THE IMMUNOCHEMISTRY OF *SERRATIA MARCESCENS*

A thesis submitted by

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for the degree of

DOCTOR OF PHILOSOPHY

at ASTON UNIVERSITY

July 1986

TO MY PARENTS

WITH LOVE

ASTON UNIVERSITY

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by HELEN L JESSOP

Submitted for the degree of Doctor of Philosophy
JUNE 1986

Protein profiles of the outer membranes (OMs) of Serratia marcescens NewCDC O14:H12, isolated from cells grown in various iron-rich and iron-restricted media, were investigated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Three major proteins were separated of 43.5 and 42 kDal (the porins), and 38 kDal (the OmpA protein), and a group of iron-regulated proteins (IRMPs) of 63-70 kDal. Immunoblotting of whole cells or OMs using antiserum raised against whole cells, revealed similar complex patterns of antigens. The OmpA protein was the major immunogen, although other OM proteins were also detected; the porins and IRMPs reacted only weakly with antibodies in this system. Immunoabsorption of antiserum with whole cells showed that only the O antigenic chains of lipopolysaccharide (LPS) and the H (flagella) antigens were accessible to antibody on the cell surface. Failure to detect the OmpA and other envelope proteins suggests that their antigenic sites are not able to react with antibodies and are possibly masked by the O antigen.

The sensitivities of two O14:H12 strains, New CDC and 4444-60, to a range of antimicrobial agents were very similar, the third O14:H12 strain, S1220, being particularly resistant suggesting the presence of a plasmid. The response of the cells to killing by normal pooled human serum were very different. New CDC was either sensitive or resistant according to the media it was grown in, whereas 4444-60 was resistant, and S1220 sensitive, independently of growth media.

Complement binding, studied by measuring hydrophobicity and using rocket immunoelectrophoresis with antihuman C3, showed the sensitive cells (S1220) rapidly bound complement whereas the resistant cells (4444-60) bound less C3b. Crossed immunoelectrophoresis suggested in S1220 cells, the polysaccharide material including LPS was less antigenic and present in smaller amounts than in 4444-60. Examining extracted polysaccharide material chemically and by SDS-PAGE, showed the resistant strain had 33% more material than the sensitive strain, comprising LPS with longer O antigen chain lengths and/or a microcapsule of O antigen polysaccharide. The extra polysaccharide material on the surface of 4444-60 cells probably prevents complement components binding and reaching the hydrophobic membrane where lytic lesions occur.

Key words — Serratia marcescens; surface antigens; serum killing; lipopolysaccharides.

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Acknowledgements

I would like to greatly thank my supervisor, Dr Peter A Lambert, for all his encouragement, advice and support throughout the course of this study. I am also grateful to Professor M R W Brown and Dr S G Wilkinson of Hull University for their help and useful discussions.

My thanks go to my friends and colleagues in the Microbiology Research Group for giving me unending support and to Roy Tilling and Dorothy Townley for their technical assistance.

I am gratefully obliged to Aston University, who provided the finance for this research project.

Lastly, I would like to thank Sue Kelly for typing this thesis.

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Abbreviations

CDMFe+	chemically defined medium with added iron
CDMFe-	chemically defined medium without added iron
CIE	crossed immunoelectrophoresis
CM	cytoplasmic membrane
°C	degrees centigrade
ECA	enterobacterial common antigen
EDTA	ethylene diamine tetra-acetic acid
EGTA	ethylene glycol-bis-(β-amino-ethylether)-N:N'-tetra-acetic acid
g	gramme
GPS	guinea pig serum
HS	heat-inactivated horse serum
IRMP	iron-regulated membrane protein
KDO	3-deoxy-D-manno-2-octulosonic acid
L	litre
LPS	lipopolysaccharide
M	moles per litre
MAC	membrane attack complex
µg	microgramme
mg	milligramme
MIC	minimum inhibitory concentration
min	minute
µl	microlitre
ml	millilitre
mm	millimetre
nm	nanometre
NPHS	normal pooled human serum

OD	optical density
OM	outer membrane
OMP	outer membrane protein
rpm	revolutions per minute
SA	soluble antigen
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TSB	tryptone soy broth
v/v	volume by volume
w/v	weight by volume

Thesis Publications

Jessop, H L and Lambert, P A (1985)

Immunochemical characterisation of the outer membrane complex of Serratia marcescens and identification of the antigens accessible to antibodies on the cell surface.

J. Gen. Microbiol. 131, 2343-2348.

Jessop, H L and Lambert, P A (1986)

The role of surface polysaccharide in determining the resistance of Serratia marcescens to serum killing.

J. Gen. Microbiol. (in press).

Tilling, R K, Jessop, H L and Lambert, P A (1986)

A note on the use of a scanning laser densitometer to analyse silver-stained lipopolysaccharide patterns on polyacrylamide gels and antigenic profiles on nitrocellulose sheets produced by immunoblotting.

Lett. Appl. Microbiol. 2, 43-46.

1. INTRODUCTION

1.1 SERRATIA - HISTORY AND TAXONOMY

Bizio in 1823 first described a red-pigmented organism as the cause of "bleeding polenta" which he named *Serratia marcescens*. Originally it was classified with the fungi, but later it was properly placed with the bacteria (De Toni and Trevisan, 1889). The genus *Serratia* contains Gram-negative, small, mobile, bacilli that can exist as pigmented (red) or non-pigmented strains.

Over the years, many different species names have been included in the genus *Serratia*. In 1959, because of similarities to the genera *Klebsiella* and *Enterobacter*, *Serratia* was placed in the tribe Klebsielleae of the family Enterobacteriaceae (Ewing et al, 1959; Ewing, 1967), with only one species included, *S. marcescens*, and one variety, *S. marcescens* var *kilensis*. In 1972, the genus *Serratia* was enlarged to include two more species, *S. liquefaciens* and *S. rubidaea* (Ewing et al, 1972). Four main species are now known, *S. marcescens*, *S. liquefaciens*, *S. marinorubra* (Ewing's *S. rubidaea*) and *S. plymuthica* (Grimont and Grimont, 1978), with *S. odorifera*, *S. fonticola*, *S. ficaria*, *S. proteamaculans* and *S. grimesii* being recently described (Grimont et al, 1978; Gavini et al, 1979; Grimont et al, 1979; Grimont et al, 1982).

1.2 CLINICAL FEATURES AND EPIDEMIOLOGY OF SERRATIA MARCESCENS INFECTIONS

S. marcescens has traditionally been considered non-pathogenic for man. Serratia is a common saphrophyte in air, soil, water and sewage and has been isolated from many plants, insects and animals (Grimont and Grimont, 1978). Because of the red pigment production by some strains, S. marcescens was until recently used as a biological marker, but since the mid-1960s its rise as a human pathogen has been overwhelming, it being implicated in every conceivable kind of infection (Yu, 1979).

Except for intravenous drug abusers (Yu, 1979), healthy human beings have little risk of being infected outside of the hospital environment. S. marcescens is a typical opportunist having a predilection for compromised hosts. The most common factors predisposing to colonisation and nosocomial infections are: severe underlying disease (eg diabetes, cancer), immunosuppression, previous or concurrent antibiotic therapy, severe trauma, prematurity, renal failure, the post-operative state, intravascular or bladder catheterisation, intubation of the trachea, preceding inhalation therapy, prolonged hospitalisation, clustering of patients, burns, age beyond 50, and as mentioned before, drug addiction (Cabrera, 1969; Wilkowske et al, 1970; Maki et al, 1973; Leading Articles, 1977ab; Yu, 1979; von Graevenitz, 1980; Gutschik et al, 1980; Young et al, 1980; Platt and Sommerville, 1981; Bullock et al, 1982; Goldstein et al, 1982).

Non-pigmented strains are isolated most often from clinical infections (although pigmented strains can be pathogenic) usually from the urinary and respiratory tract (Clayton and von Graevenitz, 1966; Maki et al, 1973; Johnson and Ellner, 1974; Madduri et al,

1976; Maskell et al, 1977; Meers et al, 1978; Brooks et al, 1979; von Graevenitz, 1980; Mutton et al, 1981; Goldstein et al, 1982). Although not all strains isolated are clinically significant in terms of infection, in some cases septiceamia can follow (Kahl and Rodnan, 1984) which can be associated with endocarditis (Balikian et al, 1980; Khan, 1983). Primary cellulitis and meningitis have also been reported (Bonner and Meharg, 1983; Muhlbauer et al, 1983). S. marcescens has also been implicated in soft tissue infections, arthritis, osteomyelitis, occular infections (keratitis) and even thyroiditis (von Graevenitz, 1980; Lafuente et al, 1981; Lass et al, 1981; Reichling et al, 1984). However, infections of the urinary tract, (lower) respiratory tract, blood and wounds account for 90% of all nosocomial infections (Daschner, 1980).

Apart from urinary wards and intensive care units, neonatal units and nurseries are often sites of S. marcescens epidemics, usually associated with high mortality (Lewis et al, 1983; Smith et al, 1984; Duggan et al, 1984; Montanaro et al, 1984). In these cases the reservoir of infection appears to be throat, gastrointestinal and rectal colonisation of the babies. It is believed that after entering the bloodstream of neonates, S. marcescens becomes pathogenic, especially to the central nervous system, and can cause fatal meningoencephalitis (Nakamura et al, 1984; Duggan et al, 1984), as well as septiceamia, pneumonia, urinary tract infection, diarrhoea and conjunctivitis (Duggan et al, 1984; Montanaro et al, 1984). In hospitalised adults, in contrast to infants and to other Gram-negative bacteria causing nosocomial infections, S. marcescens rarely colonises the stool (Farmer et al, 1976). Only in epidemic situations have faeces been implicated as a reservoir for infections (Grimont and Grimont, 1978).

The mode of entry of S. marcescens into the hospital is not well known, but once introduced it may colonise (a) equipment, such as ultrasonic nebulisers (Ringrose et al, 1968), urometers (Kocka et al, 1977; Rutala et al, 1981; Marrie et al, 1982), and in-line filters during IV therapy (Holmes et al, 1980); (b) disinfectants and hand washing solutions (Ehrenkranz et al, 1980; Sheets, 1981; Marrie and Costerton, 1981; Marrie et al, 1982); and/or (c) patients' urinary tracts (Maki et al, 1973), which can all lead to hospital spread. If it becomes endemic, usage of antibiotics will select antibiotic resistant 'substrains' (Farmer et al, 1976) so that if the reservoir of colonised or infected patients cannot be eliminated, eventually a multiply-resistant 'substrain' resistant to almost all antimicrobial drugs emerges (Yu, 1979; Daschner, 1980). The principal mode of spread within the hospital environment is cross-infection via the hands of personnel (Maki et al, 1973; Schaberg et al, 1976a; Meers et al, 1978; Brooks et al, 1979; Balikian et al, 1980; Mutton et al, 1981), and an investigation showing that S. marcescens can form new cells in an airborne state might also be linked to transmission (Dimmick et al, 1979).

Methods to prevent cross-infection and eliminate the source of S. marcescens include isolating colonised or infected patients (especially those who are catheterised), removing catheters as soon as possible, restricting prophylactic and heavy usage of antibiotics, screening high-risk patients, and ensuring frequent hand washing (chlorhexidine being superior to alcohol - Aly and Maibach, 1980) and the use of gloves when manipulating infected sites (Maki et al, 1973; Schaberg et al, 1976b; Madduri et al, 1976).

1.3 COMPOSITION AND STRUCTURE OF THE GRAM-NEGATIVE CELL ENVELOPE

The cell envelope of Gram-negative bacteria confers shape and rigidity on the cell and regulates the organism's interaction with its environment. The envelope protects the cytoplasm from harmful substances, regulates the uptake of essential nutrients, and is capable of undergoing composition changes in response to environmental alterations. The ability to change in this way confers major survival advantages on the cell (Lugtenberg and van Alphen, 1983; Brown and Williams, 1985).

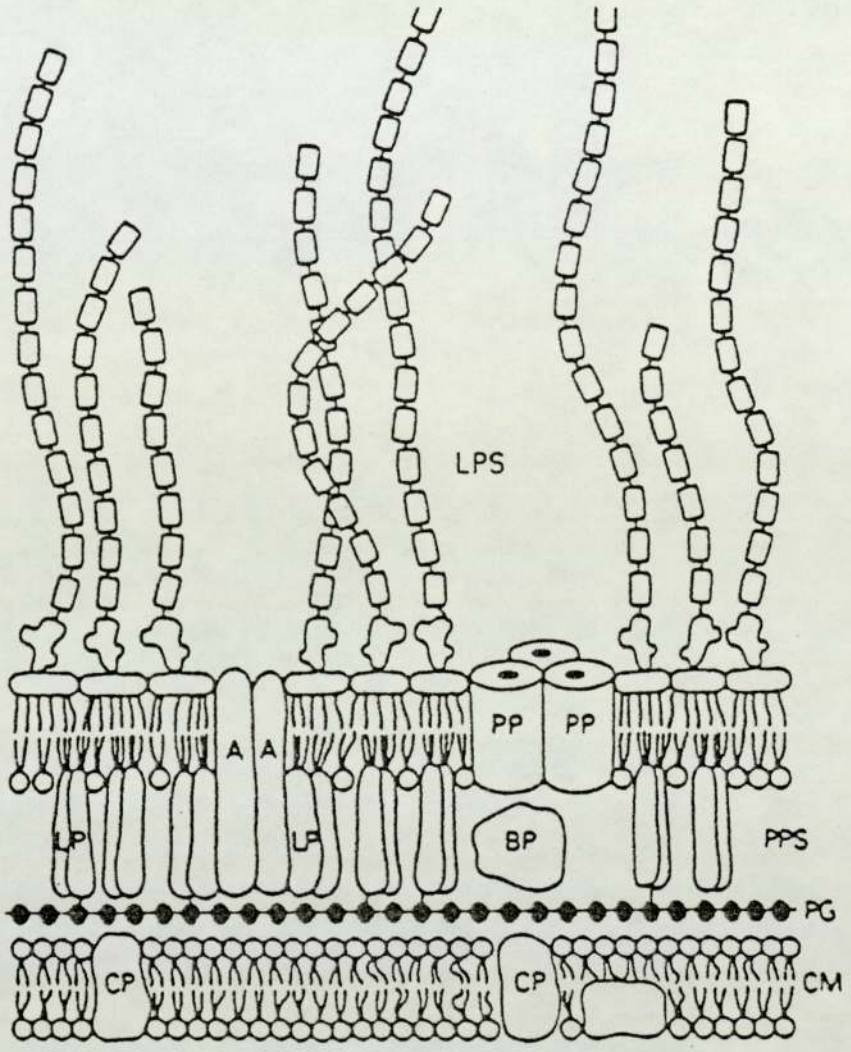
The envelope of Gram-negative bacteria consists of three layers which support different functions; an inner cytoplasmic membrane (CM), a peptidoglycan (PG) layer and an outer membrane (OM) (Figure 1.1).

The CM is composed of phospholipids and proteins, the latter carrying out a number of transport functions. The CM contains many of the cell envelope enzymes and it acts as an anchor for DNA, at least during replication (Inouye, 1979). The CM and OM are interconnected by the 'zones of adhesion', estimated at 200-400 per cell (Bayer, 1979).

The PG layer is a rigid polymer consisting of a backbone of N-acetylmuramic acid and N-acetylglucosamine cross-linked by peptide chains. It confers rigidity and shape to the bacteria and enables the cells to withstand the osmotic pressure of the cytoplasm. Hobot et al (1984) have proposed that the outer part of the PG molecule is more highly cross-linked than the inner and that the molecule is hydrated so that it forms a gel filling the space between the outer and cytoplasmic membranes. The periplasmic gel contains oligosaccharides and proteins which carry out transport and enzymatic

Figure 1.1

Schematic diagram of the cell envelope of Gram-negative bacteria



from Lugtenberg & van Alphen (1983)

Key.

LPS - lipopolysaccharide

A - outer membrane protein, Omp A type

PP - porin protein

LP - lipoprotein

BP - binding protein

PPS - periplasm

PG - peptidoglycan

CP - transport ptotein

CM - cytoplasmic membrane

functions (Hammond et al, 1984).

Covalently linked to the PG via a lipoprotein is the OM which is composed of phospholipid, protein and lipopolysaccharide (LPS) (Lugtenberg and van Alphen, 1983). A capsular layer of polysaccharide and/or protein may cover the OM, and appendages like flagella, fimbriae and pili may be anchored in the cell envelope (Costerton et al, 1974; Lugtenberg and van Alphen, 1983).

The OM acts as a selective barrier that permits permeability of nutrients from the environment into the cell, but restricts entry of a wide range of antibiotics, detergents and other noxious molecules, contributing to the inherent resistance of Gram-negative bacteria to these toxic compounds. The OM is also involved in assisting cells to multiply in hostile environments, such as those found in vivo, aiding resistance to host defence mechanisms (complement and phagocytosis) and contributing to the pathogenicity of the bacteria (Lugtenberg and van Alphen, 1983; Hammond et al, 1984; Ogata, 1983).

1.3.1 Individual constituents of the OM

1.3.1.1 Phospholipids

The phospholipids of Enterobacteriaceae are essentially located on the inner side of the OM; phosphatidylethanolamine (PE) being the major species, but substantial amounts of phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) also being found (Lugtenberg and van Alphen, 1983). Phospholipid composition can vary with growth conditions (Gilbert and Brown, 1978) and may alter the resistance of bacteria to antimicrobial agents (Noy, 1982). Winshell and Neu (1974) found that there was no appreciable difference in the lipid/

phospholipid content of resistant and susceptible pigmented and non-pigmented strains of S. marcescens, as did Button et al (1975). However, Suling and O'Leary (1977) showed that on extracting lipid from various resistant strains of S. marcescens, a minor phospholipid component (phospholipid X, possibly acylphosphatidylglycerol) increased in concentration concomitantly with a decrease in PE concentration, and a lower PG/DPG ratio was observed, compared to susceptible strains.

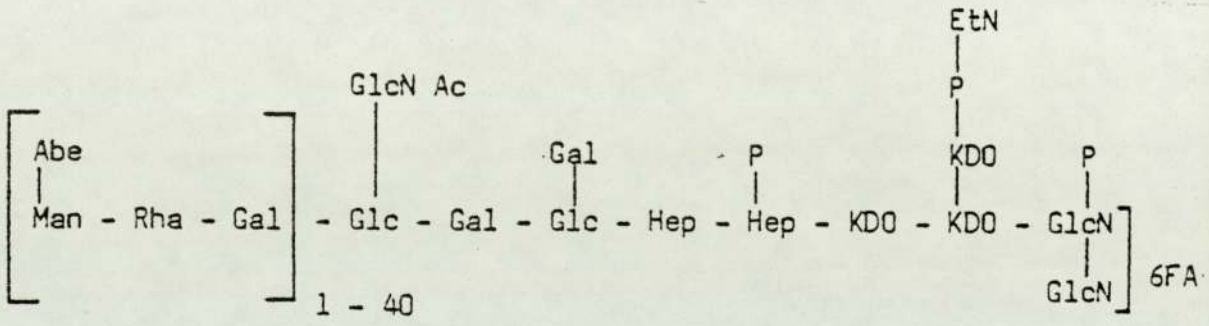
1.3.1.2 Lipopolysaccharide

LPS is located exclusively on the outer face of the OM (Mülhradt and Golecki, 1975) and consists of three regions covalently linked (see Figure 1.2), namely: lipid A (hydrophobic, embedded in the OM and highly conserved in bacterial species), an R specific polysaccharide core (similar composition in many bacterial species), and the O specific polysaccharide side chain (widely variable in composition, often within a single species). The disappearance of the latter component gives rise to the morphological smooth to rough transformation (Westphal et al, 1983).

Lipid A of Enterobacteriaceae is responsible for a large number of the endotoxic activities of LPS (Westphal et al, 1981). Lipid A is a glycolipid comprising a β-(1→6) linked phosphatidyl glucosamine disaccharide unit to which fatty acids are attached by ester and amide bonds (Hammond et al, 1984). The occurrence of hydroxy fatty acids is a unique feature of lipid A; saturated fatty acids also occur but unsaturated fatty acids are not found. The basic structure is the same for numerous bacterial strains, but heterogeneity exists in the composition of ester-linked fatty acids and in substitution of

phosphate groups, as Figure 1.3 shows (Mattsby-Balter et al, 1984). The biphosphorylated β-(1→6) linked glucosamine disaccharide backbone is present in S. marcescens, with β-hydroxy-myristic acid as the major fatty acid amide linked to the glucosamine units (Adams and Singh, 1970). Other fatty acids identified include lauric, myristic and palmitic acid (Alaupovic et al, 1966). Lipid A seems to assume a highly ordered conformation on the surface of bacteria, with the fatty acid chains being tightly packed in a dense hexagonal lattice, and the diglucosamine backbone forming domains in a parallel arrangement in the membrane. The presence of hydroxy fatty acids and absence of unsaturated fatty acids are important in maintaining the organised state of the OM. The high state order of the lipid A might be an important factor in the structural role and permeation barrier functions of LPS in the OM (Labischinski et al, 1985).

The LPS core contains the unique sugars 3-deoxy-D-manno-2-octulosonic acid (KDO) and L-glycero-D-mannoheptose, in addition to more common sugars. S. marcescens cores studied so far are similar to those of other Enterobacteriaceae consisting of D-glucose, D-galactose, D-glucosamine, heptose and KDO, but containing more D-glucose and heptose and less KDO than Salmonella and Escherichia coli cores (Wang et al, 1974). The actual composition does seem to vary slightly with strains, the major core components of LPS from S. marcescens CDC 4444-60 being D-glucose and L-glycero-D-mannoheptose, and lesser ones being KDO, D-glycero-D-mannoheptose and 2-amino-2-deoxyglucose (glucosamine) (Brigden and Wilkinson, 1983). Linkage to lipid A is via KDO, possibly in the form of a dimer (Luderitz et al, 1983). Intact cores from two S. marcescens strains were found to have two residues of KDO, one of these being bound through an acid-labile, and the other through an acid-stable, glycosidic linkage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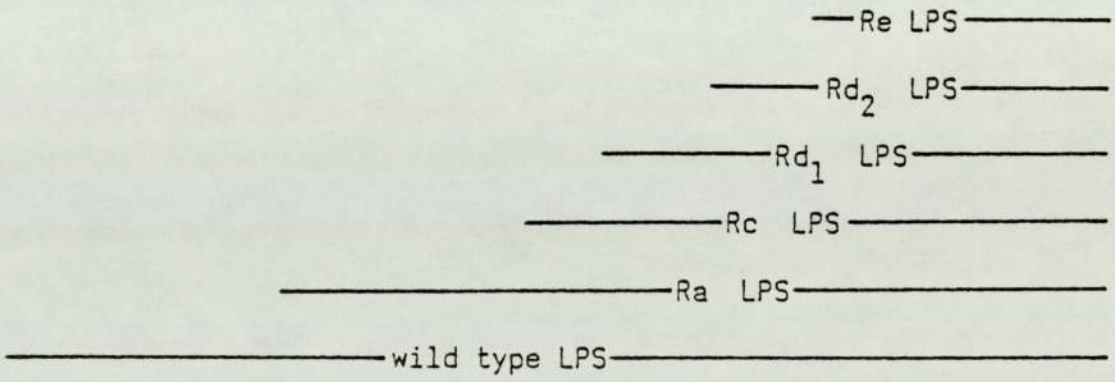
P
GlcN Ac
Abe
Gal
P
KDO
P
Man - Rha - Gal - Glc - Gal - Glc - Hep - Hep - KDO - KDO - GlcN
GlcN
6FA
1 - 40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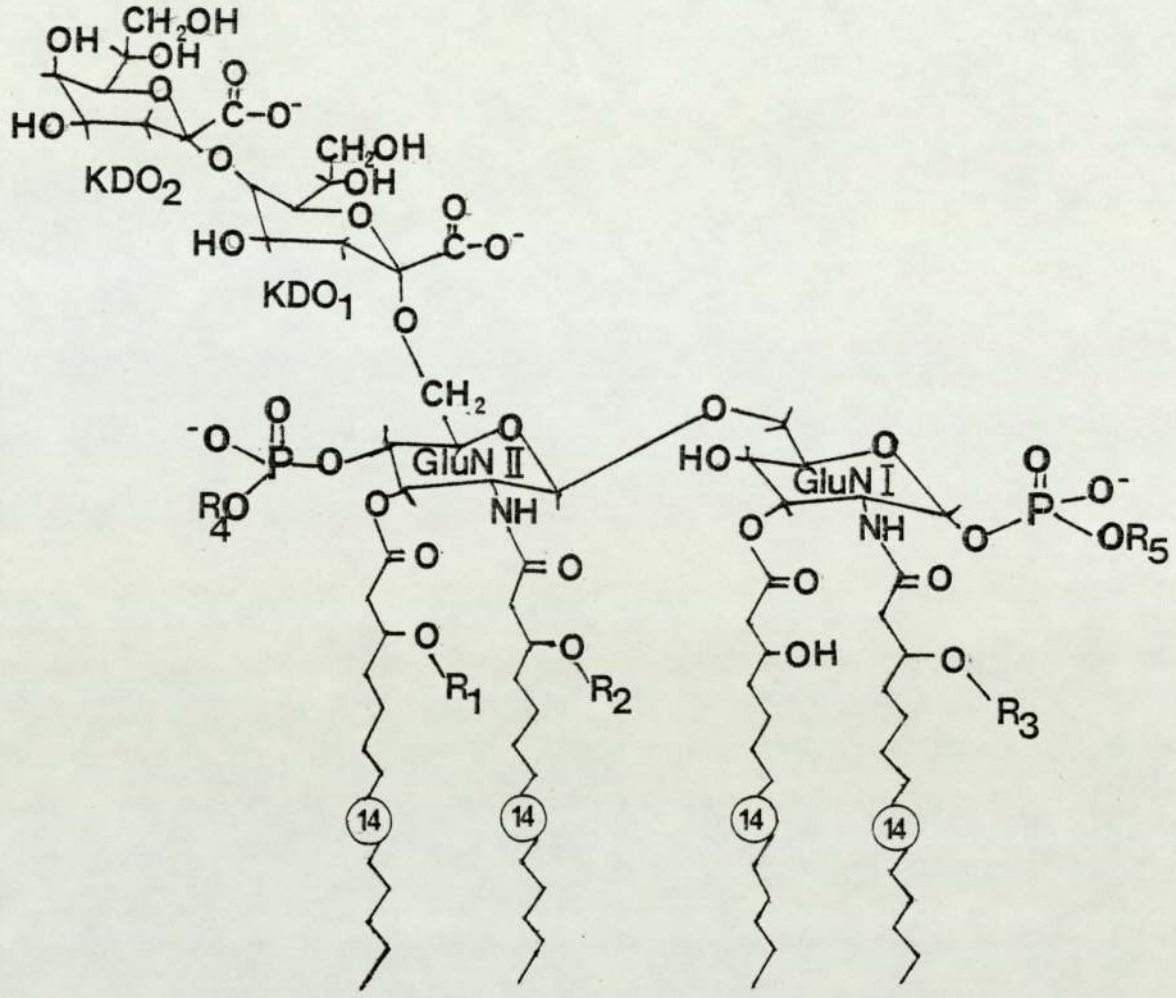
Rd2 LPS
Rd1 LPS
Rc LPS
Ra LPS
wild type LPS



Adapted from Rietschel et al. (1984)

(Wang et al, 1974). The acid-labile bond allows the hydrophobic lipid A region to be easily separated from the core and O side chains in isolated LPS by acid hydrolysis.

The O antigen side chain is a long chain polysaccharide with hydrophilic properties, built up from between 1 to 40 repeating units of oligosaccharides each containing between 1 to 7 homo- or hetero-polysaccharides with or without short chain branches (Westphal et al, 1983). This composition allows for extreme diversity in structure, even within a single genus, and is used as a basis for O serotyping, an immunological method used to identify sub-strains of one species in great detail (see section 1.4). In contrast to lipid A, the O specific chains seem to assume a low ordered, heavily coiled conformation on the bacterial surface (Labischinski et al, 1985).

1.3.1.3 Enterobacterial common antigen (ECA)

ECA is a minor common component of the OM, shared by most Enterobacteriaceae. A majority (70%) of the molecule is a polymer of N-acetyl-D-glucosamine and N-acetyl-D-mannosaminuronic acid partly esterified by palmitic and acetic acids, the rest being an uncharacterised lipid moiety (Wicken and Knox, 1980; Hammond et al, 1984). It has recently been reported to have a cyclic structure (Dell et al, 1984).

1.3.1.4 Outer membrane proteins (OMPs)

Protein components are mainly identified using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Growth and environmental conditions can alter the OMP composition, this

adaptation playing an important role in bacterial survival and cell proliferation. OM profiles often show a few prominent protein bands, others either being in amounts below levels of detection or being inducible only under certain growth conditions (Lugtenberg and van Alphen, 1983). There are three major classes of proteins: lipoprotein, OmpA and the porin proteins. A major protein is an arbitrary term: a minor protein may become major when it is fully induced (Lugtenberg and van Alphen, 1983).

Lipoprotein

First isolated by Braun and Rehn (1969) this is the most abundant protein of the cell and is highly conserved among Gram-negative bacteria, one-third being covalently and two-thirds non-covalently attached to the peptidoglycan (Inouye et al, 1972). Lipoprotein is not essential for cell survival, but it appears to be involved in maintenance of rod shape, in stabilising the OM structure and in anchoring the OM to the peptidoglycan (Lugtenberg and van Alphen, 1983). The nucleotide sequences of the lpp genes of S. marcescens have been determined (Nakamura and Inouye, 1980) and cloning experiments have shown that the Serratia lipoprotein is present exclusively in the OM (Lee et al, 1981) on the inner side (Lugtenberg and van Alphen, 1983).

OmpA Protein

The OmpA protein is a heat-modifiable, mainly non-peptidoglycan bound protein. The temperature used to solubilise the OMs with 2% SDS affects the molecular weight obtained on SDS-PAGE, being higher when the protein is in its heat-modified form. This implies that a conformational change has taken place in the molecule and not a

degradation. Heller (1979a) found that the OM protein profile of S. marcescens not only depended on solubilisation temperature, but also upon the concentration of acrylamide in the gel.

The OmpA protein appears to play a role in F pilus mediated conjugation and, together with Brauns lipoprotein, it maintains the structural integrity of the OM and the rod shape of the cell (Lugtenberg and van Alphen, 1983; Sonntag et al, 1978). Non-physiologically, it serves as a receptor for certain phages (Datta et al, 1977) and is required for the action of a colicin (Chai and Foulds, 1974).

S. marcescens OmpA protein shows significant cross-reactivity with that of Escherichia coli, a phenomenon readily observed in Enterobacteriaceae (Hofstra and Dankert, 1980; Hofstra et al, 1980). Cole et al (1982) identified the ompA gene in S. marcescens and on cloning it into E. coli K12 found that the OmpA protein was fully expressed. This suggests that the antigenic structure and amino acid sequence have been well conserved during evolution. However, Braun and Cole (1984) showed that this S. marcescens polypeptide was not functionally equivalent to the E. coli OmpA protein, displaying only characteristics of heat-modifiability and sensitivity to cleavage by proteases, and not being involved in conjugation, colicin uptake and serving as a phage receptor. Since these latter properties reflect responses to external agents, it was concluded that the domains of the proteins exposed on the cell surface were significantly different. DNA sequence analysis of the gene showed that three regions of the protein likely to be surface exposed not only differed extensively from corresponding regions of the E. coli polypeptide, but also from all other sequenced OmpA proteins. Thus there appears to be constant (trans-membrane) and variable (extracellular) regions

in the OmpA protein of Enterobacteriaceae, the sequence polymorphism probably representing a safety mechanism by which various species can avoid cross-infection by noxious agents such as phages or colicins.

Peptidoglycan-associated pore proteins

Porin proteins are tightly, but non-covalently, associated with peptidoglycan. E. coli K12 contains two peptidoglycan associated proteins known as OmpC (36 kDal) and OmpF (37 kDal) which are immunologically related (Lugtenberg and van Alphen, 1983). S. marcescens has two OMPs of estimated molecular weight 42 and 41 kDal (Lugtenberg et al, 1977) with similar functions, and which share antigenic determinants and cross-react with these E. coli porins. (Sawai et al, 1982; Hofstra and Dankert, 1980; Hofstra et al, 1980).

The peptidoglycan associated proteins act as receptors for bacteriophages, their main function, however, being to form transmembrane water-filled passive diffusion pores ('porins') that allow the OM to be permeable to nutrients and other hydrophilic molecules up to a molecular weight of 600-700 kDal (Lugtenberg et al, 1977; Nikaido and Nakae, 1979). Diffusion of a molecule through the pores not only depends on its size and hydrophilicity, but also its charge, most pores being cation selective. The porin protein channels exist as trimers, associated with LPS in their biologically active forms, being homologous and heterologous combinations of OmpF and OmpC in E. coli (Ichihara and Mizushima, 1979). Electron microscopic studies suggest that each of the three monomers can form a pore at the outer surface of the membrane as distinct units, but that these coalesce and fuse into a single central channel before reaching the inner membrane surface (Dorset et al, 1984; Engel et al, 1985). If this is confirmed, it would explain the tight association of three subunits

that is necessary to form a functional channel (Nikaido and Vaara, 1985).

Other OMPs

Several proteins become induced when specific nutrients are either present or restricted, eg the bacteriophage lambda receptor protein (LamB) of E. coli in the presence of maltose, several high molecular weight proteins when iron is restricted (see section 1.3.2). Vitamin B12, being particularly large, requires a specific protein (BtuB) to facilitate its translocation across the OM. Other OMPs of unidentified function are also present (Lugtenberg and van Alphen, 1983).

1.3.1.5 Role of divalent cations

Divalent cations, particularly Ca^{2+} and Mg^{2+} help maintain the integrity of the OM by providing a stabilising force and bridging anionic groups within the LPS and protein bring about LPS protein and/or LPS-LPS interactions. Ethylenediaminetetracetic acid (EDTA) can bind these, releasing LPS-complexes from the cell (Lugtenberg and van Alphen, 1983).

1.3.1.6 OM Enzymes

The OM is poor in enzymatic activity, but phospholipase A is present, providing repair and modification to envelope phospholipids. S. marcescens excretes lipases, proteases and nucleases (see section 1.5.5) and Heller (1979b) found that the OM can contain substantial quantities of these. Furthermore, Heller also found that β-lactamase

activity (about 80%) can be present (see section 1.6).

1.3.1.7 Capsule

Many Gram-negative organisms produce extracellular polysaccharides (exopolysaccharides) existing in the form of a discrete capsule around the cell or as loose slime. Some strains of *S. marcescens* are thought to possess capsules. Adams and Young (1965, 1966) studied the extracellular and capsular polysaccharides of *S. marcescens* and found a wide variety of structures including an acidic glucomannan, a rhamnoglucan and a glucoheptan, all containing D-glucose. However, some of these may have been LPS components contaminating the preparations. The capsule acts as a barrier to protect the cell and has also been implicated in the adhesion of bacteria to cell surfaces (Costerton et al, 1978).

1.3.1.8 Flagella

The flagella of Enterobacteriaceae are composed of three distinct substructures, the filament, the hook and the basal structure. The filament is composed of one protein, flagellin, having a different amino acid sequence, composition and molecular weight in different species. The flagella is responsible for the mobility of bacteria, allowing them to exhibit chemotactic behaviour (Silverman and Simon, 1977). Most strains of *S. marcescens* possess peritrichous flagella and are mobile.

1.3.1.9 Fimbriae and pili

Fimbriae are smaller and more numerous than flagella, playing an important role in the attachment of bacteria to surfaces (Hammond et al, 1984; Ofek and Beachey, 1980). The sex pili are longer and wider than fimbriae, being involved in the transfer of genetic material during conjugation. A classification system based on the morphology and haemagglutinating properties of fimbriae divides them into 5 main types (Duguid and Old, 1980; Hammond et al, 1984).

Many strains of the genus Serratia possess peritrichous fimbriae, usually being of type 1 (mannose sensitive haemagglutinins, MS-HA) and type 3 (mannose resistant HAs restricted to Klebsiella and Serratia, MR/K-HA), and cells are often multiply haemagglutinating (ie produce two or more HAs). The presence of class MR/P-HA (similar to type 4, first described in Proteus species) has recently been reported and further subdivided into two classes in newer species of Serratia, indicating that the distribution of Serratia fimbriae is more complex than originally thought (Adegbola and Old, 1982; Old et al, 1983).

Amako et al (1981) showed that some clinical isolates of S. marcescens could agglutinate in human urine. These strains possessed fimbriae, distinct from type 1, that interacted with agglutinating factors in the urinary slime (mucin) to form large aggregates. Further studies indicated that the fimbrial antigens responsible were widely distributed among many strains of S. marcescens (Amako and Kohno, 1982; Kohno et al, 1984).

1.3.2 Effect of the environment on the cell envelope proteins of Gram-negative bacteria and the role of iron

The environmental conditions exert a profound effect on the OM

of Gram-negative bacteria. For example, growth temperature and growth medium can both alter the porin ratio of OmpF to OmpC in E. coli K12 whilst maintaining an approximately constant total amount in the membrane (Lugtenberg et al, 1976).

Nutrient-limitation may result in the induction of new OMPs. Phosphate limitation causes the expression of a 36 kDal protein (PhoE) in E. coli, which shares many of the properties of OmpF and OmpC (Overbeeke et al, 1980), and sulphate limitation induces the formation of two proteins of 15 and 19 kDal (Lugtenberg and van Alphen, 1983). Similarly, protein H2 is induced when Pseudomonas aeruginosa is grown under Mg^{2+}-depletion and is repressed when Ca or Mn is incorporated into the Mg^{2+}-deficient medium (Anwar et al, 1983a).

Iron is essential for many bacterial enzyme systems, such as those involved in the respiratory chain, DNA biosynthesis and lymphocyte transformation (Neilands, 1981b). Very little iron is present in aerobic environments because Fe^{2+} and Fe^{3+} ions complex with hydroxyl groups forming insoluble products. To overcome this, bacteria have acquired iron assimilation systems, one being of low affinity and incompletely elucidated, the other being of high affinity (Neilands, 1981b). The latter involves the synthesis of several new OMPs and low molecular weight compounds, regulated by the intracellular iron content (McIntosh and Earhart, 1977). Low constitutive levels of this system are thought to be present, however, irrespective of environmental conditions (Neilands, 1981b) and could offer an alternative explanation to the low affinity system (Lugtenberg and van Alphen, 1983).

In the high affinity system, siderophores (ferric-specific ligands having a high affinity for iron) transport iron into the

cells via specific receptor proteins (molecular weight 74-83 kDal in E. coli) located in the OM, reduction of iron being a feature of the uptake process (Leong and Neilands, 1976). The receptors are required to accommodate the large complexes, acting as pore or channel formers as well as phage receptors (Neilands, 1981ab, 1982).

Siderophores are classified into two types: phenol-catechol (eg enterochelin) and hydroxamate (eg ferrichrome). E. coli and other enteric bacteria synthesise enterochelin (chromosomally specified) and aerobactin, a citrate hydroxamate (usually associated with the ColV plasmid) (Griffiths, 1983; Neilands, 1981a). Aerobactin has a lower affinity for iron than enterochelin, but it has an advantage in that it can be re-used after delivery of iron to the cells, enterochelin (and ferrichrome) being hydrolysed (Griffiths, 1983; Braun et al, 1984).

In E. coli four iron-regulated outer membrane proteins (IRMPs) are induced of molecular weight 74 kDal, 78 kDal (TonA protein), 81 kDal (FepA protein and colicin B receptor) and 83 kDal (Braun and Hantke, 1981). The 81 kDal protein functions as the ferric-enterochelin receptor (Ichihara and Mizushima, 1978), Chart and Griffiths (1985a) finding that it is highly conserved in many E. coli strains. The 78 kDal protein functions as a ferrichrome receptor (Wayne and Neilands, 1976; Coulton et al, 1983), even though E. coli does not synthesise ferrichrome, being mainly a fungal siderophore. The role of the 74 and 83 kDal proteins are undefined. Hancock et al (1976) showed that E. coli grown in the presence of 1 mM citrate synthesises another OMP (molecular weight 80.5 kDal) that appears to be involved in citrate-mediated iron uptake. A distinct 74 kDal OMP acting as the aerobactin receptor is reported to be present in strains of E. coli harbouring a ColV plasmid (Bindereif et al, 1982;

Krone et al, 1983). Lastly, low molecular weight proteins in E. coli have been indicated in iron transport (Klebba et al, 1982) and also in Neisseria gonorrhoeae, a 37 kDal protein being conserved among pathogenic strains (Mietzner et al, 1986).

Siderophore production is temperature sensitive. At 42 °C and under conditions of iron-stress, E. coli cells have a reduction in their ability to transport ferric-enterochelin, and there is also a reduction in the number of colicin 1 (74 kDal) receptors (Worsham and Konisky, 1984).

Traub (1977) has shown that S. marcescens produces iron-chelators when grown in Fe-free defined broth medium, but receptor proteins have not been described.

1.4 SEROTYPING AND ANTIGENIC STRUCTURE OF S. MARCESCENS

Various methods of subspeciation have emerged to aid in epidemiological investigations of S. marcescens. Serotyping is the most efficient and well used, bacteriophage and bacteriocin typing, biotyping and zymogram determinations being used in epidemics, together with serotyping, to give complete strain definition (Pitt, 1982, 1983). The O (LPS) and H (flagella) antigens of the genus Serratia provide the basis for serological typing, the K (capsular) antigens being incompletely elucidated.

1.4.1 The O antigens

Basic LPS structure has been described in section 1.3.1.2. The O antigenic side chain obviously varies in its structure according to the serotype. No systematic study of the chemistry of the O antigens has been reported for S. marcescens, although the composition of LPS from various strains has been determined.

Partially acetylated acidic glucomannans have been isolated from three strains of S. marcescens O14 and one strain of the cross-reacting group O6. Individual polymers vary in their extent of O-acetylation and in substitution with glucosyluronic acid residues (Brigden and Wilkinson, 1985a). E. coli strains often have O antigens with acidic polysaccharide moieties whose structural features are like those of capsular polysaccharides, but differ by being bound to core lipid A (Westphal et al, 1983). In some E. coli strains, such acidic LPSs occur together with neutral O8 or O9 antigens, the acid polymers due to their charge, biologically behaving like capsular antigens (Westphal et al, 1983). In S.

marcescens, however, the acidic glucomannans appear to be truly (micro)capsular in origin, and if they represent the antigens common to serogroups O14 and O6, it suggests the current scheme of O serogroups is not being based on the organisms LPS (Brigden and Wilkinson, 1985a). Various neutral polymers have been isolated in the LPSs of these strains, but one common to all has yet to be identified. A polymer of ribose and N-acetylgalactosamine (polymer a) is present in two O14 strains (Brigden and Wilkinson, 1983) and a polymer of glucose, galactose and N-acetylglucosamine (polymer b) in a third (Brigden and Wilkinson, 1985b). A fourth O14 strain contains an acetylated glucorhamnan (polymer c) (Brigden et al, 1985). To complicate matters further, polymer a has also been found in an O12 strain and as a minor component in an O6 strain, polymer b (differing only slightly in the configuration of the glucose residue) in an O8 strain (Tarcsay et al, 1973) and an E. coli O18 LPS, and polymer c in an O6 strain (Brigden et al, 1985). All of these shared polymers between strains provide possibilities for serological cross-reactions, a problem so often encountered in the serotyping of S. marcescens. The results of Wilkinson and co-workers reinforce the work of Adams and Young, who found in 1966 that strains of S. marcescens produce a complex range of cellular and extracellular polysaccharides. The only serotype so far studied that contains individual polymers not reported in other S. marcescens LPSs is strain O1. Both the neutral polymer (of N-acetylglucosamine and rhamnose) and an acidic polymer, are therefore potential candidates for the O1 antigen (Furn and Wilkinson, 1985).

The O antigen scheme in universal use is that of Ewing et al (1962) incorporating 15 O groups. LeMinor and Pigache (1978) have described 5 additional O types, O16 to O20, confirmed by Traub

(1981b) with an additional one, O21. New serogroups are still being discovered, the two most recent bringing the total to 23 and 24 (LeMinor et al, 1983; Traub, 1985). Typing is performed by slide or tube agglutination, sera being raised in rabbits by conventional methods, commercial kits being available in America. Broth cultures are used after heating to remove the masking effect of any capsular substances which might be present (Pitt, 1982). For a full account of the different typing methods, see Pitt and Erdman (1984).

The predominant serotype is O14, accounting for over 35% of all strains typed (Pitt, 1982; Rubin, 1980). Distribution of other O serogroups varies graphically, types O2, O3, O6 and O11 being relatively common. The generally poor discrimination of O serogrouping probably reflects the complex surface polysaccharides of S. marcescens, the predominance of O14 strains either representing enhanced virulence and an overall prevalence in nature, or the presence of many shared polymers and antigenic determinants.

Antigenic cross-reactions between O groups often occur, suggesting the presence of shared antigens, particularly with types O6 and O14 which sometimes cannot be resolved (by absorption of antisera) thus giving the serogroup complex O6/O14 (Traub and Fukushima, 1979a; Traub and Kleber, 1978). Traub (1981a) suggests that additional analysis is required for O6 (and O9) antigens, perhaps with further subdivisions. In fact, LeMinor and Pigache (1978) and LeMinor and Sauvegeot-Pigache (1981) have attempted to subdivide O6/O14, O9, O5, O10 and O16 serogroups. It is also of interest that S. marcescens O antigens cross-react with bacterial antigens of other Enterobacteriaceae (Traub, 1981c). The complexity of the O serotypes is further enhanced by a report that variation in somatic antigens can occur. Traub (1978) found a strain originally

O6/O14 had variants that lost O6 reactivity and acquired O12 whilst retaining O14. This is very rare and would probably only be detected in a few clinical isolates.

1.4.2 The H antigens

Ewing et al (1959) described 13 H antigens of S. marcescens, LeMinor and Pigache (1977) and Traub and Klebber (1977) discovering 7 more, all 20 being used to give a comprehensive result. The latest study has brought the total known to 26 (LeMinor et al, 1983).

If agglutination is used, sera have to be exhaustively absorbed because of test cross-reactions and therefore the immobilisation test is exploited as the simplest means for H type identification (LeMinor and Pigache, 1977; Pitt et al, 1980). Any cross-reactions which do occur can sometimes be attributed to antibodies to fimbrial antigens (Pitt et al, 1980). However, diphasic variation has been recognised in S. marcescens (Young et al, 1980a), showing a complexity in the H antigens, and this is more important in influencing the accuracy of H typing.

H12 is the predominant serotype reported by LeMinor and Pigache (1977), an O14:H12 combination being the most frequently isolated from clinical infections (Pitt et al, 1980; Pitt, 1982, 1983; Rubin, 1980; Bullock et al, 1982).

O groups form the primary serotype division of the species, H typing (or phage typing) being of real value and necessary to sub-divide strains from an outbreak involving a common O serogroup (Pitt et al, 1980; Pitt, 1982).

1.4.3 The K antigens

Capsulated strains and K antigens have been noted (Ewing et al, 1959), and even isolated from various strains (Traub et al, 1985), but K antigenic types have not been determined and await further elucidation, the complex of surface polysaccharides hindering progress in this area.

1.5 GRAM-NEGATIVE BACTERIAL INFECTIONS, HOST DEFENCE MECHANISMS AND VACCINES

To cause an infection, pathogens must gain entry into the host, survive and multiply, resist host defense mechanisms and damage host tissue. The microbial products responsible for these processes are the determinants of pathogenicity, many of them being surface components (Smith, 1977). There is a vast diversity of virulence mechanisms that are an accumulation of individual genetic traits which help the organisms to survive in host tissue.

The virulence determinants of S. marcescens are not well characterised but appear to include LPS, especially the O antigens O6 and/or O14 (Simberkoff, 1980; Traub, 1982a). Resistance to serum bactericidal activity is important and may be one of the many factors responsible for the seeming increase in S. marcescens infections over the past few years. The proteases of S. marcescens may also be linked to virulence (Doerr and Traub, 1982).

1.5.1 Adherence

The first step in creating an infection is usually attachment of the bacterial cells to the injury site or to a mucous membrane. This prevents the organisms from being swept away in fluids bathing the tissues, or by mechanical actions such as coughing (Ofek and Beachey, 1980). Bacteria can adhere non-specifically, Harber, MacKenzie and Asscher (1982) showing that urinary pathogens infecting the bladder of man can attach in this manner, but usually specific interactions occur between the surface components of bacteria and host tissues. Hydrophobic molecules on the bacterial cell surface allow approach to

the negatively charged epithelial cells, where bonds of high affinity form between the ligands of bacterial cells and receptor molecules in the tissue membrane (Ofek and Beachey, 1980). Bacterial ligands include fimbriae and/or glycocalyx material, the latter being polysaccharide or protein in nature (Costerton et al, 1978; Orskov et al, 1985).

Surface growth is now recognised to be important in many infections, perhaps initiated by fimbriae and maintained within a glycocalyx matrix (Brown and Williams, 1985). The latter allows adherent bacterial microcolonies to grow enmeshed in polysaccharide that protects the organisms from antimicrobial agents, as well as humoral and cell-mediated immune defences (Brown and Williams, 1985). The surface microcolony forms its own environment, the matrix acting as an ion-exchange material, trapping essential nutrients for bacterial growth whilst excluding noxious molecules (Gristina et al, 1985). The microenvironment therefore enhances chances of survival compared to replication in the uncontrolled environment of a liquid suspension.

The different fimbriae of E. coli associated with virulence and specific infections include the P-fimbriae (several separate fimbriae associated with pyelonephritis in man, Rhen et al, 1983); specific colonisation factors, CFA (fimbriae with separate properties to those of type 1, associated with the intestine of man causing diarrhoea, Thoren, 1983); K88 fimbriae (associated with piglet diarrhoea) and K99 fimbriae (diarrhoea in calves and lambs, Ofek and Beachey, 1980). Fimbriae (with mannose-resistant and/or mannose-sensitive haemagglutination patterns), as well as hydrophobic bacterial surfaces, are also thought to be virulence factors in urinary tract infections caused by some Enterobacteriaceae (Ljungh and Wadström, 1984).

S. marcescens has been shown to possess fimbriae (see section 1.3.1.9), but no ecological role in colonisation in vivo leading to an infection is definitely known yet. Kohno et al (1984) suggest that S. marcescens strains bearing fimbriae known to react with mucin in urine to form large aggregates, might not be seen in UTIs. The infecting bacteria could be excreted as clumps of cells and urinary mucin by voiding, the agglutination therefore acting as a host defence mechanism. The urinary mucin is thought to be derived from the mucous layer of the urinary tract. Yamamoto et al (1985) recently found that the same S. marcescens strain was able to adhere to the human bladder surface. This was mediated by the MS fimbriae, which could therefore act as a colonisation factor in urinary tract infection. The high affinity of the strain for the bladder surface was assumed to be the result of an interaction between the fimbriae and the same substance as urinary mucin on the mucous surface. The surface of the urinary tract might therefore have a high affinity for any strain of S. marcescens bearing MS fimbriae.

1.5.2 Iron and infection

The body iron is mainly located intracellularly in haemoglobin and ferritin, and extracellularly it is sequestered by the iron-binding proteins transferrin (TF) in blood and lymph, and lactoferrin in secretions. This gives a free iron concentration of $\sim 10^{-18}$ M, E. coli requiring 10^{-6} M for growth and overcoming its nutrient limitation in infections by inducing the high affinity uptake system and competing for the TF bound iron (see section 1.3.2) (Ogata, 1983). In response, vertebrate hosts reduce the amount of iron in the circulating TF-iron pool (known as the hypoferraemia of

infection). Polymorphonuclear leukocytes (PMNs) contain lactoferrin which is released during phagocytosis and removes iron from TF, the resulting Fe^{3+}-lactoferrin complex being taken up by macrophages and rapidly cleared from the circulation by the reticulo-endothelial system - RES (Ogata et al, 1983; Griffiths, 1983). Intestinal absorption of iron is also decreased, and fever itself hampers iron-sequestering mechanisms by decreasing the available iron in host tissue and interfering with the bacteria's ability to use it (Lugtenberg and van Alphen, 1983; Ogata, 1983; Worsham and Konisky, 1984). On the other hand, the host can produce a low molecular weight iron-binding factor that potentiates bacterial growth *in vivo* and *in vitro* (Jones et al, 1980). This 'host-associated iron transfer factor' is found in serum and tissues, and can bind iron and promote iron uptake by bacteria.

Direct proof that bacteria grown *in vivo* are iron-restricted comes from experiments using organisms isolated from infections without subculture, those recovered from the sputum of cystic fibrosis patients or from infected human urines expressing high molecular weight IRMPs (Anwar et al, 1984; Lam et al, 1984; Shand et al, 1985). The proteins expressed *in vivo*, however, are not necessarily identical to those induced *in vitro* (Sciortino and Finkelstein, 1983; Williams et al, 1984), indicating that factors other than simple iron deprivation must influence the regulation of these proteins. The antibody respose of cystic fibrosis patients and burns patients to *Ps. aeruginosa* infections shows that in patient's serum there are IgG antibodies present to all the major OMPs, including the IRMPs (Anwar et al, 1984, 1985). In fact, Griffiths et al (1985) have recently found that normal serum from adult humans contains antibodies to IRMPs that are mainly of the IgG class.

Bacteria that grow in vivo and that are iron-restricted tend to multiply at a slower rate to in vitro grown iron-replete bacteria, which may be crucial in the outcome of the infection. Iron restriction is a general host defence, but is not necessarily growth rate limiting. The long in vivo replication times may be the result of deprivation of nutrients other than iron, or of inhibitory substances including antibodies, some of which could be directed specifically to structures involved in nutrient uptake.

Animals injected with iron and bacteria are much more susceptible to infections, the iron acting as a nutrient and interfering with host defence mechanisms (Ogata, 1983; Griffiths, 1983; Weinberg, 1984). Iron overload inhibits phagocytosis and saturates TF, the latter then being less efficient in giving the metal to lymphocytes for initiation of DNA synthesis (Weinberg, 1984). Iron overload in patients could be treated by giving a siderophore, such as desferoxamine, but there are conflicting reports as to whether certain pathogens can use it to acquire iron, thus increasing the patient's risk of gaining an infection (van Asbeck et al, 1983; Brock and Ng, 1983). Siderophore-like compounds that do not promote iron uptake could be exploited as antimicrobial agents in vivo, by restricting growth of bacteria through iron sequestration (Jones and Grady, 1983). In vitro work has already shown that desferoxamine and ascorbic acid, and also parabactin, inhibit bacterial growth (van Asbeck et al, 1983; Bergeron et al, 1983).

Withholding iron from the diet can decrease susceptibility to infection. Mevissen-Verhage et al (1985) found that infants fed with cows milk preparations containing a low iron-content had enchanced resistance to gut colonisation. If dietary iron-restriction is too severe, however, susceptibility to infection may increase because

there is no iron available for humoral and cell-mediated immunity reactions (Weinberg, 1984).

Bacteria grown in iron-depleted conditions before injecting into experimental animals multiply more rapidly and have a greater invasive action than bacteria grown in iron-rich media (Weinberg, 1984). Sokol and Woods (1984) found that when Ps. aeruginosa was grown in low iron medium before infection, it caused more pathological changes in the lung than when grown in high iron medium, indicating iron concentrations of culutre media influence pathogenesis of chronic respiratory infection. Enterochelin production can enhance bacterial virulence. Yancey et al (1979) showed that the inability of Salmonella typhimurium to synthesise enterochelin reduced its virulence in laboratory animals and its capacity to grow in human serum. Benjamin et al (1985), however, found that although enterochelin production was necessary for S. typhimurium to grow in mouse serum, it did not affect the ability of the cells to cause mouse typhoid and was therefore not a virulence factor. Thus a more comprehensive investigation of the importance of siderophores in intracellular infections may be warranted.

Enterochelin manufacture requires a very high expenditure of cell energy and it is chemically unstable, undergoing oxidation and hydrolysis after delivering iron to the cell. It is not very water soluble and it adheres to proteins acting as a hapten for antibody production (Neilands, 1981b). Antibodies to enterochelin inhibit iron uptake by enterochelin, but not by other siderophores (Weinberg, 1984). Moore et al (1980) showed that enterochelin specific immunoglobulins exist in normal human serum which may act synergistically with TF to effect bacteriostasis and limit growth of enterochelin producing pathogens.

Aerobactin suffers none of the problems of enterochelin conferring advantages to cells already producing enterochelin. Aerobactin in E. coli is linked to virulence and the presence of the ColV plasmid. These bacteria are particularly pathogenic, Braun et al (1984) showing that they have an increased growth rate in calf serum compared to E. coli cells without a plasmid. The absence of a plasmid, however, should not infer the absence of aerobactin-mediated iron sequestration, an E. coli strain isolated from a neonate with meningitis having the genes for this sytem encoded on its chromosome (Volvano and Crosa, 1984). The presence of aerobactin may be a significant factor in the invasion of the bloodstream, E. coli isolated from blood being more aerobactin-positive than strains isolated from other sites (Montgomerie et al, 1984). Both enterochelin and aerobactin transport systems are probably required to give bacterial cells greater flexibility for a given environment (Ogata, 1983).

Haemolysins are proteins which can disrupt the membranes of erythrocytes liberating haemoglobin. The secretion of haemolysins by bacteria probably potentiates the ability of the cells to acquire iron in the blood by the high affinity uptake system (Griffiths, 1983; Ogata, 1983).

Traub (1977) showed that S. marcescens was resistant to the non-specific host defence mechanism of unsaturated transferrin, and that it does produce siderophores. However, further experiments on iron uptake systems of Serratia are limited.

1.5.3 Complement and serum bactericidal activity

Complement mediated serum killing constitutes an important

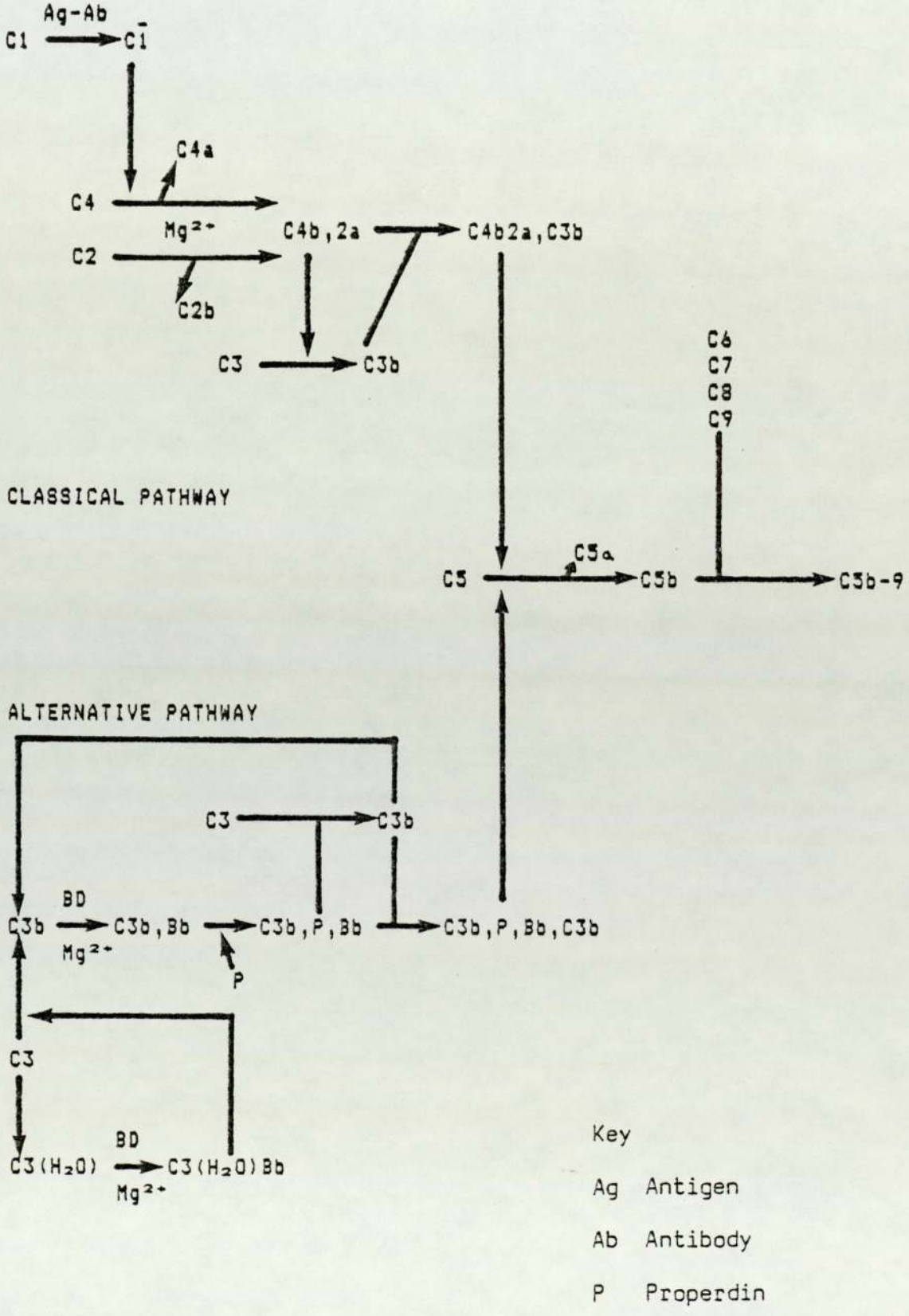
component of the host defence in combating infections with Gram-negative bacteria. Complement can directly kill bacteria and stimulate host inflammatory response and cellular defence mechanisms, having a critical role in the non-immune host (Ogata, 1983; Winkelstein, 1983).

The complement system is comprised of a series of circulating glycoproteins that interact in a precise sequence of reactions leading to the production of biologically active cleavage fragments capable of interacting with bacteria, promoting opsonisation on the one hand and direct cell damage on the other. Two major pathways of complement activation are recognised (see Figure 1.4), the cleavage of component C3 being the central event of each.

The classical pathway (CP) is generally activated by the interaction of antibody with an antigenic surface. In humans, IgG 1, 2 and 3, and IgM antibodies are capable of activating the CP. Activation involves binding of the macromolecule C1 to the Fc region of the antigen-bound antibody, with subsequent activation of the C1 molecule. A single IgM molecule is potentially able to fix C1, whereas at least a pair of IgG molecules are required for this purpose. C1 is composed of three subunits held together by Ca^{2+} ions, and activation leads to the generation of an enzyme, C1 esterase, capable of interacting with and cleaving the next two components in the complement cascade, C4 and C2. C1 esterase binds and cleaves C4 with the release of the small cleavage fragment C4a from the C4 molecule. The C4b molecule is capable of interacting with the antigenic particle surface where it can bind covalently via a thioester bond, thus continuing the complement cascade. The next component, C2, complexes with C4b in the presence of Mg^{2+} ions, when it is cleaved by C1 esterase to release a smaller C2a fragment. The

Figure 1.4

Schematic representation of the Classical and Alternative pathways of complement (Taylor, 1983)



larger C2b fragment joins with C4b to form C4b2b (the classical pathway C3 convertase enzyme), capable of cleaving the next component in the cascade, C3. C3 is cleaved into the small and large fragments, C3a and C3b. The former has various inflammation-promoting properties, and recently has been shown to inhibit C3 cleavage by the CP C3 convertase, suggesting it has a role in controlling C3 activation (Strunk and Webster, 1985). The release of C3a reveals a thioester reactive binding site on the larger C3b fragment. Through this, C3b can bind covalently to a number of substrates on the bacterial surface forming an opsonic coat, only one favourably sited C3b molecule combining with C4b2b to form C4b2b3b. The latter complex is capable of cleaving C5, leading to the relase of another biologically important fragment, C5a. The larger C5b fragment can interact with the next component in the cascade, to form a protein complex, C5b6, which can associate non-covalently with biological membranes. On addition of C7, the complex becomes hydrophobic, and insertion of the C5b67 complex into lipid bilayers is initiated. Interaction with C8 yields C5b678, which causes the polymerisation of C9 to form a tubule traversing the membrane. The tube may remain associated with C5b678, and is referred to as the membrane attack complex (MAC). The tubule or cylinder possesses a hydrophilic central channel and a hydrophobic outer surface, insertion into a cell membrane leading to free communication of small molecules between the inside and outside of the membrane leaflet. The cell cannot maintain its osmotic stability, swells and dies, or lyses (Joiner, Brown and Frank, 1984; Frank, 1985).

The second major pathway of complement activation is termed the alternative pathway (AP), which involves a different set of proteins. C3b, thought to be formed continuously in small amounts by

a 'tickover mechanism' possibly involving proteolytic enzymes in body fluids, is able to combine with a protein termed factor B to form the complex C3b,B in the presence of Mg^{2+} ions. On attachment to a cell surface favouring activation, another protein, factor D, can cleave C3b,B releasing Ba to yield C3b,Bb. The latter is the alternative pathway C3 convertase which can cleave C3 fixing more C3b to the activation surface so that more B binding sites are exposed, and a feedback cycle is formed. The C3,Bb tends to be unstable, increased stability being provided by the binding of an additional protein, properdin, in the presence of Mg^{2+} ions to form C3b,Bb,P potentiating the action of the AP C3 convertase. Addition of another C3b molecule to C3,Bb(P) gives C3b,Bb(P),3b (the AP C5 convertase), which is capable of cleaving C5 initiating MAC assembly as before (Joiner, Brown and Frank, 1984; Winkelstein, 1983).

C3b generated via the CP that binds to the cell surface and is not involved in CP C3 convertase formation has the potential to interact with proteins of the AP, generating the AP C3 convertase. The interaction of C3b, formed spontaneously or by either pathway, with factors B, D and P leading to amplification of complement action on a bacterial surface, is regulated by proteins of the C3 inactivator system. Factor H competes with factor B for its combining site on C3b, blocking the formation of the catalytically active C3 convertase of the feedback loop. Which factor is preferentially bound to C3b depends on the nature of the surface to which C3b is attached. Certain surfaces, usually polysaccharides, are also called 'activator' surfaces, and favour the uptake of factor B onto the chain of C3b, with the corresponding displacement of factor H. On such surfaces, C3b is protected from inactivation, but can still interact with AP components to assemble an AP convertase.

On the other hand, the C3 in sites that are not protected is rapidly cleaved and destroyed. When factor H interacts with C3b, factor 1, a serine protease, can rapidly cleave C3b to release a very small peptide fragment. The resulting inactivated C3b(iC3b) remains bound to the bacterial surface, and can be further cleaved by non-specific proteases to release a major fragment called C3c, leaving C3dg covalently bound. Further proteolytic digestion releases another small fragment, with C3d still covalently bound to the surface (Joiner, Brown and Frank, 1984). Phagocytic cells possess receptors for complement components C3b (CRI) and iC3b(CR3), and lymphocytes possess receptors for C3dg/C3d (Ross and Atkinson, 1985). Opsonisation by these molecules is therefore very important in the host defence mechanism. The capacity of bacteria to activate the AP and consume C3 from the serum, has a great influence on the susceptibility of the organisms to phagocytosis and in vivo clearance. Lam et al (1985) even suggest the high susceptibility of the peritoneum to infection by gut flora, despite a normal phagocytic response, may be partly explained by the low level of functional complement which is inadequate for optimal opsonisation of the bacteria. Newman and Mikus (1985) studied five bacterial species and found that only a small percentage of bound C3b was in the form of iC3b, even after 2 hours incubation, and there was no conversion of iC3b to C3dg or C3d. Their data suggests that the predominant form of C3 encountered by inflammatory phagocytes may be C3b. Binding of C3b or iC3b-coated particles to phagocytic cells immobilises the particles, thereby facilitating phagocytosis; however, second signals are required to trigger ingestion. These include activation processes, or interaction of other ligands with their specific phagocytic cell membrane receptors, eg IgG-Fc or fibronectin (Frank,

1985).

Bacteria can activate the complement pathways by a number of different mechanisms. Specific (immune or natural) antibodies to bacterial cell-surface constituents can bind and activate serum C1, leading to activation of the entire CP. It also seems that the CP can be activated in the absence of antibody in Gram-negative bacteria by the lipid A portion of LPS (Shafer et al, 1984; Joiner, Brown and Frank, 1984). The isolated porins of Salmonella have also been shown to activate the CP independently of antibody (Galdiero et al, 1984) but how important a mechanism this is in whole cells remains unknown. The AP can be activated on the surface of Gram-negative bacteria in the absence of specific immunoglobulin, although antibodies can increase AP activation and the rate of assembly of the AP convertase via unknown mechanisms (Winkelstein, 1983; Joiner, Brown and Frank 1984). The polysaccharide portion of the LPS appears to be involved in AP activation (Morrison and Kline, 1977), Grossman and Leive (1984) showing that activation is sensitive to slight variations in chemical structure, but not to large variations in the length of O polysaccharide side chains in Salmonella. Both complement pathways can interact, but they can be differentiated by their requirements for divalent cations; activation of the classical pathway requires Mg^{2+} and Ca^{2+} ions, whilst the alternative pathway needs Mg^{2+} ions only (Taylor, 1983). This allows for the use of Mg-EGTA to select for AP activation only.

In summary, the complement system performs three vital functions, either pathway giving the same end result:

1 Opsonisation. C3b and its inactivated products are deposited on bacterial surfaces which mark the cells for

phagocytosis, receptors for these opsonins being present on the phagocytic cell surfaces.

2 Cytolysis. Early complement components mark the cells for direct killing by the later complement components, the MAC together with lysozyme causing cell lysis.

3 Cell activation. C3a and C5a produced during complement activation are anaphylatoxin factors, having powerful effects on inflammatory cells. They are capable of releasing histamine from mast cells, contracting smooth muscle, and promoting increased capillary permeability (Winkelstein, 1983). C5a especially is important, also acting as a chemotactic factor attracting phagocytic cells to the infection site (Ogata, 1983).

Resistance to the bactericidal activity of complement is through bacterial surface components. Membranes have marked phenotypic variations depending on *in vitro* growth conditions, which influence the sensitivity of the cells to complement (Taylor, 1983), and these variables may also affect the serum sensitivity of organisms grown *in vivo*. Thus, the sensitivity of bacterial cells determined *in vitro* need not necessarily reflect that *in vivo* (Dalhoff, 1985). Smooth bacteria, having a surface layer of LPS, are generally more resistant to killing than their rough counterparts (Taylor 1983; Ogata, 1983). Many smooth strains show a delayed serum killing response, possibly because the long O side chains give a low membrane fluidity, reducing the rate of insertion of functional MACs into the cell envelope.

Results by Kato and Bito (1978) do seem to suggest a rigid membrane inhibits insertion of the MAC. Certain O antigen sidechains are superior to others in mediating resistance to complement. E. coli O18 strains seem to have LPS that hinders deeper structures on the bacterial surface that can activate the CP in absence of antibody, whereas E. coli O1 strains, even though they have complete LPS, are able to activate complement by this mechanism (Pluschke et al, 1983). Joiner et al (1983a and b) suggest that one reason smooth Salmonella and E. coli strains are resistant to serum killing as opposed to their rough counterparts, is because the LPS acts as a physical barrier to MAC insertion into the hydrophobic OM. Thus, activation of complement proceeds in the resistant strains, but the subsequently formed MACs are shed from the cells without causing damage. Coverage of lipid A core with O antigen chains also seems to be important, the greater the coverage, the more resistant the organism because of the inability of complement components to penetrate to the hydrophobic membrane regions (Goldman et al, 1984). Lastly, the length of O antigen affects the cell's response to killing. In S. montevideo, C3b attaches to the longest polysaccharide chains, because they sterically hinder access of C3b to shorter molecules. When bound to short-chain-length LPS, the C3b provides a focus of attachment for MAC near the OM, facilitating complement-mediated killing (Joiner et al, 1986).

The presence of an acidic polysaccharide capsule can act as a non-specific barrier independently of LPS, so that the presence of both gives an additive effect. In most cases though, there appears to be substantial evidence against a major role for capsules as mediators of serum resistance (Taylor, 1983). Williams et al (1983) showed that both K and O antigens of K. aerogenes were required to

protect the cells from complement killing in the absence of specific antisera. Serum resistance is often K antigen specific. The K1 capsule of E. coli contains sialic acid that inhibits complement amplification and AP activation by degrading cell-bound C3b and preventing additional C3 fixation (Fearon, 1978). The sialic acid acts as an inactivator surface, increasing the affinity of factor H and decreasing the affinity of factor B for C3b. Specific anti-capsular antibodies can overcome this by activating the CP, but K1 capsules are poor immunogens (Ogata, 1983). The K1 capsule of E. coli, regardless of the O serotype, always inhibits AP activation, thereby partially blocking serum killing and preventing the cells being opsonised by C3b for phagocytosis. Complete resistance to complement in the absence of antibodies also requires the presence of specific O antigens, O18 being superior to O1 (Pluschke et al, 1983).

An increase in the number of OMPs, giving surface exclusion, is also associated with resistance. E. coli strains harbouring a multiple-antibiotic-resistance plasmid R6-5 (an FII type R factor), which has a gene product encoding for the TraT protein in the OM, have selective resistance to serum killing (Moll et al, 1980). Another gene conferring serum resistance, designated iss, has been located in the colicin-producing plasmid, ColV. The iss gene product might be an OMP, but the increase in resistance in smooth strains (like that of traT) is via an unknown mechanism (Ogata, 1983).

Traub subdivided strains of S. marcescens, with regard to their resistance to killing by fresh human serum, into four different categories: PSS (promptly serum sensitive), DSS (delayed serum sensitive, covering the majority of strains), NSS (resistant to serum killing) and PSR (pseudo serum resistant; susceptible to serum after addition of homologous anti-O IgM antibodies) (Traub and Kleber,

1975; Traub and Fukushima, 1979b; Traub, 1980b). *In vitro* experiments showed that the PSS and DSS strains principally activated the classical and alternative pathways of complement respectively, although each category was shown later to possibly activate both pathways (Traub and Kleber, 1975; Traub and Fukushima, 1979c). Traub (1980a) then found homologous rabbit anti-O IgG immune sera blocked the activity of fresh human serum against DSS and PSS strains, either by consumption of complement or by steric hindrance of IgM antibodies, the latter being shown to enhance complement activity towards two NSS strains giving the PSR group (Traub 1980b). On removing the anti-O antibodies by absorption to protein A, the blocking effect was abolished. Traub (1978) had previously shown that an O6/O14:H12 serotype could undergo phenotypic variation, opaque varients being in the class DSS and grey ones PSS. These findings suggested that LPS did not determine serum sensitivity and resistance in *S. marcescens*, and that maybe some other structural feature, a protein new or otherwise, was involved. However, this blocking activity of IgG anti-O antibodies was opposite to the result found by Simberkoff et al (1976b) and Traub (1982b) on repeating his experiments did not find an antagonising effect. Thus it appears the factors determining serum resistance in *S. marcescens* are complex and yet to be fully determined.

The relationship between virulence and serum resistance in *S. marcescens* is not well documented. Strains associated with bacteremia are often serum resistant, infections usually being fatal (Simberkoff et al, 1976b). Gutschik et al (1980) showed that the ability of *S. marcescens* to establish endocarditis in rabbits was increased the more serum resistant the organism, but only in the presence of an indwelling catheter. Traub (1982a) found that

virulence of O6 and/or O14 serotypes for juvenile NMRI mice following IP injections did not correlate with susceptibility or resistance to human serum, but it was undetermined whether mice could phagocytose cells efficiently regardless of human serum susceptibility, which might have affected the results.

1.5.4 Phagocytosis

The ingestion and killing of invading bacteria by phagocytic cells is a critical determinant of resistance to infectious disease. The polymorphonuclear leukocytes (PMNs) are the major cells defending the host against organisms which have elicited an inflammatory response, passing through capillary walls in response to a chemotactic stimulus. Mononuclear phagocytes are distributed throughout the body circulating in blood (monocytes) and being fixed in tissues of the spleen, liver, bone marrow and lymph nodes and at the portal entry sites of many bacteria (fixed macrophages), the total body pool of macrophages constituting the reticuloendothelial system (RES) (Skamene and Gros, 1983).

Phagocytosis is a multi-step phenomenon involving adherence, ingestion and destruction (Skaemene and Gros, 1983), the bacteria then disintegrating due to the action of phagocytic acids, enzymes and other antibacterial compounds, eg phagocytin. The mechanisms by which PMNs recognise and ingest bacteria involve surface components (Stendahl, 1983), two main recognition mechanisms existing. One is general, depending on physiochemical surface properties such as surface charge and hydrophobicity. If bacteria are more hydrophobic than the phagocytes, they are readily phagocytosed (Ofek and Beachey, 1980). The other involves more specific ligand-receptor

interactions, such as those between opsonins coating the bacteria (eg IgG and the complement component C3b) or specific bacterial surface components (eg fimbriae), and receptors in the leukocyte membrane (Stendahl, 1983).

Once uptake of the bacterium in initiated, a phagocytic vacuole is formed. The latter is initially surrounded by the interiorized cell membrane and some extracellular fluid, and contains the bacterium. Lysosomes of antibacterial substances are then transported intracellularly and fuse with the phagocytic vacuole, thereby emptying their contents into the latter without spilling any harmful enzymes etc into the cytoplasm of the leukocyte itself. The resulting organelle is the phagolysosome, made up of the phagocytic vacuole and lysosome, microbial killing and digestion of susceptible bacteria then proceeding (Hahn, 1983).

Resistance to phagocytosis involves surface components. Capsules may interfere with the attachment of bacteria to phagocytes or restrict the access of opsonins to deeper structures (Ofek and Beachey, 1980; Cross et al, 1984; Verweij-van Vught et al, 1984). In one study, mouse virulence was assumed to be related to encapsulation of an S. marcescens strain, which protected against phagocytosis (Ohshima et al, 1984). Most encapsulated organisms require specific anticapsular antibodies for effective opsonisation (Ogata, 1983). For example, Ames et al (1985) found the antiphagocytic properties of the mucoid polysaccharide of Ps. aeruginosa could be overcome by specific antibodies. Often complement, as well as antibody , is required for efficient opsonisation. K positive strains of E. coli were shown to have a reduced interaction with phagocytes because the capsules restricted activation of the classical pathway of complement (Verweij-van Vught et al, 1984). Antibodies alone did overcome the

antiphagocytic properties of the capsules to some extent, but complement and antibodies were much more efficient. Smooth bacteria are generally more resistant to phagocytic killing than their rough counterparts (Ogata, 1983), Henricks and Verhoef (1983) showing this was dependent on the ratio of lipid A to polysaccharide in LPS. Williams et al (1983), however, found that O antigens, unlike K, are not antiphagocytic in K. aerogenes. The outer membrane protein TraT, encoded by a plasmid in E. coli, has also been associated with a reduced sensitivity of cells to phagocytosis by macrophages, independently of bacterial capsule. The protein antagonises complement opsonisation so that less C3 is deposited and phagocytosis is less efficient. Ps. aeruginosa produces extracellular proteases which can act as virulence factors in infections by interfering with one of the major antimicrobial systems in PMNs (Kharazmi et al, 1984). Some Serratia strains can also secrete proteases, but whether they interfere with phagocytic function is undetermined. Lastly, the environment can influence the response of bacterial cells to phagocytosis. Anwar et al (1983) found the rate of killing of Ps. cepacia by PMNs and serum factors in whole blood increased with a rise in temperature from 33 °C to 41 °C. The same group also showed the nutrient conditions under which the cells were grown affected killing, iron-limited bacteria being fairly sensitive. Both of these findings have important implications for the effects of fever and iron-restriction in vivo on the sensitivity of bacterial cells to phagocytic killing.

