Erwinia carotovora : Lipopolysaccharides and Serogroups.

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CONTENTS.

PUBLICATIONS

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In memory of my late grandparents.

"Endotoxins possess an intrinsic fascination that is nothing less than fabulous. They seem to have been endowed by Nature with virtues and vices in the exact and glamorous proportions needed to render them irresistible to any investigator who comes to know them".

I.L. Bennett (1964)

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Abbreviations

a.m.u. :	atomic mass units
DAFS :	Department of Agriculture and Fisheries for Scotland
DNA :	deoxyribonucleic acid
DTT :	dithiothreitol
ECA :	Enterobacterial common antigen
Eca :	*Erwinia carotovora* subsp. *atroseptica*
Ecb :	*Erwinia carotovora* subsp. *betavasculorum*
Ecc :	*Erwinia carotovora* subsp. *carotovora*
EDTA :	ethylenediaminetetraacetate
ELISA :	enzyme-linked immuno sorbent assay
GLC :	gas liquid chromatography
GLC-MS:	gas liquid chromatography-mass spectrometry
HRP :	horse radish peroxidase
KDO :	2-keto-3-mannooctulosonic acid
LPS :	lipopolysaccharide
Mr :	molecular weight
OMP :	outer membrane protein
PAGE :	polyacrylamide gel electrophoresis
SCRI :	Scottish Crop Research Institute
SDS :	sodium dodecyl sulphate
TEMED :	N,N,N',N'-tetramethylethylenediamine
Tris :	Tris (hydroxymethyl) aminomethane

SUMMARY

SUMMARY

This research on the surface antigens of the plant pathogenic bacterium, Erwinia carotovora, concentrated on the characterisation of the lipopolysaccharide as a serogroup determinant.

1. Different methods for the extraction of the lipopolysaccharide component from E. carotovora bacteria were compared. The phenol/water method was shown to be the most reproducible method of E. carotovora lipopolysaccharide extraction and also gave a preparation of sufficient purity for further studies.
2. Electrophoresis of E. carotovora lipopolysaccharides using SDS-PAGE followed by silver staining was found to be a relatively inexpensive, easy to use and reproducible system capable of differentiating lipopolysaccharides from many serogroups. Variations on this basic system of SDS-PAGE were tried but were found to be unsatisfactory for the examination of lipopolysaccharide structure.
3. SDS-PAGE analyses gave information about E. carotovora lipopolysaccharide structure. E. carotovora lipopolysaccharides gave a regular banding pattern up the gel. This pattern was

different to that observed for Escherichia coli lipopolysaccharides and Salmonella lipopolysaccharides, the most studied members of the family Enterobacteriaceae with respect to their lipopolysaccharide component. By comparison, E. carotovora lipopolysaccharides lacked the very high molecular weight components observed for E. coli and Salmonella lipopolysaccharides.

4. Comparisons of E. carotovora lipopolysaccharide structure using this electrophoretic technique within and between serogroups were made. A direct relationship was observed between lipopolysaccharide SDS-PAGE profile and serogroup of strain from which the lipopolysaccharide was extracted. Lipopolysaccharides extracted from serogroups I, IV, XII, XVIII (Ecc), XX, XXII, XXV, XXVIII, XXIX, XXXIII, XXXIV and XXXV all gave different electrophoretic patterns. Lipopolysaccharides extracted from the same serogroup were found to produce the same electrophoretic pattern. Serogroups I, XVIII (Eca and Ecc), XX, XXII and XXXV were examined in some detail. Clearly, the serogroups of E. carotovora appeared to be based on the lipopolysaccharide antigens, supporting previous findings of De Boer et al (1985). The serotyping systems of E. coli and Salmonella are also based on lipopolysaccharide antigens.

5. E. carotovora lipopolysaccharide electrophoretic behaviour was compared to that of lipopoly-saccharides from other plant pathogenic Erwinia species and also with several commonly found gram-negative soil organisms. The lipopolysaccharide from E. carotovora bacteria possessed a different structure to all those examined in this study.
6. Using lipopolysaccharide SDS-PAGE as a method of serogroup analysis, strains that had been wrongly serotyped were identified and in several cases, on the basis of this electrophoretic method could be assigned to the correct serogroup. This reassignment was confirmed by immunological tests.
7. Lipopolysaccharide SDS-PAGE appeared to be a viable method for identifying serogroups of E. carotovora strains and applicable to diseased potato tissue. The system possesses several advantages over the present system of immunodiffusion used by De Boer for the serotyping of E. carotovora strains.
8. Of the forty or so serogroups of E. carotovora identified to date, De Boer has identified four serogroups (I, XVIII, XX and XXII) which are associated with potato pathogenic strains of E. carotovora subsp. atroseptica. In the temperate climate of Scotland, it is also the atroseptica subspecies which is responsible for the production

of blackleg in the potato plant and tuber.

SDS-PAGE analysis of lipopolysaccharides from atroseptica strains belonging to these four serogroups produced only three patterns: serogroup XVIII (Eca) and serogroup XX lipopolysaccharides gave the same pattern.

9. Reciprocal lipopolysaccharide immunoblots developed with antiserum raised against whole cells from representative strains belonging to the four potato pathogenic serogroups determined there to be only three different immunological groups (I, XX and XXII)

10. Absorptions of polyclonal E. carotovora antisera with lipopolysaccharide identified that lipopoly-saccharide from serogroup I and serogroup XXII atroseptica strains had epitopes in common. Lipopolysaccharide from serogroup XVIII and XX atroseptica strains were immunologically identical. Conceivably, monoclonal antibodies could be raised against the common epitopes from these two groups and used in a dipstick test for the detection of the blackleg organism. This dipstick would have the potential for detecting all potato pathogenic strains of atroseptica in Scotland.

