frequency by *Staphylococcus aureus* (20-30%), *S. bovis* and *S. faecalis* (10-15% each) (Kaye et al, 1961; Parker and Ball, 1976; von Reyn et al, 1981; Wilson and Geraci, 1983 and 1985; Mandell, 1984; Sheld and Sande, 1985 King and Harkness, 1986a). An increase in use of invasive medical procedures has dramatically widened the range of organism causing IE (Scheld and Sande, 1984). Nosocomial IE may be acquired during, for example, cardiac surgery, catheterization or haemodialysis, microorganisms gaining access to the heart either directly, or via the bloodstream from a local infection. Intravenous drug abuse has also contributed to an increase in the number of cases of IE involving organisms not originating from the patients own flora (Karchmer and Schwarz, 1977; Parker, 1984b; Arbulu and Asfaw, 1987). The range of causal agents in IE has now expanded to include amongst others - chlamydiae (Ward and Ward, 1974), rickettsiae (Palmer and Young, 1982), *Candida* species, *Aspergillus* species (Lerner and Weinstein, 1966), brucellae, clostridia (Parker, 1984), actinomycetes, actinobacilli, *Haemophilus* species (Page and King, 1966), basidiomycetes (Speller and M^{c}Iver, 1971) and possibly even viruses (Scheld and Sande, 1985).

It has become apparent that certain factors predispose a patient to IE. These include congenital heart disease, rheumatic heart disease and rheumatic fever which all may result in lesions on heart valves/septa; replacement of valves with prosthetic devices; insertion of intravenous or intraarterial cannulae or a pacemaker; intravenous drug abuse; long-term haemodialysis; and immunosuppression (Hayward, 1973a; Kaye, 1976; Bayliss et al, 1983; Scheld and Sande, 1985; Baddour and Bisno, 1986; King and

Harkness, 1986a). In some cases the valvular lesion is due to regurgitant or jet-stream flow of blood through the heart valve or narrowed blood vessel, such as in idiopathic hypertrophic subaortic stenosis (Hayward, 1973a; Chagnac et al, 1982). Certain circumstances favour colonization of the heart by a particular species of microorganism. For example, dental procedures resulting in transient bacteraemia may lead to colonization of the endocardium by oral streptococci; whereas operative/manipulative procedures carried out on the gastrointestinal or genitourinary tracts may predispose to enterococcal, or less commonly, enterobacteriaceal IE (Durack et al, 1977; Sipes et al, 1977; Kaye, 1982: Simmons et al, 1982; Shulman et al, 1984). The question still remains as to why microorganisms comprising the normal commensal flora of the body should, upon entry into the circulation, lodge primarily on the endocardium and establish a potentially fatal infection.

1.4.2 Colonization of the heart valve

Bacteria causing IE usually originate from the body's own commensal flora and reach the endocardium via the bloodstream (Sipes et al, 1977; Kaye, 1982; King and Harkness, 1986a). These organisms, especially Gram-positive cocci, show a marked tissue tropism for the endocardium (Gould et al, 1974; Scheld et al, 1978; Vercellotti et al, 1984). The endothelial surface of the heart valves presents a surface to which these bacteria readily adhere with ensuing colonization of the valve (Durack and Beeson, 1972a). Gould et al (1974) demonstrated that the probability of bacteria successfully colonizing a heart valve was related to the duration and magnitude of bacteraemia and to the ability of the bacteria to adhere to the surface of the heart valve. Bacteraemia does not automatically result in IE, however. Gram-positive bacteraemia is more likely to culminate in IE than bacteraemia caused by Gram-negative organisms (Gould et al, 1974). The surface characteristics of both the circulating organism and the cardiac

endothelium may give a clue as to why a relatively small proportion of microbial species, normally of low virulence, should show a predilection for colonizing heart valves with potentially disasterous effect. The surface characteristics of bacteria in relation to adherence have been discussed elsewhere (see section 1.2.2, 1.2.3 and 1.2.4); therefore this section will concern those aspects of the surface of the heart valve which render it a choice location for microbial colonization.

It was noted as early as 1928, that modification of the heart valves resulted in fibrin-platelet deposition and infection (Grant, 1928). Experiments using animal models of endocarditis have since demonstrated that initial adherence of bacteria indeed occurs on sterile vegetations on heart valves, formed from fibrin and platelets (Durack and Beeson, 1972a; Durack et al, 1973; Durack, 1975a). These avascular vegetations (known as non-bacterial thrombotic endocarditis (NBTE)) are thought to form on an area of endocardium damaged through stress, congenital defect or heart disease (Angrist et al, 1960; Angrist and Oka, 1963). The model proposed by Angrist and Oka (1963) for formation of NBTE and subsequent bacterial colonization is shown in figure 5. Durack and Beeson (1972a) showed that NBTE may be converted to IE since the vegetation offers a surface to which bacteria readily adhere.

Various proposals have been put forward as to the mechanism of bacterial adherence to the fibrin-platelet matrix of the heart-valve vegetation. These suggestions include:-

(i) mediation of adherence of oral viridans streptococci and *S. bovis* biotype II via dextrans - an adherent exopolysaccharide synthesised by these species (Durack and Beeson, 1972a and 1972b; Ramirez-Ronda, 1978 and 1980; Scheld et al, 1978; Mills et al, 1984)

(ii) mediation of adherence of *Staphylococcus aureus* and streptococci of groups A, C, D and G via binding to fibronectin - shown by

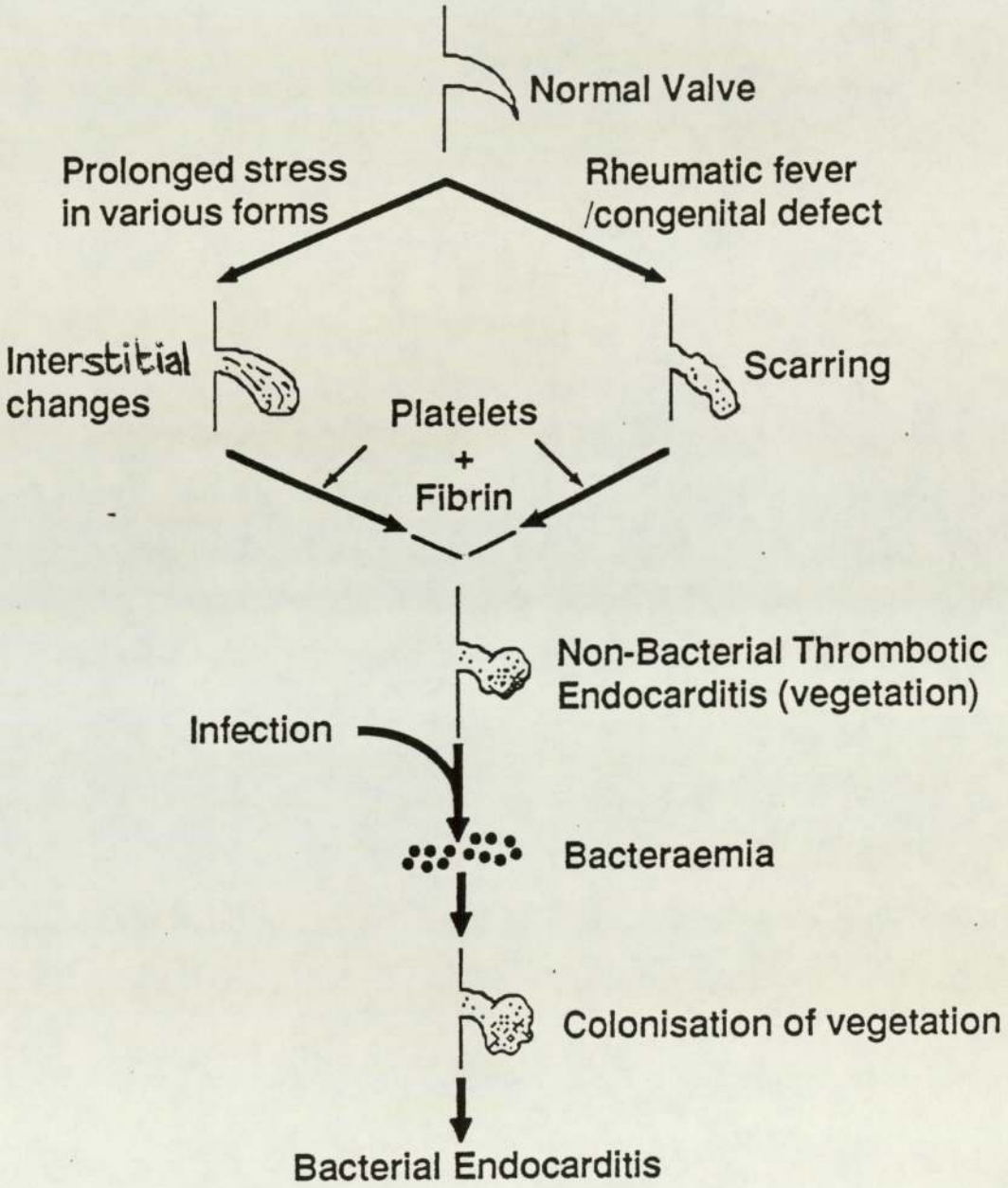


Figure 5 Proposed scheme for pathogenesis of infective endocarditis. (Adapted from Angrist and Oka, 1963)

immunofluorescence to be present on traumatised heart valves (Scheld et al, 1983; Vercellotti et al, 1984; Scheld et al, 1985; Toy et al, 1985) or

(iii) adherence of *S. mitis*, oral viridans streptococci and *Staphylococcus aureus* by binding to laminin - a consituent of the basement membrane underlying the epithelium which may become exposed in endocardial lesions (Switalski et al, 1984 and 1987). Switalski et al (1987) demonstrated that some strains of oral viridans streptococci possess high-affinity proteinaceous laminin receptors.

The degree of avidity with which bacteria adhere to the fibrin-platelet matrix of NBTE influences the likelihood of that organism to cause IE. Crawford and Russell (1986) have shown that *S. faecalis* adhered even more avidly to fibrin-platelet clots *in vitro* than viridans streptococci. Gould et al (1975) found this organism also adhered to undamaged heart valves *in vitro* better than other species. However, the degree of adhesion *in vitro* did not correlate with the frequency with which these organism caused IE (Crawford and Russell, 1986). This may indicate that the major governing factor determining the likelihood of an organism to cause IE is the frequency with which that particular species gains entry into the bloodstream. *S. faecalis* IE seems particularly prevalent in elderly males (high-risk group for genitourinary operations, especially involving the prostate gland), young women (high-risk group for gynaecological procedures) and intravenous drug abusers (origin of infecting *S. faecalis* unknown - possible lack of hygiene involved?) (Lerner and Weinstein, 1966; Mandell et al, 1970; Kaye, 1982; Parker, 1984b; Arbulu and Asfaw, 1987). It should be noted that enterococci are unusual in that they are able to adhere to heart valves showing no evidence of damage or modification (Geraci and Martin, 1954; Toh and Ball, 1960; Mandell et al, 1970; Welsby, 1978).

1.4.3 Survival of bacteria within the vegetation

A general principle of infectious disease is that after initial colonization of tissue the bacteria must survive and proliferate in order to establish an infection (Smith, 1984), and this holds true in IE. The colonizing organisms stimulate further deposition of platelets and fibrin to form a protective layer surrounding the bacteria on the heart valve (Sullam et al, 1985a). This layer also serves to provide a new surface to which circulating bacteria may adhere (Durack, 1975a), leading to enlargement of the vegetation. The deposition of fibrin onto valve-borne bacteria is thought to be activated by stimulation of the clotting cascade. *Staphylococcus aureus* can directly induce thrombin activity through synthesis of staphylococcal coagulase which activates prothrombin (Hendrix et al, 1983). Other adherent bacterial species stimulate the underlying valvular tissue to produce tissue thromboplastin (Drake et al, 1984). This results in fibrin deposition via the extrinsic clotting cascade. *S. faecalis* appears to be especially proficient at this activity (Drake et al, 1984). The exact mechanism by which the adherent bacteria stimulate expression of the tissue factor is not fully understood (Sullam et al, 1985a), but complement (C3b), antibodies (IgG) and monocytes may involved in a minor capacity (Rothberger et al, 1977; van Ginkel et al, 1979).

The coagulation pathway reactions may be enhanced if they occur on the membrane surface of platelets (Sullam et al, 1985a), this may be a secondary function of platelets within the vegetation (in addition to protection of bacteria within the vegetation and promoting further adherence of bacteria). Platelets may be bound directly by bacteria adhering to heart valves (Scheld et al, 1978; Herzberg et al, 1983). A platelet receptor has been suggested as being present on some strains of *S. sanguis*

(Herzberg et al, 1983). The vegetation is built up in this way, with layers of bacteria and fibrin-platelet matrix being deposited alternately.

Within the avascular vegetation on the heart valve, the bacteria are protected from the actions of phagocytes and antibiotics (Durack and Beeson, 1972a; Scheld and Sande, 1985) - although polymorphonuclear leukocytes may act locally on bacteria in the vegetation to inhibit bacterial growth (Sullam et al, 1985a). Durack and Beeson (1972a) liken the vegetational environment, within which the bacteria exist in IE, to a "privileged sanctuary". Bacterial proliferation thus ensues unimpeded by host defences, resulting in a large number of bacteria within the vegetation (10^{9-10} bacteria per gramme of tissue; Scheld and Sande, 1985). Durack and Beeson (1972b) demonstrated that bacteria located in the inner vegetation are metabolically inactive and though antibiotics are able to penetrate the fibrous vegetation, they are relatively ineffective against the "dormant" deep-seated bacteria (Durack and Beeson, 1978; Yersin et al, 1982; McColm and Ryan, 1985; Sullam et al, 1985b). Dextran-producing strains of bacteria tend to form larger cardiac vegetations than non-dextran-producers, thus increasing the proportion of inactive, antibiotic-insensitive bacteria within the vegetation (Mills et al, 1984; Pulliam et al, 1985). In order to eradicate these deep-lying colonies antibiotic therapy for IE must be carried out using an extended regimen of bactericidal antibiotics (King and Harkness, 1986a).

Bacteria may become dislodged from the vegetation, leading to continuous bacteraemia. The prognostic implication of vegetations on heart valves is controversial. Patients in whom cardiac vegetations are large enough to be visualized by echocardiography (>2mm) are generally held to have a poor prognosis and a high incidence of heart failure and embolism (Come et al, 1982; Pratt et al, 1978; Scheld and Sande, 1985; Stafford et al, 1985). Lutas et al (1986), however, found to the contrary, that the presence

of a detectable vegetation did not automatically mean a gloomy future for the patient. Other workers suggested 1.0cm as being the threshold vegetation size indicating probable medical failure (Robbins et al, 1986) and that the actual location of the vegetation was a more reliable prognostic indicator than its existence *per se* (Buda et al, 1986) - aortic vegetations being the most frequently fatal.

1.4.4 Clinical presentation of IE and associated complications

IE patients display a variety of symptoms differing widely from the classic symptoms of acute and subacute IE (von Reyn et al, 1981). Effects may be manifested in any organ of the body and symptoms may often be vague, or sometimes be so severe as to precipitate a medical emergency (Hayward, 1973a; King and Harkness, 1986a). In IE the health of the patient is affected by either damage to the heart valve caused directly by the colonizing organisms or by peripheral or systemic effects due to emboli or circulating immune complexes (Hayward, 1973a and 1973b; Scheld and Sande, 1985; King and Harkness, 1986a). Degeneration of heart tissue by bacterial action may result in valve perforation, valve-ring abcesses, pericarditis, mycotic aneurysms of the valve leaflet or ruptures of cardiac muscle (Scheld and Sande, 1985; King and Harkness, 1986a). Embolism is a feature of approximately 40% of IE cases (Hayward, 1973a). Pulmonary embolism following right-sided IE is especially common in intravenous drug abusers (Scheld and Sande, 1985). Fragments of vegetation become detatched and enter the circulation, resulting in embolic sequelae elsewhere in the body - mainly in the spleen, kidneys, brain, heart, lungs or bones (Weinstein and Schlesinger, 1974; Netter, 1978; Scheld and Sande, 1985). Several peripheral phenomena associated with IE which were initially thought to be of embolic origin are now known to be mainly caused by deposition of circulating immune complexes (Hayward, 1973a). These were more common in the

preantibiotic era and are no longer of such diagnostic value and include:- Janeway lesions (small areas of erythaema or haemorrhage on the palms or soles) occurring in 10-17% of patients with IE; Roth spots (retinal lesions or haemorrhage situated close to the optic disc) occurring in 5-14% of IE patients; petechiae (small skin haemorrhages) occurring in 20-60% IE patients; and Osler's nodes (raised bluish, red or pink pea-sized nodes on the pads of fingers and toes or soles of the feet) occurring in 10-25% of IE patients (Netter, 1978; Barry and Gump, 1982; King and Harkness, 1986a). Janeway lesions and Osler's nodes have been shown to contain bacteria, indicating the presence of emboli (Alpert et al, 1976; Kerr and Tan, 1979). These skin and retinal lesions are more scarce in patients with IE due to enterococci (Mandell et al, 1970).

Formation of immune complexes in IE results from the high level of circulating antibodies formed in response to the constant bacteraemia (Parker, 1984b). Initial antibody formation (and subsequent immune complex formation) occurs during the transient bacteraemia which precedes colonization of the heart valve (Sipes et al, 1977; Cabane et al, 1979). The continuous discharge of organisms from the vegetation into the bloodstream maintains a high antibody titre and the frequency of immune complex-formation increases with the duration of the illness (Scheld and Sande, 1985). Deposition of immune complexes in small blood vessels of the renal circulatory system or the glomerular basement membrane in the kidneys, results in abscess, infarction or glomerulonephritis (Iida et al, 1985). The presence of bacterial antigens and antibodies have been demonstrated in the glomeruli by several workers (Gutman et al, 1972; Keslin et al, 1973; Levy and Hong, 1973; Peres et al, 1976). Diffuse glomerulonephritis as a sequel to IE may result in death from renal failure (Hayward, 1973a). The level of circulating immune complexes may be used to gauge success of antibiotic treatment of IE - rising levels indicating inadequate treatment (Kauffman

et al, 1981), falling levels with eventual disappearance of complexes indicating successful treatment (Cabane et al, 1979). Rheumatoid factor, or anti-IgG IgM antibody, is expressed in approximately half the patients with IE of greater than six weeks duration (Williams and Kunkel, 1962). This factor may hinder IgG opsonic activity through binding to the Fc portion (Scheld and Sande, 1985), and may also be involved in the progress of IE via stimulation of phagocytes and/or contribution to microvascular damage.

Diagnosis of IE is complicated by the enormous variation in clinical findings and severity of symptoms between individuals. All or several (or indeed, few) of the cardiac, systemic and peripheral manifestations of the disease (described above) may be observed by the clinician. Additional diagnostic evidence of the disease may be gained by visualization of cardiac vegetations using echocardiography or by radiographic imaging using Gallium-67-, Technitium-99m- or Indium-111-labelling techniques (Wiseman et al, 1976; Riba et al, 1979; Come et al, 1982; Wong et al, 1982). Blood culture identification of the infecting organism is the most widely and successfully used diagnostic criterion in IE. Over two-thirds of IE patients show positive blood cultures due to constant low-grade bacteraemia (Hayward, 1973a; Scheld and Sande, 1985). Ideally the identity and antibiotic-susceptibility of the infecting microorganism should be determined.

In some cases, negative blood cultures are obtained repeatedly (Hayward, 1973a). This occurs at widely varying incidences of between 3% (Wilson and Geraci, 1983) and 30% (Hayward, 1973a). Culture-negative endocarditis may arise in several instances:-

(i) suppression of bacteraemia due to recent administration of antibiotics (Hayward, 1973a),

(ii) the endocarditis is due to nutritionally fastidious microorganisms (Wilson and Geraci, 1983),

(iii) the endocarditis is due to cell-wall deficient bacteria (Hayward, 1973a),

(iv) NBTE is present (Lopez et al, 1987),

(v) blood culture is performed towards the end of a chronic episode of IE (Scheld and Sande, 1985), or

(vi) the endocarditis is due to parasites such as chlamydiae or rickettsiae (Scheld and Sande, 1985).

The need for more specialized diagnostic tests is apparent in culture-negative endocarditis and other cases where rapid diagnosis is vital - such as when deciding upon surgical intervention (Scheld and Sande, 1985). Such tests that have been developed include detection of antibodies to:- the teichoic acid of staphylococci by counterimmunoelectrophoresis (Tuazon and Sheagren, 1976), whole cells of *Staphylococcus aureus* by ELISA (Jarløv et al, 1985) and whole cells of streptococci by fluorescent detection (Shanson et al, 1985); and blood-culturing using vitamin B_6- or cysteine-supplemented media to detect presence of nutritionally variant streptococci (Bouvet et al, 1981). Clearly new, rapid diagnostic methods, such as the one for detection of *S. faecalis* IE described in this thesis, are needed, in order to speed up selection of appropriate therapy.

1.4.5 Treatment and prophylaxis with antibiotics

Antibiotic therapy has been a major factor in the cure of IE, as demonstrated by the drop in fatality from 100% to 30% with the dawning of the antibiotic age. The role of antibiotics in this disease is doubly important since host defence mechanisms are ineffective against bacteria within cardiac vegetations.

Treatment of IE involves certain general principles. In conjunction with clinical diagnosis of the disease, isolation and identification of the infecting organism is preferrably the initial step, followed by determination of antibiotic susceptibility and bactericidal dose (Hayward, 1973b; King and Harkness, 1986b). These stages are of paramount importance in establishing a correct diagnosis and selecting an appropriate treatment regimen. A bactericidal rather than bacteriostatic antibiotic should be employed, and treatment should be initiated as promptly as possible (Hayward, 1973b; Wilson and Geraci, 1983). In cases where blood cultures are repeatedly negative or the condition of the patient necessitates immediate treatment before the causative organism has been identified, the treatment regimen should involve broad spectrum antibiotics that are active against the most resistant organisms likely to be encountered - *S. faecalis* or *Staphylococcus epidermidis* (Hayward, 1973b; Scheld and Sande, 1985; King and Harkness, 1986b).

Wilson and Geraci have studied the treatment of IE, and have published recommendations for the antibiotic treatment of IE caused by streptococci (1983 and 1985) and other microbial species (1983). These workers suggest antibiotic treatment schemes for IE caused by specific species of microorganisms; for prosthetic ,valve IE and for culture-negative endocarditis. The regimens are based upon knowledge gleaned from years of personal experience of managing the disease; clinical experience of other

physicians; studies of animal models of IE and *in vitro* and *in vivo* studies of antimicrobial susceptibility/bactericidal concentrations. Wilson and Geraci's antimicrobial regimens for streptococcal, staphylococcal and culture-negative endocarditis are briefly summarized in table 3.

IE due to enterococci (*S. faecalis* or *S. faecium*) presents a very different pattern to that caused by the viridans streptococci. Enterococcal endocarditis has the following characteristic features:-

(i) Enterococcal endocarditis can affect heart valves which show no evidence of previous underlying damage (Geraci and Martin, 1954; Toh and Ball, 1960; Mandell et al, 1970; Welsby, 1978).

(ii) Enterococci adhere particularly avidly to fibrin-platelet clots (Crawford and Russell, 1986).

(iii) Patients with enterococcal endocarditis present relatively few peripheral symptoms of the disease such as petechiae, Osler's nodes and Janeway lesions (Mandell et al, 1970).

(iv) Enterococci are insensitive to the antibiotic treatment regimen employed against penicillin-sensitive viridans streptococci (Wilson and Geraci, 1985; Kim and Bayer, 1987).

Clearly a separate antibiotic treatment regimen is needed for enterococcal endocarditis - this is shown in table 3. A stringent treatment is recommended, usually consisting of a penicillin plus an aminoglycoside (Kaye, 1982) - aqueous penicillin G 20-40 million units I/V daily, plus gentamicin 1mg/kg I/V every eight hours is an example of a typical anti-enterococcal regimen. This treatment is usually continued for four weeks (Wilson and Geraci, 1983), although some groups believe that treatment should be continued for a minimum of six weeks (Mandell et al, 1970; Tompsett and Berman, 1977), especially if the disease has been in progress for more than 3 months (King and Harkness, 1986b). Gentamicin is the aminoglycoside of choice to use synergistically with penicillin (Weinstein

TABLE 3: Treatment of endocarditis*

Microorganism	Antimicrobial therapy	Duration of treatment (weeks)	Alternative therapy	Duration of treatment (weeks)
Streptococcal endocarditis				
Penicillin-sensitive streptococci (MIC less than 0.2 μg/mL); non-enterococcal group D	Aqueous penicillin G (20 mill. units/day IV)	4	Cephalothin (1.5 g IV every four hours)	4
	or		**or**	
	Aqueous penicillin G (20 mill. units/day IV)	2	Vancomycin (7.5 mg/kg every six hours)†	4
	plus			
	Gentamicin (1 mg/kg IV every eight hours)†	2		
	or			
	Aqueous penicillin G (20 mill. units/day IV)	4		
	plus			
	Gentamicin (1 mg/kg IV every eight hours)†	2		
Relative penicillin-resistance (MIC greater than 0.2 μg/mL); nutritionally variant viridans streptococci	Aqueous penicillin G (20 mill. units/day IV)	4	Vancomycin (7.5 mg/kg IV every six hours)†	4
	plus			
	Gentamicin (1 mg/kg IV every eight hours)†			
Enterococcal endocarditis	Aqueous penicillin G (20–40 mill. units/day IV)	4–6	Vancomycin (7.5 mg/kg IV every six hours)†	4–6
	or			
	Ampicillin (12 g/day IV)	4–6		
	plus		**plus**	
	Gentamicin (1 mg/kg IV every eight hours)†		Gentamicin (1 mg/kg IV every eight hours)†	
Staphylococcal endocarditis				
Penicillin-sensitive staphylococci (MIC less than 0.1 μg/mL) (*Staph. aureus* or *Staph. epidermidis*)	Aqueous penicillin G (20 mill. units/day IV)	4–6	Cephalothin (2 g IV every four hours)	4–6
Penicillin-resistant (MIC greater than 0.1 μg/mL) (methicillin-sensitive *Staph. aureus* or *Staph. epidermidis*)	(Flu)cloxacillin (2 g IV every four hours)	4–6	Cephalothin (2 g IV every four hours)	4–6
	plus		**or**	
	Gentamicin (1 mg/kg IV every eight hours)†	1–2	Vancomycin (7.5 mg/kg IV every six hours)†	4–6
Methicillin-resistant *Staph. aureus* or *Staph. epidermidis*	Vancomycin (7.5 mg/kg IV every six hours)†	4–6	Rifampicin (600 mg/day by mouth)	4–6
	plus		**plus**	
	Gentamicin (1 mg/kg IV every eight hours)†		Fusidic acid (500 mg IV or by mouth every eight hours)	
Culture-negative endocarditis				
Normal valve	Penicillin G (20–40 mill. units/day IV)	4–6	Vancomycin (7.5 mg/kg IV every six hours)†	4–6
	plus		**plus**	
	Gentamicin (1.7 mg/kg IV every eight hours)†		Gentamicin (1.7 mg/kg IV every eight hours)†	
Prosthetic valve	Vancomycin (7.5 mg/kg IV every six hours)†	4–6		
	plus			
	Gentamicin (1.7 mg/kg IV every eight hours)†			

*Adapted from Wilson and Geraci. (N Engl J Med 1983) †Final dosage is dependent on the results of serum antimicrobial assays and of renal function tests. mill. = million; IV = intravenous route.

and Moellering Jr., 1973; Soriano and Greenwood, 1979; Simmons et al, 1986). Streptomycin is no longer deemed so suitable since some strains of enterococci have been reported to be resistant to streptomycin even when in synergistic combination with penicillin (Harvard et al, 1959; Standiford et al, 1970; Moellering Jr. et al, 1975; Gutschik, 1982; Indrelie et al, 1984). Recently strains of enterococci showing high-level gentamicin resistance have been noted (Mederski-Samoraj and Murray, 1983; Zervos et al, 1986), which might hint at future need for use of an alternative antibiotic.

Ampicillin, piperacillin or, in penicillin-allergic patients, vancomycin may also be used in conjunction with an aminoglycoside to combat enterococcal endocarditis (Kaye, 1982; Wilson and Geraci, 1983; Newsom, 1984; Simmons et al, 1986). The anti-enterococcal activity of newer antibiotics such as imipenem (Indrelie et al, 1984) or ciprofloxacin (Fernandez-Guerrero et al, 1987) in synergistic combination with penicillins or aminoglycosides is no better than with traditional synergistic combinations. Moellering Jr. et al, (1979) found that resistance is shown by enterococcal strains to combinations of penicillin with other aminoglycosides - *S. faecium* strains being more resistant to penicillin plus kanamycin, netilmicin or tobramycin than *S. faecalis* strains - although all enterococcal strains investigated by the same workers were killed by a penicillin-gentamicin combination. If a particular enterococcal isolate is not killed by a penicillin-gentamicin combination, then other penicillin-aminoglycoside combinations should be tried (Parker, 1984). For staphylococcal endocarditis, flucloxacillin may be added to the regimen (Simmons et al, 1986).

Bacteriological cure is effected when all infecting organisms have been destroyed. Even if this is achieved, mechanical damage to the heart acquired during the IE episode may lead to progressive heart disease or heart failure or may act as a focus for NBTE or reinfection with microorganisms

and other systemic or peripheral sequelae of the disease may lead to a serious deterioration in health (Parker, 1984b; Scheld and Sande, 1985).

In some cases of IE, surgical removal of the infected heart valve and its replacement with a prosthetic device is indicated. This situation arises if refractory congestive heart failure or other serious cardiac problems are present, or if a prosthetic heart valve already in place is infected (Black et al, 1974; Dinubile, 1980). The decision to proceed with surgery is not an easy task since surgical replacement of heart valves is associated with a high incidence of recurrent infection, often with a poor prognosis (Wilson et al, 1975; Calderwood et al, 1986). Echocardiography may be useful in deciding whether surgery is necessary (King and Harkness, 1986b). The high relapse rate of prosthetic valve endocarditis may be reduced by the use of new techniques such as implantation of a prosthetic valve incorporating gentamicin-releasing polymethylmethacrylate beads on its suture ring (Faidutti et al, 1986).

The aim of antimicrobial prophylaxis is to reduce the frequency and duration of transient bacteraemia in situations likely to facilitate entry of bacteria into the bloodstream, particularly in those patients that are predisposed to IE (Shulman et al, 1984; Kaye, 1986). Both the American Heart Association (AHA; Shulman et al, 1984) and the British Society for Antimicrobial Chemotherapy (BSAC; Simmons et al, 1982) have recently issued statements listing recommendations for prophylactic antibiotic regimens for prevention of IE in specific relevant situations. These reports are based upon studies of animal models of IE, *in vitro* experiments and circumstantial clinical evidence. Before publication of these and earlier reports (such as the statement issued by the AHA in 1977; Kaplan, 1977), antibiotic prophylaxis of IE was based mainly on hearsay and reports gleaned from medical, dental and scientific Journals (Scully et al, 1987). Now medical and dental practitioners are beginning to adopt the

prophylactic approach to IE recommended in the aforementioned reports - although not all adhere strictly to the recommended antibiotic regimens (Scully et al, 1987).

For obvious ethical reasons there has never been a major controlled study in humans to validate the clinical effectiveness of prophylaxis of IE. Its necessity still remains a controversial issue, prophylactic regimens reportedly having failed in several instances (Durack, 1975b; McGowan, 1978; Oakley and Somerville, 1981). It has not been unequivocably proven that bacteraemia leads to IE, especially when bacteraemia may arise after activities as inocuous as brushing the teeth or chewing hard sweets - without ensuing IE (Sipes et al, 1977)! Indeed Durack (1985) advocates that the prophylactic use of antibiotics against IE be restricted to "double-risk" patients - those patients undergoing surgical/dental procedures and who have existing heart defects which may predispose to IE. Durack (1985) also grades heart defects according to their considered risk-value for predisposing to IE and performs a two-dimensional risk analysis for the various surgical or dental procedures. This enables cross-reference to be made in assessing the need for antibiotic prophylaxis in a patient with specific heart defect and imminent operation. Gauging the risk potential for contracting IE in individual patients is not a precise science, and it is not possible to recommended prophylactic standard regimens to cover every clinical eventuality. However, the two-dimensional risk analysis performed by Durack (1985) goes some way to provide a useful guideline and, coupled with information gained from animal model studies (Durack and Petersdorf, 1973; Pelletier Jr. et al, 1975; Durack et al, 1977; Francioli and Glauser, 1985) and clinical reports (Sipes et al, 1977; Gould, 1984), has been useful in assessing the likelihood of a certain procedure to precipitate bacteraemia or IE - and the usefulness of antibiotic prophylaxis in these events.

Prophylaxis is aimed mainly at nosocomial IE. The rationale behind the prophylaxis of IE is based on various assumptions that have been made concerning the type of microorganism likely to be encountered in bacteraemia arising from certain dental/medical/surgical procedures, and the likely antibiotic sensitivity of that microorganism (Kaye, 1986). For instance evidence supports the assumption that patients undergoing invasive or manipulative procedures involving the oropharynx or gingival tissues, gastrointestinal or genitourinary tracts are at risk from viridans streptococci or enterococci, respectively (Hook and Kaye, 1962; Paterson, 1972; Simmons et al, 1982; Shulman et al, 1984; Kaye, 1986)., especially if an infection exists at these sites (Guze et al, 1985). Patients undergoing heart surgery are considered to be at risk from penicillin-resistant staphylococci (Paterson, 1972). The potential certain miccroorganisms have for causing IE must also be taken into account when deciding upon an appropriate prophylactic regimen of antibiotics as only a relatively small number of species account for the vast majority of IE cases (Hayward, 1973a; Scheld and Sande, 1985). Patients with pre-existing heart disease, heart valve damage or prosthetic heart devices are considered to be at a greater risk of contracting IE - as shown in table 4a (Shulman et al, 1984; King and Harkness, 1986b).

The guidelines for antibiotic prophylaxis of IE issued by the BSAC (Simmons et al, 1982) and AHA (Shulman,1984) take all the above factors into consideration and recommend provision of prophylactic cover for certain "risky" situations or procedures (listed in table 4b). The antibiotic regimens are thought to have an additional action of reducing the frequency of IE by inhibiting adherence of microorganisms to the heart valves/vegetations (Bernard et al, 1981; Sheld et al, 1981; Glauser et al, 1983; Francioli and Glauser, 1985).

Table 4a Cardiac Conditions for Which Endocarditis Prophylaxis is Recommended*

Endocarditis prophylaxis recommended
Prosthetic cardiac valves (including biosynthetic valves)
Most congenital cardiac malformations
Surgically constructed systemic-pulmonary shunts
Rheumatic and other acquired valvular dysfunction
Idiopathic hypertrophic subaortic stenosis
Previous history of bacterial endocarditis
Mitral valve prolapse with insufficiency†
Endocarditis prophylaxis not recommended
Isolated secundum atrial septal defect
Secundum atrial septal defect repaired without a patch 6 or more months earlier
Patent ductus arteriosus ligated and divided 6 or more months earlier
Postoperatively after coronary artery bypass graft surgery

* Adapted from Shulman and colleagues (1984)

† Definitive data to provide guidance in management of patients with mitral valve prolapse are particularly limited. In general, such patients are clearly at low risk of development of endocarditis, but the risk-benefit ratio of prophylaxis in mitral valve prolapse is uncertain.

Table 4b Procedures for Which Endocarditis Prophylaxis is Indicated*

Oral cavity and respiratory tract
All dental procedures likely to induce gingival bleeding (not simple adjustment of orthodontic appliances or shedding of deciduous teeth)
Tonsillectomy or adenoidectomy
Surgical procedures or biopsy involving respiratory mucosa
Bronchoscopy, especially with a rigid bronchoscope†
Incision and drainage of infected tissue
Genitourinary and gastrointestinal tracts
Cystoscopy
Prostatic surgery
Urethral catheterization (especially in the presence of infection)
Urinary tract surgery
Vaginal hysterectomy
Gallbladder surgery
Colonic surgery
Esophageal dilatation
Sclerotherapy for esophageal varices
Colonoscopy
Upper gastrointestinal tract endoscopy with biopsy
Proctosigmoidoscopic biopsy

* Adapted from Shulman and colleagues (1984)

† The risk with flexible bronchoscopy is low, but the necessity for prophylaxis is not yet defined.

Ideally administration of the prophylactic dose should be carried out a short time prior to the relevant risky procedure in order to ensure that peak serum levels of antibiotic are attained during the period of greatest risk of bacteraemia (Sipes et al, 1977; Petersdorf, 1978). The timing of administration is also geared to minimize selection of resistant flora (Durack, 1975b). An additional guard against bacteraemia in dental procedures is the maintenance of a high standard of dental hygiene. Studies have revealed the value of "degerming" the mouth with an antiseptic mouthwash such as 0.5% chlorhexidine (Jones et al, 1970; Bender et al, 1984) prior to dental manipulation - a practice that is seldom routinely used by dentists (Gould, 1984).

The most widely employed prophylactic regimen in dental procedures is a single 3g-dose of amoxycillin given 1 hour prior to the procedure (Shanson et al, 1980; Oakley and Somerville, 1981; Simmons et al, 1982; Glauser et al, 1983; Kaye, 1986). This is effective against oral streptococci (Shanson et al, 1980), with an extra margin of safety gained by repeating the dose six hours after the procedure (Kaye, 1986). Vancomycin or erythromycin may be substituted if penicillin-allergy is a problem (Oakley and Somerville, 1981; Simmons et al, 1982).

Regimens used in the prophylaxis of enterococcal endocarditis in gastrointestinal or genitourinary procedures usually involve a synergistic combination of a penicillin and an aminoglycoside (Simmons et al, 1982; Gould, 1984). A typical regimen is 1g amoxycillin plus 120mg gentamicin I/M immediately prior to procedure (Simmons et al, 1982), which may be followed by 0.5g amoxycillin orally or I/M six hours after the procedure. Penicillin-allergic patients may be given vancomycin 1g by slow I/V infusion for 20-30 minutes followed by 120mg gentamicin I/V immediately before induction of anaesthesia (Simmons et al, 1982). It has been suggested that, as in dental prophylaxis of IE, a 3g single-dose of amoxycillin should be used to cover

gastro-intestinal/genitourinary procedures, since this dose is as effective against enterococci as against oral viridans streptococci (Francioli et al, 1985; Kaye, 1986). This would result in a standard, simple regimen for prophylaxis of IE in oral, gastrointestinal and genitourinary procedures. The need for such a standard antibiotic prophylactic regimen is illustrated by recent studies which have shown that, in general, compliance with the BSAC and AHA recommendations has been low (Durack, 1975b; Simmons et al, 1982; Holbrook et al, 1983; Gould, 1984; Sadowsky and Kunzel, 1984). However, compliance with the BSAC's 1982 guidelines was marginally better than with those issued by the AHA in 1977 and 1984 (Scully et al, 1987). Gould (1984) surveyed the current practice of prophylaxis of IE in London and reached the conclusion that further education of physicians is needed in order to fully implement the recommended regimens. He also states that revision of the regimens is required regarding choice of antibiotic and dose; and that more emphasis should be placed on correct timing of the dose. Finally Gould advocates the simplification of any new recommendations and recommends that they be better publicized than their predecessors. Kaye (1986), and Scully et al (1987), in their respective analyses of the AHA and BSAC reports, reach the same general conclusions as Gould - namely that there is a need for simpler regimens, better compliance with these regimens and better education of physicians and dentists. Universal compliance with a simple standard antibiotic regimen which would give prophylactic cover for a wide range of clinical situations may (if circumstantial evidence is to be relied upon) reduce the incidence of nosocomial IE.

To summarize basic concepts of treatment and prophylaxis of IE:

1. A variety of microorganisms may cause IE, chiefly oral viridans streptococci, staphylococci, group D streptococci and enterococci.

2. Each discrete group of causal organisms possess distinct characteristics and antibiotic susceptibilities, necessitating a wide range of antibiotic treatment/prophylactic regimens.

4. Prompt selection and implementation of the appropriate treatment regimen is essential. This emphasizes the need for rapid and accurate diagnostic methods - especially in severe cases, enterococcal- or culture-negative endocarditis.

1.5 Aims and objectives of project

The initial aim of this research project was to study the surface characteristics of *S. faecalis*. This organism was of interest because of the avidity with which it adheres to the endocardium of a host, in endocarditis, following entry into the bloodstream. *S. faecalis* cells were examined for proteinaceous surface structures possibly responsible for adhesion to host tissue - either directly or via mediation of host factors such as fibronectin or other serum proteins.

It was desirable that the conditions under which the organism was grown be as close as possible to those encountered by the organism in the in-vivo environment. In the case of IE, this meant mimicking growth in the bloodstream, as such conditions are encountered by the microorganism during bacteraemia which precedes infection of the endocardium. In an attempt to achieve these conditions, *S. faecalis* was grown in serum. Whilst acknowledging that other substances are present in the blood that might influence the surface composition of a microorganism, serum is a closer approximation to the in-vivo environment in bacteraemia than is a nutritionally complex laboratory medium.

In the process of examining the surface of *S. faecalis* several observations arose, chiefly that the conditions of growth influenced the surface composition and that growth in serum stimulated expression of three major protein antigens which were specific to *S. faecalis* species. These antigens were investigated for their potential in the diagnosis of *S. faecalis* endocarditis. Immunoblotting of the *S. faecalis*-specific antigens was carried out with the major objective of developing a rapid, accurate serodiagnostic test for *S. faecalis* which would improve upon (or be used in conjunction with) present blood-culture methods of diagnosis.

The final section of this project was involved with isolation and purification of the *S. faecalis*-specific antigens with the ultimate aim of converting the immunoblotting serodiagnostic method into an ELISA or latex bead agglutination method for routine use in a clinical situation to facilitate rapid selection of appropriate antibiotic therapy for IE.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial strains

Eleven strains of *Streptococcus faecalis (S. faecalis)* were used: three strains cultured from the blood of a patient with *S. faecalis* endocarditis (EBH1, 9112 and SFDK); four strains isolated from patients with urinary tract infections (741, 777, 790 and SFBG); two strains isolated from patients with septicaemia (SFBA and SFSQ); a laboratory strain of *S. faecalis* subspecies *zymogenes* (Facklam, 1972; Deibel and Seeley, 1974) kindly donated by Dr. N.J. Parsons at Birmingham University (SFZ) and a reference strain, *S. faecalis* subspecies *zymogenes* NCTC 5957, obtained from the National Collection of Type Cultures, Central Public Health Laboratory, Collindale, London (deposited in the NCTC in 1940 from the American Type Culture Collection; originally isolated from pasteurized milk by J.M. Sherman, 1937).

Other NCTC strains used were: *S. milleri* 10708, deposited in the NCTC by G. Colman in 1970, originally obtained from O. Guthof in 1963 (Guthof, 1956; Colman, 1968); *S. faecium* 7171, deposited in the NCTC by A. Grumbach in 1946; *S. mutans* 10449, deposited in the NCTC by W. Sims in 1966, isolated from carious dentine (Clarke, 1924); *S. sanguis* 7863, isolated from subacute bacterial endocarditis and deposited in the NCTC in 1949 from the ATCC: *S. bovis* 11436 isolated from blood culture and deposited in the NCTC in 1982 by G. Colman (Facklam, 1972; Parker, 1978) and *S. hominis* 8618, deposited in the NCTC in 1950 from the ATCC (Sherman et al, 1943)..

A strain of *Staphylococcus aureus* cultured from the blood of a patient with endocarditis and a reference strain, *Staphylococcus aureus* NCTC 6571 (Oxford strain) was also used.

2.1.2 **Growth media**

Brain-heart infusion agar was obtained from Oxoid Ltd., Basingstoke, Hants and brain-heart infusion broth (BHI) from Lab M, Salford, Manchester and Difco, East Molesey, Surrey. When reconstituted, Lab M BHI contained, per litre: brain and heart infusion solids 17.5g, "lab M" tryptose 10g, dextrose 2g, NaCl 5g and di-sodium phosphate 2.5g, pH 7.4. Difco BHI contained, per litre: infusion from calf brains 200g, infusion from beef heart 250g, proteose peptone 10g, dextrose 2g, NaCl 5g and di-sodium phosphate 2.5g, pH 7.4. Heat-inactivated, mycoplasma-tested horse serum (HS) was obtained from Gibco, Paisley, Glasgow. HS was used neat or diluted to 60%v/v with sterile 0.9%w/v NaCl (*S. faecalis* antigens were fully expressed at this dilution; figure 6, lane 3). These media and nutrient agar and broth (Lab M) were prepared according to manufacturers instructions and sterilized by autoclaving at 121°C for 15 minutes. Blood agar was prepared by adding defibrinated horse blood (Gibco) 7%v/v to sterile blood agar base (Lab M).