Fimbriae of some bacteria can bind to specific receptors on the leukocyte surface, ingestion then depending on the hydrophobicity of the bacterial surface (Silverblatt and Ofek, 1983). To overcome the problem of the same mechanism being involved in colonisation and

termination of infection, organisms adapt *in vivo* being heavily fimbriated in the urinary tract, but not in tissues and blood (Ofek and Beachey, 1980).

The phagocytic process on the expression of virulence in *S. marcescens* is important. Depression of phagocytic cell number or functions increases the susceptibility to infection (Simberkoff, 1980). Generally for efficient opsonisation and phagocytosis of *S. marcescens*, apart from natural or immune antibodies, complement activity is required (Traub, 1982b), both neutrophil granulocytes and complement being necessary in mice for murine defence against intraperitoneal infections (Traub et al, 1983a).

Traub (1981a) found that strains from all four human serum susceptibility categories (see section 1.5.3) were phagocytosed efficiently by human blood leukocytes. Previously, Simberkoff et al (1976b) showed that serum resistant *S. marcescens* cells were ingested and killed by phagocytes in normal human or rabbit sera, opsonins being generated via the alternative pathway and involving IgM globulins in the serum of healthy humans. Traub (1982b), however, found that natural IgG antibodies in combination with classically or alternatively activated complement were required for optimal opsonisation of *S. marcescens* cells, strains CDC O6:H3 and New CDC O14:H12 being the exception and proving refractory to IgG anti-O natural opsonins. O6 and O14 serotypes are known to be particularly virulent (Traub, 1982a) and this might be linked to resistance to opsonisation by IgG antibodies.

Although Traub and Simberkoff et al found that killing by phagocytosis was relatively complete, Miller and Buckler (1969) suggest a small number of *S. marcescens* cells can survive and multiply within the phagocytes.

Addition of IgG natural immunoglobulin preparations (see section 1.5.6), despite O agglutinin activity, do not seem to enhance combined phagocytic and complement killing by normal blood (Traub, 1983a) and do not passively protect mice from S. marcescens challenge (Traub, 1983b). Thus, it appears natural IgG antibodies have no beneficial use in patients with residual functional antibodies and complement, but may be of use in compromised patients.

1.5.5 Culture filtrates of S. marcescens - extracellular enzymes

Members of the Enterobacteriaceae usually do not secrete proteins into the surrounding medium. Excreted proteins either remain in the periplasmic space or are firmly integrated into the OM. For example, MacGregor et al (1979) found that an OMP of an E. coli strain was responsible for proteolytic activity controlling in vivo processing of the major OMPs. S. marcescens is one of the few exceptions that truly secretes a range of extracellular protein enzymes, including lipases, nucleases and proteases. Braun and Schmitz (1980) showed that proteases are excreted during log, but mostly stationary phase. They appear to be immediately secreted into the medium, no intracellular cell-bound proteases being detected by Schmitz and Braun (1985). Heller (1979b) found that the exoenzymes of S. marcescens can be purified with the OM, activity extracellularly increasing in the stationary phase, and that in the OM decreasing as they are released.

Concerning the role of extracellular enzymes as virulence factors, Traub and Kleber (1974) studied those produced by various clinical isolates of S. marcescens and although they showed fibrinolytic activity, there was no correlation with virulence. Lyerly and

Kreger (1979), however, isolated a pure metalloprotease of molecular weight 52.5 kDal, that caused tissue damage without the involvement of complement and leukocytes when introduced into rabbits by intracorneal, intratracheal and intradermal administration. Other reports include: corneal damage in S. marcescens keratitis due to a protease (Lyerly et al, 1981); purification of nucleases from a S. marcescens strain isolated from a patient with a corneal ulcer, and four proteases from the same strain that were immunologically non-identical of molecular weight 56 kDal, 60 kDal (metalloenzymes), 73 kDala and 73 kDalb (thioproteases) (Yonemura et al, 1983; Matsumoto et al, 1984). The 56 kDal protease was shown to cause vascular permeability enhancement followed by oedema formation when injected into guinea pig peripheral corneas and subconjunctival space (or skin), by directly activating the Hageman-factor-kallikrein-killin pathway in the tissue (Kamata et al, 1985); the production of pulmonary oedema and haemorrhage on the introduction of a protease into the lungs of experimental animals, and detection of the enzyme in lung tissue of animals dying with Serratia pneumonia (Lyerly and Kreger, 1983); and the isolation of an IgA protease that could possibly be a virulence factor cleaving IgA in respiratory and urogenital tract secretions (Milazzo and Delisle, 1984). So overall, Serratia proteases appear to be involved in pathogenesis of disease and to be true bacterial aggressins.

Although there is no evidence that Gram-negative bacteria enhance or circumvent the bactericidal action of serum in vivo by producing proteases, Ward et al (1973) showed that a S. marcescens proteinase could cleave complement component C3 resulting in leukotactic fragments inducing chemotaxis of PMNs. Doerr and Traub (1982) then found a protease that attacked complement components C3,

C4, C5 and C9, but they regarded their results as tentative because human serum was used and Miyata et al (1981) has shown that plasma contains a protease inhibitor, α_2 macroglobulin. The enzyme becomes trapped in its matrix, enzyme activity then being hindered because of the enclosed environment. Cooper et al (1981) also found that proteases could attack the surface of fixed and fresh washed human platelets. It appears that only some proteases are true virulence factors, Traub et al (1983a) finding that one particular S. marcescens isolate had protease-independent virulence and did not give rise to guinea pig keratoconjunctivitis.

Lastly, Roig et al (1979) showed that S. marcescens produces two extracellular antigens, one identified as the LPS specific for each serotype, the other not identified, but carried by all strains of the genus Serratia and no other Gram-negative bacteria.

1.5.6 Natural antibodies, specific defence mechanisms and vaccines

Infection or vaccination induces the stimulation of specific host defence mechanisms with the formation of antibodies that can interact with bacterial antigens, allowing rapid removal of the cells from the circulation by various methods. The first antibodies synthesised are of subclass IgM, secondary responses being of IgG which function in opsonisation and complement mediated bactericidal killing. IgA antibodies are found in mucosal secretions and might function in inhibiting adhesion. IgM antibodies can fix complement and are the first line of defence in bacteremia, being too big to penetrate tissues (Penn, 1983).

The microflora of the intestine in humans provides the most continuous contact with antigens and induces the formation of 'natural' antibodies in the blood of low titre. These may then cross-react in low affinity with many antigens of pathogenic organisms (Penn, 1983). Traub (1981c) showed that a surprising number of adults had significant agglutinin titres to various O antigens of S. marcescens, suggesting that either they had clinical or sub-clinical infections, or numerous cross-reactions with Gram-negative bacteria occur. Griffiths et al (1977) found that the background level of antibodies to 130 antigens had not changed in the general population from 1951 to 1975, suggesting that in America at least the public were not more exposed to S. marcescens antigens. They also studies patients with malignant disease to show that some could respond immunologically to S. marcescens despite the underlying condition. It is not known how long anti-O antibodies persist in humans, and it is assumed that O and H antigens induce the formation of antibodies only when invasive forms of S. marcescens are present

(Freitag and Caselitz, 1975), although their absence does not necessarily mean the strain is clinically insignificant (Rubin, 1980).

Immunisation, either passive or active, to S. marcescens could be of beneficial use especially when the strains involved are multiply drug resistant (see section 1.6). Whole attenuated cells or extracted surface components of S. marcescens have been used and studied for their effectiveness in experimental animals. Simberkoff et al (1976a) showed that animals can be protected from lethal intra-peritoneal infections with bacteria suspended in 5% hog mucin, either by active or passive immunisation. Mice could also be actively immunised by exposure to whole heat-killed bacteria or hot phenol-water (LPS) extracts. Immunity was O specific, even when strains with the same H antigens were used for immunisation and challenge. Passive protection by administration of cell-free sera from animals immunised with the challenge O serotype was also found protective, sera containing high titres of complement-independent opsonising antibody (IgG) and O-specific haemagglutinating antibody (largely IgM). Traub (1983b) studied passive protection of NMRI mice to intra-peritoneal challenge with several strains of S. marcescens, using commercially available human IgG preparations (natural antibodies), selected anti-H-K-O-protease preparations and anti-live cell rabbit immune sera. He found the natural, anti-K, and anti-protease antibody preparations did not give passive protection. However, anti-O and anti-H antibodies did give protection with homologous challenge strains, anti-O failing to protect with heterologous strains. The rabbit immune sera gave excellent protection because of antibodies to the O antigens. Traub et al (1985) then studied active immunisation of mice with phenol-water

extracted LPS, K antigen extracts and isolated metalloproteases. The O and K antigens from the more virulent O14 and O6/O14 S. marcescens strains were good immunogens, protecting mice against intraperitoneal challenge with homologous strains and affording modest cross-protection. The metalloproteases of strain O6/O14 effected active immunisation, but there are problems associated with the exploitation of proteases as protective antigens. Not all strains produce them, and it is unknown if they would cross-react or even be immunogenic in some cases. Traub concludes that the search for a vaccine should begin with protective antigens from separated and well characterised LPS fractions of representative strains of S. marcescens, particularly those with the O antigens O6 and/or O14. Monoclonal antibodies to the O side chains of LPS confer protection against homologous challenge with E. coli and Salmonella (Kirkland and Ziegler, 1984; Colwell et al, 1984) and this is an area that could perhaps be exploited in the future for Serratia.

Other studies show that anti-protease antibodies can give full or partial protection from Serratia pneumonia in experimental animals (Lyerly et al, 1983). Laforce and Boose (1980) found that macrophage activation, PMN recruitment and antibody enhancement occurred on aerosol immunisation of mice, that heightened lung antibacterial activity to Serratia challenge.

Common antigens within Gram-negative bacteria can be exploited for protection against heterologous challenge. The lipid A/core regions of many bacteria have similar structures so that antigenic determinants are shared between species providing the possibility of cross-reactions. For example, Perez-Perez et al (1986) raised antisera to Campylobacter strains and found they shared lipid A determinants with the core region of LPS from several other Gram-

negative organisms. Salmonella minnesota Re595 has its core glycolipid exposed and has been used in active and passive immunisation studies against lethal challenge with other bacteria including Serratia. Young et al (1975) found protection was afforded, but it was limited, especially when serum-resistant heterologous strains were used. The problem is that antibodies to lipid A or the inner core of LPS are probably poorly accessible to their binding sites in whole living cells. Thus these antibodies do not prevent bacterial invasion in infection, but do neutralise free endotoxin shed from the cells, protecting against lethal sepsis (Mattsby-Baltzer and Alving, 1984). Kuhn et al (1981) suggested that enterobacterial common antigen complexed with protein may be a good vaccine. However, Peters et al (1985) found that monoclonal antibodies to ECA did not generally react with smooth and/or encapsulated whole cells, indicating the antigen is not accessible to antibodies. This suggests these common envelope structures may not be useful antigens for exploitation as vaccines or producing antibodies for therapy. Homma et al (1977) showed that active immunisation with heat-killed S. marcescens or Ps. aeruginosa gave heterologous and homologous protective activity in animals, suggesting the presence of common antigens among Enterobacteriaceae and Pseudomonas. Further work is required to determine the exact usefulness of these common antigens as vaccines.

Other surface components have been tested for their potential use as vaccines in Gram-negative bacteria and could be borne in mind when searching for a Serratia vaccine. E. coli and Gonococci fimbriae were found to be fairly immunogenic and effective as vaccines, blocking adhesion and enhancing phagocytosis (Korhonen and Rhen, 1982). Capsular antigens of E. coli can give rise to

protective antibodies (Kaijser and Ahlstedt, 1977) and the high molecular weight polysaccharide slime of Ps. aeruginosa has been shown to protect mice from burns infections (Pollack et al, 1984). Similarly, Cryz et al (1984) purified the capsular antigen of a K. aerogenes strain and found it was immunogenic in mice, protecting against fatal bound wound sepsis with the homologous organism. Studies on the capsular antigens of Serratia can only really begin in earnest when the K antigens have been fully characterised. OMPs might act as protective antigens. The purified porins of Ps. aeruginosa were found to elicit a specific humoral antibody response in immunised animals (Gilleland Jr et al, 1984). Chart and Griffiths (1985a), using polyclonal antibodies to the ferric-enterochelin receptor (81 kDal), found that the protein was conserved among many strains of E. coli and also in S. typhimurium and K. aerogenes (83 kDal). Cross-protection may therefore be possible if IRMPs are used as immunogens. Lastly, the extracellular antigen discovered by Roig et al (1979, 1983), which the workers exploited in a coagglutination test, might have potential for a vaccine, being immunogenic and carried by all strains of S. marcescens, but no other Gram-negative organism. Gulig et al (1984) isolated two proteins in the culture fluid after growth of H. influenzae type b. One was also associated with the cell surface (protein 1) whereas the other was only found in the medium (protein II). Both were immunogenic and the workers suggest they could be used in vaccine development. Antibodies to protein 1 could function as opsonising or bactericidal antibodies. Antibodies to protein II could neutralise its function, protecting in infection if it is involved in growth. Further work is required to see if the Serratia extracellular antigen would provide a useful vaccine.

1.6 RESISTANCE TO ANTIMICROBIAL DRUGS

The OM allows the uptake of nutrients and at the same time functions as a permeability barrier preventing the penetration of many antimicrobial agents (Lambert, 1983). Wild strains of *E. coli* are naturally sensitive to low Mr hydrophilic compounds, eg ampicillin, cephalothin, which enter the cells via porin channels regardless of whether the strains have complete or defective LPS side chains. Hydrophobic molecules (eg rifampicin, erythromycin) enter cells by diffusion across the OM, smooth strains being resistant to these molecules, the O-side chains acting as a barrier and protecting hydrophobic patches (eg phospholipids) on the surface (Nikaido, 1979). Most antibiotics are small hydrophilic molecules, rate of passage through the porin channels depending on size, hydrophobicity, charge and availability (Lambert, 1983). Sawai et al (1982) found that porin deficient mutants of *E. coli* had an increased resistance to cephalosporins. Other factors affecting antibiotic penetration include nutrient and growth conditions, known to alter the OM composition and capsule production. The exact role of the latter is unknown. The presence of a capsule and O antigen in *K. aerogenes* does not seem to affect the response of the cells to low molecular weight, charged, hydrophobic or hydrophilic antibiotics (Williams et al, 1986). In *Ps. aeruginosa*, the negatively charged exopolysaccharides may impede the passage of cationic compounds (Slack and Nichols, 1982). The trapping of antibiotics within the capsule, however, could lead to a high concentration around the cells which might then be available for transport into the cells if any subsequent dissociation of bound antibiotic should occur.

One factor of major significance elevating *S. marcescens* to its

present status as an important nosocomial pathogen, is its ability to be multiply resistant to many antibiotics and rapidly acquire resistance to new antimicrobial agents as they emerge (Farrar Jr, 1980; Fernandes et al, 1983). It appears to be naturally resistant to some antibiotics, possibly because of the barrier function of the OM. Heller (1979c) showed that changes in carbohydrate and lipid composition altered hydrophobic and hydrophilic interactions in the OM and were responsible for permeation or exclusion of antibacterial agents. Gutmann and Chabbert (1984) found multiple mechanisms were involved in resistance of S. marcescens to moxalactam, a reduced permeability barrier being associated with modification of OMPs, and an increase in β-lactamase production and alteration of penicillin binding proteins also occurring. A decrease in the permeability of the cell envelope due to a reduced amount of a porin protein can give a broad spectrum of resistance to many antibiotics. A clinical isolate of S. marcescens was resistant to both cefotaxime and amikacin by this one-step resistance mechanism (Goldstein et al, 1983), and mutants of Klebsiella, Enterobacter and Serratia were cross-resistant to nalidixic acid, trimethoprim and chloramphenicol by an identical process (Gutmann et al, 1985).

Resistance to penicillins and cephalosporins is often associated with β-lactamases, most strains producing small amounts of inducible enzymes which hydrolyse cephalosporins but not penicillins, the genes involved being chromosomally located. Strains resistant to ampicillin have, in addition, a broad spectrum β-lactamase that is not inducible and possibly associated with a plasmid (Farrar Jr and O'Dell, 1976). Heller (1979b) showed that penicillinase can be co-purified with the OM of S. marcescens, perhaps a contributing factor in the resistance to β-lactam antibiotics. When enzyme production

is not the primary resistance mechanism, surface lipid content and the barrier function of LPS may be involved (Tsang et al, 1975), Hiruma et al (1984) finding that the LPS polysaccharide chain contributes to the barrier preventing permeation of hydrophobic β-lactams.

S. marcescens was originally sensitive to aminoglycosides, especially gentamicin, but resistance has been acquired through high use (Yu et al, 1979; Farrar Jr, 1980; Lewis et al, 1983). Ruhen and Wetherall (1983) found that a strain of S. marcescens isolated from the blood of a patient receiving antibiotics exhibited three unstable characteristics - resistance to aminoglycosides, colony size and pigment production. The first two were related, small colonies generally being more resistant to aminoglycosides, reversion to large sensitive colonies occurring spontaneously. Polk et al (1983) found that aminoglycosides given systemically can penetrate burn eschar, but drug-resistant S. marcescens infecting the wound can persist, causing superinfection or bacteremia. Gentamicin-resistant strains can possess aminoglycoside acetyltransferase (AAC) or aminoglycoside adenylase enzymes (Maes, 1985), Lewis et al (1983) isolating an AAC enzyme from a netilmicin resistant strain that was epidemic in a special care baby unit. Most gentamicin resistant strains are susceptible to amikacin (Meyer et al, 1976; Traub et al, 1976; Markowitz and Sibilla, 1980), although resistance to this has been reported (John et al, 1982; Traub et al, 1983b) and will increase with use. Arroyo et al (1981) found that an amikacin-resistant S. marcescens strain caused urinary tract infections in hospitalised patients, even when the patients had received no prior administration of amikacin or other aminoglycosides. The chronically infected patients acted as the reservoir of infection, transmission to others

then occurring. Resistance to amikacin is associated with AAC enzymes, a new type acetylating amikacin at the 6'-amino group recently being described in S. marcescens (Morohoshi et al, 1984).

S. marcescens strains that are particularly resistant to aminoglycosides often call for the use of second or third generation cephalosporins (Neu, 1984). The latter are less toxic than aminoglycosides with fewer side effects, Bergin et al (1985) showing ceftazidime is effective in treating severe soft tissue infections due to S. marcescens and Ps. aeruginosa. However, monitoring is required to make sure resistance does not develop, plasmid-borne resistance determinants to newer types of cephalosporins already being found in one strain (Knothe et al, 1983).

Various combinations of antibiotics have demonstrated synergy against a high proportion of strains (Gray et al, 1978; Traub and Fukushima, 1979d; Markowitz and Sibilla, 1980) but none have been predictable enough to use in serious infections (Farrar Jr, 1980). Maslow et al (1985) suggest that Serratia and Pseudomonas strains resistant to aminoglycosides and cephalosporins might respond to the combination of amikacin and cefotaxime that acts synergistically in vitro to give a good response to these multiply-resistant species. Aztreonam (a monobactam antibiotic), either alone or in combination with amikacin, may also be useful in treating infections by aminoglycoside-resistant S. marcescens and Ps. aeruginosa strains, especially in neutropenic patients (van Laethem et al, 1984).

Plasmid mediated against chromosomal resistance remains to be defined in S. marcescens. Plasmids carrying resistance determinants to ampicillin, chloramphenicol and tetracyclines have all been described (John Jr and McNeill, 1981; Hedges, 1980). Miller and Bevill (1983) report cephalosporin resistance is not associated with

a plasmid, but Knothe et al (1983) showed that one S. marcescens strain transferred determinants to newer types of cephalosporins and concluded the genes involved were plasmid-located. Most papers suggest aminoglycoside resistance is plasmid-mediated (Miller and Bevill, 1983; John Jr and McNeill, 1981; Mendoza et al, 1984), John et al (1982) putting forward the idea of a chromosomal locus co-existing with plasmid-mediated genes for manufacture of modifying enzymes in some strains.

Transfer of plasmids from S. marcescens to E. coli is reported to be inefficient in vitro, non-transferring genes being carried on autonomous plasmids that are not self transmissible (Hedges, 1980). It was suggested that because Serratia rarely colonises the gut, where transfer often occurs, it is not adapted to the process (Hedges, 1980). However, Alvarez and Regueiro (1980) found that S. marcescens could transfer R factors to E. coli, mediating resistance to ampicillin, some aminoglycosides, and chloramphenicol, similar results also being shown by Bhujwala et al (1984). Olexy et al (1982) even reported plasmid transfer from S. marcescens to Ps. aeruginosa and Rubens et al (1981) discovered plasmids containing a common transposable DNA sequence for gentamicin resistance were present in outbreaks of infections caused by S. marcescens and Ps. aeruginosa in one hospital over a 5 year period. Schaberg et al (1977) found that in urine, R plasmids were transferred from multiply-drug resistant S. marcescens strains to E. coli, suggesting that the urinary catheter collection bag may be a potential site for resistance transfer among Enterobacteriaceae, especially for Serratia which rarely colonises the gut. The same was reported to occur in urine, faeces, blood, plasma and ascitic fluid by Mendez et al (1982), this process allowing partially resistant strains to become

very resistant and prevalent in the hospital environment.

Virulence associated with the presence of plasmids in S. marcescens is not well documented. Meyer et al (1976) found that strains resistant to gentamicin were as virulent as sensitive strains, and Traub et al (1983a) showed that variants of a multiple drug resistant isolate which had lost part or all of the R plasmid, were as virulent for mice as the parent strain. So there does not seem to be a virulence link with R factors coding for antibiotic resistance.

Lastly, antibiotics may also have an effect on adherence and phagocytosis, sub-lethal concentrations of antimicrobial drugs altering the ability of some bacteria to adhere to epithelial cells and possibly phagocytes (Ofek and Beachey, 1980). In general, aminoglycosides can decrease chemotaxis, and β-lactam exposed E. coli are more susceptible to phagocytosis (Milatovic, 1983). However, Lorian and Atkinson (1979) found sub-minimal inhibitory concentrations of mecillinam did not significantly enhance or reduce the effect of serum and phagocytes on the killing of S. marcescens, and Traub (1982c) found sub-inhibitory and inhibitory concentrations of aminoglycosides significantly enhanced the combined phagocytic/serum bactericidal activity of human blood against S. marcescens strains.

1.7 AIMS OF THE PROJECT

The aim of the project was to explore the immunochemistry of *S. marcescens*, trying to relate its serology and its interaction with host defence mechanisms to its immunochemical and chemical composition. Considering the problems involved with cross-reactions in serotyping (Pitt and Erdman, 1984), possible sub-divisions, or a totally new typing method based on the immunochemical findings were borne in mind. A further objective was to identify possible antigens as candidates for exploitation as broad spectrum protective vaccines. One of the first line host defence mechanisms is the complement system. Simberkoff (1980) suggested one reason for the rapid rise in *S. marcescens* infections was its ability to resist serum killing. Therefore, another aim was to explore whether resistance or sensitivity to complement could be related to its surface composition.

A majority of clinical isolates are serotype O14:H12. *S. marcescens* New CDC O14:H12 was therefore chosen as a reference strain to determine the protein, lipopolysaccharide and antigenic profiles of its outer membranes, whole cells and isolated LPS, using the techniques of SDS-PAGE and immunoblotting. A range of other serotypes and strains were then used for comparisons and to investigate any cross-reactions.

Growth media were chosen to investigate the effects of iron on the profiles, bacteria being shown to grow under conditions of iron-restriction *in vivo* (Brown and Williams, 1985). Tryptone soy broth (TSB) represented a laboratory medium which was iron-plentiful, whereas horse serum simulated a bacteremia, containing transferrin and being naturally iron-restrictive. Chemically defined medium with

and without additional iron was used to specifically determine the effects of iron.

Cells were grown to log phase, defined as two generations before the onset of stationary phase. Williams et al (1984) found that K. aerogenes rapidly responded to extracellular iron concentrations so that expression of IRMPs occurred early in log phase before the cells were truly iron depleted. It was considered that stationary phase would have been the best to observe the full effects on the outer membranes and whole cells of iron-restriction, but the main concern of this project was to try to mimic in vivo infections and conditions as closely as possible. As opposed to stationary phase cells, log phase cells are young, growing, virulent and in this state overcoming host defence mechanisms when acute infections originally occur.

Having established the total antigenic profiles, the antigens exposed on the surface and accessible to antibodies were investigated for whole cells of New CDC 014:H12 using an immunoabsorption technique. It was also decided to explore the relationship between the whole cell, outer membrane and soluble (extracellular) antigens of a range of serotypes of S. marcescens, using a variety of immunochemical techniques, with an aim to establishing the identity of the Serratia specific extracellular antigen discovered by Roig et al (1979).

The problems of cross-reactions in serotyping were investigated by using three 014:H12 serotypes of S. marcescens. Any differences in their surface proteins were explored using autoradiography after SDS-PAGE of ^{125}I-labelled whole cells. Differences in their response to antibiotics and serum killing were also tested. Two of the 014:H12 strains were found to have opposite reactions to the bactericidal effect of the complement. The relationship between their surface composition and sensitivity to serum was studied by a variety of chemical and immunochemical techniques.

2. MATERIALS, METHODS AND PRELIMINARY RESULTS

2.1 MATERIALS

2.1.1 Bacteria

The strains used were: New CDC O14:H12, CDC O3:H1, CDC O6:H3, SF133 O3:H12 (obtained from Dr W H Traub, Institut für Hygiene und Mikrobiologie, Universität des Saarlandes, Homburg-Saar, West Germany); 4444-60 O14:H12 (reference strain used in serotyping), 1783-57 O14:H9, 874-57 O14:H12, S818 O14:H12, S1220 O14:H12 (Oxford - adult urinary tract isolate), O1, O2, O3, O4, O5, O6, O7, O8, O9, O10, O11, O12, O13, O15 reference strains used in serotyping (obtained from Dr T L Pitt, PHLS, Colidale, London); 6358, 6380I, 6380II, 9623, 10682, 14715, 14815, 16191, 32201 (clinical isolates obtained from Mr G Domingue, Newcastle-upon-Tyne Freeman Hospital, Newcastle). The organisms were all non-pigmented and maintained on nutrient agar slopes (Oxoid Ltd, Southwark, London) at 4 °C and subcultured monthly.

For comparisons Escherichia coli GH126, Klebsiella aerogenes NCTC 5055, Pseudomonas aeruginosa NCTC 6750 and PAOI, and Proteus mirabilis 196 were used (obtained from the Microbiology Research Group culture collection, Department of Pharmaceutical Sciences, Aston University).

2.1.2 Media

Complex media used were: tryptone soy broth, TSB (Lab M, Salford, Manchester) made up according to manufacturer's instructions; heat-inactivated horse serum (Gibco, Paisley, Glasgow) diluted 1:1 with 0.9% w/v NaCl, and the same medium containing additional iron (10^{-4} M $FeSO_4.7H_2O$). For antibiotic sensitivity testing, nutrient agar (Lab M, Salford, Manchester) or diagnostic

sensitivity test agar (DST agar, Oxoid Ltd, Basingstoke, Hants) were used.

Low iron chemically defined media (CDMFe-) contained (per litre double distilled water): $K_2HPO_4.3H_2O$, 3.93 g; KH_2PO_4, 1.0 g; NH_4Cl, 5.0 g; Na_2SO_4, 2.0 g; $MgSO_4.7H_2O$, 0.1 g; glucose, 5.0 g; vitamins (nicotinic acid 50 μg, Ca-d-pantothenate 50 μg, thiamine 50 μg, biotin 0.3 μg); trace elements (see below) 1.0 ml; pH 7.4. Chemically defined media with added iron (CDMFe+) contained 0.5 mg/l of $FeSO_4.7H_2O$.

Trace element solution contained (per 500 ml) $CaCl_2.6H_2O$, 54.77 mg; H_3BO_3, 15.46 mg; $COCl_2.6H_2O$, 5.95 mg; $CuSO_4.5H_2O$, 1.25 mg; $ZnSO_4.7H_2O$, 14.5 mg; $MnSO_4.7H_2O$, 1.153 mg; $(NH_4)_6MO_7O_{29}.4H_2O$, 3.1 mg.

All media, apart from the horse serum and vitamin solution (the latter being sterilised by filtration through a 0.22 μm filter), were sterilised by autoclaving at 121°C for 15 minutes.

2.1.3 Chemicals

The chemicals used were of Analar grade or equivalent obtained from British Drug Houses (BDH) Chemicals Ltd, Poole, Dorset, or Fisons Laboratory Reagents, Loughborough, Leics, or Sigma Chemical Company, Poole, Dorset.

For immunoprecipitation techniques Agarose M and Tris-barbiturate buffer sachets were obtained from LKB Ltd, Croydon, Surrey. Each sachet contained diethylbarbituric acid 2.24 g, tris 4.43 g, calcium lactate 0.053 g, sodium azide 0.065 g, which when reconstituted to 500 ml with distilled water gave Tris-barbiturate buffer pH 8.6.

Antibiotic sensitivity was undertaken using Neosensitab antibiotic discs (A S Rosco, Tarstrup, Denmark), and MIC and

antibiotic agar assays were performed using amikacin (Mead Johnson Labs, Langley, Slough), chlorhexidine (chlorhexidine gluconate solution BP 20% w/v, Hibitane, ICI Ltd, Macclesfield, Cheshire) and benzlkonium chloride (benzalkonium chloride solution BP, MacCarthys, Romford, Essex).

2.1.4 Equipment

Equipment and apparatus used in this study and not specified in the text was supplied by:-

Automatic pipettes - Gilson Pipetman, P-200 and P-1000, Anachem, Luton, Beds.

Balances - Oertling HC22, Oertling, Orpington, Kent.
Sartorius Type 1702, Sartorius Instruments Ltd, Belmont, Surrey.

Blender - Kenwood Model A524 blender, Fisons Scientific Apparatus, Loughborough, Leics.

Blood collecting tubes - Polypropylene stoppered tubes, Sterilin Ltd Teddington, Middlesex.

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 rotar head ultracentrifuge, Measuring and Scientific Equipment, Crawley, Surrey.

Freeze dryer - Edwards Modylo freeze dryer, Edwards High Vacuum Ltd, Crawley, Surrey.

French press - Amicon Corp, Gloucester, Glos.

Gamma counter - ICN Gamma Set 500, ICN Tracerlab Division, Cleveland, USA.

Gas liquid chromatography equipment - Chromatograph series 204 attached to PM 8222 dual-pen recorder, Philips/Pye-Unicam, Cambridge, Cambs.

Gel electrophoresis apparatus - made in house by Aston services.

Gel dryer - Bio-Rad model 224 gel slab drier, Bio-Rad Laboratories Ltd, Watford, Herts.

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 - Mickle reciprocating water bath, Cam Lab Ltd, Cambridge. Gallenkamp orbital shaking incubator, Gallenkamp, London.

Laser densitometer - Ultroscan Laser Densitometer LKB 2202, LKB Instruments Ltd, Croydon, Surrey, run by an Apple II Europlus computer (Gelscan software).

Membrane filters - Gelman Acrodisc, Gelman Sciences, Brackmills, Northampton.

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 pack - Bio-Rad Model 500/200 (electrophoresis); Bio-Rad model 250/2.5 (blotting); Bio-Rad Laboratories Ltd, Watford, Herts.

Rotary evaporator - Buchi Rotavapor-R, Fisons Scientific Apparatus, Loughborough, Leics.

Sealable tubes - Screw top culture tubes with teflon lined caps, Sterilin, Teddington, Middlesex.

Sonicator - Daive Soniprobe, Dawe Instruments Ltd, Acton, London.

Spectrophotometers - 1) Unicam SP600, Pye-Unicam Instruments, Cambridge, Cambs.

2) Cecil CE 292 Digital, Cecil Instruments, Cambridge. Plastic cuvettes for spectrophotometry, Brand, Gallenkamp, Loughborough, Leics.

3) Scanning UV Spectrophotometer, Unicam 8000, Pye-Unicam Instruments, Cambridge, Cambs. Quartz cuvettes for UV spectrophotometry, Hellma, Westcliff-on-Sea, Essex.

Syringes - Gillette Surgical, Isleworth, Middlesex.

UV Lamp - Hanovia Lamps, Slough, Berks.

Water cooler - Cooling Unit, Grant Instruments Ltd, Cambridge, Cambs.

Whirlimixer - Fisons Scientific Apparatus, Loughborough, Leics.

2.1.5 Serum

To prepare hyper-immune rabbit antisera, the method of Driver and Lambert (1984) was used. Whole cells of bacteria grown to log or stationary phase in either TSB, diluted horse serum, CDMFe- or CDMFe+, were harvested by centrifugation (5000 g, 10 minutes, 4° C), washed twice with 0.9% w/v NaCl and resuspended in saline to an OD_{470} of 1.0 (10^9 organisms/ml). The cells were killed by exposure to UV irradiation (λ 254 nm, distance 10 cm) for 5 minutes, this method

being used to maintain the antigenicity of the surface components. Half-lop rabbits (Ranch Rabbits, Crawley Down, Sussex) were immunised with 1 ml of the UV-treated cells, or 1 ml of a 1 mg/ml solution of soluble antigens (see section 2.2.2.5), intradermally at weekly intervals for 4 weeks. The rabbits were bled from the marginal ear vein at weekly intervals over the following 4 weeks, with intervening weekly booster injections, and then bled out by cardiac puncture. After clotting at 37 °C for 30 minutes, serum was separated from the blood by slow centrifugation (3000 rpm, 10 minutes, room temperature), samples being pooled and stored at -20 °C.

Normal pooled human serum (NPHS) was obtained from 6 healthy volunteers (3 female, 3 male, aged 22-47). Blood taken by venipuncture was allowed to clot (37 °C, 30 minutes) and the serum separated by centrifugation (3000 rpm, 10 minutes), samples being pooled and stored in 1 ml aliquots at -20 °C. Serum from guinea-pig blood was obtained by the same method (GPS).

Serum heated to 56 °C for 30 minutes to destroy complement was known as heat-inactivated.

To absorb goat-anti-human C3 antiserum (Sigma), whole cells of 4444-60 grown to log phase in TSB (100 ml) were harvested by centrifugation (10,000 rpm, 10 minutes), washed, and resuspended in 0.9% w/v NaCl to 0.5 ml. The cell suspension was added to 2 ml of the antiserum and after mixing for 1 hour at 4 °C, the cells were removed by centrifugation (10,000 rpm, 30 minutes, 4 °C) and the absorbed supernatant stored at -20 °C in 100 µl aliquots.

2.1.6 Glassware

Glassware was Pyrex brand (Corning Glass Ltd, Sunderland, Tyne and Wear). For cleaning, it was immersed in 5% w/v Extran 300 (BDH) at room temperature for 12 hours. It was then rinsed once in distilled water, once in 1% v/v hydrochloric acid, six times in distilled water and finally twice in double distilled water. Glassware was dried at 60 °C, covered with aluminium foil and sterilised by dry heat at 160 °C for 3 hours.

2.2 METHODS AND PRELIMINARY RESULTS

2.2.1 Growth experiments

2.2.1.1 Growth conditions

Cells were grown at 37 °C with agitation at 150 rpm in an orbital shaker, or in a mickle reciprocating water bath at 100 strokes/minute. A sample of cells grown in CDMFe- was scanned from wavelength 600 to 300 nm with a spectrophotometer. The trace (Fig 2.1) shows that there was no pigment produced by the cells that could interfere with the measurement of growth by optical density (OD) readings. This was assumed to be true for all strains grown in all media. A wavelength of 470 nm was chosen to monitor subsequent growth curves, an OD of 1.0 at 470 nm indicating a concentration of $\sim 10^9$ cells/ml.

2.2.1.2 Growth measurements

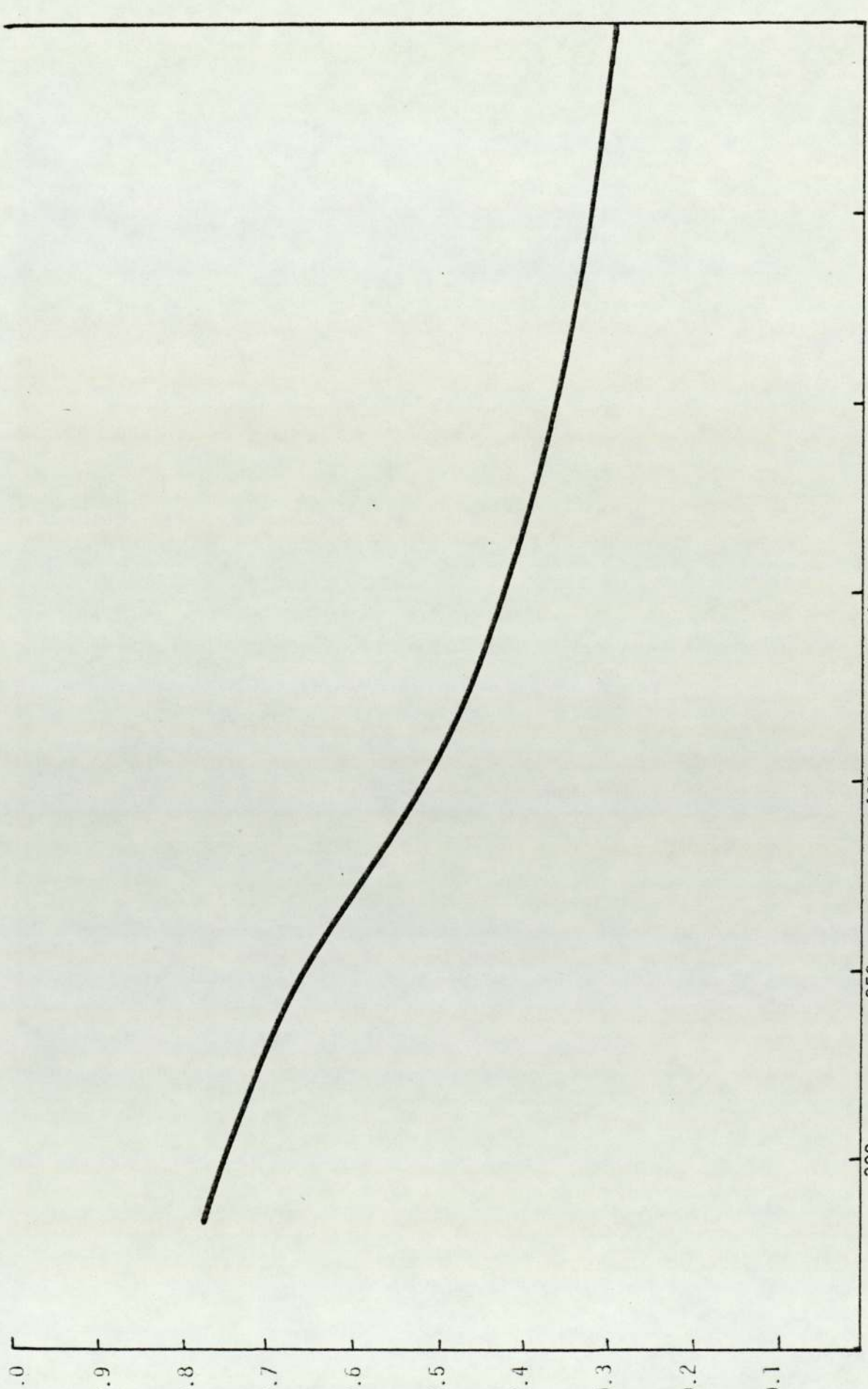
Growth was followed by measuring changes in the optical density of the medium with time. At low cell concentrations the OD is directly proportional to cell concentration (Kenward, 1975). This relationship is expressed by the Beer-Lambert law

$$OD \propto \log(Io/I)$$

where Io = incident light and I = emergent light. The relationship obeys the Beer-Lambert law up to an OD of 0.3 (Kenward, 1975). Above this absorbance the OD increases less than proportionally with increasing cell concentration due to secondary scattering of light. However, if the cell suspension is diluted with fresh media to an absorbance less than 0.3, proportionality is restored.

Media pre-warmed to 37 °C were inoculated with an overnight

Figure 2.1 Scanning Spectrophotometer Recording of S. marcescens New CDC 014:H12 cells in water ($OD_{470} \sim 0.2$)



culture of New CDC O14:H12, grown in the corresponding media, to give an OD_{470} of ~0.01. Growth was followed by aseptically taking samples for absorbance readings every 30 minutes, dilutions being made when the OD_{470} was more than 0.3. Comparison of time vs log OD_{470} curves (Figs 2.2a and b) show that growth is reduced in iron-restricted media, although growth rate is similar to that in the equivalent iron-plentiful media.

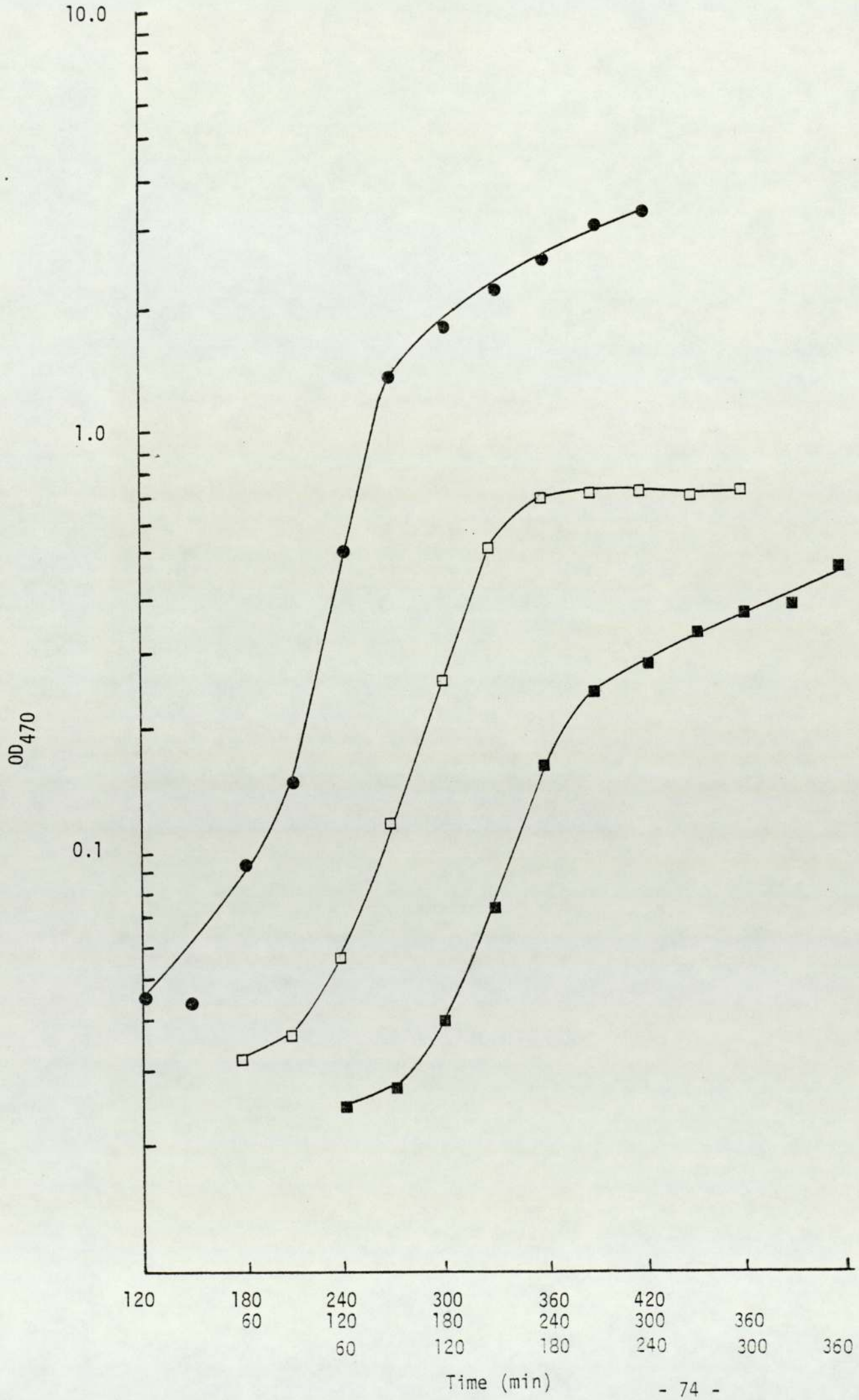
In future work cells were either grown to log phase or stationary phase. Log phase was defined as the point on the curve that was two generations before onset of stationary phase (OD_{470} = 0.3 for TSB; 0.16, diluted horse serum with iron; 0.15, diluted horse serum; 0.5, CDMFe+; 0.4, CDMFe-). Stationary phase was taken as 18 hours' growth from inoculation.

2.2.2 Preparative techniques

2.2.2.1 Isolation of outer membranes

OMs were isolated using (1) a modification of the method developed by Tsang et al, 1976 (Fig 2.3), or (2) by the technique of Filip et al, 1973, using 2% w/v sodium-N-lauroyl sarcosinate (Sarkosyl, Sigma).

In the second method, cells were harvested (10,000 rpm, 10 min, 4 °C), washed with 0.9% w/v NaCl, and resuspended in 9 ml distilled water. After sonication for two 1 minute bursts in an ice bath, with a 30 second interval to allow for cooling inbetween the bursts, 1 ml of 20% w/v Sarkosyl was added. Following incubation for 30 minutes at room temperature, the solution was centrifuged (5,000 rpm for 10 min) to remove unbroken cells. The supernatant containing solubilised cytoplasmic membrane but intact OM was then centrifuged


10.0
1.0
0.1
OD_{470}
120
180
60
240
120
60
300
180
120
360
240
180
420
300
240
360
300
360
Time (min)

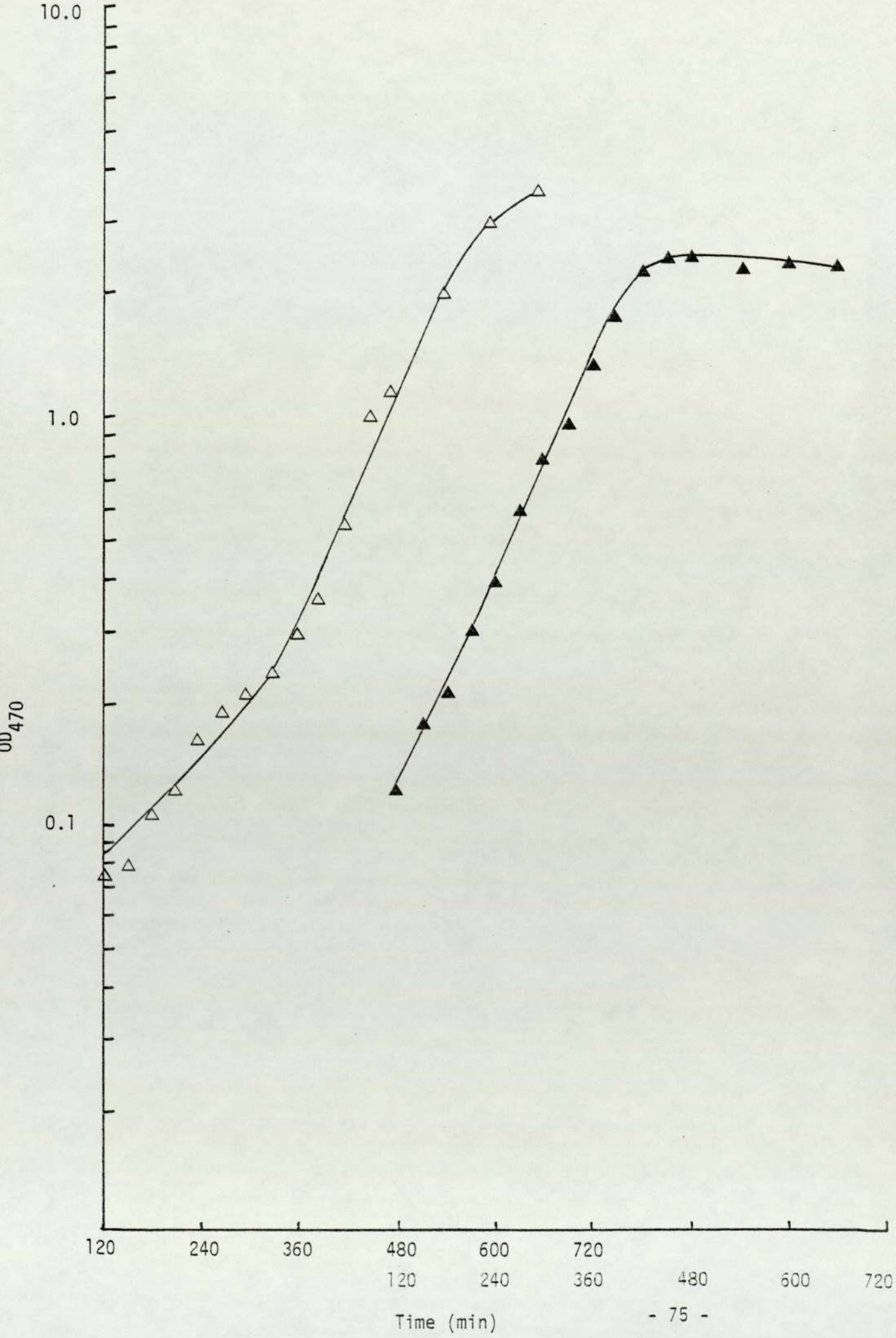
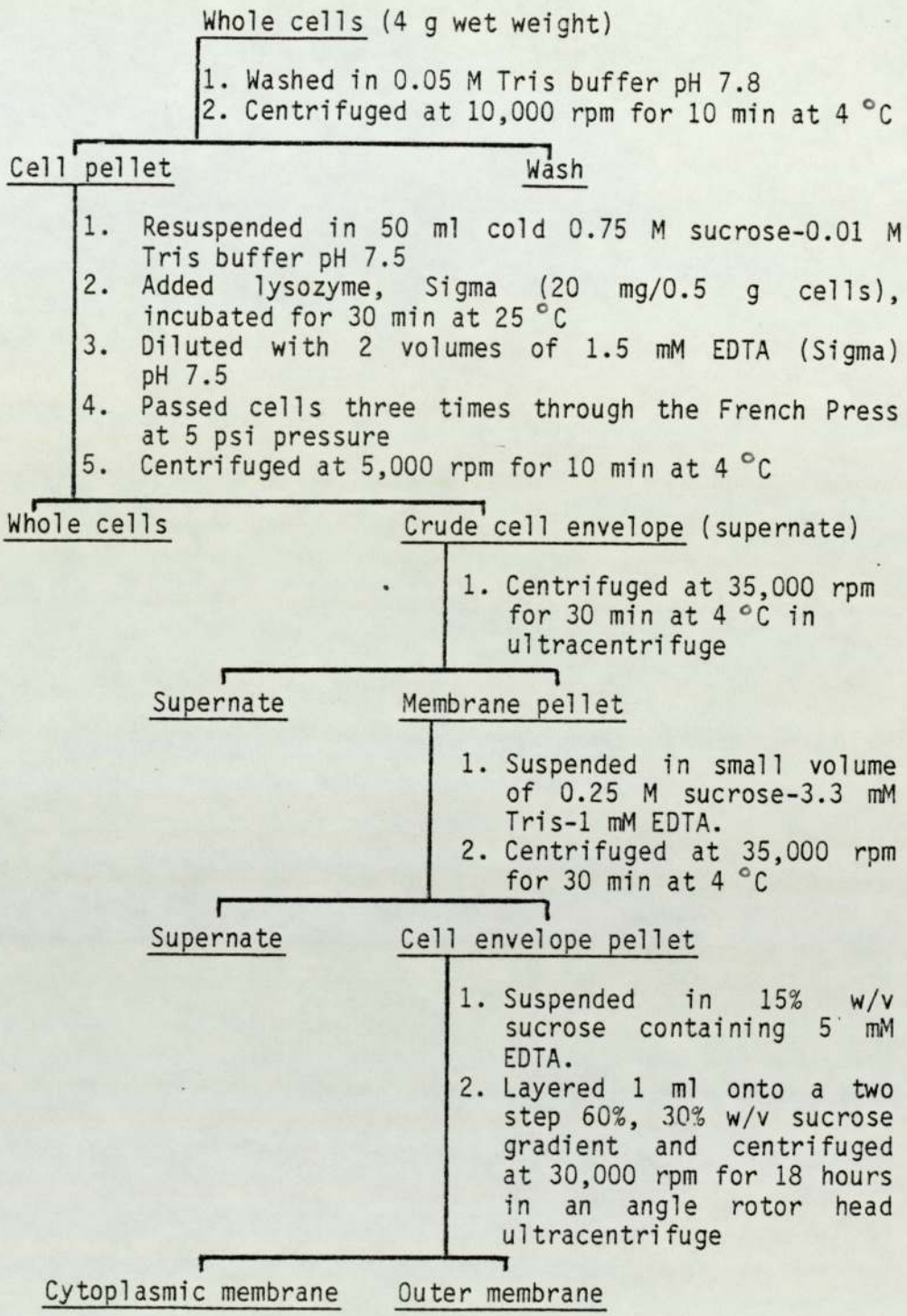

10.0
1.0
0.1
OD_{470}
120
240
360
480
600
720
120
240
360
480
600
720
Time (min)

Figure 2.3

Modified Procedure of Tsang et al (1976) for Isolation of Outer Membranes from S. marcescens



at 20,000 rpm for 1 hour at 4 °C. The resulting pellet of OMs was washed, resuspended in 0.5 ml of distilled water, and stored at -20 °C.

2.2.2.2 Isolation of lipopolysaccharide

The hot phenol-water technique of Westphal and Jann (1965) was used. A 2L culture of bacteria grown in TSB to log phase, harvested (10,000 g, 15 mins, 4 °C) and washed, was suspended in 50 ml distilled water and warmed to 65 °C. An equal volume of 90% w/v phenol (Fisons) at the same temperature was then added and the mixture stirred for 5 minutes at 65 °C until glutinous strands of DNA had dispersed. After cooling and centrifugation to permit phase separation (5,000 g, 30 mins), the upper aqueous phase was carefully removed with a pipette taking care not to disturb the proteinaceous material at the interface. This solution was dialysed against running tap water for 24 hours to remove any contaminating phenol and then ultracentrifuged (40,000 g, 4 hours, 4 °C). The pellet of LPS obtained was purified by repeating the centrifugation step twice more. It was then resuspended in 0.5 ml of water and stored at -20 °C.

2.2.2.3 Isolation of flagella

Flagella were prepared as described by McDonough (1965), omitting the final acid dissociation stage. Bacteria grown in a 2L culture of TSB were harvested (10,000 g, 10 mins, 4 °C), washed and resuspended in 100 ml water. The suspension was blended for 20 seconds to shear the flagella. Cells were separated from the flagella by centrifugation (10,000 g, 30 mins, 4 °C), the cells washed once and the washings added to the original supernatant. The

flagella were concentrated in the ultracentrifuge (40,000 g, 1 hour, 4 °C) and purified by alternative slow (15,000 g, 1 min) and fast (40,000 g, 1 hour) centrifugation until a clear pellet was obtained. This was resuspended in water (~ 0.5 ml) and stored at -20 °C.

2.2.2.4 Preparation of surface antigens able to interact with antibodies by immunoabsorption

Surface exposed antigens were isolated using a modification of the immunoabsorption technique described by Swanson (1981). Cells of New CDC 014:H12 were grown to log phase in 150 ml of media, harvested and washed with phosphate buffered saline (10 mM phosphate buffer, 0.9% w/v NaCl, pH 7.4, PBS). After resuspending with 3 ml of PBS, either 500 µl of heat-inactivated hyper-immune rabbit antisera or PBS (control) was added to 1 ml of the suspension and the whole mixed for 30 minutes at 5 °C. The cells were harvested by centrifugation (10,000 rpm, 5 mins, 4 °C) and washed twice with 10 ml PBS and then incubated with 3 ml of 1% w/v Zwittergent (Calbiochem, Cambridge, Cambs) at 37 °C for 1 hour to lyse the cells without dissociating antigen-antibody complexes. Any unlysed cells were removed by centrifugation (10,000 rpm, 5 mins, 4 °C). Surface antibody-antigen complexes were then separated from the supernatant by passing it 3 times through a column of protein A-sepharose CL-4B (Pharmacia, Milton Keynes, Bucks), 40 mg dry weight, 500 µl bed volume, packed in a Pasteur pipette. A control of rabbit serum (10 µl in 3 ml 1% w/v Zwittergent) was also passed over a column. Any cell debris included in the sepharose gel but not bound via IgG to protein A was separated by exhaustive elution with 20 bed volumes of 1% w/v Zwittergent and

3 x 20 bed volumes of distilled water. A control of untreated sepharose was also washed by this method. After elution, the protein A-sepharose was divided into two, one half being stored at -20 °C, the other having 1 ml of ethanol added to it. The ethanol samples were left overnight at -20 °C, the alcohol then being removed by aspiration. These samples of sepharose-protein A-antibody-antigen complexes and controls were suspended in 500 µl water, denatured with sample denaturing buffer and subjected to SDS-PAGE and immunoblotting (section 2.2.3.2), together with whole cells and OMs.

2.2.2.5 Preparation of soluble antigens

The method of Roig et al (1983) was used. TSB (200 ml) was inoculated with bacteria and after 18 hours' growth, cells were harvested to obtain whole cell and OM preparations. Ammonium sulphate (BDH) was dissolved in the TSB supernatants to a concentration of 60% w/v and left overnight at 4 °C. The resulting precipitates were dissolved and dialysed against water for 18-24 hours, the solutions then being freeze-dried. After weighing, 1 ml of distilled water was added to dissolve the powders to give the soluble antigen preparations which were stored at -20 °C. They were subjected to SDS-PAGE (section 2.2.3.1) and immunoblotting (section 2.2.3.2) or other immunochemical techniques.

The experiment was repeated using CDMFe- media and after freeze-drying and weighing, the soluble antigens were reconstituted with water to give 10 mg/ml - or 2 µg/µl of protein- solutions (section 2.2.2.8).

2.2.2.6 Opsonisation of bacteria

NPHS was added to log phase cells resuspended in 0.9% w/v NaCl (OD_{470} = 1.0) to a concentration of 10% v/v. The mixture was incubated with shaking at 37 °C and at various time intervals up to 15 minutes, samples were harvested, the supernatant retained and the opsonised cells retained after washing thoroughly.

Heat-inactivated NPHS was used as a control.

2.2.2.7 Solubilisation of bacteria for rocket- and crossed-immunoelectrophoresis

Opsonised, normal or ^{125}I-labelled cells were solubilised using 4% v/v Triton X-100 in 50 mM Tris-HCl pH 8.6 containing 5 mM ethylene diamine tetraacetic acid (Triton-EDTA).

The protein content of the whole cells was determined using the Lowry assay (section 2.2.2.8). Solubilisation to a protein concentration of 2 mg/ml for rocket- and 10 mg/ml for crossed-immunoelectrophoresis was then performed by incubating the cells in Triton-EDTA for 2 hours at 25 °C. Any unsolubilsed cells were removed by centrifugation (12,500 rpm, 5 mins, Eppendorf centrifuge), and the remaining soluble antigen extracts analysed.

2.2.2.8 Lowry (Folin-Ciocalteau) protein assay

The assay used was that developed by Lowry et al (1951). Bovine serum albumin standards (containing 10 to 100 µg, Sigma) and samples were made up to 0.35 ml with distilled water, 0.35 ml of 1 M NaOH was added and the solutions boiled at 100 °C for 5 minutes. On cooling, 3 ml of a solution made by mixing 1 ml of 1.0% w/v copper sulphate solution with 1 ml of 2% w/v potassium tartrate solution and 100 ml of 2% w/v sodium carbonate in 0.1 M NaOH was added. After leaving

15 minutes at room temperature, 0.3 ml of Folin Ciocalteau reagent (BDH) diluted 1:1.5 with water was pipetted into each tube and the resulting blue colour developed for 30 minutes before reading the absorbance at 750 nm. A calibration curve of albumin concentration against OD_{750} was used to determine the protein content of the samples (Fig 2.4).

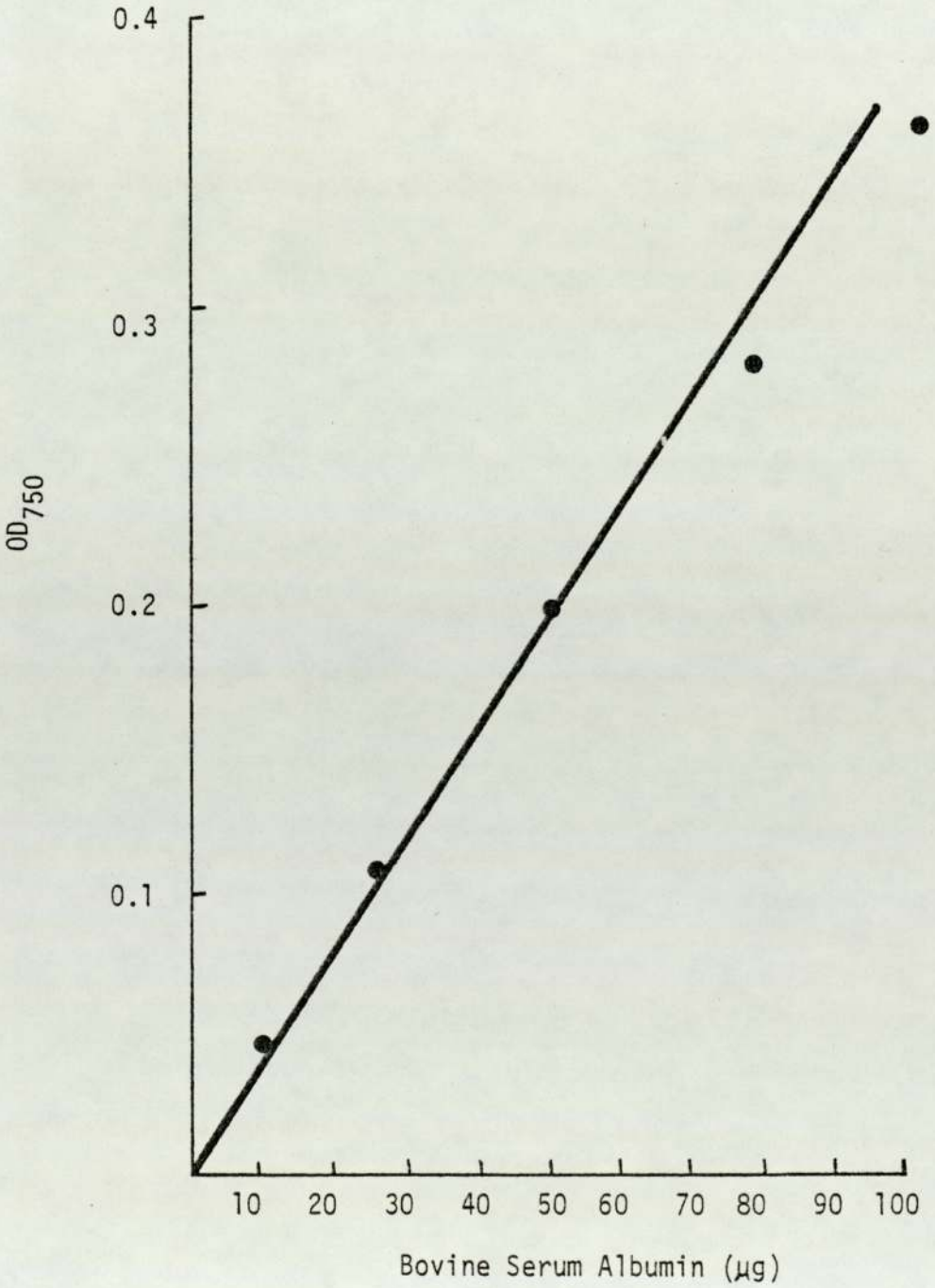
Triton X interferes with this normal Lowry method. Therefore, one volume of 20% w/v SDS was added after the 15 mins incubation with the alkaline copper reagent and before the Folin reagent to prevent precipitates forming. The total size of the incubation was scaled down for this, eg protein samples were made up to 0.2 ml, 1.0 ml alkaline copper reagent was added, 1 ml 20% w/v SDS and 0.1 ml Folin (diluted to 2.5 times its original volume with H_2O).

2.2.2.9 Iodination of cells

The method of Lambert and Booth (1982) was used. Cells were grown to log phase, harvested (10,000 rpm, 10 mins, 4 °C) and washed once with PBS (0.1 M phosphate buffer pH 7.2, 0.9% w/v NaCl). To 1 ml of cells resuspended in PBS to an OD_{470} of 10 (10^{10} cells/ml), 0.5 ml lactoperoxidase (0.1 mg/ml in PBS, Sigma) was added and 10 µl of $Na^{125}I$ (10 µCi/ml in H_2O, Amersham International plc, Amersham, Bucks). Four 100 µl quantities of H_2O_2 (10 mM in H_2O, Thornton and Ross Ltd, Huddersfield, Yorks) were added at 2.5 minute intervals and the reaction stopped with 8 ml cysteine (10 mM in H_2O, Sigma) after 10 minutes. The cells were harvested and washed four times with PBS. Two controls were performed which included omitting the lactoperoxidase or hydrogen peroxide. The whole labelling procedure was repeated on cells that had been opsonised (section 2.2.2.6).

Iodination was also carried out by replacing the lactoperoxidase

Figure 2.4 Calibration Curve for the Estimation of Total Protein by the Folin-Ciocalteau Assay



with one Iodo-bead (Pierce (UK) Ltd, Cambridge, Cambs), incubation for 10 minutes with the Na ^{125}I occurring with omission of H_2O_2 before cysteine was added.

Activity of the labelled cells was measured with a γ counter. The cells were either denatured and subjected to SDS-PAGE (section 2.2.3.1) or solubilised and separated by CIE (section 2.2.3.9). In the former case, the gels were dried between acetate sheets before autoradiography. Singul XRP X-Ray film (Cea, Ceaverken AB, Strangnas, Sweden) was used to develop autoradiographs at room temperature for between 2 to 7 days (D19 Developer and Kodafix supplied by Kodak Ltd, Hemel Hempstead, London).

2.2.3 Experimental techniques

2.2.3.1 Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

OMs, whole cells and flagella were subjected to SDS-PAGE using the system of Lugtenberg et al (1975) with a 12% or 10% w/v acrylamide concentration in the separating gel. The solutions and chemicals used to prepare the gels and buffers required for SDS-PAGE are shown in Table 2.1.

The separating gel solutions were mixed in a 250 ml beaker and stirred with a magnetic stirrer. Polymerisation was started with the addition of TEMED then AMPS, and the solution was poured inbetween two glass plates (dimensions 20 cm wide x 25 cm long) separated with 10 mm x 1 mm spacers. After setting, the stacking gel was prepared in the same way and poured inbetween the plates on top of the running gel. A comb which gave 12 or 25 wells was inserted and the stacking gel left to set. On removing the comb, the gel was fitted into an

Table 2.1 Solutions and Chemicals used to Prepare the Gels and Buffers required for SDS-PAGE

	Separating gel		Stacking gel	Sample denaturing buffer	Electrode buffer
	12%	10%			
Stock I	15 ml	12.5 ml			
Stock II			5 ml		
SDS 10% w/v (sodium dodecyl sulphate specially purified for biochemistry, BDH)	1.5 ml	1.5 ml	0.3 ml	5 ml	20 ml
Tris 1.5M pH 8.8 (Tris [hydroxymethyl] aminomethane, Sigma)	18.5 ml	18.5 ml			
Tris 0.5M pH 6.8			7.5 ml	2.5 ml	
Distilled water	23.5 ml	26 ml	16 ml	5 ml	to 2L
TEMED (N,N,N^1,N^1, tetra-methylene diamine, BDH)	0.14 ml	0.14 ml	0.08 ml		
AMPS 10% w/v freshly prepared (Ammonium persulphate, BDH)	0.2 ml	0.2 ml	0.1 ml		
Glycerol (BDH)				2.5 ml	
2-mercaptoethanol (Sigma)				0.25 ml	
5% Bromophenol blue (BDH)				0.2 ml	
Tris (Sigma)					6 g
Glycine (Biochemical grade, BDH)					28.8 g

Stock I - Solution of 44% w/v acrylamide (Sigma) and 0.8% w/v Bis(N,N^1 methylene bisacrylamide, Sigma)

Stock II - Solution of 30% w/v acrylamide and 0.8% w/v Bis

electrophoresis tank and electrode buffer was added to the top and bottom of the tank.

Samples to be analysed were mixed in a 1:1 ratio with sample buffer and boiled for 10 minutes at 100 °C. Complete heat-modification of S. marcescens OMPs was obtained under these denaturing conditions (Fig 2.5) which is in contrast to Hofstra and Dankert (1980) who found 30-40 minutes boiling was required. On cooling, the sample solutions were loaded into the wells of the stacking gel and electrophoresis was carried out at a constant current of 40 mA until the tracking dye had run 12 cm (about 4 hours).

Gels were either stained overnight (0.1% w/v coomassie brilliant blue R-250, Sigma, in 50% v/v methanol - 10% v/v glacial acetic acid solution) and then destained to reveal proteins (5% v/v methanol - 10% v/v glacial acetic acid solution), or they were subjected to immunoblotting (section 2.2.3.2).

Molecular weights of OMPs were determined by comparison with standard marker proteins (Sigma); bovine serum albumin (66 kDal), ovalbumin (45 kDal), pepsin (34.7 kDal), trypsinogen (24 kDal), lysozyme (14.3 kDal); or by running Ps. aeruginosa PAOI OM and using molecular weights of the OM proteins described by Hancock and Cary (1979). Destained gels were photographed using diffuse transmitted light.

2.2.3.2 Immunoblotting

Electroblotting of the polyacrylamide gels onto nitrocellulose membranes (0.45 μm pore size, Bio-Rad Laboratories Ltd, Watford, Herts) using the TransBlot apparatus, was carried out by the Western Blotting method of Towbin et al (1979). The gel and nitrocellulose

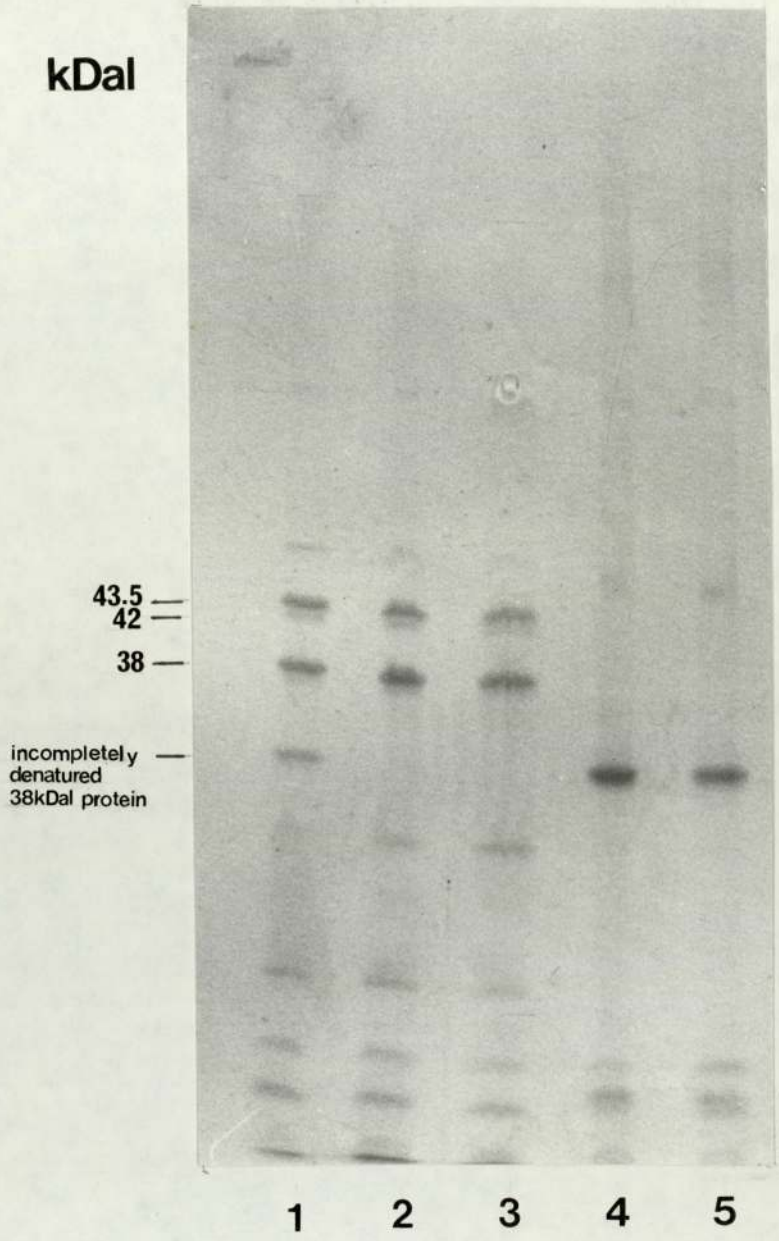
Figure 2.5

Coomassie Blue Stained Gel of Outer Membranes Isolated from New CDC O14:H12 and Denatured under Different Conditions before SDS-PAGE

OMs were isolated by the Sarkosyl method after growing the cells in TSB to stationary phase.

Key: Temperature, time of denaturation

1	100 °C, 2 mins
2	100 °C, 10 mins
3	100 °C, 30 mins
4	37 °C, 10 mins
5	37 °C, 30 mins


kDal
43.5
42
38
incompletely denatured 38kDal protein
1
2
3
4
5

paper were sandwiched between sheets of chromatography paper (Whatman Ltd, Maidstone, Kent), then Scotch-brite pads (Bio-Rad) and lastly perforated plastic support grids. The sandwich was placed in the TransBlot cell containing transfer buffer (25 mM Tris, Sigma, 192 mM Glycine, BDH, 20% v/v methanol, pH 8.6), blotting conditions being 30V for 18-24 hours at 4 °C. After transfer, initial blots were stained with 0.8% w/v amido black (naphthol blue black, Sigma) in 0.7 % w/v glacial acetic acid (Fisons) and the resulting protein profiles compared to those on coomassie blue staining of the gel after blotting. Protein transfer efficiency is known to be a function of molecular weight (Burnette, 1981; Vaessen et al, 1981), the results (Fig 2.6) showing that transfer was essentially complete. The rest of the blots were immunodetected. Soaking for 1 hour at 37 °C in Tween 20 (0.3% v/v, polyoxyethylene sorbitan monolaurate, Sigma) in Tris buffered saline (10 mM Tris-HCl, 0.9% w/v NaCl, pH 7.4, TBS) was used to block unbound sites in the nitrocellulose. Incubation with specifically raised hyper-immune rabbit antisera diluted 1 in 50 in Tween-TBS was then carried out for 3 hours at 37 °C. After washing 3 times with TBS, goat anti-rabbit IgG horse radish peroxidase conjugate (Miles-Yeda, Slough, Bucks) diluted 1 in 2000 in Tween-TBS was added for 2 hours at 37 °C. Alternatively, protein A-peroxidase (Sigma), 25 μg in 100 ml of Tween-TBS, was used. After rinsing with TBS, 4-chloro-1-naphthol 25 μg/ml (Sigma) and hydrogen peroxide (0.01% w/v (Thornton and Ross Ltd, Huddersfield, Yorks) in 10 mM Tris-HCl, pH 7.4, was used to visualise antigenic sites, the enzymatic colour reaction being terminated after 20-30 minutes by removing the solution and adding distilled water. Blots were dried between filter paper and stored in the dark.

Lectin blotting was performed to identify polysaccharide

Figure 2.6

Efficiency of Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Membranes

A Amido black stained blot

B Coomassie blue stained gel after blotting

The cells were New CDC O14:H12 grown to log phase. OMs were isolate using the Sarkosyl method.

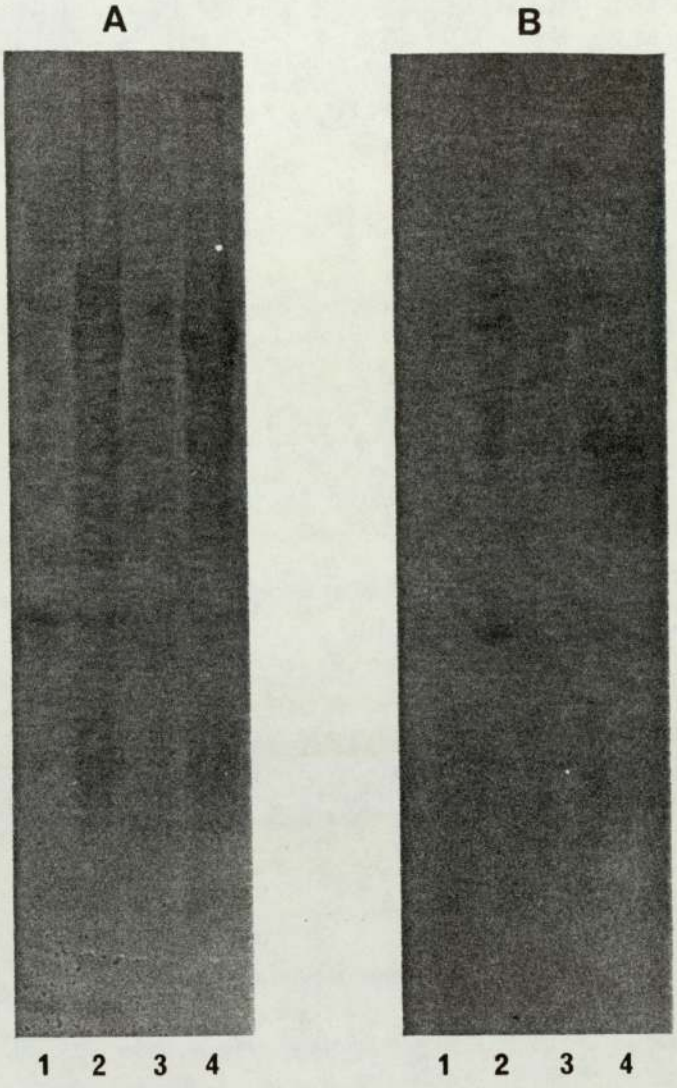
Key: Cell preparation, media

1 OM, TSB

2 OM, diluted HS

3 Whole cells, TSB

4 Whole cells, diluted HS


A
B
1 2 3 4
1 2 3 4

antigens or glycoproteins. After blocking with Tween-TBS the blots were incubated for 2 hours at 37 °C with concanavalin A-peroxidase 50 μg, or wheat germ agglutinin-peroxidase 25 μg, or soya bean agglutinin-peroxidase 25 μg, or asparagus pea lectin-peroxidase 25 μg (all from Sigma Chemical Company) in Tween-TBS (100 ml). Development with 4-chloro-1-naphthol was then carried out as before. Concanavalin A has an affinity for terminal α -D-mannosyl and α -D-glucosyl residues (Reeke et al, 1974), wheat germ agglutinin for N-acetyl-β-D-glucosamine residues (Nagata and Burger, 1974), soya bean agglutinin for N-acetyl-D-galactosamine residues (Lis et al, 1970) and asparagus pea lectin for α -L-fucosyl residues (Pereira and Kabat, 1974). By blotting with the horse radish peroxidase linked lectins, the presence of these sugars in whole cells could be identified.