11. Sugar analyses were performed on the O-chain fraction of lipopolysaccharide from the four potato pathogenic serogroups of Eca. The respective

alditol acetates of the constituent monosaccharides of the lipopolysaccharide O-chain were produced and analysed by GLC and/or GLC-MS.

12. The lipopolysaccharides of Erwinia carotovora subsp. atroseptica have structures based upon the sugars rhamnose, mannose, galactose and glucose. Unidentified sugars were found to be present in the GLC profiles of alditol acetates of Erwinia carotovora subsp. atroseptica lipopolysaccharides. They could not be identified from mass spectral fragmentation patterns for known sugars. Overall, the compositions determined by this method did not differentiate all serogroups.

(continued on next page)

13. Although a correlation between sugar composition and all serogroups was not found, sugar analyses did correlate with the three groupings (I, XX and XXII) observed for lipopolysaccharides from the four potato pathogenic serogroups of Erwinia carotovora subsp. atroseptica in SDS-PAGE and immunological analyses.

E. carotovora subsp. atroseptica strains:

	O-chain sugars					
Serogroup	Rh	Fuc	Rib	Man	Gal	Glc
I	+	+	+	+	+	+
XVIII	+	-	+	-	+	+
XX	+	-	+	-	+	+
XXII	+	-	-	-	-	+

\+ : sugar detected
\- : sugar not detected
Rh : rhamnose
Fuc: fucose
Rib: ribose
Man: mannose
Gal: galactose
Glc: glucose

INTRODUCTION

1. An Introduction To Plant Disease

After the realisation over one hundred years ago that certain microorganisms were responsible for the cause and effect of plant disease there has been a concerted effort to understand and thus control the diseases of plants. One of the most notable instances in our history was the Irish potato famine which was responsible for the deaths of over one million people. The fungus Phytophthora infestans was later identified as the causative agent responsible for the failure of the potato crop. Fungi, bacteria, viruses and nematodes have all since been found to constitute the major group of causative agents responsible for the diseases of plants. Insects, slugs, snails and animals can act as vectors for the transmission of these pathogens. Extreme environmental conditions such as drought and frost are also detrimental to plant health.

Awareness of the causes of plant disease and its transmission has led to its better management and improved agricultural practices. To try to prevent the initial infection of plants, plants are bred to produce resistant cultivars. A more modern, alternative approach is to use recombinant DNA techniques to produce transgenic plants. However, this fight to overcome plant disease will always be a continual process as pathogens develop new ways of overcoming the resistances

of the new cultivars. Methods for the prevention of the spread of the pathogens are therefore of equal importance. To successfully prevent the spread of the different pathogens, the manner in which they are transmitted and cause their pathogenic effects must be understood. The problem with fungal pathogens is that they produce spores which can be dispersed over great distances and survive in the soil for long periods of time e.g. Synchitrium endobioticum, the fungus responsible for potato wart disease can survive for up to thirty years in the soil (Hampson, 1975). Bacteria can be transmitted by water as they are motile, by insects, nematodes and animals. Certain species can remain viable in the soil for several years as a potential source of inoculum. Viruses are totally dependent on vectors for their spread. Insects are the major group of viral vector but fungi and nematodes also act as transmission vectors. Many of the pathogens produce extracellular enzymes which degrade the plant cell wall (Figure 1.1) and can ultimately lead to the death of the plant. The bacterium Erwinia carotovora is a classical example of such a pathogen (Basham & Bateman, 1975). Some of the pathogens can also produce phytotoxins of which amylovorin is one example. Amylovorin is produced by Erwinia amylovora, the fire-blight pathogen of pears and apples, and causes its pathogenic effect by blocking the vascular tissue of the plant (Goodman et al, 1974).

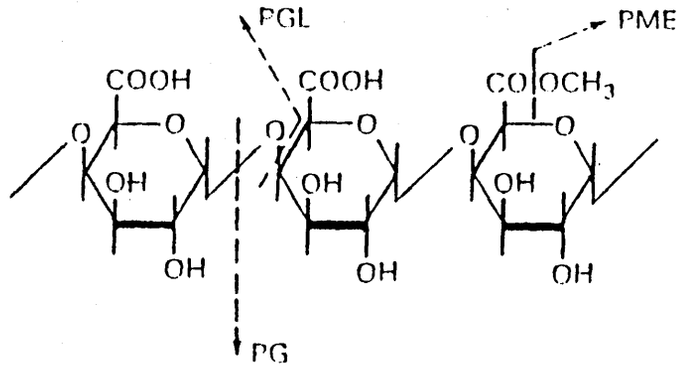


Figure 1.1. Cleavage of a representative plant cell wall polysaccharide by several different pectic enzymes: endo-polygalacturonase(PG), endo-polygalacturonide lyase(PGL), and pectin methylesterase(PME); the plant cell wall consisting mainly of polysaccharide (Cooper,1984).