A chemically defined medium (CDM) developed by Dr. S. Prior, CAMR, Porton Down, Salisbury, Wilts (personal communication, 1986) was used. The CDM was prepared by supplementing a minimal salts medium with 1% v/v horse serum. The minimal salts medium contained per litre: KH_2PO_4 400mg; K_2HPO_4 300mg; Na_2HPO_4 26.65g; NaH_2PO_4 16.45g; glucose 20.0g; sodium acetate 6.0g; ammonium sulphate 600mg; magnesium sulphate 200mg; sodium chloride 10mg; ferrous sulphate 10mg and manganese sulphate 10mg. The minimal salts medium was autoclaved at 121°C for 15 minutes. Glucose and ferrous sulphate

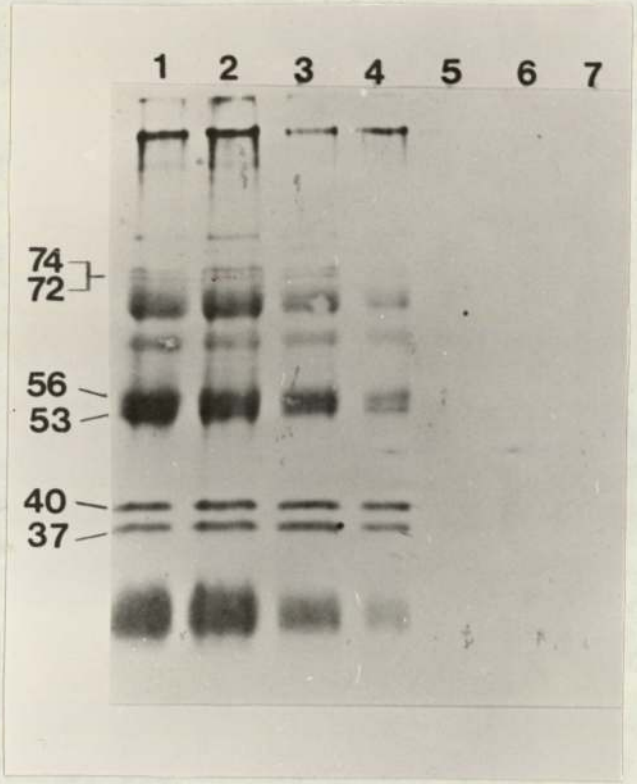


Figure 6 Immunoblot analysis of EBH1 grown in diluted horse serum. Whole cells of strain EBH1 were grown in HS diluted with sterile saline, subjected to SDS-PAGE and the separated antigens electrophoretically transferred onto nitrocellulose. The blot was reacted with serum from IE patient, Mr. P. Dilutions of HS were as follows:
Lane 1, 100% HS; 2, 80% HS; 3, 60% HS; 4, 40% HS; 5, 20% HS; 6, 10% HS; 7, 5% HS

were sterilized separately and then added aseptically together with the HS, to the minimal salts medium.

2.1.3 Chemicals

All chemicals used were of Analar grade or equivalent and were obtained from British Drug Houses (BDH) Chemicals Ltd, Poole, Dorset or Fisons Laboratory Reagents, Loughborough, Leics, unless otherwise stated. Solutions were made up with double-distilled water. Reagents for immunoblotting such as protein A-peroxidase and lectin-peroxidase conjugates were obtained from Sigma Chemical Company Ltd, Poole, Dorset.

2.1.4. Glassware

Glassware (Pyrex brand, Corning Glass Ltd, Sunderland, Tyne and Wear) was rinsed in tap water, fully immersed in Extran 5%v/v and allowed to stand overnight at room temperature. The glassware was then rinsed once with distilled and finally twice with double-distilled water before being dried at 60°C and sterilized by dry heat at 160°C for 3 hours.

2.1.5 Human sera

Serum was collected by venipuncture from patients at East Birmingham Hospital, Bordesley Green East, Birmingham. Several serum samples were taken from each patient during the course of their infection (infective endocarditis, septicaemia or urinary tract infection). Solid matter was removed from the sera by low-speed centrifugation. Sera were stored at -20°C until required.

2.1.6 **Equipment**

Equipment used in this study, if not specified in the text, was supplied by:-

Autoclave -	Astell, Astell Hearson, Catford, London
Automatic pipettes -	Gilson Pipetman, P-200, P-1000 and P-5000, Anachem, Luton, Beds.
Balances -	1. Oertling HC22, Oertling, Orpington, Kent. 2. Sartorius Type 1702, Sartorius Instruments Ltd, Belmont, Surrey.
Blood collecting tubes -	Polypropylene stoppered tubes, Sterilin Ltd Teddington, Middx.
Centrifuges -	1. Beckman J2-21 high-speed centrifuge, Beckman RIIC Ltd, High Wycombe, Bucks. 2. Eppendorf centrifuge 5412, Anderman & Co Ltd, East Molesey, Surrey. 3. MSE bench centrifuge and 4. MSE superspeed 50 angle rotor head ultra-centrifuge, Measuring and Scientific Equipment, Crawley, Surrey.
Fluorescence microscope -	F. M. Vickers Photoplan with epifluorescence optics, Vickers Instruments, UK.
Freeze drier -	Edwards Modylo freeze drier, Edwards High Vacuum Ltd, Crawley, Surrey.
Gamma counter -	ICN Gamma Set 100, ICN Tracerlab Division, Cleveland, Ohio, USA.

Gel Electrophoresis - Apparatus	1. Made in-house by Aston University 2. Bio-Rad Model 360 minivertical slab-cell and Bio-Rad Model 361 casting chamber (designed and built for Bio-Rad by Hoefer Scientific), Bio-Rad Laboratories Ltd, Watford, Herts. 3. Mini Protean II cell, Bio-Rad.
Gel Drier -	Bio-Rad model 224 gel slab drier, Bio-Rad.
Gel filtration - equipment	1. Superose 12 HR 10/30 column, Pharmacia. Laboratory Separation Division, Uppsala, Sweden. 2. Sephacryl S-200 SF column, Pharmacia. 3. High precision pump P-500, Pharmacia. 4. UV monitor, 2138 Uvicord, LKB Instruments Ltd, Croydon, Surrey.
Immunoblotting tank -	Trans-blot cell, Bio-Rad Laboratories Ltd, Watford, Herts.
Immunoelectrophoresis - equipment	Flat bed 2117 multiphor II electrophoresis unit, LKB Instruments Ltd, Croydon, Surrey.
Incubators -	1. LTE incubator, Laboratory Thermal Equipment, Greenfield, Lancs.
Incubators -	2. Mickle reciprocating water bath, Cam Lab Ltd, Cambridge, Cambs. 3. Gallenkamp orbital shaking incubator, Gallenkamp, London. 4. Grant water bath, Grant Instruments Ltd, Cambridge, Cambs.

Membrane filters -	1. Gelman Acrodisc, Gelman Siences, Brackmills, Northampton.
	2. Millipore membrane filter 0.22µm pore size, Millipore UK, Harrow, Middx.
Microscope slides -	Hendley-Essex multispot microscope slides, PTFE-coated, C.A. Hendley Ltd, Loughton, Essex.
Pasteur pipettes -	Fisons Scientific Apparatus, Loughborough, Leics.
pH meter -	Pye Unicam 290 pH meter, Cambridge, Cambs.
Photography equipment -	Nikon camera FM, Nippon Kogaku KK, Tokyo, Japan with Ilford Pan F film or Kodak technical pan film 2415.
Power packs -	Bio-Rad Model 500/200 (electrophoresis; Bio-Rad Model 250/2.5 (immunoblotting) and Bio-Rad Model 3000/300 (crossed-immunoelectrophoresis), Bio-Rad Laboratories Ltd, Watford, Herts.
Sealable tubes -	Screw-top culture tubes with teflon-lined caps, Sterilin, Teddington, Middx.
Sonicator -	MSE Soniprep 150 ultrasonic disintegrator, Measuring and Scientific Equipment, Crawley, Surrey.
Spectrophotometers -	1. Unicam SP600, Pye-Unicam Instruments, Cambridge, Cambs.
	2. Cecil CE 292 Digital, Cecil Instruments, Cambridge, Cams.Plastic cuvettes for spectrophotometry, Brand, Gallenkamp, Loughborough, Leics.

Spectrophotometers -	3. Scanning UV Spectrophotometer, Unicam 8000, Pye-Unicam Instruments, Cambridge, Cams. Quarz cuvettes for UV spectrophotometry, Hellma, Westcliff-on-Sea, Essex.
Syringes -	Gillette Surgical, Isleworth, Middx.
UV Lamp -	Hanovia Lamps, Slough, Berks.
Vortex mixer -	Whirlimixer, Fisons Scientific Apparatus, Loughborough, Leics.
Water cooler -	FH15 and FC15 flow coolers, Grant Instruments, Cambridge, Cambs.
X-ray film -	Medical X-ray film, Caeverken AB, Strägnäs, Sweden.

All addresses are in U.K. unless otherwise stated.

2.2 EXPERIMENTAL METHODS

2.2.1 Growth experiments

2.2.1.1 Growth conditions

Bacterial strains were maintained on nutrient agar slopes at 4°C and subcultured monthly. The bacteria were grown at 37°C with agitation at 120 revolutions per minute (rpm) in an orbital shaker or in a Mickle reciprocation water bath at 80 strokes/minute. In any of the media used, cells of *S. faecalis* did not produce any pigment that may have interfered with optical density (OD) measurement. 470nm was chosen as an appropriate wavelength at which to monitor subsequent growth curves, an OD at 470nm of 1.0 (OD_{470} = 1.0) indicating a concentration of approximately 10^9 cells/ml. Medium constituents did not interfere with measurements at this wavelength and none of the organisms produced extracellular products which absorbed light at 470nm.

2.2.1.2 Measurement of bacterial concentration

The method of indirect counting was used to measure bacterial cell concentration. This was thought to be a more appropriate method than performing total counts or viable counts for monitoring changes in cell concentration during the growth phases of an organism (Greenwood, 1977). Measurement of turbidity or light scattering properties of the bacterial suspension was made using a spectrophotometer. At relatively low cell concentrations the OD is directly proportional to the bacterial cell concentration. This is expressed by the Beer-Lambert law:

$$OD \propto \log_{10}(I_o/I)$$

where I_o = incident light and I = emergent light.

At higher cell concentrations this relationship does not apply. Due to the secondary scattering of light, the OD increases less than proportionally with increasing cell concentration (Meynell and Meynell, 1970; Kenward, 1975). To avoid this effect bacterial suspensions were diluted with an appropriate medium to an OD of between 0.05 and 0.3.

Media, prewarmed to 37°C, were inoculated with stationary phase *S. faecalis* grown in corresponding media to give OD_{470} = 1.0. Samples were removed aseptically at 30-minute intervals and the OD measured, diluting samples appropriately if the OD_{470} was greater than 3.0. Time versus $\log_{10} OD_{470}$ was plotted to give growth curves. Growth in several media was monitored.

2.2.2 Preparative techniques

2.2.2.1 Preparation of whole cells and cell walls

Bacteria were grown to mid-log phase in BHI (defined as being the point on the growth curve that was two generations before onset of stationary phase) or early stationary phase in HS. Cells were harvested by centrifugation at 10,000 rpm, washed three times and resuspended in 10mM Tris-HCl buffer, pH 7.4 to OD_{470} = 5.0. The resuspended bacteria were stored at -20°C until required.

Cell walls were prepared by ultrasonic disintegration of whole cells. Ice-cooled suspensions were subjected to six one-minute bursts of ultrasound interspersed with 30-second intervals. The efficiency of cell breakage was estimated by light-microscopy to be ≃ 80%. Unbroken cells were removed by centrifugation at 5,000 rpm for 2 × 5 minutes. Cell walls were obtained from the resultant supernatant by centrifugation at 20,000 rpm for 45 minutes. The resulting pellet consisted of crude cell walls and

was resuspended in 10mM Tris-HCl, pH 7.4 to OD_{470} = 5.0 and stored at -20°C until required.

2.2.2.2 **Extraction of protein antigens**

Whole-cell preparations were subjected to various treatments in an attempt to extract protein antigens. 200µl aliquots of whole-cell suspension OD_{470} = 5.0 diluted to 1ml with 10mM Tris-HCl, pH 7.4 were incubated with either: lysozyme 100µg (Laible and Germaine, 1985; Sigma); 8M urea 100µl; 15mM Na_2EDTA 100µl; or 10%w/v sodium dodecyl sulphate (SDS) 200µl each for 1 hour at 37°C (Jenkinson, 1986). Following treatment, cell suspensions were spun in an Eppendorf centrifuge for 5 minutes (urea-treated cells were first dialysed overnight against water). Supernatants were retained and pellets were resuspended to 200µl in 10mM Tris-HCl, pH 7.4.

2.2.2.3 **Extraction of lipoteichoic acid**

The method of Moskowitz (1966) as modified by Hamada et al (1985) was used. Mid-log phase cells of *S. faecalis*, strain EBH1 were suspended in double-distilled water to 10mg (dry weight)/ml. An equal volume of 95%w/v phenol was added and the mixture was stirred for 1 hour at 25°C. The phenol and aqueous layers were separated by centrifugation at 8,000 rpm for 20 minutes, and the aqueous layer was carefully removed by suction and retained. The extraction was repeated by the addition of an equal volume of double-distilled water to the phenol layer and the mixture was again stirred for 1 hour at 25°C and the aqueous layer removed as before. The two aqueous portions were combined and dialysed overnight against distilled water and then lyophilized.

To remove nucleic acid contaminants the extract was digested with nucleases. The lyophilized extract was dissolved in 10mM Tris-HCl, pH 7.4 containing 0.1mM $MgCl_2$ and nuclease (RNAse and DNAse 1:1; Sigma) The

weight ratio of extract to nuclease was 150:1. This mixture was incubated for 24 hours at 37°C, dialysed against distilled water, lyophilized and redissolved in 0.2M ammonium acetate, pH 7.0. Particulate matter was removed by centrifugation at 5,000 rpm for 10 minutes. The supernatant was applied to a Sepharose 6B column (Pharmacia Fine Chemicals, Uppsala, Sweden) and pumped upwards at a rate of 15ml/hour. Fractions were collected and their absorbance at 260nm measured. The phosphate content of the fractions comprising the two resulting phosphorus peaks was assayed (Chen et al, 1956). The phosphate-containing fractions were pooled for each peak (one was assumed to be LTA and the other deacylated LTA) and accepted as being crude preparations since no further purification was carried out to remove any contaminating nuclease.

2.2.2.4 Solubilization of bacterial components

Bacterial cell components were solubilized for crossed-immunoelectrophoresis and chromatographic separation either by digestion with mutanolysin or treatment with sodium N-lauroyl sarcosinate (Sarkosyl). Mutanolysin is a muramidase isolated from *Streptomyces globisporus* and has been used to digest cells of *S. mutans* (Siegel et al, 1981), *S. sanguis* (Morris et al, 1985) and *S. salivarius* (Weerkamp et al, 1986). Williamson et al (1986) have also used this enzyme to lyse enterococci to obtain penicillin-binding proteins. Sarkosyl is a detergent that traditionally has been used to isolate outer membranes of Gram-negative organisms (Filip et al, 1973) and has more recently been used in the extraction of surface proteins of *S. sanguis* (Jenkinson, 1986).

2.2.2.4.1 Mutanolysin digestion

Whole cells were suspended to OD_{470} = 10.0 in 50mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) buffer (Sigma), pH 7.2 containing 0.02%w/v sodium azide. Mutanolysin (Sigma) was added to give a

concentration of 50 units/ml. To inhibit proteolysis, phenylmethylsulphonyl fluoride (PMSF; Sigma), 1mM was included. The reaction mixture was incubated overnight at 37°C (resulting in a reduction in turbidity of approximately 80%, agreeing with the findings of Morris et al, 1985). Controls were performed in which no mutanolysin was included. The suspension was centrifuged at 15,000 rpm for 10 minutes and the supernatant dialysed against water and lyophilized. For future work the mutanolysin digest was resuspended in water to 20mg/ml unless otherwise stated.

2.2.2.4.2 **Sarkosyl extraction**

Early stationary phase HS-grown whole cells were harvested from a 1L culture, washed three times in 10mM Tris-HCl, pH 7.4 and resuspended in 5ml of a 1%w/v solution of Sarkosyl (Sigma) in 10mM Tris-HCl, pH 7.4 containing 1mM Na_2EDTA. The suspension was vortex-mixed and incubated for 20 minutes at room temperature, without lysis occurring. Unbroken cells were removed by centrifugation at 5,000 rpm for 2 × 10 minutes. Sarkosyl extracts were stored at -20°C unil required. This procedure removed surface proteins, polynucleotides, LTA and PSs from the bacterial cell (Jenkinson, 1986).

2.2.2.5 **Immunization of rabbits**

2.2.2.5.1 **Preparation of anti-whole cell antisera**

Freshly harvested cells of strain EBH1 were washed and resuspended in 25ml of 0.9%w/v NaCl to OD_{470} = 1.0. Bacteria were killed by exposure to UV light for 10 minutes. Antiserum was raised by injecting pairs of rabbits (3kg, half-lop males) with 1ml of the killed-cell suspension at three sites in the neck. After eight weekly injections a small quantity of blood was obtained from the rabbits' ears and the anti-EBH1 antibody titre was determined by slide agglutination tests. A titre of 128 or more was taken

to indicate that the rabbit was hyperimmune to EBH1. Hyperimmune rabbits were bled by cardiac puncture and sera stored at -20°C until required.

2.2.2.5.2 **Preparation of monospecific antisera**

The method of Knudsen (1984) was used to prepare monospecific, polyclonal antibodies. Whole cells of strain EBH1 were separated by SDS-PAGE (see section 2.2.3.1) on a 12% single-track polyacrylamide gel. The separated cell components were electrophoretically transferred onto a nitrocellulose membrane (see section 2.2.3.2). Narrow strips were removed from both ends and the middle of the blot and developed with antiserum to reveal the antigenic profile. The nitrocellulose bearing the required antigens was then excised from the corresponding undeveloped region of the blot. This was then macerated and dissolved in approximately 500µl of dimethyl sulphoxide (DMSO). An equal volume of Freund's complete adjuvant (Sigma) was added to a small portion of the suspension and an equal volume of Freund's incomplete adjuvant was added to the remainder. The antigen suspensions (100µl) were injected at weekly intervals at three sites in the neck of 3kg, half-lop male rabbits beginning with the suspension made with complete adjuvant. The subsequent 7 injections were of the suspension made with the incomplete adjuvant. Blood was obtained from the rabbits by cardiac puncture and sera were stored at -20°C until required.

2.2.2.6 **Radioiodination of surface proteins**

The method of Booth (1980) was used to radiolabel surface proteins of *S. faecalis*. Bacteria grown to early stationary phase in 60% HS were suspended in 0.1M phosphate buffer in 0.9%w/v NaCl (phosphate buffered saline, PBS) to OD_{470} = 8.0. To 500µl portions of this suspension was added 250µl lactoperoxidase (0.1mg/ml in PBS, purified grade, 100 IU/mg protein; Sigma) plus 5µl carrier-free $Na^{125}I$ (10mCi/ml in water; Radiochemical

Centre, Amersham International, Amersham, Bucks). Four 50µl portions of H_2O_2 (10mM in water; Thornton and Ross, Huddersfield, Yorks) were added at 2½-minute intervals. The iodination was stopped by addition of 4ml L-cysteine (10mM in water) 10 minutes after the first addition of H_2O_2. Activity of the labelled cells was measured using a γ-counter.

2.2.3 Experimental techniques

2.2.3.1 Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The gel system described by Lugtenberg et al, 1975 was used for SDS-PAGE. The system consisted of a stacking gel and a running gel in which bacterial components were electrophoretically separated according to molecular weight. Optimum separation of *S. faecalis* proteins was achieved using 12%w/v acrylamide in the running gel. Formulae for the stacking and running gels are shown below:-

12% RUNNING GEL		STACKING GEL	
Stock I	15.00ml	Stock II	5.00ml
SDS 10%w/v	1.50ml	SDS 10%w/v	0.30ml
1.5M Tris pH 8.8	18.50ml	0.5M Tris pH 6.8	7.50ml
Distilled water	23.75ml	Distilled water	16.00ml
TEMED	0.14ml	TEMED	0.08ml
AMPS	0.20ml	AMPS	0.10ml

where:

Stock I = 44%w/v acrylamide (Sigma) and 0.8%w/v Bis (N,N' methylene bisacrylamide; Sigma) in water

Stock II = 30%w/v acrylamide and 0.8%w/v Bis in water

TEMED = N,N,N,N' tetramethylethylene diamine (BDH)

AMPS = ammonium persulphate (BDH)

(volumes shown are sufficient for one 20cm × 25cm × 1mm gel)

Solutions for the separating gel were added in the order shown to a 250ml beaker and stirred with a magnetic stirrer. Polymerization was initiated upon the addition of TEMED and AMPS (Hames, 1981). The running gel was cast by pouring the solution between two glass plates separated with plastic spacers (Bio-Rad Mini Protean II gel apparatus, gel dimensions 10cm × 7cm × 0.75mm; Aston in-house gel apparatus, gel dimensions 20cm × 25cm × 1mm) or between a glass plate and an alumina plate (Hoefer minivertical slab gel apparatus, gel dimensions 10cm × 7cm × 0.75mm). After the running gel had set the stacking gel was prepared in a similar way and cast above the running gel. Wells were formed in the stacking gel by the insertion of a teflon comb (1, 10 or 15 wells per comb). The depth of the stacking gel was at least 5mm greater than that of the wells.

Whole-cell or cell-wall suspensions (OD_{470} = 5.0) were added to an equal volume of sample buffer, the formula of which is shown below:

SAMPLE BUFFER, pH 6.8

0.5M Tris pH 6.8	5.0ml
SDS 10%w/v	10.0ml
Mercaptoethanol	0.5ml
Glycerol	5.0ml
Distilled water	10.0ml
Bromophenol blue 0.5%w/v	0.4ml

Samples were denatured by boiling with an equal volume of sample buffer for 10 minutes and applied to the wells in the stacking gel. Electrophoretic separation was carried out at room temperature in electrode buffer (containing per 2L disilled water: Tris 6g; glycine 28.8g; and 20ml SDS 10%w/v) at a constant current of 15mA for 1-1½ hours (Hoefer minivertical slab gel apparatus) or 3-4 hours (Aston in-house gel

apparatus) or at a constant voltage of 200V for approximately 1 hour (Bio-Rad Mini Protean II gel apparatus). Gels were stained overnight for protein with Coomassie brilliant blue R-250 in methanol 50%v/v, glacial acetic acid 10v/v in water and then destained with methanol 5%v/v, glacial acetic acid 10%v/v in water.

To determine the molecular weight of proteins separated by SDS-PAGE a calibration curve was constructed (Weber and Osborn, 1969). Proteins of known molecular weight (either *Pseudomonas aeruginosa* PA01 outer membrane proteins or commercially available molecular weight markers; Sigma) were subjected to SDS-PAGE and their Rf values were determined (Rf value = distance moved by protein through the running gel ÷ distance moved by bromophenol blue marker dye through the running gel). A plot of $\log_{10}$ (molecular weight) versus Rf value gave a calibration curve from which the molecular weight of unknown proteins were determined.

Native PAGE was also carried out on *S. faecalis*. To maintain the native configuration of proteins during electrophoresis mild conditions were required (Hames, 1981). The method used followed the same basic procedure as SDS-PAGE except that no SDS was added to the running gel, stacking gel, electrode buffer or sample buffer. Mercaptoethanol was also omitted from the sample buffer. No denaturation of samples occurred. Solubilized bacteria (see section 2.2.2.4) were added to an equal volume of sample buffer and incubated at room temperature for 30 minutes, and then separated by native PAGE on a running gel containing 10%w/v polyacrylamide, at 100V for 2 hours (Mini Protean II gel apparatus). Molecular weight markers (Sigma) specifically designed for use in native PAGE were used to construct a calibration curve as for SDS-PAGE.

2.2.3.2 **Immunoblotting**

This procedure is also known as Western blotting, protein blotting, electroblotting or electroimmunoblotting. Separated bacterial components on polyacrylamide gels were electrophoretically transferred onto nitrocellulose membranes (pore size 0.45μm, Bio-Rad) as described by Towbin et al, (1979). Transfer was carried out in an ice-cooled transfer buffer containing 25mM Tris, 192mM glycine and 20%v/v methanol, pH 8.3 (Bio-Rad Transblot apparatus). The nitrocellulose membrane and polyacrylamide gel were rinsed briefly in transfer buffer and sandwiched between chromatography paper (Whatman Ltd, Maidstone, Kent) and Scotch-brite pads (Bio-Rad) both soaked in transfer buffer (figure 7). Any air bubbles were eliminated from between the gel and the nitrocellulose sheet. The gel-nitrocellulose sandwich was placed in a plastic cassette (Bio-Rad), submerged in transfer buffer and electrophoretic transfer was carried out at a constant voltage of 30V for 18 hours. Transfer of protein to the nitrocellulose was determined by staining with amido black (1%w/v in destain - see section 2.2.3.1). Efficiency of transfer, known to be a function of molecular weight (Burnette, 1981; Vaessen et al, 1981), was found to be high under these electrophoretic conditions upon comparing Coomassie blue-stains of gels before and after transfer (which showed virtually complete transfer).

After transfer the blots were immunodetected. Initially, blots were washed by gentle agitation for 30 minutes at 37°C in TTBS (Tween-Tris buffered saline, containing 0.3%v/v Tween 20, 0.9%w/v NaCl in 10mM Tris-HCl, pH 7.4). This blocked unbound sites on the nitrocellulose (Batteiger et al, 1982). TTBS was replaced by TBS (Tris-buffered saline, containing 0.9%w/v NaCl in 10mM Tris-HCl, pH 7.4) in which the blots were rinsed three times. Subsequently the blots were probed by gentle agitation for 3 hours at 37°C in either hyperimmune rabbit anti-*S. faecalis* serum, serum from patients with IE or serum from control patients each diluted 1:50 in TTBS. The blots

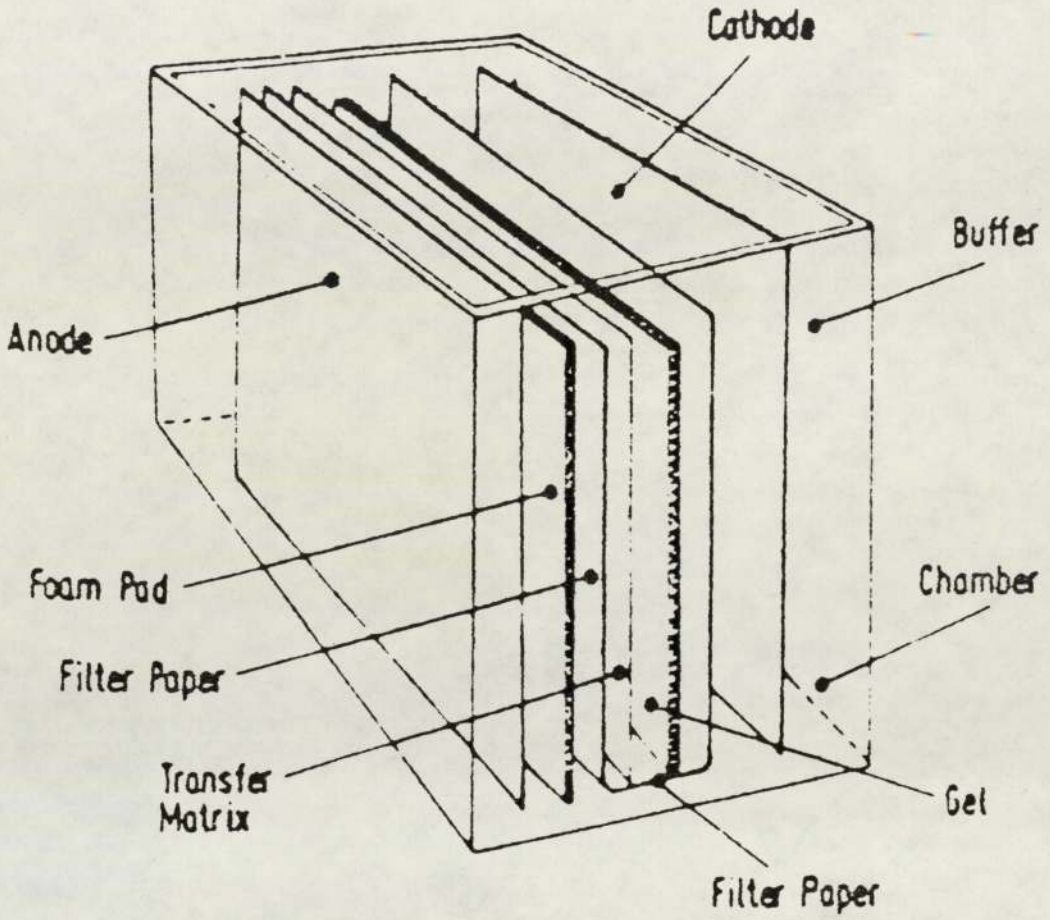


Figure 7 Assembly of apparatus for vertical transfer of proteins from polyacrylamide gels to nitrocellulose. Electrophoretic transfer takes place from cathode towards the anode Transfer matrix = nitrocellulose membrane.

were then rinsed three times in TBS and soaked for 2 hours at 37°C in TTBS containing staphylococcal protein A-horse radish peroxidase conjugate (Sigma) 0.25µg/ml (Forsgren and Sjörnquist, 1966; Goding, 1978). As an alternative to protein A-peroxidase, blots were either reacted with peroxidase-linked goat anti-rabbit IgG, IgM or IgA (Miles-Yeda, Slough, Bucks). However, control blotting experiments in which sera were omitted showed that protein A-peroxidase produced "cleaner" blots (data not shown), therefore this agent was used in the majority of blotting experiments. After a final three rinses in TBS, antigens on the nitrocellulose membrane were visualized by application of a freshly prepared solution containing 0.1%v/v H_2O_2 (Thornton and Ross Ltd, Huddersfield, Yorks) and 4-chloro-1-naphthol 25µg/ml in 10mM Tris-HCl, pH 7.4. The colour reaction was stopped after 10-15 minutes by flooding the blots with distilled water. A photographic record of results was made immediately.

Reaction of antigens from a particular organism with several sera was carried out by a technique known as strip-blotting. Whole cells were separated by SDS-PAGE on a single-well 12% polyacrylamide gel and the separated components were electrophoretically transferred onto nitrocellulose as described above. The nitrocellulose sheet was then cut into strips (5mm wide). Development of the blots was performed in plastic trays divided into compartments (9cm × 1cm; holding a volume of 5-10ml). This procedure enabled strips bearing replicate patterns of antigens to be reacted with a range of sera or ligands, thus providing a detailed antigenic profile of an organism.

Strip-blotting was also used to study the antibody response of an IE patient to the causative organism. Strips of nitrocellulose bearing the separated antigens from the organism were incubated in serially diluted patient's serum, and then visualized as above. The end point of the

titration was taken as being the highest dilution of serum to produce a visible reaction with the bacterial antigens.

2.2.3.3 **Partial characterization of antigens**

Glycosylated antigens were detected on the nitrocellulose blots either by oxidation with sodium periodate or ligand blotting with lectins.

For sodium periodate oxidation, separated bacterial antigens were immobilized on a nitrocellulose membrane and unbound sites were blocked with TTBS as in section 2.2.3.2. The blot was then incubated in 0.5M sodium periodate, pH 4.0 for 1 hour at 37°C. After washing in TBS and incubation in serum the blots were visualized with 4-chloro-1-naphthol/H_2O_2 as usual. Control experiments were performed in which TBS, pH 4.0 was substituted for sodium periodate in the initial incubation stage.

Ligand blotting with lectins followed the same basic procedure as immunoblotting (see section 2.2.3.2) using a modification of the method of Glass et al (1981). Instead of incubation in serum for 3 hours, the blots were incubated in horse radish peroxidase-conjugated lectins 0.25µg/ml in TTBS for 2 hours at 37°C. This stage was followed by visualization with 4-chloro-1-naphthol/H_2O_2. Details of the peroxidase-linked lectins, all obtained from Sigma were as follows:-

1. Concanavalin A (con A), isolated from *Canavalia ensiformis* (jack bean), has a sugar specificity for α-D-mannose and α-D-glucose (Reeke et al, 1974).
2. Wheat germ agglutinin (WGA), isolated from *Triticum vulgaris*, has a specificity for N-acetyl-D-glucosamine and N-acetyl-neuraminic acid (Greenway and Levine, 1973; Nagata and Burger, 1974).
3. Soybean agglutinin (SBA), isolated from *Glycine max*, has a specifity for N-acetyl-D-galactosamine (Lis et al, 1970).

4. Asparagus pea lectin (APL), isolated from *Tetragonolobus purpurea*, has a specificity for α-L-fucose (Pereira and Kabat, 1974).

The proteinaceous nature of *S. faecalis* antigens was examined by their susceptibility to digestion with proteolytic enzymes (trypsin, chymotrypsin and proteinase K). The method of Morris et al (1985) was used to trypsin-treat whole cells. Whole cells of strain EBH1 were suspended in 50mM HEPES buffer, pH 7.2 to OD_{470} = 5.0. The suspension was incubated for 1 hour at 37°C after the addition of trypsin (type XI from bovine pancreas; Sigma) to a final concentration of 300μg/ml. Controls were performed using trypsin that had been inactivated at 100°C for 30 minutes. Cells were recovered by centrifugation at 10,000 rpm for 10 minutes. The cells were washed twice in 50mM HEPES buffer, pH 7.2 containing 1M NaCl, and resuspended in HEPES to their original volume.

Whole cells were treated with proteinase K (Sigma) using the method of Hitchcock and Brown (1983). Cells were suspended in 10mM Tris-HCL, pH 7.4 to OD_{470} = 5.0. To 50μl of this suspension was added 30μl of sample buffer (see section 2.2.3.1). This mixture was heated at 100°C for 10 minutes and then allowed to cool to below 60°C. 10μl proteinase K (Sigma) 2.5mg/ml was added and the mixture was incubated at 60°C for 1 hour.

Chymotrypsin treatment of cells followed the method of Jenkinson (1986). 10mg α-chymotrypsin (BDH) was added to 1ml whole cells OD_{470} = 5.0 in 10 mM Tris-HCl, pH 7.4. The mixture was incubated at room temperature for 20 minutes. Distilled water was added and the digested cells were pelleted by centrifugation at 10,000 rpm for 10 minutes. The pellet was resuspended in 10mM Tris-HCl, pH 7.4 containing 1mM PMSF. Centrifugation was repeated and the resulting pellet was resuspended to 1ml in this solution.

Enzyme-treated cells were subjected to analyses by SDS-PAGE and immunoblotting.

2.2.3.4 **Autoradiography**

Radioiodinated and control whole-cell components of *S. faecalis* strains were separated by SDS-PAGE. Immunoblots were also prepared. Gels were stained with Coomassie blue (see section 2.2.3.1) and dried between cellophane and chromatography paper, using a slab gel drier (Bio-Rad). After development with hyperimmune serum, nitrocellulose blots were dried in hot air. The dried gels and blots were used to prepare autoradiographs. Autoradiography was performed using Singul X-RP medical X-ray film (Caeverken, Strängnäs, Sweden). The film was placed in contact with the gel or blot for at least a week, in the dark at room temperature, and then developed to reveal radioactive proteins. ^{125}I-lactoperoxidase is a large molecule (molecular weight 77,500) and so was assumed not to cross the cytoplasmic membrane and pass into the bacterial cell. It labelled only those proteins exposed on the cell surface which contained tyrosine or histidine residues (Lambert and Booth, 1982).

2.2.3.5 **Double diffusion immunoprecipitation**

The method of Ouchterlony (1958) was used. Tris-barbiturate buffer, pH 8.6 was prepared by adding barbitone 22.4g, Tris 44.3g, calcium lactate 0.533g and sodium azide 0.65g to 1L distilled water. This buffer was diluted 1:5 with water before use. To prepare agarose gels for use in the double diffusion immunoprecipitation technique, 1%w/v agarose M (gelling temperature 42°C: LKB) in Tris-barbiturate buffer was boiled in a covered beaker until the agarose had completely dissolved. The molten agar was cast onto the hydrophilic side of gel bond support film (LKB). The volume to be cast, which gave a suitable gel depth, was determined as follows:-

volume (ml) = area of gel bond (cm^2 × 0.132)

When set, wells were made in the gel using a No. 2 cork borer (5mm diameter). Antiserum was added to the central well; 25µl-volumes of antigen preparations were added to the peripheral wells. The gel was incubated for 18-24 hours at 4°C in moist chambers and then stained for protein to reveal precipitated antibody-antigen complex. To remove non-precipitated proteins the gel was pressed and washed several times with saline (Weeke, 1973a). Layers of moist chromatography paper (Whatman Ltd, Maidstone, Kent) were placed over the gel and a thick layer of dry absorbent tissue was placed on top. These layers were sandwiched between two glass plates and the gel was pressed to a thin film by even application of a 3kg weight for 10 minutes. The agarose gel was reconstituted by soaking in 0.9%w/v NaCl for 10 minutes. The pressing and washing stages were repeated three times. After a fourth pressing the gels were washed in distilled water to remove NaCl from the gel. The gel was then pressed for the final time and dried in hot air. Immunoprecipitin lines were revealed by staining with Coomassie blue for

10 minutes (Coomassie brilliant blue R-250:Sigma 1g, 96%v/v ethanol 90ml, glacial acetic acid 20ml and distilled water 90ml). Background staining was removed from the gel with destain (96%v/v/ ethanol 90ml, glacial acetic acid 20ml and distilled water 90ml).

2.2.3.6 Crossed immunoelectrophoresis (CIE)

CIE was performed as described by Weeke (1973b). In this study the method of CIE with an intermediate gel (CIEWIG) was used (Axelsen, 1973). A 6cm x 6cm gel (1%w/v agarose M in Tris-barbiturate buffer) was cast as described in section 2.2.3.6. Using a No. 2 cork borer a well was punched in one corner and the gel was placed on a flat-bed immunoelectrophoresis unit, water-cooled to 10°C. Complete contact between the gel bond film and the flat-bed unit was achieved by placing a drop of liquid paraffin underneath the gel bond. This ensured efficient cooling. Paper wicks (Whatman 4Chr chromatography paper) soaked in Tris-barbiturate buffer, pH 8.6 were used to connect the gel with the electrode buffer (also Tris-barbiturate buffer). Soluble bacterial antigen preparation (500µg in 25µl) and 0.5%w/v bromophenol blue solution (1µl) were placed in the well. Electrophoresis was carried out in the direction of the anode at 10 V/cm at 10°C until the dye had moved a distance of 4cm (≃ 2 hours). An incision was made across the agarose gel parallel to the direction in which electrophoresis had been carried out, approximately 1.5cm from the edge of the gel. The excess gel was removed leaving a strip of gel containing the separated bacterial antigens. A barrier formed by a strip of plastic, 1cm wide, was secured across the gel bond film adjacent to the strip of gel. Onto the free section of gel bond was cast a molten gel of 1%w/v agarose M cooled to 50°C, containing anti-*S. faecalis* serum 1:50 in Tris-barbitone buffer, pH 8.6. The antiserum was evenly dispersed throughout the gel by

prior thorough mixing. When the gel had set, the plastic barrier strip was removed and replaced with a 1%w/v agarose M gel (intermediate gel). The intermediate gel was either blank (to increase resolution) or contained standard antibody or antigen preparations for identification purposes. The gel was then returned to the flat-bed immunoelectrophoresis unit at 90° to its original position with the agarose strip carrying the separated antigens positioned towards the cathode. Electrophoresis was carried out through the intermediate gel and serum-containing gel at 2 V/cm for 18 hours at 10°C. The gel was then pressed, washed, dried and stained for immunoprecipitates as described in section 2.2.3.5.

In an attempt to reduce background staining in the upper section of the gel caused by residual serum proteins, purified immunoglobulins were use instead of whole serum. The method of Fahey and Terry (1978) was used to fractionate serum to obtain immunoglobulins. Ion exchange chromatography was performed using a "batch" centrifugation method (Stanworth, 1960). DEAE cellulose (diethylaminothyl microgranular preswollen cellulose, DE 52; Whatman Biochemicals Ltd, Maidstone, Kent) was washed with distilled water on a Buchner funnel. To the DE 52 was added 5mM phosphate buffer, pH 6.5. This buffer was prepared as follows:-

Solution A = NaH_2PO_4. $2H_2O$ 31.2g, distilled H_2O to 1L

Solution B = Na_2HPO_4 (anhydrous) 28.4g, distilled H_2O to 1L

171ml Solution A + 79ml Solution B was diluted 1 in 10 with distilled water before use.

The pH of the DE 52 suspension was adjusted to 6.5 by the addition of 10mM HCl and washed with several changes of buffer. The DE 52 slurry was placed in a 50ml Beckman centrifuge tube and sedimented by centrifugation at 2,000 rpm for 5 minutes. Serum to be used in CIE was

dialysed against 5mM phosphate buffer, pH 6.5 at 4°C for 18 hours. The dialysed serum was added to the DE 52 sediment and mixed by gentle rotation for 1 hour at room temperature. The cellulose was then sedimented by centrifugation at 2,000 rpm for 5 minutes and the supernatant was carefully transferred to a clean centrifuge tube. The supernatant was re-spun to remove any remaining DE 52 and the resulting supernatant containing immunoglobulins was decanted and retained at -20°C for use in CIE.

2.2.3.7 **Fluorescence microscopy**

The technique of fluorescence microscopy used a fluorochrome-labelled antibody to demonstrate the presence of antigenic determinants on the surface of *S. faecalis* in its native state. EBH1 was grown in BHI (Difco) to late log phase. This growth medium/growth phase provided a compromise between maximal numbers of cocci per chain and maximal antigen expression. Cells were taken directly from the growing culture and 10µl samples were placed in wells on a PTFE-coated Hendley-Essex microscope slide (12 × 10µl wells per slide). The bacterial film dried quickly and was fixed with acetone. The cells were labelled with fluorescein indirectly. In this method the fixed bacteria were first treated with unlabelled test human or rabbit serum. This was followed by treatment with fluorescent species-specific anti-immunoglobulin. The presence in the test serum of antibodies specific against bacterial surface antigens caused the fluorescent antibody to be fixed to the complex and to fluoresce under UV illumination.

Test or control serum was diluted 1:2 in PBS (phosphate buffered saline containing NaCl 8.5g, Na_2HPO_4 1.28g, NaH_2PO_4 0.156g and distilled water to 1L), pH 7.6. This dilution of serum had been previously determined by titration experiments. Aliquots of the diluted serum were

pipetted onto the fixed bacterial film in the wells on the Hendley-Essex slides. The slides were incubated at 37°C for 30 minutes in humid chambers. Excess serum was removed, taking care that cross-contamination did not occur between different sera. The slides were washed by gentle agitation in two changes of PBS for a total of 10 minutes, followed by a final rinse in distilled water. Excess liquid was blotted from around the wells with tissue, taking care not to disturb the antigen film. Fluorescent antibody in PBS was then added to each well and the slides were incubated at 37°C for 30 minutes in humid chambers. If the test serum was of human origin, the labelled serum used was fluorescein isothiocyanate (FITC)-conjugated goat anti-human immunoglobulin (polyvalent; Tissue Culture Services Ltd, Slough, Bucks), diluted 1:110 in PBS containing 2%v/v Tween 80. If rabbit antiserum had been used initially, the second incubation was in FITC-protein A conjugate (Sigma), diluted 1:100 in PBS containing 2%v/v Tween 80. After incubation in the FITC-labelled antiserum the slides were washed in PBS, rinsed in distilled water and blotted dry as before. Slides were mounted in buffered glycerol (1 volume of PBS, pH 7.2 (NaCl 7.65g, Na_2HPO_4 0.21g, distilled water to 1) + 9 volumes of "analaR" grade glycerol), pH 7.2. Slides were examined as soon as possible using a fluorescence microscope.

2.2.3.8 **Isolation of *S. faecalis*-specific antigens**

Isolation of the *S. faecalis*-specific 73, 40 and 37K antigens was attempted with the eventual aim of developing an ELISA method for the serodiagnosis of endocarditis. Two approaches were used: firstly, protein precipitation using high salt concentrations and secondly, gel filtration chromatography of soluble antigen preparations.

2.2.3.8.1 **Ammonium sulphate precipitation**

This method was based on the fact that at high salt concentrations proteins differ in solubility (Scopes, 1982a).

Early stationary-phase cells of EBH1 grown in 60% HS were harvested, washed and resuspended in 10mM Tris-HCl, pH 7.4. Cells were digested with mutanolysin or extracted with Sarkosyl as described in section 2.2.2.4. To 10ml of digested cells (2mg/ml) was added 100mg streptomycin sulphate (Glaxo Laboratories Ltd, Greenford, Middx) The suspension was incubated at room temperature for 30 minutes and then centrifuged at 15,000 rpm for 10 minutes. The pellet was reconstituted in distilled water and then dialysed for 48 hours against water. To the supernatant was added an amount of $(NH_4)_2SO_4$ equivalent to a 30% saturated solution (see calculation below). The suspension was vortex-mixed, incubated for 30 minutes at room temperature and then centrifuged at 15,000 rpm for 10 minutes. The pellet was again reconstituted in distilled water and dialysed. The precipitation was repeated using saturated concentrations of $(NH_4)_2SO_4$ of 60 and 90%. The final 90% supernantant was dialysed against distilled water for 48 hours, as were the pellets obtained at each precipitation stage. Alternative saturated concentrations of $(NH_4)_2SO_4$ used were 40, 60, and 80%. Following dialysis the pellets and final supernatant were lyophilized, resuspended in 200µl distilled water and stored at -20°C.

The amount of solid $(NH_4)_2SO_4$ to be addded to a solution already at S_1% saturation to take it to S_2% saturation (Scopes, 1982a) was calculated as follows: weight (g) = $533(S_2 - S_1) \div (100 - 0.3S_2)$

2.2.3.8.2 **Fast protein liquid chromatography**

This method involved separation of proteins according to molecular size.

Two chromatography columns were used:

1. Superose 12 HR 10/30 chromatography column (cross-linked agarose; optimal separation range molecular weight 1,000 - 3×10^5; average particle size 8-12μm; column length 30cm, diameter 1.5cm).
2. Sephacryl S-200 SF chromatography column (cross-linked dextran/bisacrylamide; optimal separation range molecular weight 1×10^3 - 8×10^4; average particle size 40-105μm; column length 25cm, diameter 2cm).