The blots could either be photographed or scanned with a laser densitometer. If rapid fading of the colour occurred, the photographic negatives were used for scanning.

2.2.3.3 SDS-PAGE of purified LPS

Gels were either prepared as in section 2.2.3.1 or had 4M urea (Sigma) included in the running and stacking gels. In both cases, after denaturing the LPS samples, electrophoresis was carried out at 20 mA and the gels silver stained (section 2.2.3.5) or subjected to immunoblotting (section 2.2.3.2). By rapidly photographing the gels before fading of the silver stain occurred, a laser densitometer could be used to scan the negatives.

2.2.3.4 SDS-PAGE after proteinase K digestion of whole cells

The basic method of Hitchcock and Brown (1983) was used to digest the proteins of whole cells leaving polysaccharide material

for analysis. 50 µl of cells ($OD_{470} \sim 0.5$) and 30 µl of sample denaturing buffer were heated to 100 °C for 10 minutes. On cooling, 20 µl of sample buffer containing 2.5 mg/ml of proteinase K (Sigma), or sample buffer as a control, was added. After warming to 60 °C for 1 hour, the samples were analysed by SDS-PAGE as for purified LPS (section 2.2.3.3).

2.2.3.5 Silver stain of LPS

The technique developed by Tsai and Frasch (1982) was used. Double distilled water was used throughout. Firstly, the LPS in the polyacrylamide gel was fixed overnight in 40% ethanol-5% acetic acid solution. 0.7% periodic acid (Fisons) in 40% ethanol-5% acetic acid was then added to oxidise the LPS for 45 minutes. After three 30 minute washes with double distilled water, freshly prepared staining reagent was poured in and the gel agitated for 45 minutes at 37 °C. Staining reagent consisted of 2 ml of concentrated ammonium hydroxide (Sigma) added to 28 ml of 0.1 N NaOH. 5 ml of 20% silver nitrate (Hopkin and Williams Ltd, Chadwell Heath, Essex) was stirred in and the whole made up to 150 ml with double distilled water. The gel was then washed as before, and formaldehyde developer added (10 mg citric acid, BDH, 0.1 ml 37% formaldehyde, BDH, water to 200 ml). When the stain had reached the desired colour intensity, 40% ethanol-5% acetic acid was used to stop the reaction. The gel was photographed immediately.

2.2.3.6 Double diffusion immunoprecipitation

The Ouchterlony double diffusion technique was performed (Ouchterlony, 1958). Tris-barbiturate buffer pH 8.6 (see 2.1.3) was used to prepare 1% w/v agarose M (LKB, medium electroendosmosis,

Mr = -0.13, gelling temp 42 °C). After boiling to dissolve the agarose, the mixture was cast onto the hydrophilic side of gel bond support film (LKB Ltd, Croydon, Surrey). The volume cast was determined by multiplying the area of the gel bond piece by 0.132. After setting, double diffusion immunoprecipitation was carried out by punching wells in the gel (with a No 2 or No 3 cork borer), and adding 10-30 µl of antiserum to the central well and similar volumes of antigen preparations to the laterally surrounding wells. After leaving 12-18 hours in a moist chamber at 4 °C, non-precipitated proteins were removed by pressing and washing the gel (Weeke, 1973). A sandwich between glass plates of the gel, wet filter paper and a thick layer of dry absorbent paper tissues, was evenly pressed with a weight for 10 minutes to reduce the gel to a thin film. Discarding the paper and transferring the gel-bond to 0.9% w/v NaCl for 10 minutes allowed the saline to reconstitute and wash the gel. These two processes were repeated 3 times more, the last time distilled water being used to wash out the NaCl. After a final pressing of the gel, it was dried with a hair-drier. Any immunoprecipitin lines were then stained with coomassie blue for 10 minutes (coomassie brilliant blue R-250, Sigma, 1 g, ethanol 96% v/v 90 ml, glacial acetic acid 20 ml, distilled water 90 ml) excess staining solution being removed from the gel with destaining solution (ethanol 96% v/v 90 ml, glacial acetic acid 20 ml, distilled water 90 ml).

2.2.3.7 Isoelectrofocusing and blotting after IEF

Agarose IEF (0.3 g, Pharmacia, Milton Keynes, Bucks), sucrose (3.6 g, Fisons) and distilled water (27 ml) were boiled to dissolve and, on cooling to 75 °C, mixed ampholines (Pharmalyte, 1.9 ml, Pharmacia, pH range 3-10 or 3-6) were added. After mixing and

casting onto gel bond film (12 cm x 22 cm, Pharmacia), the gel was allowed to set for 1 hour at 4 °C. 20 µl of soluble antigen preparations (section 2.2.2.5) and pI standards (Pharmacia) were placed on the middle of the gel either on small pieces of filter paper or as drops. Sponge strips soaked in 1 M NaOH (cathode) or 0.05 M H_2SO_4 (anode) were then placed either side of the gel and constant power (2 watts for 18 hours or 10 watts for 2 hours) was applied on a water-cooled isoelectric focusing flat bed apparatus (Pharmacia Model FBE 3000). After focusing the gel was fixed in trichloroacetic acid (10% w/v, BDH) and sulphosalicylic acid (5% w/v, BDH) for 30 minutes. It was then placed in destain (glacial acetic acid, 10 ml; ethanol, 70 ml; distilled water to 200 ml) for 15 minutes, this step being performed twice. Lastly, the gel was pressed and dried using the procedure outlined in section 2.2.3.6, then stained for 10 minutes (coomassie blue, Sigma 0.5% w/v in destain) and destained to reveal the separated protein bands.

Immediately after IEF, blotting onto nitrocellulose paper was also performed by placing a nitrocellulose sheet and then absorbent paper tissues over the gel surface, weighting them down to ensure good contact for 18 hours. By this method, antigens were transferred by capillary action in a totally natural state, and they were immunodetected as described in section 2.2.3.2.

2.2.3.8 Rocket immunoelectrophoresis

The technique of Laurell (1966) was used. 1% w/v agarose M in Tris-barbiturate buffer pH 8.6 was prepared as in section 2.2.3.6. On cooling to ~ 50 °C, 0.1% v/v absorbed goat-anti-human C3 antisera (see 2.1.5) was added, mixed in thoroughly and the whole cast onto gel bond film. After setting, wells were punched and the gel was

placed on a flat-bed immunoelectrophoresis unit (LKB) cooled to 10 °C with a water cooler. Paper wicks (Whatman 4 Chr Chromatography paper, Whatman Ltd, Maidstone, Kent) soaked in Tris-barbiturate buffer were used to make the buffer-gel connections. Antigen samples (10-20 μl) were added to the wells and electrophoresis was carried out for 18 hours at a constant voltage of $\sim$ 5 Vcm^{-1}. The gel was then pressed, washed, dried and stained with coomassie blue as in section 2.2.3.6.

2.2.3.9 Crossed immunoelectrophoresis

CIE was performed as described by Weeke (1973). 1% w/v agarose M in Tris-barbiturate buffer was cast onto gel bond film (6 cm x 5.5 cm) as detailed in section 2.2.3.6. A well was punched in one corner and then the gel was set up for electrophoresis on the flat-bed immunoelectrophoresis unit at 10 °C (see 2.2.3.8). Antigen (20-30 μl), together with bromophenol blue solution (1 μl, BDH), was placed in the well and electrophoresis was carried out in the first direction at 10 Vcm^{-1} until the dye had run 4 cm (about 2 hours). All extracts were run this distance for standardisation so that different samples could be compared. The excess agarose gel was cut away leaving a strip of approximately 2 cm x 6 cm containing the separated antigen. A plastic spacer strip of 1 cm wide was placed across the gel bond just above this strip to form a barrier. 1% w/v agarose M in Tris-barbiturate buffer cooled to 50 °C had hyper-immune rabbit serum (200 to 350 μl) added and after thorough mixing was poured above the spacer. On setting, the spacer was removed and replaced with 1% w/v agarose M in buffer. Thus a blank intermediate gel between the first and second dimension gels was formed to help increase resolution. Electrophoresis in the second direction

(perpendicular to the first) was then carried out at 2 Vcm^{-1} for 18 hours. Gels were pressed, washed, dried and the immunoprecipitates stained with coomassie blue (see 2.2.3.6).

Tandem CIE, according to Kroll (1973), involved punching two wells 8 mm apart and adding different antigen samples to each. The samples were allowed to diffuse into the gel (about 30 to 60 mins, 25 °C) and the empty wells were then sealed with a drop of cooled 1% w/v agarose M. Electrophoresis in the first and second dimensions was continued as described above.

In one experiment, 30-40 µl of Triton-EDTA solubilised whole cells (see 2.2.2.7) were treated in various ways before CIE:

(1) Autoclaved at 121 °C for 15 minutes

(2) Digested with proteinase K, Sigma (10 µg in 5 mM Tris-HCl, pH 6.8) at 60 °C for 1 hour, a control being run omitting the proteinase K.

(3) Oxidised with 0.1 M sodium periodate (BDH) in sodium acetate buffer (pH 4.5) at room temperature for 16 hours, excess periodate then being consumed by addition of butane-2,3 diol, a control without sodium periodate also being performed.

2.2.3.10 Serum bactericidal assays

The method of Williams et al (1983) was used. Bacteria grown to log phase were harvested (10,000 rpm, 10 mins, 4 °C) washed and resuspended to OD_{470} = 0.2 in 0.9% w/v NaCl. 0.6 ml of the suspension was added to 0.6 ml of NPHS or GPS (see 2.1.5) in a glass bijou bottle, sterile glass beads being added if necessary to prevent clumping. The mixture was incubated at 37 °C in a reciprocal shaking water bath, shaking at 120 rpm. 100 µl samples were taken at various

time intervals, diluted with five tenfold dilutions of 0.9 ml sterile saline, and viable counts of the two most diluted samples made according to the method of Miles and Misra (1938). 8 x 20 µl samples were spotted onto overdried nutrient agar plates, allowed to diffuse, and incubated for 18 hours at 37 °C. Each experiment was carried out in triplicate. The bacterial counts at each time interval were expressed as a percentage of the original count and the log of this was plotted against time. Serum heat-inactivated to destroy complement was used in the assay as a control.

0.12 ml of 10 mM EGTA (ethylene glycol tetra-acetic acid, Sigma) and 10 mM Mg^{2+} in 0.9% w/v NaCl, prepared according the method of Fine et al (1972), was added to 0.6 ml of serum to isolate functional alternative complement pathway activity. 0.48 ml of bacteria (OD_{470} = 0.25) was then added and the assay performed as described.

2.2.3.11 Bacterial adherence to hydrocarbon (BATH)

The technique developed by Rosenberg et al (1980) was used. 100 µl of octane (Sigma) was added to 1.2 ml of normal or opsonised bacteria resuspended to OD_{470} = 0.3 in PUM buffer ($K_2HPO_4.3H_2O$, 22.2 g; KH_2PO_4, 7.26 g; urea, 1.8 g; $MgSO_4.7H_2O$, 0.2 g; distilled water to 1000 ml, pH 7.1). After 10 minutes pre-incubation at 25 °C, the mixture was vortexed for 120 seconds. When separation of the layers was complete (20 minutes) the OD_{470} of the aqueous phase was measured, a syringe or Pasteur pipette being used to carefully remove the suspension from under the octane. Each experiment was performed in triplicate. The absorbance reading of the suspension after vortexing was expressed as a percentage of the original absorbance, the figure obtained giving an indication as to the hydrophobicity of the cells.

2.2.4 Quantitation and chemical analysis of phenol-extractable material

2.2.4.1 Quantitation of phenol-extractable material

2L cultures of TSB were grown to log phase, the OD_{470} recorded, 40 ml taken for dry weight determination (see 2.2.4.2) and the rest centrifuged (10,000 rpm, 10 mins, 4 °C) to harvest the bacteria. After washing and resuspension of the cells in 50 ml water, polysaccharide material was extracted quantitatively using the method outlined in Section 2.2.2.2. After dialysis, however, no centrifugation was performed. Instead, ribonuclease (Bovine Pancreas type 1-AS, Sigma) and deoxyribonuclease (Bovine Pancreas type III, Sigma) were added (10 μg/ml) and the mixture was incubated overnight at 37 °C with 0.1 w/v sodium azide to digest any contaminating nucleic acid material. The polysaccharide was then re-extracted with phenol, dialysed and freeze-dried.

The total weight of phenol-extractable material was carefully recorded and after reconstitution to 10 mg/ml was analysed chemically and by SDS-PAGE (2.2.3.3) and Tandem CIE (2.2.3.9). Absence of nucleic acids and protein in the extract was confirmed by absence of UV absorption at 260 and 280 nm.

To try to separate LPS from any capsular polysaccharides, ultra-centrifugation (40,000 rpm, 4 hours, 4 °C) was used (Sutherland and Wilkinson, 1971) after addition of $MgCl_2$ solution (5 mM) to help pellet the LPS. The supernatant was freeze-dried and any resulting material reconstituted in a small quantity of water. The latter and the resuspended LPS pellet, were both analysed by SDS-PAGE (2.2.3.3).

2.2.4.2 Dry weight determination

Clean Beckman centrifuge tubes were dried to constant weight

over phosphorus pentoxide in a desiccator and used to harvest and wash the bacteria (10,000 rpm, 15 mins, 4 °C). After drying the cell pellet at 50 °C, the tubes were dried to constant weight as before.

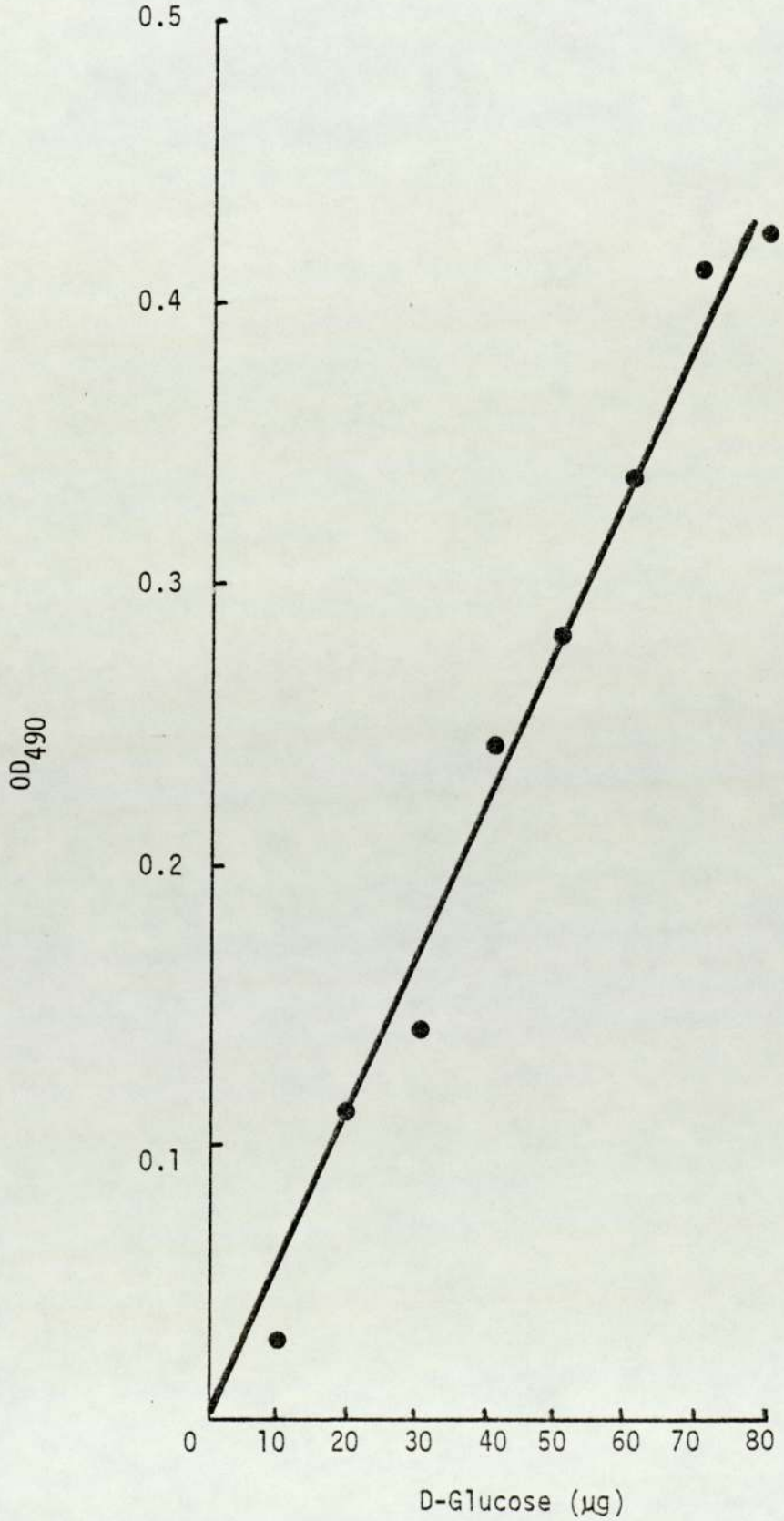
2.2.4.3 Phenol sulphuric acid assay for total hexose estimation

The method of Dubois et al (1956) was performed. 2.0 ml quantities of glucose containing 10 to 80 μg D-glucose (BDH) in distilled water were prepared. Samples of the 10 mg/ml solutions of phenol-extractable material (see 2.2.4.1) were made up to 2.0 ml in distilled water. To each, 1.0 ml of 5% w/v aqueous phenol (Fisons) was added rapidly followed by 5.0 ml of 95.5% w/v H_2SO_4 (BDH). After cooling to room temperature, the absorbance of each solution at 490 nm was recorded. A standard calibration curve of glucose concentration against OD_{490} was then used to determine the total hexose content of the samples (Fig 2.7).

2.2.4.4 3-deoxy-D-manno-2-octulosonic acid (KDO) assay

A method based on that of Osborn (1963) was used. Samples of KDO standard (10 to 100 μg, Sigma) or the 10 mg/ml solutions of phenol-extractable material (2.2.4.1) were made up to 0.25 ml with 0.05 M H_2SO_4 and hydrolysed by heating in sealed tubes at 100 °C for 30 minutes. On cooling, 0.25 ml periodic acid (0.025 M in 0.0625 M H_2SO_4) was added. After warming to 55 °C for 20 minutes, 0.5 ml of 2% w/v sodium arsenite (Sigma) in 0.5 M HCl was pipetted into each tube and the contents mixed thoroughly. 2.0 ml of 0.3% w/v thiobarbituric acid in distilled water was then added, the tubes sealed, and heated to 100 °C for 20 minutes. After cooling to room temperature, the absorbance of each solution at 550 nm was measured. By plotting a standard calibration curve of KDO

Figure 2.7 Calibration Curve for the Estimation of Total Hexose by the Phenol-Sulphuric acid Assay



concentration against OD_{550} (Fig 2.8), the KDO content of the phenol-extractable material could be estimated.

2.2.4.5 Fatty acid analysis of LPS after acid hydrolysis

Acid hydrolysis can be used to convert the fatty acids in extracted LPS to volatile derivatives for subsequent analysis by gas liquid chromatography.

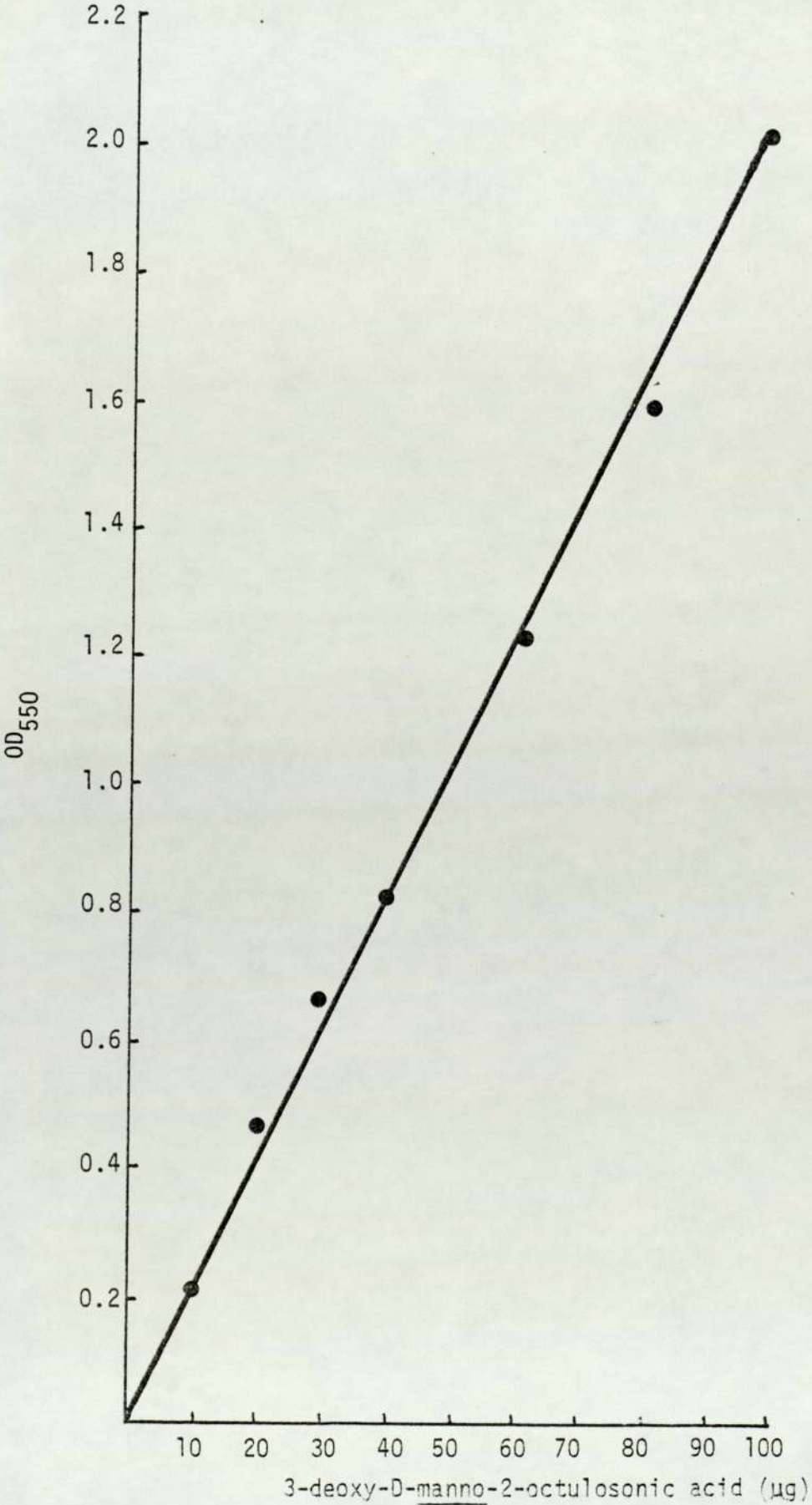
5 µl of a 1 mg/ml solution of tridecanoic acid, C13 (Sigma) in hexane was placed in sealable tubes to act as an internal standard. After the solvent had evaporated, 100 µl of the 10 mg/ml solutions of phenol-extractable material (2.2.4.1) or 100 µl distilled water as a control were added, and then 2 ml of 6N HCl to each tube. The tubes were sealed and hydrolysis carried out for 4 hours at 100 °C. After cooling, methyl esters were formed by the addition of 2 ml boron trifluoride-methanol complex (14% w/v boron trifluoride, BDH) and heating to 80 °C for 5 minutes. The fatty acid methyl esters were then extracted from the preparation with 3 ml chloroform/hexane mixture (1:4). The upper solvent layer was placed in a clean 50 ml glass round-bottomed flask and evaporated to dryness using a rotary evaporator. The residue was redissolved in 40 µl hexane and evaporated at room temperature to a final volume of approximately 10 µl. A loading of 2 µl was used for analysis by GLC.

GLC conditions were similar to those described by Lambert and Moss (1983).

Column - 3 m by 2 mm I. dia x 5 mm O. dia glass packed with 3% SP-2100 DOH on 100/120 Supelcoport (Supelco Chromatography Supplies, Suplechem, Sawbridgeworth, Herts)

Column temperature programme - 150 °C 1 min, increase at 2 °C/min to 225 °C, hold for 5 min.

Figure 2.8 Calibration Curve for the Estimation of 3-deoxy-D-manno-2-octulosonic acid



Gas pressures - Hydrogen 14.5 psi
Air 6.5 psi
} for flame ionisation detector

Nitrogen 23.5 psi - 20 ml/min - carrier gas through column

2 µl of a reference standard containing 23 fatty acids (Bacterial fatty acid methyl esters, 25 mg/ml, Supelco Inc, Suplechem, Sawbridgeworth, Herts) was run through the column to help identify the fatty acids in the samples. Knowing that the peak area of the tridecanoic acid internal standard in each sample was equivalent to 5 µg, the peak areas of the sample fatty acids could be calculated into weights.

2.2.5 Antibiotic assays

2.2.5.1 Antibiotic sensitivity with 'Neosensitab' antibiotic discs

Log phase cells were resuspended in saline to an OD of 0.2. 2 ml of this was used to flood dried DST agar plates, excess liquid being removed with a pasteur pipette. Eight different antibiotic discs were placed on the plates, incubation for one hour at room temperature enabling uniform diffusion of antibiotic from the discs. After 18 hours incubation at 37 °C, the resulting zones of inhibition around each tablet were measured.

2.2.5.2 Minimum inhibitory concentration

MICs were determined by a tube dilution method (Washington and Barry, 1974). Tubes containing a series of dilutions of the antibiotic or disinfectant in 5 ml of TSB were inoculated with a fixed inoculum of 10^5 bacteria. The lowest concentration of antibiotic which resulted in inhibition of bacterial growth (as

judged by absence of visible turbidity) after 24 hours incubation at 37 °C was considered to be the MIC. All determinations were carried out in duplicate, drug free and sterility controls being included.

2.2.5.3 Antibiotic agar assays

A range of concentrations of the antibiotic/disinfectant to be tested were mixed in nutrient agar at 40 °C and poured into petri dishes and allowed to set. 100 µl bacteria grown to log phase and diluted in sterile 0.9% w/v NaCl to approximately 10^3 organisms/ml were spread over control nutrient agar plates and those containing low concentrations of drugs, higher numbers of bacteria being added to plates containing higher concentrations of antimicrobials. After 18 hours incubation at 37 °C, the number of bacterial colonies were counted and a graph plotted of drug concentration vs percent survivors.

3. RESULTS AND DISCUSSIONS

3.1 IMMUNOCHEMISTRY OF S. MARCESCENS

3.1.1 Analysis of outer membrane proteins by SDS-PAGE

Interpretation of the results must be considered with caution because during isolation of the OMs, alterations of the basic structure to that occurring in whole cells may take place, particularly loss of individual proteins (Chopra and Shales, 1980). SDS-PAGE, followed by staining of the proteins with coomassie blue, allows a qualitative estimation of the proteins present in a sample. Figure 3.1 shows the OMP profiles of New CDC O14:H12 grown in TSB, diluted HS with and without additional iron, CDMFe+ and CDMFe- media. OMs separated by the sucrose density technique of Tsang et al (lane 1) were identical to those obtained using Sarkosyl (lane 2). Therefore, the more convenient Sarkosyl method was used in the rest of the work, giving higher yields of OMs.

Molecular weights of the proteins were determined by comparison with marker proteins. The 43.5 and 42 kDal proteins are assumed to be the porins, equivalent to OmpF and OmpC in E. coli (Sawai et al, 1982) and the 38 kDal protein the heat-modifiable OmpA protein (Cole et al, 1982). These values and those of the other OMPs were similar to molecular weights reported previously (Lugtenberg et al, 1977; Winkler et al, 1978; Hofstra et al, 1980; Gutmann and Chabbert, 1984).

Comparison of profiles 3 and 4 show that a group of high molecular weight proteins (63 to 70 kDal) were iron-regulated (IRMPs). The diffuse bands in the 63 to 70 kDal region of profile 4 are probably horse serum proteins which became attached to the OMs during growth (see section 3.2.2). A low molecular weight protein

(26.5 kDal) observed in the OMs from diluted HS grown cells (lanes 3 and 4) was not present in TSB grown cells (lane 2) and perhaps represents a nutrient binding protein or a HS protein attached to the cells.

Tracks 5 and 6 again show the induction of IRMPs after growth of the cells in CDMFe-, repression occurring after growth in medium with added iron. The profiles were obtained from stationary phase cells. Log phase cells gave identical profiles except that the IRMPs were slightly less prominent, probably because iron supplies in the CDMFe- medium were not exhausted.

OMP profiles of New CDC O14:H12 grown to stationary phase in all growth media were similar to those from log phase cells except that a protein of molecular weight 23 kDal became prominent (results only shown for cells grown in CDM, Fig 3.1, lanes 5 and 6 and Fig 3.3).

Figure 3.2 represents the OMP profiles of different strains of S. marcescens after growth of the cells to log phase in TSB or diluted HS. The patterns were very similar with a few exceptions. Small molecular weight changes were noticeable amongst the strains. For instance, the OmpA protein of CDC O3:H1 ran at 38.5 kDal and the 18.5 kDal protein at 18 kDal. The 42 kDal porin bands of CDC O6:H3, SF133 O3:H12 and CDC O3:H1 were less defined than in New CDC. Lastly, isolate 14815 appeared not to contain the 43.5 kDal porin protein.

The OMP profiles of different serotypes of S. marcescens after growth to stationary phase in CDMFe- (excluding O5 and O11 which would not grow in this medium) were similar in their major features (Fig 3.3). All showed inducement of a group of IRMPs. The porins and OmpA protein differed slightly in molecular weight in certain serotypes and also in amount (eg the porins of serotype O7). It also

appeared that the 42 kDal porin of New CDC became slightly less well defined after growth in CDMFe- possibly because of high production of the 43.5 kDal porin. The 30.5 kDal protein that was prominent after growth in TSB and diluted HS was only very weak after growth in CDMFe-. Other small molecular weight changes were seen amongst the serotypes and there were differences in the presence or absence of minor protein bands.

Comparison of the OMPs of S. marcescens New CDC O14:H12 to four other Gram-negative bacteria showed that the profiles were related (Fig 3.4). Not surprisingly, the proteins of E. coli and especially K. aerogenes were most closely linked to those of S. marcescens. The IRMPs of S. marcescens tended to be of a lower molecular weight to the other organisms, being in the 66 to 73 kDal region of the IRMPs of K. aerogenes.

Figure 3.1

SDS-PAGE Protein Profiles of Outer Membranes Isolated from S. marcescens New CDC 014:H12 after Growth to Log or Stationary Phase

Key: Medium cells grown in

1	TSB to log phase
2	TSB to log phase
3	Diluted HS to log phase
4	Diluted HS with additional iron to log phase
5	CDMFe- to stationary phase
6	CDMFe+ to stationary phase

All OMs were isolated using the Sarkosyl method, except for those i lane 1 where the sucrose density method of Tsang et al (1976) was used.

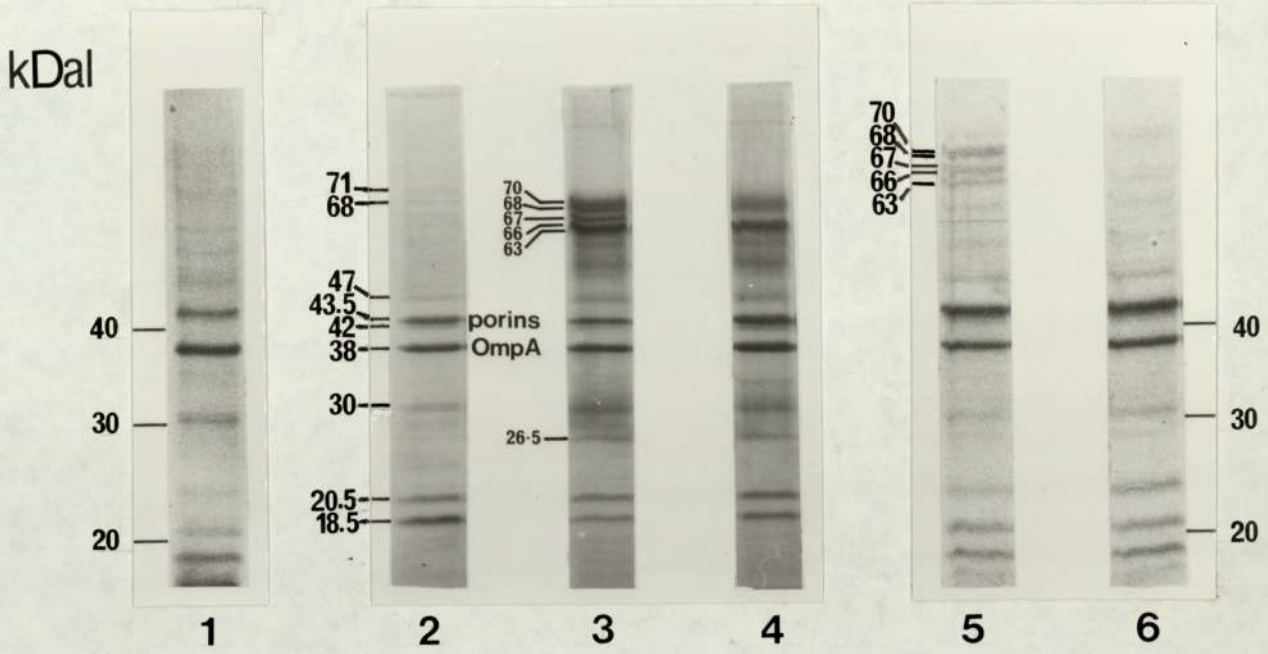
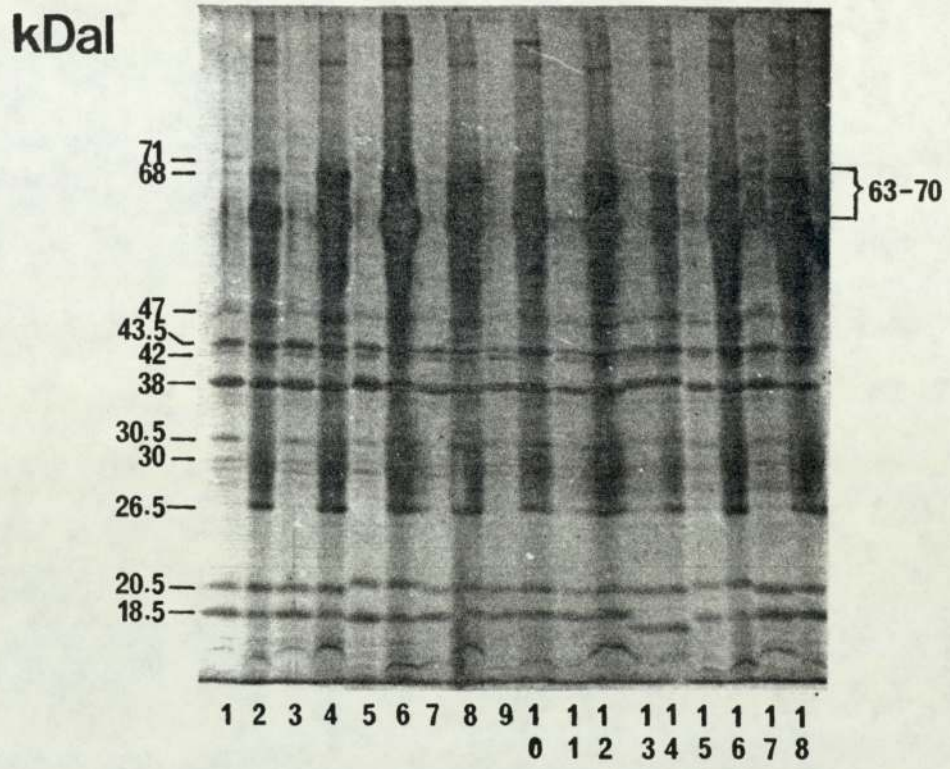

kDal
40
30
20
71
68
47
43.5
42
38
30
20.5
18.5
porins
OmpA
70
68
67
66
63
26·5
70
68
67
66
63
40
30
20
1
2
3
4
5
6

Figure 3.2

SDS-PAGE Protein Profiles of Outer Membranes Isolated from Differe Strains of S. marcescens after Growth in TSB or Diluted HS to Log Phase

The first of the pair are the OMs isolated from TSB grown cells, t second from diluted HS grown cells.

Key:	Strain
1,2	New CDC O14:H12
3,4	4444-60 O14:H12
5,6	1783-57 O14:H9
7,8	874-57 O14:H12
9,10	S818 O14:H12
11,12	S1220 O14:H12
13,14	CDC O3:H1
15,16	CDC O6:H3
17,18	SF133 O3:H12
19,20	New CDC O14:H12
21,22	6358
23,24	6380I
25,26	6380II
27,28	9623
29,30	10682
31,32	14715
33,34	14815
35,36	16191
37,38	32201


kDal
71
68
47
43.5
42
38
30.5
30
26.5
20.5
18.5
63-70
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

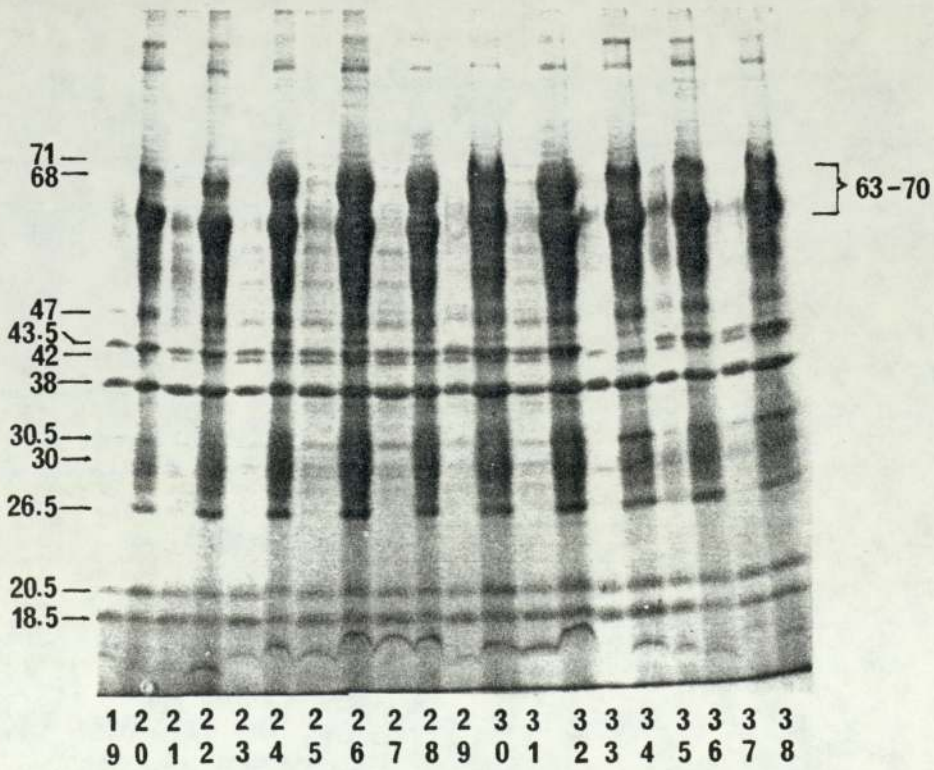

71
68
47
43.5
42
38
30.5
30
26.5
20.5
18.5
63-70
19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38

Figure 3.3

SDS-PAGE Protein Profiles of Outer Membrane Isolated from Differen
Serotypes of S. marcescens after Growth in CDMFe- to Stationary Ph

Key: Serotype

1	01
2	02
3	03
4	04
5	06
6	07
7	08
8	09
9	010
10	012
11	013
12	New CDC 014:H12
13	4444-60 014:H12
14	015

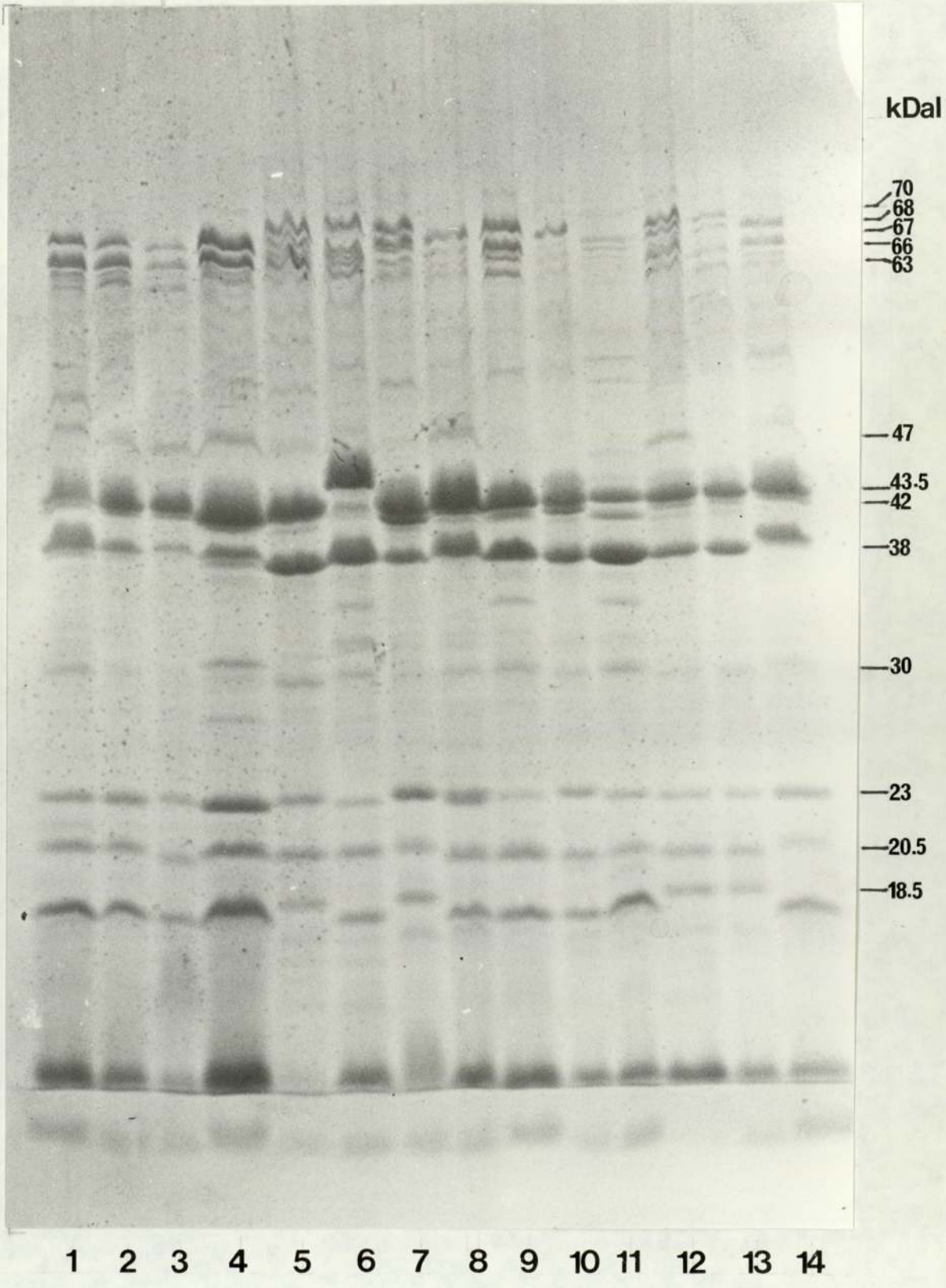

kDal
70
68
67
66
63
47
43.5
42
38
30
23
20.5
18.5
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 3.4

SDS-PAGE Protein Profiles of Outer Membranes Isolated from Five Different Gram-negative Bacteria

Cells were grown to stationary phase in iron-restricted medium (CDMFe-).

Key: Organism

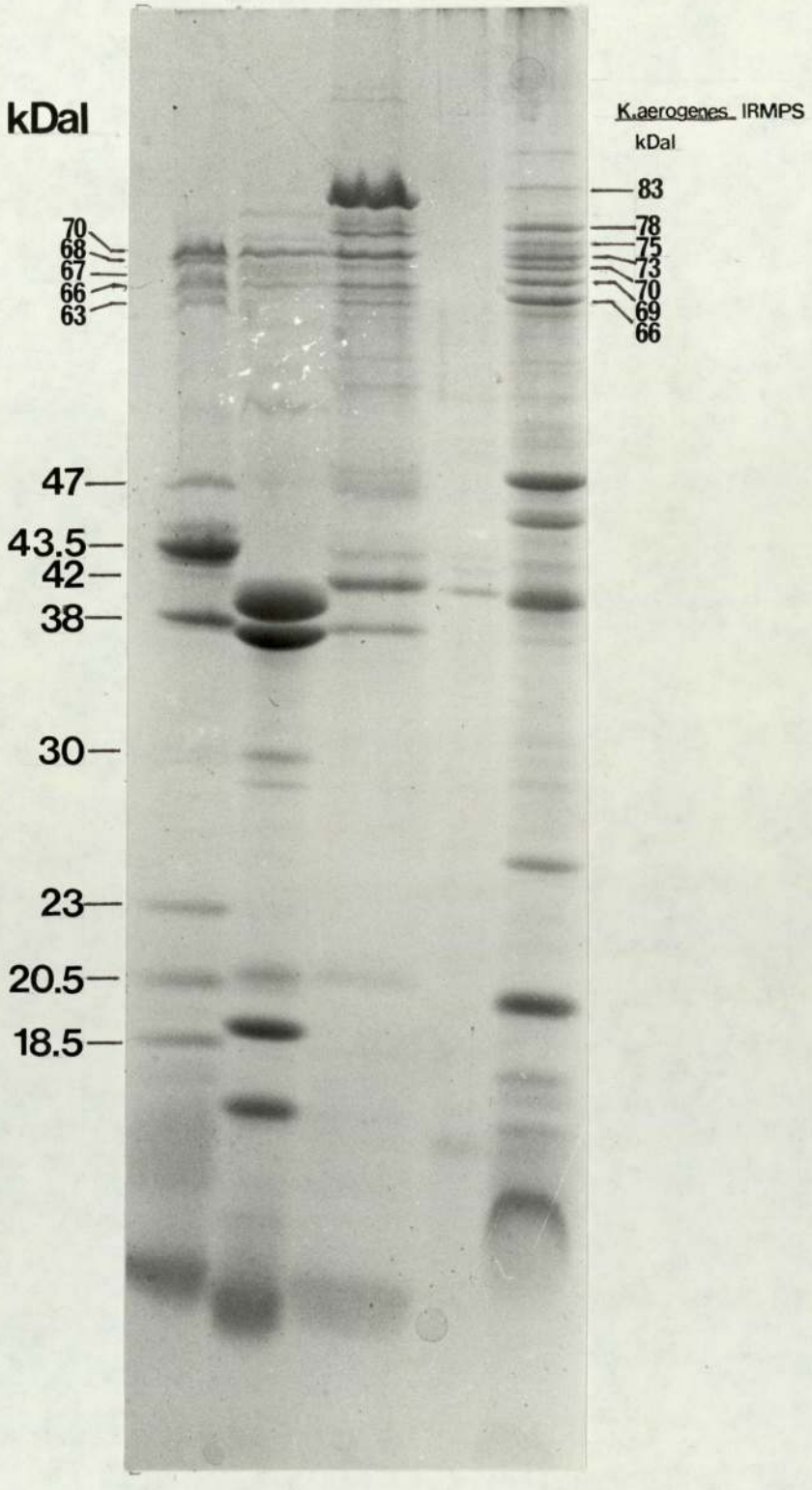
1 *S. marcescens* New CDC 014:H12

2 *E. coli* GH 126

3 *K. aerogenes* NCTC 5055

4 *P. mirabilis* 196

5 *Ps. aeruginosa* NCTC 6750


kDal
70
68
67
66
63
47
43.5
42
38
30
23
20.5
18.5
K.aerogenes IRMPS
kDal
83
78
75
73
70
69
66
1
2
3
4
5

3.1.2 Analysis of Lipopolysaccharide by SDS-PAGE

Heterogeneity in the molecular size of LPS within a strain has been well described: purified LPS from Gram-negative organisms separates by PAGE into a series of sharp bands presumed to represent varying polysaccharide chain lengths of the O antigen (Goldman and Leive, 1980; Palva and Mäkelä, 1980; Tsai and Frasch, 1982; Hitchcock and Brown, 1983). In addition, LPS with long or short chains tend to predominate (van Alphen et al, 1983).

LPS isolated from whole cells using the hot-phenol technique can be separated by SDS-PAGE. Alternatively, proteinase K may be used to digest the proteins of whole cells leaving polysaccharide material for analysis. After separation, detection can be performed using periodate oxidation followed by silver staining according to the method of Tsai and Frasch (1982), which stains LPS as well as protein. Kusecek et al (1984), however, suggest this method stains LPS more efficiently than protein.

Figure 3.5 shows the silver-stained profiles of LPS isolated from cells of New CDC 014:H12 grown to stationary phase in TSB (lane 1), and proteinase K digests of cells grown to log and stationary phase in TSB or diluted HS (lanes 2 to 5). Typical ladder patterns were revealed, the heavily stained bands at the leading edge of the gel representing rough LPS (lipid A and core) containing no O antigen. Lanes 1 and 3 confirmed the work of Hitchcock and Brown (1983) who suggested that patterns obtained from purified LPS and proteinase K digested whole cells are comparable. The LPS of log phase cells grown in diluted HS (lane 4) contained a proportion of material that separated into bands of higher molecular weight compared to the LPS from cells grown in TSB (lane 2). Figure 3.6

shows the laser densitometer profiles obtained from scanning the photographic negatives of these tracks. An accurate representation was seen with the bands forming into peaks. It therefore appears that cells grown in diluted HS contain LPS molecules with longer O antigen chain lengths. This could reflect the different growth rates in the two media (Fig 2.2a). The slower doubling time in diluted HS ($t\,1/2$ = 30 minutes) would allow longer molecules of LPS to be built up, whereas in TSB ($t\,1/2$ = 20 minutes) there would be less time before division occurred.

Silver-staining is a sensitive and quantitative technique. The proteinase K digestion was performed on the same OD_{470} of cells, the darker staining of the LPS from stationary phase cells (lanes 3 and 5) suggesting that time had allowed complete formation of LPS. The higher loading also showed up lower molecular weight bands that were present in the LPS of log phase cells, but only faintly.

Figure 3.7 represents the silver-stained proteinase K digests of different serotypes of S. marcescens grown to log phase in TSB and diluted HS. The two O14 strains gave identical profiles, both showing the higher molecular weight bands after growth in diluted HS. Other serotypes showing this effect were O1, O10, and O12, whereas strains O4 and O5 had slightly higher molecular weights when grown in TSB. With all the other serotypes there were no differences in the profiles after growth in the two media, apart from O13 (see below).

The O11 serotype had LPS that did not separate into a well-defined ladder pattern. Other workers have suggested that this represents rough mutant cells which contain only lipid A core and no O antigen (Hitchcock and Brown, 1983; Kusecek et al, 1984). Strain O11 may well have only a small proportion of O antigen which runs as a 'streak' in the background of the profiles. This would correlate

with the difficulties in the typing of it mentioned by the PHLS Laboratory, Colindale, London (personal communication). The 013 serotype after growth in TSB also gave a 'streaky' appearance, although in diluted HS separation into sharp bands occurred.

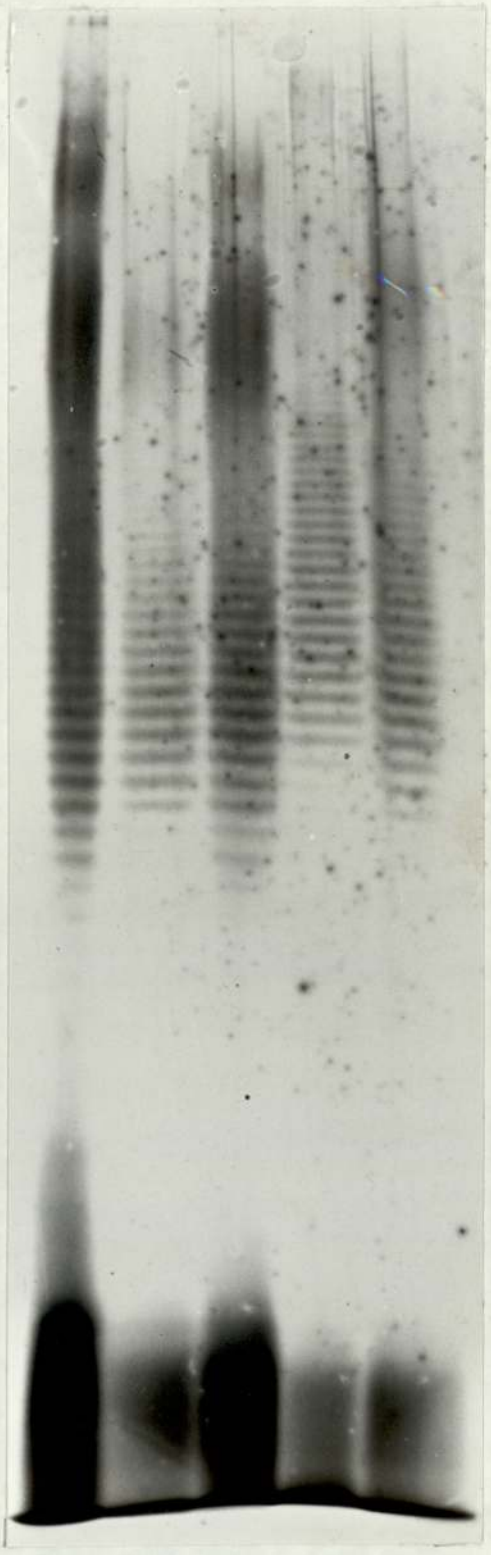
Hitchcock and Brown (1983) using two species of Salmonella, suggested that differences in LPS profiles reflect biochemical differences in LPS, including variations in the core oligosaccharides and the presence or composition of the O side chain. Kusecek et al (1984) found many isolates of E. coli that were spread among five O antigen types gave homogeneous profiles within a particular serotype, but different profiles to each other. Using 15 O serotypes of S. marcescens, it appears that some serotypes have nearly identical LPS patterns; eg O2 and O3, O6 and O7, O12 and O14.

Figure 3.5

Silver Stained LPS after SDS-PAGE of Proteinase K Digested Whole Cells of S. marcescens New CDC O14:H12

Key:	Medium cells grown in to log or stationary phas before digestion
1	Control, phenol extracted LPS from cells of New CDC grown to stationary phase in TSB
2	TSB, log (doubling time = 20 min)
3	TSB, stationary
4	Diluted HS, log (doubling time = 30 min)
5	Diluted HS, stationary

The cells were suspended to the same OD_{470} before digestion and th same loadings run on the gel.



1 2 3 4 5

Figure 3.6

Laser Densitometer Tracings of Silver Stained LPS after SDS-PAGE o
Proteinase K Digested Whole Cells of S. marcescens New CDC O14:H12

Key: Medium cells grown in to log phase before digestion

a TSB

b Diluted HS

} Photographic profiles

c Laser densitometer profiles obtained from scann the negatives of the photographs a and b

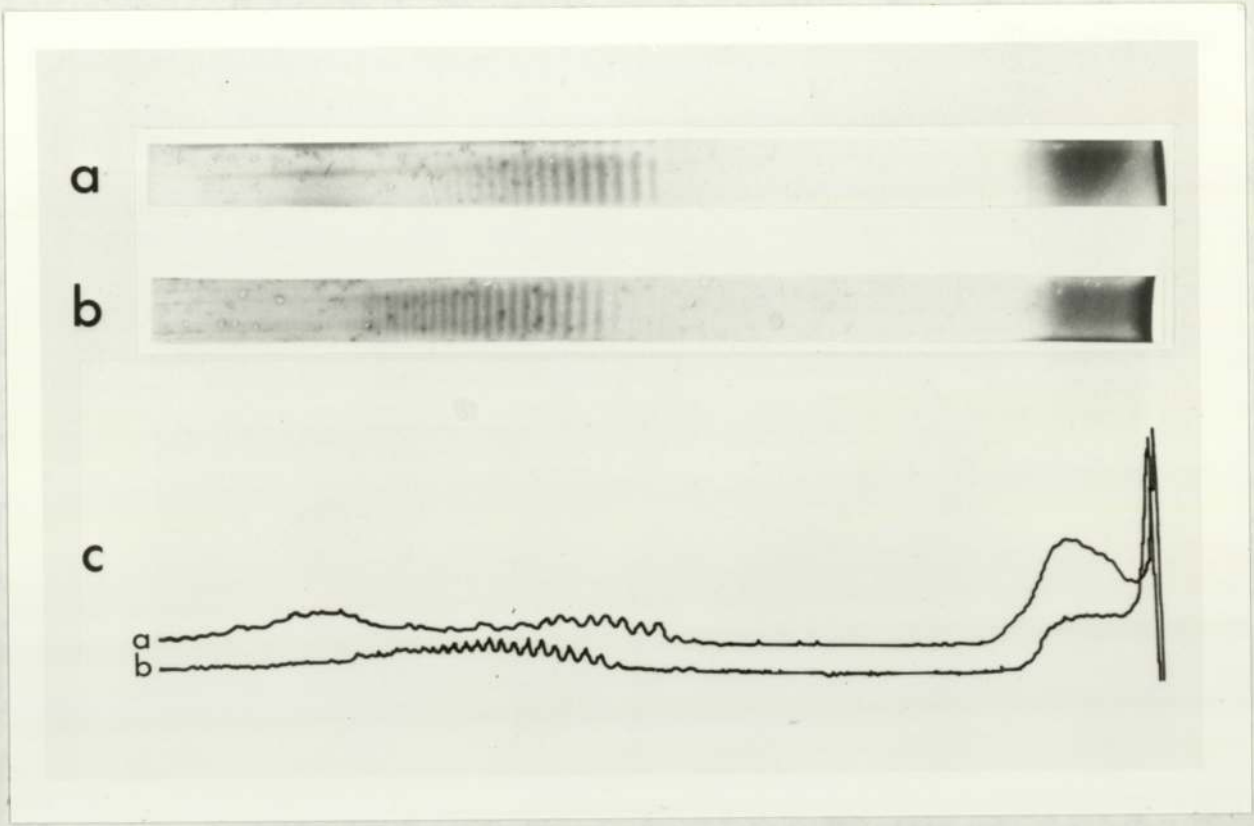

a
b
c
a
b

Figure 3.7

Silver Stained LPS after SDS-PAGE of Proteinase K Digested Whole Cells of Different Serotypes of S. marcescens

Key:	Serotype and medium grown in to log phase befor digestion
1	01, TSB
2	01, diluted HS
3	02, TSB
4	02, diluted HS
5	03, TSB
6	03, diluted HS
7	04, TSB
8	04, diluted HS
9	05, TSB
10	05, diluted HS
11	06, TSB
12	06, diluted HS
13	07, TSB
14	07, diluted HS
15	08, TSB
16	08, diluted HS
17	09, TSB
18	09, diluted HS
19	010, TSB
20	010, diluted HS
21	011, TSB
22	011, diluted HS
23	012, TSB
24	012, diluted HS
25	013, TSB
26	013, diluted HS
27	New CDC, TSB
28	New CDC, diluted HS
29	4444-60, TSB
30	4444-60, diluted HS
31	015, TSB
32	015, diluted HS

The cells were suspended to the same OD_{470} before digestion and the same loadings run on the gels.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32

3.1.3 Analysis of outer membranes and whole cells by immunoblotting

Immunoblotting using the Western Blotting technique allows transfer of OM components separated by SDS-PAGE onto nitrocellulose paper where their antigenic properties may be investigated. By using the two methods the original pattern of separated components is retained on the solid phase helping in identification of the reacting antigens (Towbin and Gordon, 1984). During the transfer components are concentrated on the surface of the nitrocellulose paper, freed from denaturing SDS in the gel, and renatured (Hjerten, 1983). However, caution must be taken when interpreting the results because antigenicity may not be restored on transfer and the transfer may not necessarily be quantitative for the proteins present on the original gel (Gershoni and Palade, 1982).

Immunisation of rabbits with UV killed whole cells results in production of antibodies to a wide range of cellular antigens, many of which are presumably revealed during phagocytic processing. A control blot (Fig 3.8) using pre-immune serum from the rabbits as specific antiserum in the immunodetection process, showed that only low levels of antibodies were present before immunisation that reacted with S. marcescens antigens. Figure 3.9 shows the immunoblot profiles of OMs and whole cells of New CDC O14:H12 grown to log phase in TSB and diluted HS immunodetected with antiserum raised against whole cells grown in TSB. Figure 3.10 represents laser densitometer profiles of the OM band, scanning of both the blot and negative of the blot showing similar peak patterns. The enzymatically produced colour tends to fade rapidly and once photographed, if scans are required, the negative may be used as opposed to the actual blot.

The OmpA protein (38 kDal) was seen to be the predominant antigen. The porins (43.5 and 42 kDal) reacted relatively weakly, showing that they were either weakly antigenic, or that their antigenic sites were destroyed during SDS-PAGE and/or transfer. The major features observed seemed to be the same in both OMs and whole cells, indicating that OM components were chiefly responsible for whole cell antigenicity. Other prominent antigen bands at 71, 68, 58, 47, 30.5 and 30 kDal were visible as coomassie blue stained bands in the SDS-PAG of solubilised OMs, and are therefore presumed to be OM proteins.

The multi-banded antigenic ladder pattern of LPS is preserved on transfer to nitrocellulose by immunoblotting (Cousland and Poxton, 1983; Driver and Lambert, 1984, and see section 3.1.4). The fine bands in the background of the immunoblots of OM or whole cell preparations over the polypeptide molecular mass range 35-65 kDal probably represent this. However, there was no evidence that antibodies to the fast-migrating lipid A core were present.

Comparing the OMs and whole cells from TSB and diluted HS grown cells in figure 3.9, very little difference could be observed on the effect of growth media. Immunodetection of the blot with antiserum raised to whole cells grown in diluted HS (Fig 3.11) shows that each corresponding antigenic profile was similar to that in figure 3.9, but overall less complicated. The OmpA response was again the strongest, but the 47 and 71 kDal proteins were not antigenic. Some of the low molecular weight components below the OmpA protein in figure 3.9 were not antigenic in figure 3.11, including the 30.5 kDal protein. Lastly, the antigen just above the 38 kDal band could represent one of the porins.

Figures 3.12 and 3.13 are the immunoblots of outer membranes from different strains of S. marcescens detected with antiserum

raised to New CDC cells. The profiles obtained were nearly identical to those already described for New CDC (Figs 3.9 and 3.11) showing that the major protein antigens were cross-reactive. It is interesting that when antiserum raised to New CDC grown in diluted HS was used, a stronger antigenic reaction occurred with at least one of the porins (Figs 3.13 and 3.11) compared to when antiserum raised to TSB grown cells was used (Figs 3.12 and 3.9).

Although iron-restriction caused induction of a group of high molecular weight IRMPs after growth in diluted HS (Figs 3.1 and 3.2), the induced proteins were not seen to be strongly antigenic (Figs 3.11 and 3.13). Horse serum proteins attaching to the cell membrane could have interfered with the immunoblotting response (see section 3.2.2). Thus cells grown in CDMFe- and CDMFe+ were used to raise antisera (Fig 3.14). However, the decreasing iron content had little effect upon the antigenic composition. The latter experiment was conducted on log phase cells, but using cells grown to stationary phase when most of the available iron in the media was consumed, the IRMPs showed as being antigenic on immunoblotting (Fig 3.15, lane 12).

Blots using antiserum raised to stationary phase New CDC cells grown in TSB were identical to those using log phase cells. The 23 kDal stationary phase induced protein did not even show as being antigenic (results not shown).

An immunoblot of the OMs of different serotypes of S. marcescens, detected with antiserum raised to New CDC cells after growth to stationary phase in CDMFe- is shown in figure 3.15. Most of the proteins were cross-reactive giving profiles that were similar across the serotypes apart from small molecular weight variations, the two O14 strains having identical patterns. The OmpA (38 kDal)

protein again was the dominant antigen. The porins, 23 kDal protein (stationary phase induced) and 18.5 kDal protein seemed to form a 'negative shadow', which possibly represented inadequate transferance onto the nitrocellulose paper and the proteins not fully regaining their antigenicity, because using whole cells, the porins and 23 kDal protein showed as being antigenic (Fig 3.16). The IRMPs of the two 014 strains and 012 serotype were predominant, but only a weak cross-reaction occurred with the other serotypes. It has been shown that LPS is associated with the IRMPs of E. coli on SDS-PAGE and immunoblotting (Chart and Griffiths, 1985). The immunogenicity of these proteins might, therefore, represent a reaction of antibodies in the rabbit antiserum with LPS antigen that has co-migrated with the IRMPs. In support of this theory, Edwards and Ewing (1972) report 012 and 014 are major cross-reacting antigens of S. marcescens, although on testing the antiserum to New CDC 014:H12 no rapid agglutination of 012 cells was observed.

At the leading edge of the gel in figure 3.15, there was a band that had the appearance of lipid A and core LPS (compare to Fig 3.7). This only showed in stationary phase cells grown in CDMFe- and not in cells grown in TSB and diluted HS.

Immunoblotting whole cells of the various serotypes (Fig 3.16) gave similar profiles to the OMs (Fig 3.15) except that there were more cross-reacting antigens and there was less of a 'negative shadow' effect. The 18.5 kDal protein did not show as being antigenic in both whole cells and OMs. The 23 kDal stationary phase induced protein was antigenic in whole cells, unlike in stationary phase TSB grown cells (results not shown). The porins of some strains in whole cells (Fig 3.16) reacted strongly, suggesting that epitopes lost on denaturation for SDS-PAGE were not restored on

transfer to nitrocellulose for other serotypes including New CDC. All of the IRMPs of O14 and O12 serotypes reacted and only a few from other strains, again suggesting interference from co-migrating LPS.

The blot of the OMs from five Gram-negative bacteria immunodetected with antiserum raised to New CDC cells grown to stationary phase in CDMFe- (Fig 3.17) shows that some antigens cross-reacted, especially within the Enterobacteriaceae family. For example, the OmpA protein, 20.5 kDal protein and even one of the IRMPs of E. coli cross-reacted, suggesting the presence of shared antigenic epitopes.

Figure 3.8

Immunoblot of Outer Membranes and Whole Cells of New CDC O14:H12 Detected with Pre-Immune Rabbit Serum

Key: Medium cells grown in to log phase/Cell preparation

1	TSB/OMs
2	Diluted HS/OMs
3	TSB/Whole cells
4	Diluted HS/Whole cells

The peroxidase-labelled conjugate used in the immunodetection proc in Figs 3.8 to 3.14 was goat-anti-rabbit IgG.

1 2 3 4

Figure 3.9

Immunoblot of Outer Membranes and Whole Cells of New CDC 014:H12 Detected with Hyperimmune Antiserum Raised in Rabbits to New CDC Cells Grown to Log Phase in TSB

Key:	Cell preparation/Medium cells grown in to log phase
1	OMs/TSB
2	OMs/Diluted HS
3	Whole cells/TSB
4	Whole cells/Diluted HS

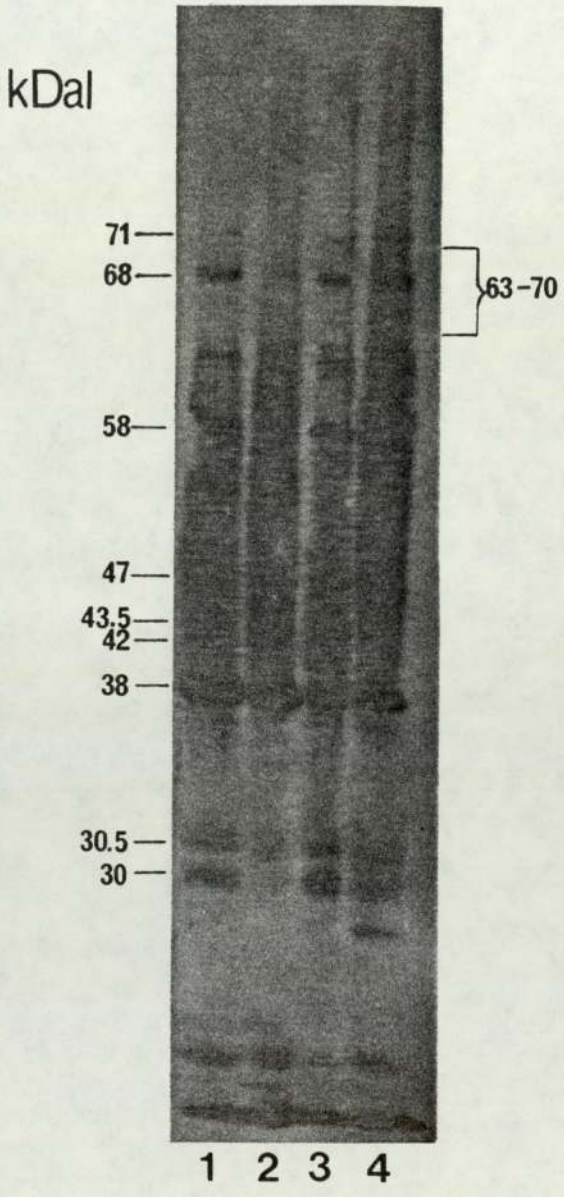

kDal
71
68
58
47
43.5
42
38
30.5
30
63-70
1 2 3 4

Figure 3.10

Laser Densitometer Scan of the S. marcescens New CDC O14:H12 Outer Membrane Immunoblot

OMs were isolated from cells grown to log phase in TSB. Immunodetection was with hyperimmune antiserum raised in rabbits to log phase TSB grown New CDC cells.

Key:

a	Photographic profile
b	Laser densitometer scan of the immunoblot
c	Laser densitometer scan of the photographic negative of the immunoblot

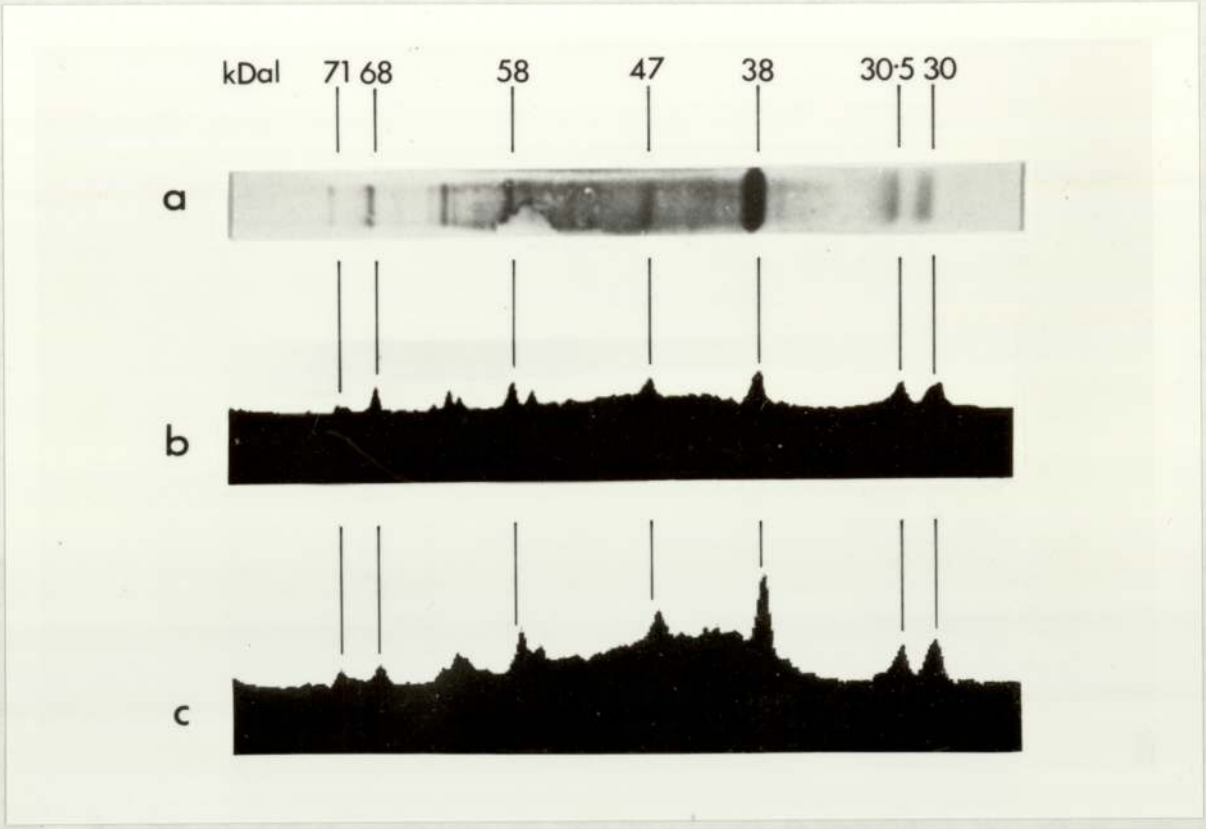

kDal
71
68
58
47
38
30·5
30
a
b
c

Figure 3.11

Immunoblot of Outer Membranes and Whole Cells of New CDC O14:H12 Detected with Hyperimmune Antiserum Raised in Rabbits to New CDC Cells Grown to Log Phase in Diluted HS

Key:	Cell preparation/Medium cells grown in to log phase
1	OMs/TSB
2	OMs/Diluted HS
3	Whole cells/TSB
4	Whole cells/Diluted HS

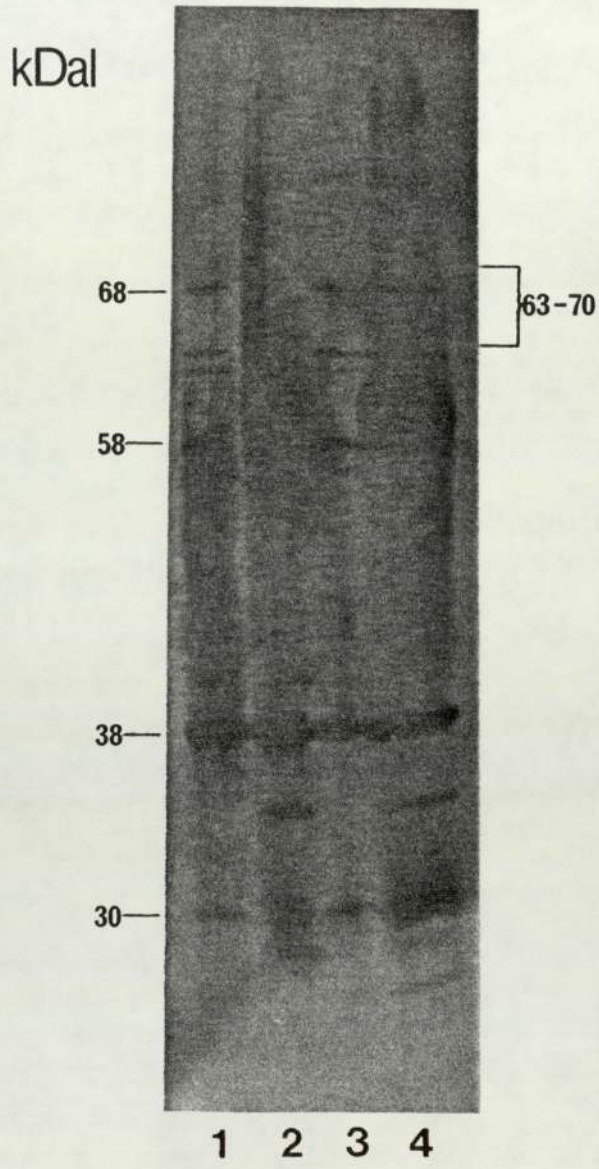
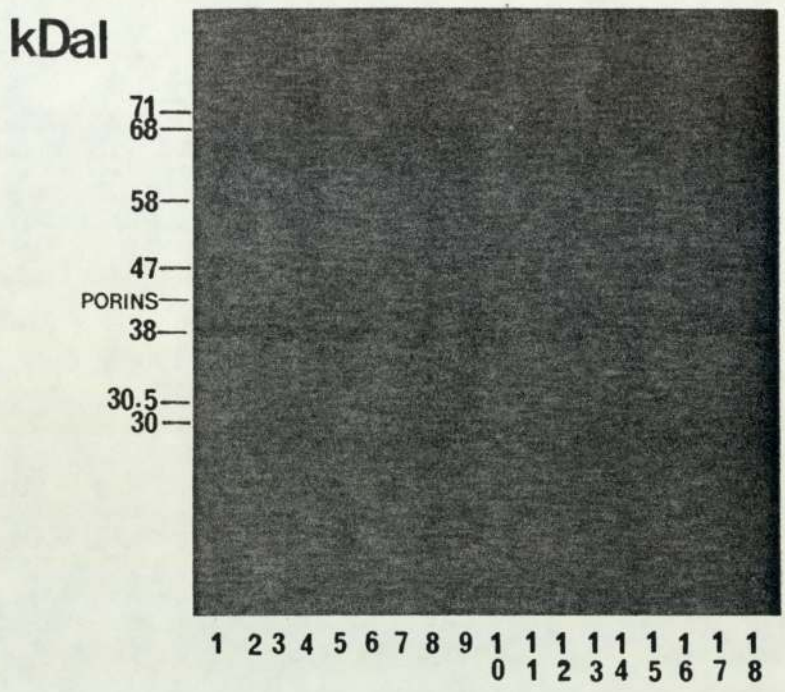

kDal
68
58
38
30
63-70
1 2 3 4

Figure 3.12

Immunoblot of Outer Membranes Isolated from Different Strains of S. marcescens Detected with Hyperimmune Antiserum Raised in Rabbits to New CDC O14:H12 Cells Grown to Log Phase in TSB

The first of the pair are the OMs isolated from log phase TSB grown cells, the second from log phase diluted HS grown cells.