2. The Genus Erwinia

2.1 Taxonomy of Erwinia

The genus Erwinia was named after the phyto-bacteriologist Erwin Frank Smith. Originally the genus was created for the classification of all gram-negative, peritrichously flagellated, plant pathogenic bacteria (Winslow et al., 1917) in the family Enterobacteriaceae (Cowan, 1974). However, since its inception in 1917 there has been much discussion about such a broad definition for the genus (Starr and Chatterjee, 1972). In an attempt to clarify the taxonomy, Waldee (1945) proposed the creation of two genera from this one genus. According to this proposal Erwinia species that cause dry necrosis (discoloured, dead plant tissue) or wilt symptoms on plants would constitute one genus. This genus would be called Erwinia, the type species being Erwinia amylovora. The other genus would contain those Erwinia species that produce pectolytic enzymes causing soft rot symptoms on plants and would be called Pectobacterium. This proposal received some support (Graham, 1964) but it has the disadvantage that there is considerable DNA homology between the two proposed genera which would argue against the separation of the bacteria into two distinct groups. Presently the genus is considered to consist of four groups based on the taxonomic studies of Dye (1968, 1969a,b,c). These

groupings may not be the best method of classification for the genus Erwinia but as yet there is no agreed alternative system of classification (Starr, 1981). The first of Dye's groups (1969a) contains those Erwinia species which produce extracellular pectic enzymes that degrade the cell walls of plants. This group is known by a variety of different names: the carotovora group, the soft rot group, or the pectolytic group. Erwinia carotovora belongs to this group of organisms.

The second of Dye's groups (1969b) consists of those Erwinia species that are yellow-pigmented of which Erwinia herbicola is an example. They are plant pathogens and plant epiphytes, and certain strains can also be found as opportunistic pathogens of man and animals. This latter group are also referred to as Enterobacter agglomerans (Ewing and Fyfe, 1972) or human clinical erwinias.

Those Erwinia species which cause dry necrotic symptoms or wilt diseases of plants constitute the third of Dye's groups (1968). Erwinia amylovora is the type species for this group and consequently the group is sometimes referred to as the amylovora group. Other names include white nonpectolytic erwinia, or the true erwinias.

The fourth and final group created from Dye's study of the genus Erwinia contains all those erwinias which do not qualify for classification into the previous three groupings (Dye, 1969c).

2.2 Taxonomy of E. carotovora subsp. carotovora and subsp. atroseptica

The soft rot erwinias are all facultative anaerobes, peritrichously flagellated, motile, gram-negative rods. They are found to be pathogenic to a wide variety of different plants e.g. carrot and chinese cabbage (E. carotovora subsp. carotovora), potato plant and tuber (E. carotovora subsp. atroseptica), sugar beet (E. carotovora subsp. betavasculorum) and Saintpaulia plants (E. chrysanthemi). The derivation of the species name can be seen as it was the practice in the early days of the taxonomy of the genus Erwinia to name the organism after the plant from which it was isolated. However, as more Erwinia organisms were isolated, some not being very plant specific, the naming system gave rise to many different species and subspecies.

Early tests used as the basis of the classification of Erwinia were based on phenotype as detected in growth tests and the phenotypic characteristics of the soft rot group have been extensively studied by Graham (1971). With recent advances, techniques based on DNA or RNA homologies have given more accurate taxonomic relationships within and between genera. Within the soft rot group there are two distinct species:

E. carotovora and E. chrysanthemi, and they share between 32% and 42% DNA homology (Brenner et al., 1973). There are three subspecies of E. carotovora: subsp. carotovora, subsp. atroseptica and subsp. betavasculorum (Skerman et al., 1980). These designations are based on phenotypic characteristics and the significance of them at the genotypic level is unclear (Verdonck et al., 1987). However, Brenner et al. (1973) have found that E. carotovora subsp. carotovora and subsp. atroseptica share between 83% to 92% DNA homology qualifying their subspecies designations (Wayne et al., 1987).

2.2.1 E. Carotovora and Potato Disease

The importance of the subspecies of E. carotovora as pathogens is dependent on environmental factors such as temperature and rainfall. In the temperate climate of Scotland, E. carotovora subsp. atroseptica is the main causative organism of both the soft rot of the potato plant (Perombelon, 1973) and of the potato tuber in storage (Graham, 1969). However, at temperatures above 22°C, E. carotovora subsp. carotovora is responsible for rotting potato tubers (Perombelon, 1979). E. chrysanthemi is responsible for the soft rot of potato tubers in warmer climates. The soft rot of the potato plant is commonly referred to as blackleg and the disease in its advanced stages causes a blackening of the stem. Rainfall plays a contributory role in the

dissemination of the soft rot organisms (Graham et al., 1977; Harrison, 1980) by producing an aerosol of the blackleg organisms from an infected plant which is then splashed onto uninfected plants, infecting them. In the past, before the introduction of better agricultural practices, heavy rainfall during the growing season could devastate a potato crop. Insects (Graham et al., 1976) and slugs (Dawkins et al., 1986) have also been identified as potential carriers of the blackleg organism. In Europe, transfer of the disease organisms by insects may be important but in Scotland this is not a major problem. This is one of the reasons for Scotland being a major grower of seed potato.

In storage, temperature, relative humidity, surface water and atmospheric conditions are all critical factors in the development of soft rot in the tuber (Lund, 1971). The source of the infecting organisms is the reservoir of ewinias found on the surface of the tuber and within the lenticels of the tuber (Perombelon, 1973). Surface washing of the tuber is therefore not an effective control measure as shown by Graham and Volcani (1961) and it may even encourage dispersal of the blackleg organisms. Fumigation with acetaldehyde has been shown in trials by the Department of Agriculture and Fisheries for Scotland (DAFS) to reduce the number of soft rot organisms on tubers and it is thought that this treatment is particularly effective

because the acetaldehyde vapour penetrates the lenticel spaces. Levels of the rot are lower in tubers stored in well aerated conditions. This reduction in disease is probably due to two factors. Firstly, control of humidity and secondly, an adequate supply of oxygen is necessary to promote those plant defence mechanisms which utilise oxygen requiring enzymatic conversions of inactive substrate to "active" compounds (Zubay, 1983). Further, Vayda and Schaeffer (1988) observed that under aerobic conditions the tuber rapidly synthesises five new proteins upon wounding. The proteins are thought to be glycolytic enzymes and other enzymes involved in carbohydrate metabolism. However, under anaerobic conditions, there is a three hour delay in the synthesis of these proteins after wounding which may reduce the tuber's defence mechanisms allowing the blackleg organism to establish itself. As Erwinia carotovora is a facultative anaerobe it is able to grow and rot the tuber at low oxygen tensions and there is evidence that Erwinia carotovora rots tubers faster under anaerobic conditions presumably because of the reduced effectiveness of the tuber's defence mechanisms (Perombelon and Lowe, 1975, Vayda and Schaeffer, 1988).