All buffers and samples used were filtered through Millipore or Gelman acrodisc 0.22μm cellulose acetate filters and degassed under vacuum. Prior to use, the column was equilibrated with at least 2 volume changes of buffer. Mutanolysin- or Sarkosyl-solubilized antigen preparations of HS-grown EBH1 (20mg (dry weight)/ml) were separated on the column. Buffer systems used were as follows:-

1. For separation of mutanolysin-digested cells - 10mM Tris-HCl, pH 7.4

   containing sodium azide 0.02%w/v with/without SDS 1%w/v.
2. For separation of Sarkosyl-extracted cells - 10mM Tris-HCl, pH 7.4 containing sodium azide 0.02%w/v and Sarkosyl 1%w/v.

Samples were applied by loop-injection to the top of the Superose 12 column or manually to the top of the Sephacryl S-200 SF column and pumped through with buffer at a rate of 36ml/hour (Superose 12) or 20ml/hour (Sephacryl S-200 SF) using a Pharmacia FPLC system with high precision pump, model P-500. Fractions were collected, dialysed and lyophilized. Each fraction was reconstituted in 10mM Tris-HCl, pH 7.4 (50μl) and stored at -20°C.

3. RESULTS AND DISCUSSIONS

3.1 Immunochemistry of *S. faecalis*

3.1.1 Growth characteristics

It is now well established that there are important differences between bacteria grown *in vivo* and those grown in complex laboratory media *in vitro* (Smith, 1977; Dalhof, 1985). In particular, surface properties related to pathogenicity are influenced by growth conditions (Costerton and Marrie, 1983; Brown and Williams, 1985). Bacteria which colonize the heart valves generally originate from bacteraemia (Parker, 1975; Sipes et al, 1977) therefore it is likely that the surface composition of these cells is influenced by growth in the bloodstream. In this project the surface composition of *S. faecalis* was studied during growth in serum as a first approximation to simulate in-vivo conditions. A comparison was made between these cells and those grown in a nutritionally complex laboratory medium (BHI; Lab M unless otherwise stated) to illustrate the influence of growth conditions upon the surface protein antigens of *S. faecalis*.

Growth characteristics of *S. faecalis* differed markedly in BHI and HS. In BHI a minimal lag phase was followed by a period of rapid growth (SFZ doubling time = 18 minutes; EBH1 doubling time = 20 minutes; figure 8). Exponential growth tailed off abruptly producing a maximum OD_{470} of $\simeq$ 2.0. Onset of stationary phase may be due to a decrease in the pH and oxygen tension of the growth medium (Dr. S.J. Prior, CAMR, Porton Down, Salisbury, Wilts) or accumulation of waste products (Davis et al, 1980). The phases of growth of *S. faecalis* in HS were poorly defined. This was because the organism grew in clumps which were difficult to disperse, preventing

accurate measurement of OD. Growth in HS was slow (EBH1 estimated doubling time ≃ 6 hours) and only reached a maximum OD_{470} of 0.5 (figure 8).

During harvesting cells grown in HS formed an ill-defined, flakey pellet which was difficult to resuspend. BHI-grown cells formed a discrete, easily resuspendable pellet. Other differences between cells grown in these contrasting media include fatty acid composition (N. Gibbons and P. Birani, 3rd year projects, Dept. Pharmaceutical Sciences, Aston University); hydrophobicity (HS-grown cells were more hydrophobic than cells grown in BHI) and ease of cell disruption (HS-grown cells were more resistant to breakage by sonication than were BHI-grown cells).

3.1.2 Antigenic composition

Until comparatively recently studies on bacterial surface proteins have focused on Gram-negative organisms. Lately, however suface proteins of oral streptococci have received considerable attention both for taxonomic purposes and in determining their biological role as salivary agglutinin receptors (Nesbitt et al, 1980; Zanders and Lehner, 1981; Applebaum and Rosan, 1984; Ogier et al, 1984; Jenkinson, 1986; Morris et al, 1986; Robinovitch et al, 1986). The fibrillar M-protein of group A streptococci has also been studied extensively in relation to its antiphagocytic property and its possible role in mediating attachment to host cells (Lancefield, 1962, Ellen and Gibbons, 1972 and 1974; Parker, 1975; Smith, 1977; Beachey et al, 1983; Oakley et al, 1985; Whitnack and Beachey, 1985). Surface proteins in *Staphylococcus aureus* and *Streptococcus pyogenes* have been related to colonization (Espersen and Clemmensen, 1982; Speziale et al, 1984). In this study SDS-PAGE, immunoblotting, ligand blotting and CIE were used to investigate the surface composition and antigenicity of *S. faecalis*, a group D *Streptococcus*.

3.1.2.1 **Protein profile of *S. faecalis***

Proteins of known molecular weight were separated by SDS-PAGE on a 12% polyacrylamide gel. Their resulting Rf values were plotted versus $\log_{10}$ molecular weight to construct a calibration curve (figure 9). Proteins used in the construction of the calibration curve were as follows:-

Electran molecular weight marker 112K; Phosphorylase B (Sigma) 97.4K; bovine serum albumin (Sigma) 68K; *Pseudomonas aeruginosa* (PA01) outer membrane protein (OMP) D 46K; PA01 OMP F 41K; PA01 OMP G 25K; PA01 OMP H_1 21K and PA01 OMP H_2 20.5K (PA01 OMP's were kindly provided by Dr. H.L. Jessop, Aston University).

For construction of a calibration curve for native PAGE the following proteins were separated on a 10% polyacrylamide gel using non-denaturing conditions:-

Jack bean urease tetramer 480K; jack bean urease dimer 240K; bovine albumin dimer 132K; bovine albumin monomer 66K chicken egg albumin 45K; carbonic anhydrase 29K and α-lactalbumin 14.2K (nondenatured protein molecular weight marker kit; Sigma).

Preliminary SDS-PAGE analysis of *S. faecalis* whole cells and cell walls revealed that cells grown in HS exhibited a simpler protein profile than cells grown in a complex laboratory medium (figure 10, lanes 1-4). Cell walls had fewer proteins than whole cells (lanes 5 and 6). Further purification of cell walls to remove cytoplasmic and membraneous contaminants proved unsuccessful (data not shown). It was therefore accepted that together with genuine cell-wall proteins, the protein profile of *S. faecalis* cell walls may show bands of cytoplasmic or membrane origin.

The whole-cell protein profiles of five strains of *S. faecalis* grown in BHI were basically similar (figure 11, lanes 1 and 5-8). Major proteins of molecular weight 77,000 (77K), 73, 65, and 53K appeared in all strains of *S. faecalis* but not in *Staphylococcus aureus* NCTC 6571 (lane 9). Growth of

strain EBH1 in HS produced a simpler pattern (lane 3). Some proteins were of similar molecular weight to those of BHI-grown cells:- 77. 73, 65, 60, 56, 53, 44, 43, 40 and 37K. Two high molecular weight proteins (97 and 96K) were detected only in HS-grown cells and were possibly serum proteins bound to the cells during growth. A third group of proteins :- 69, 47, 38, 28.5, 23 and 21K were not detected by Coomassie blue in HS-grown cells.

Extraction of proteins with lysozyme, Na_2EDTA or urea was unsuccessful (figure 12, lanes 1-6). These treatments did not release any more protein from whole cells of *S. faecalis* than did washing with 10mM Tris-Hcl, pH 7.4 (lanes 9 and 10). SDS did, however, extract most of the protein from the whole cells (lanes 7 and 8). But since analysis of cells by SDS-PAGE includes in-process treatment with SDS, it was decided that in future SDS-PAGE and immunoblotting studies, whole cells of *S. faecalis* would be used without further treatment.

3.1.2.2. **Protein antigens**

For immunoblotting experiments hyperimmune rabbit serum or serum from human patients was used to probe blots prepared from separated *S. faecalis* antigens. Rabbit antiserum was raised as described in section 2.4, against strain EBH1 grown in BHI (first pair of rabbits; rabbits 1 and 2) or in HS (second pair of rabbits; rabbits 3 and 4). The anti-EBH1 antibody titre was determined by slide agglutination test performed on serum collected from the rabbits' ears. After 8 weekly injections of antigen preparation the titre was as follows:-

rabbit 1 128

rabbit 2 256

rabbit 3 128

rabbit 4 128

where titre = reciprocal of highest serum dilution producing a visible slide agglutination reaction.

There was a slight variation between antiserum from rabbit 1 and 2 in the recognition of antigens (figure 13a and 13b). Whereas rabbits 3 and 4 had a similar immunological perception of *S. faecalis* (figures 14a and 14b). In subsequent experiments using hyperimmune rabbit serum, antisera from rabbits 1 and 2 were pooled as were antisera from rabbits 3 and 4 (henceforth referred to as rabbit 1/2 serum and rabbit 3/4 serum respectively).

Sera were collected from the four rabbits prior to immunization (pre-immune sera). Strip-blotting was used to compare the pre-immune with the post-immune antibody response (figures 15a and 15b). Before the immununization regime was started, rabbit 4 showed no antibody response to *S. faecalis* (lanes 5 and 8). Rabbit 2 and 3 pre-immune serum however, reacted very weakly with a 21K antigen in the BHI-grown cells (lanes 6 and 7), and rabbit 1 with a 63K antigen (lane 5). This may have been due to cross-reaction with an antigen contained in the rabbits' own commensal flora or in an organism previously or currently infecting the rabbits.

Similar strip-blots were used to compare pre-immune and post-immune sera collected from pairs of rabbits immunized to raise monospecific antisera, with antigens excised from nitrocellulose blots (as described in section 2.2.2.5.2). Rabbits A and B were immunized with nitrocellulose bearing the 56 and 53K antigens of HS-grown EBH1; rabbits C and D were immunized with the 40 and 37K antigens. Production of antisera monospecific against the 56 and 53K antigens in rabbits A and B was achieved with a fair degree of success (figure 16, lanes 1 and 2, post-imm section), the sera only reacted faintly with an antigen in the 70-75K region in addition to the 56/53K antigens (rabbit A serum less so than rabbit B serum). Pre-immune sera from rabbits A and B did not react with any EBH1 antigens

(lanes 1 and 2, pre-imm section). Monospecific serum raised against the 40 and 37K EBH1 antigens that had been produced in rabbit D (lane 4, post-imm section) only reacted with the 40 and 37K antigens. But anti-40/37K rabbit serum from rabbit C reacted with a 56K antigen in addition to the 40 and 37K antigens(lane 3, post-imm section). This was explained by the fact that pre-immune serum from rabbit C reacted faintly with the 56K (lane 3, pre-imm section) antigen. Immunization with the nitrocellulose bearing the 40 and 37K antigens apparently heightened the immunological response of rabbit C to the 56K antigen. Henceforth if monospecific antisera were used for immunoblotting or CIE experiments, rabbit A (anti-56/53K) and rabbit D (anti-40/37K) sera were used.

The antigenic profile of *S. faecalis* was revealed using hyperimmune rabbit serum and serum from patients with *S. faecalis* endocarditis in immunoblotting experiments.

Rabbit 1/2 antiserum was used to probe a nitrocellulose blot prepared from samples separated by SDS-PAGE as shown in figure 11. *S. faecalis* was shown to have a complex antigenic profile (figure 17). A major antigen of 53K was prominent amongst numerous other bands and was present in all *S. faecalis* strains but not in *Staphylococcus aureus* NCTC 6571. This antigen, however, was not a major feature of Coomassie blue-stained gels. This demonstrates that immunoblotting is an extremely sensitive technique, a fact highlighted by comparison of figures 11 and 17: the same loading volumes of each sample was used in each case but the gel is much less well visualized than the immunoblot, thus indicating that the proteins involved are very strongly antigenic and/or Coomassie blue is a comparatively insensitive stain for *S. faecalis* proteins and/or possibly a non-protein moiety is the immunodominant component of the antigen. Several other antigens were also common to BHI-grown *S. faecalis* strains, in particular antigens of molecular weight 87, 77, 75, 65, 63, 59, 49.5, 47, 30 and 21K.

Rabbit 1/2 serum reacted with several similar antigens in HS-grown cells in the 47-87K molecular weight range.

The antigenic pattern revealed by rabbit 3/4 serum was much simpler (figure 18). Two major antigens were a prominent feature of the immunoblot (56 and 53K), and were present in cell walls (lanes 2 and 4). The reaction produced with rabbit 3/4 serum was much stronger in whole cells and cell walls of HS-grown *S. faecalis* than with BHI-grown whole cells and cell walls. Other bands revealed in the HS-grown cells and walls were 73, 40 and 37K (lanes 2 and 4).The reaction of serum-grown cells/walls with homologous serum was so strong that perhaps other bands were masked, especially in the 56-100K region.

A range of *S. faecalis* strains were separated by SDS-PAGE. The separated antigens were electrophoretically transferred onto nitrocellulose and reacted with serum collected from the "hyperimmune" IE patient, Mr. P. The antigenic profile of strain EBH1 grown in HS was very similar to that of EBH1 grown in NHS (normal human serum; figure 19, lanes 1 and 2). Only minor differences in band intensities were observed. Also very similar to EBH1 were the antigen patterns of HS-grown *S. faecalis* strains SFZ, NCTC 5957 and 777 (lanes 3-5). Strain 790 differed from these strains only in that the 40K antigen was recognised relatively faintly (lane 7). The other *S. faecalis* and *S. faecium* strains were antigenically heterogenous. Urinary tract isolate strain 741 was poorly recognised by Mr. P's serum, only the 68, 56, 53 and 37K antigens reacting (lane 6). In the antigen profiles of the septicaemia isolates *S. faecalis* strains SFBA and SFSQ, the 37K antigen elicited the strongest reaction with the serum (lanes 8 and 9). The 65K antigen of SFBA (lane 8) and the 68 and 56K antigens of SFSQ (lane 9) were also visualized. *S. faecalis* strain SFBI, isolated from a wound swab, possessed the 73, 68 and 53K antigens (lane 10). Lane 11 contained *S. faecalis* strain 9112, isolated from the bloodstream of Mr. P. Even though this patient's serum was used to probe the blot, antigens of strain 9112 were not strongly recognised. Strain 9112 possessed the 73, 68, 56, 53, 43 and 37K antigens which, along with the 40K antigen (absent in this case) appeared to form a pattern characteristic to *S. faecalis* species. Lanes 12 and 13 contain *S. faecalis* and *S. faecium* respectively, both isolated from the blood of a patient with IE. The 68 and 56K antigens were very weakly present in the *S. faecalis* strain (lane 12). The *S. faecium* strain also weakly expressed these antigens in addition to an antigen of 40K (lane 13). Only antigens of 68 and 56K were observed in the reference strain of *S. faecium* NCTC 7171 (lane 14).

Other streptococcal species were examined by immunoblotting to compare antigen patterns. Figure 20 shows an immunoblot prepared from separated antigens of several strains of *S. faecalis* and also other streptococcal species obtained from a variety of sources. Probing the blot with rabbit 3/4 serum revealed that the *S. faecalis* strains had antigenic profiles that were basically similar (lanes 1-6). The other streptococcal species differed from *S. faecalis* in that the 73, 40 and 37K antigens were absent (lanes 7-14). The β-haemolytic group A *Streptococcus* did, however, appear to posses the 73K antigen (lane 11). The *Staphylococcus aureus* IE isolate (lane 15) possessed five antigens which were also common to the streptococci (87, 68, 62, 56 and 26K). The 73, 40 and 37K antigens therefore appeared to be characteristic of *S. faecalis* (although not all three were possessed by certain strains) and were absent in other streptococcal species and *Staphylococcus aureus*.

It was noted that during the course of this study a change in antigen expression had occurred. Upon repeated subculture the antigenic dominance shifted from the 56 and 53K antigens to the 40 and 37K antigens of HS-grown cells. This can be seen by comparing figures 19 and 18.

The antigenic profile of HS-grown EBH1 in an undenatured state was revealed by using rabbit 3/4 serum, monospecific sera A and D, and serum collected from a patient with *S. faecalis* endocarditis to probe strip-blots of HS-grown EBH1 antigens separated by native PAGE. The antigenic profile thus revealed (figure 21) was vastly different to that of cells separated by SDS-PAGE. Patient and rabbit 3/4 sera showed the native antigens to be high molecular weight, separating as two diffuse bands concentrated approximately in the 800K and 140K region (lanes 1 and 2). Patient serum also reacted with a band of lower molecular weight (lane 1). Monospecific

antisera A and D both reacted with discrete bands of molecular weight ≈ 500K and 140K (lanes 3 and 4). This suggested that the 56, 53, 40 and 37K antigens, possibly with others, existed as part of a high molecular weight complex in their native state.

3.1.2.3 **Glycosylated antigens**

To provide a detailed investigation of the antigenic composition of *S. faecalis*, replicate strip blots were prepared from separated components of IE isolate strain EBH1. The strips were reacted with sera collected from two patients with *S. faecalis* endocarditis (from one of whom strain EBH1 was originally isolated; Mr. W), hyperimmune rabbit serum or lectins. Protein transfer to nitrocellulose was efficient; even proteins of low molecular weight were detected on the amido black-stained strip (figure 22). This was in contrast to the findings of Lin and Kasamatsu (1983) who reported that low molecular weight proteins passed through nitrocellulose (pore size 0.45μm).

The antigenic and lectin-receptor patterns of whole cells of strain EBH1 grown in BHI are shown in figure 22. A strong reaction (lane 9) was obtained with serum collected from Mr. P. This patient was undergoing a third episode of IE when this serum sample was collected. In all three episodes of IE, *S. faecalis* strain 9112 was isolated as the causative organism. Following consultation with the physician attending Mr. P, the conclusion was drawn that antibiotic therapy (benzyl penicillin in combination with gentamicin) had failed to eradicate this organism from the vegetation on the heart valve. Thus, strain 9112 remained as a slow growing focus for re-infection. So a situation was presented which was analagous to an immunization programme and to all intent and purpose Mr. P may be regarded as "hyperimmune"! This patient's serum reacted with antigens of EBH1 with a level of intensity similar to rabbit 1/2

serum (lane 10). Rabbit 1/2 serum and serum from Mr. P both recognised many antigen bands in common. Two antigens of low molecular weight (16 and 17K) detected as strong bands with the human serum were not detected in the strip probed with rabbit antiserum. Several other dissimilarities existed between the immunological response to BHI-grown EBH1 antigens of the rabbit and human. These mainly involved different reaction intensities of the sera to individual antigen bands (87, 77, 65, 45, 33, 27, 23 and 21K).

Serum from Mr. W showed a much weaker response (lanes 6-8) to BHI-grown EBH1, even though his IE had been caused by this organism. Only bands of molecular weight 87, 80, 77, 43, 37 and 25K were recognised. The final serum sample collected from this patient two weeks after commencement of antibiotic therapy appeared to produce a stronger reaction with the 80K antigen (lane 8). The immune response of IE patients to *S. faecalis* will be discussed further in section 3.2.1.

Several of the antigen bands that reacted with rabbit and human sera also bound lectins. Most particularly the 43, 37, 34 and 25K antigens reacted with all four of the lectin-peroxidase conjugates used (lanes 2-5); the strongest reaction was with APL. In control experiments, the reaction of the lectin-peroxidase conjugates with antigen bands on blots was completely inhibited by incubating the lectin conjugate with the sugar specific for the particular lectin, for 1 hour at 37°C prior to development of the blot (concentration of sugar = 2.5µg/ml ≡ 10 × concentration of lectin-peroxidase conjugate). In control studies:- APL-peroxidase was pre-incubated with α-L-fucose; WGA-peroxidase with N-acetyl-D-glucosamine; SBA with N-acetyl-D-galactosamine, and con A with α-D-mannose and α-D-glucose.

Other BHI-Grown EBH1 antigens reacting with the four lectins included those of molecular weight 73, 56, 33, 25 and 21K. WGA (lane 2), con A (lane 3) and SBA (lane 5) reacted to a lesser extent than APL (lane 4) with the 43 and 37K antigens. General intensity of reaction of lectins with *S. faecalis* antigens, in order of decreasing strength, was as follows:

APL > WGA > SBA > con A

This did not necessarily represent the relative quantities of corresponding sugar residues present in the antigens, however, due to the differing affinities of the lectins for their specific sugars and the differing activities of the lectin-peroxidase conjugates (product data sheets; Sigma). A summary of the glycosylated antigens identified in *S. faecalis* by this method is shown in table 5.

The technique of strip-blotting was also used to examine the antigenic composition of whole cells of strain EBH1 grown in HS (figure 23). Considerably fewer antigens of HS-grown EBH1 reacted with rabbit 3/4 serum than did antigens of BHI-grown cells with rabbit 1/2 antiserum (figure 22). The main antigens revealed were 73, 56, 53, and 37K. Also diffuse regions of >200K and 73-100K were "lit-up" by rabbit 3/4 serum. Serum collected from the "hyperimmune" patient, Mr. P, reacted with a similar level of intensity to rabbit 3/4 serum (lane 9) and revealed a simple antigenic pattern. The 56 and 53K antigens were a prominent feature of this strip. Indeed, apart from a very faint reaction wiith antigens in the 73-100K region, these were the only antigens with which serum from Mr. W reacted (lanes 6-8). Mr. W's antigenic response increased as antibiotic treatment for IE progressed.

The simplicity of the antigenic pattern of HS-grown EBH1, as revealed by rabbit and human sera, was mirrored by the reaction of antigen bands with the lectins (figure 23, lanes 2-5). A noticeable feature was the very strong reaction of con A with antigens in the 60-100K region (lane 3). Con A also reacted with high molecular weight material and also with the 43K

TABLE 5

Glycosylated Antigens of *S. faecalis* Identified by Ligand Blotting With Lectins

BHI-grown EBH1		HS-grown EBH1	
MOLECULAR WEIGHT OF ANTIGEN (10^3)	GLYCOSYL RESIDUE	MOLECULAR WEIGHT OF ANTIGEN (10^3)	GLYCOSYL RESIDUE
77	FUC. GLUC. MAN. Ngluc. Ngal.	100-60	GLU. MAN.
		100	Ngluc.
73	FUC. GLUC. MAN. Ngluc. Ngal.	73	FUC. GLUC. MAN. Ngluc. Ngal.
56	FUC. GLUC. MAN. Ngluc. Ngal.		
43	FUC. GLUC. MAN. Ngluc. Ngal.	43	FUC. GLUC. MAN. Ngluc. Ngal.
34	FUC. GLUC. MAN. Ngluc. Ngal.		
33	FUC. GLUC. MAN. Ngluc. Ngal.		
25	FUC. GLUC. MAN. Ngluc. Ngal.		
21	FUC. GLUC. MAN. Ngluc. Ngal.		

KEY:

FUC. = ∝-L-fucose

GLUC. = ∝-D-glucose

MAN. = ∝-D-mannose

Ngluc. = N-acetyl-D-glucosamine

Ngal. = N-acetyl-D-galactosamine

antigen. The high molecular weight material also provided a strong reaction with rabbit 3/4 serum and was assumed to be glycosylated serum proteins which had bound to EBH1 cells during growth. This assumption arose from preliminary SDS-PAGE and immunoblotting experiments using cells of HS-grown *S. faecalis* which had not been thoroughly washed after harvesting. Further washing of cells reduced the amount to the steady level shown. Other lectins reacted with antigens of 100, 73 and 37K, indicating the presence of sugar residues in these antigens (summary given in table 5. But only a very faint reaction was observed with SBA.

Because of the lack of previous reports of α-L-fucose-containing antigens in *S. faecalis*, the reaction of APL with this organism was examined further. A blot containing separated whole cells and cell walls of *S. faecalis* strains was probed with APL-peroxidase (figure 24). APL reacted strongly with the 73K antigen in whole cells and cell walls of EBH1 grown in serum (lanes 3 and 4). Also a weaker reaction occurs with the 37K antigen. The latter reaction was common to whole cells and cell walls of BHI-grown EBH1 (lanes 1 and 2); and whole cells of BHI-grown SFZ (lane 5) and two of the urinary isolates (lanes 6 and 7). It was noted with interest that α-L-fucosyl residues were also found in the 45, 40 and 34K antigens of the *Staphylococcus aureus* strain (lane 9).

Similar strip-blot analyses were obtained for *S. faecalis* strain 9112, (figure 25a) and for strain EBH1, probing with different sera to the above (figure 25b). Mr. P's sera (collected at intervals during his third episode of *S. faecalis* endocarditis) reacted with antigens of 73, 56, 53 and 40K (figure 25a, lanes 1-5); the reaction reaching a steady level of intensity after a weak initial reaction by the first serum sample collected during the episode of IE (lane 1). Sera collected from patients with IE caused by organisms other than *S. faecalis* reacted very weakly, if at all (figure 25a, lanes 6-10). Similarly, antigens of EBH1 did not react (or only reacted

very weakly) with serum taken from a range of patients infected with organisms other than *S. faecalis* (figure 25b, lanes 6-10). It was of interest to note that avidin bound to receptors pesent in strains 9112 (figure 25a, lane 15) and EBH1 (figure 25b, lane 15). This may mean that the receptor actually bound avidin or that the avidin bound to biotinylated components of the bacterial cells.

3.1.2.4 LTA-associated antigens

It was thought possible that some of the *S. faecalis* antigen bands that were revealed with serum on immunoblots, but that were not well defined on Coomassie blue-stained gels might represent streptococcal group D antigen, ie. LTA (Wicken et al, 1963). To establish whether the glycosylated antigens of *S. faecalis* were LTA or LTA-associated proteins, the binding of anti-group D antiserum was investigated. A TTBS-washed nitrocellulose blot bearing separated antigens from group D streptococcal strains, was incubated in anti-group D streptococcal grouping serum (raised in rabbits; Wellcome Diagnostics, Wellcome Foundation Ltd, Dartford, Kent) 1: 50 in TTBS for 3 hours at 37°C. Development of the immunoblot was carried out as described in section 2.2.3.2. Several antigens reacted with the streptococcal grouping serum. In *S. faecalis* strains EBH1, 777 and SFZ, and *S. faecium* NCTC 7171 the 73, 68 and 43K antigens reacted (figure 26, lanes 2-4 and 7), and also the 45K band in strains EBH1 and SFZ (lane 9). These antigens were therefore associated with LTA. Attempts to ascertain whether the LTA moiety of these antigens was a prerequisite for antigenicity (Knox and Wicken, 1973; Wicken and Knox, 1979) proved unsuccessful. The method of Moskowitch (1966) was used to extract the group D antigen from cells of *S. faecalis* (see section 2.2.2.3). There was no difference between the reaction of hyperimmune rabbit serum with antigens of extracted cell and those of unextracted, whole cells. This indicated that either antigenicity was not dependent upon presence of the LTA moiety or that not all the LTA had been extracted from the cells. But oxidation with sodium periodate did not result in a loss of antigenicity (see section 3.1.3), thus indicating the former possibility to be likely.

3.1.2.5 **Antigens revealed by CIE**

The technique of CIE combines initial electrophoretic separation of antigens through an agarose gel followed by electrophoretic separation in a second dimension through agarose containing antibodies (Laurell, 1965). Precipitin lines develop upon the deposition of insoluble antigen-immunoglobulin complexes. The distance through which electrophoresis occurs before deposition of the complex and the intensity of the immunoprecipitin arc are a function of the relative quantities of antigen and antibody present (Laurell, 1972). A precipitin line is formed for each antibody-antigen complexation reaction and appears to be stable to continuing electrophoresis once deposited (Weeke, 1973b). CIE patterns of many bacterial species have been studied (Wadström, 1974; Lam et al, 1983; Smyth et al, 1978; Owen and Kaback, 1979; Niskasaari et al, 1983; Ogier et al, 1985). In this study *S. faecalis*, strain EBH1 solubilized by digestion of whole cells with mutanolysin (Weerkamp and Jacobs, 1982) was analysed by CIE.

Figure 27 shows CIE profiles of EBH1 grown in BHI (figure 27a) and HS (figure 27b). The immunoelectrophoresis of solubilized cells of EBH1 resulted in a complex pattern of precipitin lines. In both HS- and BHI-grown cells a major intense precipitin arc was predominant (designated A, figure 27). Several other, less intense peaks had a similar area (denoting their presence in the cell in similar quantities, but with a lesser antigenicity). Numerous other intese smaller arcs were also present. The differences in antigen expression of EBH1 when grown in contrasting media was not so marked upon CIE analysis as by immunoblot analysis. Approximately 20 precipitin lines were shown in either CIE pattern but slight variations in peak height, shape and intensity were observed. CIE of BHI-grown EBH1 resulted in a large diffuse area of precipitin (designated B, figure 27a), which was not observed in the CIE pattern of HS-grown cells.

Control CIE runs were performed upon cells that had not been subjected to digestion with mutanolysin (figure 28). Essentially the only components of these cells to enter the agarose gel would be soluble and excreted cellular products. The control CIE profile of EBH1 grown in HS (figure 28b) consisted of only 9 immunoprecipitin peaks. The major peaks that were seen in the CIE profile of mutanolysin digested cells (figure 27b) were either absent or were reduced in height and intensity. With BHI-grown EBH1 immunoelectrophoresis of control samples resulted in a CIE profile (figure 28a) fairly similar to that of mutanolysin digested cells (figure 27a). Only one of the major peaks was absent from the control CIE pattern, which consisted of approximately 15 peaks. The other major difference between the patterns was that in the control run, precipitin arc B was not so intense or large.

In an attempt to reduce background staining of residual protein, purified immunoglobulins were used in the upper (reference) section of the agarose gel. CIE patterns thus revealed were similar to those obtained using whole sera. Figure 29 shows an example of EBH1/HS mutanolysin digest revealed with immunoglobulins purified from Mr. P serum. The CIE pattern thus obtained (figure 29a) is comparable to that obtained with whole serum (figure 29b)

Several techniques have been established by which it is possible to identify individual precipitin peaks in CIE profiles (Axelsen, 1973; Weeke, 1973b; Owen, 1981). Two methods were chosen for use in this study - tandem CIE and CIEWIG. In tandem CIE a known antigen is applied in a second well punched in the agarose gel adjacent to the first well. Immunodiffusion is allowed to proceed for 30 minutes at 4°C and then the known and sample antigens are co-electrophoresed. Precipitin peaks related to the known antigen form a double fused peak. The area of the peak formed by the unknown antigen should also be increased since peak area is directly

related to the concentration of antigen (Krøll, 1973). In this study tandem CIE was performed using crude LTA as the reference antigen. Unfortunately no double fused peak was formed. This was possibly due to the fact that LTA did not seem to run successfully on CIE, either against anti-group D streptococcal grouping serum or with patient's serum. The reason for this was not understood, especially since in immunodiffusion experiments the LTA formed precipitates with both of these antisera (figure 30). CIE runs were performed with the well in a central position on the gel to establish whether the LTA moved in a cathodal direction. This was found not to be so.

CIEWIG may be carried out with the addition of either a reference antibody or antigen to the intermediate gel. If an antiserum against a specific antigen is incorporated into the intermediate gel the result is that the precipitin peak formed by this (or a related) antigen will partially or completely drop from the upper gel into the intemediate gel, since peak height/area is inversely related to the amount of antibody present (Axelsen, 1973). In this manner the group D antigen, LTA, was identified in the CIE profile of *S. faecalis*. Anti-group D streptococcal grouping serum was incorporated into the intermediate gel. Figure 31 shows that this caused the depression of one immunoprecipitin peak (marked with arrow in figure 31a; in figure 31b - intermediate gel contains no antibodies - the arrow denotes the previous position of the peak). The shape and position of this peak corresponded approximately to those of the precipitin arc formed upon CIE analysis of mutanolysin-digested EBH1 against anti-group D streptococcal grouping serum (contained in the reference gel; figure 32). Possibly the LTA was now in the digest because it was complexed with protein (cf. lipopolysaccharide of Gram-negative cells; Jessop and Lambert, 1985).

CIEWIG was also performed with the aim of identifying the immunoprecipitin peaks corresponding to the 56/53 and 40/37K antigens of

S. faecalis. The immunoelectrophoresis was carried out with monospecific antisera to the pairs of antigens incorporated into the intermediate gels. However, no marked depression of peaks occurred compared to control runs. A possible explanation for this is in CIE, the antigens under investigation were in their native, undenatured state. But the monospecific antisera had been raised against antigens that had been subjected to the denaturing process involved in SDS-PAGE Waeheneldt, 1975). These antigens may not have regained their full natural configuration (or their full biological activity) during immunoblotting (Gershoni and Palade, 1982; Hjerten, 1983). Ideally therefore, antigens used to raise the antisera for incorporation into the intermediate gel should by purified by non-denaturing procedures.

3.1.3 Partial characterization of antigens

Some experimental methods and results shown elsewhere in this thesis also contribute to the partial characterization of *S. faecalis* antigens:- ligand blotting with lectins (section 2.2.3.2); glycosylated antigens (section 3.1.2.3); LTA-associated antigens (section 3.1.2.4) and antigens revealed by CIE (section 3.1.2.5). Additional evidence that *S. faecalis* possessed glycosylated antigens was obtained by showing susceptibility of the antigens to oxidation by sodium periodate. Blots prepared from separated *S. faecalis* antigens were incubated in 0.5M sodium periodate, pH 4.0 for 1 hour at 37°C prior to development. Reaction of hyperimmune rabbit serum with the 37K antigen of strains EBH1 and SFZ was drastically reduced (figure 33). Also the 56/53 and several lower molecular weight antigens reacted with a lesser intensity following periodate treatment. An attempt was made to pre-treat whole cells of EBH1 with sodium periodate before analyses by SDS-PAGE and immunoblotting but these cells would not run on a polyacrylamide gel without distortion of bands occurring.

Blots were prepared from whole cells of EBH1 that had been treated with the proteolytic enzymes trypsin, chymotrypsin and proteinase K. Proteinase K has a powerful proteolytic activity on proteins, glycoproteins and peptides (Ebeling et al, 1974). The activity of this enzyme is increased by SDS (Hilz and Fanick, 1978), and therefore by SDS-PAGE. Trypsin hydrolyses proteins and peptides specifically at the carboxyl end group of basic amino acids (eg. lysine and arginine; Brown and Wold, 1973). Chymotrypsin preferentially hydrolyses peptide bonds involving the L-isomers of tyrosine, phenylalanine and tryptophan (Beauman et al, 1970) and also catalyses the hydrolysis of leucyl, methionyl, asparaginyl and glutamyl resudues.

Proteinase K digestion destroyed all the antigens of EBH1 on the blot (figure 34, lane 3), indicating that all the antigens previously reacting with rabbit or human sera on the immunoblots were proteinaceous in nature or required a protein moiety for antigenicity. Digestion of EBH1 with trypsin and chymotrypsin affected antigen bands to varying degrees. Neither enzyme had any effect on the 40 and 37K *S. faecalis*-specific antigens (lanes 2 and 4). The 73K antigen was sensitive to chymotrypsin but not to trypsin. This showed that the 73K antigen was a glycoprotein containing one, several or all of the following components:- tyrosine, phenylalanine and/or tryptophan; leucyl, methionyl, asparaginyl and/or glutamyl residues. The 40K antigen was a protein that contained none of the above amino acids or residues. The 37K antigen was a glycoprotein that contained none of the above amino acids or residues.

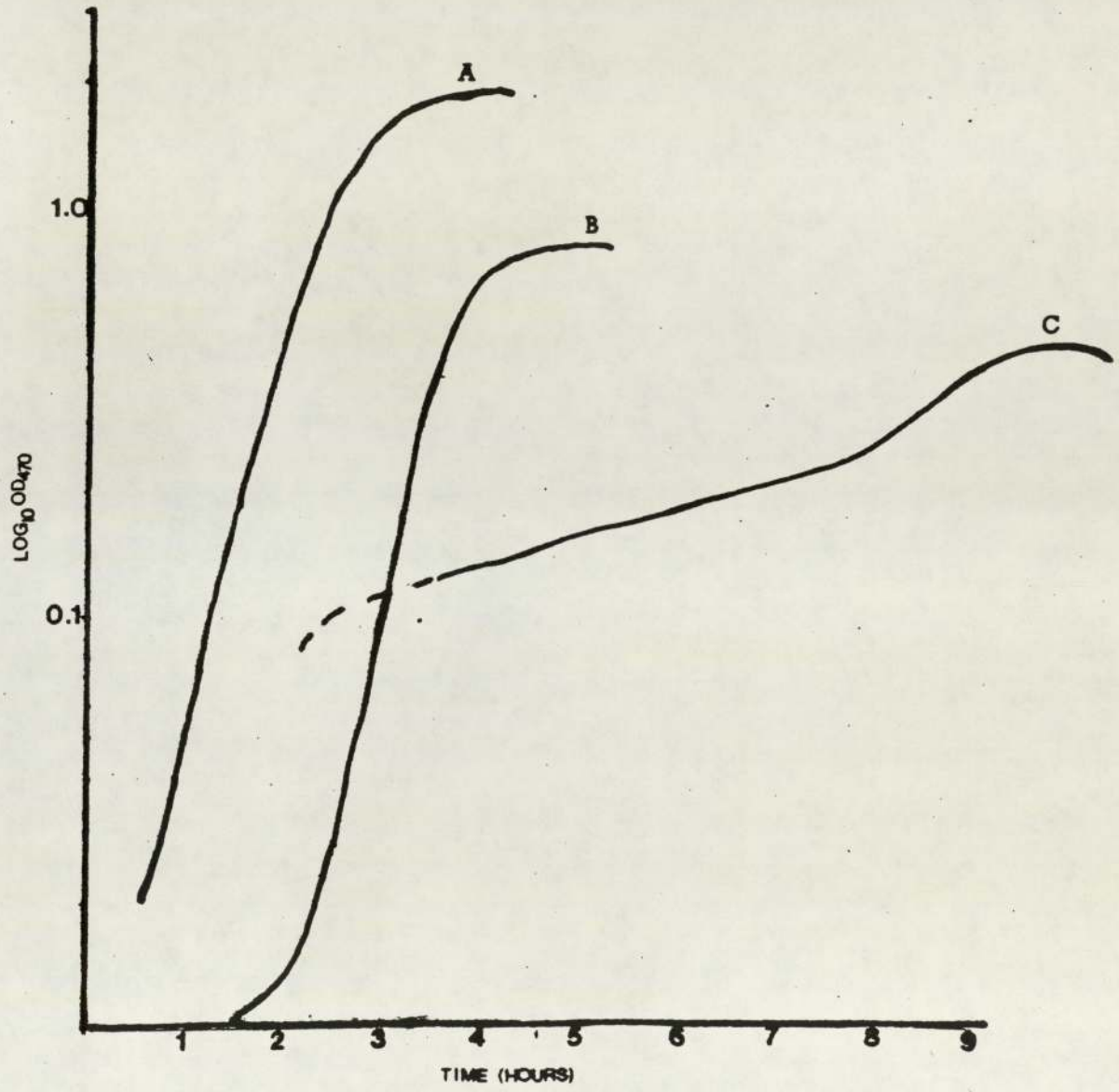


Figure 8 Growth curves of *S. faecalis* strains in BHI or HS at 37°C in batch culture, at 180 rpm on orbital shaker.

Key:

A SFZ/BHI
B EBH1/BHI
C EBH1/HS

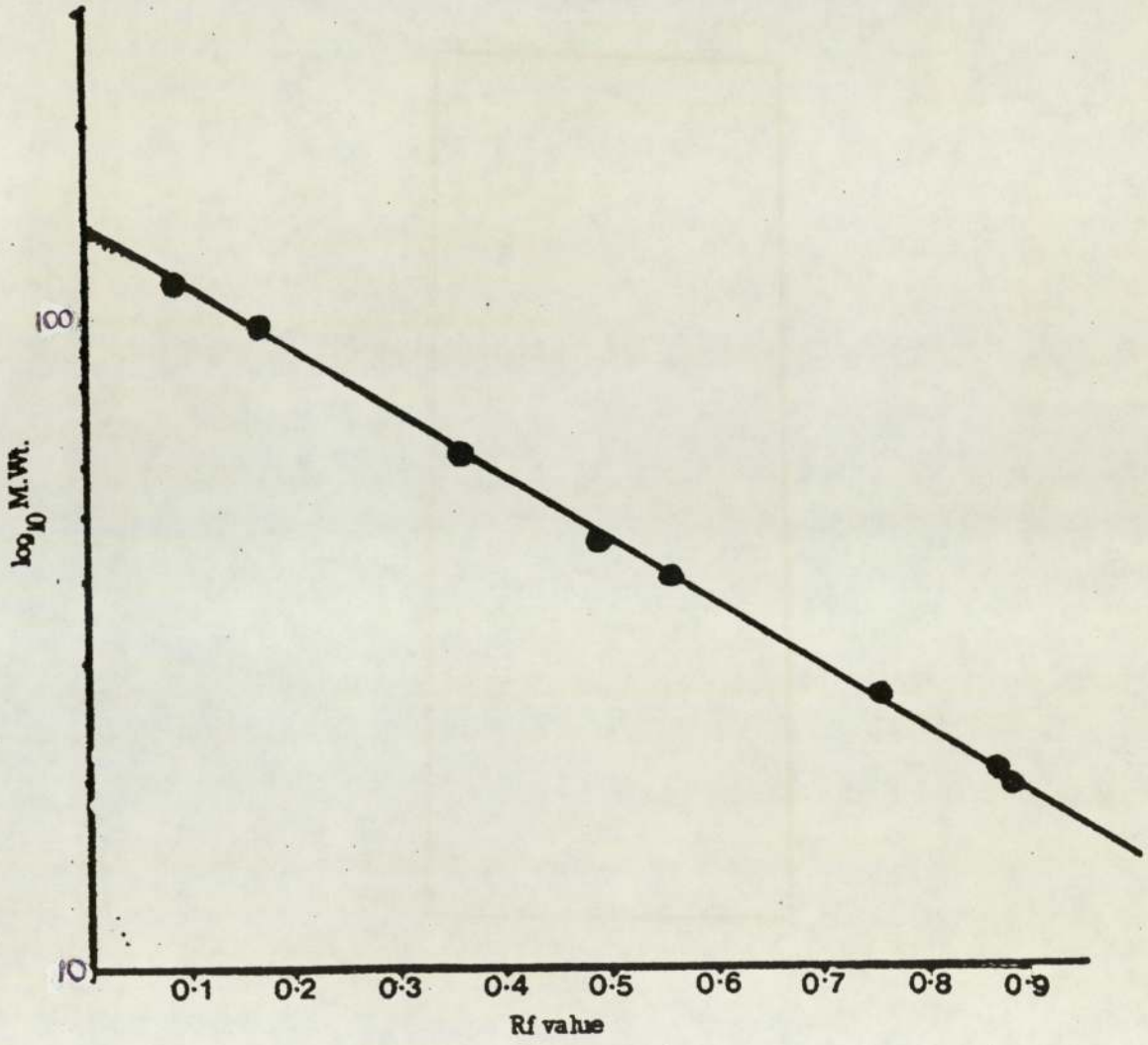


Figure 9 Calibration curve for determination of molecular weights by SDS-PAGE.
Molecular weight markers used in construction of curve: Electran molecular weight marker 112K; phosphorylase B (Sigma) 97.4K; bovine serum albumin (Sigma) 68K; *Pseudomonas aeruginosa* (PA01) outer membrane protein (OMP) D 46K; PA01 OMP F 41K; PA01 OMP G 25K; PA01 OMP H1 21K; and PA01 OMP H2 20.5K. Markers were electrophoretically separated by SDS-PAGE on a 12% polyacrylamide gel at a constant current of 40mA. Graph shows plot of Rf values versus $\log_{10}$ molecular weight.

Figure 10 SDS-PAGE protein profiles of *S. faecalis* whole cells and cell walls after growth in BHI or HS.
Lanes:
1, EBH1/BHI whole cells
2, SFZ/BHI " "
3, EBH1/HS " "
4, SFZ/HS " "
5, EBH1/BHI cell walls
6, SFZ/BHI " "
Gel was stained with Coomassie blue.

Figure 12 SDS-PAGE protein profile of *S. faecalis* after various extraction procedures. Strain SFZ whole cells grown in HS, and treated with lysozyme, Na_2EDTA, urea and SDS. Pellets and supernatants were separated by SDS-PAGE. Lanes:

1, supernatant from cells treated with lysozyme (100µg/ml)
2, pellet " " " " " "
3, supernatant " " " " Na_2EDTA (1.5mM)
4, pellet " " " " " "
5, supernatant " " " " urea (800mM)
6, pellet " " " " " "
7, supernatant " " " " SDS (2% w/v)
8, pellet " " " " " "
9, supernatant from untreated cells
10, pellet from untreated whole cells
Gel was stained with Coomassie blue.