Key:	Strain
1,2	New CDC O14:H12
3,4	4444-60 O14:H12
5,6	1783-57 O14:H9
7,8	874-57 O14:H12
9,10	S818 O14:H12
11,12	S1220 O14:H12
13,14	CDC O3:H1
15,15	CDC O6:H3
17,18	SF133 O3:H12
19,20	New CDC O14:H12
21,22	6358
23,24	6380I
25,26	6380II
27,28	9623
29,30	10682
31,32	14715
33,34	14815
35,36	16191
37,38	32201


kDal
71
68
58
47
PORINS
38
30.5
30
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

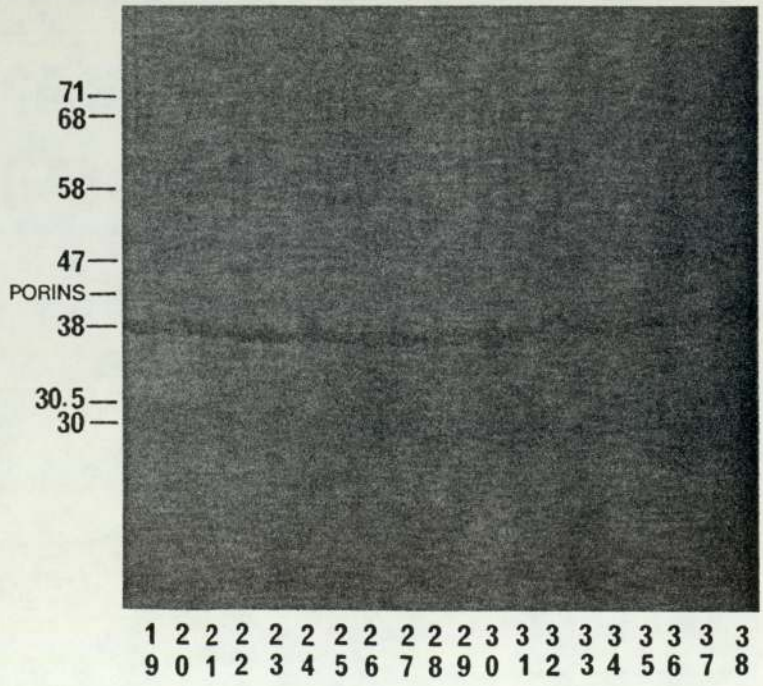
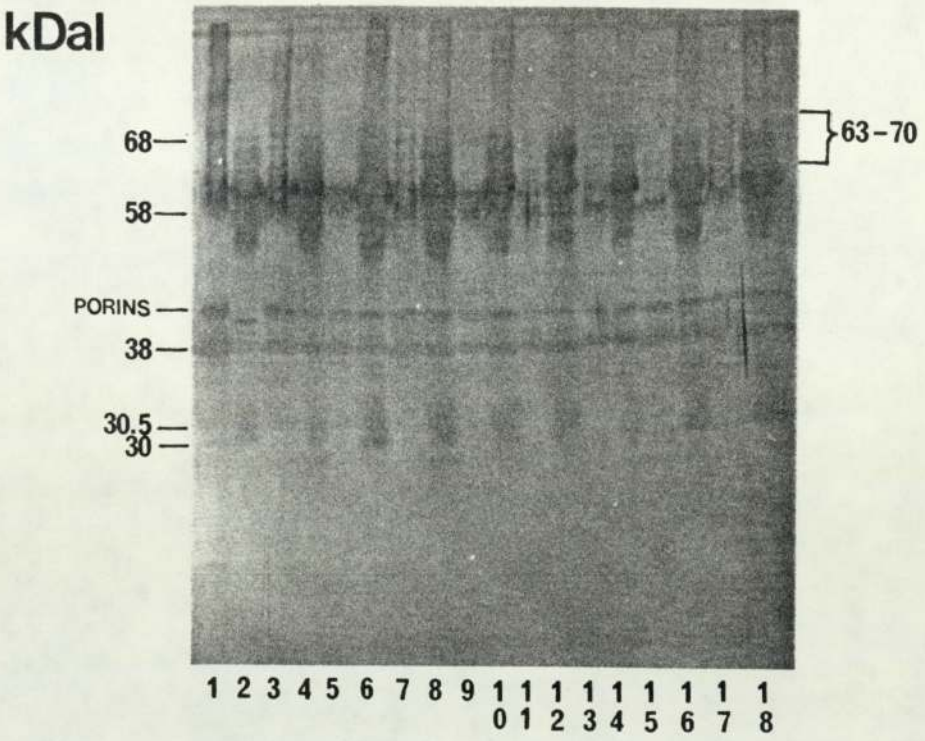

71
68
58
47
PORINS
38
30.5
30
19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38

Figure 3.13

Immunoblot of Outer Membranes Isolated from Different Strains of S. marcescens Detected with Hyperimmune Antiserum Raised in Rabbits to New CDC O14:H12 Cells Grown to Log Phase in Diluted HS

The first of the pair are the OMs isolated from log phase TSB grown cells, the second from log phase diluted HS grown cells.

Key:	Strain
1,2	New CDC O14:H12
3,4	4444-60 O14:H12
5,6	1783-57 O14:H9
7,8	874-57 O14:H12
9,10	S818 O14:H12
11,12	S1220 O14:H12
13,14	CDC O3:H1
15,16	CDC O6:H3
17,18	SF133 O3:H12
19,20	New CDC O14:H12
21,22	6358
23,24	6380I
25,26	6380II
27,28	9623
29,30	10682
31,32	14715
33,34	14815
35,36	16191
37,38	32201


kDal
68
58
PORINS
38
30.5
30
63-70
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

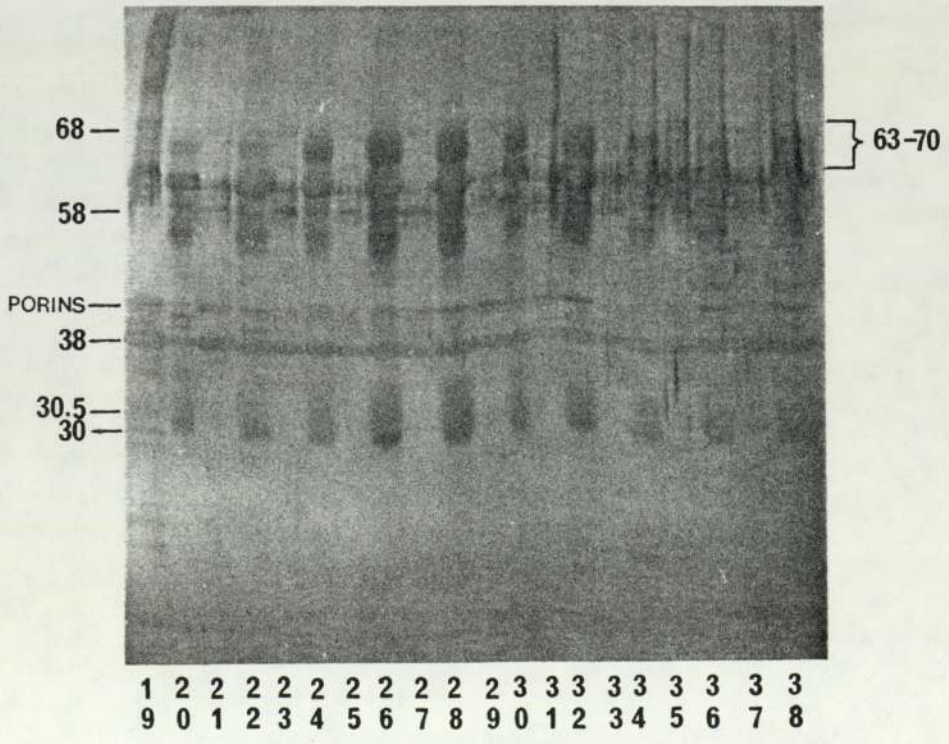

68
58
PORINS
38
30.5
30
63-70
19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38

Figure 3.14

Immunoblot of Outer Membranes and Whole Cells of New CDC O14:H12 Detected with Hyperimmune Antiserum Raised in Rabbits to New CDC Cells Grown to Log Phase in CDMFe+ or CDMFe-

A Antiserum raised to cells grown in CDMFe+

B Antiserum raised to cells grown in CDMFe-

Key: Cell preparation/Medium cells grown to log phas

1 OMs/CDMFe+

2 OMs/CDMFe-

3 Whole cells/CDMFe+

4 Whole cells/CDMFe-

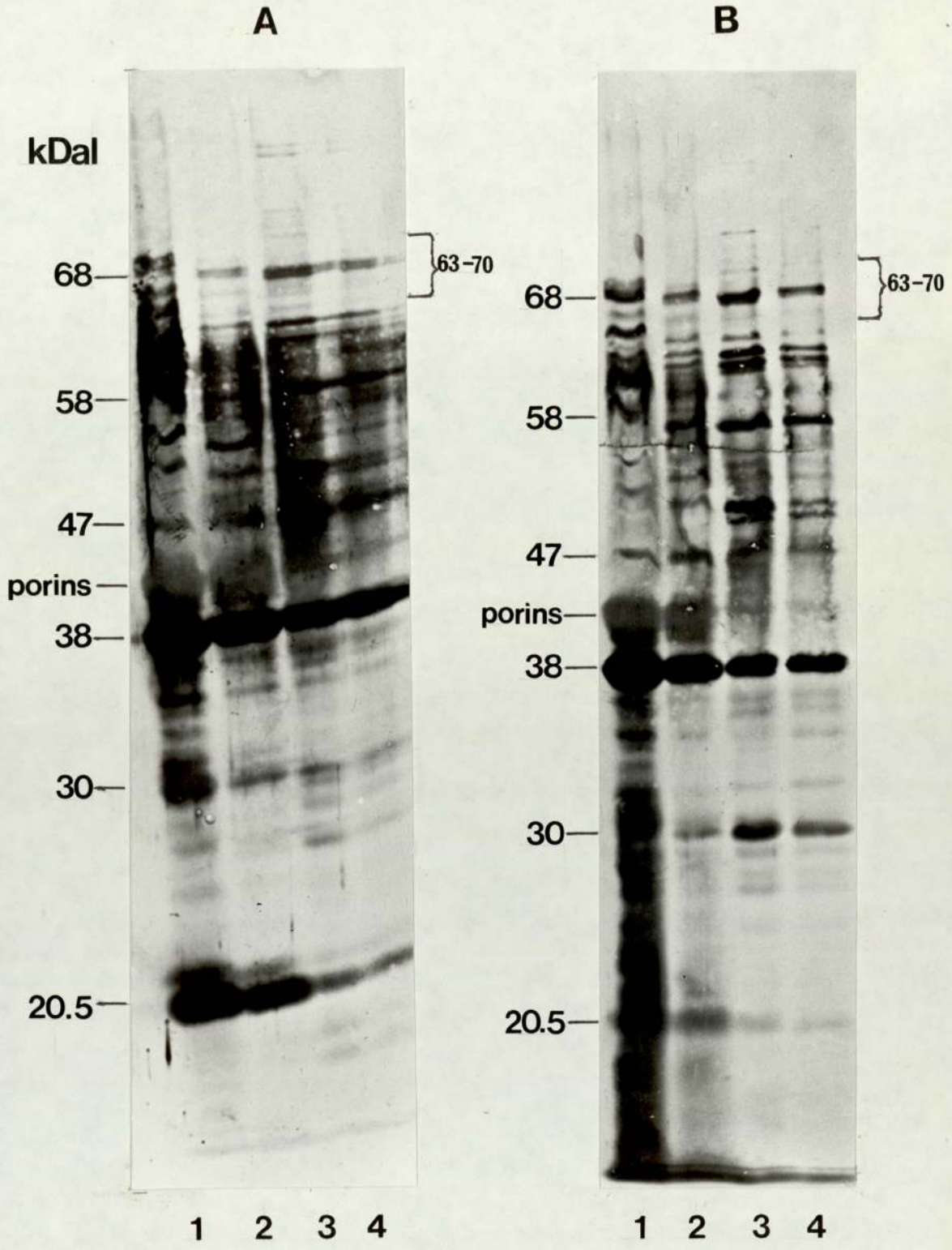

A
B
kDal
68
58
47
porins
38
30
20.5
63-70
1 2 3 4

Figure 3.15

Immunoblot of Outer Membranes Isolated from Different Serotypes o

marcescens Detected with Hyperimmune Antiserum Raised in Rabbits

New CDC Cells Grown to Stationary Phase in CDMFe-

Outer membranes were prepared from cells grown to stationary phas

CDMFe-.

Key:	Serotype
1	01
2	02
3	03
4	04
5	06
6	07
7	08
8	09
9	010
10	012
11	013
12	New CDC 014:H12
13	4444-60 014:H12
14	015

The peroxidase-labelled conjugate used in the immunodetection proc

in Figs 3.15 to 3.17 was Protein A.

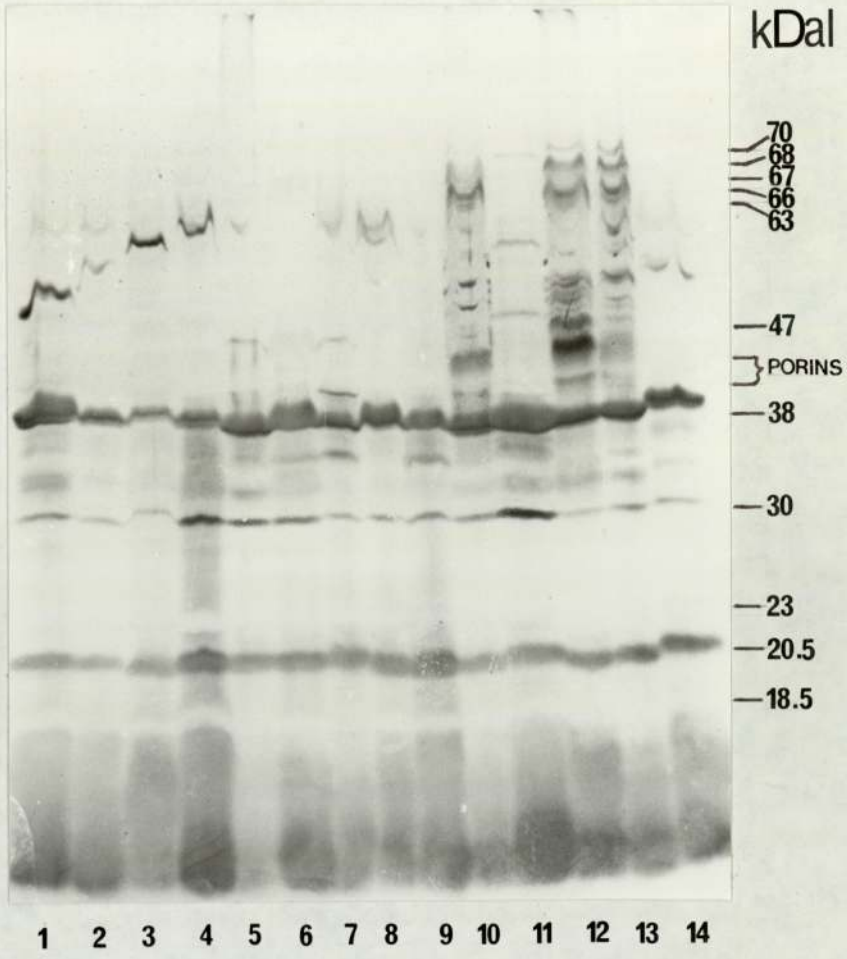

kDal
70
68
67
66
63
47
PORINS
38
30
23
20.5
18.5
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 3.16

Immunoblot of the Whole Cells of Different Serotypes of S. marces
Detected with Hyperimmune Antiserum Raised in Rabbits to New CDC
Cells Grown to Stationary Phase in CDMFe-

Cells were grown to stationary phase in CDMFe-.

Key:	Serotype
1	01
2	02
3	03
4	04
5	06
6	07
7	08
8	09
9	010
10	012
11	013
12	New CDC 014:H12
13	4444-60 014:H12
14	015

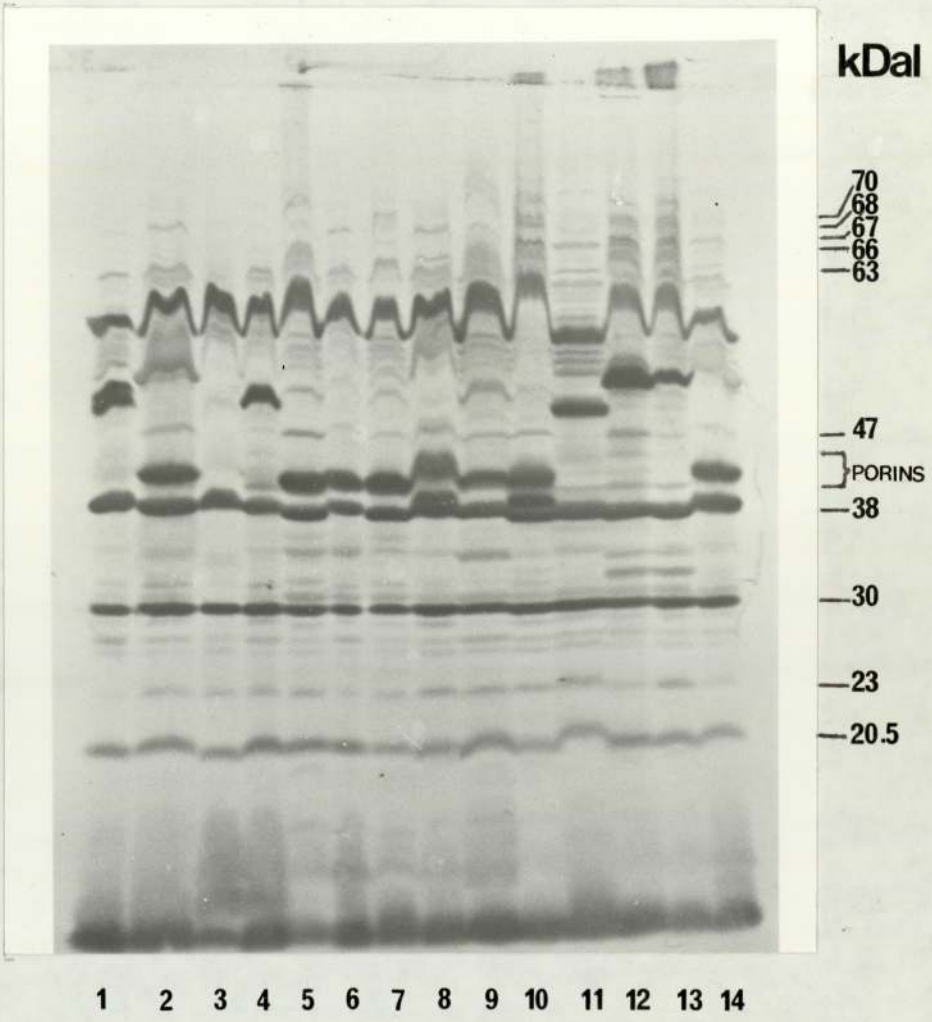
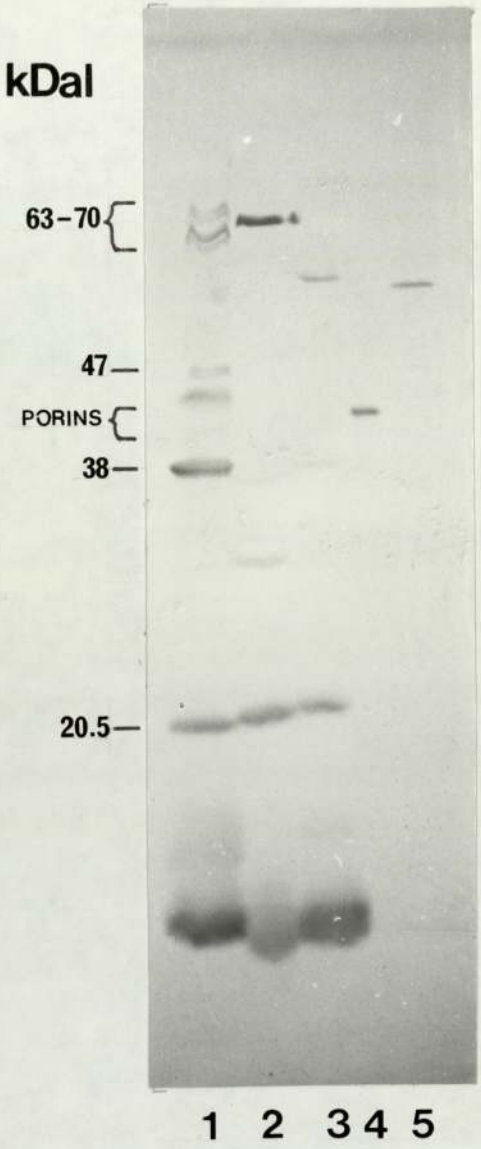

kDal
70
68
67
66
63
47
PORINS
38
30
23
20.5
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 3.17

Immunoblot of Outer Membranes Isolated from Five Different Gram-negative Bacteria Detected with Hyperimmune Antiserum Raised in Rabbits to New CDC Cells Grown to Stationary Phase in CDMFe-

OMs were isolated from cells grown to stationary phase in iron-restricted medium (CDMFe-).

Key:	Organism
1	*S. marcescens* New CDC 014:H12
2	*E. coli* GH 126
3	*K. aerogenes* NCTC 5055
4	*P. mirabilis* 196
5	*Ps. aeruginosa* NCTC 6750


kDal
63-70
47
PORINS
38
20.5
1 2 3 4 5

3.1.4 Antigens accessible to antibodies on the cell surface

Figure 3.18 shows photographic prints and figure 3.19 laser densitometer scans of the OMs, whole cells, LPS, flagella and surface accessible antigens of S. marcescens New CDC 014:H12 after growth in TSB to log phase, separated by SDS-PAGE and immunoblotting.

The results of separating OMs and LPS (lanes 1 and 2) by SDS-PAGE have been described previously (see 3.1.1 and 3.1.2). Isolated flagella (lane 3) ran as a single major band of flagellin (50 kDal) with several minor bands of lower molecular weight just detectable by coomassie blue staining of heavily overloaded gels. A corresponding 50 kDal band was not detected in the OM preparations (lane 1) presumably because most of the flagella were released during sonic disruption and Sarkosyl extraction.

The results of immunoblotting OMs and whole cells (lanes 4 and 5) have been discussed in section 3.1.3. Isolated LPS on immunodetection (lane 6) showed a faint ladder pattern, but there was no evidence that the fast migrating rough LPS was antigenic, even though it was present in large quantities in the isolated LPS (lane 2). The antibodies were therefore directed against the O antigen rather than the LPS core region. The major band in isolated flagella (50 kDal) was strongly antigenic (lane 7) and could just be identified in the whole cell and OM immunoblots. Other minor bands in the isolated flagella were also antigenic.

Lane 8 represents antigens exposed on the surface of whole cells and able to react with antibodies. Immunoabsorption of antiserum by whole cells followed by recovery and analysis of the immune complexes shows that very few of the whole cell antigens were accessible to immunoglobulins on the cell surface. The only antigens detected in

this way were the major flagella band (50 kDal) and the LPS (lane 8). Heavy chains of IgG run as a molecular weight band of 50 kDal on SDS-PAGE. Passing a volume of rabbit serum over a protein A-sepharose column and subjecting this to blotting showed that although bands representing the heavy and light chains of IgG (50 and 25 kDal) were detected on the nitrocellulose by amido black staining, they did not give positive bands on immunodetection with goat-anti-rabbit IgG peroxidase (results not shown). This proves that the 50 kDal surface exposed antigen was the flagella and not rabbit IgG in the sample. Other controls showed that little antigenic material was derived from cell debris included in the sepharose gel (lane 9) or from the protein A (lane 10).

Repeating the experiment with cells and antiserum of New CDC grown in diluted HS, CDMFe- and CDMFe+ to log phase, gave identical results with only the flagella and LPS antigens being accessible to antibodies on the cell surface.

Figure 3.18

Antigens Accessible to Antibodies on the Cell Surface of New CDC O14:H12

Cells and components were isolated after growth of New CDC O14:H1 TSB to log phase.

Key:

1	OMs separated by SDS-PAGE and stained with coomassie blue
2	LPS separated by SDS-PAGE and stained with sil
3	Flagella separated by SDS-PAGE and stained wit coomassie blue
4	OMs
5	Whole cells
6	LPS
7	Flagella
8	Antigens surface accessible to antibodies raise in rabbits to log phase TSB grown New CDC O14:H cells isolated on Protein A-sepharose after Zwittergent lysis of whole cells (see 2.2.2.4)
9	Control for 8 using whole cells absorbed with buffer in place of serum
10	Control consisting of untreated protein A-sepharose washed with Zwittergent, water and ethanol

4 to 10 are immunoblots detected with hyperimmune antiserum raised rabbits to log phase cells of New CDC O14:H12 grown in TSB. The peroxidase-labelled conjugate used was as in Fig 3.8.

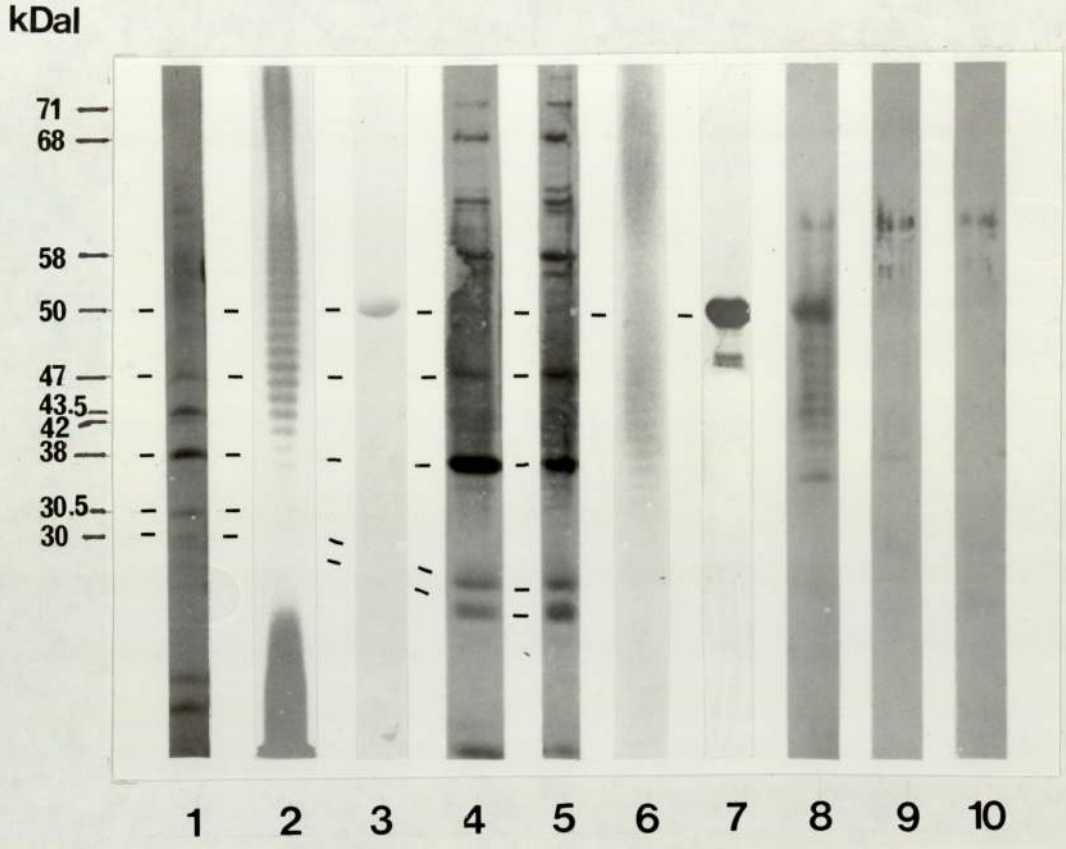
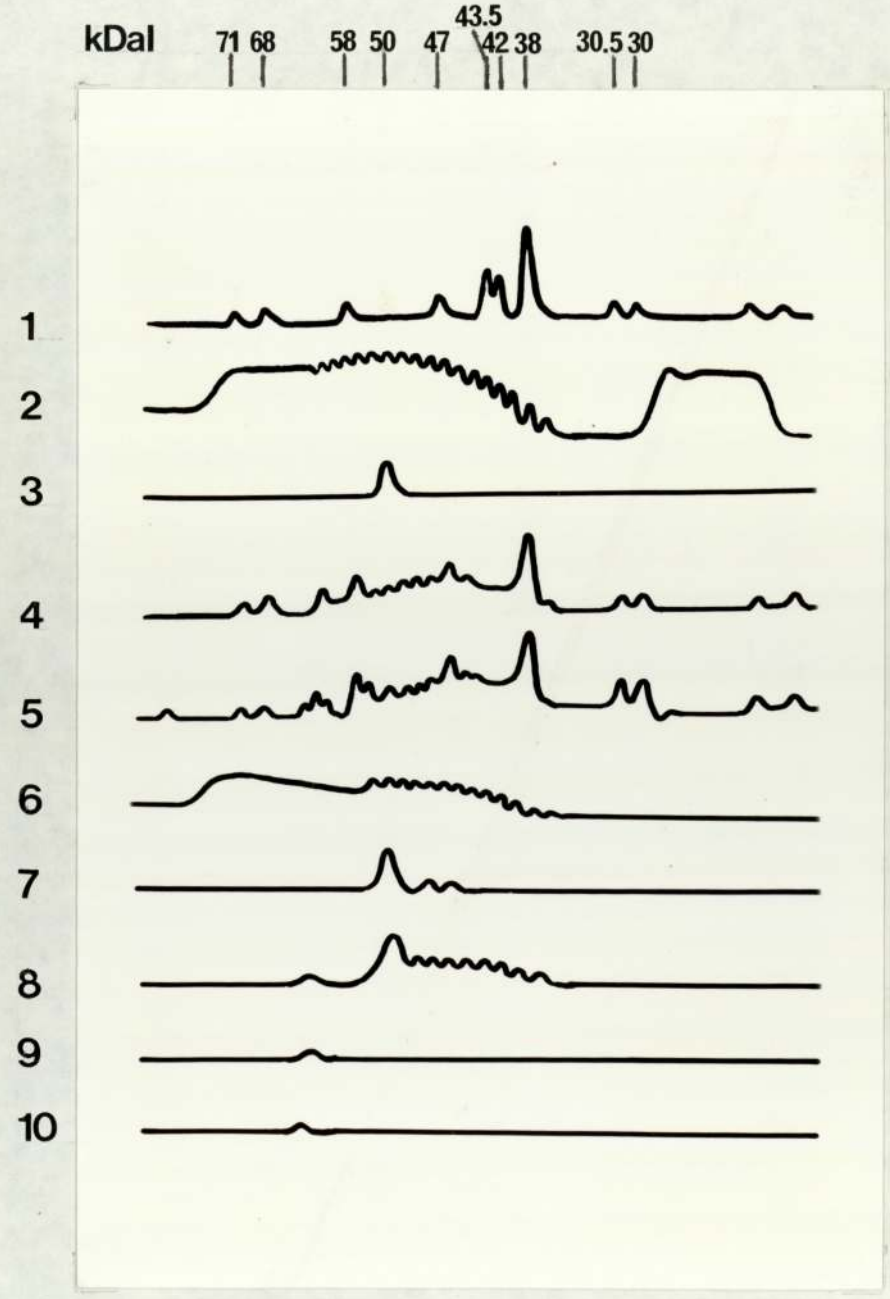

kDal
71
68
58
50
47
43.5
42
38
30.5
30
1
2
3
4
5
6
7
8
9
10

Figure 3.19 Laser Densitometer Scan of Antigens Accessible to Antibodies on the Cell Surface of New CDC O14:H12



See Fig. 3.18 for legend

3.1.5 Soluble antigens of S. marcescens

Figures 3.20 to 3.22 are results obtained by preparing soluble antigens according to the method of Roig et al (1983) after growth of cells in TSB. Different strains of serotypes O14:H12, O3:H1, O6:H3, O3:H12 and O14:H9 were used.

Double diffusion immunoprecipitation is a simple method that measures reactions of antigens and antibodies by formation of precipitin lines on diffusion in agarose containing gels. Double diffusion immunoprecipitation of the soluble antigen preparations with antiserum raised to New CDC whole cells grown in TSB (Fig 3.20), shows there was one common antigen that formed a continuous precipitin line. Additional bands were present, but only one gave complete fusion.

The results of separating the soluble antigens by SDS-PAGE and subsequently immunoblotting using antiserum raised to New CDC whole cells grown in TSB, are shown in figure 3.21 A and B. The blot (Fig 3.21, B) suggests that there were five common soluble antigens in the preparations, of estimated molecular weight 77, 61, 58, 46 and 38 kDal, SDS-PAGE profiles (Fig 3.21, A) showing that they were proteins. The soluble antigens appeared to be present in the OMs of New CDC (Fig 3.21 B, lane 12). This is not surprising considering that the antiserum used to detect the soluble antigens was raised to whole cells of New CDC. Also, the fact that Roig et al (1983) used a coagglutination test with their soluble antigens suggests surface localisation.

Isoelectrofocusing (IEF) is a technique that separates proteins in a native conformation with respect to pI. A linear and stable pH gradient is established and maintained in an agarose gel by carrier

ampholytes. When the electrical field is applied, the carrier ampholytes, having a small molecular weight in comparison with proteins, will rapidly migrate and form the pH gradient. Once established, proteins in applied samples will separate and focus at a point in the gel where the pH is equal to the pI of the proteins. The soluble antigen preparations after subjection to IEF (Fig 3.22, A) were found to possess proteins of pI values in the pH range 3 to 7. Transferring the proteins after focusing onto nitrocellulose paper by capillary blotting allowed proteins to be transferred in a totally natural state. Immunodetection with antiserum raised to New CDC whole cells grown in TSB (Fig 3.22, B) showed that a common antigen was again observed in the different preparations.

The one common soluble antigen found in double diffusion and IEF blotting experiments could possibly be the Serratia specific antigen found by Roig et al (1983). Of the five detected by immunoblotting, it is unsure which was the specific antigen. The 38 kDal protein was probably the OmpA protein, known to cross-react with other Gram-negative bacteria (Hofstra et al, 1980) and therefore not the common antigen. Candidates are therefore the 46 kDal, 58 kDal, 61 kDal or 77 kDal soluble antigens.

Figures 3.23 to 3.26 are results obtained using soluble antigen preparations from different serotypes of S. marcescens after growth in CDMFe-, as opposed to TSB. The SDS-PAGE protein profiles of the soluble antigen preparation (Fig 3.23) showed a complicated pattern of proteins that were slightly different to those obtained after growth in TSB (Fig 3.21, A). The two O14:H12 serotypes had identical profiles with a dominant protein of estimated molecular weight 50 kDal, that was possibly the flagella that had shed into the medium. The other serotypes gave similar yet individual patterns of soluble

antigens, more variation being seen than in figure 3.21 A because of the greater number of strains used.

Immunoblotting of the soluble antigen preparations and detection with antisera raised in rabbits to whole cells of New CDC (Fig 3.25), or to the soluble antigens of New CDC (Fig 3.24), gave nearly identical profiles. There was no common antigen that was readily identifiable, unlike after growth in TSB, but there were many cross-reactions that gave similar yet individual profiles for each serotype. Of interest is the fact that antiserum raised to the soluble antigens identified most of the OM components of New CDC (Fig 3.24, lane 15). Soluble antigen preparations are likely to contain antigens that have been shed or excreted into the medium. Shedding would involve mainly OM components (eg LPS, flagella), whereas other antigens would be excreted from the intracellular environment (eg proteases).

Using the soluble antigen antiserum to immunoblot whole cells (Fig 3.26), many cross-reactions were again observed and, interestingly, the porins, OmpA and 30 kDal protein were detected. The proposed flagella of the two 014 strains were antigenic, as were the IRMPs, although the antigenicity of the latter and perhaps the other proteins may have been influenced by the presence of co-migrating LPS material (see also section 3.1.3). The antiserum was raised to excreted or shed antigens only and therefore the antigenic profiles were less complicated to those using whole cell antiserum in the immunodetection process of whole cells (Fig 3.16).

Figure 3.20

Double Diffusion Immunoprecipitation of Soluble Antigens from Different Strains of S. marcescens

The soluble antigens were prepared from TSB medium after growth a removal of the cells. Antiserum in the middle well was raised in rabbits to whole cells of New CDC grown to log phase in TSB.

1	New CDC O14:H12
2	4444-60 O14:H12
3	CDC O3:H1
4	CDC O6:H3
5	SF133 O3:H12
6	1783-57 O14:H9
7	874-57 O14:H12
8	S818 O14:H12
9	S1220 O14:H12

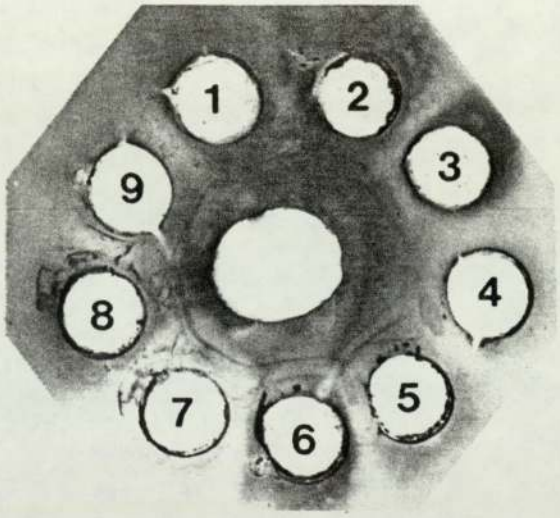

1
2
3
4
5
6
7
8
9

Figure 3.21

SDS-PAGE and Immunoblot Profiles of Soluble Antigens from Differe
Strains of S. marcescens

A SDS-PAGE protein profiles

B Immunoblot profiles using antiserum raised in
rabbits to whole cells of New CDC grown to log
phase in TSB. The peroxidase-labelled conjuga
used in Figs 3.21 and 3.22 was as in Fig 3.8.

Soluble antigens were prepared from TSB medium after gro
and removal of the cells.

Key:

1 Control, dialysed and freeze-dried TSB

2 Control, TSB treated with ammonium sulphate,
dialysed and freeze-dried

3 New CDC O14:H12

4 4444-60 O14:H12

5 CDC O3:H1

6 CDC O6:H3

7 SF 133 O3:H12

8 1783-57 O14:H9

9 874-57 O14:H12

10 S818 O14:H12

11 S1220 O14:H12

12 OM of New CDC isolated after growth of cells to
stationary phase in TSB

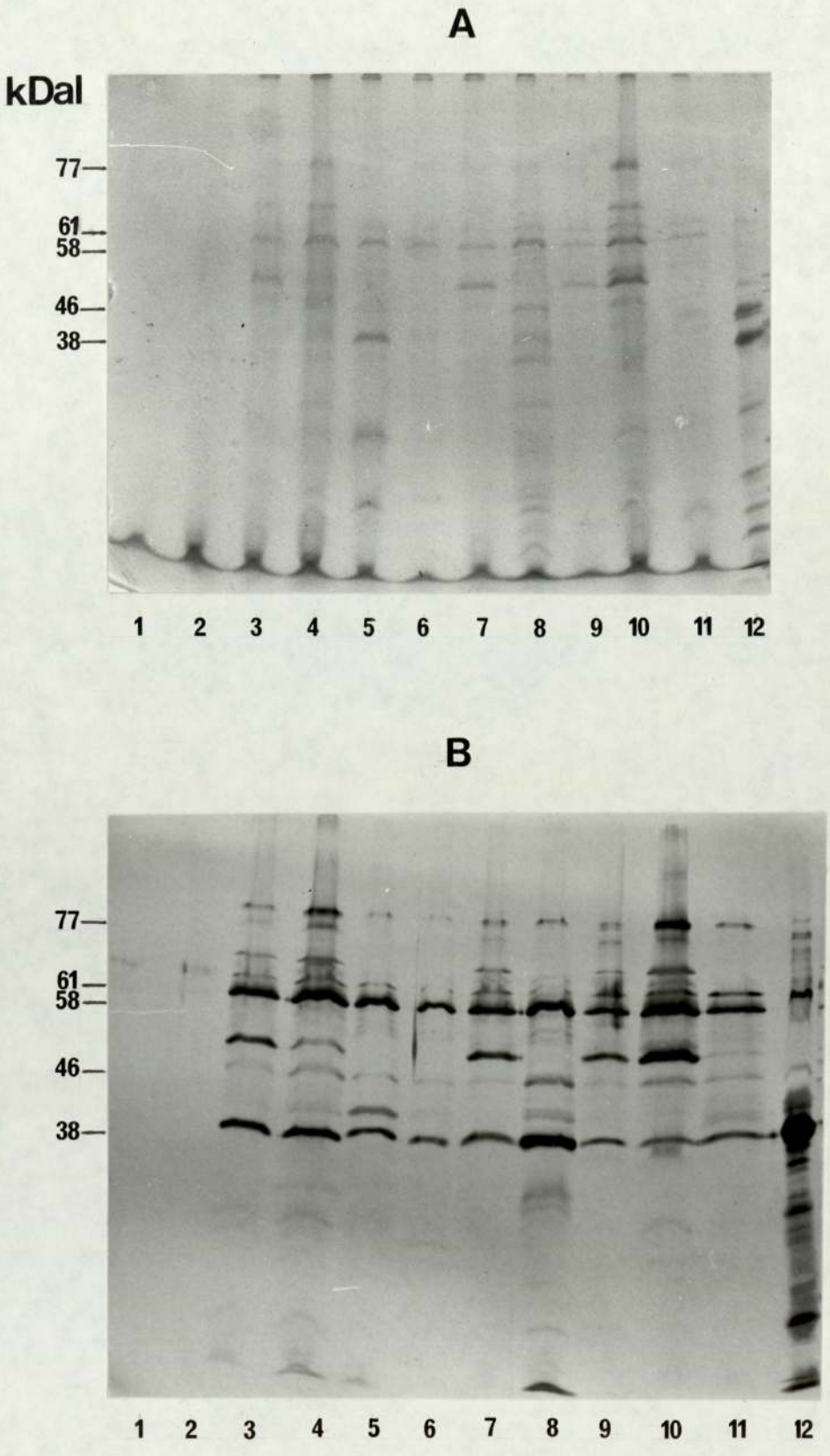

A
kDal
77
61
58
46
38
1 2 3 4 5 6 7 8 9 10 11 12
B
77
61
58
46
38
1 2 3 4 5 6 7 8 9 10 11 12

Figure 3.22

Isoelectrofocusing Protein Profiles and Blotting after IEF of Sol
Antigens from Different Strains of S. marcescens

A IEF protein profiles, pH range 3 - 10

B Blotting after separation of proteins by IEF (
range 3 - 10) using antiserum raised in rabbits
whole cells of New CDC grown to log phase in T

Soluble antigens were prepared from TSB medium after grow
and removal of the cells.

Key:

1 New CDC O14:H12

2 4444-60 O14:H12

3 CDC O3:H1

4 CDC O6:H3

5 SF133 O3:H12

6 1783-57 O14:H9

7 874-57 O14:H12

8 S818 O14:H12

9 S1220 O14:H12

10 Control, dialysed freeze-dried TSB

11 Control, ammonium sulphate treated TSB, dialyse
and freeze-dried

12 pI standard markers

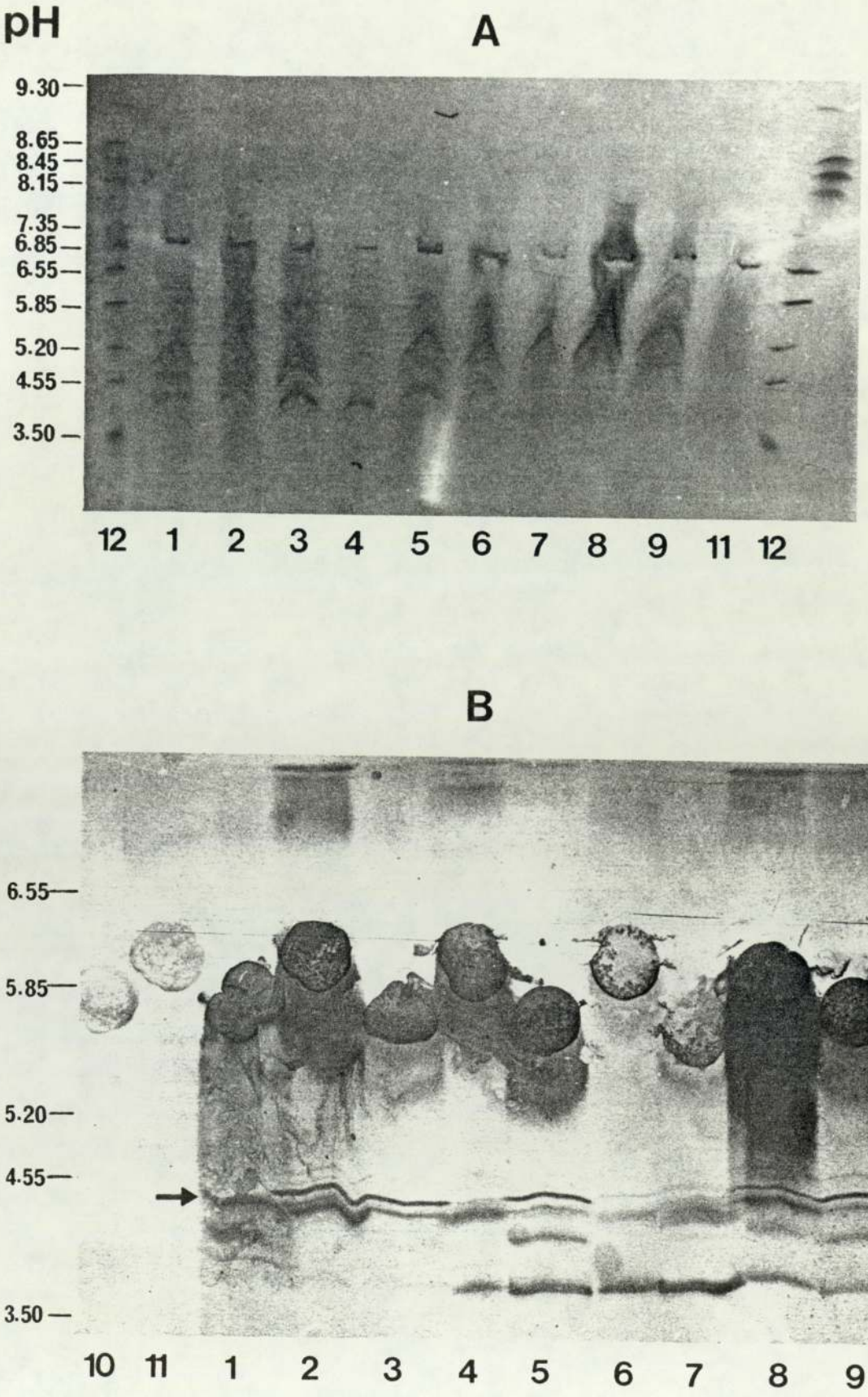

pH
A
9.30
8.65
8.45
8.15
7.35
6.85
6.55
5.85
5.20
4.55
3.50
12 1 2 3 4 5 6 7 8 9 11 12
B
6.55
5.85
5.20
4.55
3.50
10 11 1 2 3 4 5 6 7 8 9

Figure 3.23

SDS-PAGE Protein Profiles of Soluble Antigens from Different Serotypes of S. marcescens

Soluble antigens were prepared from CDMFe- medium after growth and removal of the cells.

Key:

1	01
2	02
3	03
4	04
5	06
6	07
7	08
8	09
9	010
10	012
11	013
12	New CDC 014:H12
13	4444-60 014:H12
14	015

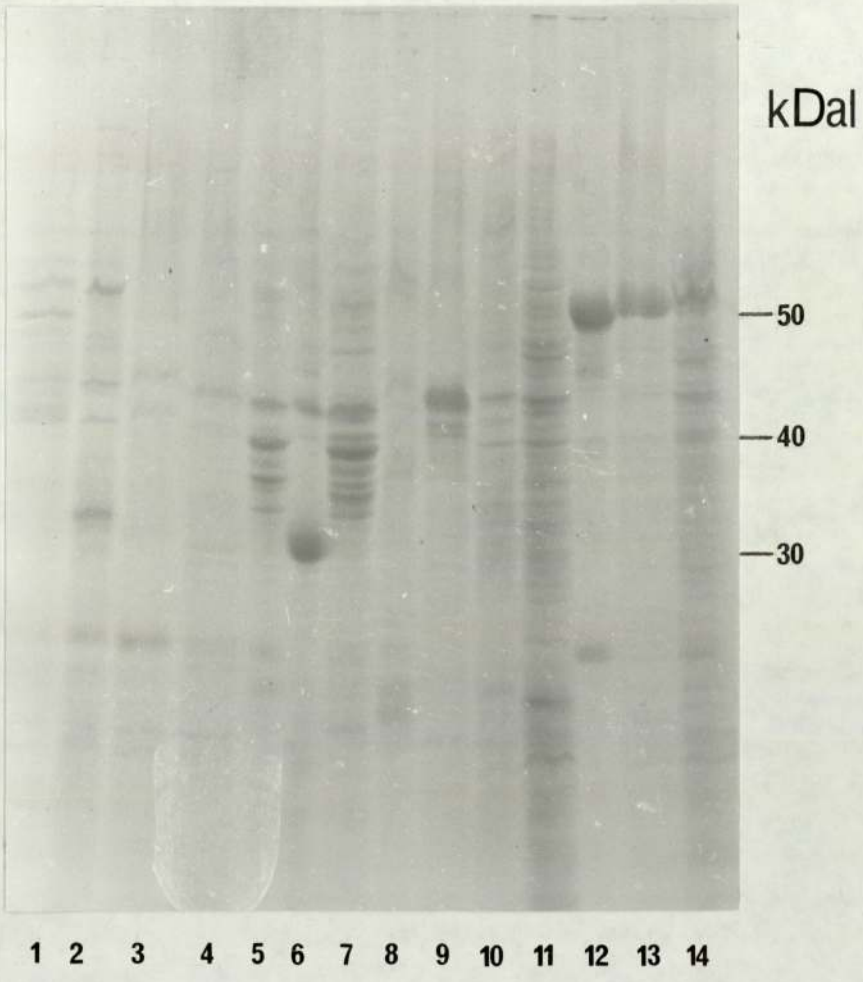

kDal
50
40
30
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 3.24

Immunoblot of Soluble Antigens from Different Serotypes of *S. marcescens* Detected with Hyperimmune Antiserum Raised in Rabbits Soluble Antigens from New CDC O14:H12

Soluble antigens were prepared from CDMFe- medium after growth and removal of the cells.

Key:

1	O1
2	O2
3	O3
4	O4
5	O6
6	O7
7	O8
8	O9
9	O10
10	O12
11	O13
12	New CDC O14:H12
13	4444-60 O14:H12
14	O15
15	OM of New CDC isolated from stationary phase ce grown in CDMFe-

The peroxidase-labelled conjugate used in Figs 3.24 to 3.26 in the immunodetection process was Protein A.

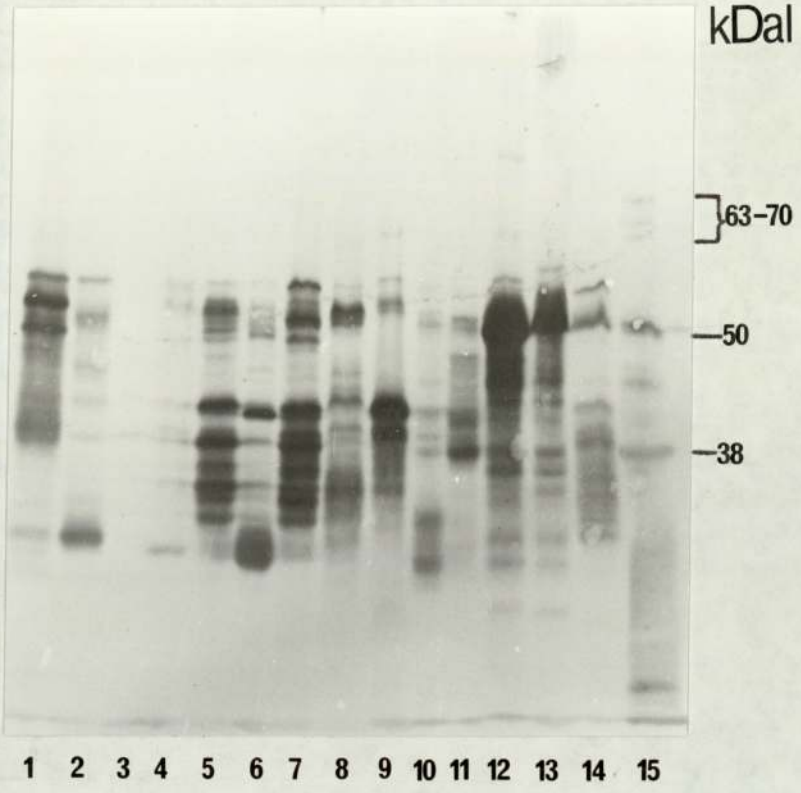

kDal
63-70
50
38
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 3.25

Immunoblot of Soluble Antigens from Different Serotypes of *S. marcescens* Detected with Hyperimmune Antiserum Raised in Rabbits New CDC Cells Grown to Stationary Phase in CDMFe-

Soluble antigens were prepared from CDMFe- medium after growth and removal of the cells.

Key:

1	01
2	02
3	03
4	04
5	06
6	07
7	08
8	09
9	010
10	012
11	013
12	New CDC 014:H12
13	4444-60 014:H12
14	015
15	OM of New CDC isolated from stationary phase ce grown in CDMFe-

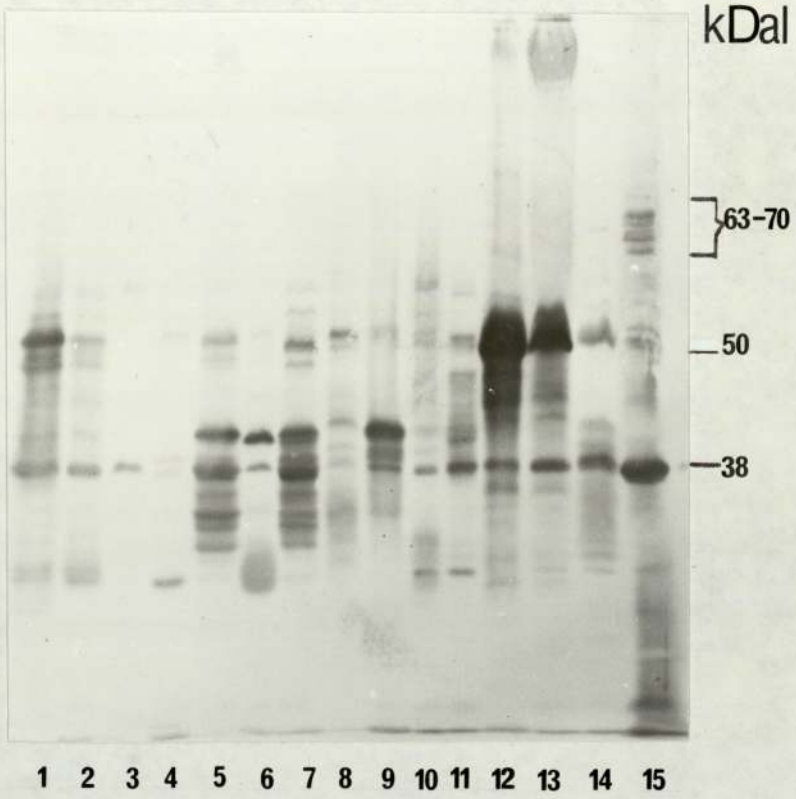

kDal
63-70
50
38
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

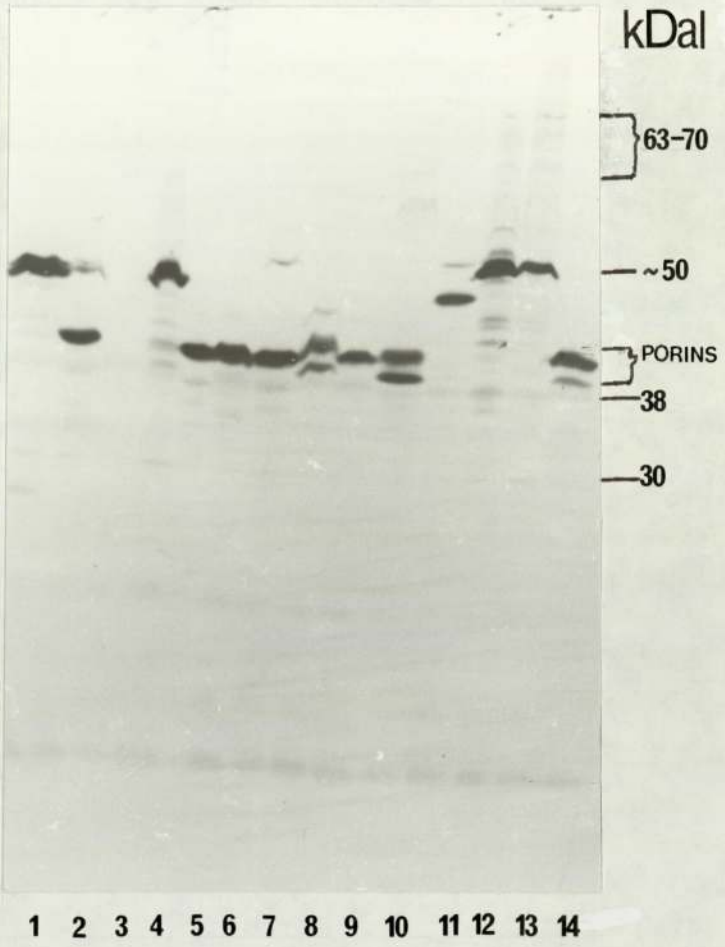
Figure 3.26

Immunoblot of the Whole Cells of Different Serotypes of S. marces
Detected with Hyperimmune Antiserum Raised in Rabbits to Soluble
Antigens Prepared from CDMFe- medium after Growth and Removal of
CDC O14:H12 Cells

Cells were grown to stationary phase in CDMFe-.

Key:

1	O1
2	O2
3	O3
4	O4
5	O6
6	O7
7	O8
8	O9
9	O10
10	O12
11	O13
12	New CDC O14:H12
13	4444-60 O14:H12
14	O15


kDal
63-70
~50
PORINS
38
30
1 2 3 4 5 6 7 8 9 10 11 12 13 14

3.1.6 Discussion

The protein composition of the OM of S. marcescens is similar to that of other Gram-negative organisms with the porins and OmpA protein representing the major proteins.

One of the nutrients essential for bacterial growth is iron. In an iron-deficient environment, growth of many Gram-negative organisms results in the induction of a number of high molecular weight OMPs (Neilands, 1982). Bacteria obtained from human CF lung infection (Brown et al, 1984) or directly from human urine without subculture (Lam et al, 1984; Shand et al, 1985), express these high molecular weight proteins in vivo. In E. coli, some of the induced OMPs are known to function as receptors for high affinity (Braun et al, 1983) and low affinity (Hartman and Braun, 1981) iron uptake systems. In comparison to other organisms, the iron uptake systems of S. marcescens are poorly studied. Traub (1977) reported S. marcescens produces Fe chelators in an iron-free defined broth medium. In this study, five IRMPs in the molecular weight range 63 to 70 kDal were found to be induced after growth in diluted HS or CDMFe-. It seems reasonable to assume that these function as receptors for iron salts or iron-siderophore complexes, but further work would be required to confirm this.

Little is known about the mechanisms by which high affinity iron transport systems are regulated. In E. coli, the biosynthesis of the 83, 81 and 74 kDal IRMPs and enterochelin are co-ordinately regulated by the intracellular iron concentration (McIntosh and Earhart, 1977). Nutrient-depleted cells characteristically are stationary phase cells. However, changes in envelope properties may occur several generations before onset of stationary phase due to depletion of a

specific nutrient. Using iron-depleted media in batch culture, K. aerogenes was found to express its high affinity uptake system about three generations before onset of stationary phase (Williams et al, 1984). Likewise with S. marcescens at log phase (two generations before onset of stationary phase), IRMPs were induced on growth in CDMFe-, but weakly compared to when the cells were totally nutrient-depleted at stationary phase. Diluted HS is naturally iron-restrictive and the IRMPs were therefore strongly expressed at exponential phase. The IRMPs seemed to be repressed by the addition of an excess of free iron to CDM and diluted HS media. Thus it appears S. marcescens is capable of rapidly responding to low iron levels in the extracellular environment, an ability which may contribute to the virulence of the organism.

Growth to stationary phase in all media saw the production of a 23 kDal protein. In batch culture, growth occurs at an optimal rate until it becomes restricted and subsequently stopped by exhaustion of some nutrient(s) or accumulation of excreted products. This protein might therefore represent a nutrient binding protein, although K. aerogenes showed no differences in its cell envelope composition after growth to early exponential and stationary phase in nutrient broth (Sterkenburg et al, 1984).

Growth rate, growth medium and specific nutrient limitation are all important in determining the composition of the cell envelope. In an iron-restricted environment, eg serum, the doubling times of Gram-negative bacteria may be increased (Bullen et al, 1974). S. marcescens had a slightly increased doubling time (30 minutes), shorter log phase and smaller cell mass after growth in diluted HS compared to TSB (DT = 20 minutes). In CDM doubling times were even greater (60 minutes). However, this did not affect the major

proteins present. Expression of the 42 kDal porin protein seemed to be reduced, and the 43.5 kDal porin increased, on growth in CDM compared to the other media, an effect more likely linked to composition of the growth medium rather than growth rate. In E. coli the OmpF/OmpC ratio is high for cells grown in minimal medium, intermediate for cells grown in complex medium without sugar, and low for cells grown in complex medium containing sugar (Lugtenberg et al, 1976). Inducement of identical IRMPs appeared to occur in the two different iron restricted/depleted media. Shand et al (1985) found the pattern of high molecular weight proteins in Ps. aeruginosa altered, depending on whether it was cultured in body fluids (serum or urine) or laboratory media. Sciortino and Finkelstein (1983) using Vibrio cholerae discovered in vivo preparations of OMs exhibited some unique proteins not present in in vitro grown iron-depleted cells. Further work is required to see if S. marcescens produces the same or different IRMPs after in vivo growth.

The ability to acquire low levels of nutrients in addition to generating a characteristic envelope, will influence growth rate and thus the attainment of critical bacterial populations crucial in determining the outcome of an infection. In vivo bacterial doubling times are reported to be slow (Smith, 1977), which is probably true in chronic infections. In acute infections, growth is likely to be faster. Sordelli et al (1985) report initial doubling times in the lungs of mice exposed to Ps. aeruginosa were approximately 30 minutes and in the peritoneal cavities 20 minutes, which are more comparable to the in vitro growth rates of S. marcescens used in this study. The overall doubling time found in vivo (usually slow) will depend upon actual growth rate and clearance. When growth rate increases beyond the point where clearance is dominant, an infection will

result. Thus bacteria usually have actual growth rates that are fast (comparable to *in vitro* rates), the influence of clearance giving an overall slow growth (Ogata, 1983).

Cells grown in diluted HS on harvesting produced a cell pellet slightly different to that from TSB grown cells. It was tightly packed, friable and more difficult to resuspend, suggesting the cells had different surface properties. Holbein (1981) using *Neisseria meningitidis* found the same occurrence on addition of normal human serum to the growth medium. Testing showed the cells bound serum proteins, including transferrin, albumin and immunoglobulins. This has important implications on the interaction of bacteria with the host in infections. Another group of workers (Korpela et al, 1984) found *S. marcescens* bound avidin, a glycoprotein induced in inflammation and secreted by macrophages. The OMP profiles of *S. marcescens* New CDC grown in diluted HS seem to suggest horse serum proteins have become bound to the cells, especially in the region of the IRMPs, and possibly in the 26.5 kDal region. The binding must be tight for the proteins to remain attached to the OMs on washing and Sarkosyl extraction. When discussing future results, the presence of these proteins will be borne in mind.

The LPS of *S. marcescens* New CDC resolved into a multibanded ladder pattern on SDS-PAGE that can be attributed to the high sensitivity of the Silver stain, which permits loading of small amounts of isolated LPS or proteinase K digests of whole cells onto the gels. Tsang et al (1976) previously reported that LPS from an O8 strain of *S. marcescens* separated into two distinct bands on staining with a carbocyanine dye, a less sensitive technique. Growth media and, more likely, growth rate affected the patterns obtained with *S. marcescens* New CDC, longer O antigen molecules being present in cells

grown in diluted HS compared to TSB.

Immunoblotting of S. marcescens New CDC showed that OMs and whole cells contain a complex pattern of antigens, the most prominent antigens being the OmpA protein and other OM proteins of 71, 68, 58, 47, 30.5 and 30 kDal after growth in TSB. The weak antigenic reaction of the porins after electrophoresis and electroblot transfer is surprising, since porins of some organisms react strongly after similar treatment. For example, protein F of Ps. aeruginosa can be detected on immunoblots (Anwar et al, 1984) even though it is transferred with low efficiency (Mutharia et al, 1982). The porins of P. mirabilis (Driver and Lambert, 1984) and Neisseria gonorrhoeae (Hook et al, 1984), also retain their antigenicity after transfer. The porins of S. marcescens have been shown to be antigenic and cross-reactive with porins of other enterobacteria by methods other than immunoblotting (Hofstra et al, 1980). It must therefore be concluded that epitopes lost on denaturation for SDS-PAGE are not restored on transfer to nitrocellulose in a majority of cases, as some immunoblots do show the porins as being antigenic.

After growth of S. marcescens New CDC in diluted HS, the IRMPs, 71, 47 and 30.5 kDal proteins gave virtually no antigenic reactions. Considering that in CDMFe-, the stationary phase iron-depleted cells had IRMPs that elicited an antigenic response, perhaps HS components bound to the cells interfered with the raising of antibodies to bacterial cell antigens, either directly, or by altering the cell surface characteristics. This has important consequences when considering potential OM components of organisms as vaccines, growth in diluted HS mimicing the in vivo environment more closely than TSB or chemically defined media.

Identification of the surface exposed antigens of New CDC

accessible to antibodies, involved the isolation of the flagella. This ran as a single 50 kDal band on SDS-PAGE which compares with similar flagellin preparations in the 50-60 kDal range for E. coli, S. typhimurium and Ps. aeruginosa (Kondoh and Hotani, 1974; Montie and Stover, 1983) and 40 kDal for P. mirabilis (Driver and Lambert, 1984). Other surface appendages of bacteria that are important as virulence factors in helping to mediate adherence of bacteria to host surfaces, are pili (Ofek and Beachey, 1980). Five different kinds of fimbriae have been identified in S. marcescens (Old et al, 1983). Kohno et al (1984) purified the pili of S. marcescens US5 which separated on SDS-PAGE as two bands of 19 and 39 kDal. Rhen et al (1983) using E. coli KS71 found the P-fimbriae consisted of 3 peptides of 22, 19 and 17.3 kDal. Electro-Blot radioimmunoassay after SDS-PAGE of cell extracts has been used to show the presence of pili (O'Donnell et al, 1983). It can be speculated that the lower molecular weight bands in the OMs of SDS-PAGE and immunoblot profiles represent pili, although their release during sonic disruption and Sarkosyl extraction (like that of flagella) may occur. Irvin et al (1984) found the fimbriae of Alysiella bovis remained associated with the cell envelope, even when sonicated or exposed to detergents (eg Triton X-100). Further work is obviously required to determine the extent of pilation of S. marcescens strain New CDC.

Work involved in determination of surface exposed antigens showed that the O antigen of isolated LPS can be transferred to nitrocellulose and immunodetected without loss of pattern, as reported by other workers (Driver and Lambert, 1984; Karch et al, 1984); the apparent lack of antigenicity of the lipid A/core structure assumes rough LPS is actually transferred in the blotting process.

The immunoabsorption technique of Swanson (1981) relies on a few general assumptions. Firstly, that only antigens exposed on the cell surface are available for interaction with antibodies, and that these antigen-antibody interactants are then bound to Protein A-sepharaose. This depends on the cells remaining intact during incubation with antiserum. Certainly unbound antibodies are removed by washing before detergent lysis, which could otherwise expose hidden antigens able to combine with antibodies. Another assumption is that individual components are solubilised by Zwittergent, which is not potent enough to disrupt antibody-antigen bonds, and that each one is physically separated from other outer membrane constituents. False positive results are obtained if membrane components have an affinity for each other that is so strong, they are not dissociated by the Zwittergent. Then antibody to one of these components will co-precipitate the other component, which may or may not be surface exposed (Loeb, 1984). This problem is more associated with the contamination of protein antigens with another protein.

The results using S. marcescens show the LPS O antigen and flagella are the only surface antigens accessible to antibodies. This is in agreement with earlier studies on surface localisation of LPS (Smit et al, 1975; Mülradt and Golecki, 1975), and is consistent with the accepted model of the OM in which LPS is present exclusively on the outer face and the flagella a surface appendage (Lugtenberg and van Alphen, 1983).

Although OM proteins dominate the antigenic profiles revealed by SDS-PAGE and immunoblotting, they are not accessible to antibody molecules on the surface of intact cells. One explanation for this is that the O antigens of LPS mask the proteins, interfering with access of antibodies to epitopes on protein antigens exposed on the

outer face of the OM. Alternatively, epitopes recognised by the antiserum by immunoblotting might not be exposed on the surface of proteins in their native configuration in the OM. Likewise, the lack of antibodies to the lipid A/core structures (assuming successful tranferance to nitrocellulose) agrees with the suggestion that the presence of core oligosaccharides and O antigen polysaccharides shields lipid A/core components from inducing the formation of antibodies and/or from reacting with antibodies (Perez-Perez et al, 1986; Luderitz et al, 1966).

Masking of antigenic epitopes on OM proteins by O antigen side chains of LPS has recently been found to occur in other Enterobacteriaceae. Van der Ley et al (1986) studied the accessibility of monoclonal and polyclonal antibodies to the PhoE porin protein of several different O serotypes and rough mutants of Enterobacteriaceae, grown under phosphate limitation. Intact cells were exposed to antibodies and after adding ^{125}I-protein A, radioactive counts were measured. Antibody binding only occurred in rough strains shown to lack O antigen side chains on SDS-PAGE of LPS. The workers concluded the O antigen side chains prevented antibodies binding to the PhoE protein on the surface of intact cells, this essentially complete shielding not being limited to one type of LPS or antigenic epitope. The PhoE protein has homology with the OmpC and OmpF proteins, and the latter are immunochemically similar to those of other Enterobacteriaceae. Therefore, assuming shielding for all pore proteins, the authors suggest the porins should not be used as vaccines, most clinical isolates of Enterobacteriaceae synthesising smooth LPS. Taken together, the results of this study together with the results of van der Ley and co-workers, suggest the O antigen side chains of LPS have the ability to mask all the

proteins of the OM and, therefore, OMPs of Enterobacteriaceae have little potential for vaccine development.

The whole cell and OM antigenic profiles of a range of different strains and serotypes of S. marcescens and different Gram-negative bacteria, showed that many antigens were cross-reactive. One of the cross-reacting proteins was the OmpA protein. Braun and Cole (1984), using cloning techniques, found the OmpA gene from S. marcescens was fully expressed in E. coli, but that it was not functionally equivalent to the OmpA protein of E. coli. They showed three regions of the protein likely to be exposed on the cell surface differed extensively from corresponding regions of the E. coli and all other sequenced OmpA proteins. They suggested this represents a safety mechanism by which enterobacterial species avoid cross-infection by noxious agents (colicins/phages). Beher et al (1980) found the OmpA proteins from all strains of Enterobacteriaceae were antigenically identical, and nearly identical in primary structure, suggesting to them that the structure and function of the OmpA protein is strongly conserved during evolution. Other workers (Hofstra et al, 1980; Hofstra and Dankert, 1980) showed that the OmpA proteins of enterobacterial strains cross-reacted by CIE independently of molecular weight variation within strains, again indicating conservation. Many cross-reacting proteins of S. marcescens were found in this study using immunoblotting. However, cross-reactivity on the blots may not represent functional cross-reactivity, and could be the result of SDS-PAGE producing increased exposure of common antigens shared between bacteria of different strains and species (Hook et al, 1984). Thus the OmpA protein may well have a variable surface exposed region and a conserved peptide region buried in the OM, solubilisation of the OMs for SDS-PAGE possibly exposing these

otherwise 'hidden' antigens shared between bacteria.

Recently there have been a number of reports indicating that many protein bands detected by immunobloting are in fact protein-LPS complexes (Poxton et al, 1985; Gulig and Hansen, 1985). Co-migrating LPS with the IRMPs, first shown by Chart and Griffiths (1985) for E. coli, may be involved in the apparent antigenicity of S. marcescens IRMPs. Considerable cross-reactions exist between the O serogroups of S. marcescens (Young and Morris, 1980). Van Alphen et al (1983) found that the LPS of different serotypes of E. coli cross-reacted because of the presence of common determinants on their lipopolysaccharides. S. marcescens O8 LPS is reputed to cross-react with E. coli O18. Both contain N-acetyl-β-D-glucosamine 1-3 galactose branches and some monoclonal antibodies raised to E. coli O18 antigen did indeed react with S. marcescens O8 antigen (Pluschke et al, 1986). In another study, Traub (1981) found S. marcescens O antigens cross-reacted with those of other Enterobacteriaceae such as Shigella and Salmonella. Further work is required to determine whether or not co-migrating LPS cross-reacting influences the antigenic protein profiles obtained.

Members of the family Enterobacteriaceae usually do not secrete proteins into the surrounding medium (Lugtenberg and van Alphen, 1983). S. marcescens characteristically produces many extracellular enzymes, such as proteases, lipases and nucleases (Braun and Schmitz, 1980; Winkler et al, 1978). They are thought to be manufactured intracellularly and transported through the cell membrane into the external environment during stationary phase growth (Braun and Schmitz, 1980). Proteolytic activity within the purified OMs of S. marcescens and E. coli has been shown (Heller, 1979; MacGregor et al, 1979), although Schmitz and Braun (1985) could not find any protease

in their extracted OM. Reported molecular weights on SDS-PAGE of isolated enzymes from various strains of S. marcescens are 73, 56 and 60 kDal (Matsumoto et al, 1984), 51 k Dal (Braun and Schmitz, 1980), 52.5 kDal (Lyerly and Kreger, 1975) and 35 kDal (Yonemura et al, 1983).

It is tempting to speculate that some of the proteins found in the soluble antigen preparations in this study are extracellular enzymes. The soluble antigens of New CDC were all antigenic. Antiserum raised to them showed cross-reactions with a range of serotypes' whole cell antigens, and likewise antiserum to New CDC whole cells reacted with the soluble antigen preparations. Gulig et al (1984) found that two proteins in the culture supernatants of H. influenzae type b had antibodies directed against them when antiserum was raised to whole cells. One protein was shown to be present on the cell surface, the other only in the culture fluid. In the case of S. marcescens, the soluble antigens may be proteins/ peptides that are physically associated with LPS and have become shed into the medium in fragments of bacterial outer membranes, eg OmpA, porins, fimbriae (Russell, 1976). Others may be proteins, such as enzymes, that are associated with the cell surface and perhaps remain with the OM on purification, as well as being secreted into the culture medium. Further work is required to determine whether any of the soluble antigens actually have proteolytic activity.

It is interesting that after growth in CDM, the flagella antigen of the O14:H12 strains gave a much stronger response. It was present in the blots of whole cells and soluble antigens detected against homologous and heterologous antisera raised to whole cells and soluble antigens. It was less obvious in the OMs, probably because of loss of the flagella on sonic disruption of the cell. The other O

serotypes do not have H12 antigens (Rubin, 1980) and indeed a corresponding protein was not observed in their profiles.

Serotyping of S. marcescens has many problems associated with it. One of the main problems concerns cross-reacting antibodies in O-sera (Pitt and Erdman, 1984). The variety of polysaccharides on the surface of cells, many with shared chemical and antigenic epitopes amongst the serotypes, interfere with the typing methods. Wilkinson and co-workers are trying to chemically analyse the LPS from various serotypes, especially that of O14 so often found in clinical infections. A partially acetylated acidic glucomannan was found to be present in three O14 strains and one strain of the cross-reacting serogroup O6 (Brigden and Wilkinson, 1985). It is suggested that this polmer may be a (micro) capsular component responsible for O specificity as presently defined. Likewise, a neutral polymer of ribose and 2-acetamido-2-deoxygalactose was found in some O14 strains and an O12 reference strain, and could be the shared antigen responsible for the serological cross-reactions of these groups (Brigden, Furn and Wilkinson, 1985). Other polymers common between cross-reacting serotypes have been found, and thus a confusing picture is emerging.

Capsular (K) antigens of S. marcescens have been incompletely elucidated (Young and Morris, 1980). Traub et al (1985) have actually isolated K antigens from strain New CDC, although there has been no evidence of their presence in this study so far, possibly because they are closely associated with the LPS. Further work on the K antigens would help to fully determine the serology of S. marcescens.

Pitt and Erdman (1984) propose that after O and H serotyping, bacteriocin typing should be used to subdivide strains further.

However, this defines broad groups and it is not as discriminating as serotyping. Biotyping, based on the biochemical variability of the species, can also be used (Grimont and Grimont, 1978), but it is not reproducible. A better typing sysem is therefore required that can clearly discriminate and subdivide clinical isolates and strains of S. marcescens.

Subtyping based on the coomassie blue-stained OM patterns on gels might be of diagnostic value with Haemophilus influenzae (Barenkamp et al, 1981). OMs of the different serotypes of S. marcescens after growth in CDMFe- were similar, but careful inspection showed small differences in molecular weight and in minor proteins. Using a defined environment of one growth medium, exploitation of these differences could then be used in typing, but maybe they are too small to be of practical purpose.

Immunoblotting suggested that many protein components of the OMs shared a common peptide region that cross-reacted among strains of S. marcescens and this, together with the lack of surface accessibility to antibodies (or lack of antigenic epitopes on the surface), indicates the proteins are not of direct value for serotyping.

LPS silver-stained profiles of the different serotypes showed that too many strains had similar patterns for exploitation as a diagnostic tool. The similar patterns perhaps link some of the cross-reactions found in serotyping (eg O2 and O3, O12 and O14, O6 and O7 (Young and Morris, 1980), each two cross-reacting strains having nearly identical profiles) with the identical chemical polymers found in the different serotypes eg O12 and O14 (Brigden, Furn and Wilkinson, 1985). This assumption, however, relies heavily on the fact that biochemical and chemical differences are really reflected in the LPS profiles, as suggested by Hitchcock and

Brown (1983).

The soluble components could be used to subtype Serratia. Grimont et al (1977) subjected supernatant fluids to agar gel electrophoresis, which produced different patterns of proteinases, dividing strains into 33 different zymotypes. The protein patterns of soluble components released into the culture medium could therefore be exploited as an epidemiological tool.

With regards to a Serratial vaccine, the inaccessibility of OMPs to antibody molecules on the surface of intact cells (or the lack of antigenic epitopes on the surface of the OMPs) suggests that they are not of direct use. Possible masking of bacterial protein antigens by components in the host body fluids binding to the cell's surface (eg serum proteins), also throws doubt on the potential of OMPs as vaccines. The accessibility of LPS O antigen to antibodies, even after growth in diluted HS, and its possible masking of other antigens, together with the predominance of the O6 and O14 serotypes of S. marcescens in infections (Traub, 1982), suggests that LPS is a prime candidate for exploitation as a protective antigen in a vaccine against S. marcescens. Traub (1983) has already shown that antibodies directed against O antigens of challenge strains afford passive protection in mice and Traub et al (1985) found active immunisation with phenol-hot water LPS extracts of S. marcescens protects NMR1 mice against challenge with homologous strains of CDC O3:H1, CDC O6:H3, New CDC O14:H12 and O6/O14:H12 and also affords moderate cross-protection.