3. Blackleg and Soft Rot of Potato Tubers

3.1 Introduction

In Scotland, rainfall, insects, weeds (diseased) and contaminated farm machinery have all been shown to be reservoirs of the blackleg organism, E. carotovora subsp. atroseptica (McCarter-Zorner, et al., 1985). However, the incidence of blackleg in the potato plant is no longer the major problem it used to be due to improved agricultural practices and to the introduction of a programme for the production of virus- and bacteria-free nuclear stock plants. This programme was introduced by the Department of Agriculture, Food and Fisheries for Scotland in the early seventies. The method of micropropagation is used to generate these pathogen-free plants. Micropropagation involves removing a piece of meristem tissue from the root of the potato plant under sterile conditions as the meristem tissue should be pathogen-free. The tissue section is then grown up on medium in a small sterile vial. Once the tissue has grown, divided and developed roots it is then transplanted into the field (Tisserat, 1985). It is at this stage when the small pathogen - free plantlets are growing in the field that they can become contaminated with the blackleg organism.

The major problem of contamination is not in the potato plant but the seed tuber produced from it. At

harvest-time, the seed tubers can be free of symptom expression but be infected with low numbers of the blackleg organism. In storage, the pathogen will still continue to increase in number (Maher and Kelman, 1986) and only when the seed tubers are planted will the blackleg manifest itself in the plant. Inability to detect these organisms in the tuber has caused problems for the trade of potato seed tubers in this country and abroad. To control outbreaks of blackleg from contaminated seed potato, DAFS rigorously test for levels of the blackleg organism in their seed potato stock. All seed potato stocks are graded and issued with a certificate which attests to the quality of that stock as part of the DAFS Official Certification Scheme.

3.2 Pathogenicity Mechanism of Soft Rot.

The soft rotting group of Erwinia organisms produce a variety of different extracellular enzymes which degrade the plant cell wall. These are pectinases, proteolytic enzymes, cellulases and phospholipases (Bateman, 1976) of which the pectinases are the most important group. The pectinases break down the pectic substances of the plant cell wall. Pectate and pectin (methyl esterified pectate) constitute the pectic substances of the plant cell wall and are predominantly polymers of galacturonic acid (McNeil, et al., 1984). Specifically, the different pectic enzymes cleave the

α-1.4-glycosidic linkages of the galacturonic acid polymers. Of the various pectic enzymes, pectate lyase, pectin lyase, and exo-polygalacturonase cleave by β-elimination and generate products with a 4,5-unsaturated galacturonide residue at the non-reducing end. Polygalacturonase and exo-poly-α-D-galacturonase cleave by hydrolysis (see review by Collmer and Keen, 1986a). The pectic enzymes are not directly pathogenic but initiate a sequence of events which if unchecked lead to the death of the plant. Degradation of the plant cell wall by the pectic enzymes results in the inability of the cell wall to support the limiting membrane of the cell (Basham and Bateman, 1975). This is followed by the leakage of electrolytes from the cell and subsequent cell death.

Both E. chrysanthemi and E. carotovora species produce several different isozymes of pectate lyase. Five isozymes of pectate lyase have been identified for E. chrysanthemi and two, sometimes three, isozymes of pectate lyase have been identified for strains of E. carotovora subsp. carotovora and subsp. atroseptica (for review see Koutojansky, 1987). The involvement of pectate lyase in the mechanism of pathogenicity has been extensively demonstrated for E. chrysanthemi. Basham and Bateman (1975) treated tobacco tissue with purified pectate lyase from E. chrysanthemi and found that it caused maceration and cell death of the tissue.

Chatterjee and Starr (1977) found that E. chrysanthemi mutants defective in their production of pectate lyase were unable to cause tissue maceration. Further, it was found that by cloning the pectate lyase genes of E. chrysanthemi into E. coli, recombinant E. coli clones were produced which possessed a limited ability to macerate potato tuber tissue (Collmer et al., 1985).

3.3 Plant Defence Mechanisms

Plants possess a variety of defence mechanisms to protect themselves against potential pathogens and it is believed that these mechanisms all work together to this end (Bell, 1981). Under aerobic conditions in the potato tuber, the plant lays down suberin in the layer of cells under damaged tissue and also produces a barrier of oxidised phenolic compounds around the damaged tissue (Lund, 1971). This only occurs in aerobic conditions as the synthesis of suberin requires the production of hydroxy fatty acids and polyhydric alcohols, which involves hydroxylation reactions (Kolattukudy et al., 1976). Maher and Kelman (1983, 1986) proposed that under these aerobic conditions, the suberin may protect the tuber tissue against further maceration by the destructive pectic enzymes of E. carotovora. Further, the oxidised phenolics may not only provide a protective barrier against the enzymes but chemically modify them, effectively inactivating them.