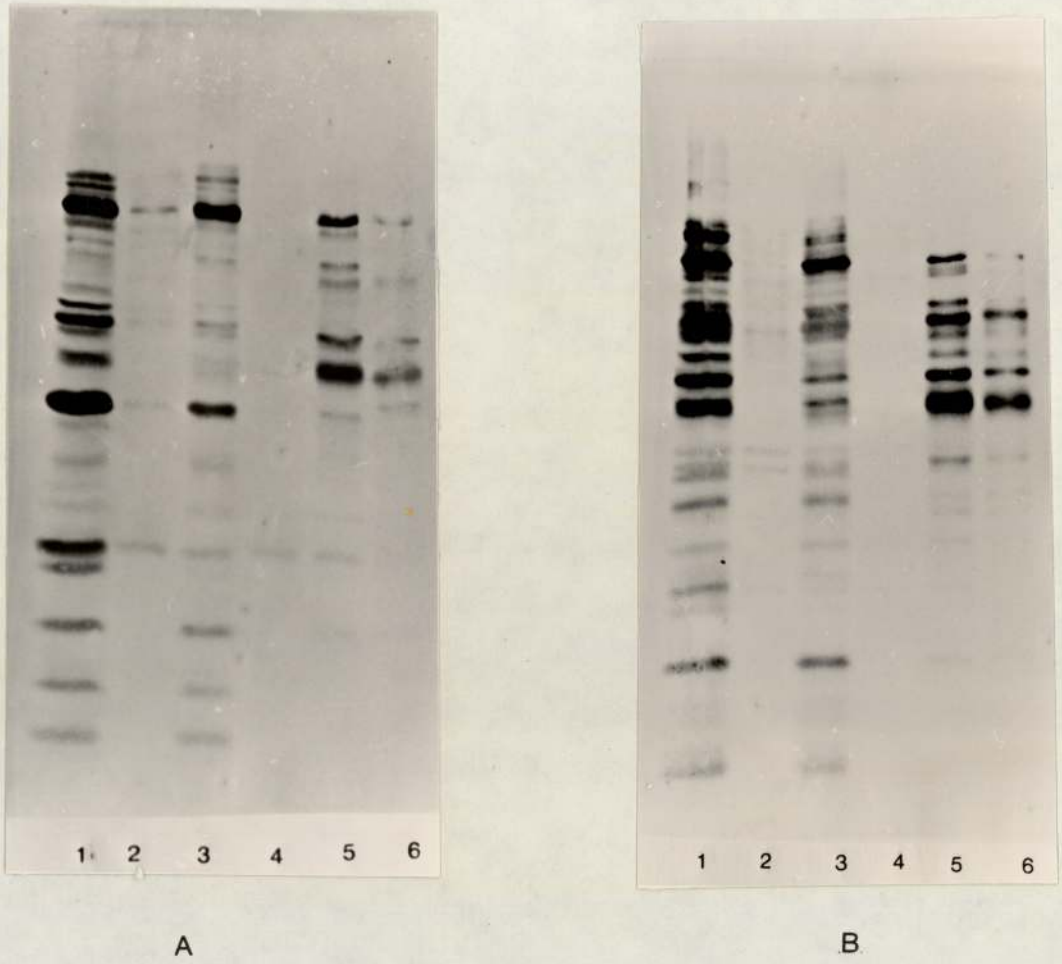


Figure 13 Immunoblot showing IgG response of rabbits immunized with EBH1 grown in BHI.
Whole cells and cell walls of *S. faecalis* strains were separated by SDS-PAGE and transfered to nitrocellulose. Blot was reacted with hyperimmune rabbit serum raised against strain EBH1 grown in BHI: figure 13a, rabbit 1 serum; figure 13b, rabbit 2 serum. Lanes:
1, EBH1/BHI whole cells
2, EBH1/HS whole cells
3, SFZ/BHI whole cells
4, SFZ/HS whole cells
5, EBH1/BHI cell walls
6, SFZ/BHI cell walls

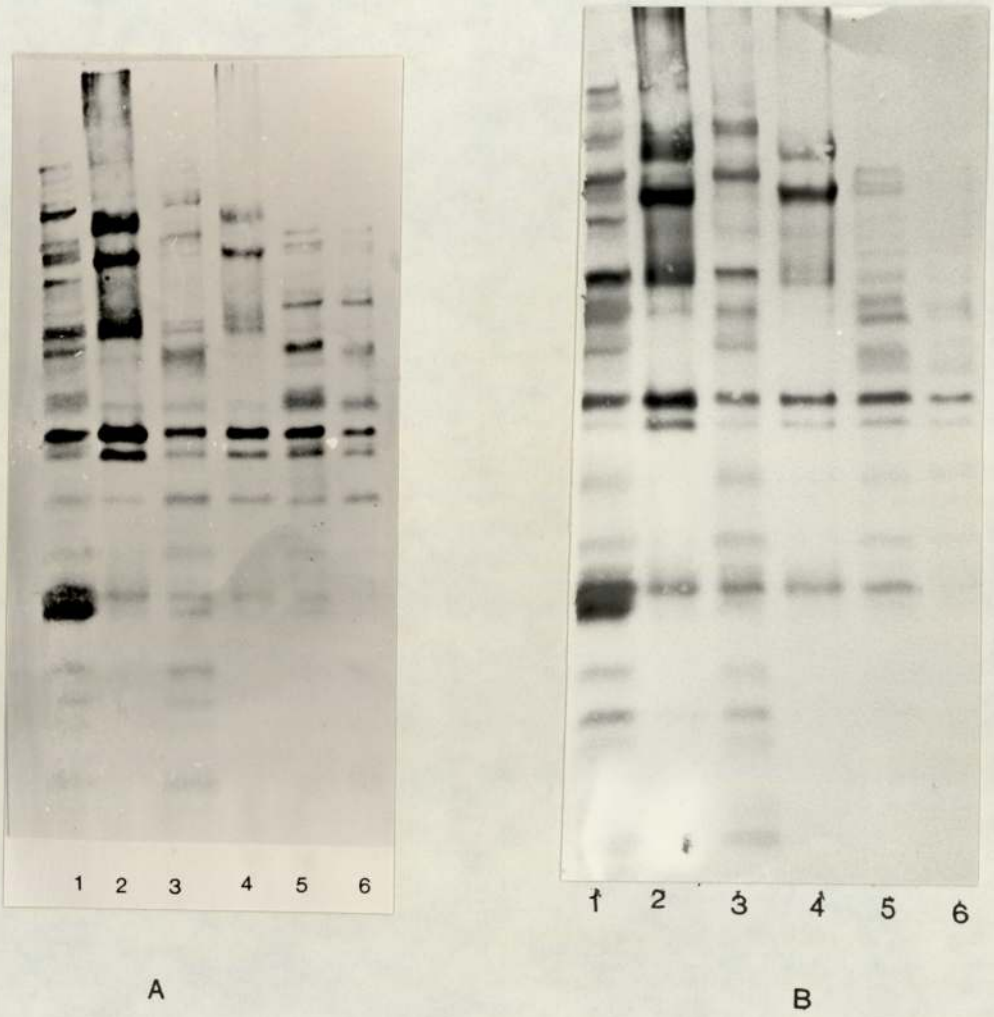


Figure 14 Immunoblot showing IgG response of rabbits immunized with strain EBH1 grown in HS.
Whole cells and cell walls of *S. faecalis* strains were separated by SDS-PAGE and transfered to nitrocellulose. Blot was reacted with hyperimmune rabbit serum raised against strain EBH1 grown in HS: figure 14a, rabbit 3; figure 14b, rabbit 4. Lanes:
1, EBH1/BHI whole cells
2, EBH1/HS whole cells
3, SFZ/BHI whole cells
4, SFZ/HS whole cells
5, EBH1/BHI cell walls
6, SFZ/BHI cell walls

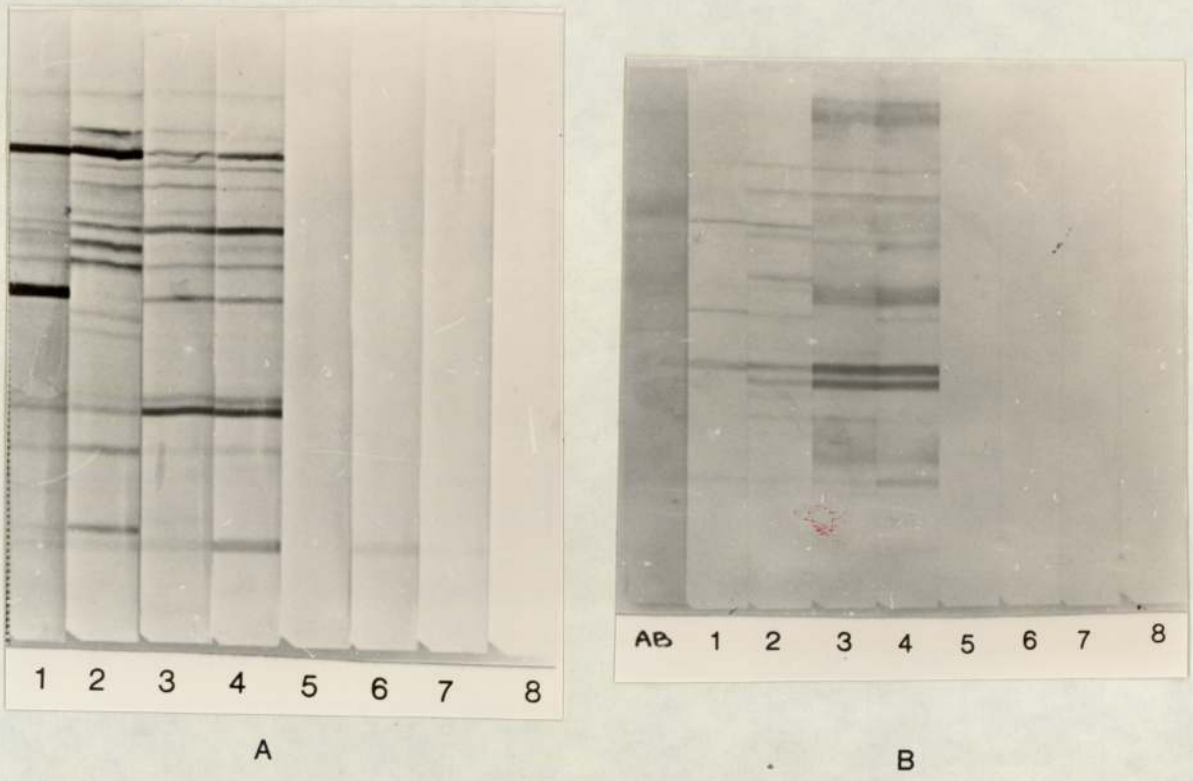


Figure 15 Strip-blots comparing pre- and post-immunization IgG response of rabbits to *S. faecalis* grown in (15a) BHI or (15b) HS. Whole cells of strain EBH1 grown in BHI (15a) or HS (15b) were separated by SDS-PAGE and transferred onto nitrocellulose. Strips of nitrocellulose were reacted with the following sera:

1, rabbit 1 hyperimmune serum
2, rabbit 2 " "
3, rabbit 3 " "
4, rabbit 4 " "
5, rabbit 1 preimmune serum
6, rabbit 2 " "
7, rabbit 3 " "
8, rabbit 4 " "
AB, amido black-stained strip

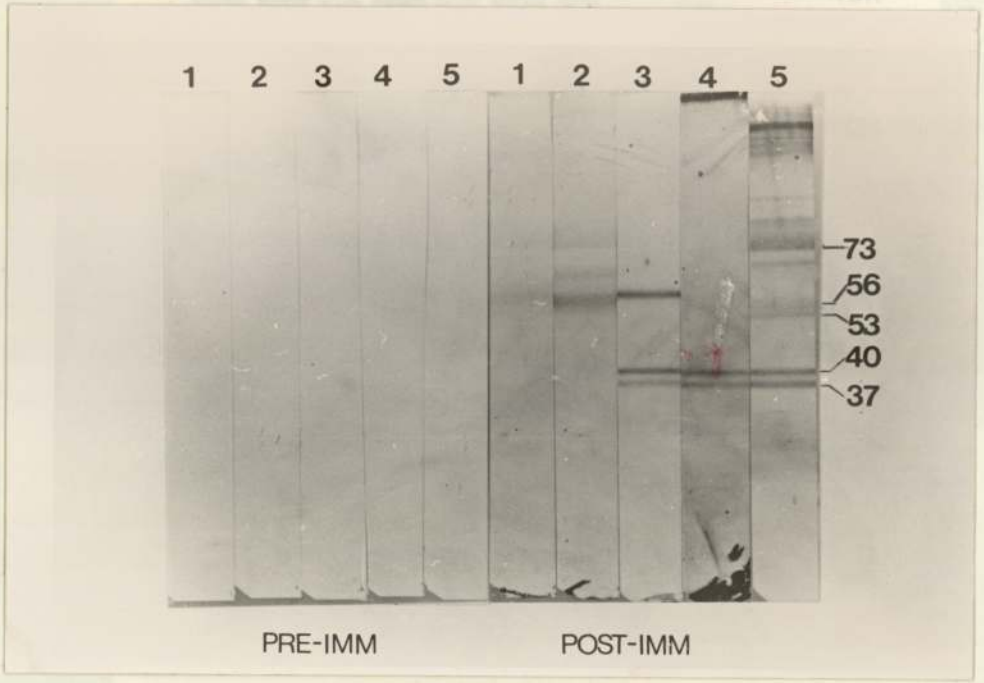


Figure 16 Strip-blots comparing pre- and post-immunization IgG response of rabbits immunized with 56/53K (rabbits A and B) or 40/37K (rabbits C and D) *S. faecalis* antigens.
Whole cells of strain EBH1 grown in HS were separated by SDS-PAGE and transferred onto nitrocellulose. Strips of nitrocellulose were reacted with the following sera:
1, rabbit A
2, rabbit B
3, rabbit C
4, rabbit D
5, rabbit 3/4 (hyperimmune serum raised against whole cells of EBH1/HS)
Pre- and post-immune sera are indicated on figure.

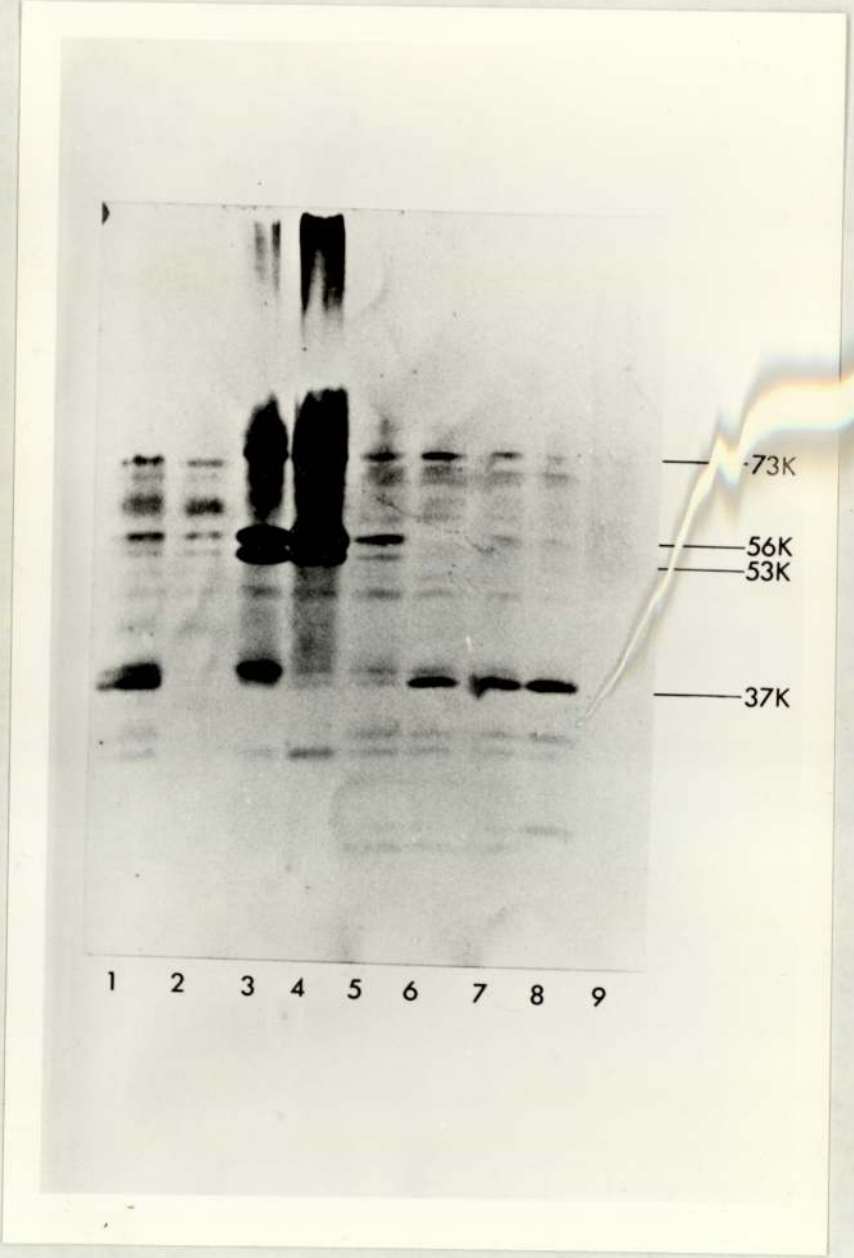


Figure 18 Immunoblot analysis of whole cells and cell walls of *S. faecalis* strains revealed by hyperimmune rabbit serum raised against whole cells of EBH1/HS.

Lanes:

1, EBH1/BHI whole cells
2, EBH1/BHI cell walls
3, EBH1/HS whole cells
4, EBH1/HS cell walls
5, SFZ/BHI whole cells
6, 741/BHI " "
7, 777/BHI " "
8, 790/BHI " "
9, *Staphylococcus aureus* NCTC 6571/tryptic soy broth

Whole cells and cell walls were separated by SDS-PAGE and transferred to nitrocellulose. Blot was reacted with rabbit 3/4 serum.
Molecular weights (10^3) are indicated vertically.

Figure 19 Immunoblot analysis of a range of HS-grown *S. faecalis* and *S. faecium* strains.

Lanes:

1, EBH1
2, EBH1 grown in normal human serum (EBH1/NHS)
3, SFZ
4, *S. faecalis* NCTC 5957
5, 777
6, 741
7, 790
8, SFBA
9, SFSQ
10, SFBI
11, 9112
12, *S. faecalis* DK
13, *S. faecium* DK (isolated from the same patient as *S. faecalis* DK)
14, *S. faecium* NCTC 7171

Whole cells were separated by SDS-PAGE and transferred to nitrocellulose. Blot was reacted with serum collected from Mr. P.

Figure 20 Immunoblot analysis of *S. faecalis*, other streptococcal species and *Staphylococcus aureus* to show species-specific antigens.
Lanes:
1, SFZ
2, 777
3, SFSQ
4, SFBI
5, EBH1
6, 9112
7-14, non-faecal streptococcal strains isolated from various infections
15, *Staphylococcus aureus* IE isolate
Whole cells were grown in HS, separated by SDS-PAGE and transferred onto nitrocellulose. Blot was reacted with rabbit 3/4 serum.

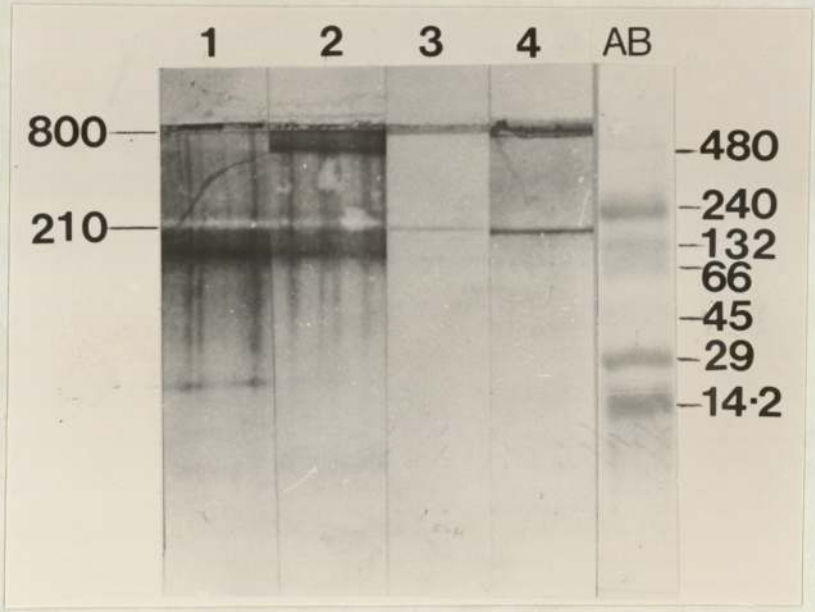


Figure 21 Strip-blot analysis of EBH1/HS antigens following native PAGE. Whole cells of EBH1/HS were separated by native PAGE and transferred to nitrocellulose. Strips of nitrocellulose were reacted with the following sera:

1, Mr. P
2, rabbit 3/4
3, anti-56/53K monospecific serum A
4, anti-40/37K monospecific serum D

AB, amido black stain of native-PAGE molecular weight markers
Numbers on right indicate molecular weights (K) of markers

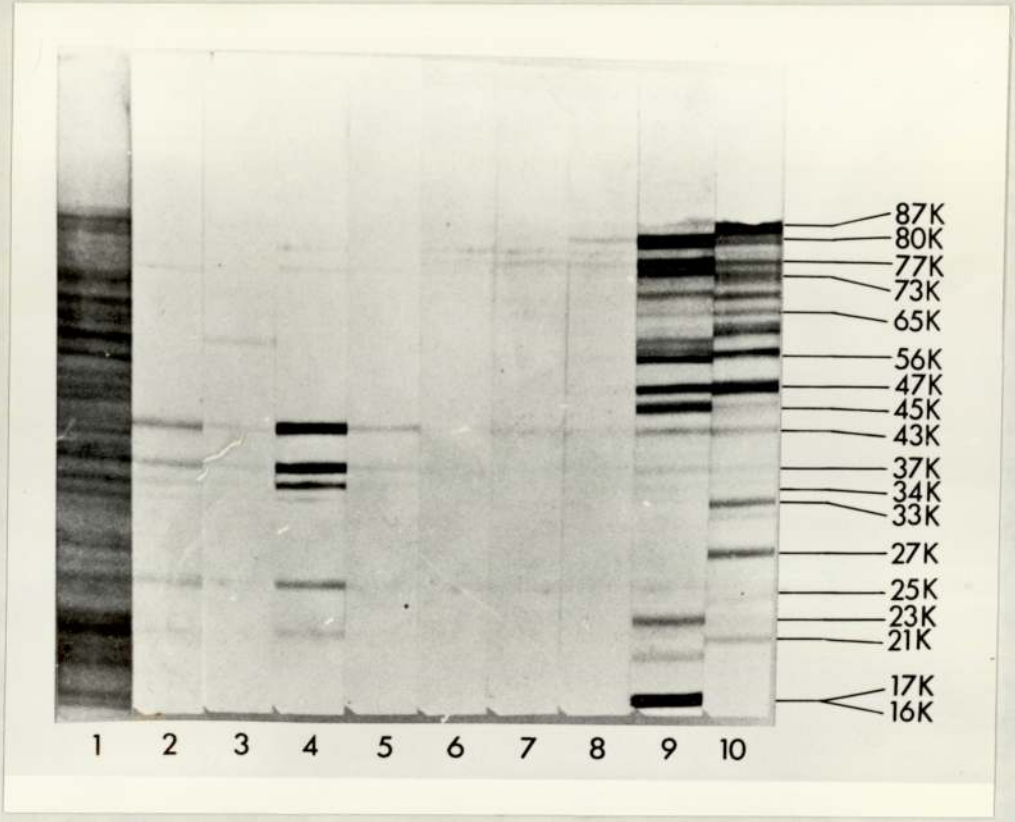


Figure 22 Strip-blots showing antigenic and lectin-receptor profiles of EBH1/BHI.
Whole cells of EBH1/BHI were separated by SDS-PAGE and transferred to nitrocellulose. Strips of nitrocellulose were reacted as follows:

1, amido black stain
2, WGA
3, con A
4, APL
5, SBA
6, serum from Mr. W collected at start of antibiotic therapy
7, serum from Mr. W collected after one week of antibiotic therapy with ampicillin and gentamicin
8, serum from Mr. W collected after two weeks of antibiotic therapy with ampicillin and gentamicin
9, serum from Mr. P collected during 3rd episode of enterococcal endocarditis
10, rabbit 1/2 serum

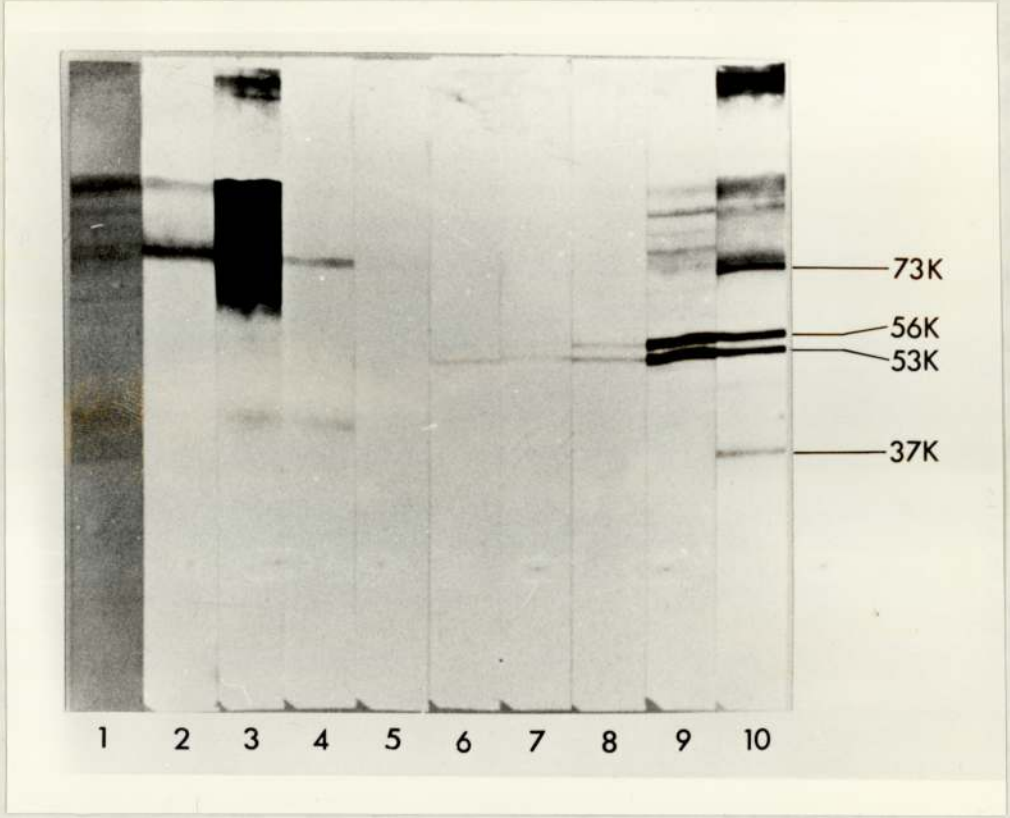


Figure 23 Strip-blots showing antigenic and lectin-receptor profiles of EBH1/HS.

Whole cells of EBH1/HS were separated by SDS-PAGE and transferred to nitrocellulose. Strips of nitrocellulose were reacted as follows:

1, amido black stain
2, WGA
3, con A
4, APL
5, SBA
6, serum from Mr. W collected at start of antibiotic therapy
7, serum from Mr. W collected after one week of antibiotic therapy with ampicillin and gentamicin
8, serum from Mr. W collected after two weeks antibiotic therapy with ampicillin and gentamicin
9, serum from Mr. P collected during 3rd episode of enterococcal endocarditis
10, rabbit 3/4 serum

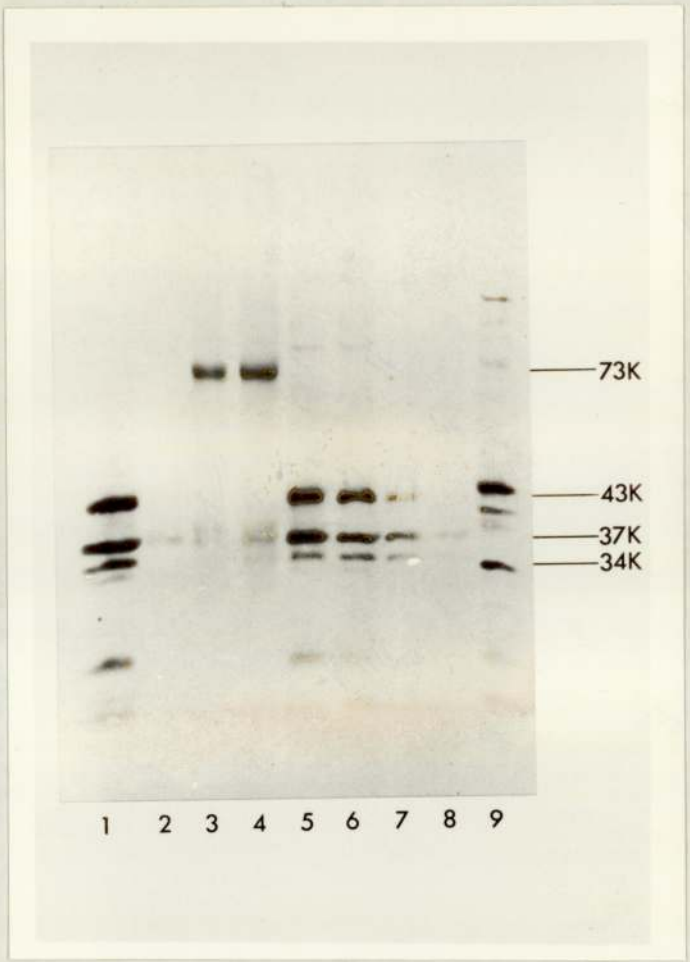


Figure 24 Immunoblot showing fucosyl-containing antigens of *S. faecalis*. Whole cells and cell walls of *S. faecalis* strains were separated by SDS-PAGE and transferred to nitrocellulose.
Lanes:
1, EBH1/BHI whole cells
2, EBH1/BHI cell walls
3, EBH1/HS whole cells
4, EBH1/HS cell walls
5, SFZ/BHI whole cells
6, 741/BHI " "
7, 777/BHI " "
8, 790/BHI " "
9, *Staphylococcus aureus* NCTC 6571/tryptic soy broth
Blot was reacted with APL-peroxidase conjugate.

Figure 25 Strip-blot analysis of *S. faecalis* strains grown in HS to show reaction with sera, lectins and avidin.
Whole cells of strain (a) 9112 and (b) EBH1 were grown in HS, separated by SDS-PAGE and transferred to nitrocellulose. Strips of nitrocellulose were reacted with sera, lectins or avidin as follows:

1, serum from (a) Mr. P or (b) Mr. W collected at start of IE episode
2, serum from (a) Mr. P or (b) Mr. W collected 3 days after sample 1
3, serum from (a) Mr. P or (b) Mr. W collected 5 days after sample 1
4, serum from (a) Mr. P or (b) Mr. W collected 10 days after sample 1
5, serum from (a) Mr. P or (b) Mr. W collected 14 days after sample 1
6a, serum from Mr. W (pooled samples collected over whole IE episode)
6b, serum from Mr. P (pooled samples collected over whole IE episode)
7, serum from patient with *S. salivarius* endocarditis
8, serum from patient with *Staphylococcus aureus* endocarditis
9, serum from patient with *S. sanguis* endocarditis
10, serum from patient with *S. pneumoniae* endocarditis
11, WGA
12, con A
13, APL
14, SBA
15, avidin

(Figure 25b overleaf)

Figure 25 Strip-blot analysis of *S. faecalis* strains grown in HS to show reaction with sera, lectins and avidin.
Whole cells of strain (a) 9112 and (b) EBH1 were grown in HS, separated by SDS-PAGE and transferred to nitrocellulose. Strips of nitrocellulose were reacted with sera, lectins or avidin as follows:

1, serum from (a) Mr. P or (b) Mr. W collected at start of IE episode
2, serum from (a) Mr. P or (b) Mr. W collected 3 days after sample 1
3, serum from (a) Mr. P or (b) Mr. W collected 5 days after sample 1
4, serum from (a) Mr. P or (b) Mr. W collected 10 days after sample 1
5, serum from (a) Mr. P or (b) Mr. W collected 14 days after sample 1
6a, serum from Mr. W (pooled samples collected over whole IE episode)
6b, serum from Mr. P (pooled samples collected over whole IE episode)
7, serum from patient with *S. salivarius* endocarditis
8, serum from patient with *Staphylococcus aureus* endocarditis
9, serum from patient with *S. sanguis* endocarditis
10, serum from patient with *S. pneumoniae* endocarditis
11, WGA
12, con A
13, APL
14, SBA
15, avidin

Figure 26 Immunoblot analysis of group D streptococcal strains showing LTA-associated antigens.
Whole cells grown in HS were separated by SDS-PAGE and transferred to nitrocellulose. Lane 1, containing EBH1 was reacted with rabbit 3/4 serum. Lanes 2-8 were reacted with anti-group D streptococcal grouping serum, and contain:

2, EBH1
3, SFZ
4, 777
5, SFSQ
6, *S. faecium* DK
7, *S. faecium* NCTC 7171
8, *Staphylococcus aureus* NCTC 6571

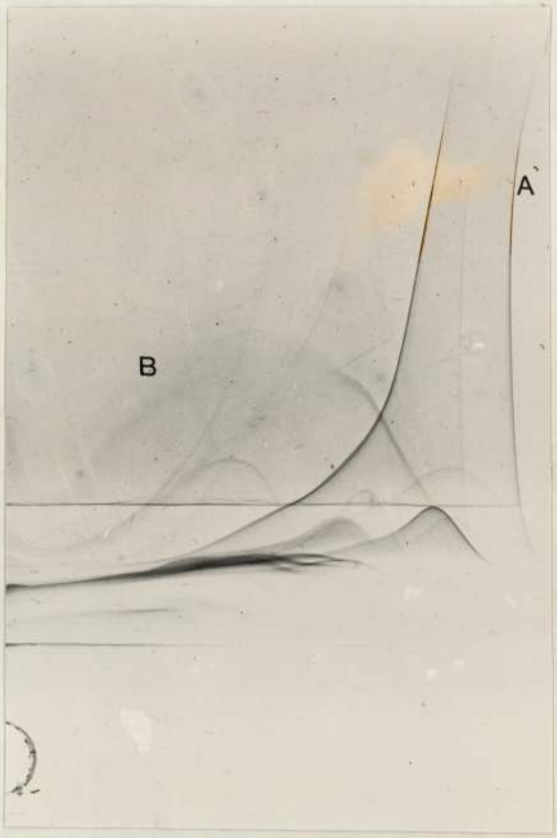


FIG. A

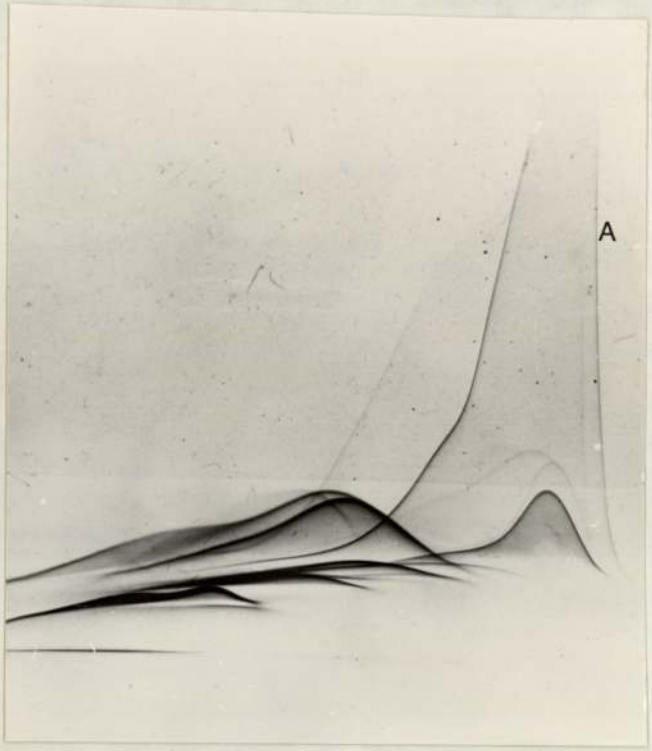


FIG. B

Figure 27 CIE patterns of mutanolysin-digested EBH1 grown in (a) BHI or (b) HS.

Well: (a) EBH1/BHI or (b) EBH1/HS mutanolysin digest (500µg in 25 µl)

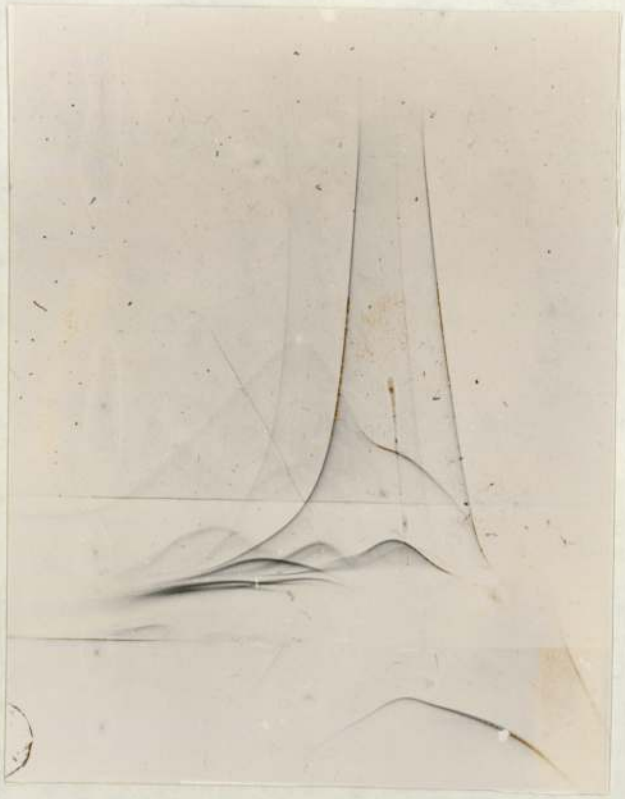
Reference gel: Mr. P serum (1:50)

Intermediate gel: no antiserum

"A" indicates major precipitin arc common to HS- and BHI-grown EBH1

"B" indicates diffuse area exclusive to BHI-grown EBH1

Eletrophoresis was carried out in the first dimension at 10 V/cm for 2 hours, and in the second dimension at 2 V/cm for 18 hours.



A

B

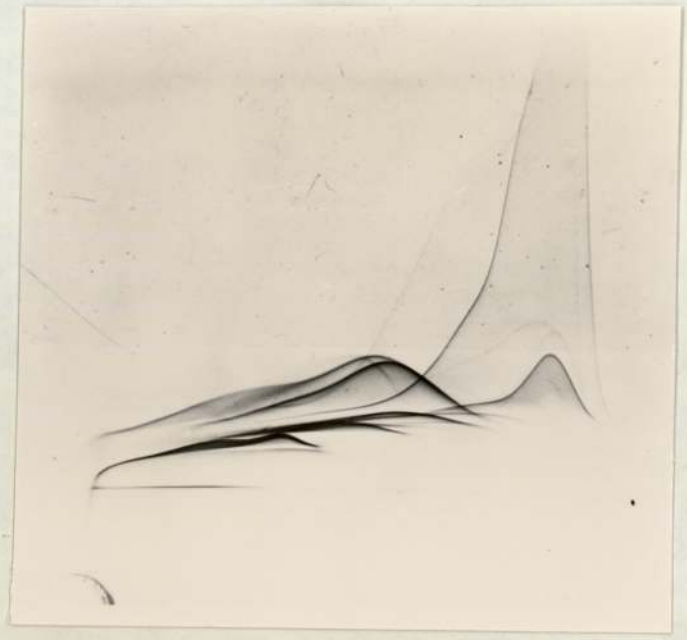
Figure 28 CIE patterns of EBH1 grown in (a) BHI or (b) HS - control supernatants undigested with mutanolysin.

Well: (a) EBH1/BHI or (b) EBH1/HS control supernatant (500µg in 25µl)

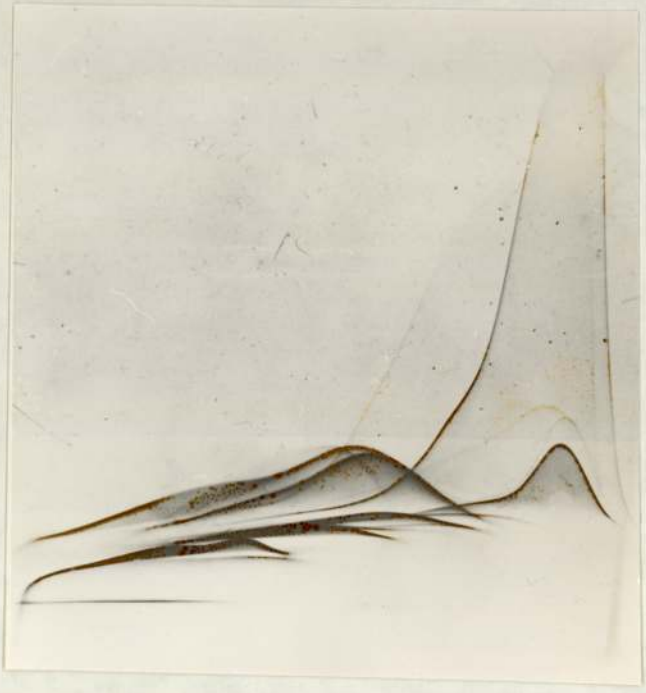
Reference gel: Mr. P serum (1:50)

Intermediate gel: no antiserum

Electrophoresis was carried out in the first dimension at 10 V/cm for 2 hours, and in the second dimension for 2 V/cm for 18 hours.



A



B

Figure 29 CIE patten of EBH1/HS revealed using purified immunoglobulins.
Well: EBH1/HS mutanolysin digest (500µg in 25µl)
Reference gel: (a) purified immunoglobulins from Mr. P serum (1:50)
(b) whole Mr. P serum (1:50)
Intermediate gel: no antiserum
Electrophoresis was carried out in the first dimension at 10 V/cm for 2 hours, and in the second dimension at 2 V/cm for 18 hours.

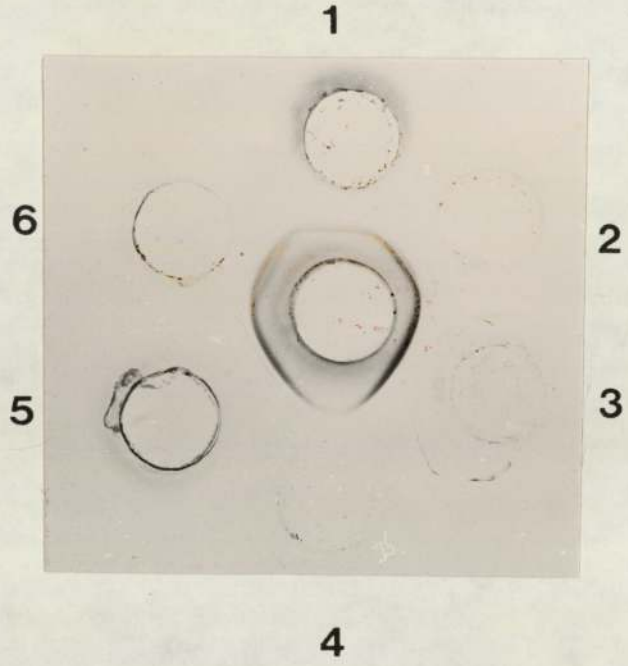
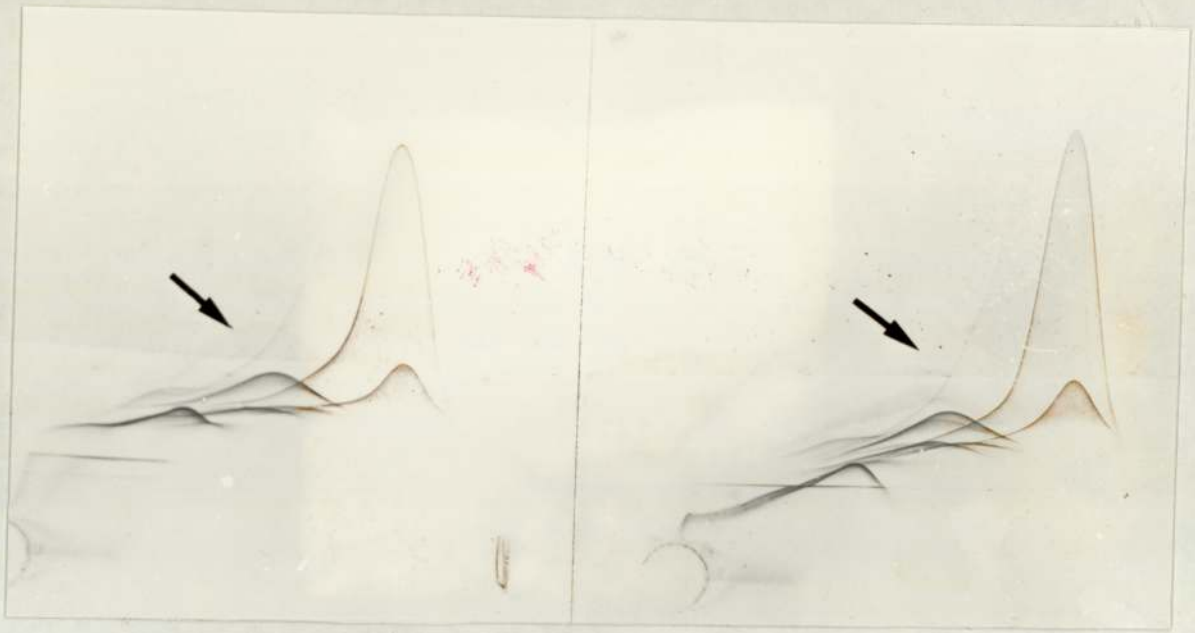


Figure 30 Double diffusion immunoprecipitation (Ouchterlony) pattern formed between anti-group D streptococcal grouping serum and *S. faecalis* strains.
Central well: anti-group D streptococcal grouping serum 30µl
Peripheral wells:

1,	EBH1/HS mutanolysin digest (500µg in 25µl)
2,	SFZ/HS " "
3,	crude LTA extract from EBH1/BHI (500µg in 25µl)
4,	9112/HS mutanolysin digest (500µg in 25µl)
5,	SFZ/BHI " "
6,	EBH1/HS " "

Immunodiffusion was allowed to proceed for 18-24 hours at 4°C.