It is unfortunate that the common soluble antigen found by Roig et al (1983) to be carried by all strains of the genus Serratia, but not by other Gram-negative bacteria, could not be identified in the soluble antigen preparations in this study. Roig et al exploited the

antigen to produce antibodies and used them in a coagglutination test to aid rapid identification of Serratia. Further work is required to isolate the common antigen and determine its usefulness as a protective vaccine.

3.2 IMMUNOCHEMISTRY, ANTIBIOTIC SENSITIVITY AND SERUM SENSITIVITY OF THREE O14:H12 STRAINS OF S. MARCESCENS

3.2.1 SDS-PAGE of OMs and ^{125}I-labelling of whole cells

The results of separating the OMs of the three O14:H12 strains by SDS-PAGE after growth in TSB and diluted HS are shown in Figure 3.27. The major features were identical, with the OmpA and porin proteins strongly staining. A group of high molecular weight IRMPs appeared in the profiles of OMs from cells grown in diluted HS, extra washing of the membranes appearing to help remove some of the horse serum proteins that attached to the cells in this region (Fig 3.27, B).

Two methods of iodination were employed to study the exposure of the major OMPs on the cell surface of the three S. marcescens strains. [^{125}I]lactoperoxidase catalyses the iodination of tyrosine and histidine residues of proteins in the presence of H_2O_2. The [^{125}I]lactoperoxidase is too large to penetrate the OM (Mr=77500) and is therefore assumed to label only those proteins that have such residues exposed on the surface. Iodo-beads consist of the oxidant N-chloro-benzenesulphonamide immobilised on non-porous polystyrene spheres. Addition of Na ^{125}I generates an iodinating species that does not readily diffuse away from the bead and is thought to be N-iodo-benzenesulphonamide. Incorporation of radioiodide into the aromatic groups of tyrosine and phenylalanine residues, or into histidine residues, of proteins by the Iodo-beads then proceeds. Retention of the iodinating species by the bead allows the solid-state reagent to be specific for surface iodination only (Markwell, 1982).

After iodination, the amount of radiolabel bound to an equivalent number of cells was measured with a γ counter, including appropriate controls (Table 3.1). The amount of ^{125}I bound to S1220 cells after growth in diluted HS was affected by cell clumping. New CDC cells did not show this phenomenon, and 4444-60 cells clumped to a limited extent. By comparing the labelling of cells grown in TSB, it can be seen there was 50% less labelling of 4444-60 cells compared to New CDC and S1220 cells. Considering that the OMP profiles as shown by SDS-PAGE were identical (Fig 3.27), it suggests that 4444-60 cells contain a non-protein surface structure that prevents [^{125}I]lactoperoxidase reaching its binding sites. Counts from the controls confirmed that in the absence of lactoperoxidase, iodination did not proceed. Omitting H_2O_2, it appeared that labelling occurred, but to a much smaller extent. The results using Iodo-beads show that this method, at least for S. marcescens New CDC, did not iodinate the cells as efficiently as the [^{125}I]lactoperoxidase method.

Figure 3.28 represents the SDS-PAGE protein profiles of the ^{125}I labelled whole cells (A) and the subsequent autoradiograph (B). The coomassie blue stained whole cells showed many protein bands, their autoradiograph profiles being less complex and similar to the OMP profiles (Fig 3.28, A, 7-10). The porins and OmpA protein were most prominent in the autoradiographs and OMP profiles, although the porins in this system did not separate into two distinct bands like in Figure 3.27. Within the whole cells (Fig 3.28, A, 1-6) the 47 kDal and 58 kDal proteins were strongly stained, but in the OMs (lanes 7-10), they were stained to a lesser extent. The autoradiograph (Fig 3.28, B) shows that the 47 kDal protein of New CDC was accessible to surface labelling, but not that from 4444-60 or S1220. The 58 kDal protein was weakly labelled in all three strains. A

protein of estimated molecular weight 54 kDal was prominent by ^{125}I-labelling, although virtually undetected by coomassie blue staining of whole cells or OMs. The IRMPs induced after growth in diluted HS showed a heavily iodinated band (Fig 28, B), its diffuse nature suggesting interference from HS proteins attaching to the cells (see 3.2.2). Weaker bands were present in this region after growth of 4444-60 and S1220 cells in TSB suggesting that total repression of the IRMPs did not occur in iron-plentiful media. Unfortunately, with New CDC grown in TSB, the top part of the profile did not stain, but Figure 3.29, B shows this comment to be also true.

Below the OmpA protein, the 30.5 kDal protein was a defined band in coomassie blue stained whole cells (Fig 3.28, A lanes 1-6) being less so in OMs (lanes 7-10) of this particular experiment. The 30 kDal protein gave an opposite trend, being defined in OMs and less so in whole cells. A protein of estimated molecular weight 29.5 kDal was present in both profiles (Fig 3.28, A, 1-10). The autoradiograph of whole cells (Fig 3.28, B) shows that the 29.5 kDal protein was labelled, whereas the 30 kDal one was not and the 30.5 kDal protein only weakly iodinated. Another protein of estimated molecular weight 24 kDal was ^{125}I-labelled, this band staining with coomassie blue in whole cell profiles, but weakly in OMP profiles. The 20.5 kDal and 18.5 kDal proteins of the OMs did not stand out in whole cells, but the autoradiograph shows the 20.5 kDal protein was strongly iodinated.

Coomassie blue is used to stain proteins qualitatively and it is not very sensitive compared to the iodination technique. It is therefore not surprising that proteins ill-defined by staining became well defined on the autoradiograph. The OMP profiles of different strains of S. marcescens (Fig 3.2) show that protein bands were

present in the 58 and 54 kDal region, confirming that these are OM labelled proteins. However, the 24 kDal protein was weakly stained in all OMP gels suggesting that it may be derived from surface components retained by the cells during harvesting and washing prior to iodination.

The intensity of the bands on the autoradiographs reflected the amount of radiolabel bound to the cells (Table 3.1). S1220 cells grown in TSB (Fig 3.28, B, lane 5) gave a slightly darker banding compared to 4444-60 cells grown in TSB (lane 3), although the contrast was not as great as expected from the amount of ^{125}I bound to the cells (Table 1).

The protein profiles of the three strains were identical after growth in both media, the only difference being the 47 kDal labelled protein of New CDC. Obviously, no comment can be made about S1220 cells grown in diluted HS, but other results allow assumption that there would be no differences in the protein composition (Fig 3.27).

It is assumed that [^{125}I]lactoperoxidase is too large to penetrate the OM and therefore that unlabelled proteins are not surface exposed. However, non-iodinated proteins could also represent molecules without tyrosine or histidine residues on surface exposed regions of their polypeptides.

Results using Iodo-beads to label the surface exposed proteins of S. marcescens New CDC are shown in Figure 3.29. [^{125}I]lactoperoxidase appeared to be the more efficient method, the autoradiographs using Iodo-beads being very weak (Fig 3.29 B, lanes 2 and 4) and representing the low counts obtained (Table 3.1). There seemed to be no major differences in the surface proteins labelled using the two techniques, unlike with Haemophilus influenzae where both methods were found to label CM as well as OM proteins (Loeb, 1984). The

porins, which showed very weakly in the coomassie blue stained protein profiles (Fig 3.29, A), and OmpA protein were again the most heavily labelled bands (Fig 3.29, B). The 47 kDal and 54 kDal proteins were iodinated, but the 58 kDal band was not, unlike in Figure 3.28, B. The IRMPs were labelled as discussed before. The 10% w/v acrylamide concentration in the gel gave a better separation of the bands in the higher molecular weight region, and some additional proteins were resolved in this region. The relatively poorer separation of the protein bands in the lower molecular weight areas did not allow the 24 kDal and 20.5 kDal proteins to be distinguished. The 29.5 kDal protein was again radiolablled, but the 30.5 kDal one was not.

Autoradiographs of the lactoperoxidase controls (Fig 3.29, B, lanes 5 and 6) confirmed the amount of radiolabel bound to the cells (Table 3.1), with a very small amount of labelling occurring in the absence of H_2O_2. All other controls (lanes 7 and 8) gave an expected blank result.

In conclusion, it appears that the outer membrane proteins from three O14:H12 strains of S. marcescens that are consistently surface exposed bearing tyrosine, phenylalanine or histidine residues, are of molecular weight 43.5 and 42 kDal (the porins), 38 kDal (OmpA protein), 54 kDal, 63 to 70 kDal (IRMPs and/or HS proteins), 29.5 kDal and 20.5 kDal. In addition, strain New CDC has a surface located protein of molecular weight 47 kDal of unknown identity.

Figure 3.27

SDS-PAGE Protein Profiles of the Outer Membranes Isolated from th
O14:H12 Strains of S. marcescens after Growth to Log Phase

Key:	Bacteria/Media
1	New CDC/TSB
2	New CDC/Diluted HS
3	4444-60/TSB
4	4444-60/Diluted HS
5	S1220/TSB
6	S1220/Diluted HS
A	OMs washed once
B	OMs washed twice

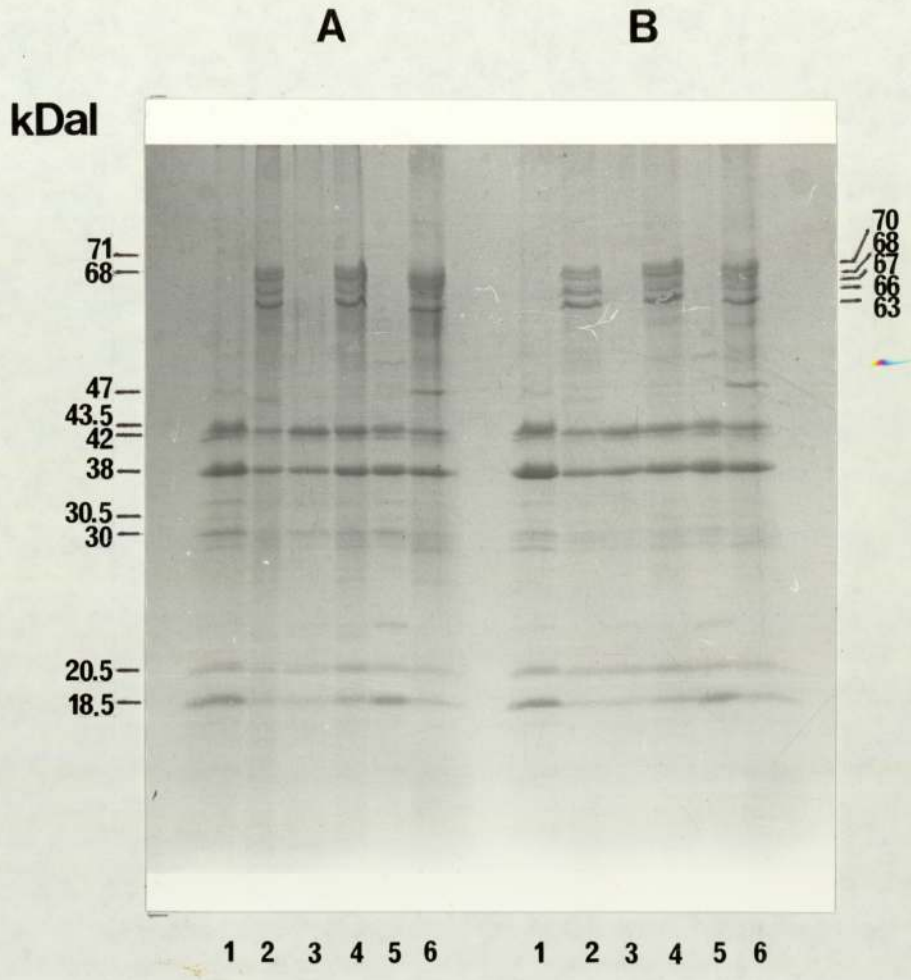

A
B
kDal
71
68
47
43.5
42
38
30.5
30
20.5
18.5
70
68
67
66
63
1 2 3 4 5 6
1 2 3 4 5 6

Table 3.1

Radiolabelling of whole cells with ^{125}I

Strain and Growth Media	Incorporation of ^{125}I into cells *				
	^{125}I-lactoperoxidase labelled	Control-omitting lactoperoxidase	Control-omitting H_2O_2	Iodo-bead labelled	Control-omitting Iodo-bead
New CDC, TSB	235056	880	14224	25324	304
New CDC, Diluted HS	285208			16540	2804
4444-60, TSB	123600				
4444-60, Diluted HS	58072				
S1220, TSB	249692				
S1220, Diluted HS	5736				

* Results are expressed as dpm of ^{125}I bound to 10^8 cells.

Figure 3.28

SDS-PAGE and Autoradiograph Profiles of ^{125}I-lactoperoxidase Labe
Whole Cells of three O14:H12 Strains of S. marcescens

A SDS-PAGE protein profiles

B Autoradiograph (96 hours) of A

All cells were grown to log phase.

Key: Strain and growth medium

1 New CDC ^{125}I-labelled whole cells, TSB

2 New CDC ^{125}I-labelled whole cells, diluted HS

3 4444-60 ^{125}I-labelled whole cells, TSB

4 4444-60 ^{125}I-labelled whole cells, diluted HS

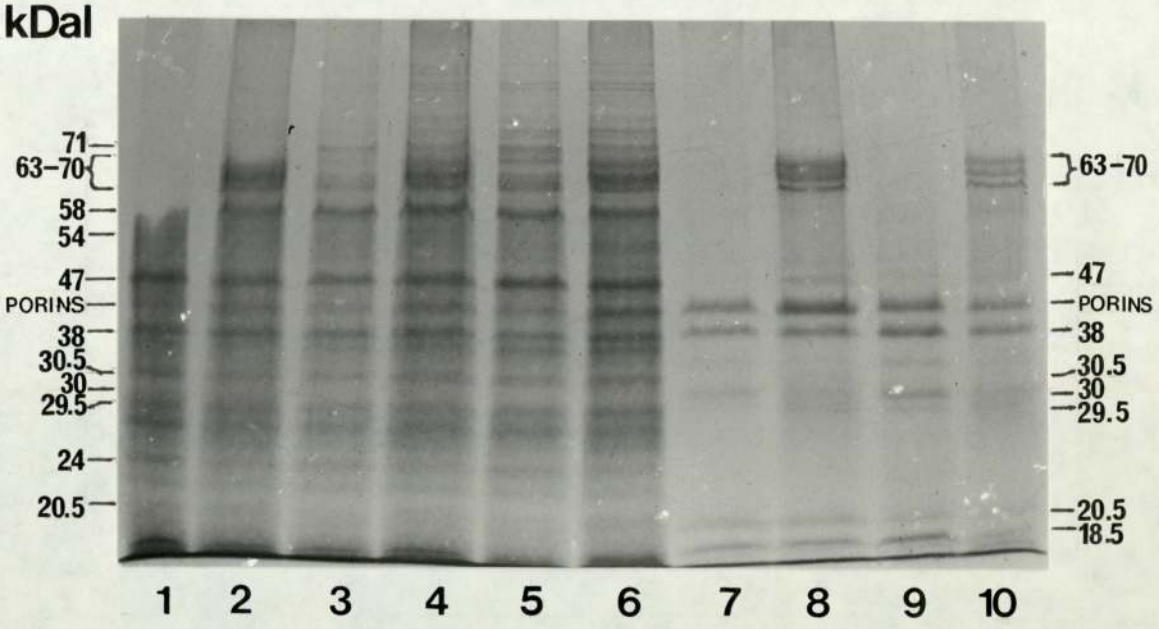
5 S1220 ^{125}I-labelled whole cells, TSB

6 S1220 ^{125}I-labelled whole cells, diluted HS

7 and 9 OMs isolated from New CDC after growth in TSB

8 and 10 OMs isolated from New CDC after growth in diluted HS

A


kDal
71
63-70
58
54
47
PORINS
38
30.5
30
29.5
24
20.5
63-70
47
PORINS
38
30.5
30
29.5
20.5
18.5
1 2 3 4 5 6 7 8 9 10

B

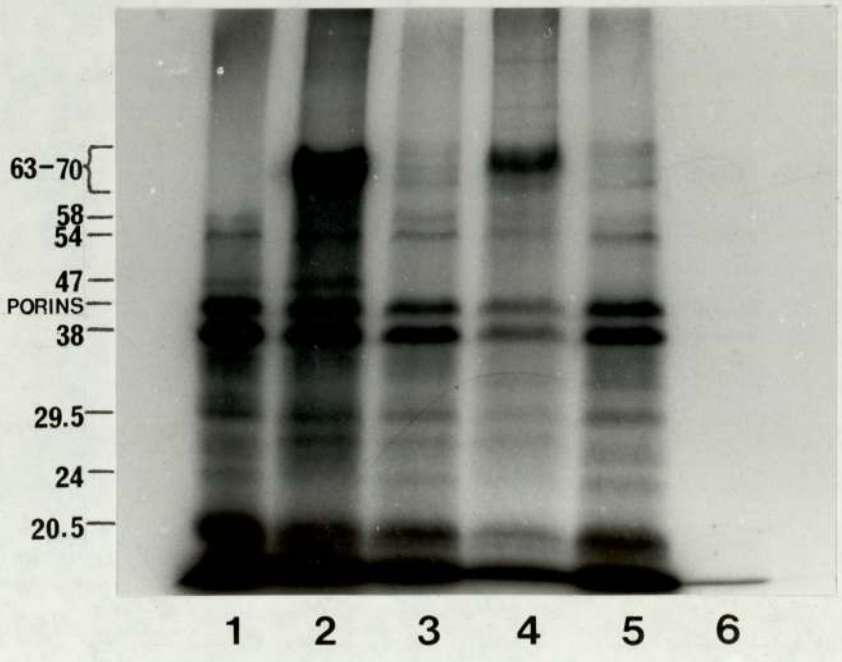

63-70
58
54
47
PORINS
38
29.5
24
20.5
1 2 3 4 5 6

Figure 3.29

SDS-PAGE and Autoradiograph Profiles of New CDC 014:H12 Whole Cel
Labelled using ^{125}I-lactoperoxidase or Iodo-beads

A SDS-PAGE protein profiles using a 10% w/v acrylamide concentration in the gel

B Autoradiograph (138 hours) of A

Key: Medium cells grown in to log phase and method to subsequently ^{125}I-label the cells

1 TSB, labelled using ^{125}I-lactoperoxidase

2 TSB, labelled using an Iodo-bead

3 Diluted HS, labelled using ^{125}I-lactoperoxidas

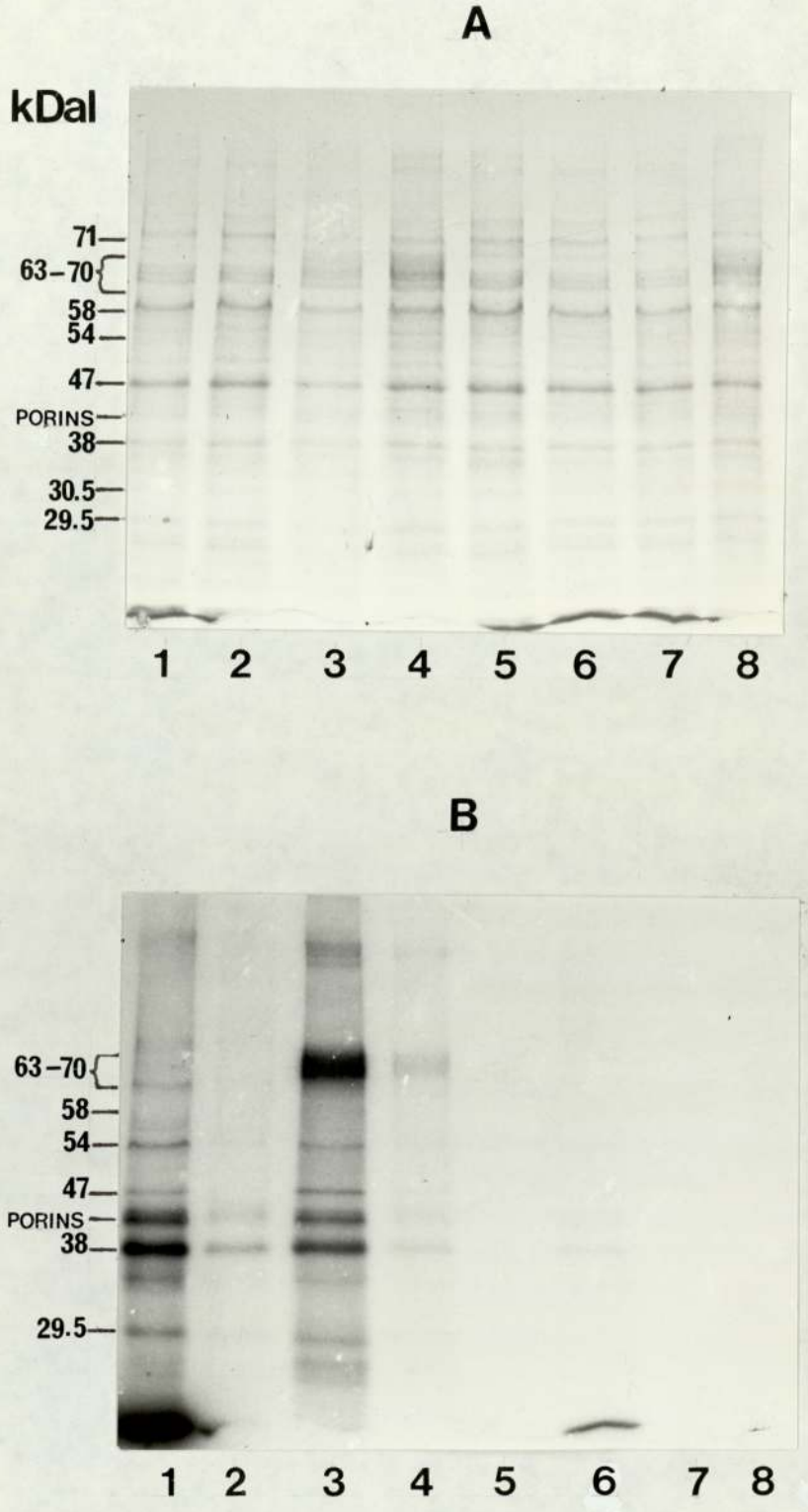
4 Diluted HS, labelled using an Iodo-bead

5 TSB, control omitting lactoperoxidase in label process

6 TSB, control omitting H_2O_2 in labelling proces

7 TSB, control omitting Iodo-bead

8 Diluted HS, control omitting Iodo-bead


A
kDal
71
63–70
58
54
47
PORINS
38
30.5
29.5
1 2 3 4 5 6 7 8
B
63–70
58
54
47
PORINS
38
29.5
1 2 3 4 5 6 7 8

3.2.2 Immunoblotting and lectin blotting of whole cells

Figures 3.30 to 3.33 show the coomassie blue stained gel, amido black stained blot, and the blots detected with hyperimmune rabbit antisera or peroxidase-linked lectins, of the whole cells of the three O14:H12 strains grown in TSB or diluted HS.

Figure 3.30 represents New CDC whole cells. After growth in both media, the OmpA protein, 58 kDal and 71 kDal proteins were the major proteins in homologous and cross-reactions. The porins showed weakly in the coomassie blue stained gel (Fig 3.30, A and B, 1) and although the amido black stain (Fig 3.30, A and B, 2) suggested all proteins were transferred onto the nitrocellulose, the porins did not regain their antigenicity. Antibodies to the 47 kDal protein only appeared in antiserum raised to New CDC cells grown in TSB (Fig 3.30, A, 3), these cross-reacting with cells grown in diluted HS (Fig 3.30, B, 3). In the IRMP region, the same blot profiles were obtained using cells grown in TSB and diluted HS (Fig 3.30, A, 4 compared to B, 4). This suggested that the IRMPs were either weakly antigenic or were not totally repressed after growth in iron-plentiful media. The 54 kDal protein was shown to be surface exposed by ^{125}I-labelling (Fig 3.28), but using immunoblotting a protein just below it became more dominant; in antisera raised to 4444-60 and S1220 cells grown in diluted HS, no antibodies to this protein appeared to be present (Fig 3.30 A and B, 6 and 8). The 30.5 kDal protein showed weak homologous and heterologous reactions after growth of New CDC in TSB, being stronger after growth in diluted HS especially when antisera raised to diluted HS grown cells was used (Fig 3.30, B, 4 and 6). A protein just below the 29.5 kDal protein was antigenic, antibodies being present to it in all antisera except that raised to 4444-60 grown in

TSB (Fig 3.30 A and B, 5).

Lectin blotting gave some interesting results. The four peroxidase-linked lectins used will attach to polysaccharides containing terminal fucosyl (asparagus pea), N-acetyl-D-galactosamine (soybean), glycosyl or mannosyl (concanavalin A), or N-acetyl-β-D-glucosamine residues (wheatgerm). Thus glycoproteins or lipopolysaccharide/surface polysaccharides with these free residues are shown on the blots. The 30.5 kDal protein might be a glycoprotein containing all of these sugars (Fig 3.30, A and B, 9 to 12). Another protein below this that was not strongly antigenic, might also be a glycoprotein containing a proportion of fucose, glucose or mannose, and N-acetyl-β-D-glucosamine. The horse serum grown cells had many more bands compared to the TSB grown cells that consisted of polysaccharides with glucosyl or mannosyl and N-acetyl-β-D-glucosamine residues and a few fucosyl residues (Fig 3.30, B, 9, 11 and 12). These were most likely horse serum components that had bound to the cells and which resisted centrifugation and washing. Strong binding occurred with the IRMPs, and the 54 kDal protein, which affected the blot profiles obtained. In the 54-58 K region of Figure 3.30, B, lanes 1-8, a band was present corresponding to a band on the lectin blots (lanes 9, 11 and 12) suggesting that these HS components were attached to the cells and were antigenic. No firm conclusions can therefore be drawn about the apparent lack of difference between the proteins of the IRMP region after growth in TSB and diluted HS of S. marcescens New CDC.

LPS has been shown to transfer nitrocellulose paper (see section 3.1.4) but there was no indication of its presence from lectin blotting. This was probably because there were no free sugars available for binding, rather than the LPS containing no fucose,

glucose or mannose, N-acetyl-β-D-glucosamine or N-acetyl-D-galactosamine residues. That from 4444-60 has N-acetyl-α-D-galactosamine, glucose and mannose in its structure (Brigden and Wilkinson, 1983), figure 3.31 confirming that lack of terminal residues available to bind to the lectin is the reason for LPS not showing.

Figure 3.31 represents 4444-60 whole cells and Figure 3.32 S1220 whole cells grown in both media. The profiles obtained were virtually identical to those using New CDC (Fig 3.30) and the comments made previously apply also to these figures. The only differences were ones regarding the strength of the reactions. It therefore appears that profiles obtained on blotting are a function of the antisera used. For instance, the 47 kDal protein of 4444-60 and S1220 grown in both media only reacted with antiserum raised to New CDC cells grown in TSB. Another example is the antibodies to the 58 kDal protein of S1220 cells which were not as pronounced as with the other two strains.

All these results are confirmed by Figure 3.33, showing the blots of whole cells after growth in TSB. There were no major differences between the whole cell immunoblot profiles obtained on subjecting the three O14:H12 strains to homologous and heterologous antisera reactions.

Figure 3.30

SDS-PAGE, Immunoblot and Lectin Blot Profiles of S. marcescens New CDC Whole Cells Grown to Log Phase in TSB or Diluted HS, using a 1 w/v Acrylamide Concentration in the Gel

Medium cells grown in:

A TSB

B Diluted HS

Key:

1 Coomassie blue protein profile

2 Amido black protein profile after blotting

3 Immunoblot using antiserum raised in rabbits to log phase TSB grown New CDC cells

4 Immunoblot using antiserum raised in rabbits to log phase diluted HS grown New CDC cells

5 Immunoblot using antiserum raised in rabbits to log phase TSB grown 4444-60 cells

6 Immunoblot using antiserum raised in rabbits to log phase diluted HS grown 4444-60 cells

7 Immunoblot using antiserum raised in rabbits to log phase TSB grown S1220 cells

8 Immunoblot using antiserum raised in rabbits to log phase diluted HS grown S1220 cells

9 Lectin blot using peroxidase linked-asparagus p lectin (attaches to α-L-fucosyl residues)

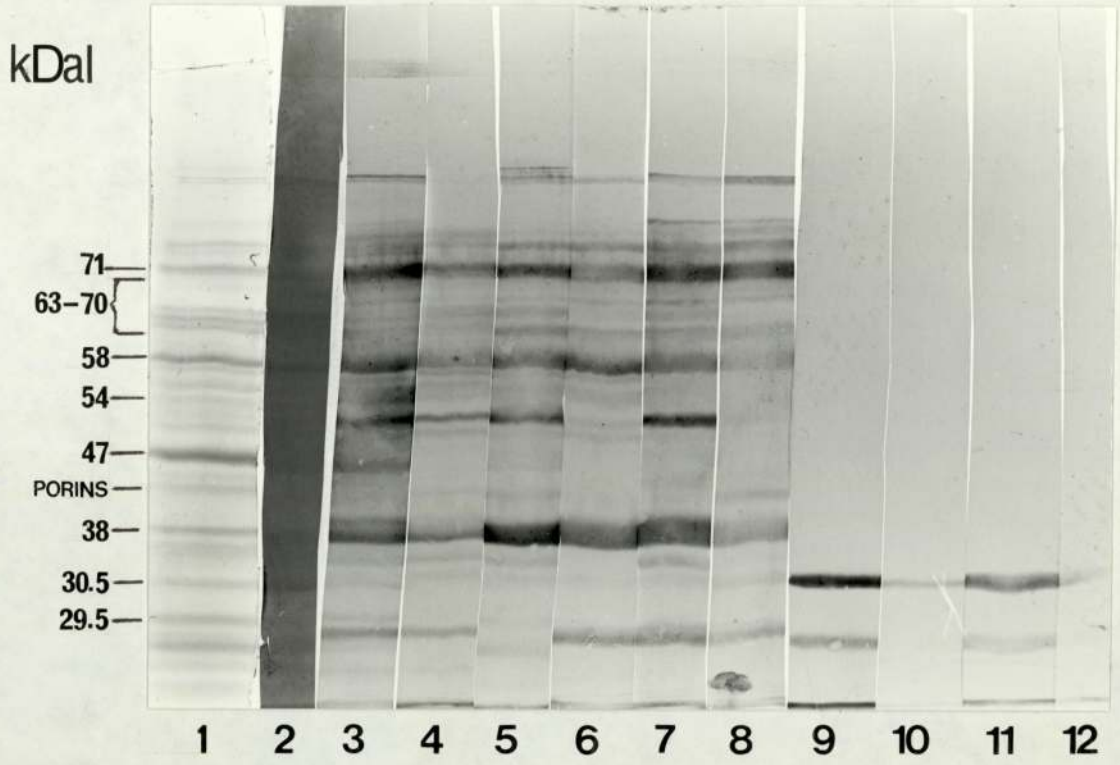
10 Lectin blot using peroxidase linked-soybean agglutinin (attaches to N-acetyl-D-galactosamin residues)

11 Lectin blot using peroxidase linked-concanavali (attaches to α-D-glucosyl or α-D-mannosyl residues)

12 Lectin blot using peroxidase linked-wheatgerm agglutinin (attaches to N-acetyl-β-D-glucosamin residues)

The peroxidase-labelled conjugate used in the immunodetection proc of 3 to 8 in Figs 3.30 to 3.32 was Protein A.

A


kDal
71
63–70
58
54
47
PORINS
38
30.5
29.5
1 2 3 4 5 6 7 8 9 10 11 12

B

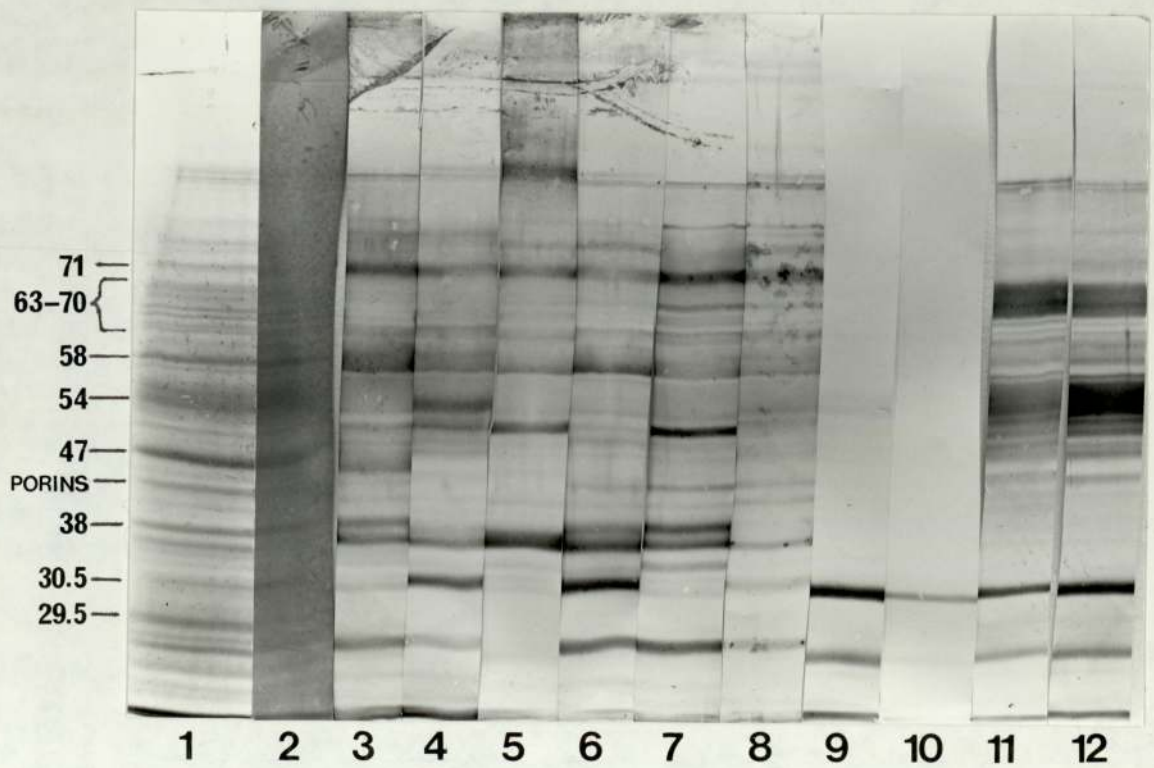
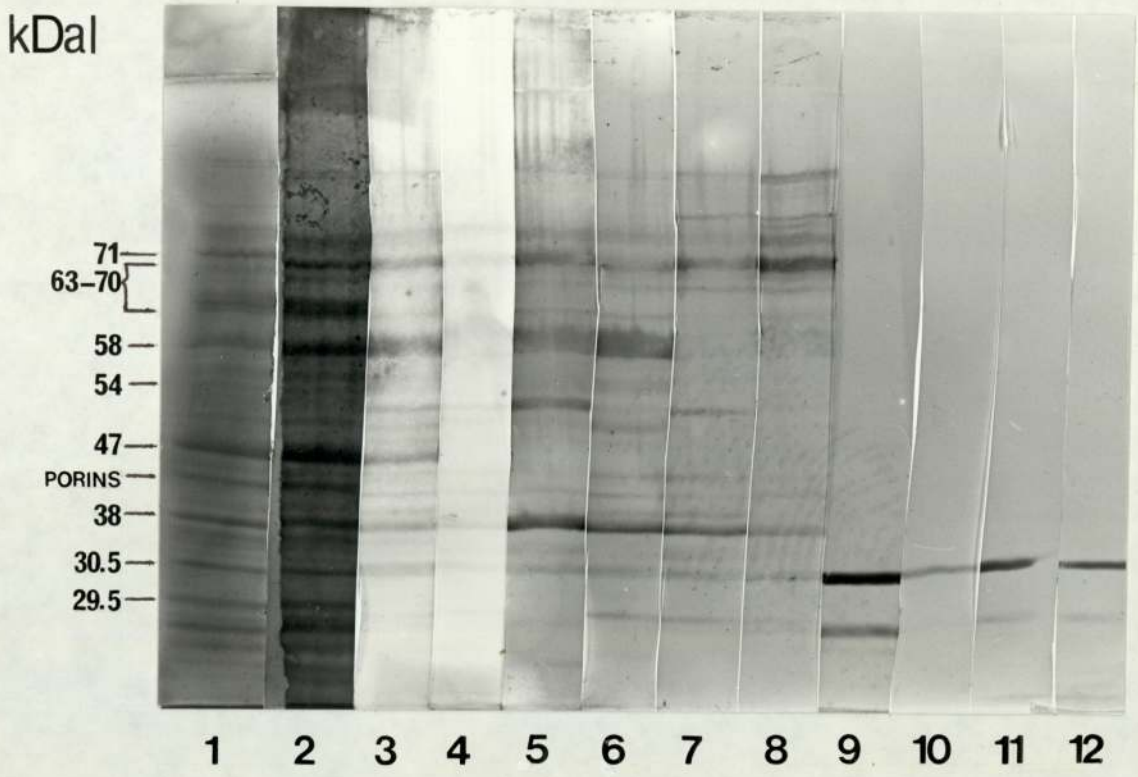

71
63–70
58
54
47
PORINS
38
30.5
29.5
1 2 3 4 5 6 7 8 9 10 11 12

Figure 3.31

SDS-PAGE, Immunoblot and Lectin Blot Profiles of S. marcescens 4444-60 Whole Cells Grown to Log Phase in TSB or Diluted HS, usin 10% w/v Acrylamide Concentration in the Gel.

For Legend see Figure 3.30

A


kDal
71
63–70
58
54
47
PORINS
38
30.5
29.5
1 2 3 4 5 6 7 8 9 10 11 12

B

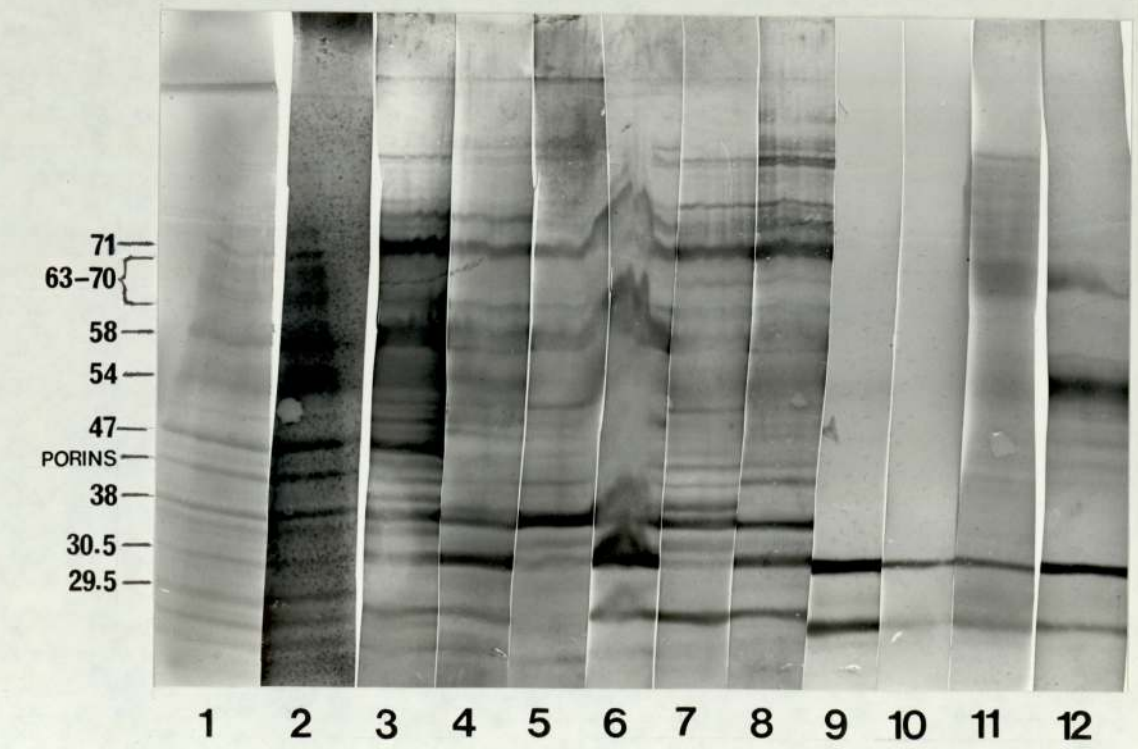
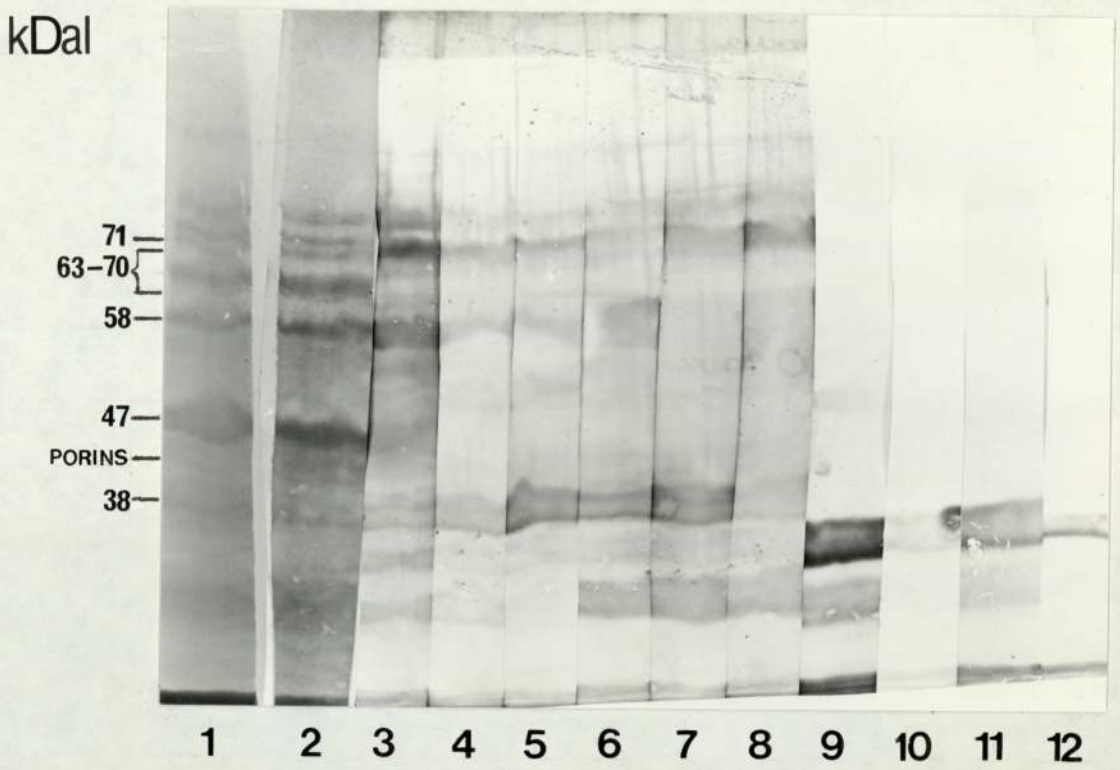

71
63–70
58
54
47
PORINS
38
30.5
29.5
1 2 3 4 5 6 7 8 9 10 11 12

Figure 3.32

SDS-PAGE, Immunoblot and Lectin Blot Profiles of S. marcescens S1 Whole Cells Grown to Log Phase in TSB or Diluted HS, using a 10% Acrylamide Concentration in the Gel.

For legend see Figure 3.30

A


kDal
71
63–70
58
47
PORINS
38
1
2
3
4
5
6
7
8
9
10
11
12

B

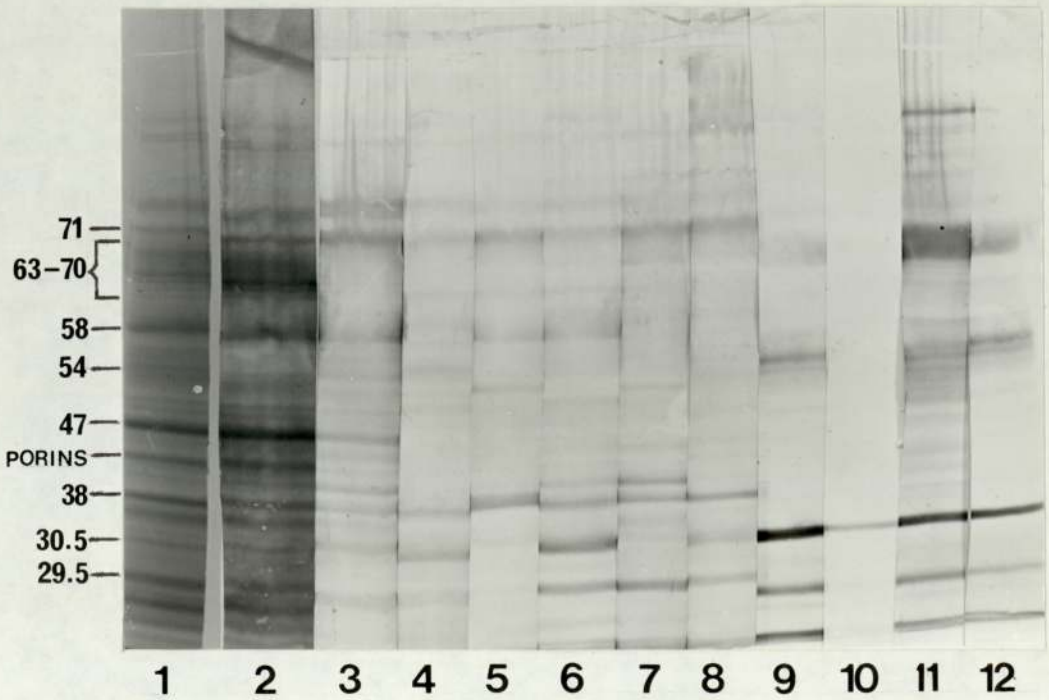

71
63–70
58
54
47
PORINS
38
30.5
29.5
1
2
3
4
5
6
7
8
9
10
11
12

Figure 3.33

Protein Profiles and Immunoblots of the Whole Cells of three O14: Strains Grown to Log Phase in TSB Detected with Hyperimmune Antis Raised in Rabbits to the three Strains

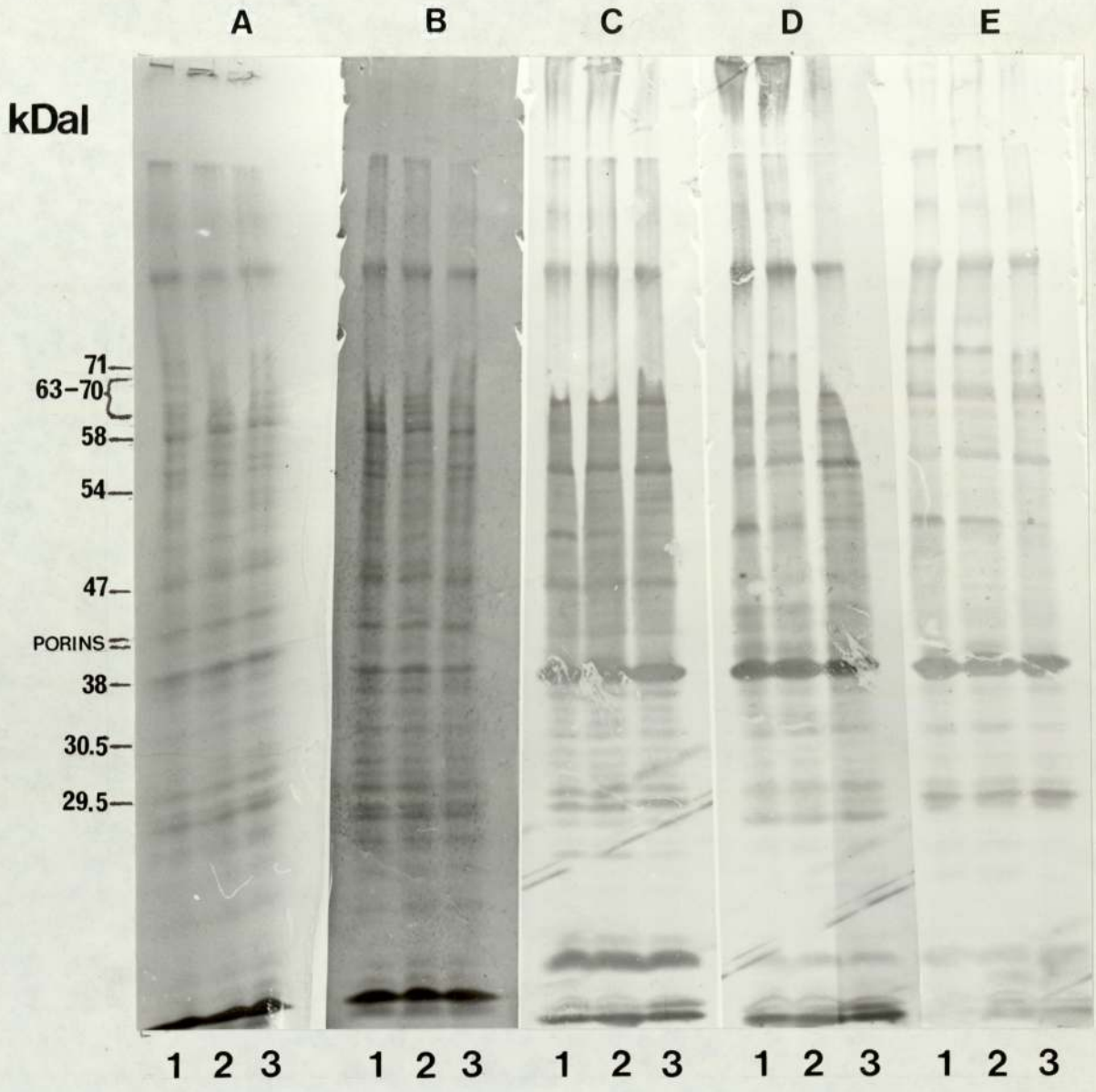
The acrylamide concentration in the gel was 10% w/v.

A	Coomassie blue stained gel
B	Amido black stained blot
C	Immunoblot detected with antiserum raised to N CDC cells grown to log phase in TSB
D	Immunoblot detected with antiserum raised to 4444-60 cells grown to log phase in TSB
E	Immunoblot detected with antiserum raised to S cells grown to log phase in TSB

The peroxidase-labelled conjugate used in the immuno-detection process of C to E was protein A.

Key:

1	New CDC whole cells
2	4444-60 whole cells
3	S1220 whole cells


A
B
C
D
E
kDal
71
63–70
58
54
47
PORINS
38
30.5
29.5
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3

3.2.3 Antibiotic sensitivity

The 'neosensitab' antibiotic disc system was used to determine the sensitivity of the three O14:H12 strains (after growth to log phase in TSB or diluted HS) to 8 antibiotics (Table 3.2). The 'zones of inhibition' recorded were calculated from the mean diameter of three separate zones, measured to the nearest whole mm of the regular circular section of the zone. The criteria upon which the organisms were classified sensitive or resistant were based on the breaking points of Casals and Penderson (1979). An estimation of the minimum inhibitory concentration (MIC) for the organisms was obtained by extrapolation of the linear regression curves of MIC against 'zone of inhibition' provided by the manufacturers. A zone of 10 mm virtually represented the diameter of the tablet (9 mm) and consequently the MICs of these resistant bacteria were not determined.

The results (Table 3.2) show that strain S1220 was resistant to all but one of the 8 antibiotics tested, that being amikacin. New CDC and 4444-60 showed the same patterns of sensitivity as each other, being resistant to azlocillin, erythromycin and polymyxin and sensitive to tetracycline, amikacin, co-trimoxazole and gentamicin. These two strains were found to have different patterns of sensitivity to chloramphenicol based on the breaking points of Casals and Penderson (1979) but the zones of inhibition and MICs suggested that these were not significant differences. In fact, with all three strains there were no significant differences in the susceptibilities of the cells grown in TSB and diluted HS to the antibiotics.

It was decided to investigate amikacin further as all the cells were sensitive to this, and to use two membrane active agents, chlorhexidine and benzalkonium chloride as indicators of differences

in membrane and envelope composition.

Determination of the MICs of amikacin, chlorhexidine and benzalkonium chloride in TSB against the three strains failed to show any significant differences (Table 3.3). Figure 3.34 illustrates the effect of increasing the concentrations of amikacin, chlorhexidine and benzalkonium chloride (in nutrient agar) on the survival of the three strains grown to log phase in TSB and diluted HS. The media the cells were grown in again had no effect on the sensitivity profiles obtained. S1220 cells showed a very small increase in sensitivity to amikacin compared to New CDC and 4444-60. There were no major differences however in the susceptibilities of the three strains to chlorhexidine and benzalkonium chloride.

Table 3.2

Antibiotic Sensitivity of three O14:H12 Strains of S. marcescens Determined using the Neosensitab Disc Method

	Antibiotic Sensitivity of Bacteria*																	
Antibiotic	New CDC TSB Zone	S/R	MIC	New CDC HS Zone	S/R	MIC	4444-60 TSB Zone	S/R	MIC	4444-60 HS Zone	S/R	MIC	S1220 TSB Zone	S/R	MIC	S1220 HS Zone	S/R	MIC
Amikacin	22	S	14	23	S	10	22	S	14	22	S	14	24	S	7	26	S	4
Azlocillin	16	R	34	19	R	15	15	R	42	17	R	25	10	R	-	10	R	-
Chloramphenicol	24	S	14	26	S	11	24	S	14	23	R	18	17	R	60	17	R	60
Cotrimoxazole (Trimethoprim & Sulphamethoxazole)	29	S	1.5T + 3.0S	30	S	1.2T + 2.5S	28	S	2.0T + 4.0S	28	S	2.0T + 4.0S	10	R	-	10	R	-
Erythromycin	13	R	85	13	R	85	13	R	85	13	R	85	11	R	150	15	R	40
Gentamicin	26	S	4.5	26	S	4.5	25	S	6	25	S	6	10	R	-	10	R	-
Polymyxin	17	R	55	17	R	55	18	R	35	18	R	35	18	R	35	18	R	35
Tetracycline	22	S	6	23	S	4.5	19	S	12	19	S	12	10	R	-	10	R	-

*The three figures represent the mean 'zone of inhibition' (mm), the sensitivity (S) or resistance (R) of the organism (Casals and Pendersen, 1979), and the MIC (μg/ml).

Table 3.3 MIC values (µg/ml) of Amikacin, Chlorhexidine and Benzalkonium Chloride against three O14:H12 Strains of *S. marcescens*

	MIC values of strains * µg/ml		
Antimicrobial agent	New CDC	4444-60	S1220
Amikacin	8	32	32
Chlorhexidine	16	32	32
Benzalkonium Chloride	8	16	32

* The MIC determinations were performed in TSB using bacteria grown to log phase in TSB.

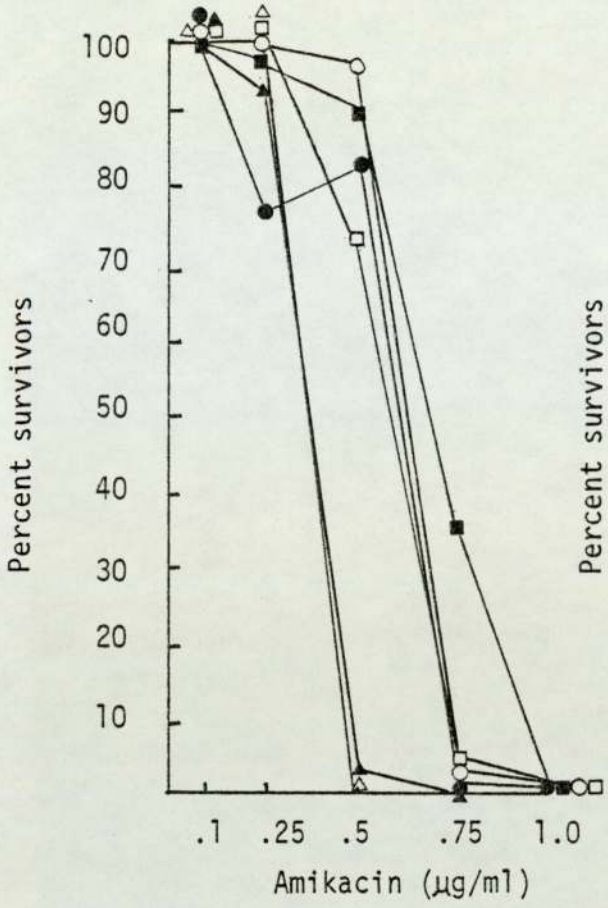
Figure 3.34

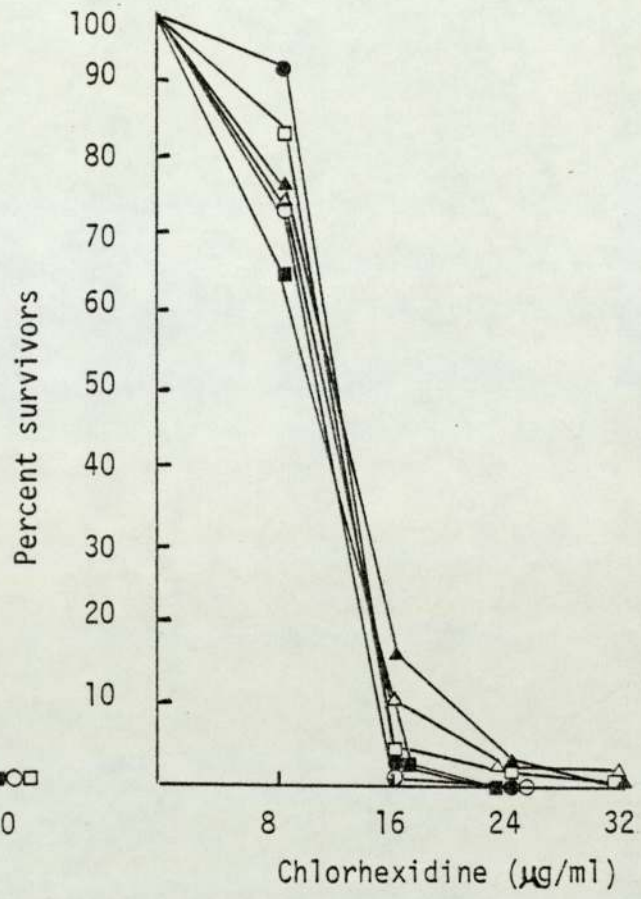
Susceptibility of three O14:H12 Strains of S. marcescens to Amika
Chlorhexidine and Benzalkonium Chloride

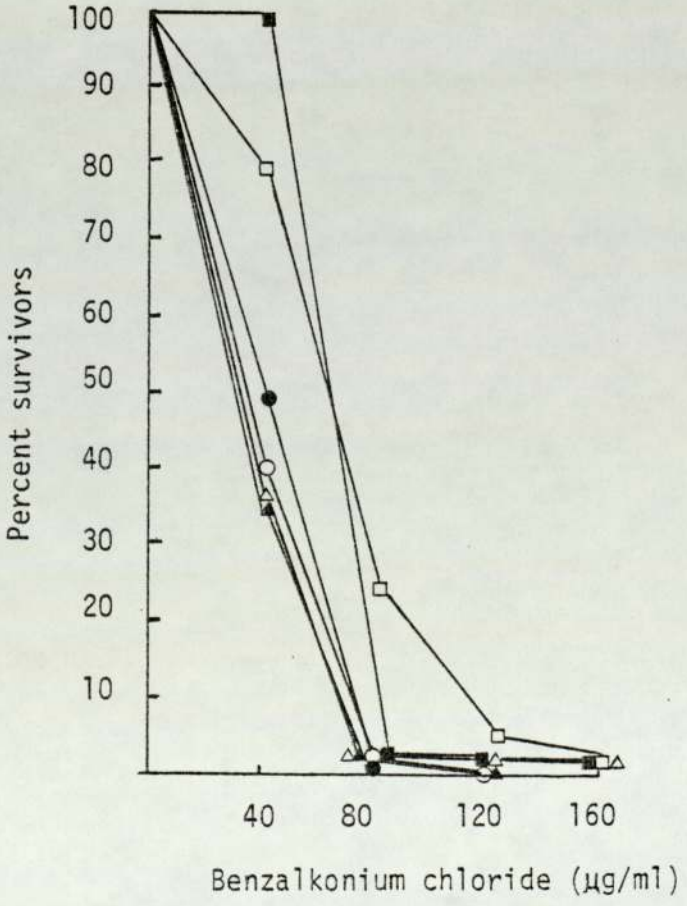
Key: Strain/Medium cells grown in to log phase

- ● New CDC O14:H12/TSB
- ○ New CDC O14:H12/Diluted HS
- ■ 4444-60/TSB
- □ 4444-60/Diluted HS
- ▲ S1220/TSB
- △ S1220/Diluted HS

The percent survival values indicate viable counts relative to th
obtained in the absence of drug.


Percent survivors
100
90
80
70
60
50
40
30
20
10
.1
.25
.5
.75
1.0
Amikacin (µg/ml)


Percent survivors
100
90
80
70
60
50
40
30
20
10
8
16
24
32
Chlorhexidine (µg/ml)


Percent survivors
100
90
80
70
60
50
40
30
20
10
40
80
120
160
Benzalkonium chloride (µg/ml)

3.2.4 Serum sensitivity

The sensitivity of the three O14:H12 strains to killing by GPS or NPHS after growth in different media was investigated. GPS was used in initial experiments because it contains high complement activity. The method performed was that of Williams et al (1983) using an OD_{470} = 0.1 of bacteria and a serum concentration of 50% v/v.

The sensitivity of New CDC to GPS after growth to log or stationary phase in TSB or diluted HS is shown in Figure 3.35. The cells grown in TSB were rapidly sensitive to complement killing whereas those grown in diluted HS were resistant, their viable counts increasing by 400 to 700%. The growth phase did not affect the overall result obtained. Log phase cells (Fig 3.35, A) tended to be more extreme in their response, viable counts either decreasing or increasing more rapidly than when stationary phase cells were used (Fig 3.35,B).

When GPS heat-inactivated to destroy complement was used in the assay (Fig 3.35), the cells cultured from both TSB and diluted HS grew.

Chelation of the serum with MgEGTA to prevent activation of the classical complement pathway whilst leaving the alternative pathway functional had no effect on the diluted HS grown cells (Fig 3.35). However, chelated GPS was not rapidly bactericidal for TSB grown cells suggesting that the classical pathway was mainly responsible for this effect, the alternative pathway having only limited killing power. On repeating the experiment on log phase cells with NPHS instead of GPS, the same trends were found (Fig 3.36).

To test that the differences in sensitivity of New CDC to serum

were not due to the iron-restriction imposed on the cells by diluted HS, cells grown to stationary phase in media with added iron were used (Fig 3.37). Resistance was observed independently of the growth media. The presence or absence of iron in CDM also had no effect on the kill-curves obtained. Cells grown in this media showed an initial 20 to 40% decrease in their viable counts, but then the counts started to increase, presumably because the resistant population began to divide and grow.

It therefore appeared the sensitivity of New CDC to complement killing was dependent upon the media the cells were grown in, being resistant in diluted HS, sensitive in TSB and inbetween in CDM, the presence or absence of iron in the environment not altering this. However, on investigating the sensitivity of the three O14:H12 strains to GPS after growth of the cells to log phase in TSB or diluted HS, a different result was found (Fig 3.38). New CDC grown in TSB became resistant and in diluted HS viable counts were reduced to approximately 10%. 4444-60 cells were resistant and S1220 rapidly sensitive to complement killing independently of growth media. The reason for the change of sensitivity of New CDC was unclear. In 25% of the experiments, this was found.

Figure 3.39 shows the kinetics of killing of 4444-60 by NPHS. There were no significant differences between cells grown in TSB and diluted HS, growth continuing when NPHS chelated with MgEGTA or heat-inactivated NPHS was used. If anything, NPHS appeared to be bacteriostatic to 4444-60 cells grown in TSB, compared to the resistance of diluted HS grown cells. When GPS was used, however, a 200% increase in the viable counts after 120 minutes was observed for cells grown in both media (Fig 3.38). It can therefore be concluded that 4444-60 is resistant to complement killing regardless of the

media used to grow the cells. This is particularly evident when these results are compared to those of S1220, which can be seen to be rapidly sensitive to killing by NPHS, again independently of growth media (Fig 3.40). When heat-inactivated NPHS was used, the S1220 cells grew. Rapid killing was not observed when NPHS chelated with MgEGTA was used (Fig 3.40). Instead viable counts dropped to about 50% suggesting that the alternative pathway was involved in killing, but the classical pathway was required for the rapid bactericidal effect. Unlike with New CDC, 4444-60 and S1220 constantly showed these resistance and sensitive patterns to complement without any significant influence of growth media.

Figure 3.41 shows a blot of OMs from New CDC cells grown in different media, immunodetected with NPHS. A weak response is shown, antibodies appearing to be present to the OmpA protein and to a few other components. The NPHS was taken from people with no history of a Serratia infection and it is assumed the immunoglobulins present are natural antibodies to enterobacterial organisms, although these are usually of the IgM subclass rather than IgG. It has already been shown S. marcescens antibodies cross-react with those of other Gram-negative bacteria (Fig 3.17). The classical pathway of complement usually requires antibody for activation, although there are reports of activation without involvement of IgG or IgM (Winkelstein, 1983). The killing of S1220 and New CDC by complement is dependent upon the classical pathway, the antibodies present in the NPHS probably playing a part in the system.

Figure 3.35

Kinetics of Killing of S. marcescens New CDC 014:H12 by Guinea Pi
Serum

A Log phase cells

B Stationary phase cells

Key: Medium cells grown in/Serum

- ● TSB/GPS alone
- ◆ TSB/Heat-inactivated GPS
- ▲ TSB/GPS chelated with 10 mM-MgEGTA
- ○ Diluted HS/GPS alone
- ◇ Diluted HS/Heat-inactivated GPS
- △ Diluted HS/GPS chelated with 10 mM-MgEGTA

The percent survival values in Figs 3.35 to 3.40 indicate viable counts relative to time zero (100% = 10^8 cells/ml).

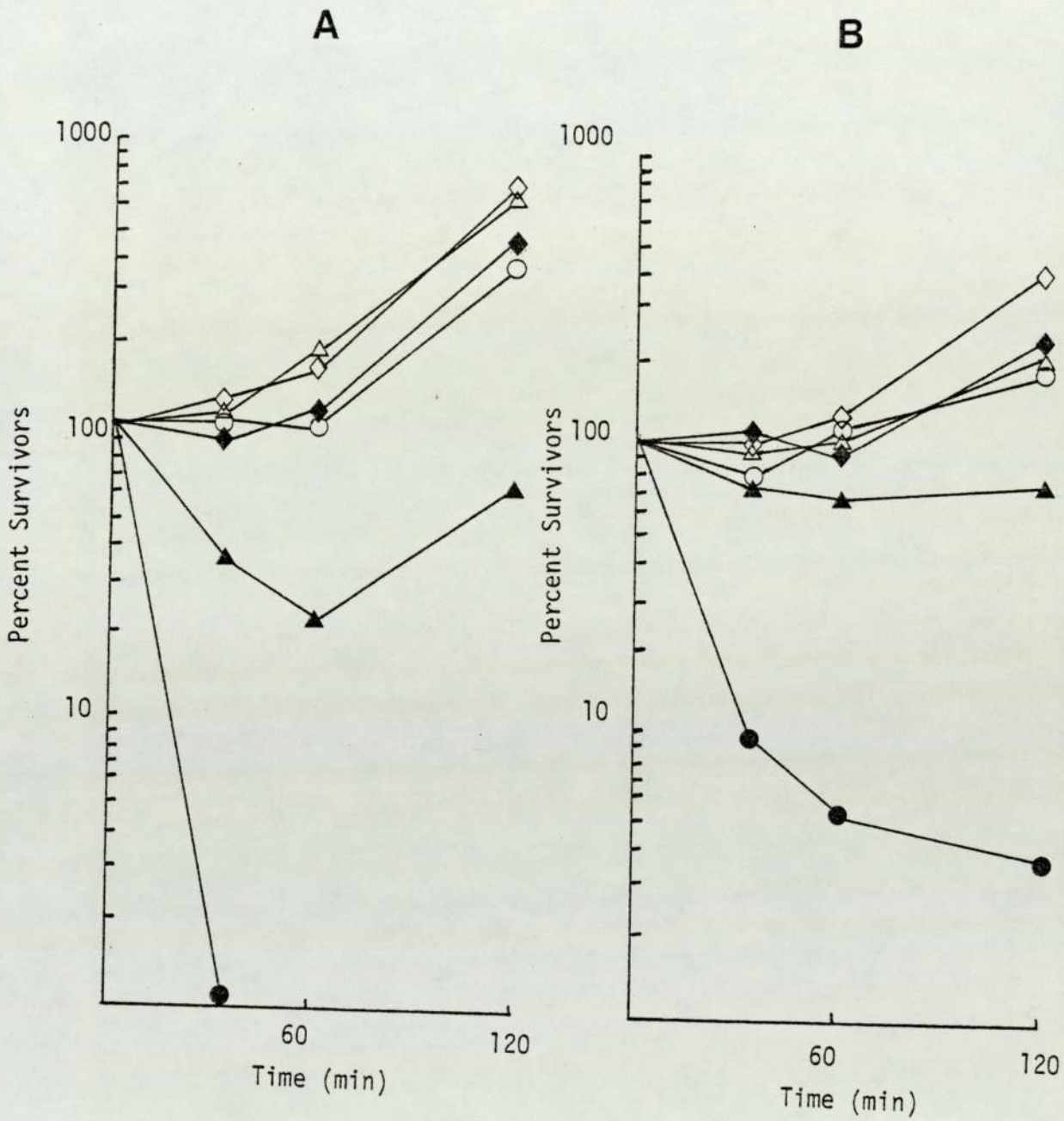

A
B
1000
100
10
Percent Survivors
60
120
Time (min)

Figure 3.36

Kinetics of Killing of S. marcescens New CDC 014:H12 by Normal Po
Human Serum

Key: Medium cells grown in to log phase/Serum

- ● TSB/NPHS alone
- ◆ TSB/Heat-inactivated NPHS
- ▲ TSB/NPHS chelated with 10 mM-MgEGTA
- ○ Diluted HS/NPHS alone
- ◇ Diluted HS/Heat-inactivated NPHS
- △ Diluted HS/NPHS chelated with 10 mM-MgEGTA

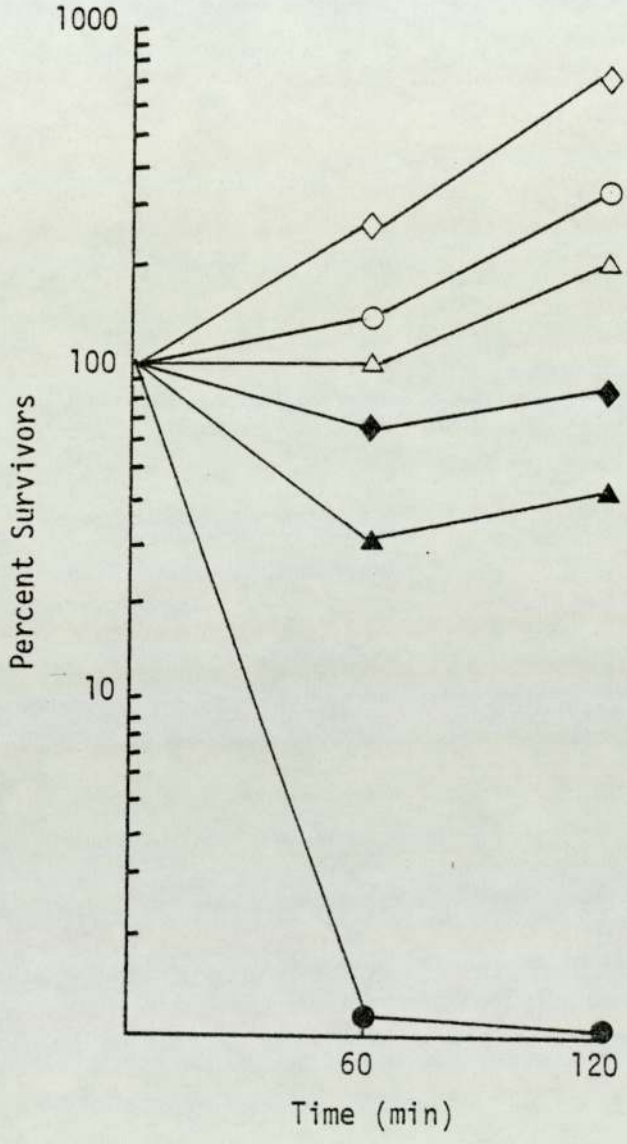

1000
100
10
Percent Survivors
60
120
Time (min)

Figure 3.37

Kinetics of Killing of S. marcescens New CDC 014:H12 Grown to Stationary Phase in Four Different Media by Normal Pooled Human S

Key: Medium cells grown in

- ● CDMFe-
- ○ CDMFe+
- ◆ Diluted HS
- ◇ Diluted HS with additional iron

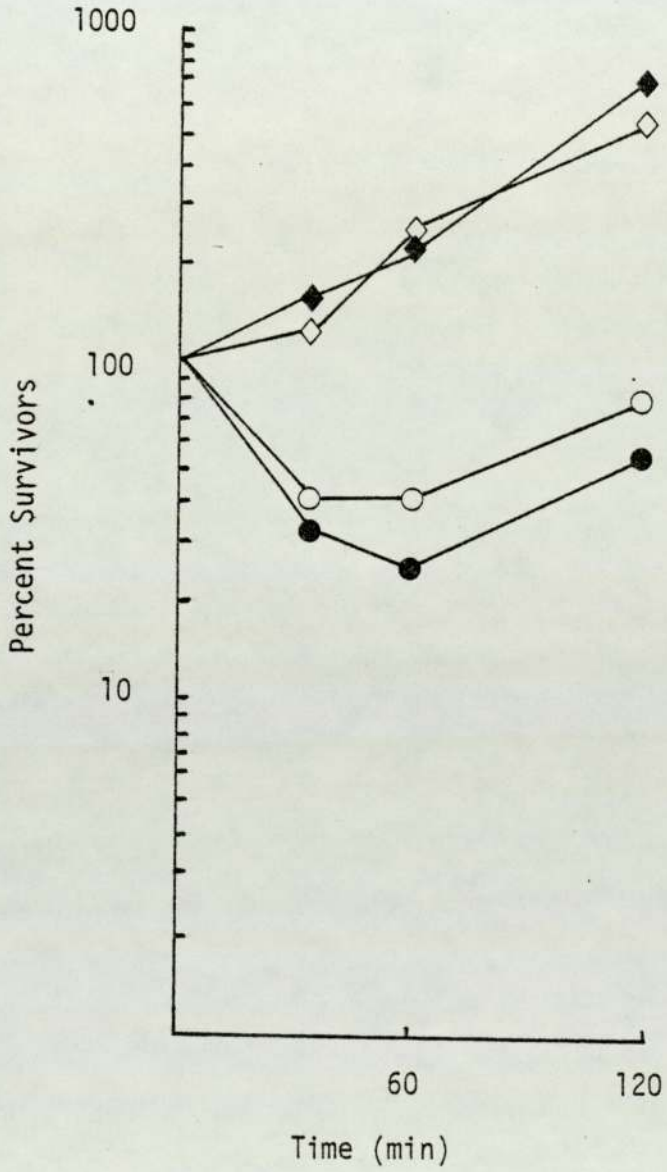

1000
100
10
Percent Survivors
60
120
Time (min)

Figure 3.38

Kinetics of Killing of three O14:H12 Strains of S. marcescens by Guinea Pig Serum

Key: Strain/Medium cells grown in to log phase

- ● New CDC/TSB
- ○ New CDC/Diluted HS
- ◆ 4444-60/TSB
- ◇ 4444-60/Diluted HS
- ▲ S1220/TSB
- △ S1220/Diluted HS

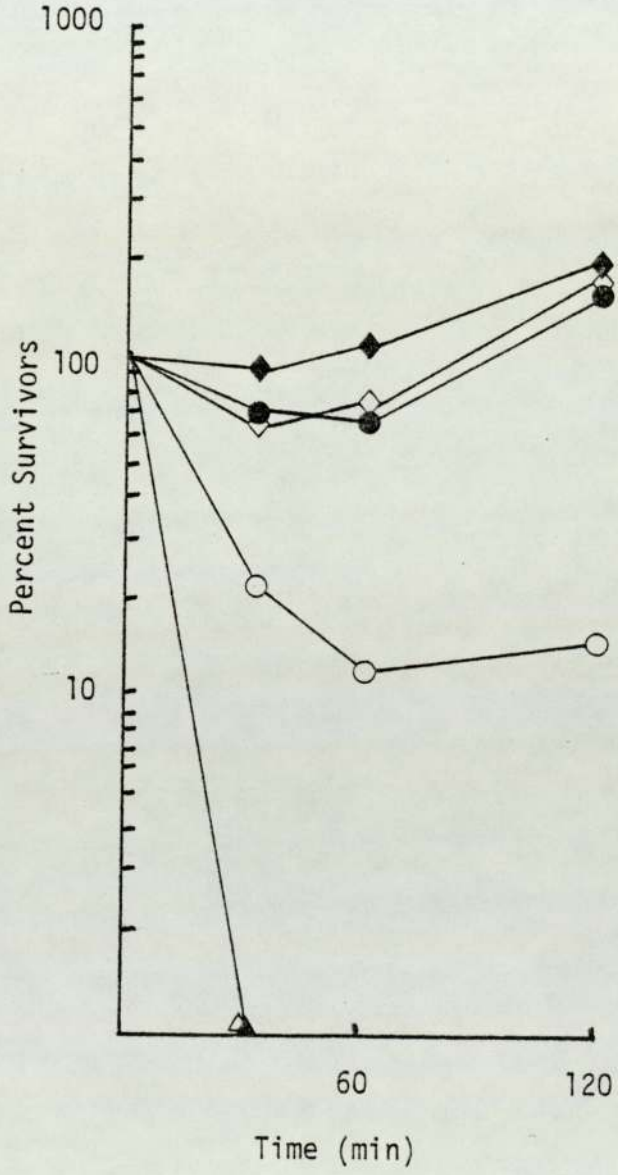

1000
100
10
Percent Survivors
60
120
Time (min)

Figure 3.39

Kinetics of Killing of S. marcescens 4444-60 by Normal Pooled Hum
Serum

Key: Medium cells grown in to log phase/Serum

- ● TSB/NPHS alone
- ◆ TSB/Heat-inactivated NPHS
- ▲ TSB/NPHS chelated with 10 mM-MgEGTA
- ○ Diluted HS/NPHS alone
- ◇ Diluted HS/Heat-inactivated NPHS
- △ Diluted HS/NPHS chelated with 10 mM-MgEGTA

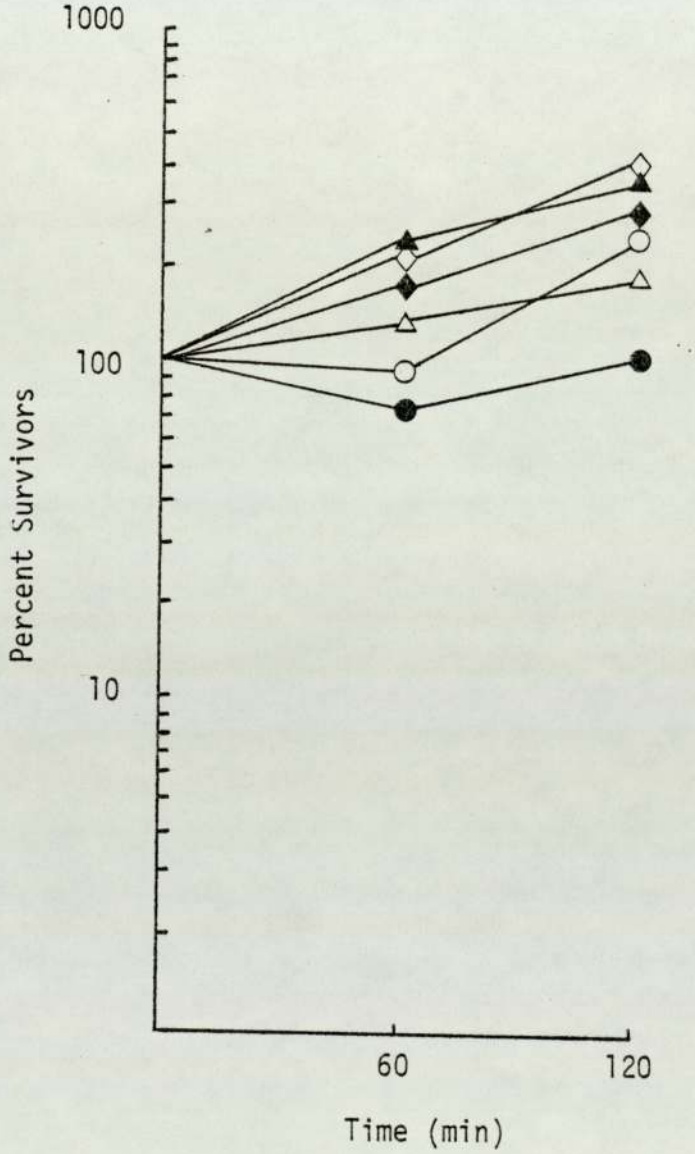

1000
100
10
Percent Survivors
60
120
Time (min)

Figure 3.40

Kinetics of killing of S. marcescens S1220 by Normal Pooled Human Serum

Key: Medium cells grown in to log phase/Serum

- ● TSB/NPHS alone
- ◆ TSB/Heat-inactivated NPHS
- ▲ TSB/NPHS chelated with 10 mM-MgEGTA
- ○ Diluted HS/NPHS alone
- ◇ Diluted HS/Heat-inactivated NPHS
- △ Diluted HS/NPHS chelated with 10 mM-MgEGTA

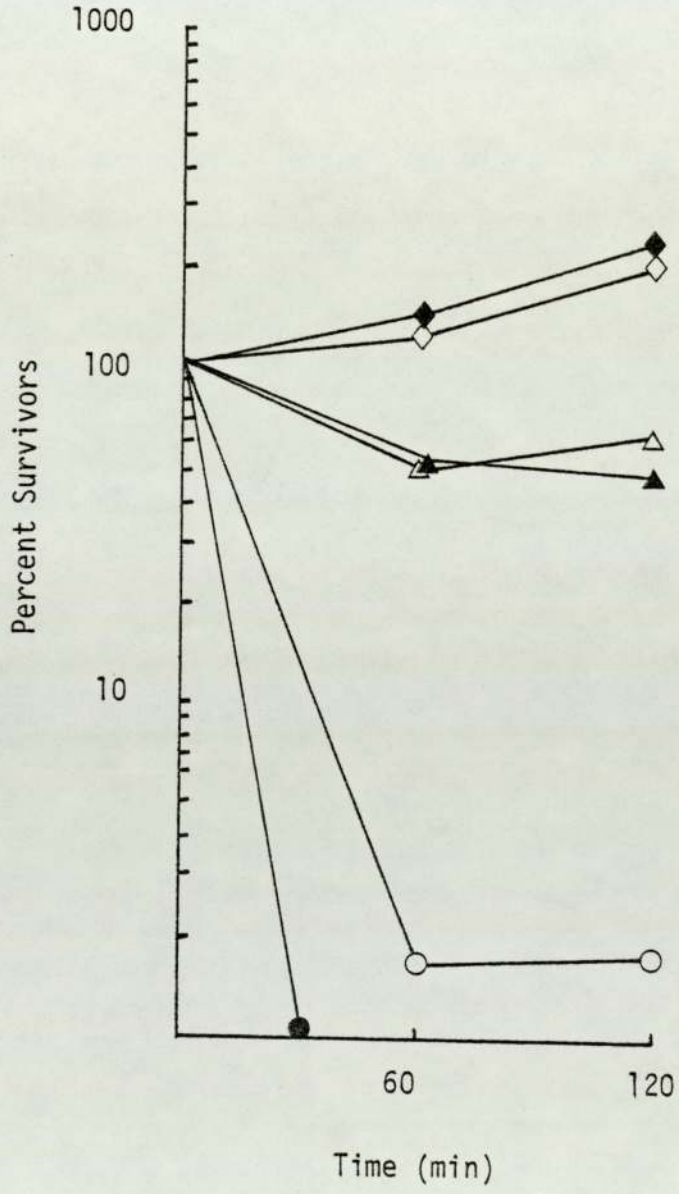

1000
100
10
Percent Survivors
60
120
Time (min)

Figure 3.41

Immunoblot of New CDC 014:H12 Outer Membranes Detected with Norma

Pooled Human Serum

Key:	Medium cells grown in to stationary phase
1	Diluted HS
2	Diluted HS with additional iron
3	Nutrient agar
4	TSB
5	CDMFe-
6	CDMFe+

The peroxidase-labelled conjugate used was Protein A.