In response to challenge by a potential pathogen plants synthesise their own antibiotics. These antimicrobial compounds are called phytoalexins and are synthesised by the plant after exposure to fungal and bacterial pathogens and accumulate at the site of injury (for review see Ebel, 1986). They belong to groups of natural compounds such as phenylpropanoids, terpenoids and fatty acid derivatives. The synthesis of phytoalexins has been shown to be induced in a number of different ways. Keen et al. 1983 (Keen and Yoshikawa, 1983), have shown that an endo-β-glucanase from soybean cotyledons will hydrolyse carbohydrate fragments from the fungal cell walls of Phytophthora megasperma which in turn elicit the synthesis of phytoalexins. The bacterium E. carotovora produces extracellular pectic enzymes which release carbohydrate fragments from the plant cell walls of soybeans. These carbohydrate fragments stimulate the synthesis of phytoalexins (Davis et al., 1984). Also, when plants are damaged they may supply an enzyme which releases carbohydrate fragments from its own cell walls. These fragments in turn induce the synthesis of phytoalexins (Albersheim and Darvill, 1985). The carbohydrate fragments are termed elicitors as they elicit the synthesis of phytoalexins. Although the exact nature of the carbohydrate fragments is still being investigated, Darvill and Albersheim (1984)

defined the minimum carbohydrate structure of the elicitor produced from the fungal cell wall of Phytophthora megasperma, necessary for the induction of phytoalexin synthesis in the soybean plant. It is a hepta-β-glucoside fragment. The effectiveness of this type of defence mechanism depends on the speed of synthesis and accumulation of phytoalexin at the site of injury. Plants have also been found to possess cell wall proteins which are specifically induced upon challenge from a potential pathogen and are believed to have a defensive function (Bohlmann et al 1988).

Resistance to pathogens is induced in plants after initial exposure to the phytopathogen. There are two types of induced resistance. One is a localised form of resistance which is produced at the site of the invading pathogen in the host. The second type is a systemic resistance which acts at sites distant to the sites of interaction of plant and pathogen (for review see Sequeira, 1983). Graham et al. (1977) observed the induction of systemic resistance in the tobacco plant upon challenge by the phytopathogen Pseudomonas solanacearum. Rough lipopolysaccharide (i.e. lipopolysaccharide without attached O-chain, see Figure 1.3) was identified as the inducer of this type of resistance. More recently, Schoonejans and Expert (1987) identified a similar inducer in E. chrysanthemi. They found upon challenging Saintpaulia plants with

lipopolysaccharide rough mutants of E. chrysanthemi, systemic resistance to the wild type strain was induced twelve hours after exposure to the mutant strains. Earlier, Mazzucchi and Pupillo (1976) isolated a protein-lipopolysaccharide complex from E. chrysanthemi. This complex prevented the induction of a hypersensitive response when used to pre-treat tobacco leaves prior to infection with an avirulent strain of Ps. syringae. A hypersensitive response is a localised type of induced resistance. It is induced in plants by plant pathogenic bacteria in incompatible host plants or by avirulent strains of the phytopathogens in their compatible host plant. The hypersensitive response results in plant tissue death in the area surrounding the pathogen which isolates the pathogen and may result in its death (Lozano and Sequeira, 1970). Whately et al. (1980) showed rough lipopolysaccharide from avirulent strains of Ps. solanacearum also to be the inducer of the hypersensitive response in the tobacco plant. Rough lipopolysaccharide would therefore appear to be able to induce both forms of resistance in plants. There is also evidence for the involvement of protein and extracellular lipopolysaccharide in the mechanism of induced resistance caused by some plant pathogens (Hendrick and Sequeira, 1984; Drigues et al., 1985).

To try to overcome the defensive mechanisms of the plant, phytopathogens possess their own strategies. The

pathogen may detoxify the phytoalexins or prevent attainment of toxic levels of the phytoalexin, either by secreting phytotoxins or other toxic metabolites (see review by Turner, 1984).

4. Identification of Erwinia and the Role of Surface Antigens in Identification.

4.1 Introduction

Every year fungi, bacteria and viruses are responsible for the spoilage of millions of pounds worth of crops. To try to curb such losses, research into the identity, source and methods of eradication of the important phytopathogens is being carried out on a world scale.

The first step in the control of plant diseases is identification of the disease causing organism and sources of contamination. Present methods of identification for the blackleg organism involve time-consuming microbiological and biochemical tests (Graham, 1971). Immunological techniques developed in the past two decades as a result of the hybridoma technology of Kohler and Milstein (1970) have offered the agricultural industry the potential for detecting plant pathogens more rapidly and with greater sensitivity than existing methods (Gatz et al., 1983). Specifically for the detection of the blackleg organism in potato tubers, a postharvest test employing monoclonal antibodies to the blackleg organism was envisaged. This test would monitor levels of the blackleg organism in seed potato stocks. This line of research has been pursued by Agriculture-Canada,

Vancouver (De Boer and McNaughton, 1987), by DAFS, Scotland and at Wageningen, The Netherlands.

Although more labour intensive to produce than polyclonal antibodies, monoclonal antibodies have several advantages which make them more desirable for use in a diagnostic assay as envisaged above. Monoclonal antibodies are antibodies produced by one cell type of which there is potentially an infinite supply. All the antibodies produced from this one cell possess identical epitope specificities (for review see Tiffin, 1987). The screening procedure for a monoclonal antibody of the desired specificity is critically important. The monoclonal antibody must be screened against a range of possible cross-reactive antigens to ensure its uniqueness. Ghosh and Campbell (1986a) found that most instances of cross-reactive monoclonal antibodies were not true cross-reactions but assay dependent. Cross-reactions which were observed in an assay which did not employ wash steps, such as an agglutination assay, were not observed in an assay which did employ wash steps, such as an ELISA assay. They also found that the sensitivity of monoclonal antibodies to bacterial antigens was assay dependent (Ghosh and Campbell, 1986b). An ELISA assay was determined to be ten times more sensitive than an agglutination assay. These factors must be considered in the development of an appropriate diagnostic assay employing monoclonal

antibodies for the detection of any pathogen.