A B

Figure 31 CIEWIG pattern of EBH1/HS to identify group D antigen precipitin arc.
Well: EBH1/HS mutanolysin digest (500µg in 25µl)
Reference gel: Mr. P serum (1:50)
Intermediated gel: (a) no antiserum
(b) anti-group D streptococcal grouping serum (1:100)
Arrow marks precipitin arc corresponding to group D antigen.
Electrophoresis was carried out in the first dimension at 10 V/cm for 2 hours, and in the second dimension at 2 V/cm for 18 hours.

Figure 32 CIE pattern of EBH1/HS revealed by anti-group D streptococcal grouping serum.
Well: EBH1/HS mutanolysin digest (500µl in 25µl)
Reference gel: anti-group D streptococcal grouping serum (1:50)
Intermediate gel: no antiserum.
Electrophoresis was carried out in the first dimension at 10 V/cm for 2 hours, and in the second dimension at 2 V/cm for 18 hours.

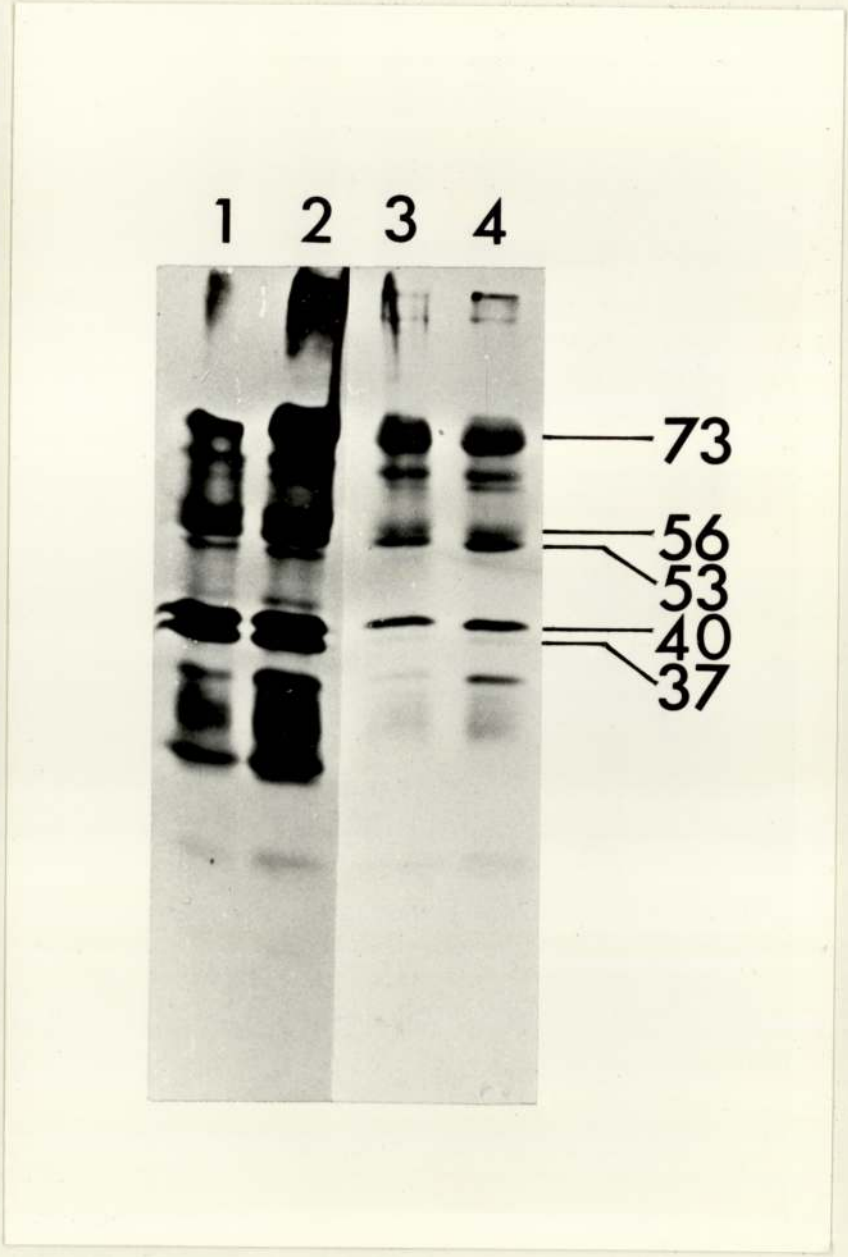


Figure 33 Immunoblot analysis of *S. faecalis*, following incubation of blot in sodium periodate.

Whole cells of *S. faecalis* were separated by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose blot was incubated in 0.5M sodium periodate, pH 4.0 for 60 minutes at 37°C prior to reaction with rabbit 3/4 serum (lanes 3 and 4). Control blots were untreated with periodate (lanes 1 and 2).

Lanes:

1, EBH1/HS (untreated blot)
2, SFZ/HS " "
3, EBH1/HS (blot treated with sodium periodate prior to development)
4, SFZ/HS " " " " " " " "

3.1.5 DISCUSSION

SDS-PAGE is a powerful technique for separating proteins from a complex mixture according to their size (Weber and Osborn, 1961; Laemmli, 1970; Hames, 1981). An identical copy of the pattern of proteins separated by SDS-PAGE can be electrophoretically transferred onto an immobilizing carrier. This transfer was first described by Renart et al (1979) and Towbin et al (1979). This sensitive technique enables the detection of single proteins in crude mixtures separated by SDS-PAGE. Biological activity is lost on SDS-PAGE (Helenius and Simons, 1975) but is regained due to elimination of SDS during the blotting procedure, thus enabling reaction of the immobilized proteins with a range of physiological ligands (Hjerten, 1983; Beisiegel, 1986). One of the intrinsic advantages that SDS-PAGE/immunoblotting has over other gel separation techniques such as CIE is that it allows the assignation of molecular weights to individual antigens (Weber and Osborn, 1969). The method of Towbin et al (1979), as modified and named "Western Blotting" by Burnette (1981), was chosen for use in this study. Efficiency of protein transfer is higher from a cross-linked polyacrylamide gel to nitrocellulose (as used by Towbin et al) than from a polyacrylamide/agarose gel to diazobenxzyloxymethyl paper (as used by Renart). The nature of the interaction between the protein and the nitrocellulose is not fully understood but is assumed to be associated with hydrophobicity and is increased by inclusion of methanol in the transfer buffer (Beisiegel, 1986). The extent to which this hydrophobic interaction affects the biological activity of the immobilized antigens is not known.

At the outset of this study little work had been done on the analysis of surface proteins of *S. faecalis* by SDS-PAGE or immunoblotting. This report showed that it was possible to separate *S. faecalis* whole-cell components by SDS-PAGE. These components were electrophoretically

transferred onto nitrocellulose and reacted with rabbit and human sera. SDS-PAGE protein profiles of *S. faecalis* contained many bands in a complex pattern. The profiles of a variety of strains, including laboratory, reference and clinical strains, were all basically similar. The banding patterns were apparently devoid of any immediately noticeable features, no single major band being noted. However upon transfer to nitrocellulose and subsequent reaction with antisera certain bands were seen to be antigenically dominant. It was of interest that bands that appeared to be of equal density on an SDS-PAGE protein profile, reacted with differing intensities with serum. This phenomenon must therefore be due to the contrasting immunodominance of such antigens. Growth of *S. faecalis* in BHI resulted in the 53K antigen being dominant amongst many bands. Growth in serum resulted in the 73, 56, 53, 40 and 37K antigens being the major bands in a relatively simple antigenic pattern. HS was chosen as a growth medium for *S. faecalis* as a first approximation to the in-vivo situation in bacteraemia. The 73, 56, 53, 40 and 37K antigens may therefore predpminate *in vivo*. However, there are several reasons why one should be cautious about making such a statement. Firstly, when *S. faecalis* was grown in Difco BHI (as opposed to Lab M) the 40 and 37K antigens became more prominent than in HS-grown cells (see figure 70, section 3.3). Also, in this medium the 73K antigen was weakly expressed. The ingredients of the two BHI preparations are given in section 2.1.2 and the considerable differences in formulation (Difco BHI is much richer in brain and heart infusion solids than the Lab M preparation) may explain the variation in stimulation of antigen expression. Secondly, the observed antigen expression may have been influenced by the phase of growth attained by the organism upon harvesting. Cells grown in BHI were harvested at mid-log phase (originally assumed to be stage of maximal antigen expression); HS-grown cells were harvested at early stationary phase (because of the difficulty in accurately pin-pointing

mid-log phase). Peak antigen expression is achieved at late stationary phase (Dr. S.J. Prior, 1987, CAMR, Porton Down, Salisbury, Wilts; personal communication). Harvesting cells grown in both media at late stationary phase might have lessened the differences between the antigenic profiles of *S. faecalis* grown in these media. Thirdly, transfer of antigens onto nitrocellulose is molecular weight-dependent and so may not necessarily be quantitative (Gershoni and Palade, 1982). But nevertheless the supposition that the slow, clumped growth exhibited by *S. faecalis* in HS represents the pattern of growth *in vivo*, is supported by the reports of other workers who have used animal models of IE (Gladstone and Glencross, 1960; Durack and Beeson, 1972b; Smith, 1976; Zak and Sande, 1982; Gengo et al, 1984; Sullam et al, 1985) and excised heart valves (Gould et al, 1975). It is now generally accepted that doubling times *in vivo* are long (Brown and Williams, 1985). Additional support for the suitability of HS as a medium for simulating in-vivo growth conditions arose from the similarity in antigenic profiles between EBH1 grown in HS and in normal human serum.

It is therefore possible that in bacteraemia and IE *S. faecalis* grows slowly, in clumps. The tendency of Gram-positive cocci to clump may increase their ability to adhere to heart valves in IE as opposed to Gram-negative organisms, which do not tend to clump and adhere less well (Gould et al, 1975). Aggregation of cells may be host-mediated or cell-mediated. Binding of host serum proteins such as fibronectin, fibrinogen, albumin or immunoglobulins (Kronvall et al, 1979; Miorner et al, 1980; Bjorck et al, 1981; Chhatwal et al, 1985) to bacterial components may lead to subsequent binding to other bacterial cells (although fibrinogen is now known not to bind to *S. faecalis* (Myhre, 1986)). Alternatively, growth in serum may favour plasmid-mediated clumping as described by Dunny et al (1978). Indeed HS-grown cells posses a high molecular weight plasmid (Dr. S.J. Prior, CAMR, Porton Down, Salisbury, Wilts, 1987; personal communication) which may be

responsible for production of CIA's (Dunny et al, 1978; Franke and Clewell, 1981; Kessler and Yagi, 1983; Tortorello and Dunny, 1985; Ehrenfeld et al, 1986).

When probing blots of *S. faecalis* antigens with rabbit 1/2 serum it became evident that this organism possessed a complex antigenic profile. But serum raised against HS-grown cells (rabbit 3/4 serum) did not react with anywhere near as many *S. faecalis* antigens, even in BHI-grown cells. This may be explained by the fact that HS-grown cells had fewer surface proteins than did BHI-grown cells, therefore fewer antigens were available for stimulation of antibody production by the rabbits' immune systems during the immunization programme. Thus, when rabbit 3/4 serum was used to probe blots it only reacted with those antigens to which the rabbits immune system had been exposed. This explanation may not be the whole story however since upon comparing the number of bands stained by amido black on nitrocellulose blots of HS-grown EBH1 with the number of bands that reacted with rabbit 3/4 serum it was noted that the serum did not react with all the proteins present on the cells. This could by due to binding of serum components to receptors on the surface of *S. faecalis* during growth (Kronvall et al, 1979; Miorner et al, 1980, Chhatwal et al, 1985). These cell-bound serum components may prevent certain *S. faecalis* antigens from being perceived by the immune systems of rabbits 3 and 4, either by steric hinderance or by direct attachment to, and masking of, the antigen. Thus, when subsequently used to probe blots, the rabbits serum only reacts with those antigens to which it had access during the process of immunization. A similar situation may exist in humans since serum from IE patients generally (except for Mr. P) reacted with fewer *S. faecalis* antigens on immunoblots.

The major antigens of *S. faecalis* (53K in BHI-grown cells; 73, 56, 53, 40 and 37K in HS-grown cells) are located in the cell walls. The surface

location of these antigens is confirmed later in this report (see section 3.3). It is reasonable to postulate their role in interaction with the environment in general and host cells in particular.

The 56K antigen may be analagous to the 56K protein of group A streptococci (Yarnall and Boyle, 1986), which acts as a type II Fc-receptor. This receptor is extractable by heat treatment at neutral pH or by digestion with mutanolysin or hyaluronidase. The role of this antigen in the pathogenicity of streptococci is not known but possibly IgG Fc-receptors inhibit phagocytosis of streptococci through interference with antibody-dependent complement-activation (Burova et al, 1982). Since all the streptococcal species used in this study possessed a 56K antigen, it may be a candidate for future exploitation as a vaccine for protection against streptococcal endocarditis. Following repeated subculturing the 56 and 53K antigens of serum-grown cells, although remaining prominent, were superseded in intensity on immunoblots by the 40 and 37K antigens. This antigenic shift is usually employed as a mechanism by which microorganisms elude host immune responses (Turner, 1980). Changes in surface properties of bacteria upon subculturing have been well documented. Loss of high molecular weight protein antigens in *S. mutans* after repeated subculture on laboratory media has been associated with a decrease in hydrophobicity (Olsson and Westergren, 1982; McBride et al, 1984), accompanied by a reduction in adhesiveness (Orstavik and Orstavik, 1982; Rundegren and Olsson, 1987). In group A streptococci repeated subculturing results in the loss of the fibrillar M protein (Lancefield, 1962) and an increased IgG Fc-receptor content (Stjernquist-Desatnik et al, 1984). Changes of the above sort may be elicited in *S. faecalis* during subculture and manifest themselves in the altered antigenic dominance shown in this report. However the reported increase in Fc receptors in group A streptoccci upon subculture is contrary to the theory that this antigen is analagous to the 56K

common streptococcal antigen reported in this study since, in *S. faecalis*, this antigen becomes less immunodominant.

Several other antigens were common to the range of streptococcal species and the strain of *Staphylococcus aureus* used - 87, 68, 62, 53 and 26K. These antigens thus all have possible protective potential. Such a vaccine could be administered prior to a manipulative or operative procedure known to elicit bacteraemia. Alternatively, production of monospecific or monoclonal antibodies against these antigens may lead to the development of passive immunization for streptococcal/staphylococcal endocarditis.

It has been well established that FN binds to group A, C and G streptococci (Switalski et al, 1982; Myhre and Kuusela, 1983) and more recently Scheld et al (1985) reported the binding of FN to *S. faecalis*. Indeed, it has been postulated that FN mediates attachment of bacteria to the fibrin-platelet matrix in NBTE (Crawford and Russell, 1986; Scheld et al, 1985). Crawford and Russell (1985) found that *S. faecalis* adhered avidly to fibrin-platelet clots *in vitro*. It is possible that one or several of the protein or glycoprotein antigens of *S. faecalis* reported in this study may be a receptor for FN.

A possible role for the 73 and 40K *S. faecalis*-specific antigens is as CIA's produced in response to a sex pheromone (Tortorello and Dunny, 1985; Tortorello et al, 1986). Tortorello et al (1986) report that surface proteins of 73 and 40K (among others) are induced by a *S. faecalis* sex pheromone determined by the conjugative tetracycline resistance plasmid, pCF10. Additional evidence for this postulation rests with the fact that these antigens are more prominent when *S. faecalis* is grown in HS, a medium in which the organism clumps.

Because of their specificity, lectins are valuable as probes for identifying sugars on the surface of cells (Sharon, 1977). Streptococci bind to lectins (Köhler et al, 1973; Ottensooser et al, 1974; Kashket and Guilmette, 1975; Hamada et al, 1977). This study has revealed the presence of several *S. faecalis* antigens containing glycosyl residues. The major glycosylated antigens are 73, 43, 37 and 34K and contain N-acetylglucosaminyl, N-acetylagalactosaminyl, glucosyl, mannosyl and fucosyl residues in varying combinations. The glycoproteins present on the surface of *S. faecalis* may be involved in colonization in IE by binding to lectin-like proteins on cardiac tissue (Banerjee et al, 1981; Izhar et al, 1982). Clumping of S. faecalis during growth in serum may also be due to lectin-ligand interactions mediated by serum glycoproteins in a similar way that aggregation of oral bacteria is mediated by salivary glycoproteins (Gibbons and van Houte, 1975; M^{c}Intire et al, 1982; Murry et al, 1982; Abaas, 1985; Abaas and Holme, 1983a and b). Several reports have been published which suggest that lectin-like proteins are present on the bacterial cell surface and may contribute to colonization by binding to glycosyl residues on host cell surfaces. These include:- the high molecular weight proteinaceous adhesins on *S. salivarius* (Weerkamp and Jacobs, 1982); proteinaceous adhesins with sialic acid- and galactose-binding lectin-like activity on the surface of *S. sanguis* (Liljemark and Bloomquist, 1981; Murry et al, 1982); lectins with unknown sugar specificities on *S. sanguis* (Kolenbrander, 1982) and the lectin-like cell surface component of group B streptococci which is a receptor for N-acetyl-D-glucosamine (Bagg, 1984). The high molecular weight material on EBH1 cells which avidly bound con A may be cell-bound glycosylated serum components.

Whether lectin-like entities are present on the surface of the host cell or bacterial cell (or both), strong specific interactions of the lectin-ligand type are likely to contribute to the tissue tropism exhibited towards the endocardium by some species of streptococci. Such interactions would aid colonization by helping to overcome the considerable haemodynamic pressures existing in the cardiac region. The mechanism of streptococcal adherence to heart valves is obviously complex. FN is known to bind to bacteria (Kuusela, 1978) and to mediate attachment to host tissue (Simpson and Beachey, 1983). FN-receptor binding proteins (Espersen and Clemmensen, 1983) and glycoproteins (Urushihara and Yamada, 1986) have been identified; although an alternative view is held that the FN-binding component of bacteria is LTA (Courtney et al, 1983; Courtney et al, 1986; Nealon et al, 1986). Attempts at identification of a FN-receptor protein in *S. faecalis* have so far proved unsuccessful.

Incubation of blots prepared from streptococcal antigens in anti-group D streptococcal grouping serum revealed the presence of several LTA-associated protein antigens in strains of *S. faecalis*. The grouping serum reacts with the glucosyl α-1→2-glucosyl determinant in the group D antigen (Wicken and Knox, 1978). In *S. faecalis* strains and *S. faecium* the 73, 68 and 43K protein/glycoprotein antigens were shown to be associated with LTA. These antigens may form complexes with extracellular LTA in a similar manner to the M protein of group A streptococci (Beachey, 1980; Ofek et al, 1982; Beachey et al, 1983). Ligand blotting with lectins indicated that glycosyl residues are contained in the 73 and 68K antigens (and to a lesser extent, the 43K antigen) of *S. faecalis*. The 73 and 43K antigens contain fucosyl, N-acetylglucosaminyl and N-acetylgalactosaminyl residues. This

implies that these two antigens are glycoproteins existing in association with LTA. The 68K antigen appears to contain glucosyl or mannosyl residues. Glucosyl residues are contained in LTA molecules therefore the glycosyl moiety reacting with the con A in lectin-blots may indeed be situated on the LTA. Thus the 68K antigen may be composed of a protein in association with LTA. LTA (and LTA-M protein complex) plays an important role in adherence of group A streptococci to host mucosal cells (Beachey, 1975; Ofek et al, 1975; Beachey 1980 and 1981) but is not so involved in attachment of group B streptococci to epitheleal cells (Bagg, 1984). The role of LTA in the pathogenicity of *S. faecalis* has yet to be fully determined.

Anti-group D streptococcal grouping serum was also used to identify an immunoprecipitin arc in the CIE profile of strain EBH1. The CIEWIG technique showed that one of the (approximately 20) peaks was LTA and that it was not a major antigen. LTA appeared to be a more prominent antigenic component of BHI-grown cells than of HS-grown cells. The LTA content of gram-positive bacterial cells is relatively independent of growth conditions (Wicken and Knox, 1975a). The difference in LTA peak size between cells grown in the two media may therefore arise from the difference in ratios of extracellular to intracellular LTA. This is influenced by a number of factors:- concentration of Mg^{2+} in the growth medium (Hay et al, 1963); growth phase of the organism (Joseph and Shockman, 1975; Markham et al, 1975) and rate of growth (Wicken and Knox, 1977). Rapid growth in rich media may result in an abundance of extracellular LTA (Wicken and Knox, 1975a). Only soluble (or solubilized) cell components may be analysed by CIE. Since mutanolysin-digestion of whole cells is not 100% efficient, an

increased LTA peak size may be an indication of an increased amount of extracellular LTA (ie. available for analysis by CIE) as opposed to the intracellular type. Indeed CIE analysis of soluble (non-enzyme-digested) cell components of EBH1 revealed that in BHI-grown cells, the precipitin arc corresponding to LTA had a large peak area. CIE analysis of soluble HS-grown cells components showed no such peak.

In the study of bacterial antigens CIE has three main advantages over immunoblotting. The antigens remain in their native configuration (Uriel, 1971), the technique is quantitative for both antigen and antibody, and the antigenic relatedness of individual antigens is revealed (Weeke, 1973b; Owen, 1981). The relationship between amounts of antibody forming a precipitin arc is expressed as follows:-

$$A = (k_i C) \div B$$

where A = area under peak

k_i = area-loading constant (dependent on antigenic nature of i)

C = amount of antigen analysed

B = amount of anti-i immunoglobulin present in serum (Owen, 1981)

In this study the relative amounts of antigens present in BHI-and HS-grown cells were compared as serum concentration was maintained at a constant value throughout CIE analyses. Thus the greater the area of a particular peak, the greater the amount of that antigen is present in the soluble cell preparation.

A major antigen formed a large precipitin peak in both BHI- and HS-grown cell preparations. This arc has not been identified but may conceivably be the 53K protein antigen that is prominent on immunoblots of *S. faecalis* grown in either media. For identification of the numerous other immunoprecipin lines on CIE profiles, purified antigens or monospecific or monoclonal antibody preparations would ideally be incorporated into the

intermediate gel in CIEWIG or purified antigen would be co-electrophoresed in tandem CIE.

An overall picture of the antigenic composition of *S. faecalis* has arisen from the SDS-PAGE, immunoblotting and CIE analyses used in this study. *S. faecalis* presents a complex antigenic structure. Over 20 protein antigens were identified both by immunoblotting and CIE, although the anitgens revealed by each method were not necessarily identical. The denaturing of proteins during SDS-PAGE may cause cleavage of some proteins resulting in some bands on gels or blots being degredation products of higher molecular weight antigens. Because CIE deals with antigens in their native state this problem would not arise. However CIE would not reveal antigens that were insoluble. The net effect would be a reduction in the number of precipitin arcs in CIE profiles compared to the number of bands in immunoblot profiles (as observed with BHI-grown cells). Native PAGE showed several *S. faecalis* antigens to exist as a complex in their undenatured, non-enzyme-digested state. Upon CIE analysis this complex reverts to discrete antigens following digestion with mutanolysin. Although the pathogenic role in IE of the surface antigens of *S. faecalis* has yet to be determined, a number of possibilities exist. To summarize:

1. The 73K and 40K antigens may be analogous to clumping-inducing agents of similar molecular weight produced in response to a plasmid-induced pheromone.
2. The 56K protein may be analogous to the Fc-receptor of similar molecular weight in group A streptococci
3. The 73, 43, 37, 34 and 25K glycosylated antigens may mediate attachment to host tissue by interaction with lectin-like proteins on host cell or via interaction with FN.

4. The 73, 68 and 43K LTA-associated (glyco)protein antigens may be involved in adherence via interaction of the hydrophobic end of the LTA molecule with host cells.

3.2 Serodiagnosis of *S. faecalis* endocarditis

3.2.1 Immunological response of IE patients to *S. faecalis*

The production of human immunoglobulins (Ig's) in response to infection with *S. faecalis* was studied by immunoblotting. IgG is the most abundant species of Ig in normal human serum, comprising 80% of the total Ig content (Roitt, 1971). Of the other major human Ig classes IgA comprises 13%, IgM 6%, IgD 1% and IgE 0.002%. IgM is a high molecular weight antibody largely confined to the bloodstream and is involved in the early stages of infection. This Ig is therefore likely to participate in the immunological response to *S. faecalis* upon the entry of the bacterium into the bloodstream and during the ensuing bacteraemia. IgA is present selectively in sero-mucous secretions (although also present in the serum) and so is likely to come into contact with *S. faecalis* in its natural habitat of the gut. IgG is the major Ig to be synthesised during the secondary immune response. For the above reasons, IgM, IgA and IgG were selected as being of interest in the human immune response to *S. faecalis* endocarditis.

In section 3.1.2.2 the IgG responses of two IE patients infected with *S. faecalis* were examined briefly. Preliminary strip-blotting experiments led to the superficial observation that this response differed widely between individuals (figure 24). Serum from Mr. P, who was infected by strain 9112, had a much stronger reaction with antigens from strain EBH1. Mr. W only weakly recognised antigens from his own infecting strain (EBH1). The difference in response originates from variation in the host and the infecting bacterial strain (Smith, 1980). Further immunoblotting experiments involved reaction of nitrocellulose-immobilized EBH1 antigens with two samples of serum collected from Mr. W. The first sample was collected at an early stage in his episode of IE (before antibiotic therapy had begun) and

the second was collected two weeks later, during treatment with gentamicin and penicillin. The blots were then probed with rabbit anti-human IgA, M or G to reveal the change in the patient's IgA, IgM and IgG response to strain EBH1 over two weeks of antibiotic therapy. In both serum samples the patient had weak IgA and IgM responses (as estimated visually by intensity of reaction of sera with EBH1 antigens immobilized on nitrocellulose blot (figures 35a, 35b, 36a and 36b); and a strong IgG response (figures 35c and 36c). All three Ig classes reacted more strongly with the antigens following antibiotic treatment (figures 36a, b and c). This was not necessarily an antibiotic-induced effect but may have been due to progression of the disease state. The precise date upon which the patient first became infected with *S. faecalis*, strain EBH1 was not known. However the weakness of reaction of the first serum sample indicated that it was likely to have been collected early in the antibody response. Two weeks later the titres of IgA, IgM and IgG had all increased. Thus, the second serum sample appeared to have been collected at a point in the immunological response time-curve prior to reduction in titre of IgM, assuming that the patient was exhibiting a traditional secondary antibody response (Roitt, 1971). Because IgG is the major Ig synthesised during the secondary immune response and because more *S. faecalis* antigens were revealed on immunoblots by probing with rabbit anti-human IgG than either IgA or IgM, subsequent immunoblotting experiments involving human sera focussed upon IgG.

The change in levels of IgG produced by a patient (Mr. W.) during the initial fortnight of an episode of *S. faecalis* IE were evaluated for diagnostic potential or for monitoring the success of treatment or progression of disease state. The increase in IgG response, which had previously been estimated visually (as above), was validated using a more quantitative method. This method was an ELISA (enzyme-linked immunosorbant

assay; Engvall and Perlman, 1971) using nitrocellulose instead of microtitre plates as an immobilization phase. Strip-blot ELISA's showed the titre in the initial serum sample to be 400 and in the second sample to be 3,200 (figure 37a and 37b). Visual comparison of the reaction of sera with antigens on nitrocellulose was therefore corroborated, and shown to be a useful gauge of antibody titre. Five serum samples collected from Mr. W, at intervals, during the first 14 days of antibiotic therapy (the first and the last of these being the aforementioned samples, with titres of 400 and 3,200 respectively), were used to probe strip-blots as above. The IgG response of this patient reached a steady level after an initial weak response (figure 38). A similar pattern of response was shown by another *S. faecalis* endocarditis patient, Mr. P. Five samples of serum were collected from this patient and used to probe strip-blots prepared with antigens from his own infecting strain, 9112. The first sample (figure 39, lane 1) was collected during the patient's second episode of IE - the IgG response was weak. The subsequent four samples, collected during the third exacerbation, showed an increase in reaction intensity which reached a steady level (figure 39, lanes 2-5). A progressive increase in the IgG response was noted against the 40K antigen of strain 9112. Serum from Mr. P. had (as mentioned in section 3.1.2.2) a stronger reaction with *S. faecalis* strain EBH1 than with strain 9112. In fact, strip-blot titrations performed on the final serum sample collected from this patient showed the anti-EBH1 IgG titre to be in excess of 12,800 (figure 40). Three serum samples collected at weekly intervals from another patient with *S. faecalis* IE, Mr. A. The anti-SFZ IgG titre in all three serum samples was 400 (figure 41a, b and c). This characteristic steady level of IgG response attained by *S. faecalis* endocarditis patients could indicate a constant or intermittent low grade leakage of *S. faecalis* into the bloodstream from the infected

vegetation on the heart valve. Such a situation would provide a recurrent stimulus for antibody production.

3.2.2 Endocarditis serodiagnosis trial

The 73, 40 and 37K antigens appeared to be exclusive to *S. faecalis* species (see section 3.1.2) so their potential as serodiagnostic agents was investigated. Serum was collected from IE patients and used to probe immunoblots prepared from a test panel of seven *Streptococcus* species and *Staphylococcus aureus*. Four *S. faecalis* strains, SFZ, 777, EBH1 and NCTC 5957 were chosen for inclusion in the reference panel of organisms. This was because these four strains strongly expressed all three of the *S. faecalis*-specific antigens. The other species used were chosen to represent those which commonly cause IE: *S. faecium*, *S. milleri*, *S. mutans*, *S. sanguis*, *S. bovis*, *S. hominis* and *Staphylococcus aureus*. These strains were grown in HS and whole cells were separated by SDS-PAGE. The separated antigens were transferred onto a nitrocellulose membrane as described in section 2.2.3. Replicate blots were prepared and stored between sheets of filter paper at -20°C until required for use.

Before embarking upon the trial, the species-specificity of the 73, 40 and 37K *S. faecalis* antigens was tested by using rabbit 3/4 antiserum to probe a blot prepared from the above strains. The antigenic profiles thus revealed are shown in figure 42. The 73, 40 and 37K antigens were lit-up in the *S. faecalis* strains (lanes 1-4) but not in the other streptococcal species (lanes 5-10) or *Staphylococcus aureus* (lane 11). This was consistent with the species specificity of these antigens.

A preliminary trial was performed using sera from 12 IE patients from East Birmingham Hospital, Bordesley Green East, Birmingham, West Midlands, kindly supplied by Dr's E.G. Smith and I.D. Farrell. The trial was performed "blind" in that neither the identity of the patient nor of the infecting

organism causing the IE was revealed to the author until a serodiagnosis had been made. Reaction of serum with the 73, 40 and/or 37K antigens in the *S. faecalis* strains indicated a positive diagnosis for *S. faecalis* endocarditis. Non-reaction of serum with these antigens indicated that the IE was caused by a species other than *S. faecalis*. The patients were assigned numbers, 1-12. The causative organism of the IE for each patient was independently identified by the Public Health Laboratory, East Birmingham Hospital. Each patient's infecting strain (kindly donated by the PHL) was included (if available) in lane 5 of the trial panel of organisms and the blot was probed with the corresponding serum. Antigens revealed by probing replicate blots with patients sera are shown in figures 43-54. A brief summary of the results of the preliminary trial is given below (PHL identification of infecting organism was made from blood cultures):-

Patient no. 1 (infecting organism, *S. faecalis*). This patient showed a positive result for *S. faecalis* endocarditis. The serum collected from patient no. 1 reacted with the 73K antigen and weakly with the 62 and 40K antigens of the four test strains of *S. faecalis* (figure 43, lanes 1-4). Other streptococcal species were not visualised by the serum (lanes 6-11). The strain of *S. faecalis* isolated from the bloodstream of this patient was very poorly recognised (lane 5). *Staphylococcus aureus* antigens were also visualised relatively weakly. This patient appeared to have a low anti-streptococcal and anti-staphylococcal IgG titre.

Patient no. 2 (infecting organism *Staphylococcus aureus*). This patient showed a negative result for *S. faecalis* endocarditis. The only antigens that the patient's serum reacted with were those of his own infecting strain (figure 44, lane 5) and the reference strain of *Staphylococcus aureus* (lane 12).

Patient no. 3 (infecting organism, *S. sanguis*). This patient showed a negative result for *S. faecalis* endocarditis. No antigens were visualised either in the test *S. faecalis* strains (figure 45, lanes 1-4) or in the other streptococcal species (lanes 6-11). This patient failed to recognise the antigens of his own infecting strain (lane 5) and the reference strain of *S. sanguis* (lane 9). *Staphylococcus aureus* antigens reacted with this patient's serum (lane 12). It became evident as the trial progressed that all human sera reacted to varying extents with *Staphylococcus aureus* antigens. This became a useful positive internal control, and will not be further remarked upon in this section.

Patient no. 4 (infecting organism, *S. faecalis*). This patient showed a positive result for *S. faecalis* endocarditis. The characteristic pattern of antigens were lit-up in the four test strains of *S. faecalis* (figure 46, lanes 1-4). Two different morphological types (producing large colonies and small colonies when grown on blood agar) of *S. faecalis* were cultured from this patient's blood. Both of these strains were included on the blot of test organisms (lanes 5 and 6). The 73 and 37K antigens of both of the isolated colonial types were recognised by the patient's serum. The isolate with the large colonies (lane 5) reacted with a lesser intensity than did the small-colonied strain (lane 6). Serum from this patient also reacted with a 68K antigen in the other streptococcal species (lanes 7-12) plus a high molecular weight antigen in the reference strain of *S. hominis* (lane 12).

Patient no. 5 (infecting organism, *Staphylococcus epidermidis*). This patient was negative for *S. faecalis* endocarditis. His serum did not react with any *S. faecalis* antigens (figure 47, lanes 1-4), or with antigens of the other streptococcal species (lanes 6-10) except for a high molecular weight antigen in *S. hominis* (lane 11). This patient's serum did not react with the antigens of the strain that had been cultured from his bloodstream (lane 5).

Patient no. 6 (infecting organism, *S. faecalis*). This patient showed a positive result for *S. faecalis* endocarditis. A strong reaction was noted with the 40K antigen in the reference strains of *S. faecalis* (figure 48 lanes 1-4), though not in the patient's own isolate (lane 5). No other streptococcal antigens were visualised (lanes 6-11).

Patient no. 7 (infecting organism, *S. bovis*). This patient was negative for *S. faecalis* endocarditis. However a very slight reaction was detected between the serum and the 40K antigen in strain EBH1 (figure 49, lane 3). The only other streptococcal antigens recognised were in the reference strains of *S bovis* (67K; lane 10) and more weakly in the *S. hominis* (75K; lane 11).

Patient no. 8 (infecting organism, *S. sanguis*). This patient showed a negative result for *S. faecalis* endocarditis. No antigens were visualised in any of the streptococcal species, including *S. faecalis* (figure 50, lanes 1-11). This patient's serum did not recognise antigens in his own infecting strain (lane 5).

Patient no. 9 (culture negative endocarditis). This patient showed a negative result for *S. faecalis* endocarditis. The serum detected no streptococcal antigens in the test panel of streptococci (figure 51, lanes 1-10). The *Staphylococcus aureus* strain was only weakly detected (lane 11). No organism was cultured from the blood of this patient. However this does not automatically rule out the possibility of a bacterium being the

aetiological cause of the endocarditis (see section 1.3.). If, indeed, this was the case then the causal organism was unlikely to have been *S. faecalis*.

Patient no. 10 (infecting organism, *S. faecalis*). This patient showed a positive result for *S. faecalis* endocarditis. Serum reacted with the characteristic antigens in the test strains of *S. faecalis* (figure 52,lanes 1-4). However, no reaction was noted between the serum and the antigens of the *S. faecalis* strain isolated from the patient's bloodstream (lane 5). None of the other streptococcal species were recognised by the serum (lanes 6-11).

Patient no. 11 (infecting organism, *S. faecalis*). This patient showed a positive result for *S. faecalis* endocarditis. The serum reacted with the 73, 40 and 37K species-specific antigens in the four test strains of *S. faecalis* (figure 53, lanes 1-4). A weaker response was produced with the strain of *S. faecalis* isolated from the blood of the patient (lane 5). A 68K antigen was recognised in all of the other test species of streptococci (lanes 6-11).

Patient no. 12 (infecting organism, *S. faecalis*). This patient showed a strong positive result for *S. faecalis* endocarditis. Many antigens, including those of 73, 40 and 37K, were recognised in the test *S. faecalis* strains (figure 54, lanes 1-4). The serum also reacted with 73 and 37K antigens, amongst others, in the patients own infecting strain of *S. faecalis* (lane 5). Except for an extremely weak reaction with a 68K antigen, no other antigens were recognised in the other species of streptococci (lanes 6-11).

As an added control, a blot prepared from the trial panel of organisms was probed with normal human serum (NHS, obtained from a single healthy donor). NHS did not react with any *S. faecalis* antigens (figure 55, lanes 1-4). Also NHS did not react with antigens of strain EBH1 that had been

grown in NHS (lane 5). The NHS did not recognise any antigens of the other streptococcal species (6-11), and only a weak reaction was shown with antigens of the *Staphylococcus aureus* strain (lane 12).

This endocarditis serodiagnosis trial had thus proved succesful for 6/6 patients with *S. faecalis* IE and 6/6 patients with non-*S. faecalis* IE. The trial was expanded to include screening of sera collected from a total of 129 patients. These patients included 32 with IE; 24 with septicaemia; 49 with *S. faecalis* infections other than IE or septicaemia; and 10 patients undergoing CAPD (continuous ambulatory peritoneal dialysis). These categories were selected to represent groups of patients who might have been expected to have raised antibody titres to microorganisms.

An abridged version of the immunoblotting method was used to screen serum samples from these patients. Strips of nitrocellulose bearing separated antigens of *S. faecalis* strain SFZ were probed. This enabled a large number of sera to be screened simultaneously. As in the preliminary trial, the criterion for a positive diagnosis of *S. faecalis* endocarditis was reaction of serum with the 73, 40 and/or 37K antigens. Figure 56 shows an example of serodiagnosis results using strip-blots prepared from separated antigens of HS-grown *S. faecalis* antigens. The first three strips were probed with sera from patients with *S. faecalis* endocarditis. The characteristic pattern of antigens were visualized on all three strips. Serum collected from patients with *S. faecalis* infections, other than IE or septicaemia, in general did not react with the *S. faecalis* antigens (strips 4 and 5). Strip 6 shows an atypical result shown by a patient with persistent *S. faecalis* kidney infection - which will be discussed below. Two patients from whom *S. faecalis* had been isolated from the blood showed a negative serodiagnosis (strips 7 and 8); one of these patients (8) was undergoing CAPD. Sera from patients with septicaemia caused by other microbial species also did not react with the *S. faecalis* antigens

(strips 9-11). A non-reaction was also shown by sera collected from patients with IE caused by organisms other than *S. faecalis* (strips 12-14).

The results of the full trial are shown in table 6. Only 3 false-positive *S. faecalis* endocarditis diagnoses arose. The first such result was shown by a second serum sample collected from patient no. 4 (as above). This second sample was collected during a second IE episode 10 months after the original *S. faecalis* endocarditis. Even though this later infection was caused by *S. bovis*, serum from this patient produced a strong reaction with the characteristic *S. faecalis* antigens (figure 57, lanes 1-4). It was interesting that this serum lit-up a 67K antigen in the patient's own infecting strain (lane 5) and also in the reference strain of *S. bovis* (lane 10). This might be a pointer towards a future serodiagnostic agent for *S. bovis* endocarditis. The second false-positive result was shown by a patient with a persistent refractory *S. faecalis* infection of the kidney. Serum from this patient reacted weakly with the 73K antigen of SFZ (see figure 56, strip 6). The third false-positive diagnosis was made for a patient with IE caused by a nutritionally variant streptococcus (NVS). Serum from this patient reacted with the 73 and 40K antigens in the test *S. faecalis* strains (figure 58, lanes 1-4). A strong reaction was also noted with the 56K *S. faecalis* antigen.

TABLE 6 SERODIAGNOSIS OF *S.FAECALIS* ENDOCARDITIS BY WESTERN BLOTTING

DISEASE STATE	INFECTING MICRO-ORGANISM (PHLS IDENTIFICATION)	ISOLATION SITE	NO. OF PATIENTS	NO. OF PATIENTS SERA SHOWING POSITIVE REACTION WITH *S. FAECALIS* - SPECIFIC ANTIGENS
IE	*S.faecalis*	blood	6	6
	S.sanguis	"	4	0
	S.bovis	"	2	1*
	S.faecium	"	1	0
	S.pyogenes	"	1	0
	S.mitior	"	1	0
	S.viridans	"	1	0
	Group G strep.	"	2	0
	Staph. epidermidis	"	2	0
	Staph.aureus	"	4	0
	P.aeruginosa	"	1	0
	Culture negative	—	3	0
	NVS	blood	1	1*
	NI	"	1	0
SEPTICAEMIA	*S.faecalis*	blood	2	0
	S.pneumoniae	"	4	0
	S.milleri	"	1	0
	S.mitis	"	1	0
	Group B strep.	"	1	0
	Group C strep.	"	1	0
	Strep. spp.	"	2	0
	Staph.epidermidis	"	1	0
	Staph.aureus	"	2	0
	Ent.aerogenes	"	1	0
	Ent. spp.	"	1	0
	E.coli	"	5	0
	Bact.fragilis	"	3	0
	Prot.mirabilis	"	1	0
	Pseud.aeruginosa	"	3	0
	Pseud. spp.	"	2	0
	"Anaerobe"	"	1	0
	Salm.paratyphi A	"	1	0
	Klebs.aerogenes	"	1	0
VARIOUS	*S.faecalis*	urine	28	0
	"	nephrostomy fluid	4	1*
	"	wound swab	5	0
	"	peritoneal swab	1	0
	"	ascitic fluid	1	0
	"	abcess drain	2	0
	"	rectal drain	1	0
	"	urinary catheter tip	1	0
	"	central venous line tip	3	0
	"	bronchial washings	1	0
	"	ear swab	1	0
	"	sputum	1	0
	"	high vaginal swab	1	0
CAPD	VARIOUS	peritoneal fluid	10	0

Key:- * = false positive result
NVS = nutritionally variant *Streptococcus*
NI = not identified

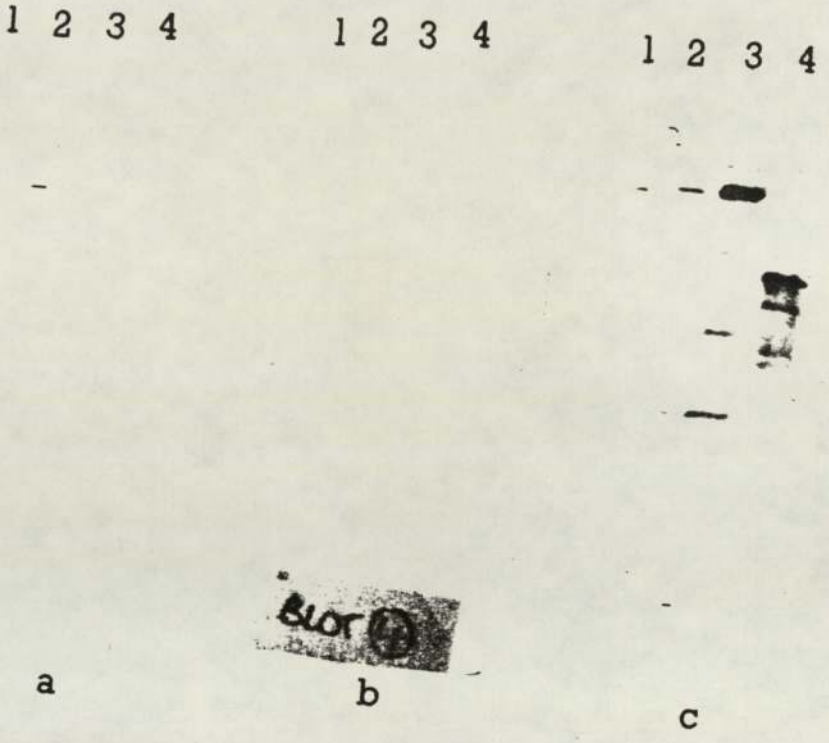


Figure 35 Immunoblot analysis of *S. faecalis* showing (a) IgA, (b) IgM and (c) IgG response of IE patient prior to treatment with antibiotics.
Lanes:
1, EBH1/BHI whole cells
2, EBH1/HS " "
3, SFZ/BHI " "
4, SFZ/HS " "
Whole cells were separated by SDS-PAGE and transferred to nitrocellulose. Blot was reacted with serum from Mr. W collected at start of IE episode, and then incubated in (a) goat anti-rabbit IgA-peroxidase, (b) goat anti-rabbit IgA-peroxidase or (c) goat anti-rabbit IgG-peroxidase (1:2000).