1 2 3 4 5 6

3.2.5 Discussion

The protein composition of three O14:H12 strains were found to be virtually identical on analysis by SDS-PAGE and ^{125}I-labelling of whole cells. The latter showed that the porins and OmpA protein have at least part of their polypeptide chain exposed on the cell surface, an observation found by Booth (1980) for the equivalent proteins of *E. coli*. Other proteins accessible to labelling were the IRMPs, even after growth in TSB. Booth (1980) also discovered that *E. coli* grown in nutrient broth had these high molecular weight proteins present that bound ^{125}I. Synthesis of constitutive levels of the high affinity uptake system, even at very high iron concentrations, is known to exist in *E. coli* (Neilands, 1981b) and it appears the same occurs in *S. marcescens*. Lectin blotting confirmed the surface binding of serum components, probably glycoproteins, to the IRMP region of cells grown in diluted HS, that consequently bound ^{125}I and gave a diffuse band on the autoradiograph.

In this study, other proteins were also found to be radiolabelled, but their function and identity are unknown. The flagella were shown to be surface exposed by an immunoabsorption technique. Their absence from the autoradiographs is perhaps because they do not bear surface tyrosine or histidine residues. Certainly, the N-terminal amino acids of the flagella from *S. marcescens* 8UK do not consist of these (Kobayashi et al, 1959).

The O antigen chains of *S. marcescens* New CDC LPS were found to interfere with access of antibodies to epitopes on protein antigens by immunoabsorption (section 3.1.4), yet the [^{125}I]lactoperoxidase managed to reach its target sites on the OMPs. [^{125}I]lactoperoxidase has a molecular weight of 77.5 kDal and IgG molecules 150 kDal. One

explanation is that the smaller complex can penetrate more easily through the LPS. Alternatively, antibodies to LPS may rapidly react in the immunoabsorption experiment, forming a further barrier that hinders IgG molecules specific for the OMPs reaching their target sites. Iodination was performed in the absence of antiserum and hence this would not occur. It may be that the second explanation of the immunoabsorption experiment is true, with epitopes recognised by the antiserum by immunoblotting not being exposed on the surface of proteins in their native configuration in the OM. Lastly, it could well be that the [^{125}I]lactoperoxidase and IgG molecules have different binding sites on the proteins and the antigenic epitopes are masked by O antigen chains of the LPS, but the exposed tyrosine/histidine residues are not.

Immunoblotting of the whole cells with homologous and heterologous antisera showed that there were no major differences in the antigenic profiles of the three S. marcescens O14:H12 strains. The only proteins that were found to be consistently surface exposed by ^{125}I-labelling and that were antigenic on immunoblotting, were the OmpA protein and the 47 kDal protein of New CDC grown in TSB. Other surface-exposed proteins were either weakly antigenic, or else did not have their antigenic epitopes renatured on the transfer to nitrocellulose paper (eg the porins). Many whole cell protein antigens were present on the blots that were not surface-exposed, proving that antibodies are manufactured to antigens released on lysis of the cells by the rabbit's host defence mechanisms (eg phagocytosis, complement killing etc).

Lectin blotting confirmed that HS components bound to the cells. This has important consequences on the adherence of the cells to bacterial surfaces in vivo, an important step in the initial

colonisation of bacteria in infection (Smith, 1977). Sugar moieties are so far the only residues identified as bacterial receptors on epithelial cells, present either as glycolipids or glycoproteins, mannose being the sugar involved for E. coli (Ofek and Beachey, 1980). The binding of such components in HS to the bacterial cells gives an indication as to why the cells formed a tight friable pellet on harvesting, the cells adhering to one another possibly via specific ligands, as they would to glycoproteins on epithelial surfaces. However, binding of HS glycoproteins and other components to bacterial surfaces might inhibit bacterial adherence to epithelial cells by either combining with specific binding sites (ligands) on the bacteria (competitive inhibition), by steric hindrance, or by simply modifying the surface hydrophobicity and charge of the bacteria (Ofek and Beachey, 1980). This would be beneficial regarding the cells' ability to resist phagocytosis, but it would not allow colonisation of the cells. In contrast, other proteins bound to bacterial surfaces would aid adherence of the bacteria to host cells, including to phagocytes eg fibronectin (Simpson and Boughton, 1984). Depending on the site of colonisation, phagocytes might not be able to reach the cells, thus such an adhering bacterial surface would be an asset. Further experiments need to be performed to find out if the HS components bound to S. marcescens cells aid adherence to epithelial cells, the fact the cells bound to one another on harvesting suggesting that they would.

Lectin blotting of three S. marcescens O14:H12 strains showed that two low molecular weight cell proteins were glycoproteins. Heller (1979a) found that carbohydrate moieties were covalently linked to proteins of S. marcescens on SDS-PAGE and suggested that the OMPs are all glycoproteins, although it is more likely many

proteins were associated with LPS. In contrast, the absence of glycoproteins in E. coli was confirmed by CIE of inner and outer membranes that showed the antigens did not react with concanavalin A or wheatgerm agglutinin lectins (Owen, 1981). In between the two extremes of Heller and Owen, the results of this study suggest two proteins at least of S. marcescens are glycoproteins.

S. marcescens has always been one of the most antibiotic resistant members of the Enterobacteriaceae and has shown uncommon ability to rapidly acquire resistance to new antimicrobial agents (Farrar, 1980). With the exception of the β-lactams, the target sites of action of the major groups of antibiotics are intracellular. Aminoglycosides, tetracyclines, macrolides and chloramphenicol all inhibit ribosome function; trimethoprim and the sulphonamides interfere with folate metabolism. β-lactams interfere with transpeptidases and carboxypeptidases (the penicillin binding proteins) located in the CM. In all cases, the antibiotics must first pass across the cell wall. Low molecular weight hydrophilic compounds (eg azlocillin, gentamicin, amikacin) presumably penetrate through the general diffusion pores, a mechanism which does not appear to be much affected by alterations in LPS structure (Nikaido, 1979). Hydrophobic compounds, however, pass through the cell wall by diffusion across the OM bilayer, a mechanism not generally available in smooth strains. For this reason, Enterobacteriaceae are often resistant to hydrophobic compounds (eg rifampacin, novobiocin, erythromycin). Hiruma et al (1984) found that LPS, based on its polysaccharide chain region, acted as a selective barrier against the permeation of hydrophobic β-lactams. Treatment with EDTA, which removes up to 50% of the LPS present in the OM, renders cells sensitive to these hydrophobic molecules (Leive, 1974). Some

antibiotics do not fit into this general pattern. Chloramphenicol and tetracyclines are hydrophobic, yet they penetrate and are active against smooth Gram-negative strains (Lambert, 1983).

Benzalkonium chloride is a cationic surface-active quaternary ammonium compound that contains both a hydrophilic and hydrophobic group. Chlorhexidine is a diguanide disinfectant that is only slightly surface-active, with cationic properties. Polymyxin is a cationic hydrophilic polypeptide antibiotic. All three compounds interact with anionic cellular components, such as LPS and phospholipids, forming lesions and disrupting the structure and function of the OM and CM with a concomitant leakage of cytoplasmic constituents.

Using the neosensitab antibiotic assay and agar dilution method, it was found that S. marcescens cells grown in TSB and diluted HS were virtually identical in their sensitivity to a range of antibiotics and disinfectants. It was expected that the increase in LPS O antigen polysaccharide chain length of cells grown in diluted HS, the IRMPs and the HS components attached to the cell surfaces would affect the penetration of antibiotics. There are a number of reasons why no differences were observed. The antibiotic/disinfectant molecules have a low molecular weight and the cell surface changes are probably too small to have a major effect on preventing the molecules reaching the hydrophobic portions of the OMs and the hydrophilic porin channels. The results may also reflect the insensitivity of the assays. Following inoculation onto nutrient agar, bacteria will only retain their particular envelope characteristics for a few generations. The assay is therefore dependent upon the action of the antibiotic on these first few generations and differences in sensitivity between TSB and diluted HS

grown cells, if any, may not become apparent. Lastly, Labischinski et al (1985) reported O antigen molecules assume a heavily coiled conformation on the surface of bacteria and if this is true, the small increase in O antigen chain length might not affect the overall properties of the LPS.

Strain 4444-60 was found to bind 50% less radiolabel (^{125}Iodine) to its cells than strains S1220 and New CDC after growth in TSB, suggesting that a non-protein surface component (eg LPS/capsule) is present that prevented the [^{125}I]lactoperoxidase reaching its binding sites. However, there were no significant differences in the MICs of the antimicrobial compounds for strains 4444-60 and New CDC, probably for the first reason outlined above on the smallness of the antibacterial molecules.

Since its earliest study, S. marcescens appears to have possessed a high degree of resistance to penicillins, cephalosporins, tetracyclines and polymyxins. In the late 1960s, only gentamicin remained effective in a majority of cases, but within 5 years resistance had appeared to this (Yu et al, 1979). Amikacin now remains the only hope, but amikacin-resistant strains have even been isolated (Morohoshi et al, 1984). The resistance of S. marcescens to antibiotics is frequently related to the presence of R plasmids, as well as to the presence of chromosomally mediated resistance determinants. The relative importance of plasmid-mediated vs chromosome-determined resistance remains to be defined (Farrar, 1980). Transmissible plasmids in the hospital environment were found to play a significant role in the resistance of S. marcescens to chloramphenicol, tetracycline, gentamicin, sulphamethoxazole and trimethoprim as well as to ampicillin (Bhujwala et al, 1984). From the results of this study, it appears that S. marcescens strain S1220

may contain an R plasmid with these determinants. It was also resistant to azlocillin and Miller and Bevill (1983) found the resistance gene for azlocillin in S. marcescens rests on a plasmid. The only antibiotic to which strain S1220 was susceptible was amikacin, and it appeared to be more sensitive to this than strains 4444-60 and New CDC. Strain S1220 was obtained from a clinical infection and hospital strains tend to be multi-resistant, the resistance pattern being related to the local customs of antibiotic usage. Strains 4444-60 and New CDC were reference strains without this history and therefore probably do not bear multi-resistant plasmids or chromosomes. Certainly Farmer et al (1976) found the S. marcescens hospital strains they tested were often resistant to several or all of the agents to which 'natural strains' with no exposure to the hospital environment were uniformly susceptible. Of their natural strains, none were highly resistant to chloramphenicol, tetracycline or gentamicin, whereas all showed resistance to polymyxin and to some penicillins and cephalosporins. Likewise in this study, S. marcescens strains New CDC and 4444-60 were sensitive to chloramphenicol, gentamicin, tetracycline and co-trimoxazole, to which strain S1220 appeared to carry resistance markers. With regards to azlocillin, New CDC and 4444-60 were naturally resistant and S1220 seemed to have a resistance gene. On using erythromycin and polymyxin, all three strains gave virtually the same response. Thus the resistance to these two antibiotics is probably characteristic of S. marcescens rather than being related to prior antibiotic exposure. A resistance determinant for polymyxin is thought to be carried by genes located on the chromosome (Hedges, 1980). Whether a similar resistance gene is present for erythromycin or whether the strains by virtue of their smooth nature prevent

penetration of the hydrophobic molecule is unknown.

A variety of methods have been reported regarding the mechanisms of resistance in S. marcescens that involve production of enzymes which inactivate the antibiotic, modification of the target site so that it is insensitive to the antibiotic, and prevention of the antibiotic reaching its target site.

Most gentamicin resistant strains possess aminoglycoside acetyltransferase (ACC) or adenyltransferase enzymes encoded on plasmids (Bullock et al, 1982). Amikacin resistant strains possessing enzymes like AAC6' have been isolated (Morohoshi et al, 1984), although amikacin resistance is also reported to be due to a permeability barrier, a condition which excludes the use of any other aminoglycoside (Shanon and Phillips, 1982). A low level permeability type aminoglycoside resistance in Ps. aeruginosa was associated with a plasmid gene that caused a markedly reduced overall length of LPS molecule, decreasing the hydrophilic nature of the bacterial surface (Bryan et al, 1984). In this study, the small changes in LPS length associated with growth media appeared to have no effect on S. marcescens' response to gentamicin and amakacin.

Most strains of S. marcescens produce small amounts of inducible β-lactamases which hydrolyse cephalosporins but not penicillins, a property mediated by chromosomal genes (Hedges, 1980). Gutman and Chabbert (1984) found multiple mechanisms caused resistance to moxalactam, which might exist to all β-lactams. The mechanisms involved a decrease in permeability associated with modification to porin protein composition, an increase in β-lactamase production and, in one case, modification of the PBPs. Winkler et al (1978) found that a S. marcescens mutant which was hypersensitive to oxacillin had one protein less than the parent strain, presumably because it was

LPS defective. This rough mutant was sensitive to hydrophobic antibiotics, but not to hydrophilic ones. Another mutant of S. marcescens was shown to be resistant to both β-lactams and aminoglycosides by a one-step mechanism involving a decrease in a major OMP reducing the permeability of the OM (Goldstein, 1983). Even resistance to nalidixic acid, trimethoprim and chloramphenicol in S. marcescens has been attributed to a decrease in the amount of a porin protein (Gutman et al, 1985). SDS-PAGE protein profiles of the three strains in this study appeared to be identical. There was no evidence that resistance to any of the antimicrobial agents was due to a reduction in the amount of a porin protein or any other OMP. Azlocillin is a penicillin that is generally resistant to inactivation by β-lactamases. In S. marcescens strain S1220, it may be speculated that the resistance gene for azlocillin causes alterations in the PBPs. It is unknown why strains 4444-60 and New CDC were naturally resistant to azlocillin but it is either because of altered PBPs or, more likely, because the molecule could not penetrate the OM. Azlocillin is a hydrophilic anionic compound and perhaps it was inhibited from passing through the OM by negative charges within a capsule/LPS or by the acidic groups lining the pores (Lambert, 1983).

Tsang and co-workers have extensively studied the interaction of polymyxin with S. marcescens. When whole cells are treated with the antibiotic, blebs appear on the OM cell envelope and if the cell is susceptible to polymyxin, there is subsequent disorganisation of the OM. Degradation of LPS occurs as well as dissociation and release of LPS/protein entities, that subsequently disrupt the barrier function of the OM. Molecules of polymyxin can then reach the CM where they form lesions breaking the selective permeability barrier of the CM

and causing leakage of cytoplasmic constituents from the cells. Polymyxin and LPS also complex in resistant cells forming blebs, but there is no subsequent disruption of the OM (Tsang, Weber and Brown, 1976a; Brown and Tsang, 1978).

The disinfectants benzalkonium chloride and chlorhexidine have similar mechanisms of action to polymyxin causing disorganisation of the OM and CM. Lannigan and Bryan (1985) found an increase in resistance to chlorhexidine was associated with an inner membrane change of unknown nature, rather than an OM barrier change. The three strains of S. marcescens in this study all gave virtually identical responses to the two disinfectants and to polymyxin, the presumed plasmid in S1220 therefore not carrying resistance determinants to these compounds.

Although there were no major differences in the sensitivity of the S. marcescens strains to amikacin and to two disinfectants by the agar dilution technique, on testing the response of the cells to serum killing, significant differences were found. This suggests the mechanisms involved in determining the sensitivity of bacterial cells to antibiotics and complement are not the same, a finding already reported for K. aerogenes (Williams et al, 1986).

The bactericidal effect of serum on the killing of S. marcescens has been studied by various workers (Traub and Kleber, 1975a; Traub and Fukushima, 1979b; Simberkoff et al, 1976b). The initial findings that after growth in diluted HS New CDC was resistant to killing by NPHS but susceptible after growth in TSB, led to the theory that the increase in LPS O antigen chain length or the presence of IRMPs or diluted HS components attached to the membrane, interfered in some way with the access of complement components to the hydrophobic OM where damage occurs.

Nutrient supply and growth phase affect serum sensitivity; Taylor (1978) showed that *in vitro* slow growing *E. coli* cells were more serum resistant than those rapidly growing. Anwar et al (1983) found that oxygen and iron depleted *Ps. cepacia* were more resistant to serum killing than were log phase or carbon depleted cells. Morse et al (1983) showed that growth conditions affected the phenotypic cell surface properties expressed by *N. gonorrhoeae*. When grown at a low dilution rate in a chemostat, the cells had 8-fold less serotype antigen and were more sensitive to serum killing than when grown at high dilution rate. Serum sensitivity may also be linked to metabolic activity. Antibody and complement inhibit uptake of iron by *E. coli* 0111 and cell death follows as a result of the absence of a species of tRNA which is synthesised via an iron-dependent enzyme (Bullen et al, 1974). It could be supposed that *S. marcescens* New CDC cells grown in diluted HS already have mechanisms to override this lack of iron, and the fact that they are slower growing compared to in TSB media, might explain their greater resistance to serum killing.

Generally, smooth bacteria tend to be more resistant to complement killing than their rough counterparts (Ogata, 1983). There is probably some unspecific shielding, as well as specific mechanisms such as unstable insertion of the MAC into the membrane (Brown et al, 1983). Reduction in side chain sugars of LPS generally has to be drastic before changes are seen in serum sensitivity (Taylor, 1983). Thus the longer O antigen chains found after growth in diluted HS are probably not different enough to those present after growth in TSB to explain the responses of the cells to complement killing, especially if the chains are heavily coiled on the surface (Labischinksi et al, 1985).

Often cells can be serum resistant *in vivo* but lose this on subculture and *in vitro* growth. The shift in serum sensitivity may be attributed to phenotypic changes in cell surface properties of *in vivo* grown bacteria (Dalhoff, 1985). A glycoprotein found to be derived from RBC membranes in GPS and human serum was the *in vivo* factor that induced *N. gonorrhoeae* to become resistant to complement killing (Patel et al, 1984a and b). The process required metabolic energy and the glycopeptide was heat- and acid-labile. Inzana and Anderson (1985) found that a low molecular weight component of plasma or serum converted *H. influenzae* type b cells to become resistant to killing, although their component was not the same as that found by Patel and co-workers, being resistant to heat. Based on these findings, it is possible that a component in the diluted HS induced *S. marcescens* New CDC cells to become resistant to NPHS. However, *N. gonorrhoeae* and *H. influenzae* have LPS consisting only of lipid A/core, whereas *S. marcescens* LPS contains O antigen chains as well, which might affect the resistance-inducing capacity of any serum components.

S. marcescens New CDC in 25% of experiments was found to be susceptible to killing after growth in diluted HS and resistant after growth in TSB. Strains 4444-60 and S1220 were either resistant or susceptible respectively to the bactericidal effects of NPHS, virtually independently of growth media. Thus, although the aforementioned theories are true for other species of bacteria and although after growth in diluted HS there was an increase in O antigen chain length and HS components did bind to the cells, other

factors must be involved in determining the response of S. marcescens to NPHS.

The presence of the TraT lipoprotein in the OMs of some Enterobacteriaceae has been linked to resistance to serum killing. It is encoded by genes in F II type R factors and cells harbouring these plasmids express a 25 kDal protein that confers resistance by a mechanism as yet unknown (Moll et al, 1980). There was no evidence of this protein in the OMs of the three S. marcescens strains based on molecular weight determinations after SDS-PAGE, confirming the results of other workers who found no TraT protein in S. marcescens (Montenegro et al, 1985). Kanukollu et al (1985) discovered no isolate of S. marcescens tested had tra T related sequences, suggesting this gene has no role in serum resistance of these strains. Taylor and Parton (1977) found that a serum-resistant mutant of E. coli produced more of an envelope protein of molecular weight 46 kDal than the smooth delayed-susceptible parent. The amount of protein was subject to variation as a result of alterations in growth conditions. The workers suggested that the protein was involved in determination of serum resistance, but it was only functional when superimposed on a full complement of LPS O side chains. The smooth S. marcescens strains in this study contained a 47 kDal protein that was shown to be surface exposed by ^{125}I-labelling in New CDC cells, but not in 4444-60 and S1220 cells. It may be that this has an equivalent function to the 46 kDal protein of Taylor and Parton, and is very sensitive to growth conditions (TSB or diluted HS) for its full expression in New CDC cells. Whether this protein is involved in the serum resistance of strain New CDC or not, SDS-PAGE, autoradiograph and immunoblot profiles suggest that a different mechanism exists in strains 4444-60 and S1220, that is not

related to protein composition.

The rapid killing of S. marcescens strains S1220 and New CDC required the classical complement pathway. Natural bactericidal antibodies are present in the serum of most normal individuals, typically being of the class IgM. IgG antibodies were also found in this study and both classes could well be involved in the killing of S1220 and New CDC via the classical pathway, very little antibody being needed to activate C1 (Taylor, 1983). It is likely antibodies were required because antibody-independent activation of the classical pathway usually occurs in rougher strains (Taylor, 1983). Traub (1981d) found an IgG preparation that contained a low titre of 'natural' IgG antibodies against O antigens, failed to augment in vitro human bactericidal activity against S. marcescens. On the other hand, natural rabbit IgM antibodies enhanced activity against some test strains (Traub, 1980b). In the NPHS of this project, it is known that there were natural IgG antibodies to S. marcescens OMPs, but whether immunoglobulins to the LPS were present remains undetermined. The fact that the classical pathway was involved in the serum killing of the two O14:H12 strains is in contrast to the result of Simberkoff et al (1976b). These workers suggested Serratia opsonins in the serum of healthy individuals are type specific IgM globulins which combine with the organism and activate complement by the alternative pathway. The alternative pathway, whether activated by antibody or not, was involved to some extent in the bactericidal reactions of this study, but it was the classical pathway that was responsible for the rapid killing effect.

Capsular or K antigens of S. marcescens have not been well studied, although a complex range of polysaccharides on the surface of S. marcescens cells have been noted since the 1960s (Adams and

Young, 1966). The three O14:H12 strains were identical in their colonial morphology on nutrient agar plates. However, on harvesting them after growth in TSB, it was noticed that the 4444-60 pellet had a 'slimy' appearance, whereas the S1220 pellet did not. The pellet of New CDC was in between these two extremes. Radiolabelling suggested that strain 4444-60 contained a non-protein surface component which prevented [^{125}I]lactoperoxidase reaching its protein binding sites. It was therefore decided to investigate the binding of C3 components to the cells and the LPS/polysaccharide content of strains 4444-60 and S1220. The results would then be related back to the sensitivity or resistance of the cells to serum killing. With regards to strain New CDC, K antigens have been isolated from it (Traub et al, 1985), but further investigations were decided not to be performed because of its fluctuating response to killing by NPHS when grown in different media. Pitt and Erdman (1984) report S. marcescens cells grown in TSB produce more O-masking substances, such as capsules, than when grown in nutrient broth. The expression of the K antigens may therefore vary according to the growth media (TSB or diluted HS) and growth conditions, affecting the response of the cells to killing by NPHS.

The O14 serotype of S. marcescens is most often isolated from clinical infections and the presence of this O antigen is related to virulence (Traub, 1982a). Often serum resistance is associated with specific O antigens (Ogata, 1983), but in the case of S. marcescens, this is not true, the three O14 strains having different susceptibilities to complement. Strain S1220 was isolated from a clinical urinary tract infection. It appears to contain an R plasmid that determines resistance to antibiotics, but not to complement. In the lower urinary tract there is little risk of killing by complement

(Taylor, 1983) and therefore S1220 cells have no need to acquire serum resistance mechanisms. The cells and infection, however, would not be able to spread via the bloodstream to cause further tissue colonisation. It may well be that the site of infection was selected to be the urinary tract because of the strain's inability to resist complement killing. 4444-60, although a reference strain, has the potential to be more virulent. Its ability to resist complement would theoretically enable it to cause septicaemia in a host, a condition which is so often fatal (Simberkoff et al, 1976b).

3.3 SERUM SENSITIVITY IN RELATION TO SURFACE POLYSACCHARIDE CONTENT OF TWO O14:H12 STRAINS

3.3.1 Binding of complement components to the cells

Bacterial adherence to hydrocarbon (BATH) is a method of measuring the hydrophobicity of bacterial cells; the more hydrophobic the cells the greater the partitioning into the octane (Rosenberg et al, 1980). Figure 3.42 shows that 4444-60 cells grown in both TSB and diluted HS remained hydrophilic after treatment with 10% v/v NPHS. In contrast, S1220 cells became hydrophobic, showing approximately 50% partitioning into octane after 15 minutes incubation with NPHS yet remaining hydrophilic when heat-inactivated serum was used. This suggests that activation, fixation and opsonisation of complement components by the sensitive cells was occurring and not by the resistant cells.

Rocket immunoelectrophoresis is a quantitative immunochemical technique based on the electrophoretic migration of soluble antigens in an antibody containing agarose gel and a specific immunoprecipitation of the antigens by the antibodies. Figures 3.43 to 3.46 show rocket immunoelectrophoresis profiles of whole cells solubilised after contact with 10% v/v NPHS or heat-inactivated NPHS, and the supernatant serum remaining after removal of the cells. To detect any C3 attached to the cells or remaining in the supernatant, the antiserum used was goat-anti-human C3 cast in 1% w/v agarose in Tris-barbiturate buffer pH 8.6. The pH is important. At 8.6 the antibodies have a net neutral charge and do not migrate or move during electrophoresis, whilst the antigen molecules with a different electrophoretic migration do move, usually towards the anode. As the

antigen molecules migrate from the wells into the antibody containing gel, the number of antigens exceeds the number of antibody molecules. Small soluble immune complexes form that continue migration towards the anode at a slower rate. As electrophoresis continues, more and more small complexes form until the equivalence point for precipitation is reached when the complexes fuse to form a precipitate. Coomassie blue can be used to stain the immunoprecipitates after firstly pressing and washing the gel to remove any non-precipitated proteins.

Figure 3.43 suggests that activation of the complement pathway and binding of C3 components to the sensitive cells after growth in TSB was rapid, occurring after 7.5 minutes. The resistant cells in comparison bound much less C3, even after 15 minutes incubation. Figure 3.44 A, lanes 3 and 7, show that after growth in diluted HS and 15 minutes contact with 10% v/v NPHS, the same effect was seen to after growth in TSB (lanes 1 and 5), with greater binding to S1220 cells. When heat-inactivated NPHS was used (Fig 3.44, lanes 2, 4, 6 and 8) there was no attachment of C3 to the cells, lanes 2, 4, 6, 8 of Figure 44B showing that the C3 in the supernatant was antigenic after inactivation, heat therefore destroying the binding capacity of C3 but not the antigenic epitopes.

Figure 3.45 (lanes 1 and 2) again shows the difference in binding of C3 to the two strains after growth in TSB and 15 minutes incubation with 10% v/v NPHS. Lanes 5 to 8 confirmed that the technique of rocket immunoelectrophoresis was quantitative, the height of the rockets being directly proportional to the concentration of C3 in the samples.

Analysis of the supernatant serum after removal of the cells suggests that the quantity of C3 actually increased after incubation

with S1220 compared to 4444-60 (Fig 3.43, lanes 19 to 24 compared to 13 to 18; Fig 3.44, lanes 5 and 7 compared to 1 and 3; Fig 3.45, lane 4 compared to 3). This probably indicates activation of the complement pathway and the splitting of C3 into C3a and C3b. Only C3b attaches to cells (Joiner et al, 1984) and presuming that the antiserum contains antibodies to both molecules , because C3a remains in the supernatant serum and also some of the C3b (not all molecules of the latter bind to surfaces), there would be an increase in the amount of antigen-antibody complex formation. Also, inactivation of bound C3b would be concurrently occurring with the release of fragment C3c into the serum, which may be involved in the immuno-precipitation reaction.

Figure 3.46 is the rocket (A) and subsequent autoradiograph (B) of cells grown in TSB or diluted HS, ^{125}I-labelled after contact with NPHS or heat-inactivated NPHS for 15 minutes, and then solubilised. The C3 proteins have become radiolabelled suggesting that some at least were surface exposed and presumably bound to outer membrane surface components of whole cells. The C3 bound to S1220 cells grown in diluted HS did not ^{125}I-label, presumably because of cell clumping (see section 3.2.1).

Figure 3.42

Affinity of Bacterial Whole Cells to Octane

Bacteria: A 4444-60

B S1220

Key: Medium cells grown in to log phase/cells

1 TSB/normal cells

2 Diluted HS/normal cells

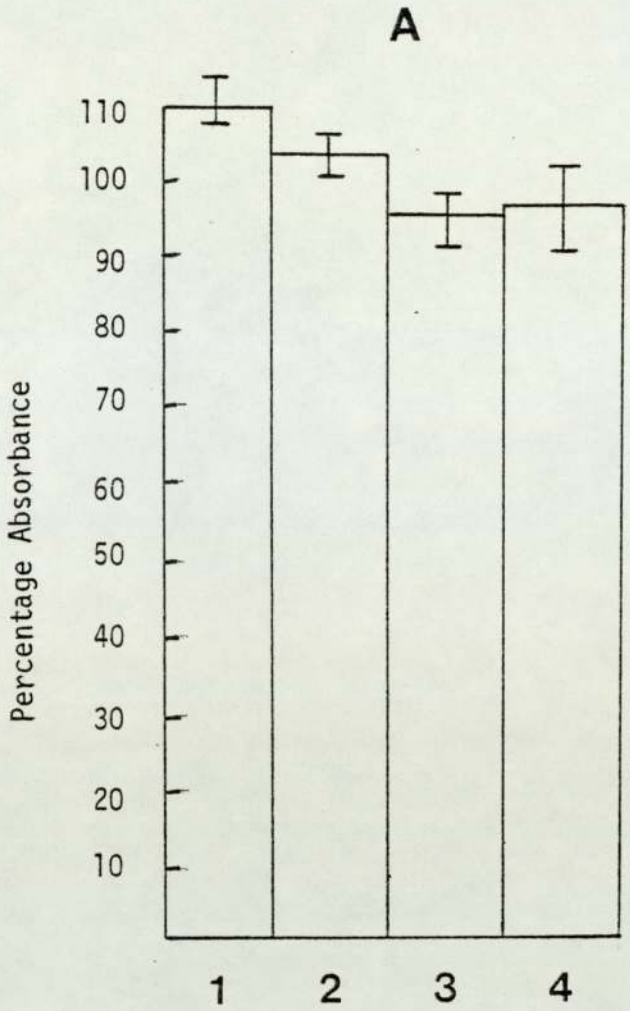
3 TSB/cells incubated with 10% v/v NPHS for 15 minutes (opsonised cells)

4 Diluted HS/cells incubated with 10% v/v NPHS 15 minutes (opsonised cells)

5 As 3, but heat-inactivated NPHS used

6 As 4, but heat-inactivated NPHS used

Results are expressed as a percentage of the initial absorbance o the aqueous cell suspension.


A
Percentage Absorbance
110
100
90
80
70
60
50
40
30
20
10
1
2
3
4

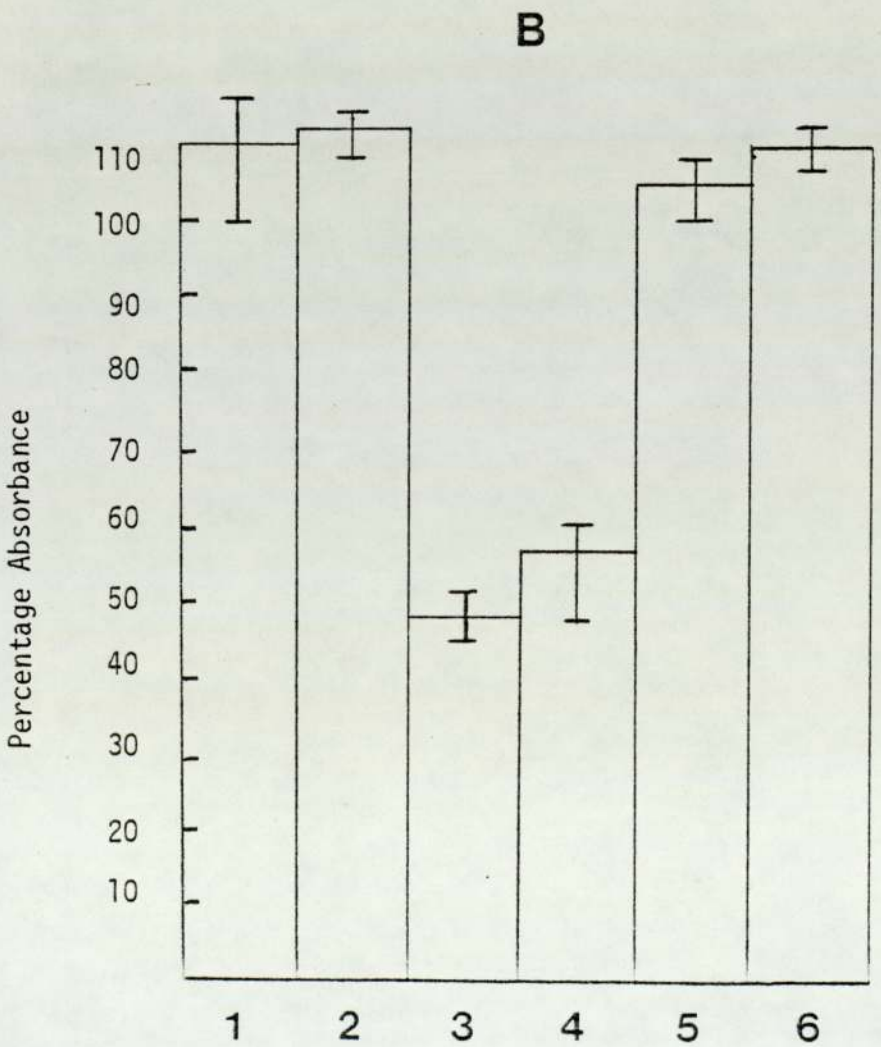

B
Percentage Absorbance
110
100
90
80
70
60
50
40
30
20
10
1
2
3
4
5
6

Figure 3.43

Rocket Immunelectrophoresis using Anti-Human C3 Antiserum of Solubilised Whole Cells and Supernatant Serum after Incubation of Cells with NPHS for Different Times

1 to 12 Whole cells solubilised after contact with 10% NPHS for various times (20 μl)

13 to 24 The supernatant 10% v/v NPHS remaining after removal of the cells (10 μl)

The cells were grown to log phase in TSB before treatment with NP

Key: Strains involved in the samples

1 to 6 & 13 to 18 4444-60

7 to 12 & 19 to 24 S1220

25 10% v/v NPHS control (10 μl)

In each set of six, the cells were in contact with the NPHS for 1 5, 7.5, 10 and 15 mins respectively.

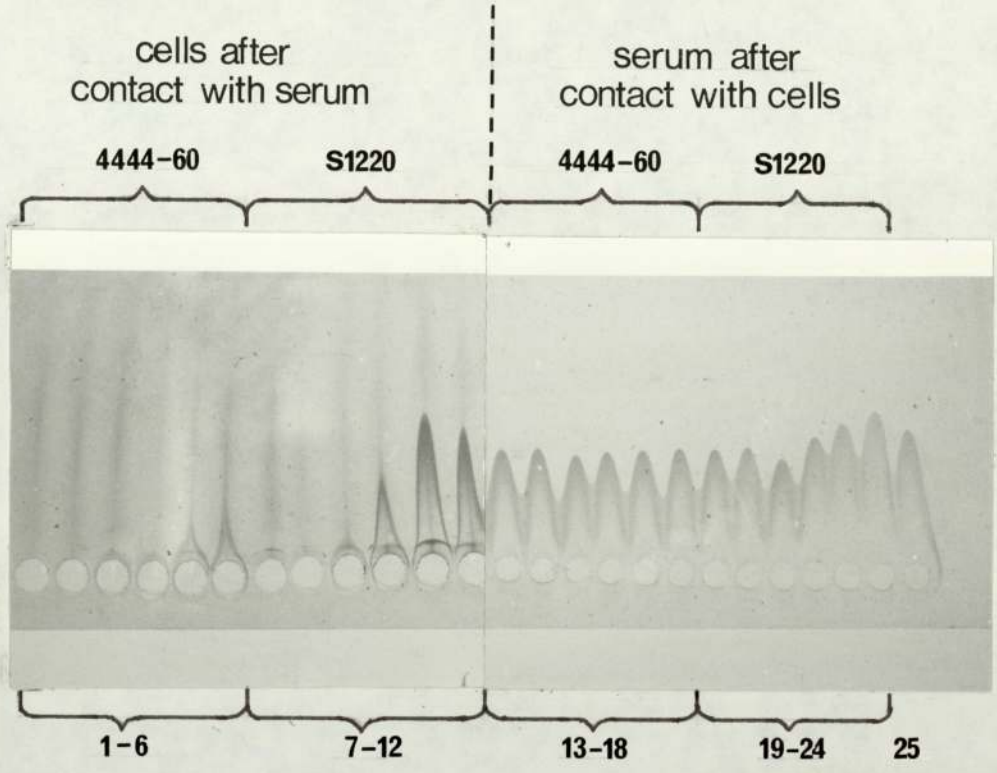

cells after
contact with serum
serum after
contact with cells
4444-60
S1220
4444-60
S1220
1-6
7-12
13-18
19-24
25

Figure 3.44

Rocket Immunoelectrophoresis using Anti-Human C3 Antiserum of Solubilised Whole Cells and Supernatant Serum after Incubation of the Cells with NPHS or NPHS Heat-inactivated for 15 minutes

A Whole cells solubilised after contact with 10% v/v NPHS or heat-inactivated NPHS for 15 mins (20 µl)

B The supernatant 10% v/v NPHS or heat-inactivated NPHS remaining after removal of the cells (10 µl)

Key: Cells and medium grown in to log phase before treatment with serum

1 4444-60, TSB, NPHS

2 4444-60, TSB, NPHS heat-inactivated

3 4444-60, diluted HS, NPHS

4 4444-60, diluted HS, NPHS heat-inactivated

5 S1220, TSB, NPHS

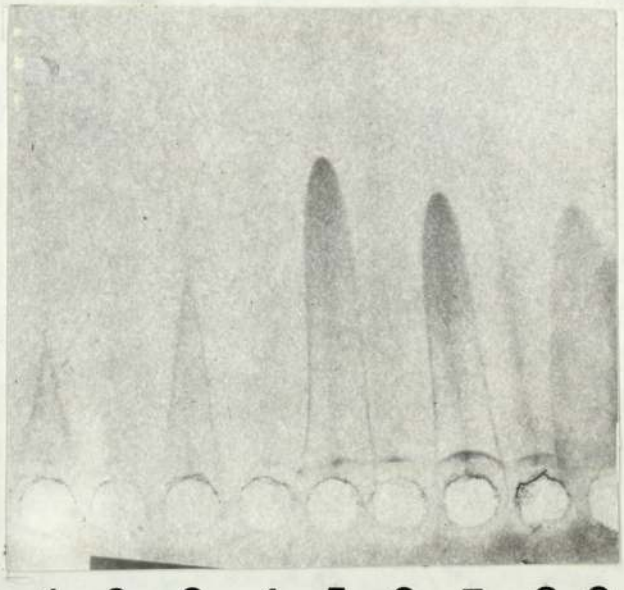
6 S1220, TSB, NPHS heat-inactivated

7 S1220, diluted HS, NPHS

8 S1220, diluted HS, NPHS heat-inactivated

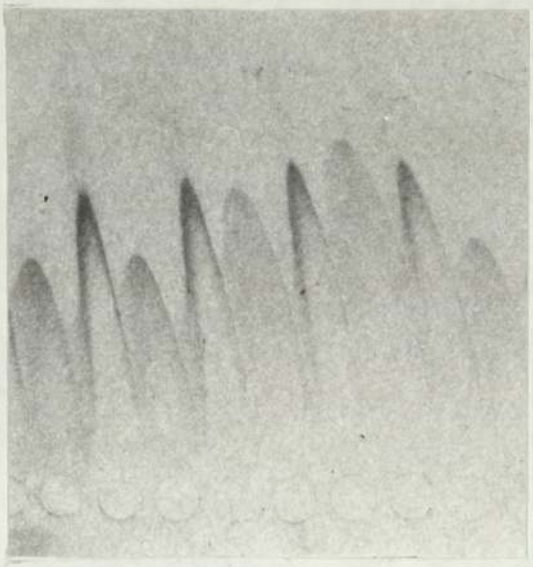
9 Control, 10% NPHS (10 µl)

A



1 2 3 4 5 6 7 8 9

B



1 2 3 4 5 6 7 8 9

Figure 3.45

Rocket Immunoelectrophoresis using Anti-Human C3 Antiserum of Solubilised Whole Cells and Supernatant Serum after Incubation of the Cells with NPHS for 15 minutes

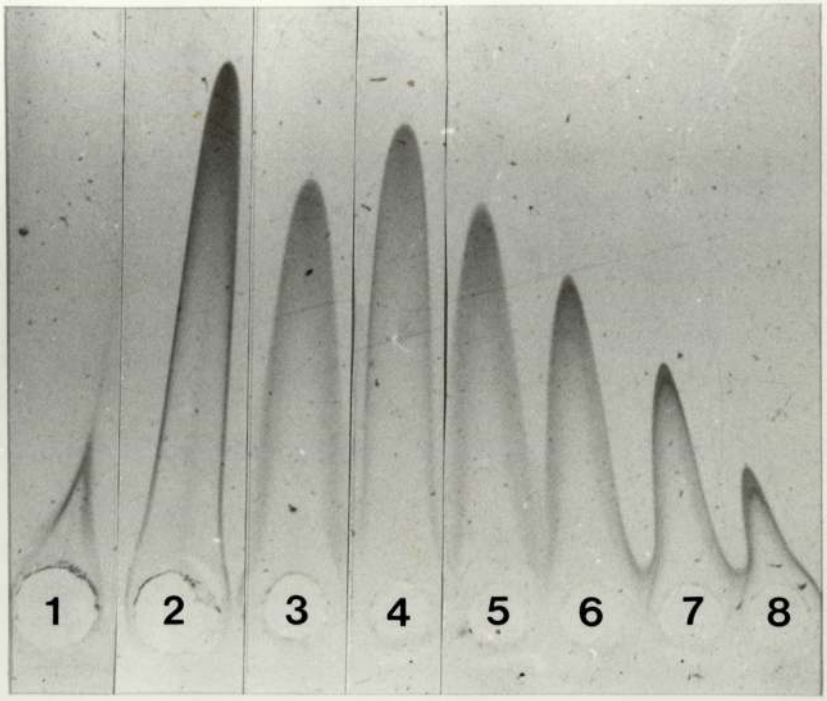
1 and 2 Whole cells solubilised after contact with 10% v/v NPHS for 15 minutes (20 µl)

3 and 4 The supernatant 10% v/v NPHS remaining after removal of the cells (10 µl)

Cells were grown in TSB to log phase before treatment with NPHS.

Key: Cells involved in the samples

1 and 3	4444-60	
2 and 4	S1220	
5	10% v/v NPHS	Controls showing quantitative response of RIE
6	7.5% v/v NPHS	
7	5% v/v NPHS	
8	2.5% v/v NPHS	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2
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7
8

Figure 3.46

Rocket Immunoelectrophoresis using Anti-Human C3 Antiserum of *S. marcescens* Whole Cells Incubated with NPHS or NPHS Heat-inactivated for 15 minutes and Subsequently ^{125}I-lactoperoxidase Labelled.

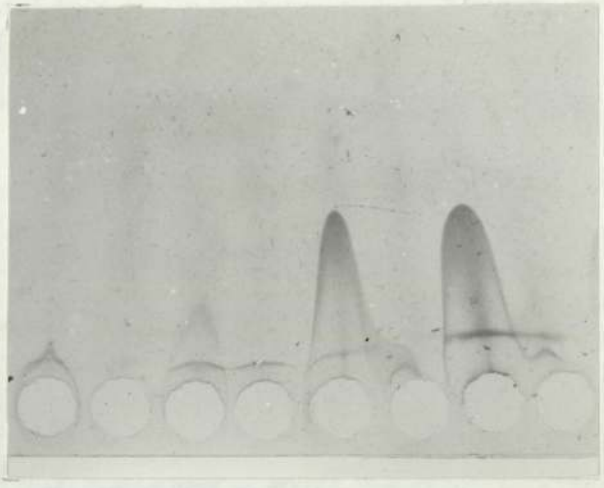
A — Coomassie blue stained rocket of whole cells treated with 10% v/v NPHS or heat-inactivated NPHS for 15 minutes and then ^{125}I-labelled and solubilised (20 µl)

B — Autoradiograph (138 hours) of A

Key: Cells and medium grown in to log phase before treatment with serum

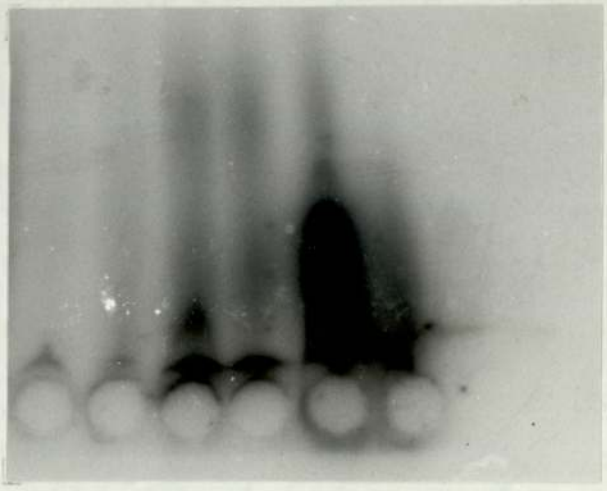
1 — 4444-60, TSB, NPHS
2 — 4444-60, TSB, NPHS heat-inactivated
3 — 4444-60, diluted HS, NPHS
4 — 4444-60, diluted HS, NPHS heat-inactivated
5 — S1220, TSB, NPHS
6 — S1220, TSB, NPHS heat-inactivated
7 — S1220, diluted HS, NPHS
8 — S1220, diluted HS, NPHS heat-inactivated

A



1 2 3 4 5 6 7 8

B



1 2 3 4 5 6 7 8

3.3.2 Chemical analysis of quantitative phenol-extractable material

Polysaccharide material was quantitatively extracted from 4444-60 and S1220 cells after growth to log phase in TSB. The results of chemically analysing it are summarised in Table 3.4, all figures being related to 1 mg dry weight of cells.

Absence of nucleic acids and proteins that could interfere with hexose analysis was confirmed by absence of UV absorption at 260 and 280 nm.

The total weight of extracted material in Table 3.4 shows that 33% more was obtained from the serum resistant strain than the sensitive one.

KDO measurements are usually used to estimate LPS in Gram-negative bacteria. In the assay according to Osborne (1963) material containing LPS is hydrolysed with sulphuric acid to release KDO and further treated with periodic acid and sodium arsenite. The formyl-pyruvic acid thus formed is reacted with thiobarbituric acid to give a chromophore having an absorption maximum at 550 nm. The estimated KDO content of 4444-60 (Table 3.4) was probably influenced by the presence of a related sugar that was found to give a positive response with the periodate-thiobarbituric acid assay by Brigden and Wilkinson (1983). It appears that this component was not present in S1220 LPS. Fatty acid analysis was therefore used as an independent measure of LPS. In this assay the material containing LPS is acid hydrolysed (6M HCl, 100 C, 4 hrs) to release amide- and ester-linked fatty acids. Boron trifluoride-methanol complex is added to allow formation of fatty acid methyl esters which are then extracted with chloroform/hexane. The results of subsequently analysing these

volatile derivatives by GLC are shown in Figures 3.47 and 3.48. The nature and relative proportions of fatty acids in each LPS were identical, viz laurate 5%, myristate 27%, β-hydroxymyristate 60%, unknown 8%. These values are close to those reported by Alaupovic et al (1966) viz laurate 8.5 - 11.8%, myristate 20.8 - 25.0%, β-hydroxymyristate 36.2 - 38.0%, although their proportion of β-hydroxymyristic acid was lower, due to their milder conditions of acid hydrolysis. In Table 3.4, the fatty acid content was expressed as a total of those present.

Carbohydrate was measured using a phenol-sulphuric acid assay. This method relies on the hydrolysis of polysaccharides to monosaccharides, dehydration and rearrangement of the monosaccharides to form furfurals (from pentoses) or hydroxymethyl-furfurals (from hexoses) and reaction of these with the 'colour developer' (phenol). In the assay used, phenol was added to the sample and then concentrated sulphuric acid, the heat produced by dilution of the strong acid being relied upon to develop the colour. The phenol reagent gives approximately the same reaction with pentoses, hexoses and heptoses, the colour produced by the latter two having an absorption maxima at 490 nm as opposed to 480 nm for pentoses (Herbert et al, 1971). Table 3.4 shows the total hexose present in the samples determined by this method.

The ratio of hexose in the phenol-extracted material to the ratio of fatty acids in lipid A gives an indication as to the average chain length of sugars in the LPS. The results in Table 3.4 suggest that in 4444-60 the average sugar chain length is longer than in S1220, or alternatively there is the presence of additional polysaccharides, possibly in the form of a microcapsule.

Table 3.4 Chemical Analysis of Phenol-Extractable Material

All values are related to 1 mg dry weight of cells.

Analysis	Strain	
	4444-60	S1220
Total Weight Phenol-Extractable Material (µg)	83	56
Total Hexose (µg)	36	20
Total Fatty Acids (µg)	1.8	1.7
Total KDO (µg)	6.7	0.84
Ratio Hexose:Fatty Acids	20:1	12:1

Figure 3.47

Profiles of Fatty Acid Reference Samples Separated by Gas Liquid Chromatography

A	Supelco bacterial fatty acid reference standard, 25 mg/ml solution (2 μl)
B	Internal tridecanoate fatty acid reference sample (2 μl)

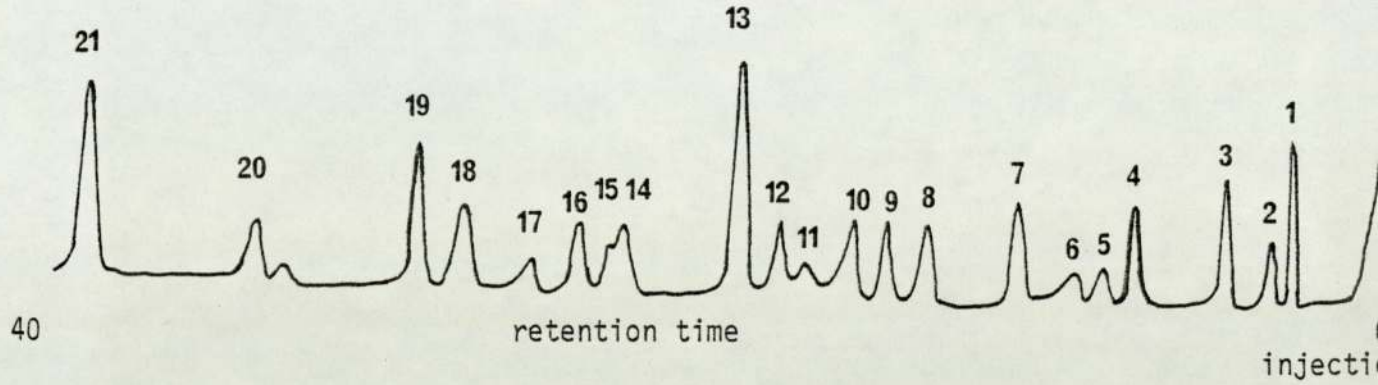
Key:	Fatty acids	
1	11:0	Methyl undecanoate
2	2OH 10:0	Methyl 2-hydroxy decanoate
3	12:0	Methyl laurate
4	13:0	Methyl tridecanoate
5	2OH 12:0	Methyl 2-hydroxy dodecanoate
6	3OH 12:0	Methyl 3-hydroxy dodecanoate
7	14:0	Methyl myristate
8	∝15:0	Methyl 12-methyl tetradecanoate
9	15:0	Methyl pentadecanoate
10	2OH 14:0	Methyl 2-hydroxy tetradecanoate
11	3OH 14:0	Methyl 3-hydroxy tetradecanoate
12	16:1	Methyl palmitoleate
13	16:0	Methyl palmitate
14	17:0Δ	Methyl∝L-cis 9,10 methyl hexadecanoate
15	17:0	Methyl heptadecanoate
16	2OH 16:0	Methyl 2-hydroxy hexadecanoate
17	3OH 16:0	Methyl 3-hydroxy hexadecanoate
18	18:1	Methyl oleate
19	18:0	Methyl stearate
20	19:0Δ	Methyl∝L-cis 9,10 methylene octadecanoate
21	20:0	Methyl arachidate

Key 16:1 = 16C 1 double bond

17 Δ = 1 cyclopropane ring created by subsitution of a methyl group across a double bond

For conditions see materials, methods and preliminary results, page 99

A


21
20
19
18
17
16
15
14
13
12
11
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8
7
6
5
4
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2
1
40
retention time
injecti

B

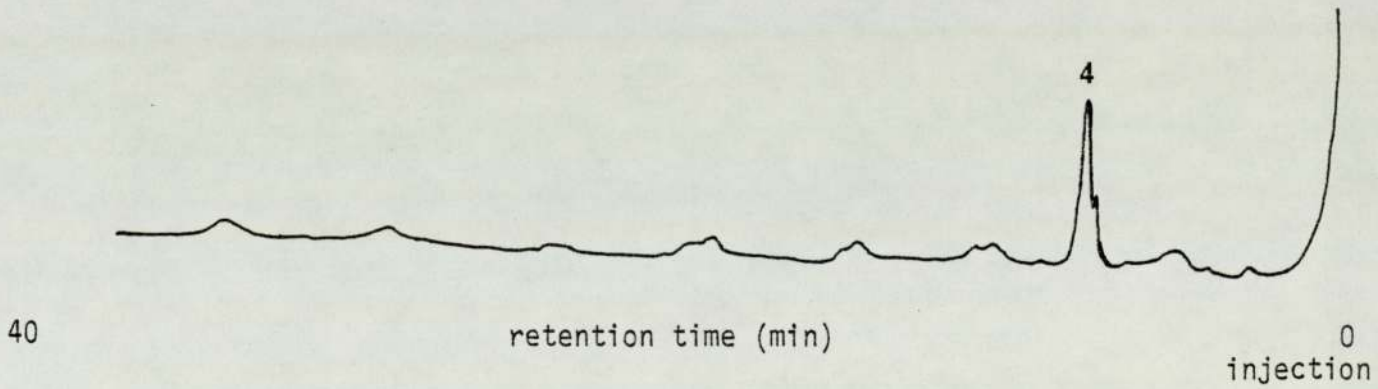

4
40
retention time (min)
0
injection

Figure 3.48

Profiles of Fatty Acid Methyl Esters Obtained from Phenol-Extractable Material Samples by Acid Hydrolysis and Separated by Gas Liquid Chromatography

Fatty acids from the phenol-extractable material of:

A 4444-60

B S1220

Key: Fatty acids

1 Laurate (5% w/w)

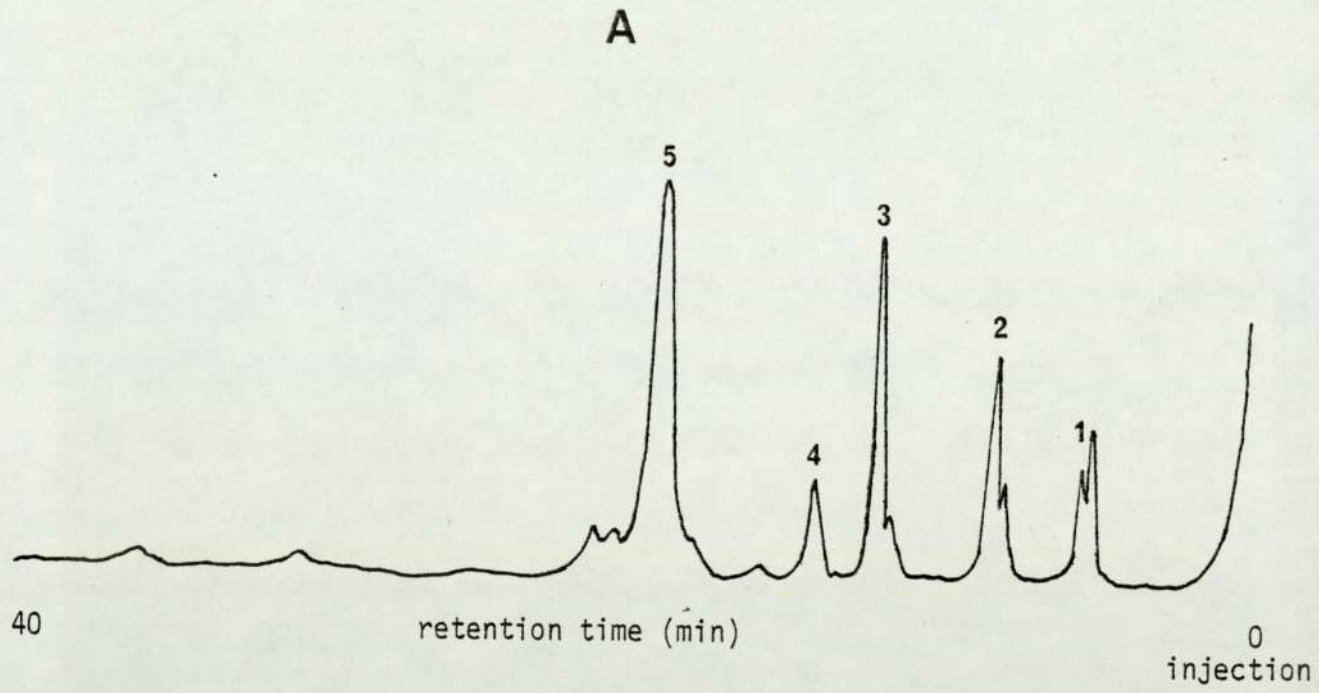
2 Internal reference - tridecanoate

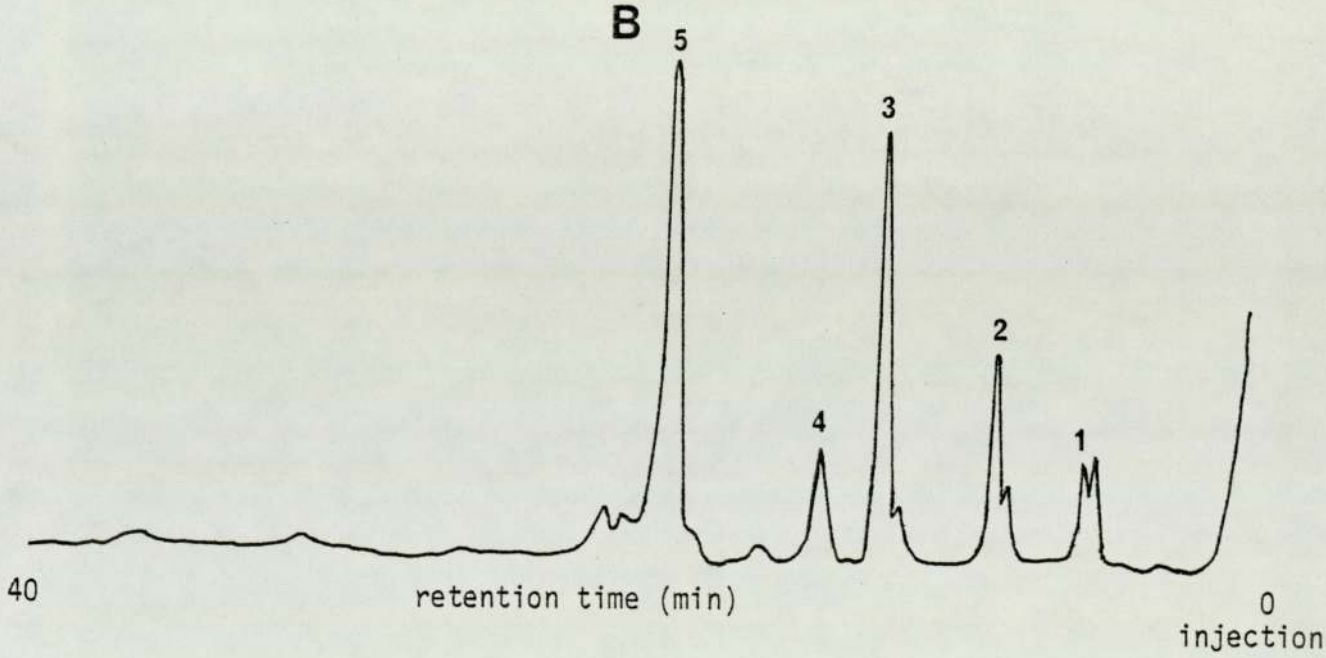
3 Myristate (27% w/w)

4 Unknown (8% w/w)

5 3-hydroxymyristate (60% w/w)

The percentages represent the relative proportions of the fatty acids in the two samples.


A
5
3
2
4
1
40
retention time (min)
0
injection


B
5
3
2
4
1
40
retention time (min)
0
injection

3.3.3 Double diffusion immunoprecipitation and crossed-immunoelectrophoresis

Diagrams of analysing the quantitatively phenol-extracted material and phenol-extracted LPS from cells of 4444-60 and S1220 after growth to log phase in TSB by double diffusion immuno-precipitation are shown in Figure 3.49. Antisera was raised in rabbits to whole cells. The LPS from both 4444-60 and S1220 cells cross-reacted to form a continuous line of fusion. That from 4444-60 gave a weaker second line with its homologous antiserum (A) which could possibly represent micro-capsular material.

Results of running double diffusion immunoprecipitation plates of phenol-extracted material and opsonised whole cells with whole cell antisera and goat-anti-human C3 antiserum are diagramatically shown in Figure 3.50. With both 4444-60 (A) and S1220 (B) a common precipitin line ran between the quantitative-extracted material (1) and presumably polysaccharide in whole cells (3) using rabbit anti-sera (2). The 'micro-capsular' material of 4444-60 did not show a line of identity in whole cells. C3 antiserum (4) formed a precipitin line with the C3 in the whole cell preparations (3), but naturally there was no identity between these goat antibodies and the rabbit antibodies of the polysaccharide precipitates.

Crossed-immunoelectrophoresis is a technique that combines electrophoretic separation of antigens in an agarose gel followed by electrophoresis perpendicular to this into an antibody containing gel. The two dimensions provide powerful resolution of the antigens. It is a quantitative technique, the strength of the reaction and the area subtended by an immunoprecipitate being proportional to the

antigen/antibody ratio (Weeke, 1973).

Crossed-immunoelectrophoresis of solubilised whole cells into pre-immune rabbit serum (Fig 3.51) showed that very few precipitin lines were formed. Owen (1981) reports that normal rabbit serum invariably contains antibodies to an acidic component found in many extracts of bacterial origin. The precipitin line in the CIEs of Figure 3.51 could well represent this.

Reaction of solubilised whole cells of 4444-60 with homologous antiserum raised in rabbits showed the presence of one dominant precipitin arc and several minor arcs (Fig 3.52, A). The major arc was not observed in the profile of S1220 with its homologous antiserum (Fig 3.52, B).

To help identify immunoprecipitin peaks in CIE profiles, Tandem CIE can be used. Tandem CIE is a technique that allows two antigen samples to be applied in the same run in such a way that related precipitin peaks form as fused double peaks in the final pattern (Kroll, 1973). Running solubilised cells and quantitative phenol-extracted material from 4444-60 in this way, suggested that the dominant arc was polysaccharide (Fig 3.52, C). In comparison, the polysaccharide peak of S1220 was a small weakly formed precipitin line (Fig 3.52, D).

To help identify the chemical nature of the immunoprecipitates, the solubilised whole cell antigens can be treated in various ways before CIE. Subjecting solubilised 4444-60 to heat (121 ^{o}C for 15 minutes) destroyed all antigens apart from one that appeared to correspond with the dominant peak (Fig 3.53, B). The polysaccharide arc remained intact after digestion with proteinase K, and the fainter broader arc disappeared (Fig 3.53, C). The two precipitin lines that formed in the intermediate gel become very pronounced

suggesting they were not protein material, but their appearance on the CIE plates was irregular (Fig 3.53, D, the proteinase K control). Periodate treatment caused the disappearance of the major antigen arc, confirming that it was polysaccharide in nature (Fig 3.53 E, and F the control).

Immunoelectrophoresis of solubilised iodinated 4444-60 whole cells (Fig 3.54, A) followed by autoradiography (Fig 3.54, B) showed that the slower migrating portion of the polysaccharide peak was labelled, and that the fainter broader arc was not. This suggested that part of the major peak was a surface protein/polysaccharide (LPS) complex, although the antigenicity was due to the polysaccharide.

Cross-reactions by CIE of 4444-60 antigen with S1220 antiserum showed that the polysaccharide peak was indistinct (Fig 3.55, A) but could be located by running authentic phenol-extracted material in tandem (Fig 3.55, C). With S1220 antigen and 4444-60 antiserum, the peak became more defined than with homologous antiserum (Fig 3.55, B and D).

CIE is a quantitative technique and all the results therefore suggest that in 4444-60 the polysaccharide material and LPS is more antigenic and present in greater amounts than in S1220.

CIEs of opsonised whole cells into homologous antisera with and without goat-anti-human C3 in the intermediate gel are shown in Figure 3.56. The C3 in the preparation from 4444-60 formed a small separate immunoprecipitate when anti-C3 was included in the intermediate gel (Fig 3.56, B) without altering the precipitin lines formed by the rabbit antiserum (Fig 3.56, A). Thus it appeared that the C3, on solubilisation of the cells, became dissociated from whole cell components, or else there was so little C3 bound to the cells

that no change was apparent. S1220 cells bound much more C3 in comparison, reflected by the dominant peak in profile D. It appeared that some of the arcs formed by the rabbit antiserum (marked by arrows in C), one of which may be the polysaccharide arc, were precipitated by the anti-C3 in the intermediate gel (Fig 3.56, D), suggesting that they were cell components to which C3 had bound. It is difficult to draw any firm conclusions, however, because of the weakness of the immunoprecipitin lines.

Figure 3.49

Double Diffusion Immunoprecipitation of 4444-60 and S1220 Phenol-Extractable Material

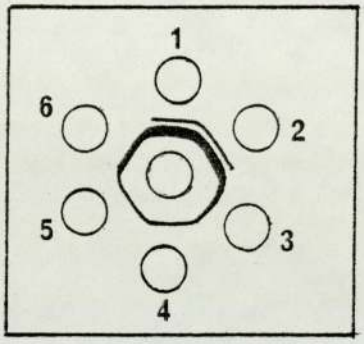
Middle well contains hyperimmune antiserum raised in rabbits to whole cells of A, 4444-60 and B, S1220 grown to log phase in TSB.

Phenol-extractable material and LPS were isolated from cells grown to log phase in TSB.

Key:

1	Quantitative phenol-extractable material of 4444-60
2	LPS isolated from 4444-60
3	LPS isolated from 4444-60, with 2 hour ultra-centrifugation
4	Quantitative phenol-extractable material of S1220
5	LPS isolated from S1220
6	LPS isolated from S1220, with 2 hour ultra-centrifugation

A



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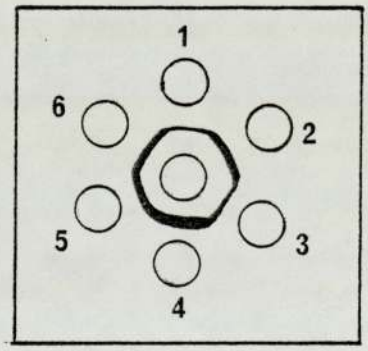


Figure 3.50

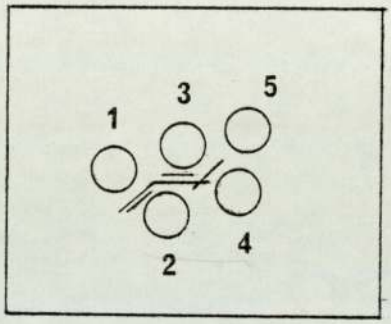
Double Diffusion Immunoprecipitation of 4444-60 and S1220 Whole Cells Solubilised After Contact with NPHS (10% v/v) for 15 minutes, Together with Phenol-Extractable Material

A	Antiserum, whole cells and phenol-extractable material from 4444-60 after growth to log phase in TSB
B	Antiserum, whole cells and phenol-extractable material from S1220 after growth to log phase in TSB

Key:

1	Phenol-extractable material
2	Antisera raised in rabbits to whole cells
3	Whole cells solubilised after contact with NPHS for 15 minutes
4	Anti-human C3 antiserum
5	As A.

A



B

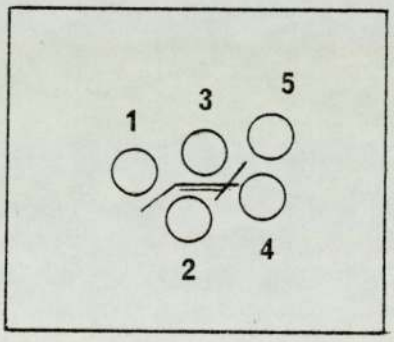


Figure 3.51

Crossed Immunoelectrophoresis Patterns Obtained by Running 4444-60 and S1220 Solubilised Whole Cells into Pre-Immune Rabbit Serum

Key:

A	4444-60 (200 μg protein)
B	S1220 (300 μg protein)

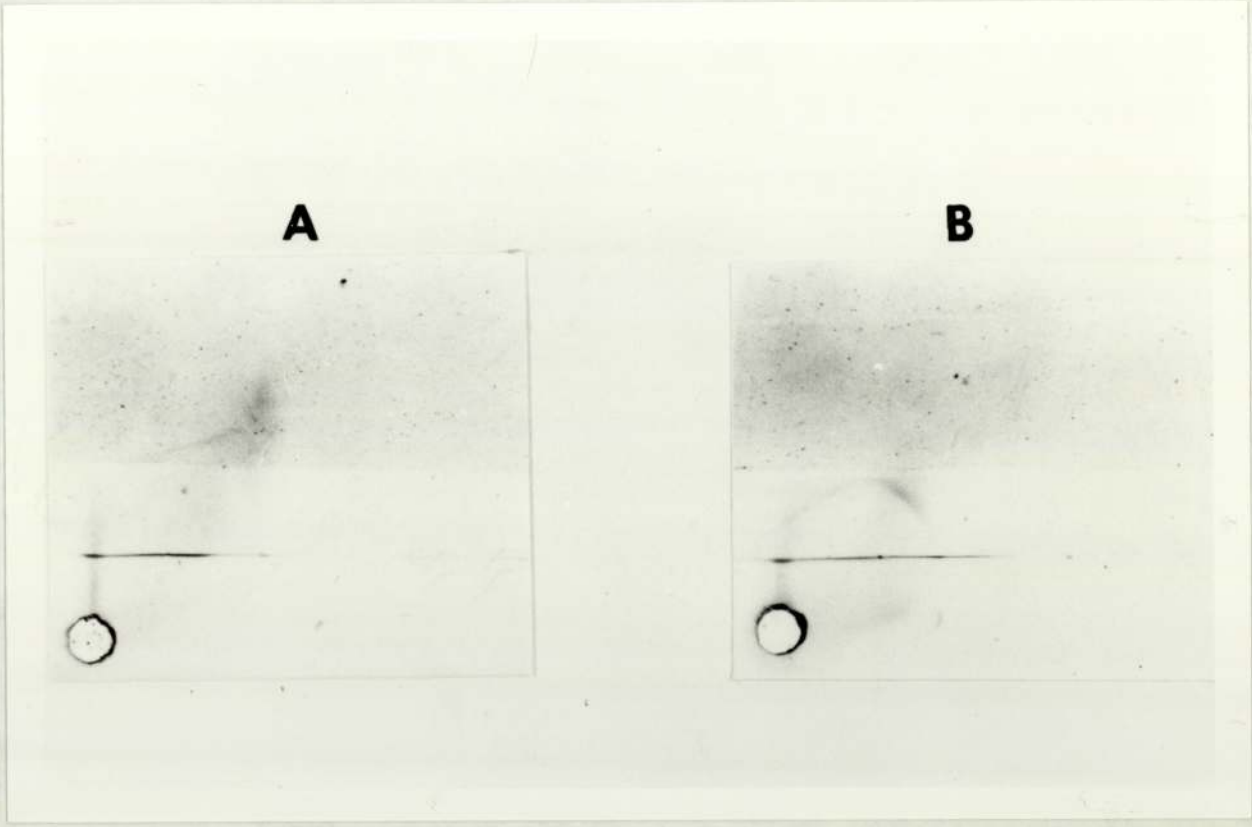

A
B

Figure 3.52

Crossed Immunoelectrophoresis Patterns of 4444-60 and S1220 Solublised Whole Cells with Homologous Antiserum and Tandem CIEs of Solubilised Whole Cells with Quantitative Phenol-Extractable Material

Antiserum was raised in rabbits to log phase cells grown in TSB.