For the detection of plant pathogens, the practice is to raise monoclonal antibodies against the surface structures of the phytopathogen. The presence of the pathogen can then be tested for *in planta*. *Erwinia* are gram-negative bacteria and as such their major surface antigens are lipopolysaccharide and outer membrane protein (Hammond *et al.*, 1984). This should allow the use of either purified lipopolysaccharide, outer membrane protein or membrane fractions for the raising of monoclonal antibodies. Monoclonal antibodies can be raised against purified lipopolysaccharide (Gustaffsson *et al.*, 1982) but generally this is not done because of the associated problems of endotoxic shock for the animal (Milner *et al.*, 1971). Also, purified lipopolysaccharide is found to be less immunogenic than lipopolysaccharide complexed with protein in the membrane. Membrane fragments therefore tend to be used for the raising of monoclonal antibodies against phytopathogens. The antibodies raised can then be screened against the antigen or antigens of interest to obtain the monoclonal antibody of the desired specificity. De Boer and McNaughton (1987) using this approach screened against purified lipopolysaccharide from different serogroups of *E. carotovora* to obtain serogroup I-specific monoclonal antibody. Lin *et al.*, (1987) screened their monoclonal antibodies against

membrane fragments of E. amylovora as they were interested only in the general structures of the bacterial surface unique to E. amylovora.

Other systems of detection for Erwinia plant pathogens have also been developed. Moline et al. (1983) developed a system of two dimensional polyacrylamide gel electrophoresis of proteins for the identification of soft rotting bacteria. From the two dimensional SDS-PAGE profile produced from ribosome-enriched proteins, species of plant pathogenic bacteria could be identified. A very sensitive technique for identifying E. amylovora was developed by Falkenstein et al. (1988). E. amylovora was detected in bacterial colonies from field isolates by modification of Southern blotting techniques where the probe used was plasmid DNA carrying E. amylovora specific DNA sequences. In choosing which system of detection to use, its application must be considered. The methods of Moline et al. (1982) and Falkenstein et al. (1988) are both very sensitive techniques for use in the laboratory and better suited for confirmatory analyses. For routine analyses and use in the field, a diagnostic assay such as a dipstick based on monoclonal antibodies would be easiest to use (Dewey et al., 1989).

Once the plant pathogen has been identified and its importance assessed, the next step is its control. With recent advances in recombinant DNA technology there are

a variety of approaches that can be taken (Buck, 1989). One method is to take advantage of the natural competitive mechanism of bacteria in the phyloplane, and exploit the beneficial genes of one organism to the detriment of the pathogen. E. herbicola is frequently found on plant surfaces in niches similar to those of the fire-blight pathogen, E. amylovora (Hattingh et al., 1986) and it was found that certain strains of E. herbicola could limit the growth of E. amylovora (Beer et al., 1980). It was found that these strains of E. herbicola achieved this effect by producing antibiotics toxic to E. amylovora (Vanneste et al., 1987). Attempts are being made to improve this approach by identifying the genes responsible for antibiotic production and then manipulating them to produce more antibiotic or transferring them to a more abundant, harmless bacterium found on the plant of interest.

Another method of control of plant disease is the production of new cultivars upon which some type of resistance has been conferred. Traditionally this was done by breeding new varieties of plants with resistance to the disease to which the other varieties of the plant were susceptible (Day, 1986). As a result of advances in recombinant DNA techniques, new cultivars of plants can also be produced by the introduction into the plant cells of exogenous DNA encoding some form of resistance. There are several techniques available for

the production of these transgenic plants. One technique exploits the phytopathogen, Agrobacterium tumefaciens (Horsch et al, 1985). This bacterium causes the plant disease, crown gall (Lippincott and Lippincott, 1975). The infection produces tumours on the plant at the site of infection. Ti-plasmid DNA is transferred from the A. tumefaciens bacterial cell to the plant cell during the course of infection. Exogen ous DNA can be introduced into the Ti-plasmid which is then transferred to the plant cell. A segment of the Ti-plasmid then integrates into the plant DNA with the resultant production of new varieties of plant (Pua et al., 1987; Radke et al., 1988). Transgenic plants can also be produced by the technique of DNA segment hybridisation (Guangyu, 1988). This method introduces DNA from disease-resistant plants into disease-sensitive varieties via the pollen tube pathway of the plant directly into the nucleus of the egg cell. Electroporation is another method (Guerche et al., 1987). It entails using a pulse of electric current to open up channels in the membrane of the plant cell. Exogen ous DNA can then enter the plant cell through these channels and transform the plant DNA.

Genetic engineering can also be applied to the phytopathogen in an attempt to control plant disease. This approach involves the identification of those genes responsible for the pathogenicity of the organism. The

genes are then deleted or interrupted to prevent their expression. To obtain biological control over the wild type phytopathogen the host plants are treated with the genetically altered phytopathogen. This is to try and reduce the population size of the wild type strain by competition between the wild type pathogen and the mutant form of the pathogen for the same ecological niche. This strategy has been successfully applied to the bacterium, Ps. syringae. In its wild type form, Ps. syringae is the major organism responsible for frost damage to crops. The bacterium acts as a nucleation centre for the formation of ice crystals (Lindow et al., 1982). The genes responsible for this phenomenon were mutagenised and inactivated to produce ice-minus mutants (Lindow, 1987). The ice-minus forms of Ps. syringae were one of the first genetically engineered organisms to be tried outside the greenhouse in field trials (1987) with a certain degree of success. It is too soon to determine the effect these genetically engineered organisms may have on the environment. However, a common agricultural practice since the beginning of this century has been the inoculation of the seeds of leguminous plants with Rhizobium with no apparent adverse effect on the environment. Also, there is no guarantee that such engineered organisms which are viable in the laboratory will be able to compete with other organisms in planta and establish themselves.