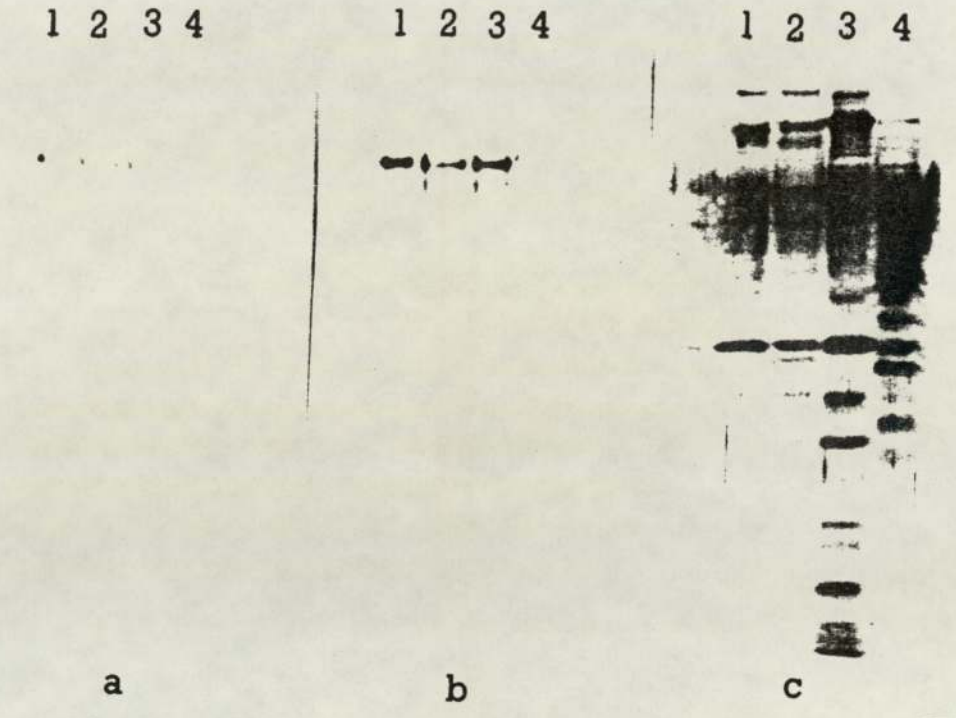


Figure 36 Immunoblot analysis of *S. faecalis* showing (a) IgA, (b) IgM and (c) IgG response of IE patient after two weeks of antibiotic therapy with ampicillin and gentamicin.

Lanes:

1, EBH1/BHI whole cells
2, EBH1/HS " "
3, SFZ/BHI " "
4, SFZ/HS " "

Whole cells were separated by SDS-PAGE and transferred to nitrocellulose. Blot was reacted with serum from Mr. W collected after two weeks therapy with ampicillin and gentamicin, and then incubated in (a) goat anti-rabbit IgA-peroxidase, (b) goat anti-rabbit IgM-peroxidase or (c) goat anti-rabbit IgG peroxidase (1:2000).

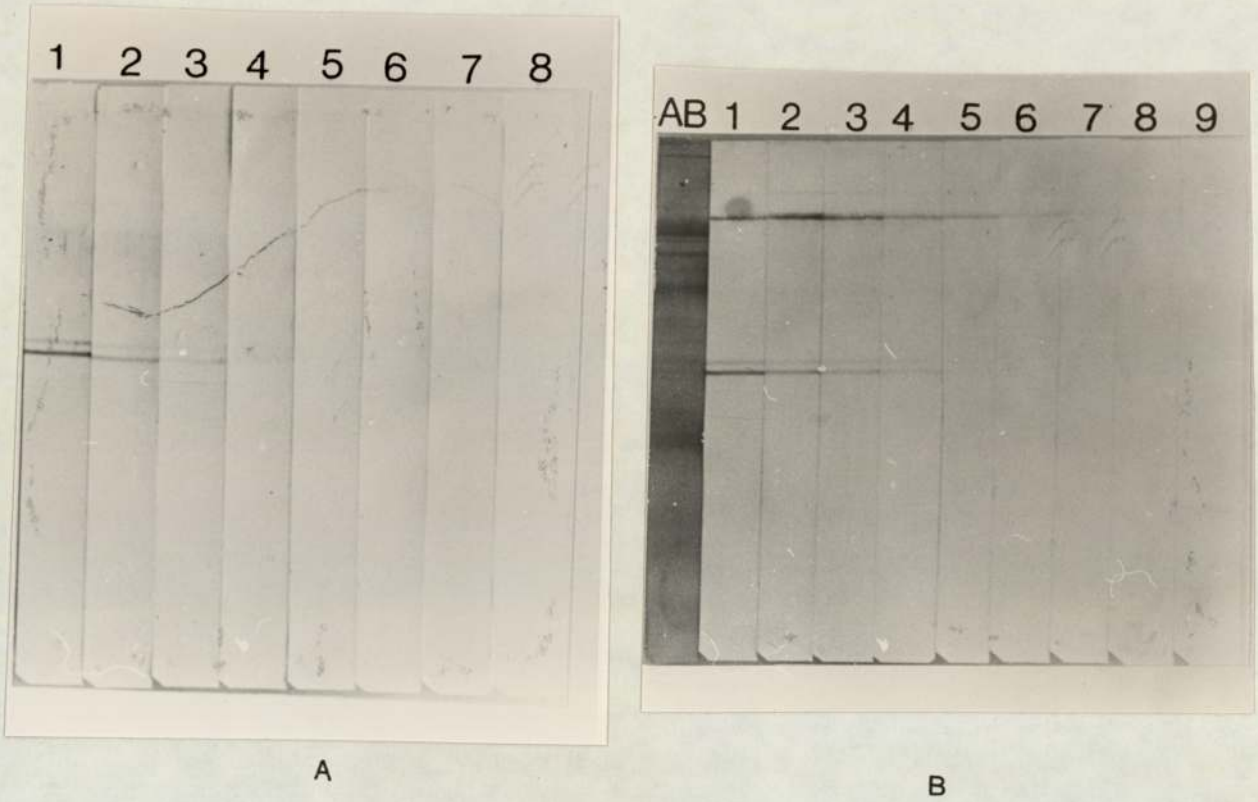


Figure 37 Strip-blot ELISA showing anti-EBH1 IgG titre of IE patient before and after two weeks of antibiotic treatment.
Whole cells of EBH1/HS were separated by SDS-PAGE and transferred to nitrocellulose. Strips of nitrocellulose were reacted with serial dilutions of serum from Mr. W collected (a) prior to antibiotic therapy and (b) after two weeks of treatment with ampicillin and gentamicin. Dilution of serum was as follows:

1, 1:25
2, 1:50
3, 1:100
4, 1:200
5, 1:400
6, 1:800
7, 1:1600
8, 1:3200

AB, amido black stain
Titre = (a) 400 before treatment and (b) 3200 after 14 days treatment

Figure 38 Strip-blots showing anti-EBH1 IgG response of IE patient over 14 days during an episode of IE.
Whole cells of EBH1/HS were separated by SDS-PAGE and transferred onto nitrocellulose. Strips of nitrocellulose were reacted with sera collected sequentially from Mr. W at intervals during 14 days of an episode of IE as follows

1, serum collected upon hospitalization of patient
2, serum collected 3 days after hospitalization of patient
3, serum collected 5 days " " " "
4, serum collected 10 days " " " "
5, serum collected 14 days " " " "

Patient was undergoing antibiotic treatment with ampicillin and gentamicin during collection of serum samples 2-5,

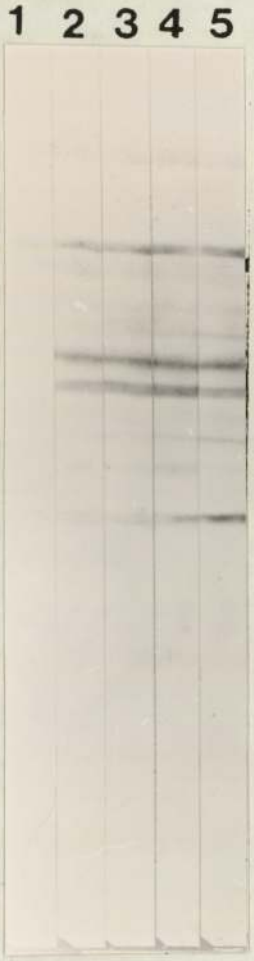


Figure 39 Strip-blots showing anti-9112 IgG response of IE patient over 14 days during an episode of IE.
Whole cells of 9112/HS were separated by SDS-PAGE and transferred to nitrocellulose. Strips of nitrocellulose were reacted with sera collected sequentially from Mr. P at intervals during 14 days of an episode of IE as follows:

1, serum collected upon hospitalization of patient
2, serum collected 3 days after hospitalization of patient
3, serum collected 5 days " " " "
4, serum collected 10 days " " " "
5, serum collected 14 days " " " "

Patient was undergoing antibiotic treatment with ampicillin and gentamicin during collection of serum samples 2-5.

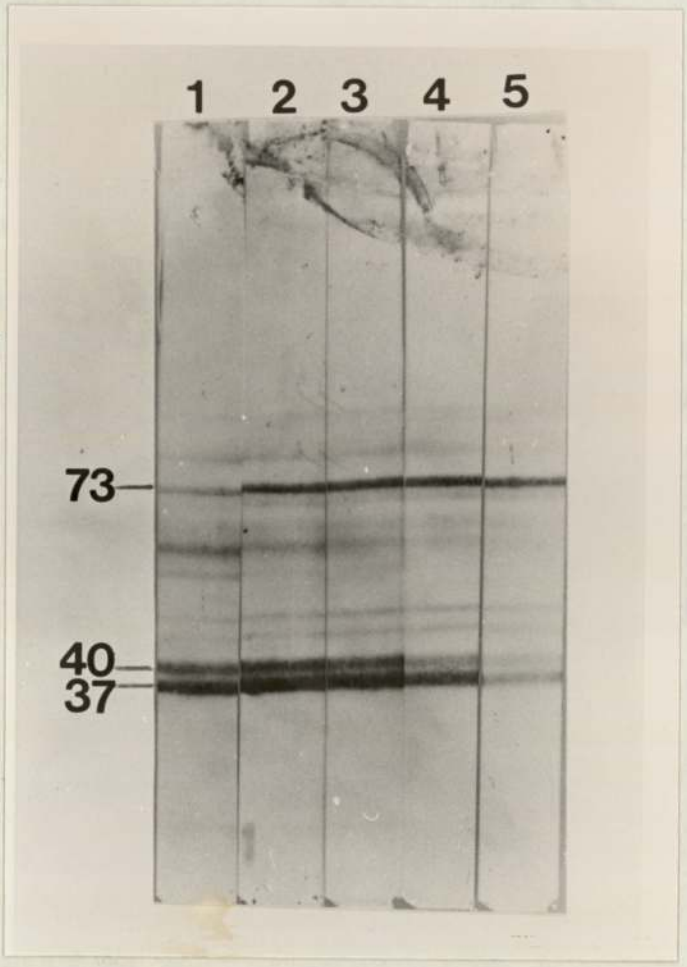
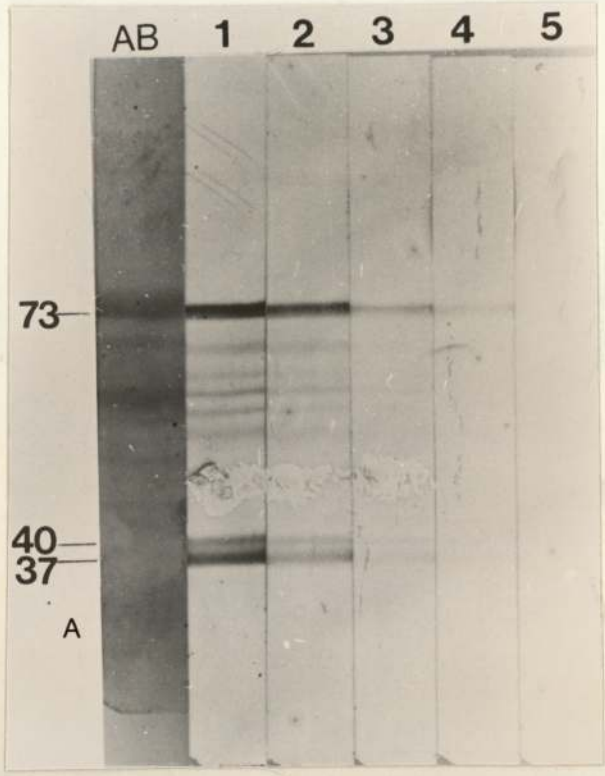
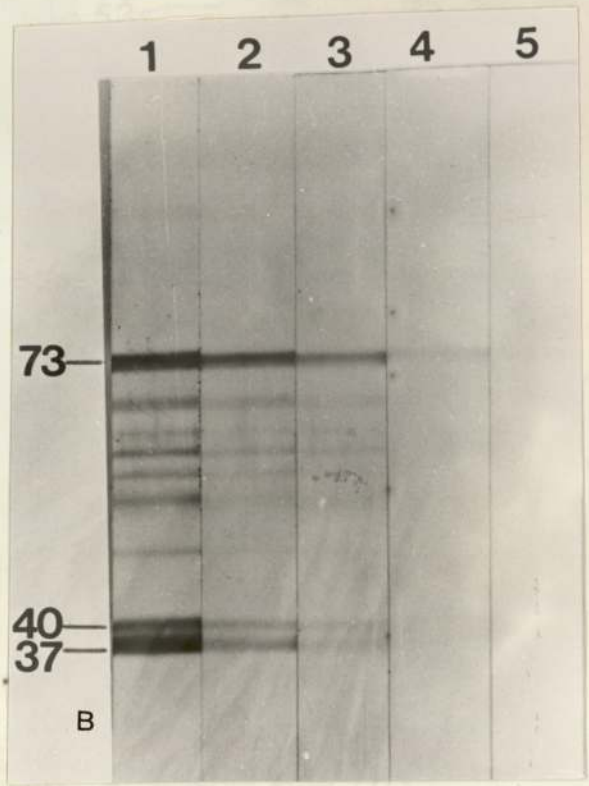


Figure 40 Strip-blot ELISA showing anti-EBH1 IgG titre of IE patient.
Whole cells of EBH1/HS were separated by SDS-PAGE and transferred to nitrocellulose. Strips of nitrocellulose were reacted with serial dilutions of serum collected from Mr. P on 14th day of 3rd episode of IE. Dilutions of serum were as follows:

1, 1:800
2, 1:1600
3, 1:3200
4, 1:6400
5, 1:12800
Titre = >12800





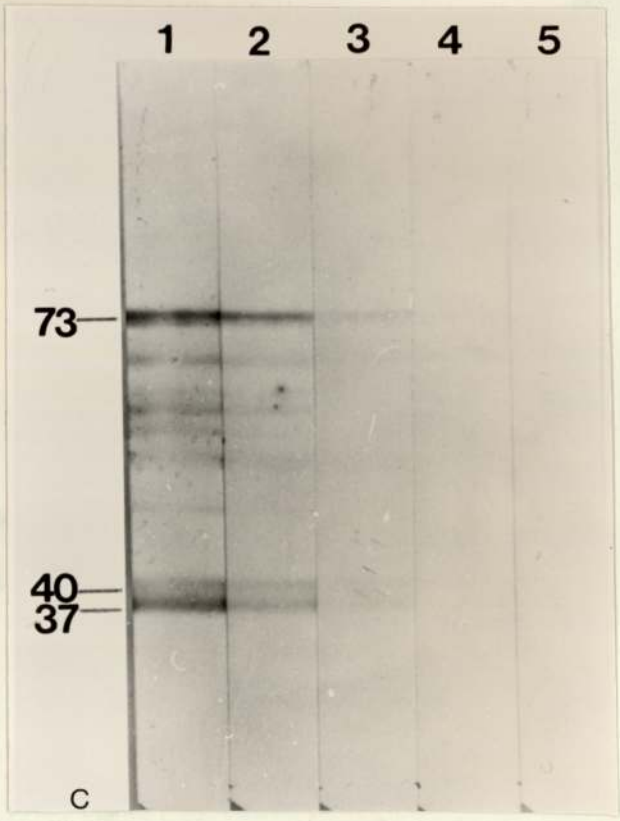


Figure 41 Strip-blot ELISA showing anti-SFZ IgG titre of IE patient during 14 days of IE episode.
Whole cells of SFZ/HS were separated by SDS-PAGE and transferred to nitrocellulolse. Strips of nitrocellulose were reacted with serial dilutions of serum collected from patient No. 4 (see section 3.2.2) (a) upon hospitalization, (b) one week later and (c) two weeks later. Dilutions of serum were as follows:
1, 1:50
2, 1:100
3, 1:400
4, 1:800
AB, amido black stain
Titre in all three samples = 400

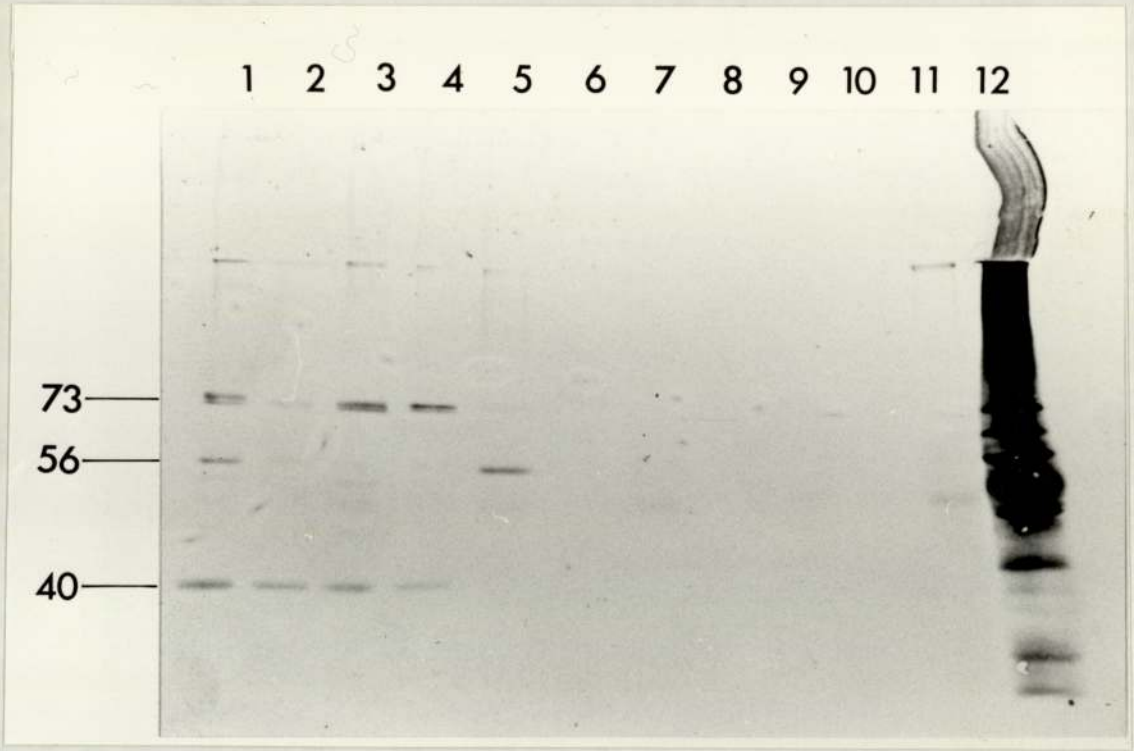


Figure 43 Immunoblot analysis of trial panel showing reaction of serum from patient No. 1 (infected with *S. faecalis*)
Lanes:
1, SFZ
2, 777
3, EBH1
4, *S. faecalis* NCTC 5957
5, Patient No. 1 isolate
6, *S. faecium* NCTC 7171
7, *S. milleri* NCTC 10708
8, *S. mutans* NCTC 10449
9, *S. sanguis* NCTC 7863
10, *S. bovis* NCTC 11436
11, *S. hominis* NCTC 8618
12, *Staphylococcus aureus* IE isolate
Blot was reacted with pooled serum samples collected from trial IE patient No. 1.

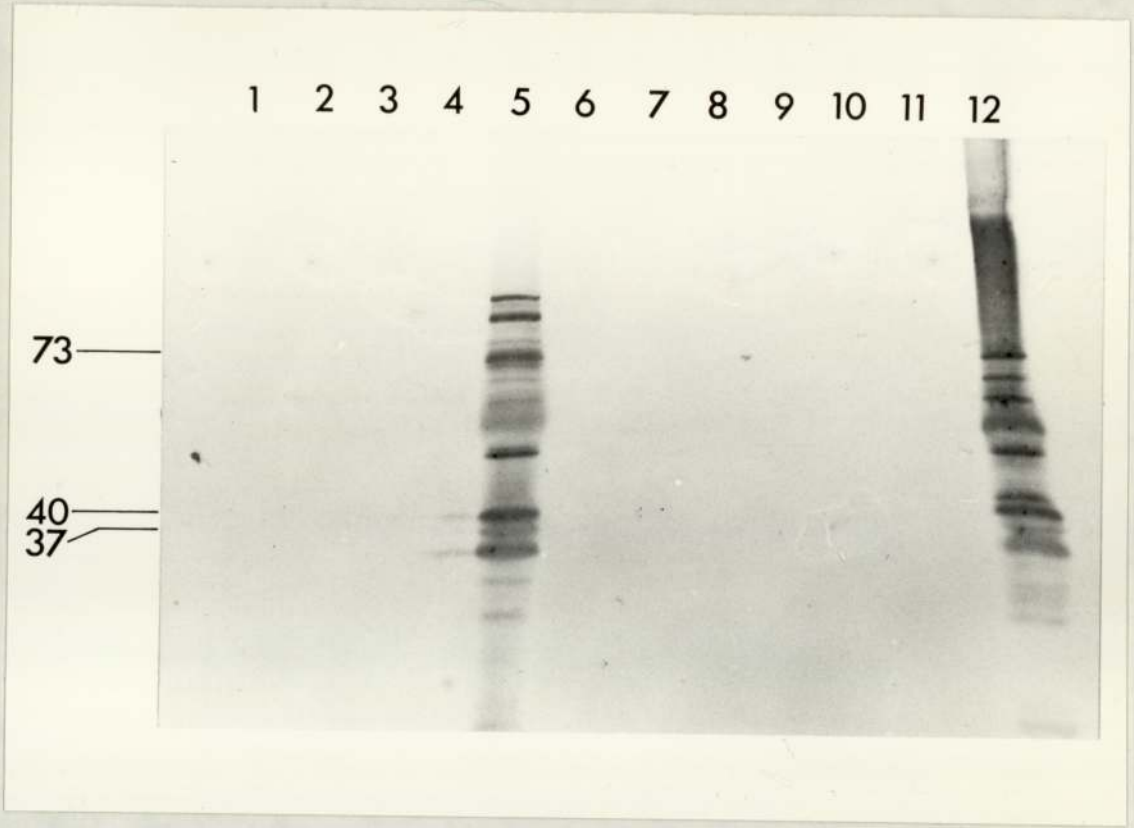


Figure 44 Immunoblot analysis of trial panel showing reaction with serum from patient No. 2 (infected with *Staphylococcus aureus*).
Lanes:
1, SFZ
2, 777
3, EBH1
4, *S. faecalis* NCTC 5957
5, Patient No. 2 isolate
6, *S. faecium* NCTC 7171
7, *S. milleri* NCTC 10708
8, *S. mutans* NCTC 10449
9, *S. sanguis* NCTC 7863
10, *S. bovis* NCTC 11436
11, *S. hominis* NCTC 8618
12, *Staphylococcus aureus* IE isolate
Blot was reacted with pooled serum samples collected from trial IE patient No. 2.

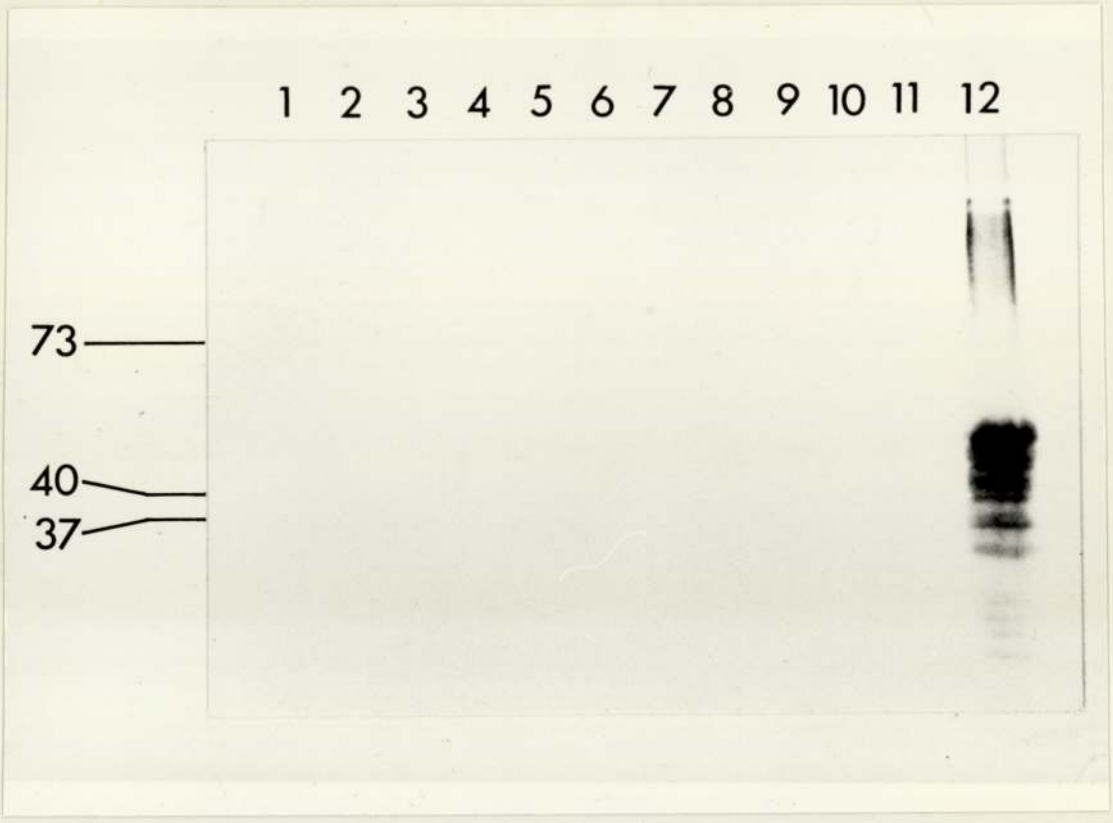


Figure 45 Immunoblot analysis of trial panel showing reaction with serum from patient No. 3 (infected with *S. sanguis*).
Lanes:
1, SFZ
2, 777
3, EBH1
4, *S. faecalis* NCTC 5957
5, Patient No. 3 isolate
6, *S. faecium* NCTC 7171
7, *S. milleri* NCTC 10708
8, *S. mutans* NCTC 10449
9, *S. sanguis* NCTC 7863
10, *S. bovis* NCTC 11436
11, *S. hominis* NCTC 8618
12, *Staphylococcus aureus* IE isolate
Blot was reacted with pooled serum samples collected from trial IE patient No. 3.

Figure 47 Immunoblot analysis of trial panel showing reaction with serum from patient No. 5 (infected with *Staphylococcus epidermidis*).
Lanes:
1, SFZ
2, 777
3, EBH1
4, *S. faecalis* NCTC 5957
5, Patient No. 5 isolate
6, *S. faecium* NCTC 7171
7, *S. milleri* NCTC 10708
8, *S. mutans* NCTC 10449
9, *S. sanguis* NCTC 7863
10, *S. bovis* NCTC 11436
11, *S. hominis* NCTC 8618
12, *Staphylococcus aureus* IE isolate.
Blot was reacted with pooled serum samples collected from trial IE patient No. 5.

Figure 48 Immunoblot analysis of trial panel showing reaction with serum from patient No. 6 (infected with *S. faecalis*).
Lanes:
1, SFZ
2, 777
3, EBH1
4, *S. faecalis* NCTC 5957
5, Patient No. 6 isolate
6, *S. faecium* NCTC 7171
7, *S. milleri* NCTC 10708
8, *S. mutans* NCTC 10449
9, *S. sanguis* NCTC 7863
10, *S. bovis* NCTC 11436
11, *S. hominis* NCTC 8618
12, *Staphylococcus aureus* IE isolate
Blot was reacted with pooled serum samples collected from trial IE patient No. 6.

Figure 51 Immunoblot analysis of trial panel showing reaction with serum from patient No. 9 (with culture-negative endocarditis).
Lanes:
1, SFZ
2, 777
3, EBH1
4, *S. faecalis* NCTC 5957
5, *S. faecium* NCTC 7171
6, *S. milleri* NCTC 10708
7, *S. mutans* NCTC 10449
8, *S. sanguis* NCTC 7863
9, *S. bovis* NCTC 11436
10, *S. hominis* NCTC 8618
11, *Staphylococcus aureus* IE isolate

Blot was reacted with pooled serum samples collected from trial IE patient No. 9.

Figure 53 Immunoblot analysis of trial panel showing reaction of serum from patient No. 11 (infected with *S. faecalis*).
Lanes:
1, SFZ
2, 777
3, EBH1
4, *S. faecalis* NCTC 5957
5, Patient No. 11 isolate, large colonial type
6, Patient No. 11 isolate, small colonial type
7, *S. faecium* NCTC 7171
8, *S. milleri* NCTC 10708
9, *S. mutans* NCTC 10449
10, *S. sanguis* NCTC 7863
11, *S. bovis* NCTC 11436
12, *S. hominis* NCTC 8618
13, *Staphylococcus aureus* IE isolate
Blot was reacted with pooled serum samples collected from trial IE patient No. 11.

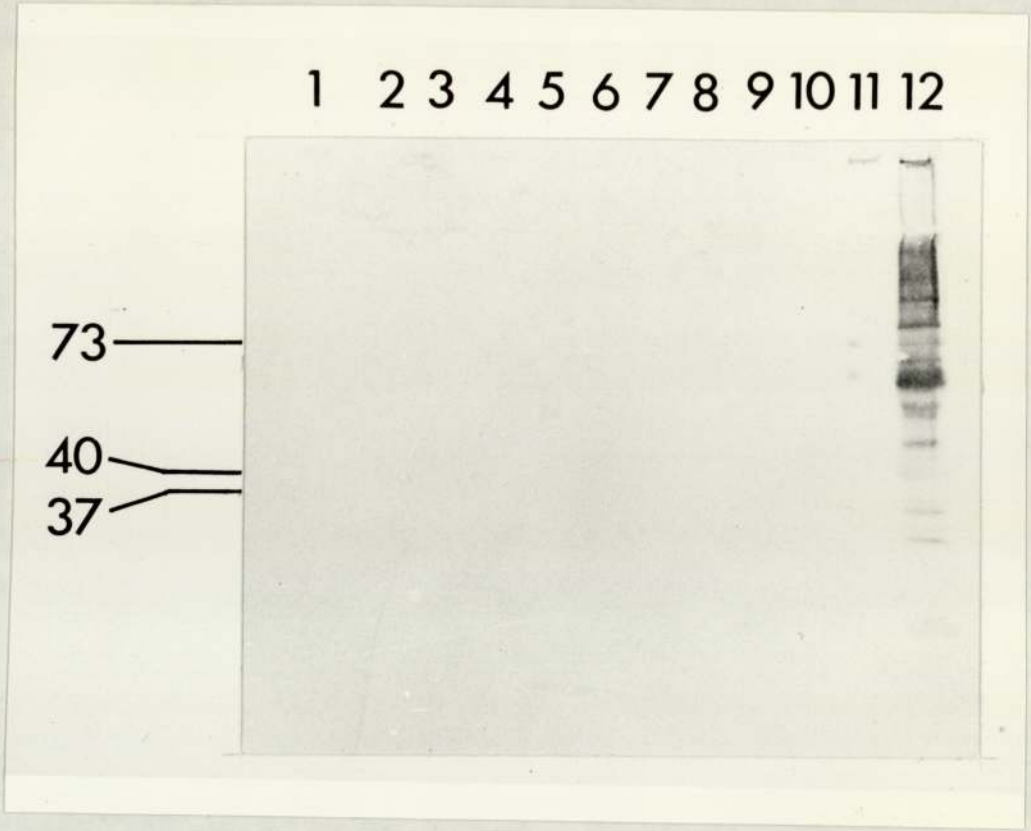


Figure 55 Immunoblot analysis of trial panel showing reaction with normal human serum.
Lanes:
1, SFZ
2, 777
3, EBH1
4, EBH/NHS
5, *S. faecalis* NCTC 5957
6, *S. faecium* NCTC 7171
7, *S. milleri* NCTC 10708
8, *S. mutans* NCTC 10449
9, *S. sanguis* NCTC 7863
10, *S. bovis* NCTC 11436
11, *S. hominis* NCTC 8618
12,*Staphylococcus aureus* IE isolate
Blot was reacted with normal human serum (NHS).

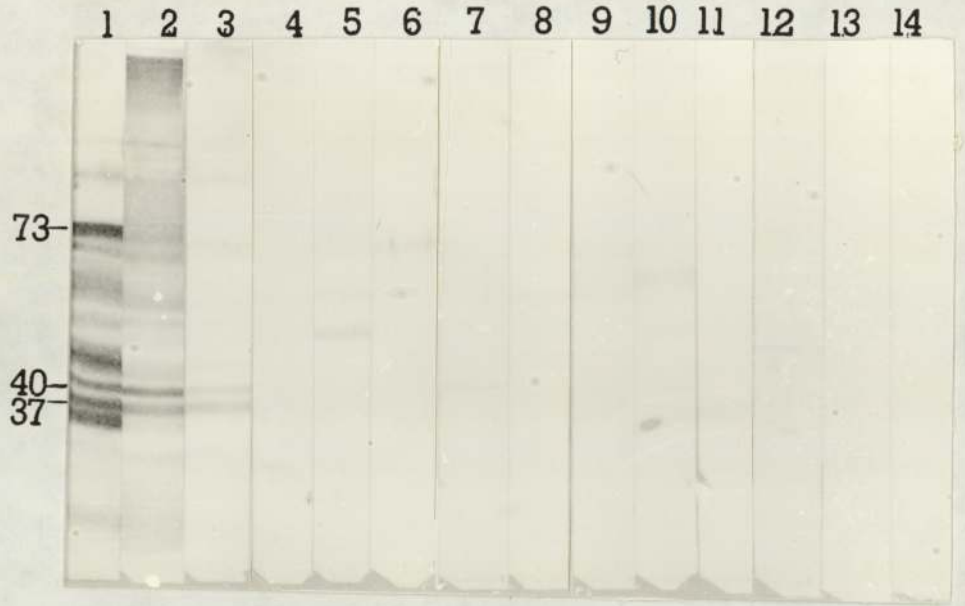


Figure 56 Example of abridged version of immunoblotting serodiagnosis trial. Whole cells of EBH1/HS were separated by SDS-PAGE and transferred to nitrocellulose. Strips of nitrocellulose were reacted with sera from patients with a range of infecting organisms and infection sites, as follows:

1, *S. faecalis* - endocarditis
2, *S. faecalis* - endocarditis
3, *S. faecalis* - endocarditis
4, *S. faecalis* - wound swab
5, *S. faecalis* - catheter tip
6, *S. faecalis* - rectal drain
7, *S. faecalis* - septicaemia blood culture
8, *S. faecalis* - septicaemia (CAPD patient)
9, *Enterobacter aerogenes* - blood culture
10, *Escherichia coli* - blood culture
11, *S. sanguis* - blood culture
12, *S. sanguis* - endocarditis
13, *Staphylococcus epidermidis* - endocarditis
14, *S. sanguis* - endocarditis

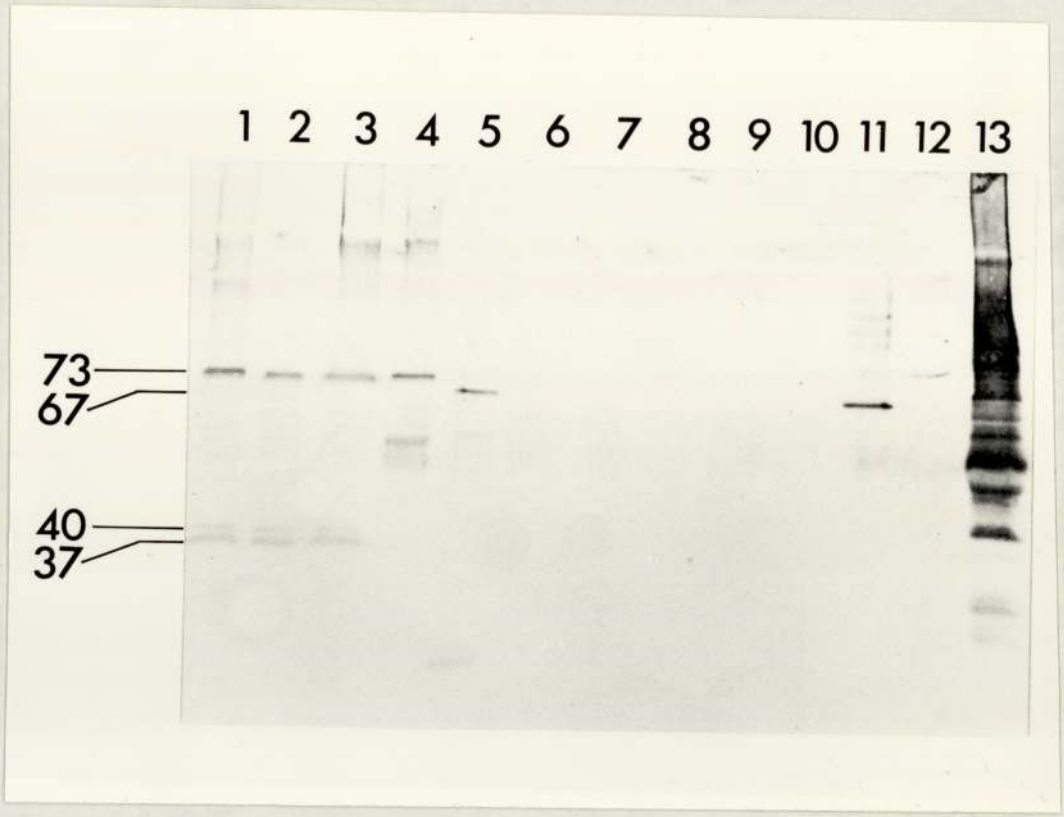


Figure 57 Immunoblot analysis of trial panel showing reaction of sera collected from patient during an episode of *S. bovis* endocarditis, following an episode of *S. faecalis* endocarditis 10 months previously.
Lanes:
1, SFZ
2, 777
3, EBH1
4, *S. faecalis* NCTC 5957
5, Patient no. 4 isolate from present infection (*S. bovis*)
6, Patient no. 4 isolate from previous infection (*S. faecalis*)
7, *S. faecium* NCTC 7171
8, *S. milleri* NCTC 10708
9, *S. mutans* NCTC 10449
10, *S. sanguis* NCTC 7863
11, *S. bovis* NCTC 11436
12, *S. hominis* NCTC 8618
13, *Staphylococcus aureus* IE isolate
Blot was reacted with pooled serum samples collected from trial IE patient No. 4 during an episode of *S. bovis* endocarditis occurring 10 months subsequently to an episode of *S. faecalis* endocarditis (see figure 46).

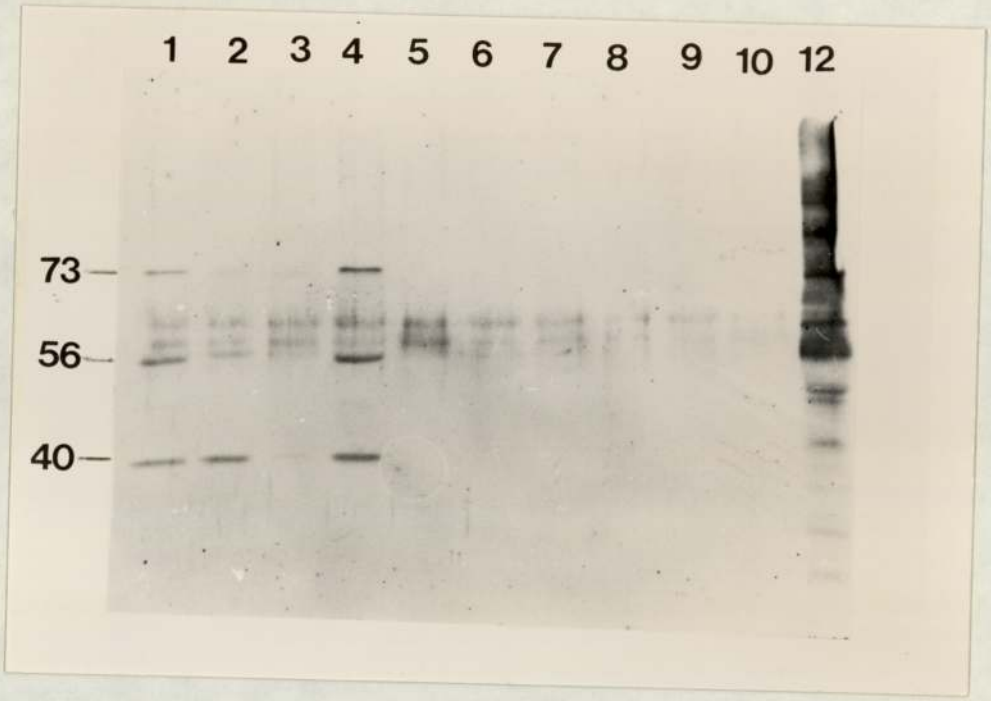


Figure 58 Immunoblot analysis of trial panel showing reaction with serum from IE patient (infected with nutritionally variant *Streptococcus*).
Lanes:
1, SFZ
2, 777
3, EBH1
4, *S. faecalis* NCTC 5957
5, Patient's isolate
6, *S. faecium* NCTC 7171
7, *S. milleri* NCTC 10708
8, *S. mutans* NCTC 10449
9, *S. sanguis* NCTC 7863
10, *S. bovis* NCTC 11436
11, *S. hominis* NCTC 8618
12, *Staphylococcus aureus* IE isolate
Blot was reacted with pooled serum samples collected from patient.

3.2.3 Discussion

When examined by SDS-PAGE, strains of *S. faecalis* were shown to have basically similar protein profiles (see section 3.1.2). But immunoblotting experiments showed that IE patients differed in their immunological response to this organism. Sera from two *S. faecalis* endocarditis patients, Mr. P and Mr. W, reacted with differing intensity and with different antigens contained in individual strains of *S. faecalis* . Both patients had a weak antibody response to their own infecting strain. Mr. P's serum reacted strongly with many antigens of the strain infecting Mr. W (EBH1) but reacted more weakly with fewer antigens of his own infecting strain (9112). The explanation for this is complex: a multitude of variations exist both within the immune response of an individual and the antigenicity of the infecting microorganism. These include host-host variation, both in the genetic make-up of the host and in the internal environment within the host; variation in the internal environment within an individual host throughout an infection; inter-strain microbial variation; and antigenic variation within a microbial species or strain. Such variations are briefly discussed below.

Invasion of a host by a pathogenic microorganism results in development of a cellular and humoral immune response (Smith, 1977). This response, involving direct attack on the microbe and an increase in the efficiency of phagocytosis, may be aimed specifically against a single invading agent or non-specifically against invading pathogens in general (Smith, 1977; Scheld and Sande, 1985). In IE the point of bacterial invasion is considered as being the moment of entry into the bloodstream. Upon entering the circulation the bacterium is challenged with non-specific immunity resulting in elimination of many bacteria. For this reason, bacteraemia does not automatically develop into septicaemia or IE. The role

of pre-formed antibodies in IE is unclear. Initially it was thought that circulating antibodies might increase the likelihood of heart valve-colonization due to their property of bacterial agglutination (Mair, 1923). However, this property might also have the opposite effect of decreasing the probability of colonization by lowering the number of "free" bacteria in the bloodstream (Scheld and Sande, 1985). Nowadays it is the commonly held view that these antibodies confer protection against infection (Durack et al, 1978; Scheld et al, 1979). A few days after initial entry into the host, specific antibodies are formed against antigens of the invading microorganism. IgG, IgM and IgA have been reported to be involved in the response to microbial challenge in IE in their capacity as opsonins (IgG), agglutinins (IgG and IgM) and complement-fixators (IgG and IgM) and as cryoglobulins (IgG, IgM and IgA; Laxdal et al, 1968). In IE the invading bacteria avoid destruction by these antibodies by concealment within the fibrin-platelet vegetations on heart valves, thus giving rise to the development of chronic infection - subacute bacterial endocarditis.

Once the infection is established, inflammatory and pathological processes occur which provide an almost continuously changing environment within which the micro-organism dwells (Smith, 1977 and 1980). Clinical isolates readily adapt to their environment (Brown and Williams, 1985). The selective pressure of specific antibodies may induce the appearance of antigenic variants; although antibodies to those variants will develop eventually (Dalhof, 1985). The mechanism of antigenic shift is employed to the advantage of microorganisms within the infected host (Mäkelä, 1980; Turner, 1980). This involves the alteration of surface antigens to avoid destruction of the bacterial cell by antibodies produced against the original surface antigens. It has long been known that phenotypic change caused by variation in growth environment can alter the antigenicity of bacteria (Lacey, 1954 and 1961). An example of this is the phenotypic

phase-variation of *Bordetella pertussis* (Robinson, 1984). This organism shifts antigenically from a virulent form to an avirulent variant in response to decreasing Na^{+} ions, increasing Mg^{2+} ions or increasing nicotinic acid in the growth medium. Meningococci and gonococci are also known to exhibit antigenic shift to evade host defences (Zak et al, 1984; Tinsley and Heckels, 1986). The poor IgG response of Mr. P and Mr. W (to their own infecting organisms) may be a manifestation of such a phenomenon. Perhaps *S. faecalis*, strains EBH1 and 9112 were originally similar in antigenic make-up, thus accounting for the strong recognition of strain EBH1 by Mr. P. Subsequent antigenic variation in strain 9112 during the course of the episode of IE, would thus result in decreasing efficiency in the immunological response of Mr. P. to strain 9112, accompanied by a change in the antigens recognised. Inter-host genetic variation could account for the difference in strength of the reaction of the two patients' sera with the original antigenic composition of *S. faecalis*. This could also have been due to another effect of antigenic shift, suppression of immune response, in a similar manner to group A streptococci in which an immunosuppressant is induced, which reduces IgM and IgG response (Schwab, 1975).