Key: CIE patterns

A 4444-60 solubilised whole cells (200 µg protein) with homologous antiserum (200 µl)

B S1220 solubilised whole cells (300 µg protein) with homologous antiserum (250 µl)

Key: Tandem CIE patterns

C As in A, with 4444-60 phenol-extractable material (200 µg) in 2nd well

D As in B, with S1220 phenol-extractable material (200 µg) in 2nd well

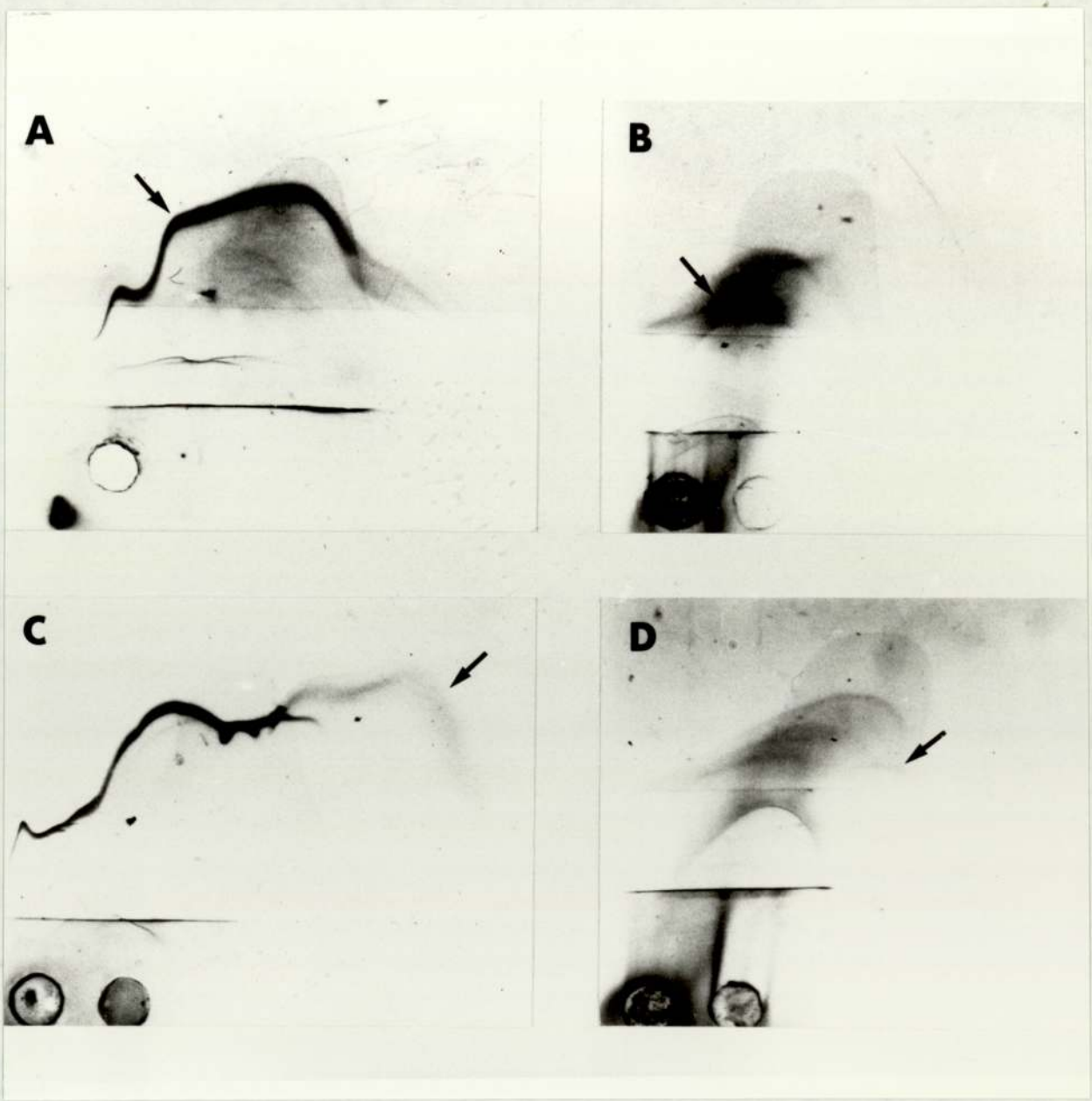

A
B
C
D

Figure 3.53

Crossed Immunoelectrophoresis Patterns of 4444-60 Solubilised Whole Cells Treated with Heat, Proteinase K or Sodium Periodate before CIE into Homologous Antiserum

Antiserum was raised in rabbits to 4444-60 whole cells grown in TSB to log phase.

Cells were grown in TSB to log phase before solubilisation.

Key:

A	Solubilised whole cells (200 µg protein)
B	As A, but extract treated first with heat (121°C for 15 mins)
C	As A, but extract treated first with Proteinase K (10µg in 5 mM Tris-HCl pH 6.8, 60°C for 1 hour)
D	As C, but omitting Proteinase K
E	As A, but extract treated first with 0.1 M sodium periodate in sodium acetate buffer (pH 4.5) overnight
F	As E, but omitting sodium periodate

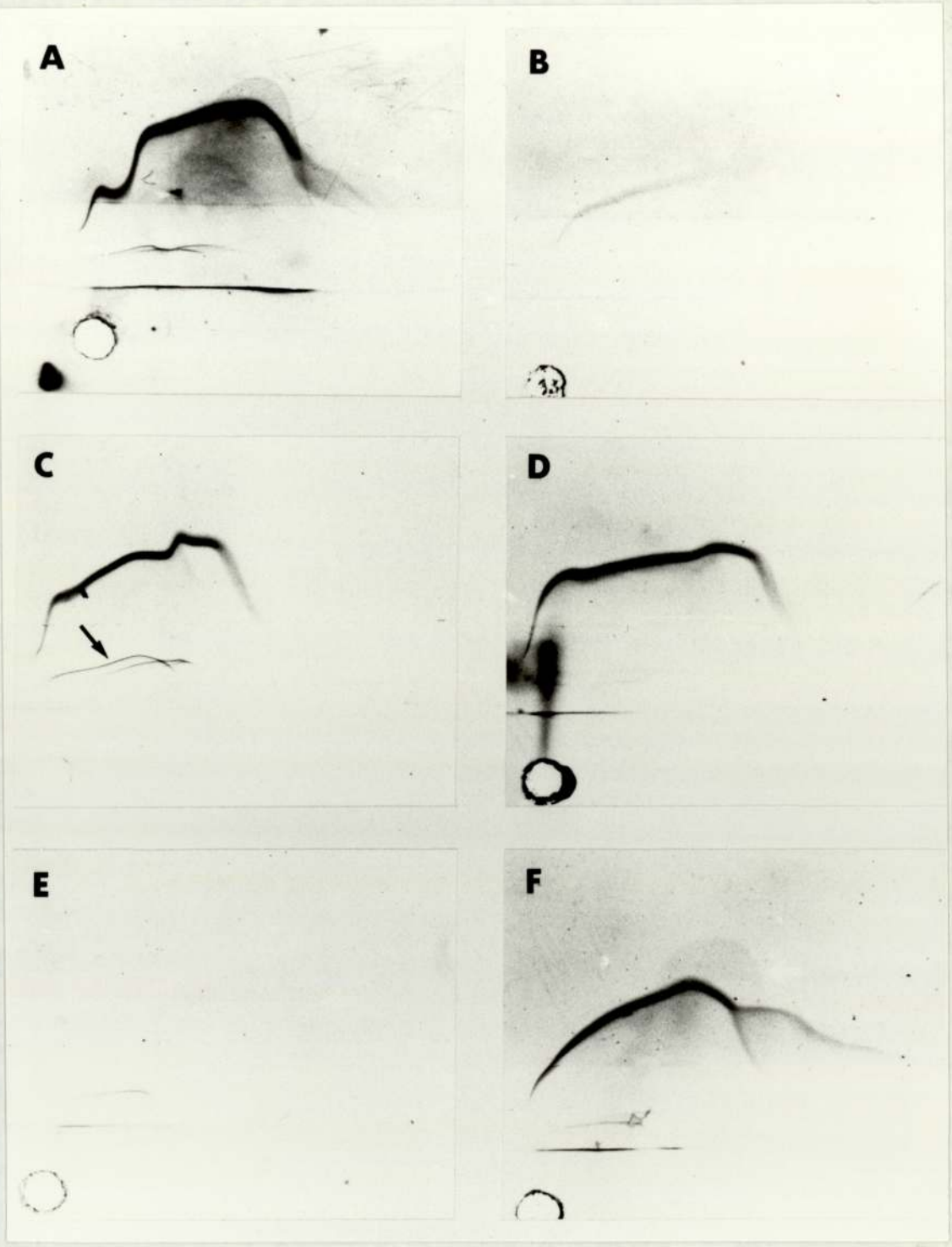

A
B
C
D
E
F

Figure 3.54

Crossed Immunoelectrophoresis Patterns with Homologous Antiserum of 4444-60 Whole Cells Solubilised after Labelling with ^{125}I-Lactoperoxidase

Antiserum was raised in rabbits to 4444-60 whole cells grown in TSB to log phase.

Cells were grown in TSB to log phase before iodination and solubilisation.

A CIE pattern of solubilised whole cells (200 µg protein) with homologous antiserum (200 µl)

B Autoradiograph of A, developed after 7 days

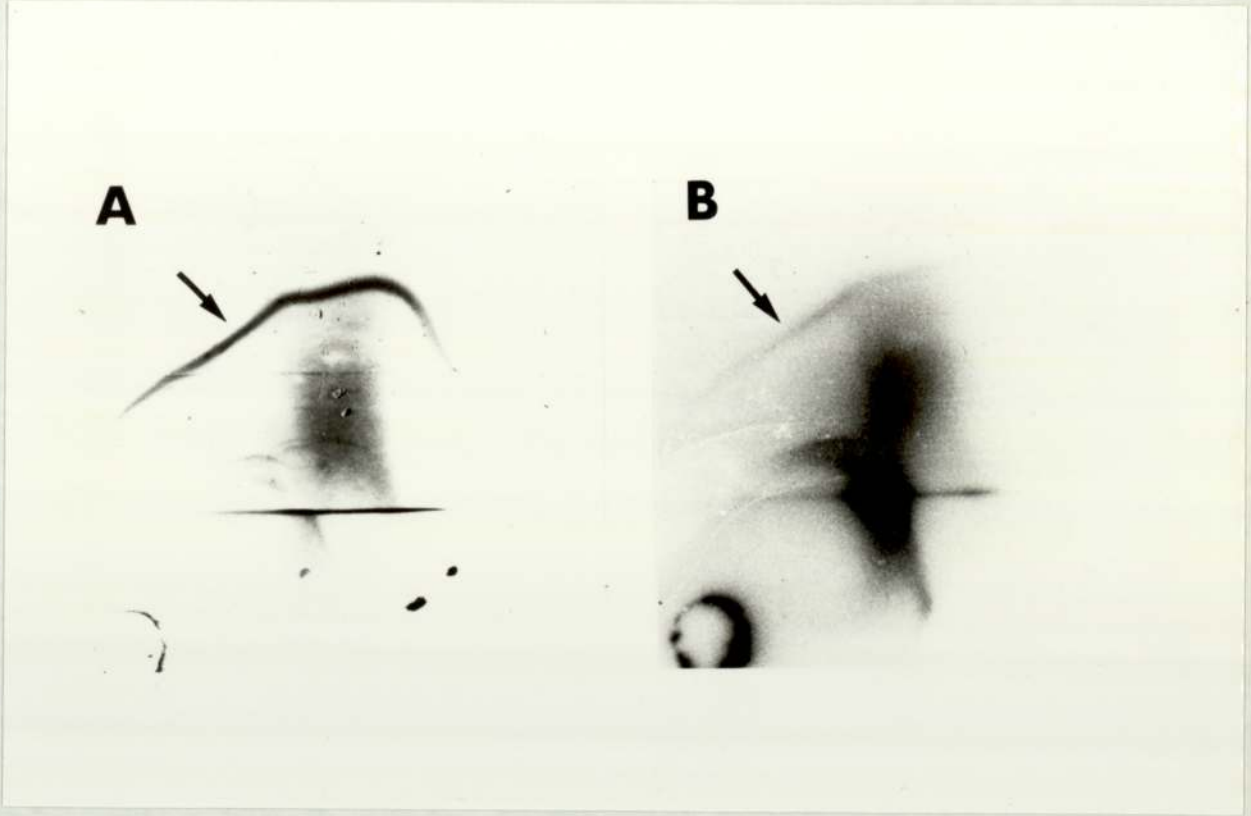

A
B

Figure 3.55

Crossed Immunoelectrophoresis Patterns of 4444-60 and S1220 Solubilised Whole Cells with Heterologous Antiserum and Tandem CIEs of Solubilised Whole Cells with Quantitative Phenol-Extractable Material

Antiserum was raised in rabbits to log phase cells grown in TSB.

Key: CIE patterns

A 4444-60 solubilised whole cells (200 μg protein) with S1220 antiserum (250 μl)

B S1220 solubilised whole cells (300 μg protein) with 4444-60 antiserum (200 μl)

Key: Tandem CIE patterns

C As in A, with 4444-60 phenol-extractable material (200 μg) in 2nd well

D As in B, with S1220 phenol-extractable material (200 μg) in 2nd well

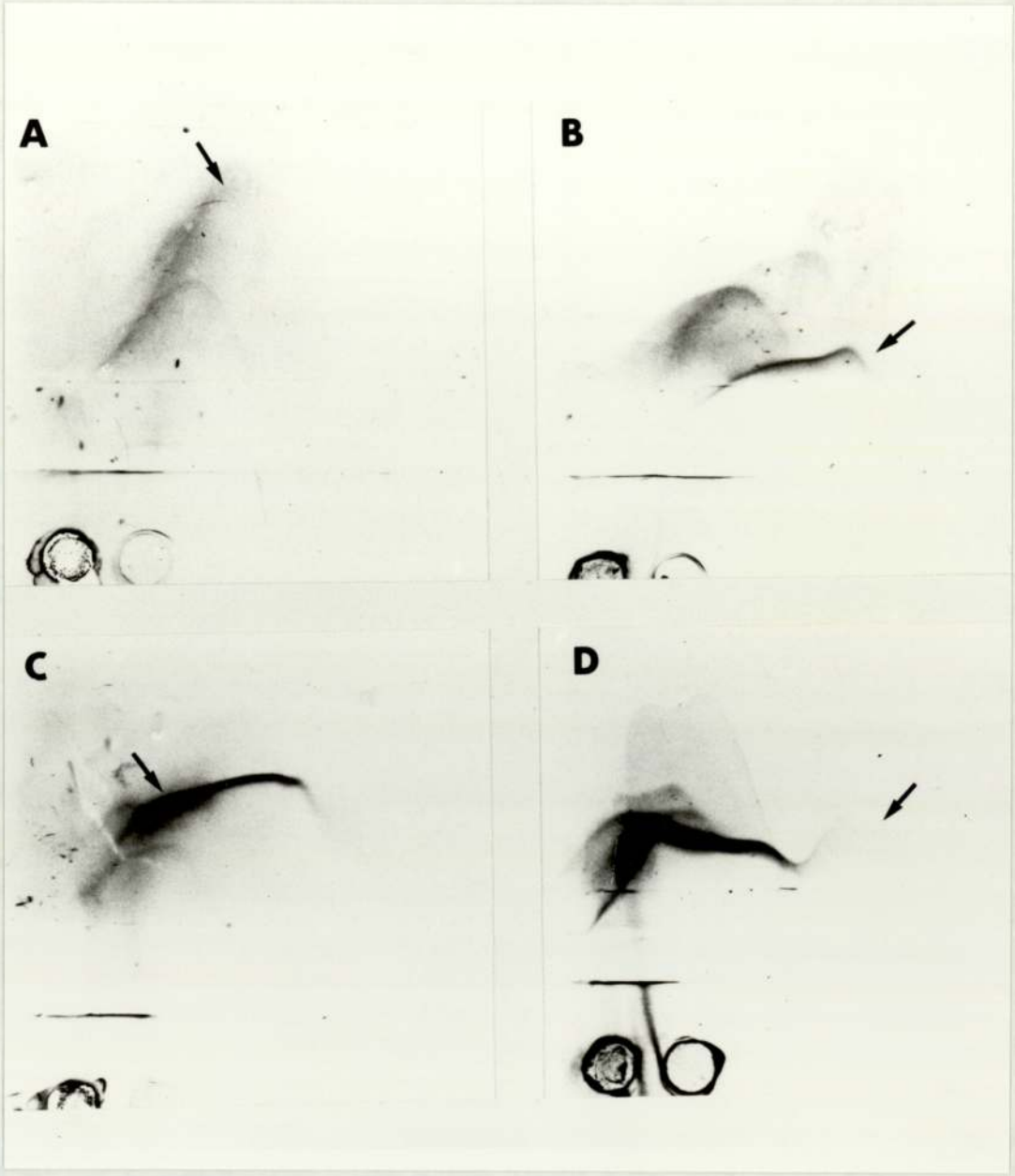

A
B
C
D

Figure 3.56

Crossed Immunoelectrophoresis Patterns of 4444-60 and S1220 Whole Cells Solubilised after Incubation with NPHS for 15 minutes using Homologous Antiserum with Anti-Human C3 in the Intermediate Gel

Antiserum was raised in rabbits to log phase cells grown in TSB.

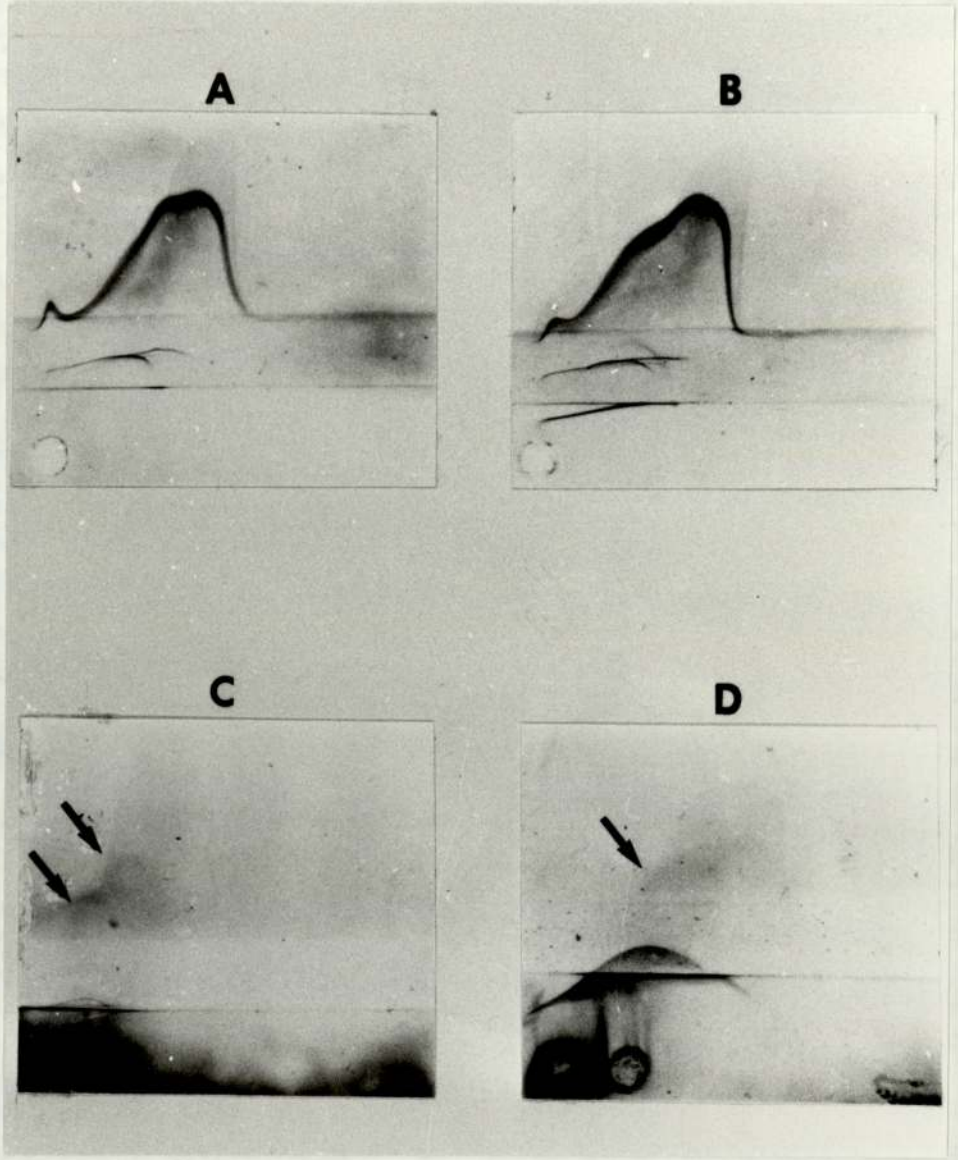
Cells were grown to log phase in TSB before opsonisation with serum.

A — 4444-60 whole cells (200 μg protein) solubilised after 15 minutes contact with NPHS, run into homologous antiserum (200 μl)

B — As A, but with anti-human C3 (10 μl/ml) in the intermediate gel

C — S1220 whole cells (300 μg protein) solubilised after 15 minutes contact with NPHS, run into homologous antiserum (250 μl)

D — As C, but with anti-human C3 (10 μl/ml) in the intermediate gel.


A
B
C
D

3.3.4 SDS-PAGE of phenol-extractable material

Silver stains of the quantitative phenol-extracted materials after SDS-PAGE are shown in Figure 3.57. Identical amounts were loaded to each track. It appears that 4444-60 contained a proportion of material which migrated slowly, was not resolved into the familiar LPS 'ladder' pattern and is thought to be molecules with long O antigen chains (Peterson and McGroarty, 1985). S1220 contained far less of this material and the ladder pattern was more pronounced near the fast migrating lipid A region probably representing rougher LPS. Attempts to resolve the slow migrating material into discrete bands by increasing the SDS concentration in the gel (Peterson and McGroarty, 1985) were unsuccessful, confirming that it was not dimers of short LPS chains but true high molecular weight long chained LPS.

Capsular polysaccharides can usually be separated from LPS by ultracentrifugation, the LPS being pelleted and capsular polysaccharides remaining in the supernatant (Sutherland and Wilkinson, 1971). On subjecting samples of phenol-extracted material to this, a characteristic ladder pattern was obtained from both the freeze-dried reconstituted supernatant (probably monomeric LPS) and pellet fractions on SDS-PAGE (Fig 3.58). Even in the presence of $MgCl_2$, which helps the LPS to pellet by promoting formation of micelles, separation could not be obtained on ultracentrifugation. Therefore, any capsular material in the supernatant could not be distinguished.

Results of digesting the same OD_{470} of whole cells with proteinase K and subsequently separating the remaining polysaccharide material by SDS-PAGE are shown in Figure 3.59. S1220 gave a fainter LPS ladder pattern on silver-staining. Assuming a quantitative response to the stain, it appears that the sensitive cells contain

less polysaccharide material than the resistant ones, which confirms previous results. The patterns obtained from both strains by proteinase K digestion were different to those from isolated polysaccharide material (Fig 3.57) probably because the samples contained less concentrated material.

Figure 3.57

Silver Stained Phenol-Extractable Material of S. marcescens after SDS-PAGE

Key:	Material from strain
1	4444-60 (50 µg)
2	S1220 (50 µg)

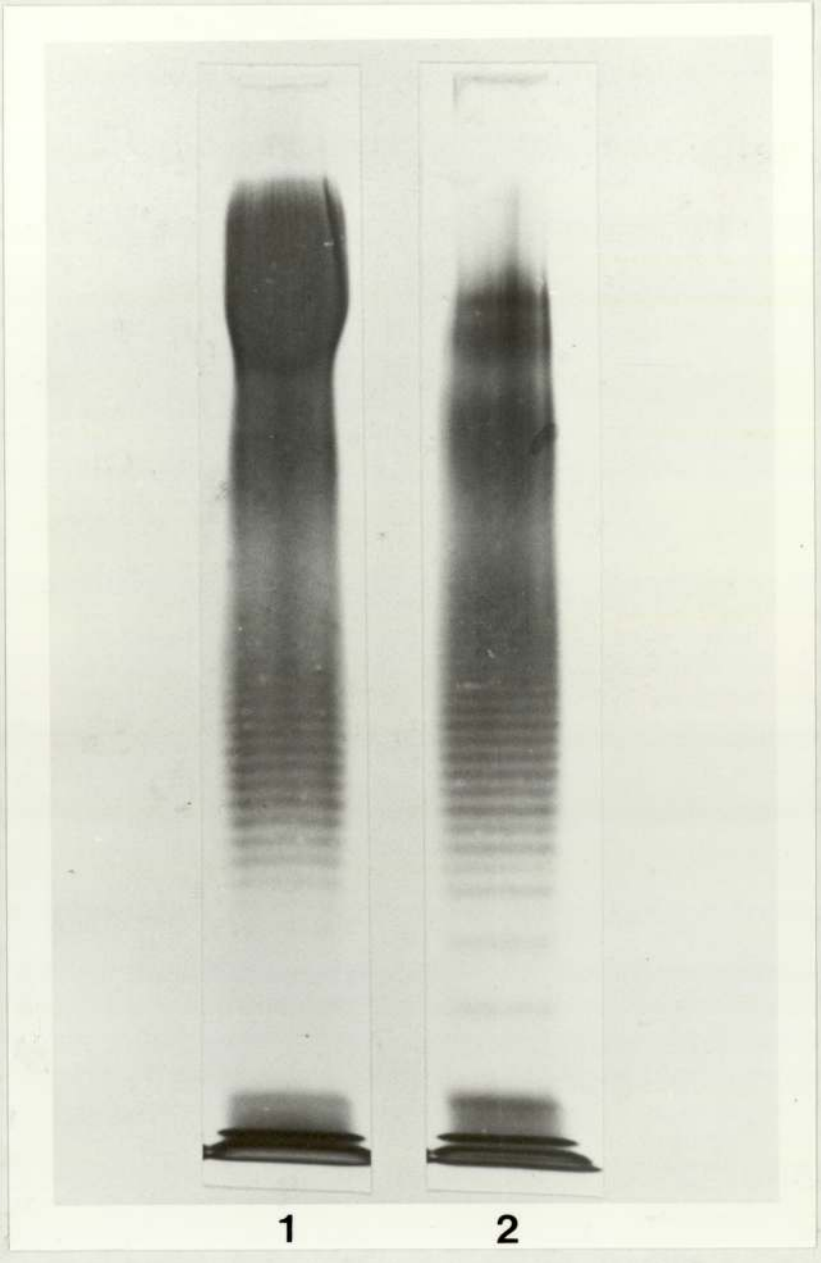

1
2

Figure 3.58

Silver Stained Gel after SDS-PAGE of the Pellet and Freeze-Dried Supernatant Obtained by Ultracentrifugation of the Phenol-Extractable Material in the presence of $MgCl_2$

Key:

1	4444-60 pellet (25 μg)
2	4444-60 supernatant (25 μg)
3	S1220 pellet (25 μg)
4	S1220 supernatant (25 μg)

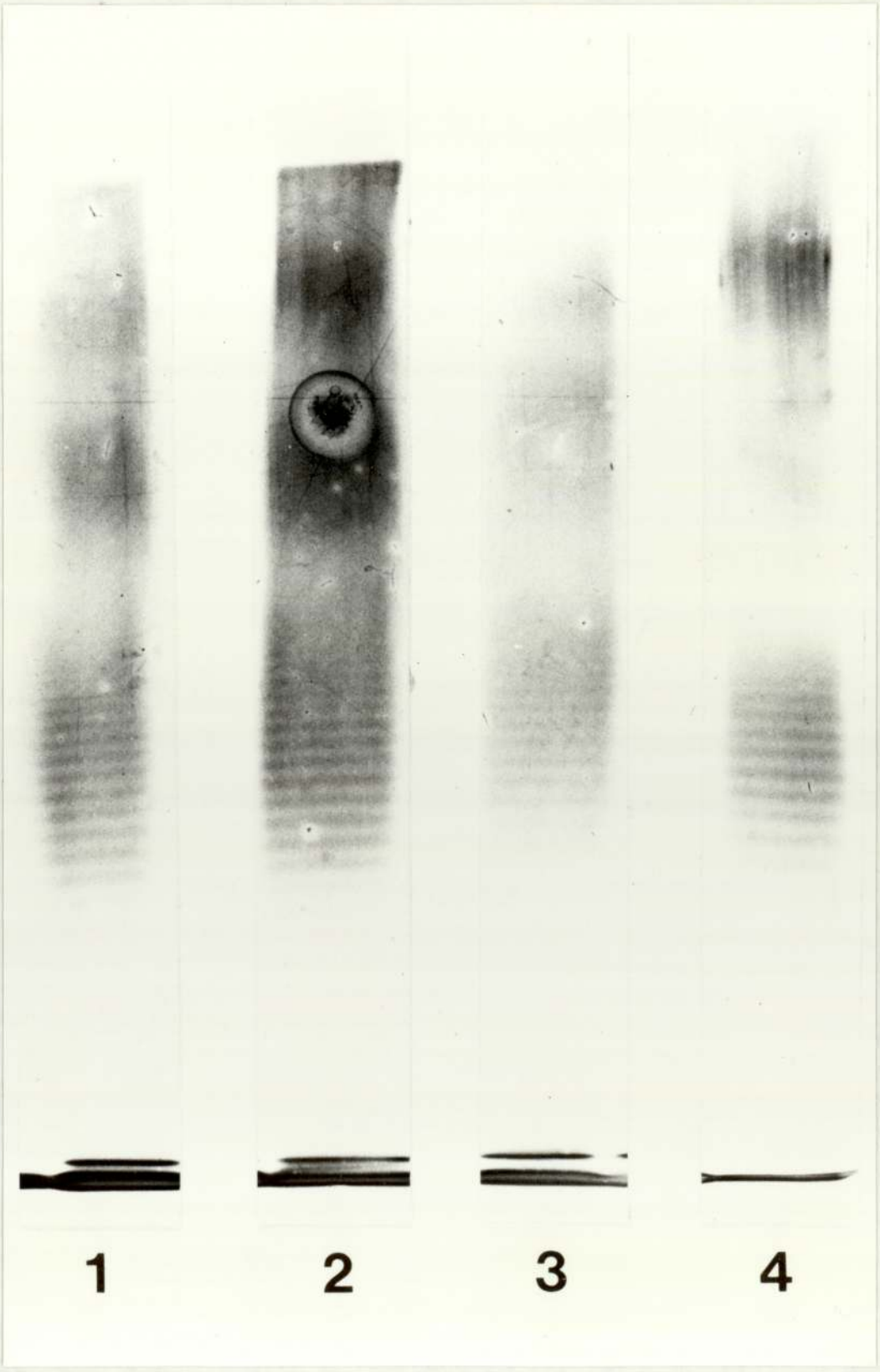

1
2
3
4

Figure 3.59

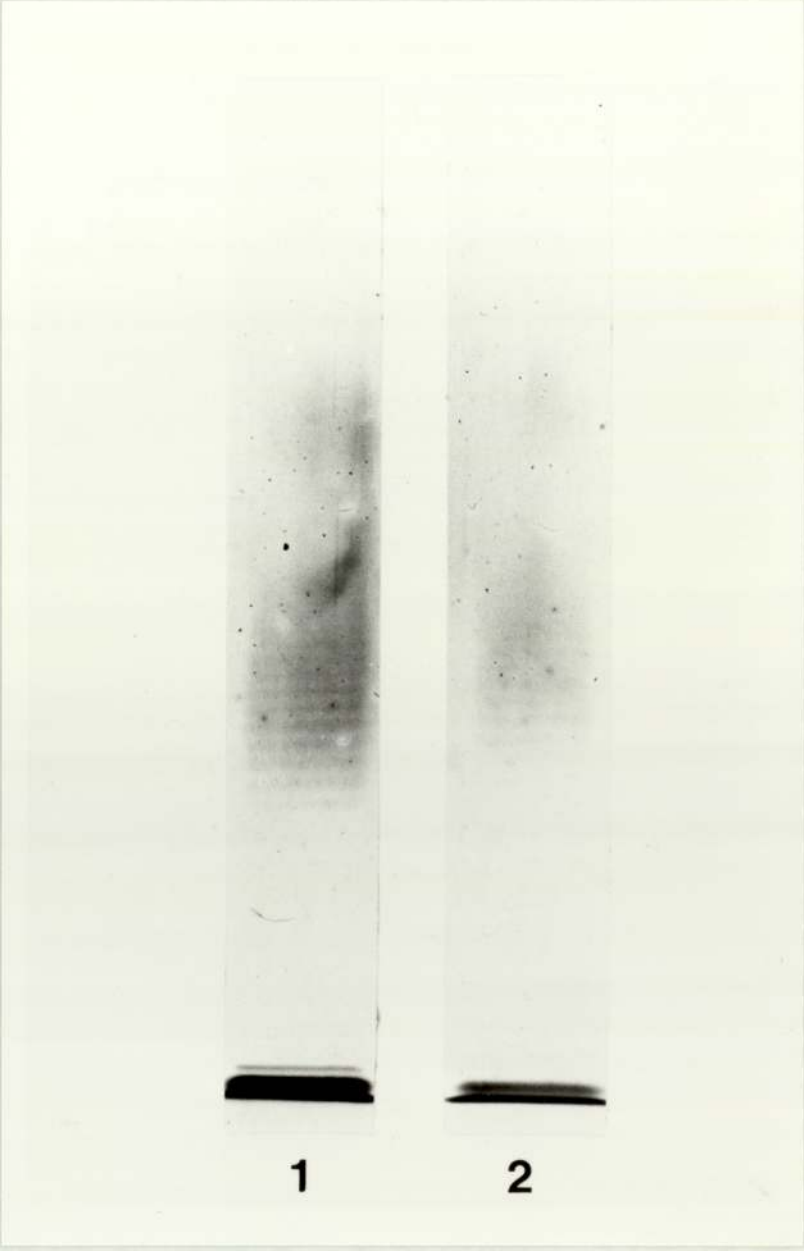
Silver Stained Gel after SDS-PAGE of Proteinase K Digested Whole Cells of 4444-60 and S1220

Cells were grown to log phase in TSB before digestion.

Key:

1	4444-60
2	S1220

The cells were suspended to the same OD_{470} (1.0) and the same loadings run on the gel.


1
2

3.3.5 Discussion

Analysis of two O14:H12 strains of S. marcescens with different sensitivities to serum killing showed that the sensitive strain rapidly bound large quantities of C3 to its cells and activated complement. Strain 4444-60 in comparison bound much less C3b to its cells, the results suggesting that it could not efficiently activate complement. Growth media (TSB and diluted HS) had no effect on the cells' sensitivity to complement or on the binding of C3b to the cells. As discussed in the previous section, the proteins of the two strains were virtually identical and therefore it was decided to concentrate on growth in TSB and look for any differences in the polysaccharides of the cells.

Intact C3 has an internal thioester linkage between a glutamine and cysteine within the α chain of this two chain molecule, forming a tight ring (Pangburn and Müller-Eberhard, 1980). On loss of the C3a portion of the molecule leaving C3b, the thioester is exposed (activation). Since it is highly reactive, it will either be hydrolysed, or bind to an appropriate nucleophilic substrate, forming a covalent ester or amide bond with antibody, capsule, LPS etc (Joiner et al, 1984). C3b bound to early acting complement components initiates the cascade responsible for MAC formation and direct cell death. C3b bound on its own coats the bacteria, serving as an opsonin. It may interact with components of the C3-inactivator system (factors H and I) that cleave C3b to form inactivated C3b (iC3b). Further cleavage by non-specific proteases releases fragment C3c from the bacterial surface, leaving C3dg covalently bound (Joiner et al, 1984). Phagocytic cells possess receptors for C3b and for iC3b (Ross and Lambris, 1982). Newman and Mikus (1985) tested five

species of bacteria and found that only 16-28% of bound C3b was in the form of iC3b, even after 2 hours incubation, suggesting that the predominant form of C3 encountered by phagocytes on bacterial surfaces may be C3b. The increased quantity of C3 bound to S. marcescens strain S1220 suggests that it would be more sensitive to phagocytic killing than strain 4444-60. The bound complement components increased the hydrophobicity of the S1220 cells, an effect that renders cells more sensitive to phagocytic engulfment (Ofek and Beachey, 1980), the C3b itself also acting as a ligand for specific receptors.

Other workers have found, like with S. marcescens, that more C3b binds to cells sensitive to complement than to resistant cells. Taylor and Kroll (1985) used rough strains of E. coli and discovered by rocket immunoelectrophoresis, that covalent binding of C3b to the target cell occurred during the early stages of the reaction and at the end of a 10 min lag period, large amounts of C3b could be detected on the OM. This is virtually identical to the results using the smooth S. marcescens strain S1220. Taylor and Kroll also measured the binding of C5b-9 components and found that these were first complexed to the OM after 10 min serum exposure (a point in time coinciding with the onset of viability loss) and then rapidly increased during the active killing phase reaction.

Gram-negative bacteria can activate complement by both the alternative and classical pathways in the absence (and presence) of antibody (Joiner et al, 1984). Whereas the lipid A portion of LPS is capable of direct classical pathway activation without antibody, the polysaccharide portion of LPS can activate the alternate pathway (Morrison and Kline, 1977). LPS provides a 'protected' site for C3 deposition, a site at which inactivation by factors H and I is

relatively inefficient (Joiner et al, 1984). Grossman and Leive (1984) demonstrated LPS molecules bearing as few as five O antigen repeats are as efficient as molecules bearing eighteen to forty O antigen repeats in activating the alternative pathway.

Natural antibodies appear to initiate complement-mediated opsonisation of most strains of Ps. aeruginosa. In general, the classical pathway is involved in opsonisation of the cells with C3b, and to a lesser extent the alternative pathway. Engels et al (1985) used three mutant strains of Ps. aeruginosa which differed in their content of O antigen. They found the amount of C3 attached to the cells' surfaces correlated with phagocytosis. The rough strain bound much more C3, was phagocytosed well and was sensitive to complement killing. The strain with long O polysaccharide side chains bound much less C3 and was resistant to serum killing. An intermediate strain (shorter O chains) bound as much C3 as the rough strain, but was not serum sensitive. Opsonisation of the latter two depended on natural or immune antibodies to LPS, even in serum with functioning alternative pathway activity only. What was surprising was that these two strains both had O antigen side chains, yet the ones with longer chains bound less C3 and consumed more complement than the one with shorter chain lengths. The results are similar to those found for S. marcescens. The SDS-PAGE profiles of isolated LPS from 4444-60 and S1220 resembled the LPS profiles of the longer chained and shorter chained Ps. aeruginosa strains respectively. Miller and Nussenzweig (1975) demonstrated a solubilising effect of the complement system on preformed immune complexes. Alternative pathway C3 convertase assembles on immune complexes, generating accelerated activation of C3. Large amounts of C3b then bind to immune complexes, contributing to disruption of primary antigen-antibody

bonds and the subsequent dissociation of immune complexes. Engels et al (1985) suggested that this might explain why the strain containing long O antigen chains was less opsonised by C3b and IgG. They could not suggest, however, why the cells with shorter O side chains did not lose bound IgG and became well opsonised by C3b. In conclusion, their results demonstrated that the O antigen structure is critical to evade host defence mechanisms.

In the case of S. marcescens, a similar mechanism may be occurring, but the role of antibody in the serum killing process can only be speculated. Certainly natural IgG antibodies to some S. marcescens proteins were shown to be present and the classical pathway was involved in the killing, and therefore presumably in opsonisation, of strain S1220. Bactericidal antibody is required for direct complement-mediated killing of most Gram-negative bacteria. Winkelstein and Shin (1974) found naturally occurring antibodies cross-reacting with a wide variety of bacteria significantly increased the ability of the bacteria to activate the alternative pathway. Likewise, there is the potential for these antibodies to be involved in classical pathway activation (Winkelstein, 1983).

Substantial differences exist between different LPS O polysaccharides in their capacity to activate complement. Wilson and Morrison (1982) suggested differences in the degree of LPS aggregation played a role in the ability of cells to activate complement. Grossman and Leive (1984) showed that LPS from two Salmonella strains varying only in the substitution of abequose for tyvulose (epimers of one another) in the O antigen repeat unit, affected the activation of the alternative pathway. S. marcescens 4444-60 and S1220 are both O14 serotypes, but the presence of a different sugar in the LPS of the resistant strain not associated

with the O antigen epitope, could be the reason for its inability to activate and bind complement.

Regarding the resistance of S. marcescens to serum killing, chemical analysis (and CIE) showed that strain 4444-60 had 33% more polysaccharide/LPS than the serum sensitive strain S1220. SDS-PAGE suggested that the resistant strain contained more high molecular weight LPS, presumably being longer O antigen chains, which was supported by the hexose:fatty acid ratio. Taylor (1975), however, found mutations to serum resistance occurred independently of quantitative changes in E. coli LPS content. In his review (1983) he suggests that although strains with a low LPS content may generally be susceptible to serum, it is unlikely that the total polymer in the envelope is the major factor determining resistance. Many susceptible clinical isolates are completely smooth by cultural, morphological and serological criteria and are indistinguishable in this respect from serum-resistant organisms, as found for S. marcescens 4444-60 and S1220. S1220 did have a lower polysaccharide content, possibly with shorter O antigen chain lengths in the LPS. However, the reduction of the O side chain sugars was not drastic enough to make the strain have characteristics of a rough organism, which is usually required for serum susceptibility (Taylor, 1983). The degree of substitution of the core stubs by O side chains in the two strains is unknown. Kato and Bito (1978) have suggested that membrane fluidity is obligatory for the formation of functional complement lesions. It may well be that a low degree of substitution by shorter O side chains in S1220 makes the membrane fluidity higher than in 4444-60, thus allowing an increased rate of insertion of functional MACs into the cell envelope.

Joiner and colleagues (1982 a and b) found that a smooth

resistant strain of S. minnesota bound more C3 molecules to its cells than a rough sensitive strain, demonstrating that the resistance mechanism was not an inability to activate complement efficiently through C3. Much larger amounts of terminal components were deposited onto the resistant cells, binding reaching a peak after 10 mins and a progressive loss of bound components being observed with prolonged incubation. The sensitive strain in comparison bound fewer MAC components, but did so rapidly and irreversibly. This resistance was not due to a failure in MAC formation, but failure of the MAC to integrate into the hydrophobic domains of the OM, through the smooth LPS acting as a physical barrier. Similarly, Kroll et al (1984) showed that complement-mediated killing was a consequence of stable binding of the MAC to the OM (but not to the CM) of susceptible strains. Although terminal components were not studied in S. marcescens, more C3 was covalently bound to the sensitive cells than to the resistant ones, indicating 4444-60 might not be able to activate complement efficiently. Resistance is probably also linked to failure of any MACs to insert into the OM, the extra polysaccharide material forming more of a physical barrier in 4444-60 cells compared to S1220 cells.

Apart from reduction in the rate of insertion of functional MACs into the cell envelope, it has been suggested that long O antigen side chains cause antibody-mediated and antibody-independent activation of complement at some distance from the site of lesion formation, and intermediates therefore decay before they can be incorporated into functional MACs (Muschel and Larson, 1970; Rowley, 1973). The possible longer O antigen chains of S. marcescens 4444-60 compared to S1220 suggest this mechanism may play a part in its resistance, although overall 4444-60 appeared to be a poor activator

of the complement system.

Reports in the literature on the role of LPS and resistance to complement are immense. Lee and Scott (1980) analysed E. coli in early exponential phase of batch culture and found a mutant, subcultured in serum to become serum resistant, produced twice as much LPS as the parent strain. The mutation was not due to the synthesis of longer polysaccharide chains, but an increase in synthesis of the whole LPS molecule. They suggested the extent of bacterial surface coverage by LPS determined the degree of serum sensitivity. Other workers found a smooth serum resistant stain of E. coli was more resistant to killing than its rough serum sensitive counterpart, because the smooth O antigen chains blocked antibody binding sites on the OM, thereby preventing activation of the classical pathway (Sansano Jr et al, 1985). Odumeru et al (1985) using Haemophilus ducreyi found the classical pathway was indicated in the complement killing of sensitive strains, natural antibodies being involved in this. They also discovered that the LPS of the sensitive cells showed anti-complementary activity. In smooth and rough Salmonella and E. coli mutants, anti-complementary activity is attributed to lipid A via the classical pathway. However, polysaccharide modifies the potential anti-complementary activity of lipid A, which decreases with an increase in the length of the polysaccharide moiety (Morrison and Kline, 1977). The presence of polysaccharide may prevent the lipid A achieving required aggregate surface density of functional groups necessary for complement activation via the classical pathway (Morrison and Kline, 1977). Inzana and Anderston (1985) found H. influenzae type b was sensitive to killing by antibody to LPS and complement on growth in broth, but was phenotypically converted to resistance on brief incubation with

low molecular weight components of plasma or sera. The resistant phenotype was found to contain more LPS that bound less antibody. The workers suggested that maybe the increase in LPS was accompanied by a change in orientation of the LPS molecules, or in their interaction with each other, or with some other surface molecules, which caused steric hindrance or other interference with antibody binding. Gildsdorf and Ferrieri (1986) passaged H. influenzae type b in rats to obtain a strain with altered LPS that was resistant to killing by the classical pathway of non-immune rat sera. Their findings suggested normal rat sera had natural antibodies against antigens on both strains, but at a lower level to the serum resistant one. They proposed that the resistant strain, by possessing antigens (eg LPS) not recognised by natural antibodies, escaped complement killing, or alternatively the different LPS affected the surface structure of the resistant cells so that the MAC could not be inserted into the OM.

Joiner and co-workers have studied many resistance mechanisms, and have quantified binding of complement components to cells. Most of their findings suggest complement resistance does not represent inefficient complement activation, but subversion of the membrane attack process at a later stage. Thus, many of the serum resistant strains they have used bind C3 in non-immune serum to a similar or greater extent than the serum sensitive strains (Joiner et al, 1984). E. coli O111B4 contains an O antigen capsule that is a surface polysaccharide with biochemical and immunochemical similarities to O antigen chains of LPS, that confers the property of a capsule to the cell (Joiner et al, 1984a). On studying the binding of C3 in absorbed NPHS to E. coli O111B4, they found 75% attached to the O antigen capsule and the rest to the OM. C3 on the OM bound to both

LPS and OMPs. On presensitisation of the cells with immune IgG, there was an increase in the overall amount of C3 bound, but the distribution did not change. The reason for the preferential binding to the O antigen capsule was its greater surface exposure, for in a mutant with no O antigen capsule, the fraction of LPS molecules binding C3 increased 3 fold. The O antigen capsule was found to activate and consume C3, a direct demonstration that the polysaccharide moiety of LPS can activate the alternative pathway. On trying to determine whether C3b bound to the polysaccharide of S. marcescens by incorporating anti-C3 antiserum into the intermediate gel of CIEs, inconclusive results were obtained. On reflection, it appears that the ester- or amide- links covalently binding the C3b to cell surface components were not able to withstand treatment with Triton X-100-EDTA, so that the C3b surprisingly became totally solubilised. If this is true, the rockets also show totally solubilised C3b that was originally bound to the cells. Radiolabelling showed that the C3b iodinated, and therefore the only conclusion to be reached is that the C3b attached to the OM.

The presence of an O antigen capsule does not seem to affect serum sensitivity or resistance, the extent of lipid A coverage with O antigen in LPS being far more important. Goldman et al (1984) found serum-resistant mutants of E. coli O111 were of two types. Completely resistant mutants had no O antigen capsule, but contained 50% more LPS with 30% fewer lipid A core molecules devoid of O antigen than the serum sensitive parent strain. Partially resistant mutants still had an O antigen capsule, but contained 40% more LPS than the parent, the extent of lipid A coverage being unchanged. The mutants bound antibody and activated complement, yet were resistant to killing. The increased lipid A core and increased coverage of

lipid A core with O antigen, probably restricted access of MAC to lethal sites on the hydrophobic membrane. Also, C3b seems preferentially to attach to the longest O antigen chains in LPS (Joiner et al, 1986), thus the increased coverage of lipid A core with O antigen may have served to direct complement deposition to sites distant from the cell surface where a lethal event could not occur. The long chained LPS of S. montevideo sterically hinders C3 from attaching to shorter LPS molecules, direct proof that the O polysaccharide hinders access of large molecules to the OM, ie the LPS functions as a barrier against large protein molecules (Joiner et al, 1986). S. montevideo was found to become sensitive to complement killing when the O antigen chain length and percent coverage of lipid A core were decreased. The C3 bound to shorter chain molecules of LPS, providing a focus of attachment for C5b-9 near the OM, facilitating complement-mediated killing (Joiner et al, 1986).

Joiner and co-workers have also shown that the presence of IgG in the bactericidal reaction does not cause redistribution of C3 to different target sites in E. coli O111, eg from O antigen capsule to OM (Joiner et al, 1985). Neither does it function primarily to increase the extent of complement activation at the bacterial surface (Joiner et al, 1983a and b). The IgG instead seems to bind covalently C3, the C3b-IgG complex being more efficient in presensitising E. coli O111 for serum killing. Fries et al (1984) have shown C3b bound to IgG is protected from inactivation by factors H and I. Thus it appears IgG serves as an acceptor site for deposition of C3b molecules that are critical for subsequent formation, and/or localisation of the bactericidal MAC.

Little is known about the capsular antigens of S. marcescens, although Traub et al (1985) have recently isolated K antigen extracts

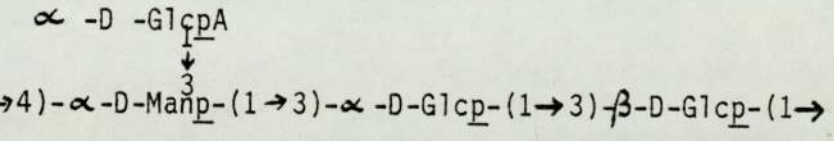
from four strains. Ohshima et al (1984) found a S. marcescens strain that was encapsulated proved to be mouse virulent, the capsule protecting the cells from phagocytosis. Acidic polysaccharides were thought to influence the serum bactericidal reaction by impeding antibody binding and the subsequent attachment of complement components to the bacterial surface (Glynn and Howard, 1970). More recent evidence has tended to suggest that capsules of Gram-positive bacteria do not represent a diffusion, permeability, or adsorption barrier to IgG or other proteins (King and Wilkinson, 1981), whereas the K antigens of some Gram-negative bacteria do restrict the access of opsonins to deeper structures (Hammond et al, 1984).

Other workers have failed to find any correlation between exo-polysaccharide production and serum resistance. Klebsiella strains are frequently serum susceptible (Gower et al, 1972), even though most members produce copious amounts of acidic polysaccharide. Thus there appears to be evidence against a major role for acidic poly-saccharides as mediators of serum resitance (Taylor, 1983).

Some capsules, such as the K1 capsule of E. coli, contain sialic acid. Fearon (1978) demonstrated that membrane-bound sialic acids inhibit complement amplification and alternative pathway activation by facilitating intrinsic complement inactivation mechanisms which degrade cell-bound C3b and prevent additional C3 fixation. K1 strains tend not to be opsonised for phagocytosis by the alternative pathway, implying C3b is not deposited on the K1 capsule by alternative pathway activation (Joiner et al, 1984). Specific anticapsular antibody is usually required to activate complement by the classical pathway, but these capsules tend to be poor immunogens (Ogata, 1983). Verweij-van Vught et al (1984) found the major factor influencing the interaction of K antigens of E. coli with phagocytic

cells, considering the inability of K+ strains to activate the alternative pathway, was the ability of the strains to activate complement by the classical route during opsonisation. Even this was reduced, however, for most K+ strains compared to K- strains. Often, the presence of LPS and capsule are additive, Pluschke et al (1983) finding the K1 and 018 antigens of E. coli restricted antibody-independent classical pathway activation, and the K1 antigen alone, antibody-independent alternative pathway activation.

S. marcescens produces a complex range of surface carbohydrates, Adams and Young first showing in 1966 that acidic glucomannans can be present as extracellular and capsular products. Brigden and Wilkinson (1985a) isolated a partially acetylated acidic glucomannan from the LPS of three 014 serotypes (4444-60, CDC 1783-57, CDC 874-57) and one strain of cross-reacting serogroup 06, of structure:



Individual polymers varied in their extent of O acetylation and substitution with glucosyluronic acid residues. Although no strains were visibly encapsulated, there were doubts as to whether these acidic glucomannans were integral components of true LPS, ultra-centrifugation of phenol-extracted LPS leaving a mannose containing polymer in supernatant fluids. The structure may constitute the common antigen defining the 014-06 complex, and if a (micro)capsular origin is confirmed, the authors will show that the current scheme of O serogroups is not based entirely on the chemistry of the organisms' LPS.

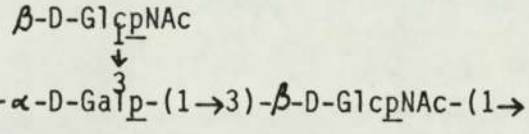
Various neutral polymers have been detected in S. marcescens

LPSs that pellet on ultracentrifugation and have LPS core-specific components associated with them. A ribose and N-acetylgalactosamine repeat was found in the LPS of 4444-60 (O14:H12), CDC 874-57 (O14:H12), an O12 and O13 strain, and as a minor component in an O6 strain:

→4)-α-D-GalpNAc-(1→2)-β-D-Ribf-(1→

(Bridgen and Wilkinson, 1983; Bridgen, Furn and Wilkinson, 1985).

Another neutral polymer was found in the LPS of S. marcescens CDC 1783-57 (O14:H9), an O8 strain, and in E. coli O18, containing 2-acetamido-2-deoxyglucose, galactose and glucose (molar ratios 2:1:1):



(Brigden and Wilkinson, 1985b).

Lastly, an acetylated glucorhamnan in the LPS of one O14 (NCTC 1377) and one O6 strain was detected:

→4)-α-D-Glcp-(1→3)-β-L-Rhap-(1→

(Brigden, Furn and Wilkinson, 1985).

All of these polymers provide the possibility of serological cross-reactions, and show that the surface carbohydrates of S. marcescens are very complicated.

In this study, the increase in phenol-extractable material and the hexose:fatty acid ratio in 4444-60 compared to S1220, could reflect the presence of a (micro)capsular acidic glucomannan.

Although Bridgen and Wilkinson suggest such an acidic glucomannan is the O14 serotype antigen, cross-reactions may have serotyped S1220 as O14, or alternatively there is less polymer present in S1220 compared to 4444-60.

CIE of the whole cells of 4444-60 and S1220 was performed by solublising the cells with Triton X 100/EDTA. Proteins of the OMs are difficult to solubilise with detergents and therefore a chelating agent (EDTA) is incorporated for optimal solubilisation (Owen, 1981). EDTA can also bind to divalent cations that maintain the integrity of the OM (by bridging anionic groups within the LPS and protein giving LPS/protein interactions) thus releasing LPS complexes from the cells. It is conceivable that immunoprecipitates from the detergent extracts represent a complex association of membrane components which resist dissociation by virtue of strong cohesive interactions in localised membrane microenvironments (Owen, 1981). Proteins and LPS are known to be tightly associated in whole cells (Lugtenberg and van Alphen, 1983) and Triton-EDTA did not appear to separate them completely, radiolabelling of S. marcescens 4444-60 showing that part of the polysaccharide peak was a protein/polysaccharide complex. However, the faster-migrating half of the polysaccharide peak of 4444-60 was totally separated from protein and this could represent the (micro)capsule polymer. Although the results obtained on CIE will depend on the experimental conditions employed, a few workers have identified polysaccharide peaks of various bacterial species. Owen and Kaback (1978) identified the LPS of E. coli membrane vesicles as a slow cathodally migrating precipitin line. Baek et al (1985) showed that the LPS of Ps. aeruginosa slowly migrated anodally on CIE, forming a precipitin line in the region of the intermediate gel, similar to S. marcescens S1220. Tsang et al (1974) in their

experiments, found phenol-water extracted LPS of S. marcescens exhibited a single precipitin arc close to the antigen well on immunoelectrophoresis. Cousland and Poxton (1984) discovered the phenol-extracted LPS of two Bacteroides fragilis strains migrated anodally on CIE to produce three major precipitin arcs. The LPS was fairly fast migrating, like that of S. marcescens strain 4444-60, and one of the B. fragilis strains was known to possess a capsule. Lastly, the K1 and K2 capsules of K. aerogenes have been identified as fast anodally migrating immunoprecipitates (J Lodge, personal communication). The presence of a polysaccharide (micro)capsule in 4444-60 that is closely associated with the LPS, could explain the higher electrophoretic mobility of the polysaccharide peak compared to strain S1220. Seeing as this possible capsule would have the properties of an O antigen capsule, it would also be immunologically indistinguishable from the LPS on CIE.

Goldman et al (1982) fractionated the phenol-extracted LPS of E. coli O111 to obtain isolated O antigen capsule and showed that it would not separate by SDS-PAGE, having no lipid A/core to bind SDS. Cousland and Poxton (1983) ran LPS isolated from B. fragilis on SDS-PAGE and suggested that some high molecular weight silver-stained bands might represent smooth LPS and others contaminating polysaccharides (possibly capsules), which according to their laboratory had a high molecular weight on gels. Similarly, the slowly migrating material found on SDS-PAGE of 4444-60 phenol-extractable material may represent the (micro)capsule polymer rather than long chained LPS. The material would migrate on SDS-PAGE, being associated with LPS and not isolated by fractionation.

On subjecting the phenol-extractable material of 4444-60 to ultracentrifugation, even in the presence of magnesium chloride, no

such (micro)capsular material could be separated. Therefore, it cannot be concluded whether the CIE, chemical analysis and SDS-PAGE results show the presence of a (micro)capsule or a greater quantity of LPS with longer O antigen sugar chain lengths in S. marcescens strain 4444-60 compared to strain S1220.

The phenol-extractable material of 4444-60 seemed to contain a sugar not present in the material of S1220, that gave a reaction with the periodate thiobarbituric acid (TBA) assay. This assay is a method for quantitative determination of 2-deoxy and 3-deoxy sugars. Bridgen and Wilkinson (1983) also found interference with their TBA assay of 4444-60 LPS and called the component involved (that was related to KDO) compound X. It contained no phosphorus and was not a simple glycosyl derivative or a degradation product of KDO. Kuwahara and Snetting (1979) found sialic acid produced an interfering colour in the TBA assay that gave a falsely elevated KDO level when sialic acid was present in the LPS fraction tested. Sialic acid is the component of the E. coli K1 capsule that inhibits alternative pathway activation, but whether it (or a related compound) is present in 4444-60 LPS remains undetermined.

Overall, a number of conclusions can be drawn regarding the possible mechanisms of resistance in S. marcescens 4444-60 cells compared to S1220 cells. Strain 4444-60 contains additional surface polysaccharide material compared to strain S1220 which is in the form of more LPS with longer O antigen chain lengths, a (micro)capsule, or a combination of both. Goldman et al (1984) found the possession of an O antigen capsule in E. coli O111 B4 did not affect the serum sensitivity, the quantity of LPS being more important. Whatever the form of the extra polysaccharide material, the surface of 4444-60 cells will probably provide more of a permeability barrier against

large protein molecules than that of S1220.

Overall, strain 4444-60 did not seem to be able to activate complement as efficiently as strain S1220. Assuming that natural antibodies are involved in the killing of S1220 cells via the classical pathway, the extra polysaccharide material of strain 4444-60 may sterically hinder the access of antibodies to binding sites on the OM, thereby preventing classical pathway activation. The increase in polysaccharide of 4444-60 cells may also change the orientation of LPS molecules, or alter their interaction with each other, or some other surface molecules, so that the antibodies cannot bind, restricting classical pathway activation and C3b deposition. It could be that there are less natural antibodies to the LPS of 4444-60, because even though both strains are serotyped O14, Wilkinson and co-workers have shown the complexity of different polymers within strains. If antibodies are not involved in the classical pathway killing of strain S1220, it may be that the extra polysaccharide of 4444-60 cells prevents lipid A achieving aggregates of functional groups which would cause direct classical pathway activation.

C3b covalently bound to IgG is protected from inactivation, and the IgG-C3b complexes serve as a marker for MAC formation (Joiner et al, 1985). Thus, natural antibodies to OM components near the hydrophobic regions of the OM (eg lipid A/core, OMPs etc) would direct the MAC to its target site. Such antibodies would have restricted access in 4444-60. Although complement activation in 4444-60 appears to be limited, if natural antibodies to LPS are present which can bind, leading to some classical pathway activation, any MACs formed would be directed to sites further from the cell surface where lethal events cannot occur. It is also possible that if any complexes

formed between natural antibodies and LPS, they might be solubilised by the alternative complement pathway, releasing C3b (Miller and Nussenzweig, 1975).

The high proportion of polysaccharide material on the surface of 4444-60 cells suggests that any activation of complement is probably via the alternative pathway rather than the classical pathway. Because of steric hindrance, C3b attaches to the longest O antigen chains available. Therefore, in 4444-60 cells, C3b is directed to sites further from the cell surface, whether it be to long O antigen chains or to the O antigen (micro)capsule. The C3b then serves as a marker for the site of MAC formation, which would be far away from the hydrophobic membrane. Thus the increased polysaccharide content would restrict access of the complement components C3b and C5b-9 to the hydrophobic domains of the OM in 4444-60, making the cells resistant to killing.

Lastly, it may be that activation of the alternative pathway is even inhibited in strain 4444-60 by the presence of a polysaccharide sugar that has a similar action to sialic acid, preventing deposition of C3b onto the cells.

S1220 cells have contrasting properties to 4444-60 cells, containing less polysaccharide material that would not form such a permeability barrier to complement components. Thus C3b, the MAC and any natural antibodies would more easily be able to reach the hydrophobic membrane where lethal events can occur killing the cells. The decreased polysaccharide content would also enable the cells to activate the classical complement pathway more efficiently, either with or without antibody. A schematic diagram of the OMs of strains 4444-60 and S1220 showing these proposed mechanisms of interaction with complement is shown in Figure 3.60.

Wretlind et al (1985) found the presence of plasmids in Ps. aeruginosa PAOI led to an increased sensitivity of the cells to human serum bactericidal activity that was not attributed to a single factor such as the tra gene. Only curing of the entire plasmid restored serum resistance. On studying the sensitivity of S. marcescens strains S1220 and 4444-60 to neosensitab antibiotics (section 3.2.3), it was suggested that S1220 contains a plasmid, whereas 4444-60 does not. It may be that in addition to the decreased polysaccharide content, the plasmid increases the serum sensitivity of strain S1220. Whether this is the case or not, the results of CIE, chemical analysis and SDS-PAGE show that surface polysaccharide content is very important in determining the sensitivity or resistance of S. marcescens to complement killing.

Traub and colleagues (1985) have recently shown that capsular polysaccharides and LPS from a range of S. marcescens serotypes separately afford protection against challenge with homologous strains in mice. Therefore, further information is required both on their antigenicity and on their role in protecting the cells from serum killing.

Figure 3.60

Schematic Diagram of the Outer Membranes of S. marcescens Strains 4444-60 and S1220 and their Interaction with Serum Components

Key:

1 and 2 Natural antibodies to OMPs (1) or to core/lipid A regions of LPS (2) are sterically hindered from reaching their binding sites in 4444-60 by extra surface polysaccharide material, whereas the antibodies can bind and activate the classical complement pathway in S1220.

3 Natural antibodies to O antigen side chains of the LPS and/or (micro)capsule are prevented from binding in 4444-60, perhaps by altered orientation of the LPS molecules, whereas they can bind and activate the classical complement pathway in S1220.

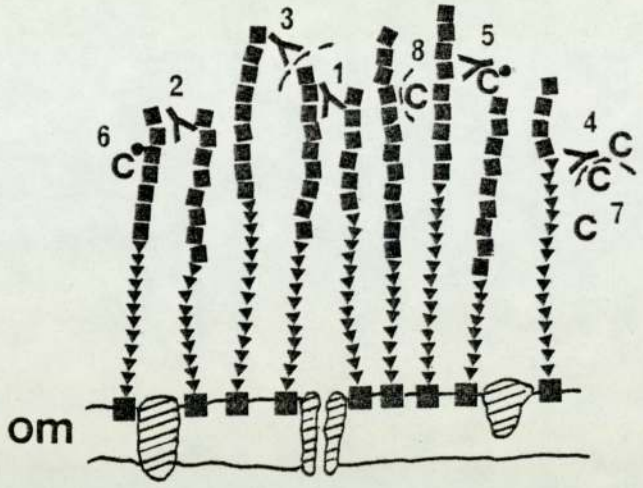
4 The alternative pathway of complement solubilises C3b/IgG immune complexes in 4444-60, whereas in S1220 it does not.

5 and 6 Activated classical pathway IgG/C3b (5) or activated alternative pathway C3b (6), attach to sites further away from the hydrophobic membrane in 4444-60 compared to S1220 because of steric hindrance by extra surface polysaccharide material. The same mechanism prevents or allows subsequently formed MACs to reach their target sites in the hydrophobic domains.

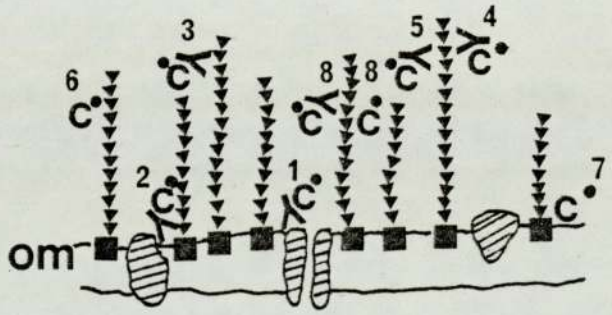
7 The classical complement pathway is not activated in 4444-60 in absence of antibody because C3 cannot come into contact with lipid A portions of the LPS, whereas in S1220 activation can proceed.

8 Sialic acid (which could be anywhere in the polysaccharide chain) prevents deposition and activation of C3 via the alternative pathway in 4444-60 and also via the classical pathway, it being very unlikely that natural antibodies to this component are present. The absence of sialic acid in S1220 allows complement activation to proceed by both pathways.

4444-60



S1220



Key

Protein

LPS lipid A / core

LPS O antigen side chains

Extra polysaccharide material (longer O antigen side chains of the LPS and/or (micro)capsule)

Antibody molecule

C — Complement components (• denotes they are activated)

4. CONCLUSIONS

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The results in this thesis describe the basic immunochemical composition of S. marcescens. In common with other Enterobacteriaceae the major antigens of S. marcescens comprise the outer membrane proteins, including the porins and OmpA protein; flagella and LPS. The OMP antigens are common to all O serotypes. No antigens were identified that might readily help to sub-divide the established O-serotypes.

Other in vitro work described in this study indicates how important bacterial surface components are in determining the response of S. marcescens cells to an in vivo environment.

The in vivo environment is naturally iron-restrictive, this being an important component of the host defence. Growth of S. marcescens in iron-restricted media caused the induction of several new OMPs of molecular weight 70, 68, 67, 66 and 63 kDal. IRMPs of S. marcescens have not been described before, and they seem to be of slightly lower molecular weight to those of other Enterobacteriaceae. It can only be assumed that they function as receptors for siderophore-iron complexes, as in other Gram-negative bacteria, allowing the cells to sequester iron for growth.

An important factor in determining whether bacterial organisms will cause an infection is the ability of the cells to resist one of the first line in vivo host defense mechanisms, the complement system. Resistance to the bactericidal effect of complement appears to be related to the surface polysaccharide material of S. marcescens. Rocket immunoelectrophoresis, CIE and chemical analysis showed that the resistant cells bound less C3 and contained a greater quantity of antigenic polysaccharide material than the sensitive

cells. It was undetermined whether the extra material was in the form of longer O antigen chains, a (micro)capsule, or a combination of both. Restricted access of complement components to the hydrophobic OM is probably one reason the cells were able to resist killing. Whether extra surface polysaccharide material causes resistance to complement in other Gram-negative bacteria can only be speculated. It could well be of significance in organisms causing sepsis, which is so often fatal, where resistance to serum killing is necessary for growth and survival. In bacteria causing chronic infections of, for example, the urinary tract, the presence of this particular virulence mechanism would not be required.

In studying the immunochemistry of S. marcescens many protein antigens, including the OmpA protein, were shown to be immunogenic, reacting with antibodies in immune rabbit serum on immunoblotting. However, using immunoabsorption experiments before immunoblotting, it was found that the LPS of S. marcescens masked the antigenic epitopes of surface proteins in whole cells, preventing antibodies reacting with their binding sites. This has important consequences in vaccine development and suggests that separated and purified OMPs would not provide useful protective antigens. Instead, the surface exposure of the LPS, together with the possible role of polysaccharides in mediating resistance to complement, suggests that these components are ideal candidates for vaccine development, substantiating the work of Traub et al (1985). Based on the results of this thesis, antibodies to the polysaccharides would successfully opsonise the cells, whereas antibodies to the OMPs would not. Most clinical isolates of S. marcescens are serotype O14:H12 (Pitt, 1982) and, considering the cross-reactions which occur in serotyping, the LPS/ polysaccharides of only a few well characterised serotyped strains of

S. marcescens would be sufficient to provide good all-round protective antigens for active or passive immunisation. The dramatic rise of S. marcescens infections over recent years in (immuno)compromised patients (Yu, 1979), together with its ability to acquire rapidly resistance to antimicrobial agents (Farrar Jr, 1980), suggests that such immunisation or immune therapy in high risk groups would be beneficial.

A last conclusion to be made from this project is the importance of using a combination of techniques to study microbial surfaces. The results of immunoblotting alone suggest that the OMPs are the major cellular antigens and would be useful for vaccine development; however, a combination of immunoabsorption and immunoblotting show this may not be true. The two different methods of CIE and chemical analysis show quantitative differences in the polysaccharides of S. marcescens strains which are not immediately noticeable from the results of SDS-PAGE alone. These differences have a significant influence upon the resistance of the organisms to serum killing.

5. REFERENCES

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ADAMS G A and SINGH P P (1970). The chemical constitution of lipid A from Serratia marcescens. Can. J. Biochem. 48, 55-62.

ADAMS G A and YOUNG R (1965). Capsular polysaccharides of Serratia marcescens. Can. J. Biochem. 43, 1499-1512.

ADAMS G A and YOUNG R (1966). Constitution of extracellular and capsular polysaccharides of Serratia marcescens. Ann. NY Acad. Sci. 133, 527-545.

ADEGBOLA R A and OLD D C (1982). New fimbrial hemagglutinin in Serratia species. Infect. Immun. 38, 306-315.

AGÜERO M E, ARON L, DE LUCA A G, TIMMIS K N and CABELLO F C (1984). A plasmid-encoded outer membrane protein, TraT, enhances resistance of Escherichia coli to phagocytosis. Infect. Immun. 46, 740-746.

ALAUPOVIC P, OLSON A C and TSANG J (1966). Studies on the characterisation of lipopolysaccharides from two strains of Serratia marcescens. Ann. NY Acad. Sci. 133, 546-565.

ALVAREZ J S and REGUEIRO B (1980). R plasmids from clinical isolates of Serratia marcescens. J. Hosp. Infect. 1, 133-139.

ALY R and MAIBACH H I (1980). A comparison of the antimicrobial effect of 0.5% chlorhexidine (Hibistat) and 70% isopropyl alcohol on hands contaminated with Serratia marcescens. Clin. Exp. Dermatol. 5, 197-201.

AMAKO K and KOHNO K (1982). Haemagglutination pattern and the distribution of pili antigen among clinically isolated strains of Serratia marcescens. Kansenshogaku Zasshi 56, 560-565.

AMAKO K, OHSHIMA H, YASUNAKA K and KONO K (1981). Pili mediated agglutination of Serratia marcescens in human urine. Microbiol. Immunol. 25, 981-992.

AMES P, DES JARDINS D and PIER G B (1985). Opsonophagocytic killing activity of rabbit antibody to Pseudomonas aeruginosa mucoid exopolysaccharide. Infect. Immun. 49, 281-285.

ANWAR H, BROWN M R W, DAY A and WELLER P M (1984). Outer membrane antigens of mucoid Pseudomonas aeruginosa isolated directly from the sputum of a cystic fibrosis patient. FEMS Microbiol. Lett. 24, 235-239.

ANWAR H, BROWN M R W and LAMBERT P A (1983). Effect of nutrient depletion on sensitivity of Pseudomonas cepacia to phagocytosis and serum bactericidal activity at different temperatures. J. Gen. Microbiol. 129, 2021-2027.

ANWAR H, LAMBERT P A and BROWN M R W (1983a). Effect of divalent metal ions on the production of outer membrane protein H_2 in mucoid and non-mucoid strains of Pseudomonas aeruginosa. FEMS Microbiol. Lett. 16, 247-250.

ANWAR H, SHAND G H, WARD K H, BROWN M R W, ALPAR K E and GOWAR J (1985). Antibody response to acute Pseudomonas aeruginosa infection in a burn wound. FEMS Microbiol. Lett. 29, 225-230.

ARROYO J C, MILLIGAN W L, POSTIC B, NORTHEY J, PARKER E and BRYAN C S (1981). Clinical, epidemiologic and microbiologic features of a persistent outbreak of amikacin-resistant Serratia marcescens. Infect. Control 2, 367-372.

BAEK L, HØIBY N, HERTZ J B and ESPERSEN F (1985). Interaction between Limulus amoebocyte lysate and soluble antigens from Pseudomonas aeruginosa and Staphylococcus aureus studied by quantitative immunoelectrophoresis. J. Clin. Microbiol. 22, 229-237.

BALIKIAN J P, HERMAN P G and GODLESKI J J (1981). Serratia pneumonia. Radiology 137, 309-311.

BARENKAMP S J, MUNSON R S and GRANOFF D M (1981). Subtyping isolates of Haemophilus influenzae type b by outer membrane protein profiles. J. Infect. Dis. 143, 668-676.

BAYER M E (1979). The fusion sites between outer membrane and cytoplasmic membrane of bacteria: their role in membrane assembly and virus infection. In: Bacterial Outer Membranes, Biogenesis and Function (Inouye M, Ed.). John Wiley and Sons, New York, pp 167-202.

BEHER M G, SCHNAITMAN C A and PUGSLEY A P (1980). Major heat-modifiable outer membrane protein in Gram-negative bacteria: comparison with the OmpA protein of Escherichia coli. J. Bacteriol. 143, 906-913.

BENJAMIN Jr W H, TURNBOUGH C L, POSEY B S and BRILES D E (1985). The ability of Salmonella typhimurium to produce the siderophore enterobactin is not a virulence factor in mouse typhoid. Infect. Immun. 50, 392-397.

BERGERON R J, ELLIOTT G T, KLINE S J, RAMPHAL R and St JAMES III L (1983). Bacteriostatic and fungostatic action of catecholamide iron chelators. Antimicrob. Agents Chemother. 24, 725-730.

BERGIN C J, PHILLIPS P, CHAN R M T, RUEDY J and CRICHTON E P (1985). Treatment of Pseudomonas and Serratia infections with ceftazidime. J. Antimicrob. Chemother. 15, 613-621.

BHUJWALA R A, SHRINIWAS and DUTTA S (1984). Transfer of antimicrobial resistance in Serratia marcescens. Indian J. Med. Res. 79, 22-27.

BINDEREIF A , BRAUN V and HANTKE K (1982). The cloacin receptor of ColV-bearing Escherichia coli as part of the Fe^{3+}-aerobactin transport system. J. Bacteriol. 150, 1472-1475.

BIZIO B (1823). Lettera di Bartolomeo Bizio al chiarissimo canonico Angelo Bellani sopra il fenomeno della polenta porporina, Biblioteca Italiana o sia Giomale di Letteratura Scienze e Arti (Anno VIII), 30, 275; in J. Bacteriol., 9, 1924, 527.

BONNER M J and MEHARG Jr J G (1983). Primary cellulitis due to Serratia marcescens. JAMA 250, 2348-2399.

BOOTH B R (1980). Cell surface proteins of E. coli. Biochem. Biophys. Res. Commun. 94, 1029-1036.

BRAUN G and COLE S T (1984). DNA sequence analysis of the Serratia marcescens ompA gene: implications for the organisation of an enterobacterial outer membrane protein. MGG 195, 321-328.

BRAUN V, BRAZEL-FAISST C and SCHNEIDER R (1984). Growth stimulation of Escherichia coli in serum by iron (III) aerobactin. Recycling of aerobactin. FEMS Microbiol. Lett. 21, 99-103.

BRAUN V, GROSS R, KOSTER W and ZIMMERMAN L (1983). Plasmid and chromosomal mutants in the iron (III) aerobactin transport system of Escherichia coli. Use of streptonigrin for selection. MGG 192, 131-139.

BRAUN V and HANTKE K (1981). Bacterial cell surface receptors. In: Organisation of Prokaryotic Cell Membranes, Volume II (Ghosh B K, Ed.). CRC Press Inc, Boca Raton, Florida, pp 1-73.

BRAUN V and REHN K (1969). Chemical characterisation, spatial distribution and function of a lipoprotein (murein-lipoprotein) of the E. coli cell wall. The specific effect of trypsin on the membrane structure. Eur. J. Biochem. 10, 426-438.

BRAUN V and SCHMITZ G (1980). Excretion of a protease by Serratia marcescens. Arch. Microbiol. 124, 55-61.

BRIGDEN C J, FURN S and WILKINSON S G (1985). Structural studies of neutral polymers isolated from the lipopolysaccharides of Serratia marcescens O6 (strain CDC 862-57) and O12 (CDC 6320-58). Carbohydr. Res. 139, 298-301.

BRIGDEN C J and WILKINSON S G (1983). Lipopolysaccharide from the O14 type strain of Serratia marcescens: structural studies of a polymeric fraction. Carbohydr. Res. 115, 183-190.

BRIGDEN C J and WILKINSON S G (1985a). Structural studies of acidic glucomannans from strains of Serratia marcescens O14 and O6. Carbohydr. Res. 138, 267-276.

BRIGDEN C J and WILKINSON S G (1985b). Structural studies of a neutral polymeric fraction from the lipopolysaccharide of Serratia marcescens CDC 1783-57 (O14:H9). Carbohydr. Res. 145, 81-87.

BROCK J H and NG J (1983). The effect of desferrioxamine on the growth of Staphylococcus aureus, Yersinia enterocolitica and Streptococcus faecalis in human serum: uptake of desferrioxamine-bound iron. FEMS Microbiol. Lett. 20, 439-442.

BROOKS H J, CHAMBERS T J and TABAQCHALI S (1979). The increasing isolation of Serratia species from clinical specimens. J. Hyg. 82, 31-40.

BROWN D A and TSANG J C (1978). Chemical and electrophoretic changes induced by polymyxin B on outer membrane components from Serratia marcescens. J. Antibiot. 31, 603-609.

BROWN E J, JOINER K A and FRANK M M (1983). The role of complement in host resistance to bacteria. Springer Seminars Immunopathol. 6, 349-360.

BROWN M R W and WILLIAMS P (1985). The influence of environment on envelope properties affecting survival of bacteria in infection. Annu. Rev. Microbiol. 39, 527-556.

BRYAN L E, O'HARA K and WONG S (1984). Lipopolysaccharide changes in impermeability-type aminoglycoside resistance in Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 26, 250-255.

BULLEN J J, ROGERS H J and GRIFFITHS E J (1974). Bacterial iron metabolism in infection and immunity. In: Microbial Iron Metabolism: A Comprehensive Treatise (Neilands J B, Ed.). Academic Press, New York, pp 517-555.

BULLOCK D W, BIDWELL J L, REEVES D S, WHITE L O, TURNER A, SPELLER D C E and WILKINSON P J (1982). Outbreaks of hospital infection in south-west England caused by gentamicin-resistant Serratia marcescens. J. Hosp. Infect. 3, 263-273.