With the ice-minus forms of Ps. syringae this has not appeared to be a significant problem as they were found to markedly reduce the population size of the wild type form (Lindow, 1987). Scanferlato et al. (1989) conducted a similar study. They compared the survival rates of a population of wild type E. carotovora with the population of a genetically engineered mutant derivative in an artificially constructed aqueous environment. Both the populations of the wild type form and the mutant form declined in this environment. Conclusions could therefore not be made as to which form was more successful at colonising this niche. However, the natural habitat of E. carotovora is not a simple laboratory aqueous environment but is to be associated with a variety of living and decaying plant material.

4.2 The Gram-Negative Bacterial Surface

4.2.1 Introduction

Bacteria can be classified as either gram-positive or gram-negative depending on whether or not they retain the Gram stain. This is due to differences in the structure of the cell wall of the two groups of bacteria. The cell wall of gram-positive organisms consists of a cytoplasmic membrane and a thick layer of peptidoglycan which is located to the exterior of the cytoplasmic membrane. The cell walls of gram-negative

bacteria differ from those of gram-positive bacteria in that they possess an additional membrane, the outer membrane, which is located to the exterior of a thin layer of peptidoglycan (Figure 1.2a). Erwinia, as are all members of the family Enterobacteriaceae, are gram-negative organisms.

4.2.2 Molecular Organisation of the Gram-Negative Cell Wall

The outer membrane of the gram-negative cell wall acts as a barrier to hydrophobic compounds such as certain hydrophobic dyes, antibiotics and detergents (Havekes et al. 1976), and acts as a molecular filter for hydrophilic compounds into the cell (Benz and Bauer, 1988). The outer membrane is an asymmetric bilayer composed of phospholipid, protein and lipopolysaccharide (Nikaido and Vaara, 1985). Shukla et al. (1980) have shown the outer leaflet of the lipid bilayer of E. carotovora cells contains 4% phosphatidylethanolamine and the inner leaflet contains 30% phosphatidyl-ethanolamine. Phosphatidylethanolamine constitutes 95% of the total phospholipid content of E. carotovora cells. Kamio and Nikaido (1977) made similar observations for the distribution of phospholipid in the outer membrane of S. typhimurium. In the same study, Shukla et al. (1980) determined the overall phospholipid content of the cytoplasmic membrane to be higher than

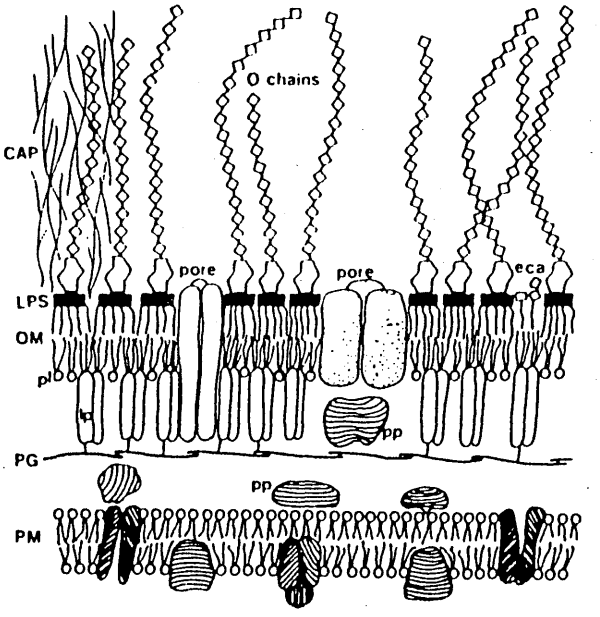
Figure 1.2

a. Gram-negative bacterial cell surface (Freer, 1985)

OM : outer membrane
LPS : lipopolysaccharide
pl : phospholipid
eca : enterobacterial common antigen
pore : porin protein
PG : peptidoglycan
lp : lipoprotein
pp : periplasmic protein
PM : plasma membrane
CAP : acidic polysaccharide capsule.

b. Cell surface of the bacterium, E. carotovora (Shukla et al., 1980)

a.


O chains
CAP
pore
pore
eca
LPS
OM
pl
PG
pp
pp
PM

b.

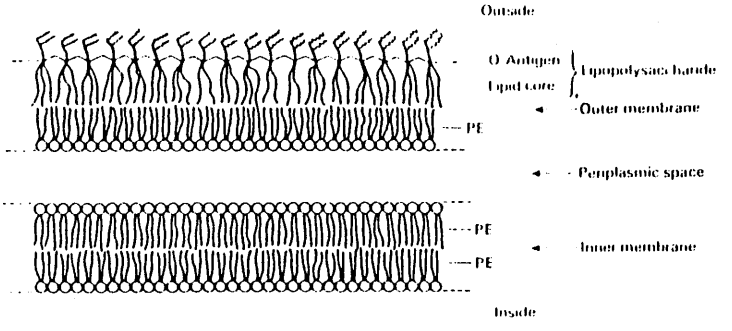

Outside
O Antigen
Lipopolysaccharide
Lipid Core
Outer membrane
PE
Periplasmic space
PE
PE
Inner membrane
Inside

Figure 1.2.

that of the outer membrane, 65% and 34%, respectively. This was consistent with the cytoplasmic membrane being more fluid than the outer membrane. From these observations, Shukla et al. (1980) proposed a model for the molecular organisation of the cell wall of E. carotovora (Figure 1.2b).