When studying the human response to an invading pathogen it would be ideal to use bacteria isolated directly from an infection (Brown and Williams, 1985; Dalhof, 1985). All the variabilities incumbent in in-vivo-grown microorganisms as well as the practicalities involved in doing this preclude such a step in this case. The antigenic alteration noted in *S. faecalis* upon subculturing on laboratory media (see section 3.1) resulted in development of a pattern of dominant antigens characteristic for the species. This pattern formed by the initial, rapid antigenic shift, has thus far remained stable for more than 2 years during which time subcultures have been performed at monthly intervals. This antigenic pattern has formed

a constant template allowing comparison of antibody response by challenge with IE patients' sera. The monitoring of the IgG response of individual patients during the course of an IE episode appeared to follow a standard course: an initial weak response, which increased rapidly to a plateau level of intensity. It was not known whether this was an indication of successful antibiotic treatment or of progression of the underlying disease state. Such observations were therefore of questionable value. A more useful gauge of response to treatment would be gained by measuring levels of immune complexes formed. The levels of immune complexes formed in chronically infected patients have been found to fall with successful treatment in IE and to rise if treatment fails (Cabane et al, 1979)

Despite being of limited value in following the progress of disease state or success of treatment, the antibody response of patients proved to be a valuable asset in the serological diagnosis of *S. faecalis* endocarditis. It became apparent that within a few days of hospitalization *S. faecalis* endocarditis patients had a sufficient level of anti-*S. faecalis* antibodies to be detectable by immunoblotting. All IE patients infected with *S. faecalis* recognised at least one of the characteristic species-specific antigens. The non-reaction with these antigens of sera from patients infected with other species provided a marked contrast. An unambiguous diagnosis could be made, with little danger of confusing a positive result with a negative result. It was, perhaps, surprising that negative results were displayed by patients with *S. faecalis* infections other than IE. This was especially so with *S. faecalis* septicaemia since prolonged presence of *S. faecalis* in the bloodstream would be expected to elicit an immune response against this organism. Possibly the patients used in this trial had serum titres of anti-*S. faecalis* IgG antibody that were too low to be detected (for various reasons). It may emerge in future studies that *S. faecalis* septicaemia patients do possess detectable antibody levels that

react with the 73, 40 and 37K antigens. Another unexpected bonus was the absence of false-positive results in the CAPD patients studied. These patients frequently present a high incidence of peritonitis - rarely less than one episode per 12 patient-treatment-months (Verbrugh and van der Muelen, 1986). Gram-positive bacteria are responsible for ≈ 70% of these peritonitis episodes (Rubin et al, 1980; Gokal et al, 1982). Infection in the CAPD patient is thought to arise from lack of hygiene, bag or catheter leakage or growth of microorganisms along the catheter from the external environment. Resistance to infection of the peritoneal cavity is reduced in the CAPD host but this is only a local effect in the peritoneum however, and there is no clear evidence of a generalised systemic reduction in host resistance (Verbrugh and van der Muelen, 1986). At any rate, this type of patient did not exhibit an abnormally high anti-*S. faecalis* antibody titre which could potentially confuse the serodiagnosis of *S. faecalis* endocarditis.

The incidence of false-positive results (3/129) in the serodiagnostic trial was very low. Of the three patients exhibiting such results, the least expected was the one having NVS endocarditis. This patient had no previous history of infection with *S. faecalis*. NVS therefore may possess some similar antigenic determinants to *S. faecalis*. There may be also a slight chance that the organism causing this patient's IE was a nutritionally variant *S. faecalis*-like organism, although no reports have been made of the existence of such a species - all NVS seem to be of the "viridans" type (Carey, 1984). There is, however, a tenuous link between NVS and *S. faecalis* in their similar response to deprivation of nutrients: Bouvet et al (1981) reported that NVS grown in a medium enriched with pyridoxal appeared to have thicker cell walls than NVS grown in the corresponding unenriched medium. Higgins et al (1974) had previously reported a similar occurrence in *S. faecalis* deprived of valine.

NVS were originally described by Frenkel and Hirsch (1961). These organisms require excess cysteine or vitamin B6 for growth and can grow as satellite colonies surrounding other organisms secreting these nutrients (Frenkel and Hirsch, 1961; Cayeux et al, 1971), or in media supplemented with these nutrients (Chapman, 1972; Bouvet et al, 1981). NVS are responsible for approximately 5-10% of streptococcal endocarditis cases (Roberts et al, 1979; Bouvet et al, 1982; Wilson and Geraci, 1985). There are several serological types of NVS, grouped according to expression of surface proteins (van de Rijn and George, 1984). As well as possessing serotype-specific proteins, the serotypes of NVS share several common antigens with other viridans streptococci (van de Rijn and George, 1984). Indeed the NVS strains studied by van de Rijn and George appeared to possess surface proteins with molecular weights in the 73 and 37K region. These might account for the false-positive diagnosis for *S. faecalis* endocarditis shown by the NVS-infected patient.

Some workers consider the penicillin G sensitivity of NVS to be similar to that of other viridans streptococci (Wilson and Geraci, 1985). Others have reported that these organisms are relatively resistant to the standard anti-viridans streptococci antibiotic treatment (Carey et al, 1977). If the latter holds true, a (false-)positive *S. faecalis* diagnosis obtained in a genuine clinical situation would have had no untoward effect on the patient concerned since the stringent treatment subsequently selected would have been equally appropriate against resistant NVS. This also applies to the patient with refractory *S. faecalis* infection of the kidney. The infection was deep-seated, chronic and resistant to treatment with penicillin G. So possibly this patient would benifit from the recommended treatment for enterococcal endocarditis - penicillin G 20-40 M units IV daily plus streptomycin 7.5mg/Kg IV every 12 hours (or if the organism is

streptomycin-resistant, gentamicin 1mg/Kg IV every eight hours) for at least 4 weeks (Wilson and Geraci, 1983 and 1985; Scheld and Mandell, 1984). The third false-positive result was shown by a patient with a previous history of *S. faecalis* endocarditis. This indicated that, were this type of serodiagnostic test to be used in hospitals, then such patients should be ruled inelligible for serodiagnostic tests and their infecting organism be identified using traditional culturing methods. Conflicting evidence exists as to how long the capacity for producing anti-*S. faecalis* antibodies persists in patients. Lancefield (1959) studied sera from a few patients that had been bled serially over periods of 10-32 years and concluded that "bactericidal" antibody persisted for many years in at least half the subjects. However, Bergner-Rabinowitz et al (1971) observed that titres had fallen to a low figure 7 months after initial infection in another group of patients. It is therefore difficult to set a time limit beyond which patients may again become suitable to undergo serodiagnosis after an episode of *S. faecalis* endocarditis.

Hayward (1973a) stated that "the traditional criteria for diagnosis (of IE) are no longer adequate". Although the ideal way to diagnose streptococcal endocarditis is to isolate the causative organism from the bloodstream (Shafi and Heimer, 1982), this is not always the most appropriate or most rapid way. Traditional blood-culture techniques may take a matter of days to reveal the identity of the infecting agent. Also this method falls short in culture-negative cases of endorcarditis or those where organisms are isolated from only one of several blood cultures. A serological method of diagnosis is thus a logical progression. Shanson and Hince (1978) suggested the use of a fluorescent antibody test for diagnosis of viridans streptococcal endocarditis. This test was further developed to define its usefulness and limitations (Shafi and Heimer, 1978; Shanson et al, 1985). It emerged from these studies that by the time streptococcal

endocarditis is clinically diagnosed, patients have high antibody titres (> 400) against the causative microorganism. This bodes well for serodiagnosis. However, Shafi and Heimer found that some sera from culture negative patients reacted with a number of strains and that the likelihood of making a correct diagnosis in culture negative case was decreased if the endocarditis was due to *S. mitior* or *S. milleri*. So ultimately the fluoresent antibody method of serodiagnosis has its major value in rapidity and is of limited value in culture negative cases. The immunoblotting method of serodiagnosis reported in this thesis is also rapid, the use of pre-prepared nitrocellulose blots reduces the time of diagnosis to 5 hours. The likelihood of cross-reaction is also lessened since the blot is examined for reaction of serum with discrete antigens. Thus, for the above reasons, this immunoblotting method deserves a place in the diagnosis of *S. faecalis* IE, either in conjunction with existing techniques, or in its own right in culture-negative cases. Such a method will prove useful in guiding the physician towards selection of proper therapy

Certain antigens are common to streptococcal and staphylococcal species and may contribute to cross-reaction of sera with heterologous strains as observed by Shafi and Heimer (1978) and Shanson et al (1985). Although titres of anti-*S. faecalis* antibody vary widely in *S. faecalis* endocarditis patients (Laxdal, 1968), most endocarditis patients presenting clinical symptoms of the disease have antibody titres of at least 400 (Shafi and Heimer, 1978; Shanson et al, 1985). Therefore it may be prudent to reduce the serum dilution to 1: 400 in the serodiagnostic immunoblotting test. This might reduce even further the incidence of false-positive results. Also this test could be simplified and be made even more specific by purification of the *S. faecalis*-specific antigens. These antigens could then be used for serodiagnosis of *S. faecalis* endocarditis by ELISA techniques; latex-bead agglutination methods; or could be separated by

SDS-PAGE and used to prepare strip-blots for use in a "dip-stick" rapid blotting test. The future ideal situation would be the use of species-specific antigens from all species of streptococci in serodiagnostic kits. Such kits could not only be used for the rapid identification of the causative organism in IE but also to record the patients antibody titre against that organism.

3.3 Isolation of *S. faecalis* antigens and determination of their cellular location

3.3.1 Surface antigens of *S. faecalis*

Preliminary immunoblotting experiments revealed that several major protein antigens were contained in the crude cell wall preparations of *S. faecalis* (see section 3.1.2). Cell-wall antigens included the serum-induced, *S. faecalis*-specific antigens that were used as the basis of a serodiagnostic test for *S. faecalis* endocarditis (described in section 3.2.2). The crude cell wall preparations may have also included proteins originating from the cytoplasm or the cell membrane. This necessitated the use of other methods to determine the location of the *S. faecalis*-specific antigens within the bacterial cell. Cell-surface antigens were labelled either directly using radioactive iodine (^{125}I), or located indirectly using fluorescein-conjugated antibody. Both methods were selective for identification of proteins/antigens which were exposed on the surface of the bacterial cell.

3.3.1.1 Radioiodination of cell-surface proteins

Cell-surface proteins were labelled with ^{125}I using the lactoperoxidase method (Heckels, 1978; Booth, 1980). ^{125}I-lactoperoxidase catalyses the iodination of tyrosine and histidine resudues of proteins in the presence of H_2O_2 (Morrison, 1974). Because the molecule has a large size (77.5K) it was assumed not to pass through the cell wall and into the bacterial cell. Thus only the surface proteins were labelled.

Figure 59 shows that not all proteins were accessible for radiolabelling on the surface of *S. faecalis* cells. Only the 93, 86, 82, 73, 68, 56, 53, 47, 40 37, 30 and 24K proteins were labelled by ^{125}I-lactoperoxidase. This indicated that these proteins (or parts of these

proteins) contained tyrosine or histidine residues and were exposed on the surface of the cell and accessible to iodination. No proteins were labelled when either lactoperoxidase or H_2O_2 were ommitted from the radioiodination procedure. Strain EBH1 (lane 4, gel; lane 8, autoradiograph) contained the greatest number of surface-exposed proteins. Strains SFZ (lane 1, gel; lane 5, autoradiograph) and 777 (lane 2, gel; lane 6, autoradiograph) had fewer surface-exposed proteins. *S. faecalis* strain NCTC 5957 unfortunately was neither stained well by Coomassie blue on the gel (lane 3) nor showed up on the autoradiograph (lane 7). This was due to a substantial reduction in the number of cells caused by accidental loss of part of the pellet during the harvesting of the labelled cells.

Several of the surface proteins were recognised as antigens by IE patient or rabbit serum (figure 60). Surface protein antigens included the 73, 40 and 37K *S. faecalis*-specific antigens in strain EBH1 (figure 60; lane 4, blot; lane 8, autoradiograph) and the 73 and 37K antigens in strains SFZ (lane 1, blot; lane 5, autoradiograph) and 777 (lane 2, blot; lane 6, autoradiograph). Several proteins were available on the surface of the cell for labelling with ^{125}I-lactoperoxidase but did not react with antibodies. These included the 100, 86, 82 and 30K proteins.

3.3.1.2 Immunofluorescence microscopy: fluorochrome-labelling of surface proteins

Whole cells of *S. faecalis*, strain EBH1 grown in BHI (Difco) were incubated in serum (rabbit or human) and labelled with FITC-conjugated antibody. Emission of fluorescence under UV illumination indicated that the corresponding antigens were accessible on the bacterial cell surface. Obtaining a good quality photographic record of immunofluorescence from the microscope proved difficult (see figure 61). It was therefore decided to record fluorescence intensity as a series of grades relative to strong

positive and negative results. Rabbit 3/4 serum provided a strong positive control. The value assigned to the fluorescence emission when EBH1 whole cells were incubated in this serum followed by labelling with FITC-protein A was "++++". The fluorescence was observed solely on the surface of the bacterial cell, producing a "halo" effect around the cell. Incubation of the EBH1 cells in normal human serum followed by goat anti-human immunoglobulin resulted in an extremely weak emission of fluorescence and was assigned a value of "-". Fluorescence emission obtained with all other sera were assigned values relative to these.

Table 7 shows the comparative values of intensity of fluorescence emission observed under UV illumination using a fluorescence microscope, with a magnification of ×1000. Sera from two *S. faecalis* IE patients, Mr. P. and Mr. W. showed positive results for immunofluorescence of EBH1 cells. These two sera had previously shown positive reactions with *S. faecalis* antigens in immunoblotting experiments. As before, serum from Mr. P. showed the stronger reaction - his serum bound to cells of EBH1 and was labelled with fluorochrome-conjugated antibody to a greater extent than serum from Mr. W. Monospecific sera raised against the 56/53K and 40/37K antigen pairs (sera A and D respectively) bound to the surface of the cells in patches. This had the effect of making the cocci appear as incomplete fluorescent spheres under microscopic UV illumination. These pairs of antigens must therefore be antibody-accessible on the bacterial cell surface - the 40/37K pair to a greater extent than the 56/53K pair. The group D antigen, LTA, was also shown to be accessible on the cell surface. This is in agreement with the proposed model of the Gram-positive cell wall (Wicken and Knox, 1980; Hammond et al, 1984). Serum from a patient with non-*S. faecalis* endocarditis (caused by *S. sanguis*) did not adhere to the surface of EBH1 cells, as shown by a lack of fluorescence when cells were illuminated with UV light.

TABLE 7

Relative Intensity of Fluorescence Emission of Intact Cells of EBH1 Labelled With FITC-conjugate Following Incubation in Antisera

SERUM	FLUOROCHROME LABEL	FLUORESCENCE INTENSITY
Rabbit 3/4	FITC-protein A	++++
Mr. P	FITC-antihuman Ig	++++
Mr. W	FITC-antihuman Ig	+++
Monospecific D	FITC-protein A	++
Monospecific A	FITC-protein A	+
Anti-group D streoto-coccal grouping serum	FITC-protein A	++
S. sanguis IE patient	FITC-antihuman Ig	---
Normal Human Serum	FITC-antihuman Ig	---

3.3.2 Isolation of *S. faecalis*-specific antigens

The 73, 40 and 37K *S. faecalis*-specific antigens were used as a basis for the immunoblotting method of serological diagnosis of *S. faecalis* endocarditis (see section 3.2.2). This method was rapid and sensitive, showing a low incidence of false-positive results. The interpretation of results could be simplified and the possibility of cross-reaction between sera might be further reduced by using only the relevant *S. faecalis* antigens (ie. 73, 40 and 37K) in blotting or ELISA tests. With this aim, the isolation of these antigens was attempted. Two techniques were chosen for preliminary isolation experiments: crude separation involving precipitation of proteins at high salt concentrations and a more refined separation method involving FPLC.

3.3.2.1 Ammonium sulphate precipitation

The salting-out process was performed on solubilized whole-cell preparations of HS-grown EBH1. Solubilization of cells was carried out by extraction with Sarkosyl or digestion with mutanolysin. Nuclear material was precipitated by the addition of 1%w/v streptomycin sulphate. Subsequent precipitation with ammonium sulphate was carried out either using progressive partial saturation concentrations of 0, 30, 60 and 90% or 0, 40, 60 and 80%.

Initial addition of 1%w/v streptomycin sulphate to the mutanolysin digest resulted in precipitation of high molecular weight material (visualised as a diffuse region on the blot; figure 62, lane 2) plus a large proportion of antigens, made up the characteristic antigenic pattern of *S. faecalis*. The majority of the remaining EBH1 antigens were precipitated in the 30-60% ammonium sulphate fraction (lane 4). The 73, 40 and 37K antigens were included in this fraction along with others. The 0-30% precipitate contained these *S. faecalis*-specific antigens plus only one major contaminating protein (lane 3). Neither the 60-90% precipitate

(lane 5) nor the 90% final supernatant (lane 6) contained any of the required antigens. Precipitation of the EBH1 mutanolysin digested proteins using the 0-40-60-80% ammonium sulphate concentration scheme did not result in selective precipitation of the *S. faecalis*-specific antigens to any satisfactory degree (data not shown).

The Sarkosyl-extracted cells of EBH1 were subjected to the same treatment. In this case the 40 and 37K antigens were deposited mainly in the 60-90% ammonium sulphate precipitate (figure 63a, lane 5), together with traces of several other proteins. The 40 and 37K antigens were particularly prominent in this fraction. Unfortunately the 73K *S. faecalis* antigen was only weakly present in the 0-30% precipitate (lane 3). The greater quantity of this antigen had been precipitated by the addition of the streptomycin sulphate (lane 2). The salting-out of proteins from the Sarkosyl extract using 0-40-60-80% ammonium sulphate resulted in the 73K antigen being recovered in the 40-60% precipitate together with one major contaminant (63K;figure 63b, lane 3). Most of the other proteins, including the 40 and 37K antigens appeared to have been removed by the addition of streptomycin (lane 2).

Ammonium sulphate precipitation did not achieve isolation of the *S. faecalis*-specific antigens to a satisfactory degree of purity. However the 73, 40 and 37K antigens were obtained from mutanolysin-digested cells of *S. faecalis* by the addition of 30% ammonium sulphate with the added presence of only one other protein in the precipitate; and the 40 and 37K antigens were salted out from a Sarkosyl digest by the addition of 90% ammonium sulphate together with only a few other surplus proteins. This method may be useful as a preliminary clarification stage in the ultimate isolation of the *S. faecalis*-specific antigens.

3.3.2.2 **Fast protein liquid chromatography**

In an attempt to achieve separation of *S. faecalis* proteins with a greater degree of purity, gel filtration chromatography of soluble antigen preparations was carried out. Mutanolysin-digested EBH1 was separated on a Superose 12 HR 10/30 column. Fractions collected manually from the column on a peak-by-peak basis (see below) were analysed by immunoblotting against serum from a *S. faecalis* endocarditis patient (Mr. P.; anti-*S. faecalis* IgG titre >12800). Discrete peaks on the UV absorption elution profile (figure 64a) did not appear to correspond to single antigens (figure 64b). None of the *S. faecalis*-specific antigens were selectively obtained in any one fraction. The 73K antigen was found in peaks 6 and 7 (figure 64b, lanes 6 and 7) but most of the other EBH1 antigens were also present. The 40 and 37K antigens were present in later fractions eluted from the column (lanes 9 and 10) but additional unwanted antigens were also there (a 63K antigen being particularly persistent). When 1% w/v SDS was introduced into the sample and buffer system, a better separation resolution was obtained (figure 65). In this case fractions were collected at two-minute intervals (flow rate 36ml/hour; fraction volume 1.2ml). The higher molecular weight proteins came off the column in the early fractions (figure 65b, lanes 3-6). The 73K *S. faecalis* antigen was present in these early fractions up till fraction number 10 (lane 10). In all of these fractions other antigens were also present. The 40 and 37K antigens continued to be eluted from the column up till fraction number 15 (lane 15). In fractions 14 and 15 (lanes 14 and 15) only one other unwanted protein persisted (63K). This combination of column and buffer system therefore had some degree of potential for isolation of the 40 and 37K *S. faecalis*-specific antigens. However, too many contaminating antigens were present in

the fractions containing the 73K antigen to make this a viable method for isolation of this antigen.

Separation of (mutanolysin-digested) EBH1 antigens was also carried out using a Sephacryl S-200 SF FPLC column. Buffer flow rate through this column was slower than through the Superose 12 column, so the antigens began to come off the column after a greater time had lapsed following initial application of the sample to the column. The UV absorption elution profile (figure 66a) featured only one large peak. The total peak area corresponded to elution of all the antigens contained in the soluble EBH1 preparation. The 73K *S. faecalis* antigen was eluted from the column in large amounts in the early fractions (figure 66b, lanes 2-6) together with many other antigens. Fraction number 8 (lane 8) contained the 73K antigen without any high molecular weight contaminants but unfortunately this fraction also contained unwanted additional lower molecular weight antigens. In each of the fractions collected, the 40 and 37K antigens were strongly expressed. The fewest additional proteins were present in the later fractions (lanes 11-16). Fraction number 16 (lane 16) gave the best result for isolation of these antigens, Again, mainly the 63K antigen persisted.

Introduction of 1%w/v SDS into the sample and buffer system resulted in a simple elution profile (figure 67a). The fractions containing the required antigens contained fewer contaminants. Initial fractions contained the 73K antigen (figure 67b, lanes 2-10). Fractions 3 and 4 (lanes 3 and 4) contained the fewest lower molecular weight contaminants. However, high molecular weight material was eluted from the column in these fractions. The final fractions coming off the column contained the 40 and 37K antigens (lanes 15 and 16), plus the 63K protein.

Replacement of 1%w/v SDS with 1%w/v Sarkosyl in the system resulted in the most satisfactory separation profile thus far obtained. In this case the separation was performed on Sarkosyl-extracted antigens of EBH1. Fractions

were collected every two minutes from the Sephacryl S-200 SF chromatography column, commencing collection 40 minutes after initial application of the sample. The third fraction contained the 73K *S. faecalis*-specific antigen (figure 68b, lane 3). Only one other unwanted protein was present in this fraction (63K). The 40 and 37K antigens were also present in trace amounts in this fraction. Fractions 14, 15 and 16 (lanes 14-16) contained the 40 and 37K *S. faecalis*-specific antigens in substantial amounts, with the 63K contaminant being present in small amounts. The FPLC system using a Sephacryl S-200 SF column to separate Sarkosyl-extracted EBH1 proteins, using a buffer containing 1%w/v Sarkosyl, thus appeared to give the most promising results for isolation of the three *S. faecalis*-specific antigens. The 73, 40 and 37K antigens were obtained in discrete fractions with only one other contaminating protein present. This contaminant was the same in both cases - 63K.

In an attempt to maximise expression of *S. faecalis*-specific antigens by each bacterial cell for isolation purposes, EBH1 was grown in various media. The antigenic profile of EBH1 grown in CDM supplemented with 1% v/v HS is shown in figure 69. Growth in CDM (lane 3) resulted in satisfactory expression of the 73K antigen but inadequate expression of the 40 and 37K antigens as compared to growth in HS (lane 1). Growth of EBH1 in normal human serum appeared to enhance production of the 73K antigen (lane 2). This may possibly have implications in the in-vivo situation in IE.

When EBH1 was grown in BHI (Difco) as shown in figure 70, expression of all three *S. faecalis*-specific antigens resulted (lane 4), albeit in reduced amounts compared to growth of the organism in HS (lane 1) or NHS (lane 2). It was noted with interest that the antigenic profile of EBH1 differed in Lab M and Difco BHI (as discussed in section 3.1.5). These two media differ widely in formula (see section 2.1.2), so perhaps this is not surprising in view of the fact that growth environment influences bacterial

cell surfaces. For future large-scale production of *S. faecalis*-specific antigens, BHI (Difco) was deemed a suitable medium in which to grow *S. faecalis* since all three required antigens are expressed by the organism in this medium plus the fact that large volumes of BHI are more economical than the equivalent volume of HS.

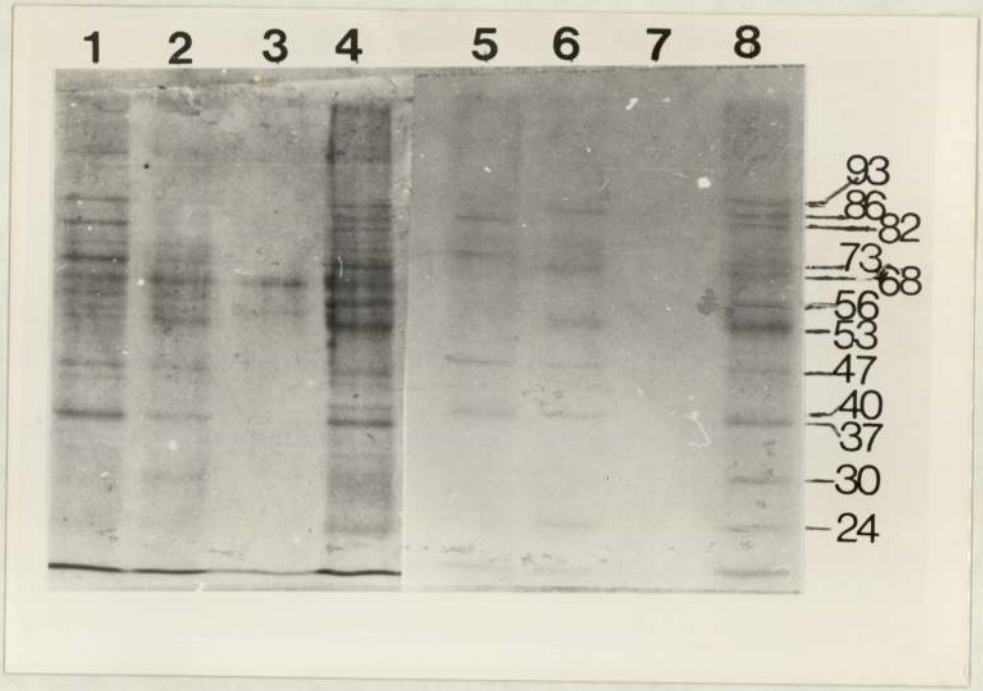


Figure 59 SDS-PAGE and autoradiograph analysis of I^{125}-labelled *S. faecalis* strains showing surface-exposed proteins.
Whole cells grown in HS were radioiodinated using the lactoperoxidase method and separated by SDS-PAGE. An autoradiograph was developed from the gel. Lanes 1-4 show gel, lanes 5-8 show autoradiograph.
Lanes:
1, SFZ - gel
2, 777 - gel
3, *S. faecalis* NCTC 5957 - gel
4, EBH1 - gel
5-8, as for lanes 1-4 - autoradiograph

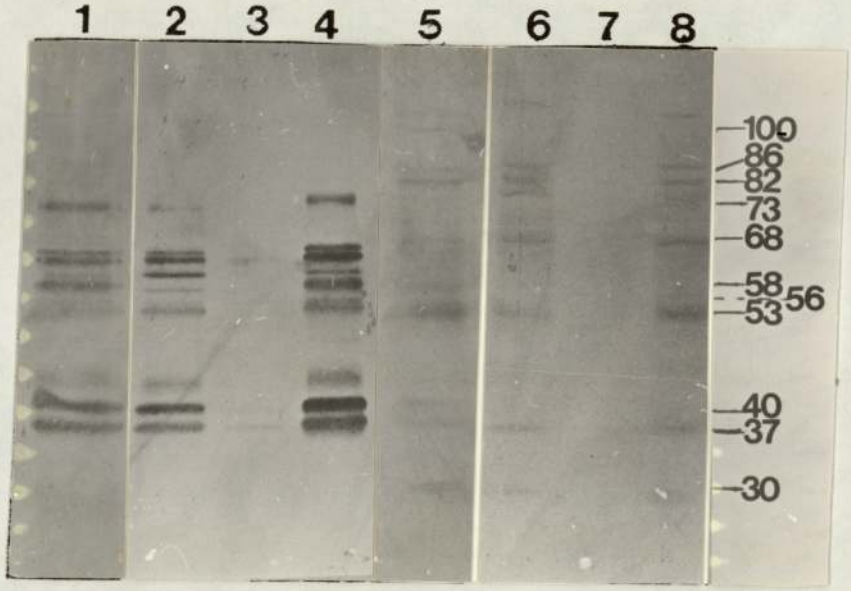


Figure 60 Immunoblot and autoradiograph analysis of I^{125}-labelled *S. faecalis* showing surface-exposed antigens.
Whole cells grown in HS were radioiodinated using the lactoperoxidase method, separated by SDS-PAGE and transferred to nitrocellulose. An autoradiograph was developed from the blot. Blot was reacted with rabbit 3/4 serum. Lanes 1-4 show blot, lanes 5-8 show autoradiograph.
Lanes:
1, SFZ - blot
2, 777 - blot
3, *S. faecalis* NCTC 5957 - blot
4, EBH1 - blot
5-8, as for lanes 1-4 - autoradiograph

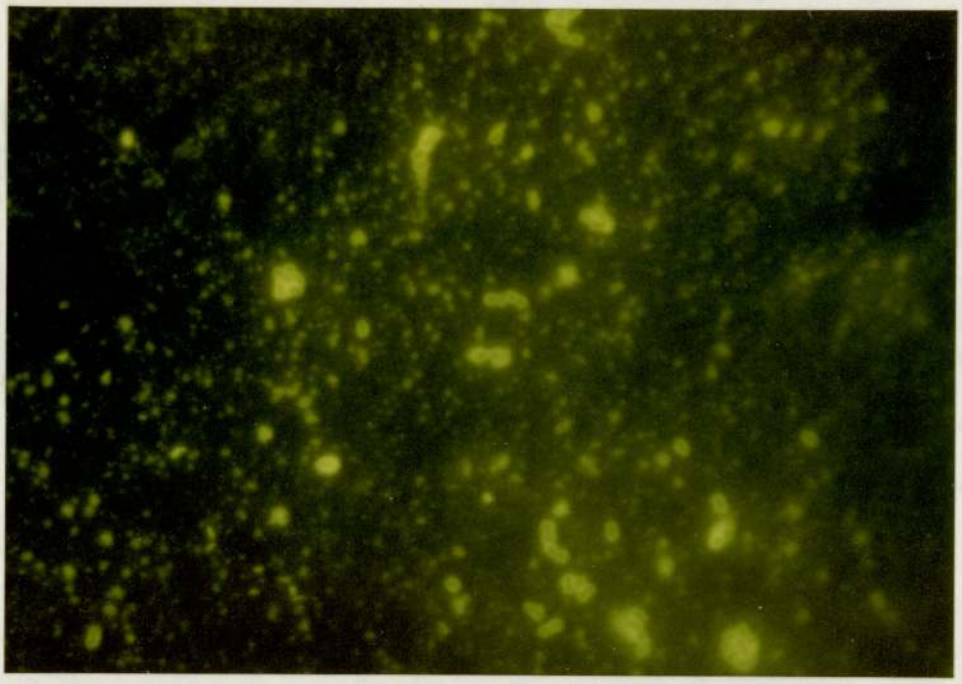
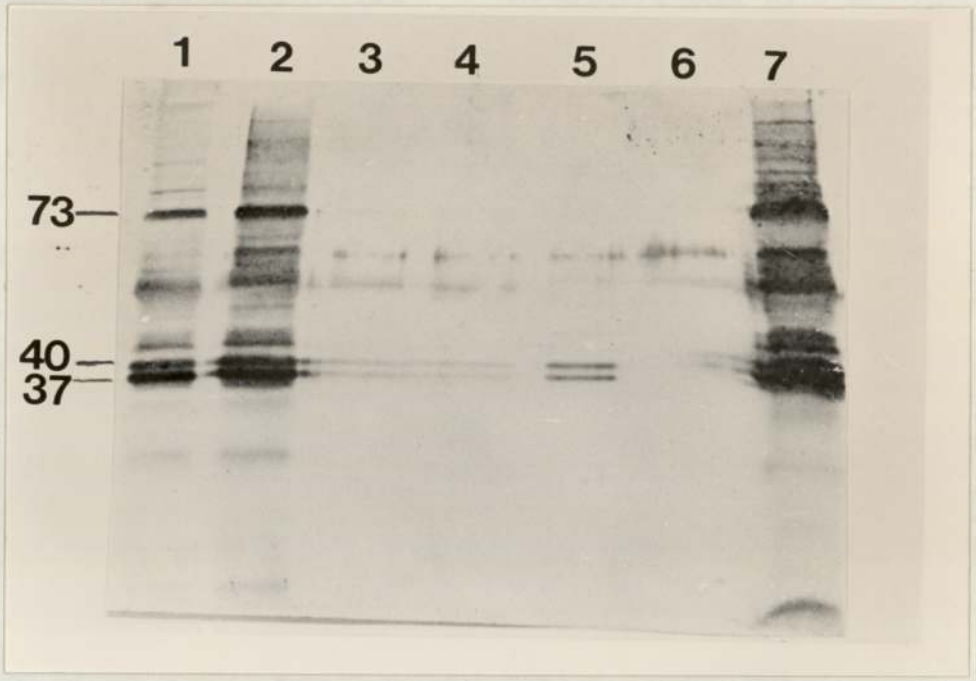


Figure 61 Immunofluorescence observation of *S. faecalis* labelled with fluorochrome to show surfac antigens.
Whole cells of EBH1 grown in HS were incubated in Mr. P serum, followed by FITC-anti-human gammaglobulin conjugate (or FITC-protein A conjugate). Cells were observed under UV illumination. Magnification × 1000.

Figure 62 Immunoblot analysis of EBH1 mutanolysin digest separated by ammonium sulphate precipitation.
EBH1/HS mutanolysin digest was precipitated by progressive additions of ammonium sulphate to partial saturation concentrations of 0, 30, 60 and 90%. The precipitates formed after each addition of salt, together with the final supernatant were subjected to immunoblot analysis.
Lanes:

1, EBH1/HS mutanolysin digest, untreated
2, precipitate formed by addition of 1% streptomycin
3, " " " " " 30% ammonium sulphate
4, " " " " " 60% " "
5, " " " " " 90% " "
6, supernatant " " " " 90% " "
7, EBH1/HS mutanolysin digest, untreated

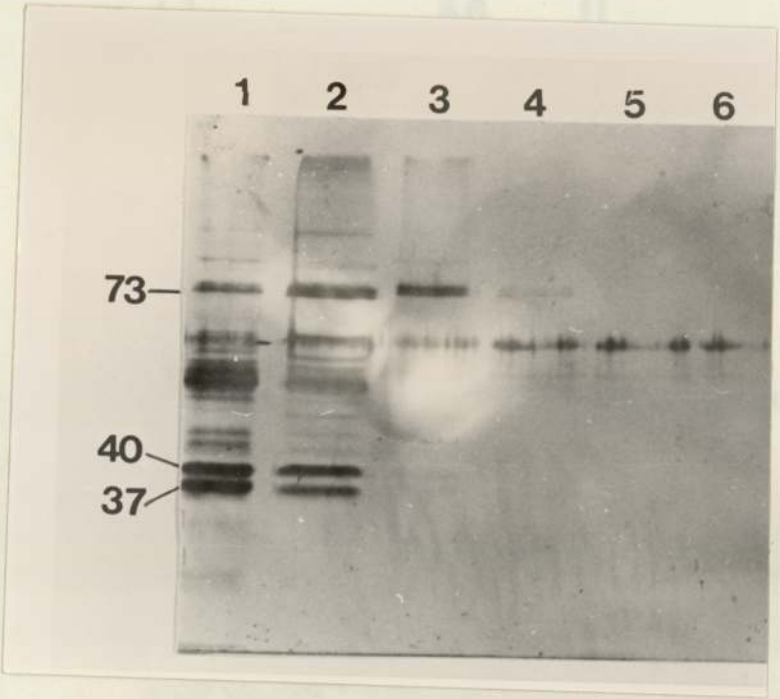
Blot was reacted with Mr. P serum.



A

Figure 63 Immunoblot analysis of EBH1 Sarkosyl extract separated by ammonium sulphate precipitation.
EBH1/HS Sarkosyl extract was precipitated by additions of ammonium sulphate to progressive partial saturation concentrations of (a) 0, 30, 60 and 90% or (b) 0, 40, 60 and 80%. The precipitates formed after each addition of salt, together with the final supernatant were subjected to immunoblot analysis.
Lanes:
1a, EBH1/HS Sarkosyl extract, untreated
2a, precipitate formed by addition of 1% streptomycin
3a, " " " " " 30% ammonium sulphate
4a, " " " " " 60% " "
5a, " " " " " 90% " "
6a, supernatant " " " " 90% " "
7a, EBH1/HS Sarkosyl extract, untreated

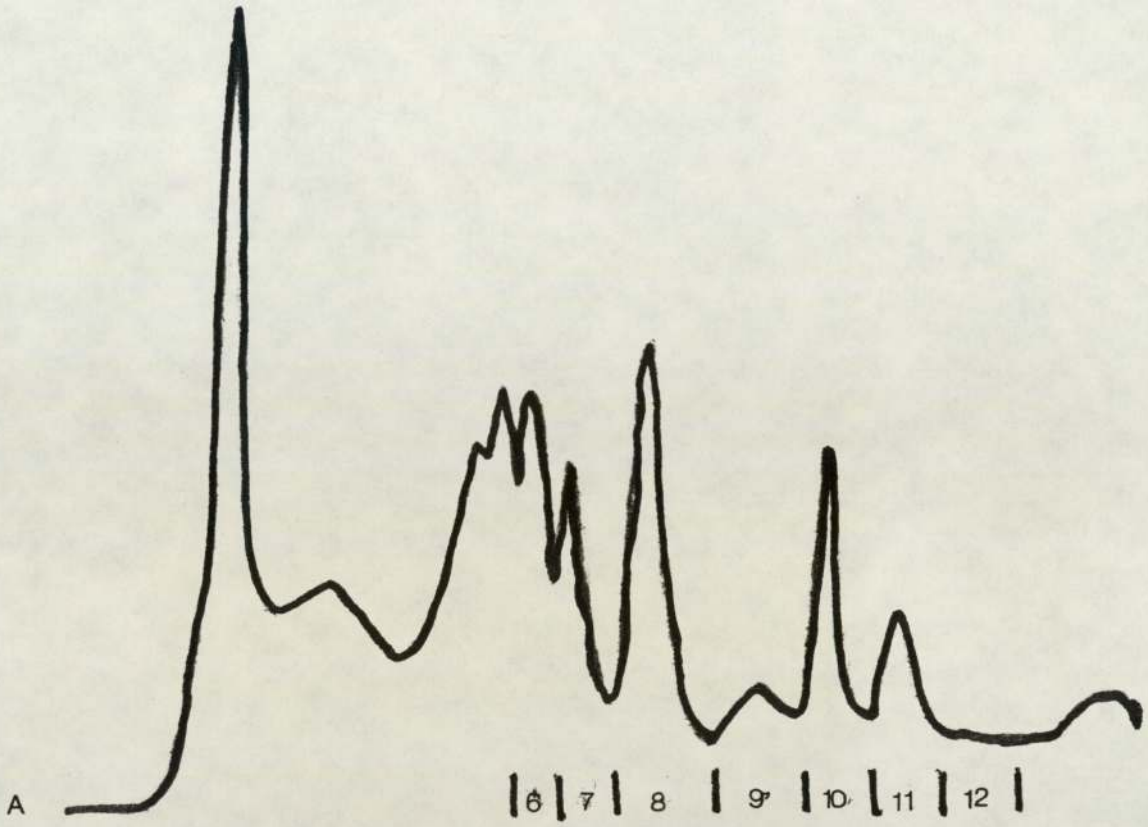
Blots were reacted with Mr. P serum.



B

Figure 63 Immunoblot analysis of EBH1 Sarkosyl extract separated by ammonium sulphate precipitation.
EBH1/HS Sarkosyl extract was precipitated by additions of ammonium sulphate to progressive partiol saturation concentrations of (a) 0, 30, 60 and 90% or (b) 0, 40, 60 and 80%. The precipitates formed after each addition of salt, together with the final supernatant were subjected to immunoblot analysis.
Lanes:

1b, EBH1/HS Sarkosyl extract, untreated
2b, precipitate formed by addition of 1% streptomycin
3b, " " " " 40% ammonium sulphate
4b, " " " " 60% " "
5b, " " " " 80% " "
6b, supernatant " " " " 80% " "
Blots were reacted with Mr. P serum.



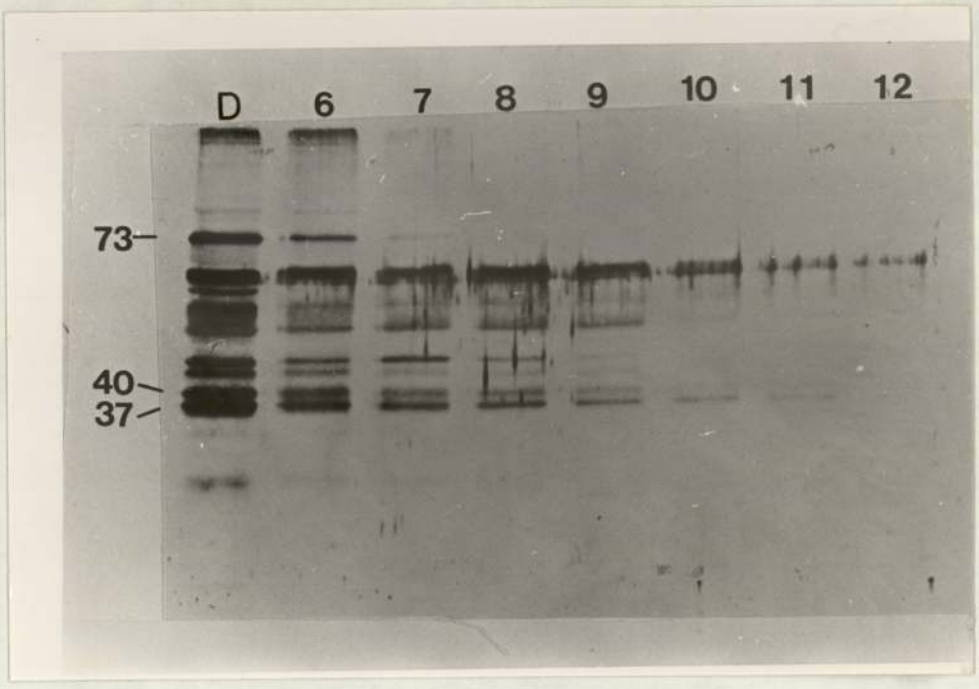


Figure 64a UV absorption profile of fractions eluted from Superose 12 HR 10/30 column following FPLC separation of EBH1/HS mutanolysin digest. Numbers indicate peaks/fractions collected.

Sample applied to column:	2mg EBH1/HS mutanolysin digest
Flow rate throuth column:	35ml/hour
Chart speed:	5mm/minute
Absorbance units full scale (AUFS):	0.01 (absorbance at 280nm)
Buffer system:	10mM Tris-HCl, pH 7.4

Figure 64b Immunoblot analysis of fractions collected from Superose 12 HR 10/30 column following FPLC separation of EBH1/HS mutanolysin digest. Samples were collected on a peak-by-peak basis as shown in figure 64a.
Lanes:
D, EBH1/HS mutanolysin digest, untreated
6-12, fractions comprising peaks 6-12 as shown in figure 64a
Blot was reacted with Mr. P serum.