BURNETTE W N (1981). Western Blotting: Electrophoretic transfer of proteins from sodium dodecyl sulphate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated Protein A. Anal. Biochem. 112, 195-203.

BUTTON G L, MILLER M A and TSANG J C (1975). Antibiogram and lipid analysis of a pigmented strains of Serratia marcescens and its non-pigmented variants. Antimicrob. Agents Chemother. 7, 219-222.

CABRERA H A (1969). An outbreak of Serratia marcescens and its control. Arch. Intern. Med. 123, 650-655.

CASALS J B and PEDERSEN O G (1979). Antimicrobial sensitivity testing using Neosensitabs. Fourth Edition (1978-1979) A/S Rosco, Denmark.

CHAI T and FOULDS J (1974). Demonstration of missing outer membrane protein in tolG mutants in Escherichia coli. J. Mol. Biol. 85, 465-474.

CHART H and GRIFFITHS E (1985). Antigenic alteration of contaminating lipopolysaccharide during extraction of Escherichia coli outer membrane proteins from polyacrylamide gels. J. Gen. Microbiol. 131, 2847-2850.

CHART H and GRIFFITHS E (1985a). Antigenic and molecular homology of the ferric enterobactin receptor protein of Escherichia coli. J. Gen. Microbiol. 131, 1503-1509.

CHOPRA I and SHALES S W (1980). Comparison of the polypeptide composition of Escherichia coli outer membranes prepared by two methods. J. Bacteriol. 144, 425-427.

CLAYTON E and VON GRAEVENITZ A. Non-pigmented Serratia marcescens. JAMA 197, 1059-1964.

COLE S T, SONNTAG I and HENNING U (1982). Cloning and expression in Escherichia coli K12 of the genes for major outer membrane protein OmpA from Shigella dysenteriae, Enterobacter aerogenes and Serratia marcescens. J. Bacteriol. 149, 145-150.

COLWELL D E, MICHALEK S M, BRILES D E, JIRILLO E and McGHEE J R (1984). Monoclonal antibodies to Salmonella lipopolysaccharide: anti-O-polysaccharide antibodies protect C3H mice against challenge with virulent Salmonella typhimurium. J. Immunol. 133, 950-957.

COOPER H A, BENNETT W P, KREGER A, LYERLY D and WAGNER R H (1981). The effect of extracellular proteases from Gram-negative bacteria on the interaction of von Willebrand factor with human platelets. J. Lab. Clin. Med. 97, 379-389.

COSTERTON J W, GEESEY G G and CHENG K J (1978). How bacteria stick. Sci. Am. 238, 86-95.

COSTERTON J, INGRAM J and CHENG K (1974). Structure and function of the cell envelope of Gram-negative bacteria. Bacteriol. Rev. 38, 87-110.

COULTON J W, MASON P and DU BOW M S (1983). Molecular cloning of the ferrichrome-iron receptor of Escherichia coli K-12. J. Bacteriol. 156, 1315-1321.

COUSLAND G and POXTON I R (1983). Analysis of lipopolysaccharides of Bacteroides fragilis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electroblot transfer. FEMS Microbiol. Lett. 20, 461-465.

COUSLAND G and POXTON I R (1984). Crossed immunoelectrophoresis and enzyme-linked immunosorbent assay of the cell surface antigens of Bacteroides fragilis. J. Gen. Microbiol. 130, 645-655.

CROSS A S, GEMSKI P, SADOFF J C, ØRSKOV F and ØRSKOV I (1984). The importance of the K1 capsule in invasive infections caused by Escherichia coli. J. Infect. Dis. 149, 184-193.

CRYZ Jr S J, FÜRER E and GERMANIER R (1984). Prevention of fatal experimental burn wound sepsis due to Klebsiella pneumoniae KP1-O by immunisation with homologous capsular polysaccharide. J. Infect. Dis. 150, 817-822.

DALHOFF A (1985). Differences between bacteria grown in vitro and in vivo. J. Antimicrob. Chemother. 15, Suppl. A, 175-195.

DASCHNER F D (1980). The epidemiology of Serratia marcescens. In: The Genus Serratia (von Graevenitz A and Rubin S J, Eds.). CRC Press Inc, Boca Raton, Florida, pp 187-196.

DATTA D B, ARDEN B and HENNING U (1977). Major proteins of the Escherichia coli outer cell envelope membrane as bacteriophage receptors. J. Bacteriol. 131, 821-829.

DELL A, OATES J, LUGOWSKI C, ROMANOWSKA E, KEENE L and LINDBERG B (1984). The enterobacterial common antigen. A cyclic polysaccharide. Carbohydr. Res. 133, 95-104.

DE TONI J B and TREVISAN V (1889). Schizomycetaceae Naeg. Sylloge Fungorum. 81, 923.

DIMMICK R L, WOLOCHOW H and CHATIGNY M A (1979). Evidence that bacteria can form new cells in airborne particles. Appl. Environ. Microbiol. 37, 924-927.

DOERR M and TRAUB W H (1982). Proteolytic activity of selected clinical isolates of Serratia marcescens against human-serum proteins. Zbl. Bakt. Hyg. A. 252, 196-207.

DORSET D L, ENGEL A, MASSALSKI A and ROSENBUSCH J P (1984). Three-dimensional structure of a membrane pore. Electron microscopical analysis of Escherichia coli outer membrane matrix porin. Biophys. J. 45, 128-129.

DRIVER K and LAMBERT P A (1984). Surface antigens of Proteus mirabilis revealed by electroblotting from sodium dodecyl sulphate-polyacrylamide gels. Microbios 40, 87-98.

DUBOIS M, GILLES K A, HAMILTON J K, REBERS P A and SMITH F (1956). Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350-356.

DUGGAN T G, LENG R A, HANCOCK B M and CURSONS R T (1984). Serratia marcescens in a newborn unit - microbiological features. Pathology 16, 189-191.

DUGUID J P and OLD D C (1980). Adhesive properties of Enterobacteriaceae. In: Bacterial Adherence, Receptors and Recognition, Series B, Vol 6 (Beachey E H, Ed.). Chapman and Hall, London, pp185-217.

EDWARDS P R and EWING W H (1972). Identification of the Enterobacteriaceae. 3rd Ed., Burgess, Minneapolis, Chap. 14.

EHRENKRANZ N J, BOLYARD E A, WIENER M and CLEARY T J (1980). Antibiotic-sensitive Serratia marcescens infections complicating cardiopulmonary operations: contaminated disinfectant as a reservoir. Lancet 2(8207), 1289-1292.

ENGEL A, MASSALSKI A, SCHINDLER H, DORSET D L and ROSENBUSCH J P (1985). Porin channel triplets merge into single outlets in Escherichia coli outer membranes. Nature 317, 643-645.

ENGELS W, ENDERT J, KAMPS M A F and VAN BOVEN C P A (1985). Role of lipopolysaccharide in opsonisation and phagocytosis of Pseudomonas aeruginosa. Infect. Immun. 49, 182-189.

EWING W H (1967). Revised definitions for the family Enterobacteriaceae, its tribes and genera. Centre for Disease Control, Atlanta, GA.

EWING W H, DAVIS B R and FIFE M A (1972). Biochemical characterisation of Serratia liquefaciens and Serratia rubidaea. Centre for Disease Control, Atlanta, GA.

EWING W H, DAVIS B R and REAVIS R W (1959). Studies on the Serratia group. Centre for Disease Control, Atlanta, GA.

EWING W H, JOHNSON J G and DAVIS B R (1962). The occurrence of Serratia marcescens in nosocomial infections. Communicable Disease Center, US Public Health Service, Atlanta, GA.

FARMER J J III, DAVIES B R, HICKMAN F W, PRESLEY D B, BODEY G P, NEGUT M and BOBO R A (1976). Detection of Serratia outbreaks in hospital. Lancet 2, 455-459.

FARRAR Jr W E (1980). Antimicrobial susceptibility of clinical isolates, synergistic effects and beta-lactamases of Serratia. In: The Genus Serratia (von Graevenitz A and Rubin S J, Eds.). CRC Press Inc, Boca Raton, Florida, pp121-138.

FARRAR Jr W E and O'DELL N M (1976). Beta-lactamases and resistance to penicillins and cephalosporins in Serratia marcescens. J. Infect. Dis. 134, 245-251.

FEARON D T (1978). Regulation by membrane sialic acid of β1H-dependent decay-dissociation of amplification C3 convertase of the alternative complement pathway. Proc. Natl. Acad. Sci. USA, 75, 1971-1975.

FERNANDES C J, STEVENS D A, MURRAY S I and ACKERMAN V P (1983). An evaluation of recently developed antibiotics. J. Antimicrob. Chemother. 12, 577-585.

FILIP C, FLETCHER G, WULFF J L and EARHART C F (1973). Solubilisation of the cytoplasmic membrane of Escherichia coli by the ionic detergent sodium-lauryl-sarcosinate. J. Bacteriol. 115, 717-725.

FINE D P, MARNEY S R, COLLEY D G, SERGENT J S and DES-PREZ R M (1972). C3 shunt activation in human serum chelated with EGTA. J. Immunol. 109, 807-809.

FREITAG V and CASELITZ F H (1975). Antibody detection in infections with Serratia marcescens. Infection 3, 74-77.

FRIES L F, GAITHER T A, HAMMER C H and FRANK M M (1984). C3b covalently bound to IgG demonstrates a reduced rate of inactivation by factors H and I. J. Exp. Med. 160, 1640-1655.

FURN S and Wilkinson S G (1985). Structure of a neutral polymer isolated from the lipopolysaccharide of Serratia marcescens O1 (strain CDC 866-57). Carbohydr. Res. 139, 293-297.

GALDIERO F, TUFANO M A, SOMMESE L, FOLGORE A and TEDESCO F (1984). Activation of complement system by porins extracted from Salmonella typhimurium. Infect. Immun. 46, 559-563.

GAVINI F, FERRAGUT C, IZARD D, TRINEL P A, LECLERC H, LEFEBVRE B and MOSSEL D A A (1979). Serratia fonticola, a new species from water. Int. J. Syst. Bacteriol. 29. 92-101.

GERSHONI J M and PALADE G E (1982). Electrophoretic transfer of proteins from sodium dodecyl sulphate polyacrylamide gels to a positively charged membrane filter. Anal. Biochem. 124, 396-405.

GILBERT P and BROWN M R W (1978). Influence of growth rate and nutrient limitation on the gross cellular composition of Pseudomonas aeruginosa and its resistance to 3- and 4-chlorophenol. J. Bacteriol. 133, 1066-1072.

GILLELAND Jr H E, PARKER M G, MATTHEWS J M and BERG R D (1984). Use of a purified outer membrane protein F (porin) preparation of Pseudomonas aeruginosa as a protective vaccine in mice. Infect. Immun. 44, 49-54.

GILSDORF J R and FERRIERI P (1986). Susceptibility of phenotypic variants of Haemophilus influenzae type b to serum bactericidal activity: Relation to surface lipopolysaccharide. J. Infect. Dis. 153, 223-231.

GLYNN A A and HOWARD C J (1970). The sensitivity to complement of strains of Escherichia coli related to their K antigens. Immunology 18, 331-346.

GOLDMAN R C, JOINER K and LEIVE L (1984). Serum-resistant mutants of Escherichia coli 0111 contain increased lipopolysaccharide, lack an O antigen-containing capsule and cover more of their lipid A core with O antigen. J. Bacteriol. 159, 877-882.

GOLDMAN R C and LEIVE L (1980). Heterogeneity of antigenic side chain length in lipopolysaccharide from Escherichia coli 0111 and Salmonella typhimurium LT2. Eur. J. Biochem. 107, 145-153.

GOLDMAN R C, WHITE D, ØRSKOV F, ØRSKOV I, RICK P D, LEWIS M S, BHATTACHARJEE A K and LEIVE L (1982). A surface polysaccharide of Escherichia coli 0111 contains O antigen and inhibits agglutination of cells by O antiserum. J. Bacteriol., 151, 1210-1221.

GOLDSTEIN F W, GUTMANN L, WILLIAMSON R, COLLATZ E and ACAR J F (1983). In vivo and in vitro emergence of simultaneous resistance to both β-lactam and aminoglycoside antibiotics in a strain of Serratia marcescens. Ann. Microbiol. (Inst. Pasteur) 134A, 329-337.

GOLDSTEIN J D, GODLESKI J J, BALIKIAN J P and HERMAN P G (1982). Pathologic patterns of Serratia marcescens pneumonia. Hum. Pathol. 13, 479-484.

GOWER P E, TAYLOR P W, KOUTSAIMANIS K G and ROBERTS A P (1972). Serum bactericidal activity in patients with upper and lower urinary tract infections. Clin. Sci. 43, 13-22.

GRAY J, McGHIE D and BALL A P (1978). Serratia marcescens: a study of the sensitivity of British isolates to antibacterial agents and their combinations. J. Antimicrob. Chemother. 4, 551-559.

GRIFFITHS E (1983). Availability of iron and survival of bacteria in infection. In: Medical Microbiology, Vol 3 (Easmon C S F, Jeljaszewicz J, Brown M R W and Lambert P A, Eds.). Academic Press Inc, London, pp153-177.

GRIFFITHS E, STEVENSON P, THORPE R and CHART H (1985). Naturally occurring antibodies in human sera that react with the iron-regulated outer membrane proteins of Escherichia coli. Infect. Immun. 47, 808-813.

GRIFFITHS E K, SURGALLA M J, FITZPATRICK J E and NETER E (1977). Antibody response to Serratia marcescens isolated from patients with malignant diseases. J. Clin. Microbiol. 6, 499-501.

GRIMONT P A D and GRIMONT F (1978). The genus Serratia. Annu. Rev. Microbiol. 32, 221-248.

GRIMONT P A D and GRIMONT F (1978a). Biotyping of Serratia marcescens and its use in epidemiological studies. J. Clin. Microbiol. 8, 73-83.

GRIMONT P A D, GRIMONT F and DULONG DE ROSNAY H L C (1977). Characterisation of Serratia marcescens, S. liquefaciens, S. plymuthica and S. marinorubra by electrophoresis of their proteinases. J. Gen. Microbiol. 99, 301-310.

GRIMONT P A D, GRIMONT F, RICHARD C, DAVIS B R, STEIGERWALT A G and BRENNER D J (1978). Deoxyribonucleic acid relatedness between Serratia plymuthica and other Serratia species, with a description of Serratia odorifera sp. nov. (type strain: ICPB 3995). Int. J. Syst. Bacteriol. 28, 453-463.

GRIMONT P A D, GRIMONT F and STARR M P (1979). Serratia ficaria sp. nov., a bacterial species associated with smyrna figs and the fig wasp Blastophaga psenes. Curr. Microbiol. 2, 277-282.

GRIMONT P A D, IRINO K and GRIMONT F (1982). The Serratia liquefaciens-S. proteamaculans-S. grimesii complex: DNA relatedness. Curr. Microbiol. 7, 63-68.

GRISTINA A G, OGA M, WEBB L X and HOBGOOD C D (1985). Adherent bacterial colonisation in the pathogenesis of osteomyelitis. Science 228, 990-993.

GROSSMAN N and LEIVE L (1984). Complement activation via the alternative pathway by purified Salmonella lipopolysaccharide is affected by its structure but not its O-antigen length. J. Immunol. 132, 376-385.

GULIG P A and HANSEN E J (1985). Coprecipitation of lipopolysaccharide and the 39,000-molecular weight major outer membrane protein of Haemophilus influenzae Type b by lipopolysaccharide-directed monoclonal antibody. Infect. Immun. 49, 819-827.

GULIG P A, McCRACKEN Jr G H, HOLMANS P L and HANSEN E J (1984). Immunogenic proteins in cell-free culture supernatants of Haemophilus influenzae type b. Infect. Immun. 44, 41-48.

GUTMANN L and CHABBERT Y A (1984). Different mechanisms of resistance to latamoxef (moxalactam) in Serratia marcescens. J. Antimicrob. Chemother. 13, 15-22.

GUTMANN L, WILLIAMSON R, MOREAU N, KITZIS M D, COLLATZ E, ACAR J F and GOLDSTEIN F W (1985). Cross-resistance to nalidixic acid, trimethoprim and chloramphenicol associated with alterations in outer membrane proteins of Klebsiella, Enterobacter and Serratia. J. Infect. Dis. 151, 501-507.

GUTSCHIK E, NORWOOD R S, MØLLER S and OLLING S (1980). Experimental endocarditis in rabbits. 4. Experiments with Serratia marcescens on the significance of serum susceptibility and proteolytic capacity of the strains and the influence of an indwelling catheter. Acta Pathol. Microbiol. Scand. [B] 88, 269-276.

HAHN H (1983). Antibacterial defense mechanisms. Infection 11, Supp 2, S112-S118.

HAMMOND S M, LAMBERT P A and RYCROFT A N. The Bacterial Cell Surface. Groom Helm Ltd, Beckenham, Kent.

HANCOCK R E W and CAREY A M (1979). Outer membrane of Pseudomonas aeruginosa: heat and 2-mercaptoethanol-modifiable proteins. J. Bacteriol. 140, 902-910.

HANCOCK R E W, HANTKE K and BRAUN V (1976). Iron transport in Escherichia coli K-12: involvement of the colicin B receptor and of a citrate-inducible protein. J. Bacteriol. 127, 1370-1375.

HARBER M J, MacKENZIE R K and ASSCHER A W (1982). Bacterial adherence and the urinary tract. Lancet 1(8285), 1352-1353.

HARTMAN A and BRAUN V (1981). Iron-uptake and iron limited growth of Escherichia coli K-12. Arch. Microbiol. 130, 353-356.

HEDGES R W (1980). R factors of Serratia. In: The Genus Serratia (von Graevenitz A and Rubin S J, Eds.). CRC Press Inc, Boca Raton, Florida, pp 139-153.

HELLER K B (1979a). Outer membrane of Serratia marcescens: Apparent molecular weights of heat-modifiable proteins in gels with different acrylamide concentrations. J. Bacteriol. 137, 670-672.

HELLER K B (1979b). Lipolytic activity copurified with the outer membrane of Serratia marcescens. J. Bacteriol. 140, 1120-1122.

HELLER K B (1979c). Further characterisation of the outer membrane of Serratia marcescens and an oxacilline-sensitive mutant: surface carbohydrates and outer membrane lipid composition. Curr. Microbiol. 2, 127-130.

HENRICKS P A J and VERHOEF J (1983). Effect of lipopolysaccharide, lipid A and polysaccharide on phagocytic cell function. Agents and Actions 13, 498-499.

HERBERT D, PHIPPS P J and STRANGE R E (1971). Chemical analysis of microbial cells. In: Methods in Microbiology, Vol 5B (Norris J R and Ribbons D W, Eds.). Academic Press Inc, London, pp209-344.

HIRUMA R, YAMAGUCHI A and SAWAI T (1984). The effect of lipopolysaccharide on lipid bilayer permeability of β-lactam antibiotics. FEBS Lett. 170, 268-272.

HITCHCOCK P J and BROWN T M (1983). Morphological heterogeneity among Salmonella lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. 154, 269-277.

HJERTEN S (1983). A study of the denaturation of membrane proteins after solubilisation in SDS or following polyacrylamide gel electrophoresis in SDS with special reference to a phosphatase from Acholeplasma. Biochim. Biophys. Acta 736, 130-136.

HOBOT J A, CARLEMALM E, VILLIGER W and KELLENBERGER E (1984). Periplasmic gel: new concept resulting from the reinvestigation of bacterial cell envelope ultrastructure by new methods. J. Bacteriol. 160, 143-152.

HOFSTRA H and DANKERT J (1980). Major outer membrane proteins: common antigens in Enterobacteriacae species. J. Gen. Microbiol. 119, 123-131.

HOFSTRA H, VAN TOL M J D and DANKERT J (1980). Cross-reactivity of major outer membrane proteins of Enterobacteriaceae studied by crossed immunoelectrophoresis. J. Bacteriol. 143, 328-337.

HOLBEIN B E (1981). Growth and surface binding of proteins by Neisseria meningitidis in normal human serum. Curr. Microbiol. 6, 213-216.

HOLMES C J, KUNDSIN R B, AUSMAN R K and WALTER C W (1980). Potential hazards associated with microbial contamination of in-line filters during intravenous therapy. J. Clin. Microbiol. 12, 725-731.

HOMMA J Y, ITO M and YABUUCHI E (1972). Cross-protective immunity in mice injected with heat-killed cells of Pseudomonas aeruginosa and Serratia marcescens Jpn. J. Exp. Med. 42, 583-585.

HOOK E W, OLSEN D A and BUCHANAN T M (1984). Analysis of the antigen specificity of the human serum immunoglobulin G immune response to complicated gonococcal infection. Infect. Immun. 43, 706-709.

ICHIHARA S and MIZUSHIMA S (1978). Identification of an outer membrane protein responsible for the binding of the ferric-enterochelin complex to Escherichia coli cells. J. Biochem. 83, 137-140.

ICHIHARA S and MIZUSHIMA S (1979). Arrangement of proteins O8 and O9 in the outer membrane of Escherichia coli K12: existence of homotrimers and heterotrimers. Eur. J. Biochem. 100, 321-328.

INOUYE M (1979). What is the outer membrane? In: Bacterial Outer Membranes, Biogenesis and Function (Inouye M, Ed.). John Wiley and Sons, New York, pp1-12.

INOUYE M, SHAW J and SHEN C (1972). The assembly of a structural lipoprotein in the envelope of Escherichia coli. J. Biol. Chem. 247, 8154-8159.

INZANA T J and ANDERSON P (1985). Serum factor-dependent resistance of Haemophilus influenzae type b to antibody to lipopolysaccharide. J. Infect. Dis. 151, 869-877.

IRVIN R T, TO M and COSTERTON J W (1984). Mechanism of adhesion of Alysiella bovis to glass surfaces. J. Bacteriol. 160, 569-576.

JOHN Jr J F and McNEILL W F (1981). Characteristics of Serratia marcescens containing a plasmid coding for gentamicin resistance in nosocomial infections. J. Infect. Dis. 143, 810-817.

JOHN Jr J F, McNEILL W F, PRICE K E and KRESEL P (1982). Evidence for a chromosomal site specifying amikacin resistance in multi-resistant Serratia marcescens. Antimicrob. Agents Chemother. 21, 587-591.

JOHNSON E and ELLNER P D (1974). Distribution of Serratia species in clinical specimens. Appl. Microbiol. 28, 513-514.

JOINER K A, BROWN E J and FRANK M M (1984). Complement and bacteria: chemistry and biology in host defence. Annu. Rev. Immunol. 2, 461-491.

JOINER K A, FRIES I F, SCHMETZ M A and FRANK M M (1985). IgG bearing covalently bound C3b has enhanced bactericidal activity for Escherichia coli O111. J. Exp. Med. 162, 877-889.

JOINER K, GOLDMAN R, HAMMER C, LEIVE L and FRANK M (1983a). Studies of the mechanism of bacterial resistance to complement mediated killing. V. IgG and F (ab)'$_2$ mediate killing of E. coli O111B4 by the alternative pathway without increasing C5b-9 deposition. J. Immunol. 131, 2563-2569.

JOINER K A, GOLDMAN R, HAMMER C, LEIVE L and FRANK M M (1983b). Studies on the mechanism of bacterial resistance to complement-mediated killing. VI. IgG increases the bactericidal efficiency of C5b-9 for E. coli O111B4 by acting at a step prior to C5 cleavage. J. Immunol. 131, 2570-2576.

JOINER K A, GOLDMAN R, SCHMETZ M, BERGER M, HAMMER C H, FRANK M M and LEIVE L (1984a). A quantitative analysis of C3 binding to O antigen capsule, lipopolysaccharide and outer membrane protein of E. coli O111B4. J. Immunol. 132, 369-375.

JOINER K A, GROSSMAN N, SCHMETZ M and LEIVE L (1986). C3 binds preferentially to long chain lipopolysaccharide during alternative pathway activation by Salmonella montevideo. J. Immunol. 136, 711-715.

JOINER K A, HAMMER C H, BROWN E J and FRANK M M (1982a). Studies on the mechanism of bacterial resistance to complement-mediated killing. 1. Terminal complement components are deposited and released from Salmonella minnesota S218 without causing bacterial death. J. Exp. Med. 155, 797-808.

JOINER K A, HAMMER C H, BROWN E J and FRANK M M (1982b). Studies on the mechanism of bacterial resistance to complement-mediated killing. II. C8 and C9 release C5b67 from the surface of Salmonella minnesota S218 because the terminal complex does not insert into the bacterial outer membrane. J. Exp. Med. 155, 809-819.

JONES R L and GRADY R W (1983). Siderophores as antimicrobial agents. Eur. J. Clin. Microbiol. 2, 411-413.

JONES R L, PETERSON C M, GRADY R W and CERAMI A (1980). Low molecular weight iron-binding factor from mammalian tissue that potentiates bacterial growth. J. Exp. Med. 151, 418-428.

KAHL L E and RODNAN G P (1984). Olecranon bursitis and bacteremia due to Serratia marcescens. J. Rhematol. 11, 402-403.

KAIJSER B and AHLSTEDT S (1977). Protective capacity of antibodies against Escherichia coli O and K antigens. Infect. Immun. 17, 286-289.

KAMATA R, YAMAMOTO T, MATSUMOTO K and MAEDA H (1985). A Serratial protease causes vascular permability reaction by activation of the Hageman factor-dependent pathway in guinea pigs. Infect. Immun. 48, 747-753.

KANUKOLLU U, BIELER S, HULL S and HULL R (1985). Contribution of the traT gene to serum resistance among clinical isolates of Enterobacteriaceae. J. Med. Microbiol. 19, 61-67.

KARCH H, LEYING H and OPFERKUCH W (1984). Analysis of electrophoretically heterogeneous lipopolysaccharides of Escherichia coli by immunoblotting. FEMS Microbiol. Lett. 22, 193-196.

KATO K and BITO Y (1978). Relationship between bactericidal action of complement and fluidity of cellular membranes. Infect. Immun. 19, 12-17.

KENWARD M A (1975). The effect of metal cations upon cell wall chemistry, drug resistance and sensitivity to cold shock of Pseudomonas aeruginosa. PhD Thesis, University of Aston.

KHAN M Y (1983). Subacute constrictive pericarditis from Serratia marcescens bacteremia. Hum. Pathol. 14, 1089-1091.

KHARAZMI A, HØIBY N, DÖRING G and VALERIUS N H (1984). Pseudomonas aeruginosa exoproteases inhibit human neutrophil chemiluminescence. Infect. Immun. 44, 587-591.

KING B F and WILKINSON B J (1981). Binding of human immunoglobulin G to protein A in encapsulated Staphylococcus aureus. Infect. Immun. 33, 666-672.

KIRKLAND T N and ZIEGLER E J (1984). An immunoprotective monoclonal antibody to lipopolysaccharide. J. Immunol. 132, 2590-2592.

KLEBBA P E, McINTOSH M A and NEILANDS S B (1982). Kinetics of biosynthesis of iron-regulated membrane proteins in Escherichia coli. J. Bacteriol. 149, 880-889.

KNOTHE H, SHAH P, KREMERY V, ANTAL M and MITSUHASHI S (1983). Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of Klebsiella pneumoniae and Serratia marcescens. Infection 11, 315-317.

KOBAYASHI T, RINKER J N and KOFFLER H (1959). Purification and chemical properties of flagellin. Arch. Biochem. Biophys. 84, 342-362.

KOCKA F E, ROEMISH E, CAUSEY W A and O'DELL A (1977). The urometer as a reservoir of infectious organisms. Am. J. Clin. Pathol. 67, 106-107.

KOHNO K, YAMAMOTO T, KUROIWA A and AMAKO K (1984). Purification and characterisation of Serratia marcescens U55 pili. Infect. Immun. 46, 295-300.

KONDOH H and HOTANI H (1974). Flagellin from Escherichia coli K12: polymerisation and molecular weight in comparison with Salmonella typhimurium flagellins. Biochim. Biophys. Acta 336, 117-139.

KORHONEN T K and RHEN M (1982). Bacterial fimbriae as vaccines. Ann. Clin. Res. 14, 272-277.

KORPELA J, SALONEN E-M, KUUSELA P, SARVAS M and VAHERI A (1984). Binding of avidin to bacteria and to the outer membrane porin of Escherichia coli. FEMS Microbiol. Lett. 22, 3-10.

KROLL J (1973). Tandem crossed immunoelectrophoresis. Scand. J. Immunol. 2, Suppl 1, 57-59.

KROLL H P, VOIGT W H and TAYLOR P W (1984). Stable insertion of C5b-9 complement complexes into the outer membrane of serum treated, susceptible Escherichia coli cells as a prerequisite for killing. Zbl. Bakt. Hyg. A. 258, 316-326.

KRONE W J A, OUDEGA B, STEGEHUIS F and DE GRAAF F K (1983). Cloning and expression of the cloacin DF13/aerobactin receptor of Escherichia coli (ColV-K30). J. Bacteriol. 153, 716-721.

KUHN H M, ADAMUS G, ROMANOWSKA E and MAYER H (1981). Effect of proteins on the immunogenicity of enterobacterial common antigen. Infect. Immun. 34, 373-377.

KUNIN C M (1962). Antibody distribution against non-enteropathogenic E. coli. Arch. Intern. Med. 110, 676-686.

KUSECEK B, WLOCH H, MERCER A, VAISÄNEN V, PLUSCHKE G, KORHONEN T and ACHTMAN M (1984). Lipopolysaccharide, capsule and fimbriae as virulence factors among O1, O7, O16, O18 or O75 and K1, K5, or K100 Escherichia coli. Infect. Immun. 43, 368-379.

KUWAHARA S S and SNETTING M C (1979). Sialic acid interference with the thiobarbituric acid method for measuring 3-deoxyoctulosonic acid. Anal. Biochem. 100, 118-121.

LABISCHINSKI H, BARNICKEL G, BRADACZEK H, NAUMANN D, RIETSCHEL E T and GIESBRECHT P (1985). High state of order of isolated bacterial lipopolysaccharide and its possible contribution to the permeation barrier property of the outer membrane. J. Bacteriol. 162, 9-20.

LA FORCE F M and BOOSE D (1980). Immune stimulation of lung bactericidal activity: evidence for both cellular and humoral participation after immunisation with Serratia marcescens. Am. Rev. Respir. Dis. 121, 921-929.

LAFUENTE MARTINEZ D, BONILLA VELASCO F A, SAMPEDRO ALVAREZ J and MENENDEZ LOZANDO A (1981). Septic arthritis caused by Serratia marcescens. Arthritis Rheum. 24, 567-568.

LAM C, SCHULZ F, LABER G and SCHÜTZE E (1985). Opsonic activity of the alternative complement pathway in infected human intra-abdominal fluid. Infection 13, 8-11.

LAM C, TURNOWSKY F, SCHWARZINGER E and NERUDA W (1984). Bacteria recovered without subculture from infected human urines expressed iron-regulated membrane proteins. FEMS Microbiol. Lett. 24, 255-259.

LAMBERT M A and MOSS C W (1983). Comparison of the effects of acid and base hydrolysis on hydroxy and cyclopropane fatty acids in bacteria. J. Clin. Microbiol. 18, 1370-1377.

LAMBERT P A (1983). The bacterial surface and drug resistance. In: Medical Microbiology, Vol 3 (Easmon C S F, Jeljaszewicz J, Brown M R W and Lambert P A, Eds.). Academic Press Inc, London, pp 1-19.

LAMBERT P A and BOOTH B R (1982). Exposure of outer membrane proteins on the surface of Pseudomonas aeruginosa PAOI revealed by labelling with [^{125}I]lactoperoxidase. FEMS Microbiol. Lett. 14, 43-45.

LANNIGAN R and BRYAN L E (1985). Decreased susceptibility of Serratia marcescens to chlorhexidine related to the inner membrane. J. Antimicrob. Chemother. 15, 559-565.

LASS J H, HAAF J, FOSTER C S and BELCHER C (1981). Visual outcome in eight cases of Serratia marcescens keratitis. Am. J. Ophthalmol. 92, 384-390.

LAURELL C B (1966). Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Anal. Biochem. 15, 45-52.

Leading Article (1977a). Serratia marcescens infections in general hospitals. BMJ 1(6070), 1177-1178.

Leading Article (1977b). The importance of Serratia marcescens. Lancet 1(8012), 636-637.

LEE G L and SCOTT G K (1980). Analysis of outer membrane components of Escherichia coli ML308 225 and of a serum-resistant mutant. Infect. Immun. 28, 387-392.

LEE N, NAKAMURA K and INOUYE M (1981). Expression of the Serratia marcescens lipoprotein gene in Escherichia coli. J. Bacteriol. 146, 861-866.

LE MINOR S, BENAZET F and MARTIN L (1983). New O(023) and H(H26) antigenic factors in Serratia marcescens. Ann. Microbiol. (Inst. Pasteur) 134B, 447-449.

LE MINOR S and PIGACHE F (1977). Antigenic study of Serratia marcescens isolated in France. 1. H-antigens: individualisation of six new H factors. Ann. Microbiol. (Inst. Pasteur) 128B, 207-214.

LE MINOR S and PIGACHE F (1978). Antigenic study of Serratia marcescens isolated in France. 2. Characterisation of O antigens and description of 5 new O factors, occurrence of serotypes and labelling of new H factors. Ann. Microbiol. (Inst. Pasteur) 129B, 407-423.

LE MINOR S and SAUVAGEOT-PIGACHE F (1981). Nouveaux facteurs antigéniques H(H21-H25) et O(021) de Serratia marcescens: subdivision des facteurs O5, O10, O16. Ann. Microbiol. (Inst. Pasteur) 132A, 239-252.

LEONG J and NEILANDS J B (1976). Mechanisms of siderophore iron transport in enteric bacteria. J. Bacteriol. 126, 823-830.

LEWIS D A, HAWKEY P M, WATTS J A, SPELLER D C E, PRIMAVESI R J, FLEMING P J and PITT T L (1983). Infection with netilmicin resistant Serratia marcescens in a special care baby unit. BMJ 287, 1701-1705.

LIS H, SELA B-A, SACHS L and SHARON N (1970). Specific inhibition by N-acetyl-D-galactosamine of the interaction between soybean agglutinin and animal cell surfaces. Biochim. Biophys. Acta 211, 582-585.

LJUNGH Å and WADSTRÖM T (1984). Fimbriation in relation to hydrophobicity of bacteria in urinary tract infections. Eur. J. Clin. Microbiol. 3, 568-570.

LOEB M R (1984). Immunoblot method for identifying surface components, determining their cross-reactivity and investigating cell topology: results with Haemophilus influenzae type b. Anal. Biochem. 143, 196-204.

LORIAN V and ATKINSON B A (1979). Effect of serum and blood on Enterobacteriaceae grown in the presence of subminimal inhibitory concentrations of ampicillin and mecillinam. Rev. Infect. Dis. 1, 797-806.

LOWRY O H, ROSEBROUGH N J, FARR A L and RANDALL R J (1951). Protein measurement with the folin-phenol reagent. J. Biol. Chem. 193, 265-275.

LUDERITZ O, FREUDENBERG M A, GALANOS C, LEHMAN V, RIETSCHEL E T and SHAW D H (1983). Lipopolysaccharides of Gram-negative bacteria. Current Topics in Membranes and Transport 17, 79-151.

LUDERITZ O, GALANOS C, RISSE H J, RUSCHMANN E, SCHELCHT S, SCHMIDT G, SCHULTE-HOLTHAUSEN H, WHEAT R, WESTPHAL O and SCHLOSSHARDT J (1966). Structural relationships of Salmonella O and R antigens. Ann. NY Acad. Sci. 133, 349-374.

LUGTENBERG B, BRONSTEIN H, VAN SELM N and PETERS R (1977). Peptidoglycan-associated outer membrane proteins in Gram-negative bacteria. Biochim. Biophys. Acta 465, 571-578.

LUGTENBERG B, MEIJERS J, PETERS R, VAN DER HOEK P and VAN ALPHEN L (1975). Electrophoretic resolution of the major outer membrane protein of E. coli K12 into four bands. FEBS Lett. 58, 254-258.

LUGTENBERG B, PETERS R, BERNHEIMER H and BERENDSEN W (1976). Influence of cultural conditions and mutations on the composition of the outer membrane proteins of Escherichia coli. MGG 147, 251-262.

LUGTENBERG B and VAN ALPHEN L (1983). Molecular architecture and functioning of the outer membrane of Escherichia coli and other Gram-negative bacteria. Biochim. Biophys. Acta 737, 51-115.

LYERLY D, GRAY L and KREGER A (1981). Characterisation of rabbit corneal damage produced by Serratia keratitis and by a Serratia protease. Infect. Immun. 33, 927-932.

LYERLY D and KREGER A (1979). Purification and characterisation of a Serratia marcescens metallo-protease. Infect. Immun. 24, 411-421.

LYERLY D M and KREGER A S (1983). Importance of Serratia protease in the pathogenesis of experimental Serratia marcescens pneumonia. Infect. Immun. 40, 113-119.

MacGREGOR C H, BISHOP C W and BLECH J E (1979). Localisation of proteolytic activity in the outer membrane of Escherichia coli. J. Bacteriol. 137, 574-583.

MADDURI S D, MAURIELLO D A, SMITH L G and SEEBODE J J (1976). Serratia marcescens and the urologist. J. Urol. 116, 613-615.

MAES P (1985). Evaluation of the resistance mechanisms of gentamicin-resistant Gram-negative bacilli and their susceptibility to tobramycin, netilmicin and amikacin. J. Antimicrob. Chemother. 15, 283-289.

MAKI D G, HENNEKENS C G, PHILLIPS C W, SHAW W V and BENNETT J V (1973). Nosocomial urinary tract infection with Serratia marcescens: an epidemiologic study. J. Infect. Dis. 128, 579-587.

MARKOWITZ S M and SIBILLA D J (1980). Comparative susceptibilities of clinical isolates of Serratia marcescens to newer cephalosporins alone and in combination with various aminoglycosides. Antimicrob. Agents Chemother. 18, 651-655.

MARKWELL M A K (1982). A new solid-state reagent to iodinate proteins. I. Conditions for the efficient labelling of antiserum. Anal. Biochem. 125, 427-432.

MARRIE T J and COSTERTON J W (1981). Prolonged survival of Serratia marcescens in chlorhexidine. Appl. Environ. Microbiol. 42, 1093-1102.

MARRIE T J, NOBLE M A, HALDANE E V, DUNCAN N H, PATERSON I, CARRUTHERS D, BARRY A, WEST A and McCORMICK S (1982). Serratia marcescens - marker for an infection control program. Infect. Control 3, 134-142.

MASKELL R, CRUMP J and LEE R (1977). Importance of Serratia marcescens. Lancet 1(8019), 1013.

MASLOW M J, SIMBERKOFF M S and RAHAL J J (1985). Clinical efficacy of synergistic combination of cefotaxime and amikacin against multi-resistant Pseudomonas and Serratia infections. J. Antimicrob. Chemother. 16, 227-234.

MATSUMOTO K, MAEDA H, TAKATA K, KAMATA R and OKAMURA R (1984). Purification and characterisation of four proteases from a clinical isolate of Serratia marcescens kums 3958. J. Bacteriol. 157, 225-232.

MATTSBY-BALTZER I and ALVING C R (1984). Antibodies to lipid A: occurrence in humans. Rev. Infect. Dis. 6, 553-557.

MATTSBY-BALTZER I, GEMSKI P and ALVING C R (1984). Heterogeneity of lipid A. Rev. Infect. Dis. 6, 444-448.

McDONOUGH M W (1965). Amino acid composition of antigenically distinct salmonella flagellar proteins. J. Mol. Biol. 12, 342-355.

McINTOSH M A and EARHART C F (1977). Coordinate regulation by iron of the synthesis of phenolate compounds and three outer membrane proteins in Escherichia coli. J. Bacteriol. 131, 331-339.

MEERS P D, FOSTERS C S anc CHURCHER G M (1978). Cross-infection with Serratia marcescens BMJ 1, 238-239.

MENDEZ F J, MENDOZA M C, LLANEZA J J and HARDISSON C (1982). Transfer of drug-resistance plasmids by conjugation from nosocomial strains of Serratia marcescens to Escherichia coli in biological fluids of human origin. J. Hosp. Infect. 3, 285-292.

MENDOZA M C, BLANCO M G, JAVIER MENDEZ F and HARDISSON C (1984). Evolution de la résistance aux aminosides chez des souches hospitalières de Serratia. Pathol. Biol. 32, 750-754.

MESHULAM T, VERBRUGH H A and VERHOEF J (1982). Opsonisation and phagocytosis of mucoid and non-mucoid Pseudomonas aeruginosa strains. Eur. J. Clin. Microbiol. 1, 112-117.

MEVISSEN-VERHAGE E A E, MARCELIS J H, HARMSEN-VAN AMERONGEN W C M, DE VOS N M, BERKEL J and VERHOEF J (1985). Effect of iron on neonatal gut flora during the first week of life. Eur. J. Clin. Microbiol. 4, 14-18.

MEYER R D, HALTER J, LEWIS R P and WHITE M (1976). Gentamicin-resistant Pseudmonas aeruginosa and Serratia marcescens in a general hospital. Lancet 1(7959), 580-583.

MIETZNER T A, BARNES R C, JEANLOUIS Y A, SHAFER W M and MORSE S A (1986). Distribution of an antigenically related iron-regulated protein among the Neisseria spp. Infect. Immun. 51, 60-68.

MILATOVIC D (1983). Antibiotics and phagocytosis. Eur. J. Clin. Microbiol. 2, 414-425.

MILAZZO F H and DELISLE G J (1984). Immunoglobulin A proteases in Gram-negative bacteria isolated from human urinary tract infection. Infect. Immun. 43, 11-13.

MILES A A and MISRA S S (1938). The estimation of the bactericidal power of blood. J. Hyg. 38, 732-748.

MILLER G W and NUSSENZWEIG V (1975). A new complement function: solubilisation of antigen-antibody aggregates. Proc. Natl. Acad. Sci. USA. 72, 418-423.

MILLER M A and BEVILL R (1983). Plasmid analysis of multiple resistant Serratia marcescens. Curr. Microbiol. 9, 329-332.

MILLER M E and BUCKLER J M (1968). Intracellular survival and growth of Serratia marcescens following phagocytosis by human leukocytes. J. Infect. Dis. 118, 149-152.

MIYATA K, NAKAMURA M and TOMODA K (1981). Interaction between Serratia protease and human plasma alpha 2 macroglobulin. J. Biochem. 89, 1231-1237.

MOLL A, MANNING P A and TIMMIS K N (1980). Plasmid-determined resistance to serum bactericidal activity: a major outer membrane protein, the traT gene product, is responsible for plasmid-specified serum resistance in Escherichia coli. Infect. Immun. 28, 359-367.

MONTANARO D, GRASSO G M, ANNINO I, DE RUGGIERO N, SCARCELLA A and SCHIOPPA F (1984). Epidemiological and bacteriological investigation of Serratia marcescens epidemic in a nursery and in a neonatal intensive care unit. J. Hyg. 93, 67-78.

MONTENEGRO M A, BITTER-SUERMANN D, TIMMIS J K, AGÜERO M E, CABELLO F C, SANYAL S C and TIMMIS K N (1985). traT gene sequences, serum resistance and pathogenicity-related factors in clinical isolates of Escherichia coli and other Gram-negative bacteria. J. Gen. Microbiol. 131, 1511-1521.

MONTGOMERIE J Z, BINDEREIF A, NEILANDS J B, KALMANSON G M and GUZE L B (1984). Association of hydroxamate siderophore (aerobactin) with Escherichia coli isolated from patients with bacteremia. Infect. Immun. 46, 835-838.

MONTIE T C and STOVER G B (1983). Isolation and characterisation of flagellar preparations from Pseudomonas species. J. Clin. Microbiol. 118, 452-456.

MOORE D G, YANCEY R J, LANKFORD C E and EARHART C F (1980). Bacteriostatic enterochelin-specific immunoglobulin from normal human serum. Infect. Immun. 27, 418-423.

MOROHOSHI T, TORIYA M, YOKOIYAMA S, FUJIMOTO K and HAYANO K (1984). The acetylation of 6'-amino group of amikacin by a new enzyme prepared from Serratia sp. J. Antibiot. 37, 1687-1691.

MORRISON D C and KLINE L F (1977). Activation of the classical and properdin pathways of complement by bacterial lipopolysaccharides (LPS). J. Immunol. 118, 362-369.

MORSE S A, MINTZ C S, SARAFIAN S K, BARTENSTEIN L, BERTRAM M and APICELLA M A (1983). Effect of dilution rate on lipopolysaccharide and serum resistance of Neisseria gonorrhoeae grown in continuous culture. Infect. Immun. 41, 74-82.

MUHLBAUER B, FATTAL A and SIEGMAN-IGRA Y (1983). A small outbreak of Serratia marcescens sepsis and meningitis. Ir. J. Med. Sci. 19, 980-981.

MÜLRADT P F and GOLECKI J R (1975). Asymmetrical distribution and artifactual reorientation of lipopolysaccharide in the outer membrane bilayer of Salmonella typhimurium. Eur. J. Biochem. 51, 343-352.

MUSCHEL L H and LARSEN L J (1970). The sensitivity of smooth and rough Gram-negative bacteria to the immune bactericidal reaction. Proc. Soc. Exp. Biol. Med. 133, 345-348.

MUTHARIA L M, NICAS T I and HANCOCK R E W (1982). Outer membrane proteins of Pseudomonas aeruginosa serotype strains. J. Infect. Dis. 146, 770-779.

MUTTON K J, BRAD L M and HARKNESS J L (1981). Serratia cross-infection in an intensive therapy unit. J. Hosp. Infect. 2, 85-91.

NAGATA Y and BURGER M M (1974). Wheat germ agglutinin. Molecular characteristics and specificity for sugar binding. J. Biol. Chem. 249, 3116-3122.

NAKAMURA K and INOUYE M (1980). DNA sequence of the Serratia marcescens lipoprotein gene. Proc. Natl. Acad. Sci. USA 77, 1369-1373.

NAKAMURA Y, NOHARA M, NAKASHIMA T, ICHIKAWA K, TOMITA T, KONDO M, KOMATSU Y, NAKASHIMA H and HASHIMOTO T (1984). Meningoencephalitis due to Serratia marcescens infection in neonates. Hum. Pathol. 15, 651-656.

NEILANDS J B (1981a). Microbial iron compounds. Annu. Rev. Biochem. 50, 715-731.

NEILANDS J B (1981b). Iron absorption and transport i micro-organisms. Annu. Rev. Nutr. 1, 27-46.

NEILANDS J B (1982). Microbial envelope proteins related to iron. Annu. Rev. Microbiol. 36, 285-309.

NEU H C (1984). Do we need the third-generation cephalosporins? J. Antimicrob. Chemother. 14, Suppl B, 1-12.

NEWMAN S L and MIKUS L K (1985). Deposition of C3b and iC3b onto particulate activators of the human complement system. Quantitation with monoclonal antibodies to human C3. J. Exp. Med. 161, 1414-1431.

NIKAIDO H (1979). Non-specific transport through the outer membrane. In: Bacterial Outer Membranes, Biogenesis and Function (Inouye M, Ed.). John Wiley and Sons, New York, pp361-340.

NIKAIDO H and NAKAE T (1979). The outer membrane of Gram-negative bacteria. Adv. Microb. Physiol. 20, 163-250.

NIKAIDO H and VAARA M (1985). Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. 49, 1-32.

NOY M F (1982). Effect of phosphate limited growth on drug resistance of Pseudomonas aeruginosa. PhD Thesis, University of Aston.

O'DONNELL I J, STEWART D J and CLARK B L (1983). Serological identification of pilus antigen and other protein antigens of Bacterioides nodosus using electroblot radioimmunoassay after electrophoretic fractionation of the proteins on sodium dodecyl sulfate polyacrylamide gels. Aust. J. Biol. Sci. 36, 15-20.

ODUMERU J A, WISEMAN G M and RONALD A R (1985). Role of lipopoly-saccharide and complement in susceptibility of Haemophilus ducreyi to human serum. Infect. Immun. 50, 495-499.

OFEK I and BEACHEY E H (1980). General concepts and principles of bacterial adherence in animals and man. In: Bacterial Adherence, Receptors and Recognition, Series B, Vol 6 (Beachey E H, Ed.). Chapman and Hall, London, pp 1-29.

OGATA R T (1983). Factors determining bacterial pathogenicity. Clin. Physiol. Biochem. 1, 145-159.

OHSHIMA Y, OHTOMO T and YOSHIDA K (1984). Encapsulation and mouse-virulence of Serratia marcescens strain SM-1 and its variants in relation to colonial morphology. Acta Microbiol. Hung. 31, 55-60.

OLD D C, ADEGBOLA R and SCOTT S S (1983). Multiple fimbrial haem-agglutinins in Serratia species. Med. Microbiol. Immunol. 172, 107-115.

OLEXY V M, KELLY D, BIRD T J, GRIEBLE H G and FARRAND S K (1982). An R plasmid of Serratia marcescens transferable to Pseudomonas aeruginosa. Chemotherapy 28, 6-17.

ØRSKOV I, BIRCH-ANDERSEN A, DUGUID J P, STENDERUP J and ØRSKOV F (1985). An adhesive protein capsule of Escherichia coli. Infect. Immun. 47, 191-200.

OSBORN M J (1963). Studies on the Gram-negative cell wall. I. Evidence for the role of 2-keto-3-deoxyoctonate in the LPS of Salmonella typhimurium. Proc. Natl. Acad. Sci. USA. 50, 499-506.

OUCHTERLONY O (1958). "Diffusion in gel" methods for immunological analysis. Prog. Allergy 5.

OWEN P (1981) Immunology of the bacterial membrane. In: Organisation of Prokaryotic Cell Membranes, Vol I (Ghosh B K, Ed.). CRC Press Inc, Boca Raton, Florida, pp73-164.

OWEN P and KABACK H R (1978). Molecular structure of membrane vesicles from Escherichia coli. Proc. Natl. Acad. Sci. USA. 75, 3148-3152.

PALVA E T and MÄKELÄ P H (1980). Lipopolysaccharide heterogeneity in Salmonella typhimurium analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Eur. J. Biochem. 107, 137-143.

PANGBURN M K and MÜLLER-EBERHARD H J (1980). Relation of a putative thioester bond in C3 to activation of the alternative pathway and binding of C3b to biological targets of complement. J. Exp. Med. 152, 1102-1114.

PATEL P V, VEALE D R, FOX J E, MARTIN P M V, PARSONS N J and SMITH H (1984a). Fractionation of guinea pig serum for an inducer of Gonococcal resistance to killing by human serum: active fractions containing glucopeptides similar to those from human red blood cells. J. Gen. Microbiol. 130, 2757-2766.

PATEL P V, MARTIN P M V, GOLDNER M, PARSONS N J and SMITH H (1984b). Red blood cells, a source of factors which induce Neisseria gonorrhoeae to resistance to complement-mediated killing by human serum. J. Gen. Microbiol. 130, 2767-2770.

PENN C W (1983). Bacterial envelope and humoral defenses. In: Medical Microbiology, Vol 3 (Easmon C S F, Jeljaszewicz J, Brown M R W and Lambert P A, Eds.). Academic Press Inc, London, pp109-135.

PEREIRA M E A and KABAT E A (1974). Discussion paper: Blood group specificity of the lectin from Lotus tetragonolobus. Ann. NY Acad. Sci. 234, 301-305.

PEREZ-PEREZ G I, HOPKINS J A and BLASER M J (1986). Lipopolysaccharide structures in Enterobacteriaceae, Pseudomonas aeruginosa and Vibrio cholerae are immunologically related to Campylbacter spp. Infect. Immun. 51, 204-208.

PETERS H, JÜRS M, JANN B, JANN K, TIMMIS K N and BITTER-SUERMANN D (1985). Monoclonal antibodies to Enterobacterial common antigen and to Escherichia coli lipopolysaccharide outer core: demonstration of an antigenic determinant shared by enterobacterial common antigen and E. coli K5 capsular polysaccharide. Infect. Immun. 50, 459-466.

PETERSON A A and McGROARTY E J (1985). High-molecular weight components in lipopolysaccharides of Salmonella typhimurium, Salmonella minnesota and Escherichia coli. J. Bacteriol. 162, 738-745.

PITT T L (1982). State of the art: typing of Serratia marcescens. J. Hosp. Infect. 3, 9-14.

PITT T L (1983). Identification and typing of Serratia marcescens. J. Med. Microbiol. 16, Abstract 8, i-ii.

PITT T L and ERDMAN Y J (1984). Serological typing of Serratia marcescens. Methods in Microbiology, 15, 173-211.

PITT T L, ERDMAN Y J and BUCHER C (1980). The epidemiological type identification of Serratia marcescens from outbreaks of infection in hospitals. J. Hyg. 84, 269-283.

PLATT D J and SOMMERVILLE J S (1981). Serratia species isolated from patients in a general hospital. J. Hosp. Infect. 2, 341-348.

PLUSCHKE G, MAYDEN J, ACHTMAN M and LEVINE R P (1983). Role of the capsule and the O antigen in resistance of O18:K1 Escherichia coli to complement-mediated killing. Infect. Immun. 42, 907-913.

PLUSCHKE G, MOLL A, KUSECEK B and ACHTMAN M (1986). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and monoclonal antibodies as tools for the subgrouping of Escherichia coli lipopolysaccharide O18 and O23 antigens. Infect. Immun. 51, 286-293.

POLK R E, MAYHALL G, SMITH J, HALL G, KLINE B J, SWENSSON E and HAYNES B W (1983). Gentamicin and tobramycin penetration into burn eschar. Arch. Surg. 118, 295-302.

POLLACK M, PIER G B and PRESCOTT R K (1984). Immunisation with Pseudomonas aeruginosa high-molecular weight polysaccharides prevents death from Pseudomonas burn infections in mice. Infect. Immun. 43, 759-760.

POXTON I R, BELL G T and BARCLAY G R (1985). The association on SDS-polyacrylamide gels of lipopolysaccharide and outer membrane proteins of Pseudomonas aeruginosa as revealed by monoclonal antibodies and Western blotting. FEMS Microbiol. Lett. 27, 247-251.

REEKE Jr G N, BECKER J W, CUNNINGHAM B A, GUNTHER G R, WANG J L and EDELMAN G M (1974). Relationships between the structure and activities of concanavalin A. Ann. NY Acad. Sci. 234, 369-382.

REICHLING J J, ROSE D N, MENDELSON M H and HIRSCHMAN S Z (1984). Acute suppurative thyroiditis caused by Serratia marcescens. J. Infect. Dis. 149, 281.

RHEN M, WAHLSTRÖM E and KORHONEN T K (1983). P-fimbriae of Escherichia coli: fractionation by immune precipitation. FEMS Microbiol. Lett. 18, 227-232.

RIETSCHEL E T, WOLLENWEBER H-W, BRADE H, ZÄHRINGER U, LINDER B, SEYDEL U, BRADACZEK H, BARNICKEL G, LABISCHINSKI H and GIESBRECHT P (1984). Structure and conformation of the lipid A component of lipopolysaccharides. In: Handbook of Endotoxin Vol 1: Chemistry of Endotoxin (Rietschel E T, Ed.). Elsevier Science Publishers BV, Holland, pp187-220.

RINGROSE R E, McKOWN B, FELTON F G, BARCLAY B O, MUNCHMORE H G and RHOADES E R (1968). A hospital outbreak of Serratia marcescens associated with ultrasonic nebulisers. Ann. Intern. Med. 69, 719-729.

ROIG J M, DORRONSORO I and DIAZ R (1979). Aplicación de las técnicas de coaglutinación y precipitación en placa de Elek para la tipificación serológica de Serratia marcescens. Laboratorio 68, 501-527.

ROIG J M, DORRONSORO I and DIAZ R (1983). Rapid identification of Serratia marcescens by coagglutination. J. Clin. Microbiol. 18, 741-742.

ROSENBERG M, GUTNIGK D and ROSENBERG E (1980). Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. 9, 29-33.

ROSS G D and ATKINSON J P (1985). Complement receptor structure and function. Immunol. Today 6, 115-119.

ROSS G D and LAMBRIS J D (1982). Identification of a C3bi-specific membrane complement receptor that is expressed on lymphocytes, monocytes, neutrophils and erythrocytes. J. Exp. Med. 155, 96-110.

ROWLEY D (1973). Antibacterial action of antibody and complement. J. Infect. Dis. 128 (Suppl), 170-175.

RUBENS C E, FARRAR Jr W E, McGEE Z A and SCHAFFNER W (1981). Evolution of a plasmid mediating resistance to multiple antimicrobial agents during a prolonged epidemic of nosocomial infections. J. Infect. Dis. 143, 170-181.

RUBIN S J (1980). Biotyping, serotyping, zymotyping and antibiogram typing of Serratia marcescens. In: The Genus Serratia (von Graevenitz A and Rubin S J, Eds.). CRC Press Inc, Boca Raton, Florida, pp101-118.

RUHEN R W and WETHERALL F M (1983). Colonial variation in Serratia marcescens together with antibiotic resistance. Pathology 15, 65-66.

RUSSELL R R B (1976). Free endotoxin - a review. Microbios Lett. 2, 125-135.

RUTALA W A, KENNEDY V A, LOFLIN H B and SARUBBI Jr F A (1981). Serratia marcescens noscomial infections of the urinary tract associated with urine measuring containers and urinometers. Am. J. Med. 70, 659-663.

SANSANO Jr M, REYNARD A M and CUNNINGHAM R K (1985). Inhibition of serum bactericidal reaction by lipopolysaccharide. Infect. Immun. 48, 759-762.

SAWAI T, HIRUMA H, KAWANA N, KANEKO M, TANIYASU T and INAMI A (1982). Outer membrane permeation of β-lactam antibiotics in Escherichia coli, Proteus mirabilis and Enterobacter cloacae. Antimicrob. Agents Chemother. 22, 585-592.

SCHABERG D R, ALFORD R H, ANDERSON R, FARMER III J J, MELLY M A and SCHAFFNER W (1976a). An outbreak of nosocomial infections due to multiply resistant Serratia marcescens: Evidence of inter-hospital spread. J. Infect. Dis. 134, 181-188.

SCHABERG D R, HIGHSMITH A K and WACHSMUTH I K (1977). Resistance plasmid transfer by Serratia marcescens in urine. Antimicrob. Agents Chemother. 11, 449-450.

SCHABERG D R, WEINSTEIN R A and STAMM W E (1976b). Epidemics of nosocomial urinary tract infection caused by multiply resistant Gram-negative bacilli: epidemiology and control. J. Infect. Dis. 133, 363-366.

SCHMITZ G and BRAUN V (1985). Cell-bound and secreted proteases of Serratia marcescens. J. Bacteriol. 161, 1002-1009.

SCIORTINO C V and FINKELSTEIN R A (1983). Vibrio cholerae expresses iron-regulated outer membrane proteins in vivo. Infect. Immun. 42, 990-996.

SHAFER W M, JOINER K, GUYMAN L F, COHEN M S and SPARLING P F (1984). Serum sensitivity of Neisseria gonorrhoeae: The role of lipopoly-saccharide. J. Infect. Dis. 149, 175-183.

SHAND G H, ANWAR H, KADURUGAMUWA J, BROWN M R W, SILVERMAN S H and MELLING J (1985). In vivo evidence that bacteria in urinary tract infection grow under iron-restricted conditions. Infect. Immun. 48, 35-39.

SHANNON K and PHILLIPS I (1982). Mechanisms of resistance to amino-glycosides in clinical isolates. J. Antimicrob. Chemother. 9, 91-102.

SHEETS R D (1981). Serratia marcescens contaminated disinfectants. Lancet, 1(8222), 727.

SILVERBLATT F J and OFEK I (1983). Interaction of bacterial pili and leukocytes. Infection 11, 235-238.

SILVERMAN M and SIMON M I (1977). Bacterial flagella. Annu. Rev. Microbiol. 31, 397-419.

SIMBERKOFF M S (1980). Experimental Serratia marcescens infection and defense mechanisms. In: The Genus Serratia (von Graevenitz A and Rubin S J, Eds.). CRC Press Inc, Boca Raton, Florida, pp157-164.

SIMBERKOFF M S, MOLDOVER N H and RAHAL Jr J J (1976a). Specific and non-specific immunity of Serratia marcescens infection. J. Infect. Dis. 134, 348-353.

SIMBERKOFF M S, RICUPERO I and RAHAL Jr J J (1976b). Host resistance to Serratia marcescens infection: serum bactericidal activity and phagocytosis by normal blood leukocytes. J. Lab. Clin. Med. 87, 206-217.

SIMPSON A W and BOUGHTON B J (1984). Fibronectin as an opsonic regulator of monocyte phagocytosis. J. Clin. Pathol. 37, 787-789.

SKAMENE E and GROS P (1983). Role of macrophages in resistance against infectious diseases. Clin. Immunol. Allergy 3, 539-560.

SLACK M P E and NICHOLS W W (1982). Antibiotic penetration through bacterial capsules and exopolysaccharides. J. Antimicrob. Chemother. 10, 368-372.

SMIT J, KAMIO Y and NIKAIDO H (1975). Outer membrane of Salmonella typhimurium: chemical analysis and freeze-fracture studies with lipopolysaccharide mutants. J. Bacteriol. 124, 942-958.

SMITH H (1977). Microbial surfaces in relation to pathogenicity. Bacteriol. Rev. 41, 475-500.

SMITH P J, BROOKFIELD D S K, SHAW D A and GRAY J (1984). An outbreak of Serratia marcescens infection in a neonatal unit. Lancet 1(8369), 151.

SOKOL P A and WOODS D E (1984). Relationship of iron and extracellular virulence factors to Pseudomonas aeruginosa lung infection. J. Med. Microbiol. 18, 125-133.

SONNTAG I, SCHWARZ H, HIROTA Y and HENNING U (1978). Cell envelope and shape of Escherichia coli: multiple mutants missing the outer membrane lipoprotein and other major outer membrane proteins. J. Bacteriol. 136, 280-285.

SORDELLI D O, CERQUETTI M C and HOOKE A M (1985). Replication rate of Pseudomonas aeruginosa in the murine lung. Infect. Immun. 50, 388-391.

STENDAHL O (1983). The physiochemical basis of surface interactions between bacteria and phagocytic cells. In: Medical Microbiology, Vol 3 (Easmon C S F, Jeljaszewicz J, Brown M R W and Lambert P A, Eds.). Academic Press Inc, London, pp138-152.

STERKENBURG A, VLEGELS E and WOUTERS J T M (1984). Influence of nutrient limitation and growth rate on the outer membrane proteins of Klebsiella aerogenes NCTC 418. J. Gen. Microbiol. 130, 2347-2355.

STRUNK R C and WEBSTER R O (1985). Inhibition of cleavage of the third component of human complement (C3) by its small cleavage fragment, C3a: inhibition occurs with the classical pathway, but not the alternative pathway C3 convertase. Mol. Immunol. 22, 37-43.

SULING W J and O'LEARY W M (1977). Lipids of antibiotic-resistant and susceptible members of the Enterobacteriaceae. Can. J. Microbiol. 23, 1045-1051.

SUTHERLAND I W and WILKINSON J F (1971). Chemical extraction methods of microbial cells. In: Methods in Microbiology, Vol 5B (Norris J R and Ribbons D W, Eds.). Academic Press Inc, London, pp345-383.

SWANSON J (1981). Surface-exposed protein antigens of the Gonococcal outer membrane. Infect. Immun. 34, 804-816.

TARCSAY L, WANG C S, LI C and ALAUPOVIC P (1973). Composition and structure of the O-specific side chain of endotoxin from Serratia marcescens O8. Biochemistry 12, 1248-1955.

TAYLOR P W (1975). Genetical studies of serum resistance in Escherichia coli. J. Gen. Microbiol. 89, 57-66.

TAYLOR P W (1978). The effect of the growth environment on the serum sensitivity of some urinary Escherichia coli strains. FEMS Microbiol. Lett. 3, 119-122.

TAYLOR P W (1983). Bactericidal and bacteriolytic activity of serum against Gram-negative bacteria. Microbiol. Rev. 47, 46-83.

TAYLOR P W and KROLL H-P (1985). Effect of lethal doses of complement on the functional integrity of target Enterobacteria. Curr. Top. Microbiol. Immunol. 121, 135-158.

TAYLOR P W and PARTON R (1977). A protein factor associated with serum resistance in Escherichia coli. J. Med. Microbiol. 10, 225-232.

THORÉN A (1983). Enterotoxin production, invasive ability and surface antigens of enteropathogenic Escherichia coli. Eur. J. Clin. Microbiol. 2, 478-479.

TOWBIN H and GORDON J (1984). Immunoblotting and dot immunoblotting - current status and outlook. J. Immunol. Methods 72, 313-340.

TOWBIN H, STAEHELIN T and GORDON J (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350-4354.

TRAUB W H (1977). Further studies on the susceptibility of Serratia marcescens to the bactericidal activity of human serum. Resistance of isolates against serum transferrin-mediated iron deprivation and chelation of cations by EDTA. Exp. Cell Biol. 45, 184-206.

TRAUB W H (1978). Phenotypic variations of bacteriocin susceptibility, O-antigen determinants and serum susceptibility among isolates of a multiple-drug-resistant, nosocomially significant strain of Serratia marcescens. Zbl. Bakt. Hyg. A. 240, 57-75.

TRAUB W H (1980a). Susceptibility of Serratia marcescens to human serum: antagonism of serum bactericidal activity by IgG immunoglobulins of homologous rabbit anti-O sera. Zbl. Bakt. Hyg. A. 6, 26-49.

TRAUB W H (1980b). Enhancement of human serum bactericidal activity by specific IgM antibodies of normal and anti-O immune rabbit sera against pseudo-serum-resistant strains of Serratia marcescens. Zbl. Bakt. Hyg. A. 246, 50-66.

TRAUB W H (1981a). Phagocytosis of Serratia marcescens by leukocytes of fresh defibrinated human blood: failure of 'natural' human specific anti-O IgG antibodies to enhance phagocytosis. Zbl. Bakt. Hyg. A. 250, 92-103.

TRAUB W H (1981b). Serotyping of Serratia marcescens: confirmation of five recently described new O-antigens and characterisation of an additional O-antigen. Zbl. Bakt. Hyg. A. 250, 307-311.

TRAUB W H (1981c). Towards serodiagnosis of Serratia marcescens infections: examination of sera from non-infected patients and from experimentally infected rabbits for anti-H and anti-O antibodies. S. marcescens O-antigen cross-reacts with those of other Enterobacteriaceae. Zbl. Bakt. Hyg. A. 250, 490-496.

TRAUB W H (1981d). Human serum bactericidal activity against Serratia marcescens: failure of human 'natural' antibodies and specific anti-O IgG antibodies to enhance serum bactericidal activity. Zbl. Bakt. Hyg. A. 250, 84-91.

TRAUB W H (1982a). Virulence of nosocomial isolates of Serratia marcescens for NMRI mice: correlation with O-antigens O6 and O14. Zbl. Bakt. Hyg. A. 252, 360-369.

TRAUB W H (1982b). Opsonisation requirements of Serratia marcescens. Zbl. Bakt. Hyg. A. 253, 204-224.

TRAUB W H (1982c). Interactions of antimicrobial drugs and combined phagocytic/serum bactericidal activity of defibrinated human blood against Serratia marcescens. II. Aminoglycosides: amikacin, gentamicin and netilmicin. Chemotherapy 28, 434-443.

TRAUB W H (1983a). Failure of the commercial human IgG immunoglobulin preparation Polyglobin to enhance combined phagocytic and serum bactericidal activity of normal blood against Serratia marcescens. Chemotherapy 29, 43-47.

TRAUB W H (1983b). Passive protection of NMRI mice against Serratia marcescens: comparative efficacy of commercial human IgG immunoglobulin preparations and rabbit anti-O,-H,-K,-live cell and -protease immune sera. Zbl. Bakt. Hyg. A. 254, 480-488.

TRAUB W H (1985). Serotyping of Serratia marcescens: identification of a new O-antigen (O24). Zbl. Bakt. Hyg. A. 259, 485-488.

TRAUB W H and FUKUSHIMA P I (1979a). Serotyping of Serratia marcescens: simplified tube O-agglutination test and comparison with other serological procedures. Zbl. Bakt. Hyg. A. 244, 474-493.

TRAUB W H and FUKUSHIMA P I (1979b). Mammalian serum susceptibility of Serratia marcescens: detection of three human serum susceptibility categories. Zbl. Bakt. Hyg. A. 245, 301-311.

TRAUB W H and FUKUSHIMA P I (1979c). Further characterisation of 'promptly' and 'delayed' serum sensitive strains of Serratia marcescens. Zbl. Bakt. Hyg. A. 245, 495-511.

TRAUB W H and FUKUSHIMA P I (1979d). In vitro additive effect of nitrofurantoin combined with trimethoprim-sulfamethoxazole against Serratia marcescens. Chemotherapy 25, 140-146.

TRAUB W H and KLEBER I (1974). Detection of fibrinolytic and elastase activity among clinical isolates of Serratia marcescens. Pathol. Microbiol. 41, 334-340.

TRAUB W H and KLEBER I (1975). Selective activation of classical and alternative pathways of human complement by 'promptly serum-sensitive' and 'delayed serum-sensitive' strains of Serratia marcescens. Infect. Immun. 13, 1343-1346.

TRAUB W H and KLEBER I (1977). Serotyping of Serratia marcescens: evaluation of LeMinor's H-immobilisation test and description of three new flagellar H antigens. J. Clin. Microbiol. 5, 115-121.

TRAUB W H and KLEBER I (1978). Serotyping of Serratia marcescens: determination of serogroup (O) antigens and serological cross-reactions. Zbl. Bakt. Hyg. A. 240, 30-56.

TRAUB W H, KLEBER I, PÜHLER A and BURKARDT H-J (1976). Characterisation of a nosocomially significant, multiple drug-resistant strain of Serratia marcescens. Chemotherapy 22, 297-312.

TRAUB W H, SPOHR M and BAUER D (1983a). Resistance-plasmid-independent and protease-independent murine virulence of a multiple drug-resistant strain of Serratia marcescens. Zbl. Bakt. Hyg. A. 256, 184-195.

TRAUB W H, SPOHR M and BAUER D (1983b). Plasmid-independent resistance of 'gray' colony variants of a strain of Serratia marcescens resistant to amikacin, cefotaxime and lamoxactam. Chemotherapy 29, 265-274.

TRAUB W H, SPOHR M and BAUER D (1985). Active immunisation of NMRI mice against Serratia marcescens. Zbl. Bakt. Hyg. A. 259, 410-425.

TSAI C M and FRASCH C E (1982). A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. 119, 115-119.

TSANG J C, BROWN D A and KRANZ D M (1976). Detection of lipopolysaccharide components in isolated outer membranes of Serratia marcescens. Microbios Lett. 1, 209-217.

TSANG J C, SANSING G A and MILLER M A (1975). Relation of beta-lactamase activity to antimicrobial susceptibility in Serratia marcescens. Antimicrob. Agents Chemother. 8, 277-281.

TSANG J C, WANG C S and ALAUPOVIC P (1974). Degradative effects of phenol on endotoxin and lipopolysaccharide preparations from Serratia marcescens. J. Bacteriol. 117, 786-795.

TSANG J C, WEBER D A and BROWN D A (1976a). Evidences for complex formation between polymyxin B and lipopolysaccharides from Serratia marcescens. J. Antibiot. 29, 735-742.

VAESSEN R T M J, KREIKE J and GROOT G S P (1981). Protein transfer to nitrocellulose filters. FEBS Lett. 124, 193-196.

VALVANO M A and CROSA J H (1984). Aerobactin iron transport genes commonly encoded by certain ColV plasmids occur in the chromosome of a human invasive strain of Escherichia coli K1. Infect. Immun. 46, 159-167.

VAN ALPHEN L, VAN KEMPEN-DE TROYE F and ZANEN H C (1983). Immunological detection of heterogeneous O-antigen-containing lipopolysaccharides in Escherichia coli. J. Gen. Microbiol. 29, 191-198.

VAN ASBECK B S, MARCELIS J H, MARX J J M, STRUYVENBERG A, VAN KATS J H AND VERHOEF J (1983). Inhibition of bacterial multiplication by the iron chelator desferoxamine: potentiating effect of ascorbic acid. Eur. J. Clin. Microbiol. 2, 426-431.

VAN DER LEY P, KUIPERS O, TOMMASSEN J and LUGTENBERG B (1986). O-antigenic chains of lipopolysaccharide prevent binding of antibody molecules to an outer membrane pore protein in Enterobacteriaceae. Microbial Pathogenesis 1, 43-49.

VAN LAETHEM Y, HUSSON M and KLASTERSKY J (1984). Serum bactericidal activity of aztreonam, cefoperazone and amikacin, alone or in combination, against Escherichia coli, Klebsiella pneumoniae, Serratia marcescens and Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 26, 224-227.

VERWEIJ-VAN VUGHT A M J J, NAMAVAR F, PEERBOOMS P G H, SPARRIUS M and MACLAREN D M (1984). The role of different K antigens of Escherichia coli in phagocytosis by polymorphonuclear leukocytes. J. Med. Microbiol. 17, 141-150.

VON GRAEVENITZ A (1980). Infection and colonisation with Serratia. In: The Genus Serratia (von Graevenitz A and Rubin S J, Eds.). CRC Press Inc, Boca Raton, Florida, pp167-186.

WANG C S, BURNS R K and ALAUPOVIC P (1974). Isolation and composition of oligosaccharide cores from endotoxins of two Serratia marcescens strains. J. Bacteriol. 120, 990-993.

WARD P A, CHAPITIS J, CONROY M C and LEPOW I H (1973). Generation by bacterial proteinases of leukotactic factors from human serum and human C3 and C5. J. Immunol. 110, 1003-1009.

WASHINGTON J A and BARRY A L (1974). Dilution test procedures. In: Manual of Clinical Microbiology, 2nd Ed (Lennette E H, Spaulding E H and Truant J P, Eds.). American Society for Microbiology, Washington DC, pp 410-418.

WAYNE R and NEILANDS J B (1975). Evidence for common binding sites for ferrichrome and bacteriophage theta 80 in the cell envelope of Escherichia coli. J. Bacteriol. 121, 497-503.

WEEKE B (1973). Crossed immunoelectrophoresis. Scand. J. Immunol. 2 (Suppl 1), 47-56.

WEINBERG E D (1984). Iron Witholding: a defense against infection and neoplasia. Physiol. Rev. 64, 65-102.

WESTPHAL O and JANN K (1965). Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. In: Methods in Carbohydrate Chemistry, Vol V (Whistler R E, Ed.). Academic Press Inc, New York, pp83-91.

WESTPHAL O, JANN K and HUMMELSPACH K (1983). Chemistry and immunochemistry of bacterial lipopolysaccharides as cell wall antigens and endotoxins. Prog. Allergy 33, 9-39.

WESTPHAL O, LÜDERITZ O, RIETSCHEL E T H and GALANOS C (1981). Bacterial lipopolysaccharide and its lipid A component: some historical and some current aspects. Biochem. Soc. Trans. 9, 191-195.

WILKINSON S G and REX M C (1983). Structural studies of the O-specific polysaccharide from Serratia marcescens NCTC 1377. Carbohydr. Res. 112, 95-103.

WILKOWSKE C J, WASHINGTON II J A, MARTIN W J and RITTS R E (1970). Serratia marcescens: biochemical characteristics, antibiotic susceptibility patterns and clinical significance. JAMA 214, 2157-2162.

WILLIAMS P, BROWN M R W and LAMBERT P A (1984). Effect of iron deprivation on the production of siderophores and outer membrane proteins in Klebsiella aerogenes. J. Gen. Microbiol. 130, 2357-2365.

WILLIAMS P, LAMBERT P A, BROWN M R W and JONES R J (1983). The role of the O and K antigens in determining the resistance of Klebsiella aerogenes to serum killing and phagocytosis. J. Gen. Microbiol. 129, 2181-2191.

WILLIAMS P, LAMBERT P A, HAIGH C G and BROWN M R W (1986). The influence of the O and K antigens of Klebsiella aerogenes on surface hydrophobicity and susceptibility to phagocytosis and antimicrobial agents. J. Med. Microbiol. 21, 125-132.

WILSON M E and MORRISON D C (1982). Evidence for different requirements in physical state for the interaction of lipopolysaccharides with the classical and alternative pathways of complement. Eur. J. Biochem. 128, 137-141.

WINKELSTEIN J A (1983). Complement and natural immunity. Clin. Immunol. Allergy 3, 421-439.

WINKELSTEIN J A and SHIN H S (1974). The role of immunoglobulin in the interaction of pneumococci and the properdin pathway: evidence for its specificity and lack of requirement for the Fc portion of the molecule. J. Immunol. 112, 1635-1642.

WINKLER U, HELLER K B and FOLLE B (1978). Pleiotropic consequences of mutations towards antibiotic-hypersensitivity in Serratia marcescens. Arch. Microbiol. 116, 259-268.

WINSHELL E and NEU H (1974). Relation of cell wall lipid content of Serratia marcescens to resistance to antimicrobial agents. Antimicrob. Agents Chemother. 6, 73-75.

WORSHAM P L and KONISKY J (1984). Effect of growth temperature on the acquisition of iron by Salmonella typhimurium and Escherichia coli. J. Bacteriol. 158, 163-168.

WRETLIND B, BECKER K and HAAS D (1985). IncP-1 R plasmids decrease the serum resistance and the virulence of Pseudomonas aeruginosa. J. Gen. Microbiol. 131, 2701-2704.

YAMAMOTO T, ARIYOSHI A and AMAKO K (1985). Fimbria-mediated adherence of Serratia marcescens strains US5 to human urinary bladder surface. Microbiol. Immunol. 29, 677-681.

YANCEY R J, BREEDING S A L and LANKFORD C E (1979). Enterochelin (enterobactin): virulence factor for Salmonella typhimurium. Infect. Immun. 24, 174-180.

YONEMURA K, MATSUMOTO K and MAEDA H (1983). Isolation and characterisation of nucleases from a clinical isolate of Serratia marcescens kums 3958. J. Biochem. 93, 1287-1295.

YOUNG L S, STEVENS P and INGRAM J (1975). Functional role of antibody against 'core' glycolipid of Enterobacteriaceae. J. Clin. Invest. 56, 850-861.

YOUNG V M, MOODY M R and MORRIS M J (1980). Distribution of Serratia marcescens serotypes in cancer patients. J. Med. Microbiol. 13, 333-339.

YOUNG V M, MOODY M R and MORRIS M J (1980a). Phase variation in the genus Serratia. J. Med. Microbiol. 13, 341-343.

YOUNG V M and MORRIS M J (1980). The antigenic structure of Serratia marcescens. In: The Genus Serratia (von Graevenitz A and Rubin S J, Eds.). CRC Press Inc, Boca Raton, Florida, pp17-30.

YU V L (1979). Serratia marcescens. Historical perspective and clinical review. N. Engl. J. Med. 300, 887-893.

YU V L, OAKES C A, AXNICK K J and MERIGAN T C (1979). Patient factors contributing to the emergence of gentamicin-resistant Serratia marcescens. Am. J. Med. 66, 468-472.