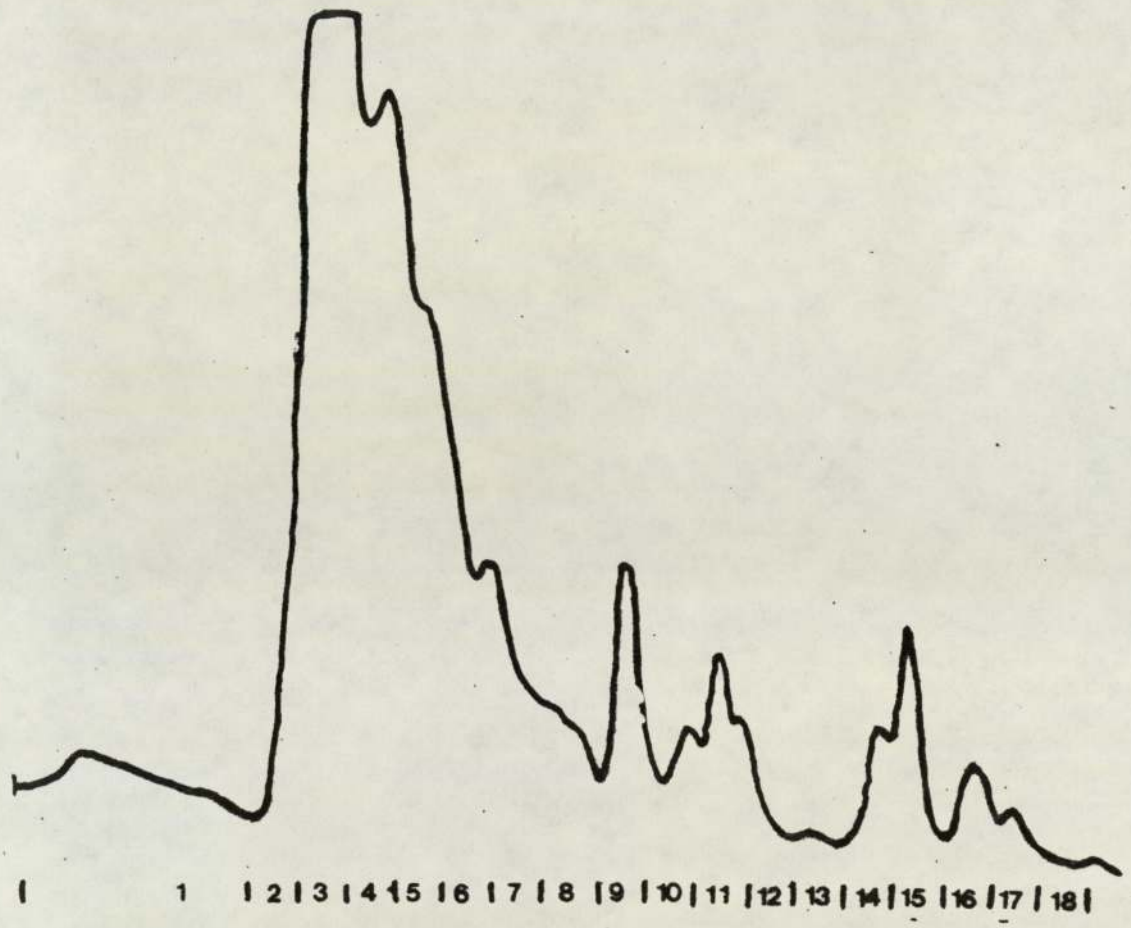
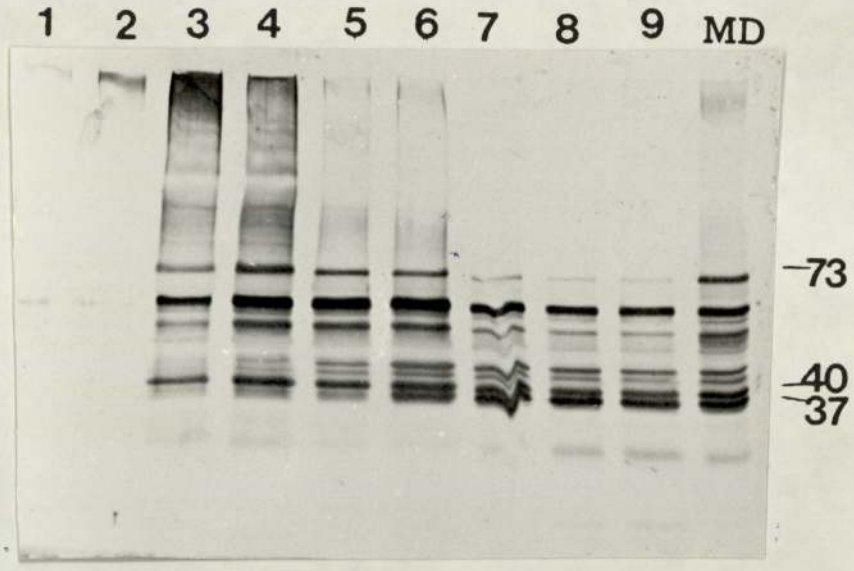


Figure 65a UV absorption profile of fractions eluted from Superose 12 HR 10/30 column following FPLC separation of EBH1/HS mutanolysin digest in 1% w/v SDS.
Fraction numbers are indicated horizontally.

Sample applied to column:	2mg EBH1/HS mutanolysin digest
Flow rate through column:	36ml/hour
Chart speed:	5mm/minute
AUFS:	0.01
Buffer system:	10mM Tris-HCl, pH 7.4 containing 1% w/v SDS
Fraction volume:	1.2ml



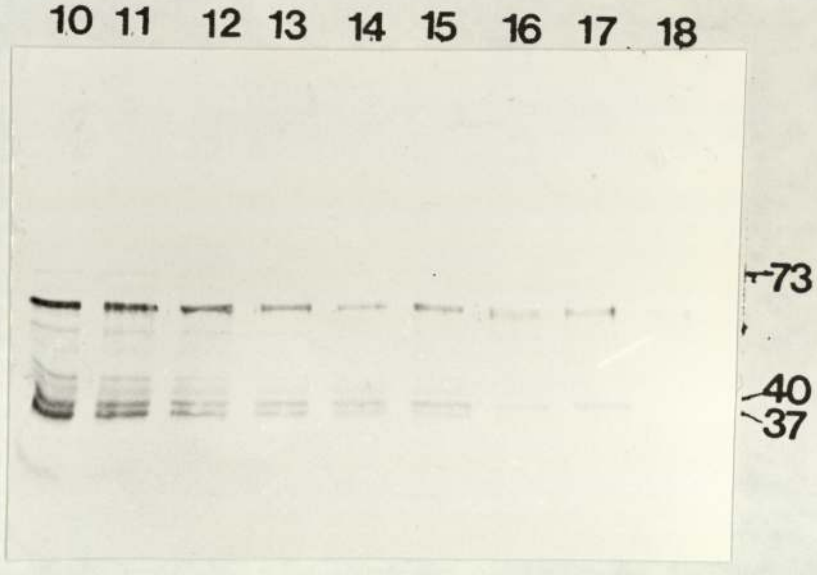


Figure 65b Immunoblot analysis of fractions collected from Superose 12 HR 10/30 column following FPLC separation of EBH1/HS mutanolysin digest in 1% w/v SDS.

Lanes:

MD, EBH1/HS mutanolysin digest in 1% w/v SDS, untreated

1-9, fractions 1-9 as shown in figure 65a

10-16, fractions 10-16 as shown in figure 65a.

Blot was reacted with Mr. P serum.

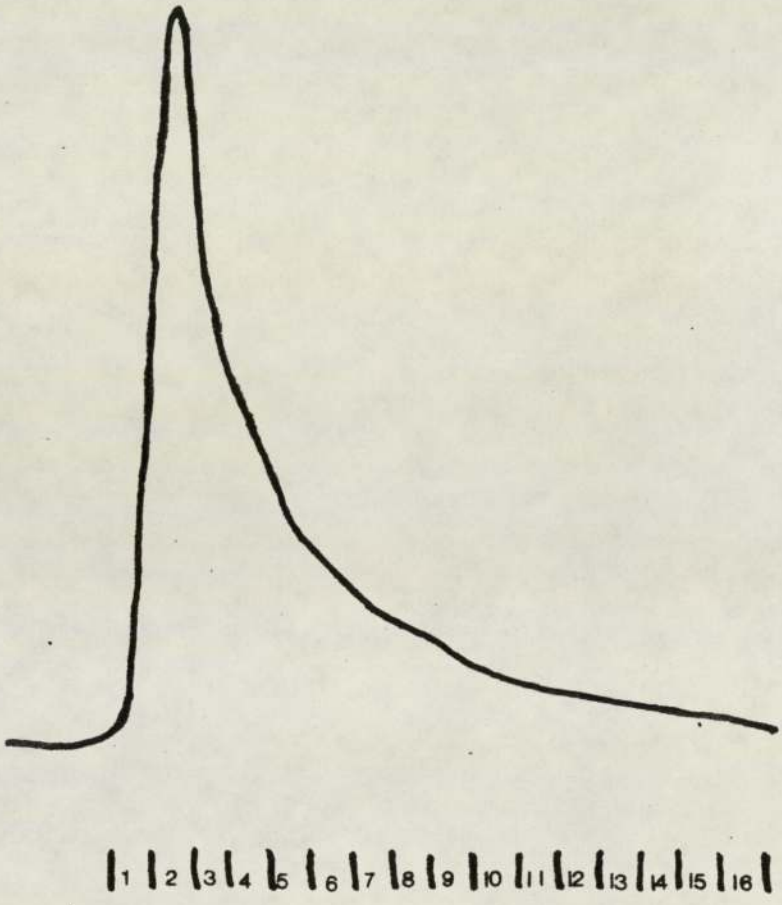


Figure 66a UV absorption profile of fractions eluted from Sephacry S-200 SF column following FPLC separation of EBH1/HS mutanolysin digest.
Fraction numbers are indicated horizontally.

Sample applied to column:	4mg EBH1/HS mutanolysin digest
Flow rate through column:	20ml/hour
Chart speed:	2mm/minute
AUFS:	0,1
Buffer system:	10mM Tris-HCl, pH 7.4
Fraction volume:	1ml

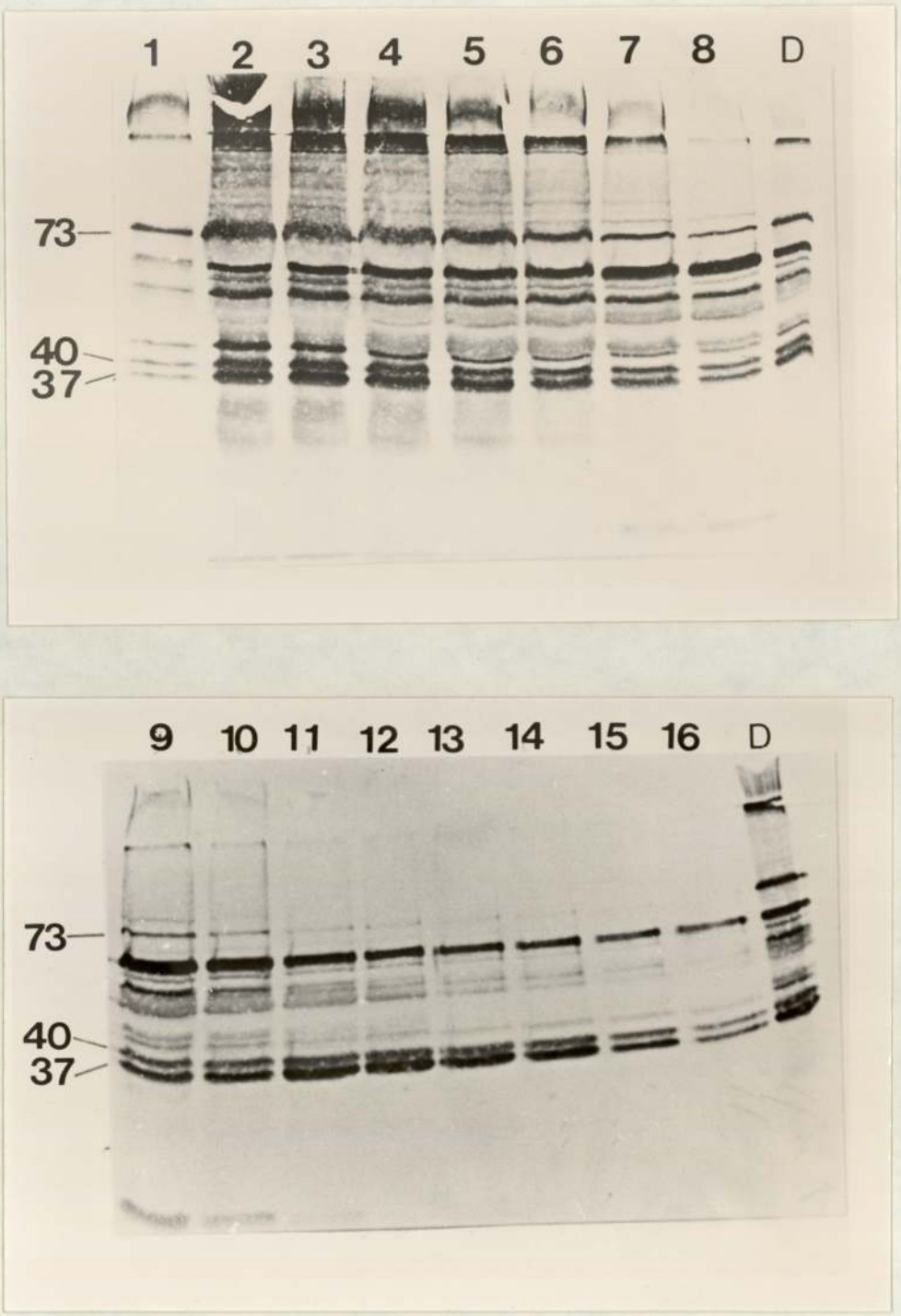


Figure 66b Immunoblot analysis of fractions eluted from Sephacryl S-200 SF column following FPLC separation of EBH1/HS mutanolysin digest.
Lanes:
D, EBH1/HS mutanolysin digest, untreated
1-8, fractions 1-8 as shown in figure 66a
9-16, fractions 9-16 as shown in figure 66a
Blot was reacted with Mr. P serum.

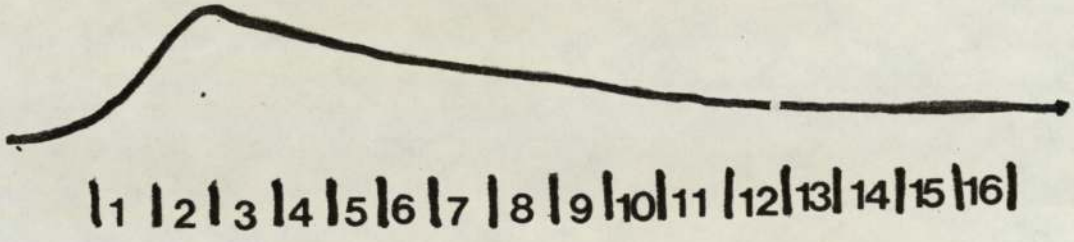


Figure 67a UV absorption profile of fractions eluted from Sephacryl S-200 SF column following FPLC separation of EBH1/HS in 1% w/v SDS. Fraction numbers are indicated horizontally.

Sample applied to column:	4mg EBH1/HS mutanolysin digest containing 1% w/v SDS
Flow rate through column:	20ml/hour
Chart speed:	5mm/minute
AUFS:	0.1
Buffer system:	10mM Tris-HCl, pH 7.4 containing 1% w/v SDS
Fraction volume:	0.495ml

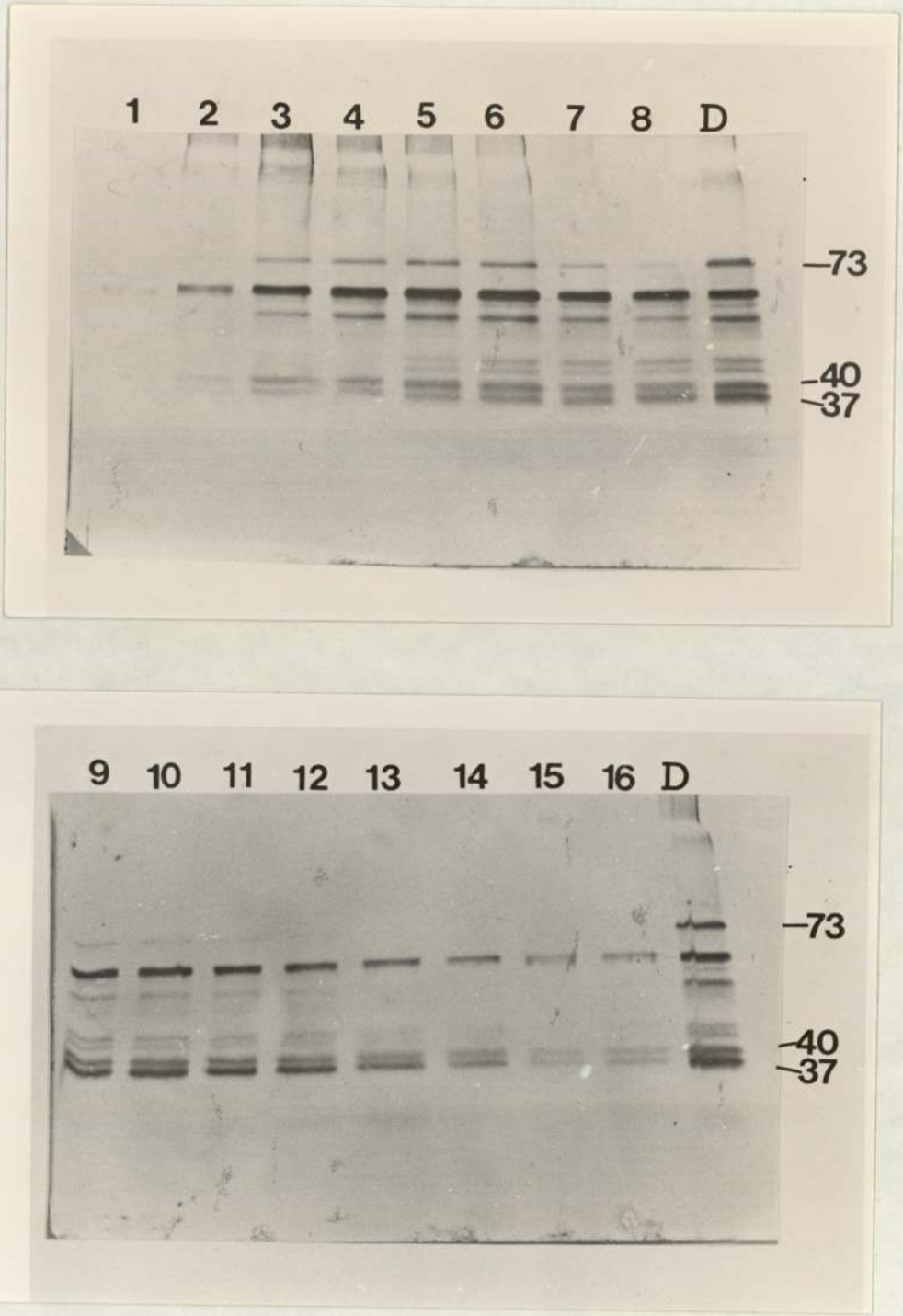


Figure 67b Immunoblot analysis of fractions eluted from Sephacryl S-200 SF column following FPLC separation of EBH1/HS mutanolysin digest containing 1% w/v SDS.

Lanes:

D, EBH1/HS mutanolysin digest in 1% w/v SDS

1-8, fractions 1-8 as shown in figure 67a

9-16, fractions 1-8 as shown in figure 67a

Blot was reacted with Mr. P serum.

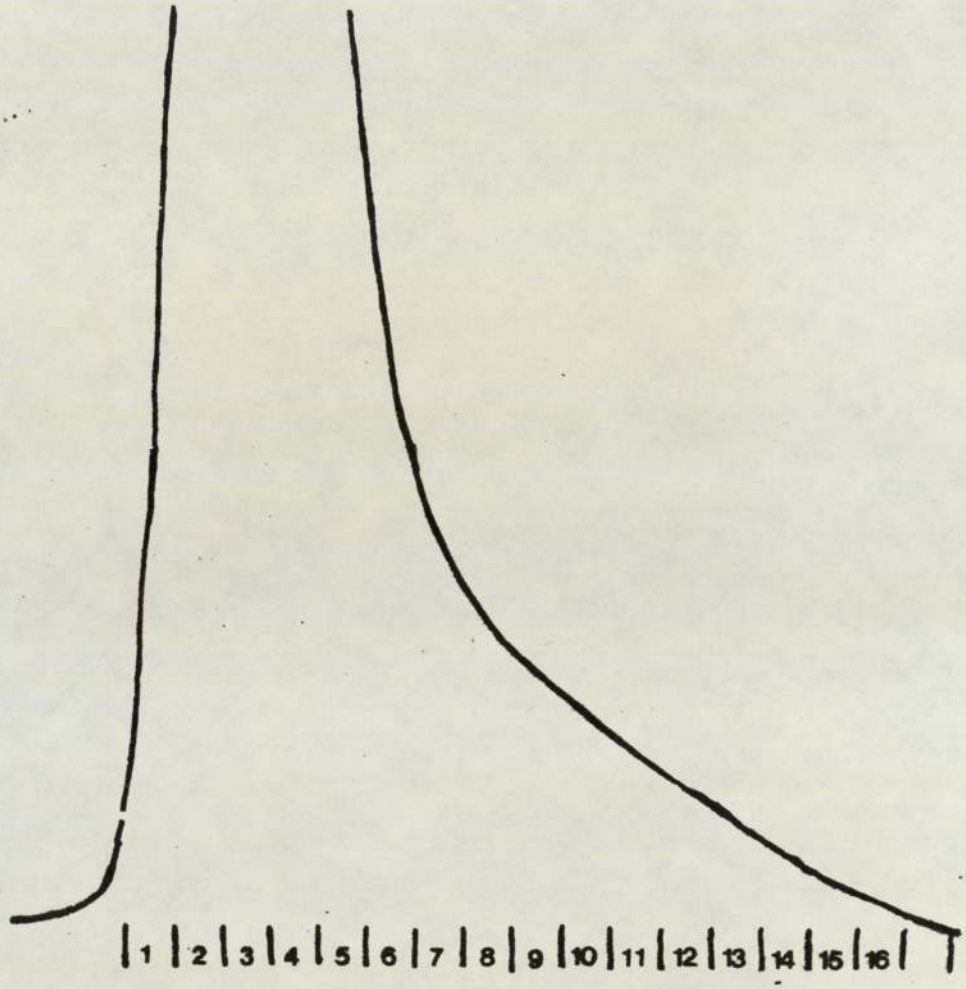


Figure 68a UV absorption profile of fractions eluted from Sephacryl S-200 SF column following FPLC separation of EBH1/HS Sarkosyl extract. Fraction numbers are indicated horizontally.

Sample applied to column:	4mg EBH1/HS Sarkosyl extract
Flow rate through column:	20ml/hour
Chart speed:	5mm/minute
AUFS:	0.05
Buffer system:	10mM Tris-HCl, pH 7.4 containing 1% w/v Sarkosyl
Fraction volume:	0.66ml

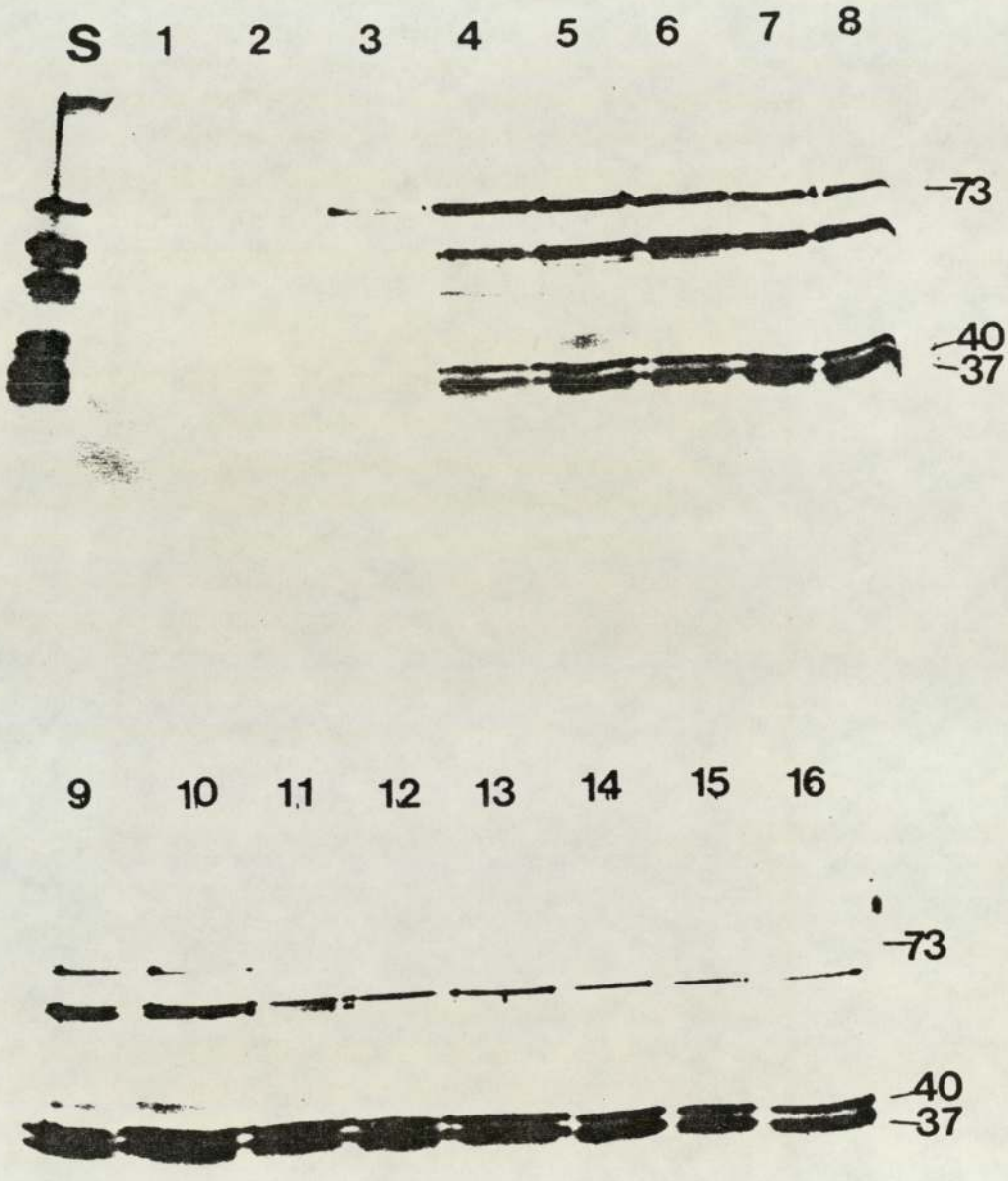


Figure 68b Immunoblot analysis of fractions eluted from Sephacryl S-200 SF column following FPLC separation of EBH1/HS Sarkosyl extract.
Lanes:
S, EBH1/HS Sarkosyl extract
1-8, fractions 1-8 as shown in figure 68a
9-16, fractions 9-16 as shown in figure 68a
Blot was reacted with Mr. P serum.

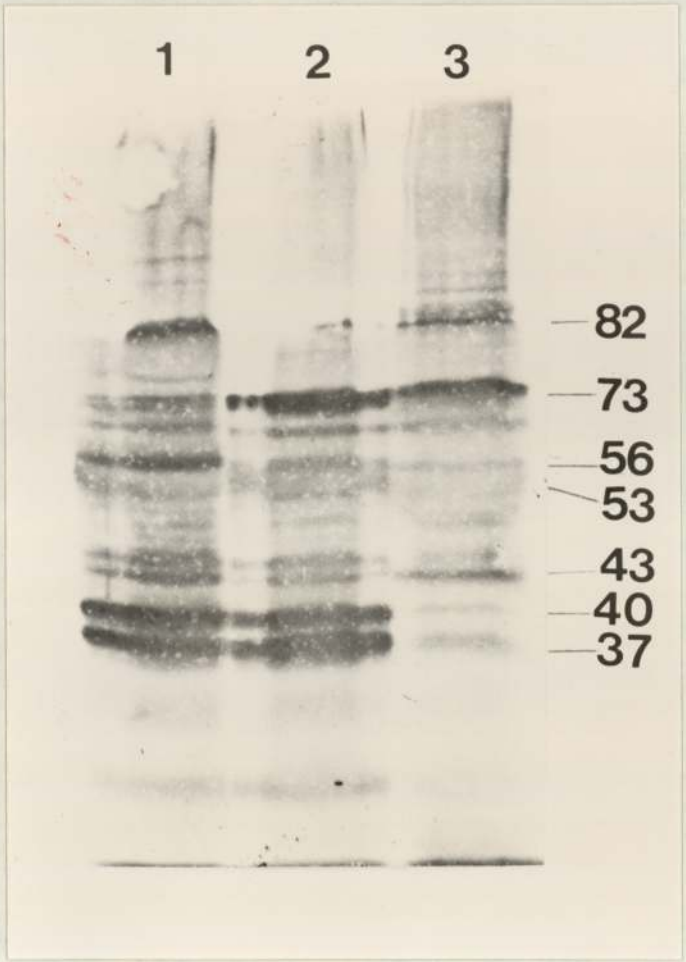


Figure 69 Immunoblot analysis of *S. faecalis* showing antigenic profile after growth in CDM.
Whole cells of EBH1 were separated by SDS-PAGE and transferred to nitrocellulose. Cells were grown in the following media:
1, HS
2, NHS
3, CDM + 1% v/v HS
Blot was reacted with Mr. P serum.

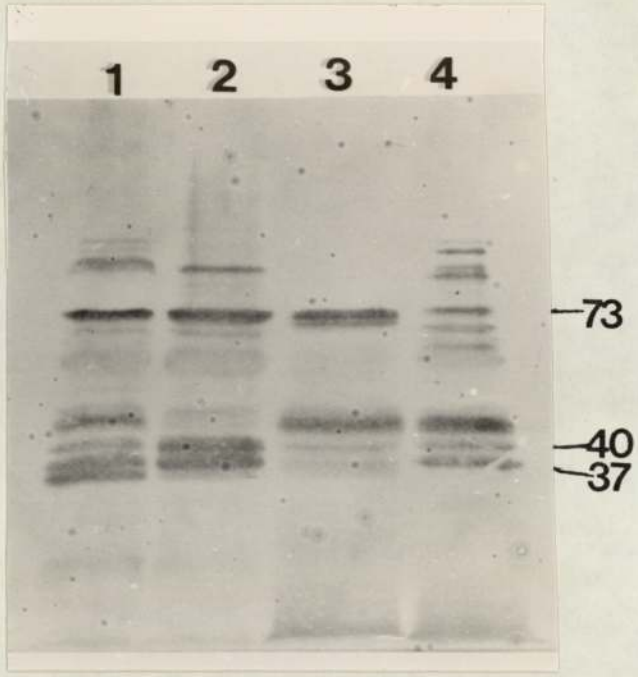


Figure 70 Immunoblot analysis of *S. faecalis* showing antigenic profile after growth in BHI of different brands.
Whole cells of EBH1 were separated by SDS-PAGE and transferred to nitrocellulose. Cells were grown in:
1, HS
2, NHS
3, BHI (Lab M)
4, BHI (Difco)
Blot was reacted with Mr. P serum.

3.3.3 **DISCUSSION**

It is important to establish which antigens are present on the surface of *S. faecalis* cells since it is these which provide the primary contact between the bacterial cell and its environment. Surface structures are likely to have a role in pathogenicity via adherence or action as virulence factors (Lancefield, 1962; Smith, 1977; Espersen and Clemmensen, 1982; Brown and Williams, 1985). To examine surface antigens of *S. faecalis* two techniques were used:- immunofluorescence microscopy and radioiodination. Immunofluorescence microscopy is a technique commonly used to diagnose diseases caused by several species including *Toxoplasma gondii, Legionella* species, *Neisseria gonorrhoeae, Treponema pallidum* and *Streptococcus pyogenes* (Fletcher, 1965; Kellogg and Mothershed, 1969; Chessum, 1970; Lind, 1975; Thomason et al, 1979; Nahm et al, 1980; McKinney et al, 1983). In this work immunofluorescence microscopy has been used to locate the binding sites of antibodies on the surface of bacteria. The cells were labelled by the indirect method. This involved the specific binding of a fluorochrome-labelled antibody to immunoglobulins bound to antigenic determinants on the surface of the organism (McKinney and Cherry, 1985). Under UV illumination (254nm) the fluorochrome (FITC) emitted green fluorescence. The Vickers fluorescence microscope used in this exeriment used incident light- or epi-illumination (first used by Brumberg, 1959 and further developed by Ploem, 1967). In this type of microscope a dichroic (beam-splitting) mirror deflects the excitation light downwards onto the specimen whilst transmitting the green fluorescence vertically to the eye-piece of the microscope. Fluorescence occurs if the wavelength of the light emitted by the fluorochrome is longer than that of the excitation light.

This technique demonstrated that serum from rabbits and humans, containing anti-*S. faecalis* antibodies, bound to antigens present on the

surface of intact *S. faecalis* cells. In these immunofluorescence experiments, the bacterial cells had been manipulated as little as possible in order to preserve their native state. The cells were taken directly from growing cultures with no centrifugation or washing procedures. Bacterial films applied to the Hendley-Essex microscope slides were allowed to dry naturally before being fixed. Essentially, therefore, it was surface antigens of native cells that were labelled with fluorochrome..

Antigenic determinants which reacted with anti-*S. faecalis* whole-cell antibodies in human and rabbit antisera appeared to be distributed evenly over the whole surface of EBH1 cells. Microscopically, the cells appeared as complete fluorescent spheres. These antibody-accessible surface antigens on the native cells are likely to include all or some of the cell-wall and whole-cell antigens shown previously on immunoblots (see section 3.1.2.1). It is useful to prove that the rabbit and patient serum does indeed react with antigens accessible on the surface of undenatured whole cells, a fact which hitherto had merely been an assumption.

Monospecific antisera raised against the 56/53K and 40/37K antigen pairs were also used in immunofluorescence experiments. These sera had been shown by immunoblotting (see section 3.1.2.2) to react solely with the homologous antigens in denatured EBH1 cells. It was not known whether these sera would recognise these antigens in native cells since originally the sera had been raised against denatured antigens excised from nitrocellulose blots. However, immunofluorescence microscopy showed that antibodies in the monospecific sera bound to cell-surface antigens, albeit weakly. EBH1 cells incubated in the monospecific sera and then labelled with FITC fluoresced in patches distributed more-or-less evenly over the surface of the cell. The weakness of the fluorescence was to be expected since the four antigens in question made up only a fraction of the total antigenic complement of *S. faecalis*. The occurrence of discrete patches of fluorescence might

suggests that the antigens are arranged together, possibly forming surface structures analogous to the surface tufts described on *S. sanguis* (Handley et al, 1985).

This method confirms the cell-surface location of the 56/53K and 40/37K antigens; with the 40/37K pair being more predominant at the surface of the cell.

Labelling of whole cells with radioactive iodine provided an additional means of determining which antigens were located on the surface of *S. faecalis* cells. As with fluorochrome labelling, in this method the antigens to be labelled were in an undenatured, native state. The use of the enzyme lactoperoxidase has a great advantage over other radiolabelling methods in that it is gentle and sensitive, binding to the bacterial proteins without appreciably disturbing their conformation (Marchalonis et al, 1971; Morrison, 1974). Lactoperoxidase catalyses the iodination of tyrosine and histidine residues of proteins in the presence of hydrogen peroxide (Morrison and Bayse, 1970; Morrison, 1974). This method of labelling is very specific, only the α-amino group participates in the reaction. When the radiolabelled cells are denatured and separated by SDS-PAGE, only those proteins which have been radioiodinated are visualized by autoradiography. The size of the lactoperoxidase molecule (77.5K) precludes it from entering the bacterial cell, since the peptidoglycan network of Gram-positive bacterial is thought to have a size exclusion limit of approximately 30-57K (Scherrer and Gerhardt, 1971; Lambert, 1984). Also, lactoperoxidase does not dissociate into subunits (Rombauts et al, 1967).

S. faecalis was shown by this method to possess several surface protein antigens including the 56, 53, 40 and 37K antibody-accessible antigens. Because of their surface location upon the bacterial cell, these antigens may act as contributing agents of bacterial pathogenicity. The

accessibility of these protein antigens to antibodies also invites speculation about their future use in vaccines. The 56/53K and 40/37K antigen pairs have already been shown to be immunogenic in rabbits (see section 3.1.2.2). Protective studies have yet to be performed. The 56K antigen may find use in a general anti-streptococcal endocarditis vaccine in view of the fact that this antigen is common to most streptococcal species (see section 3.1.2.2). The 73, 40 and 37K *S. faecalis*-specific surface antigens may have application in a vaccine specifically aimed against *S. faecalis* endocarditis. Isolation and purification of these antigens is desirable to permit such protective studies to be performed. Obtaining pure *S. faecalis*-specific antigens would also enable refinement of the serodiagnostic immunoblotting test for *S. faecalis* endocarditis (see section 3.2.3).

Many techniques exist by which proteins may be separated from crude mixtures. These include precipitation of proteins with organic solvents, high or low salt concentrations or by selective denaturation; ion exchange-, affinity adsorption- or dye-ligand-chromatography; gel filtration, electrophoresis, isoelectric focusing or ultrafiltration; and partition from liquid phases (Scopes, 1982c). Two of these methods were selected as the means of separating the *S. faecalis*-specific 73, 40 and 37K antigens from solubilized whole-cell preparations of EBH1. Precipitation by high salt concentration, one of the methods most commonly used to cause selective precipitation of proteins from solution and was used as a preliminary attempt to isolate these antigens. Ammonium sulphate was chosen for this purpose since multiply charged anions/monovalent cations are most effective for salting out (Scopes, 1982a). It is simple to recover proteins precipitated by ammonium sulphate since the precipitate so formed has a higher density than a saturated solution of this salt, and is thus recoverable by centrifugation. The salt affects the solubility of proteins

by altering the solvent properties of water via hydrophobic interactions. Protein solubility is also affected by other factors such as pH and temperature. Most proteins have maximal solubility at neutral pH and some are most likely to be precipitated when the pH of the solution approaches the isoelectric point of the protein (Czok and Bücher, 1960). Solubility also generally decreases with increasing temperature (Scopes, 1982a). Separation of EBH1 proteins was carried out at room temperature and at pH 7.4. This method wass successful up to a point in that the 73, 40 and 37K antigens of mutanolysin-digested cells were obtained in the 1-30% ammonium sulphate precipitate with only one other major contaminant. Unfortunately the 73K protein was only present in this precipitate in a minute amount. Salting out of Sarkosyl-extracted EBH1 resulted in a 30-60% precipitate which contained a substantial proportion of the 40 and 37K antigens together with a trace amount of contaminant. This situation was not improved by using other ammonium sulphate concentraion regimens. Thus it was concluded that this method was not satisfactory for obtaining pure *S. faecalis* antigens.

FPLC proved a much more promising prospect for selecting the required antigens from a soluble mixture of EBH1 antigens. This technique was first introduced by Porath and Flodin in 1959 and has since been developed for separation of a large range of molecular sizes. The process of separation is based upon seiving molecules of different sizes through pores in beads of cross-linked polymer (Scopes, 1982b). In selecting the appropriate polymer one must consider both the size range of molecules to be separated and also the desired mechanical properties of gel filtration bed. The polymer making up the Superose 12 column the polymer is cross-linked agarose; and in the case of the Sephacryl S-200 column it is dextran/bisacrylamide. Agarose gels are more porous and less rigid than dextran/bisacrylamide gels thus the latter can be operated under higher

pressure/faster flow rates. However, in this experiment the Sephacryl S-200 column was operated at a lower flow rate than the Superose 12 column because a larger loading volume of sample was used. The two columns were selected for use because both had an optimal molecular weight separation range which encompassed the molecular weights of the *S. faecalis*-specific antigens (optimal molecular weight separation range: Superose 12, 1×10^{3} - 3×10^{5}; Sephacryl S-200, 1×10^{3} - 8×10^{4}). Separation of the *S. faecalis*-specific antigens was much more satisfactory using the Sephacryl S-200 column. The best separation of all was gained using a buffer containing 1% w/v Sarkosyl. The detergent probably improved separation resolution by initially breaking up non-covalently bound protein aggregates. FPLC separation of Sarkosyl-extracted EBH1 in this buffer resulted in elution of fractions containing the 73K antigen and also the 40/37K antigen pair with only one contaminant present in small amounts. This contaminant was a 63K protein in each case. The potential therefore exists for further purification to remove this unwanted protein. It is apparent that a one-step purification process is not sufficient and that a series of stages are necessary to obtain pure *S. faecalis*-specific antigens.

An attempt was made to combine the two separation techniques described above. Initial precipitation with ammonium sulphate was used as a preliminary clarification stage followed by FPLC separation of the precipitates which contained the required antigens. After dialysis and lyophilization, the relevant precipitates were reconstituted in appropriate buffer and applied to a Sephacryl S-200 SF column. Unfortunately no fractions were eluted that contained purely the *S. faecalis*-specific antigens (data not shown).

It may prove possible to remove the unwanted 63K protein that contaminated most fractions which was the sole contaminant in most fractions containing the *S. faecalis*-specific antigens. This could be

carried out by immunoadsorption chromatography prior to gel filtration (Scopes, 1982c). Monospecific antiserum could be raised to the 63K antigen in a similar manner to that raised against the 56/53 or 40/37K *S. faecalis* antigens (see section 2.2.2.5.2). IgG purified from such serum could be immobilized on a column of insoluble matrix (eg. Protein A-Sepharose; cyanogen-bromide-activated agarose). Soluble antigen extracts of EBH1 cells could be passed down this column and the 63K antigen would theoretically be adsorbed out of the antigen mixture and bind to the immobilized anti-63K IgG. At the very least the 63K protein content of the soluble EBH1 antigen preparation would be greatly reduced, thus lowering its potential as a contaminant in fractions subsequently eluted from FPLC columns. If prior removal of this protein was achieved, then subsequent FPLC separation of the 63K-free, Sarkosyl-extracted *S. faecalis* antigens using a (Sephacryl S-200 SF FPLC column with a buffer system containing 1% w/v Sarkosyl) would result in early elution of a fraction containing pure 73K *S. faecalis* antigen and fractions at a later stage containing pure 40 and 37K *S. faecalis*-specific antigens.

If the purified *S. faecalis*-specific antigens were to be used in a serodiagnostic immunoblotting test for *S. faecalis* endocarditis, removal of detergent from the preparation should not be strictly necessary. It has been shown that patient's serum reacted with the antigens contained in the FPLC column eluate (containing Sarkosyl). The same might possibly hold true if the antigens were to be used for serodiagnostic ELISA or latex bead agglutination tests. Removal of detergent or salt would, however, be recommended of the antigens were intended for use in a vaccine against *S. faecalis* endocarditis. Also a high degree of purity would be ideal. This might necessitate further purification stages such as crystallization in order to obtain a stable, pure protein preparation.

4. Concluding Remarks

The study of proteins on the surface of bacteria has become increasingly important regarding their role in infection and as vaccine components. Surface proteins of Gram-positive cocci now challenge LTA as prime candidates for mediation of attachment to host tissue. The work in this thesis describes the surface protein composition of *S. faecalis*, an organism which has been little studied in this capacity.

In common with many bacteria, the protein antigen profile of *S. faecalis* was markedly influenced by the growth environment. Growth in a complex medium showed that *S. faecalis* had the capacity to express many proteins. These were considerably reduced in number following growth in serum. Possibly this occurs *in vivo* to aid survival of the organism by presenting fewer antigens to host defences. In the course of this study three serum-induced protein antigens were discovered that enabled distinction of *S. faecalis* from other streptococcal species.

SDS-PAGE and immunoblotting were the major methods used to study protein antigens of *S. faecalis*. These are ideal methods for characterization of proteins by molecular weight. However the intrinsic denaturing process thereby involved begs the question "do the antigens thus revealed exist in nature?" The solution would seem to be to use CIE or native-PAGE as additional means to study bacterial proteins. These techniques preserve the bacterial components in their native state with minimum alteration of their configuration. When examined by these undenaturing methods *S. faecalis* exhibited fewer antigens than revealed by immunoblotting. Denaturation must therefore result in degradation of proteins, leading to the appearance on immunoblots of additional lower molecular weight bands. The 73K *S. faecalis*-specific antigen may indeed be

a dimer, denaturation of which produces the 37K antigen. This may also occur naturally. In their natural state all three *S. faecalis*-specific antigens may associate to form a surface structure. Evidence for this supposition is provided by native-PAGE immunoblot profiles and fluorescence microscopy using monospecific antisera. The function of such a surface structure or of the discrete antigens has yet to be determined.

IE is a disease which has long fascinated clinicians and microbiologists. Since the introduction of antibiotics the fatality level of IE has remained at approximately 30%, but there is a disturbing increase in the incidence of IE caused by resistant organisms. *S. faecalis* is one such species. This organism has been shown to readily adhere to undamaged heart valves and to fibrin-platelet clots, such as are found in NBTE. It is likely that surface proteins of *S. faecalis* either independently, or in conjunction with other bacterial surface components, may be involved in the adherence mechanism. The multiple antibiotic resistance of *S. faecalis* poses a problem in the treatment of IE caused by this organism, a prolonged course of a synergistic combination of antibiotics being required. It is vital to establish the identify of the causal organism in IE in order to instigate the correct treatment. This thesis describes a method for the diagnosis of *S. faecalis* endocarditis which is rapid and accurate. The rapidity arises from the method employed being one of serodiagnosis, precluding the sometimes lengthy process of culturing a microorganism from the blood. The accuracy arises from the fact that the diagnosis relies on the reaction of a patient's serum with antigens that are exclusive to *S. faecalis* species, which are apparently not cross-reactive with antigens of other Gram-positive organisms. Furthermore all IE patients infected with *S. faecalis* appear to possess antibodies to these antigens. The immunoblotting method produces a diagnosis in 5 hours and would be extremely useful in culture-negative cases of endocarditis or in serious situations where instigation of

treatment cannot await isolation of an organism from the bloodstream. The ability to reliably discount the presence of *S. faecalis* as the infecting organism in such cases would be welcome to physician and patient alike. Such a proposition would prove cost-effective to the clinician and be of benefit to the patient who would not needlesly have to undergo prolonged therapy with antibiotics which may have adverse effects.

The specificity of the serodiagnostic technique could be further improved by use of purified 73, 40 or 37K *S. faecalis* antigens. The eventual aim is to discover antigens that are exlusive to other streptococcal species that may also be used in serodiagnosis. The screening of various growth media would be carried out to investigate stimulation of such antigens. The logical conclusion of this project is the development of an ELISA kit comprising species-specific antigens from a range of streptococci, that could be used to diagnose the identity of a causal organism in IE, or even of a particular serotype, for several species of streptococci or other Gram-positive organisms.

With the development of monoclonal antibody technology the specificity of therapeutic immunological methods is improving dramatically. If it is discovered that all or any of the *S. faecalis* proteins, or others, are involved in the organism's pathogenicity, then monoclonal antibodies produced against the relevant antigen (or indeed monospecific antisera as described in this thesis) may have application in passive immunotherapy for *S. faecalis* endocarditis. However,such therapy is of limited value in IE unless administered sufficiently early in the disease process since organisms existing in vegetations on heart valves are little affected by antibodies. Incorporation of *S. faecalis* surface proteins into vaccines may be of more value, and would be used in situations similar to those in which antibiotic prophylaxis is recommended.

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