From studies of E. coli and S. typhimurium, the most investigated members of the family, Enterobacteriaceae, the outer membrane has been shown to be less abundant in protein and has little enzymic activity, as compared with the cytoplasmic membrane (Lugtenberg and Van Alphen, 1983; Nikaido and Vaara, 1985). An earlier study by Shukla et al. (1978) showed that 42% of the total membrane protein of E. carotovora cells was located in the outer membrane and the remaining 58% in the cytoplasmic membrane. The porin proteins are organised to form pores in the outer membrane and are found complexed with lipopolysaccharide (Osborn and Wu, 1980). Such protein-lipopolysaccharide complexes have been found to act as receptors for the attachment of certain bacteriophages (Datta et al., 1977).

Lipopolysaccharide is unique to gram-negative organisms and the majority of lipopolysaccharide is found attached to the outer membrane. About 45% of the bacterial surface area is covered with lipopolysaccharide. As it is an amphiphilic molecule,

the lipopolysaccharide is anchored in the outer leaflet of the lipid bilayer by the hydrophobic interaction and/or ionic interaction of its lipid moiety with the lipid component of Braun's lipoprotein (Freer and Salton, 1971). The hydrophilic sugar chain of the molecule projects outwards from the surface of the cell. Lipopolysaccharide is found complexed in the membrane with other lipopolysaccharide molecules and with protein. The lipopolysaccharide molecules are complexed together by divalent cations, Ca^{2+} and Mg^{2+} (Galanos and Luderitz, 1975). Lipopolysaccharide is therefore amenable to extraction by the chelating agent, EDTA (Leive, 1965).

Studies have shown that the organisation of phospholipid, protein and lipopolysaccharide in the outer membrane can change. It has been observed for S. typhimurium lipopolysaccharide mutants lacking O-chain and part of the core region from their lipopoly-saccharide molecules (see Figure 1.3), that the outer leaflet of the lipid bilayer contains greatly increased amounts of phospholipid and decreased amounts of protein (Gmeiner and Schlecht, 1979; Smit et al., 1975). Such a reorganisation would alter the hydrophilicity of the outer membrane making it overall more hydrophobic in nature. Gmeiner and Schlecht (1979) proposed that this increased hydrophobicity would account for the increased permeability of the outer membrane to certain

hydrophobic dyes, antibiotics and detergents.

Between the cytoplasmic and outer membranes is the peptidoglycan and associated periplasm (Figure 1.2a). The peptidoglycan is a bacterial polymer covalently attached to the outer membrane by Braun's lipoprotein (Freer and Salton, 1971). Peptidoglycan consists of alternating residues of N-acetylglucosamine and N-acetylmuramic acid. A small peptide consisting of four amino acids is attached to the N-acetylmuramic acid residue. One of the amino acids is a diamino acid, m-diaminopimelic acid. In most gram-negative organisms, it is this amino acid which directly crosslinks the peptide sidechains in the peptidoglycan (Schleifer and Kandler, 1972). This crosslinked matrix gives mechanical strength and rigidity to the bacterial cell wall. Associated with the peptidoglycan is the periplasm. The periplasm contains peripheral proteins of the cytoplasmic membrane and periplasmic proteins. There are three different types of periplasmic protein. There are those proteins involved in the binding of sugars, amino acids and ions, there are those involved in detoxifying compounds toxic to the cell, and there are those responsible for converting solutes to a form suitable for transport into the cell (Nossel and Heppel, 1966). From electron microscopic studies of the cell wall of *E. coli* B, Hobot *et al.* (1984) have proposed a model for peptidoglycan and the associated periplasm.

They envisage the peptidoglycan to span the intracellular space between the inner and outer membrane as a periplasmic gel. This gel has a high water content and contains large pores in which periplasmic proteins could conceivably freely diffuse. They found the peptidoglycan to be more crosslinked at the outer membrane surface than the cytoplasmic surface which would account for the gap i.e. the periplasmic space, observed in electron micrographs.

The cytoplasmic membrane unlike the outer membrane consists of a bilayer of simple phospholipids (Figure 1.2a). It acts as a permeability barrier to the cell exerting control over the entry of solutes in and out of the cell. The cytoplasmic membrane contains the electron transport system proteins and is involved in the synthesis of the outer cell wall components, phospholipids, proteins, lipopolysaccharides and peptidoglycan. The outer membrane and cytoplasmic membrane are joined at several sites over the membrane. These sites are called sites of adhesion. They have been identified as sites for the export of newly synthesised lipopolysaccharide (Bayer et al., 1982; Muhlradt and Golecki, 1975), binding of certain phages for the introduction of their DNA into the host cell, and possibly for the translocation of outer membrane proteins (Datta et al., 1977).

4.2.3 Major Surface Antigens of Gram-negative Bacteria

Of the components of the outer membrane of gram-negative bacteria (Introduction 4.2.2) only the lipopolysaccharide and protein elicit an immune response and as such are regarded as surface antigens.

4.2.3.1.1 Lipopolysaccharide structure

In wild type strains of gram-negative bacteria lipopolysaccharide is composed of three different regions, each region possessing its own individual biological function (Luderitz et al., 1966). The three regions: lipid A, core and O-chain are all covalently linked (Figure 1.3). Smooth (wild type) strains of gram-negative bacteria are considered to possess a complete core region and attached O-chain. Rough strains do not possess O-chain and may not possess all the sugars of the core region (Luderitz et al., 1966). The terms rough and smooth are derived from their respective colony morphologies. Smooth strains can be differentiated from rough strains not only from their colony morphologies but also from their inability to autoagglutinate in 3.5% sodium chloride solution or 0.3% auramine solution (Luderitz et al., 1971).

4.2.3.1.1.1 Lipid A

The lipid A component of Enterobacteriaceae has been shown to consist of two glucosamine residues which are β-1-6'-linked (Gmeiner et al., 1969). This

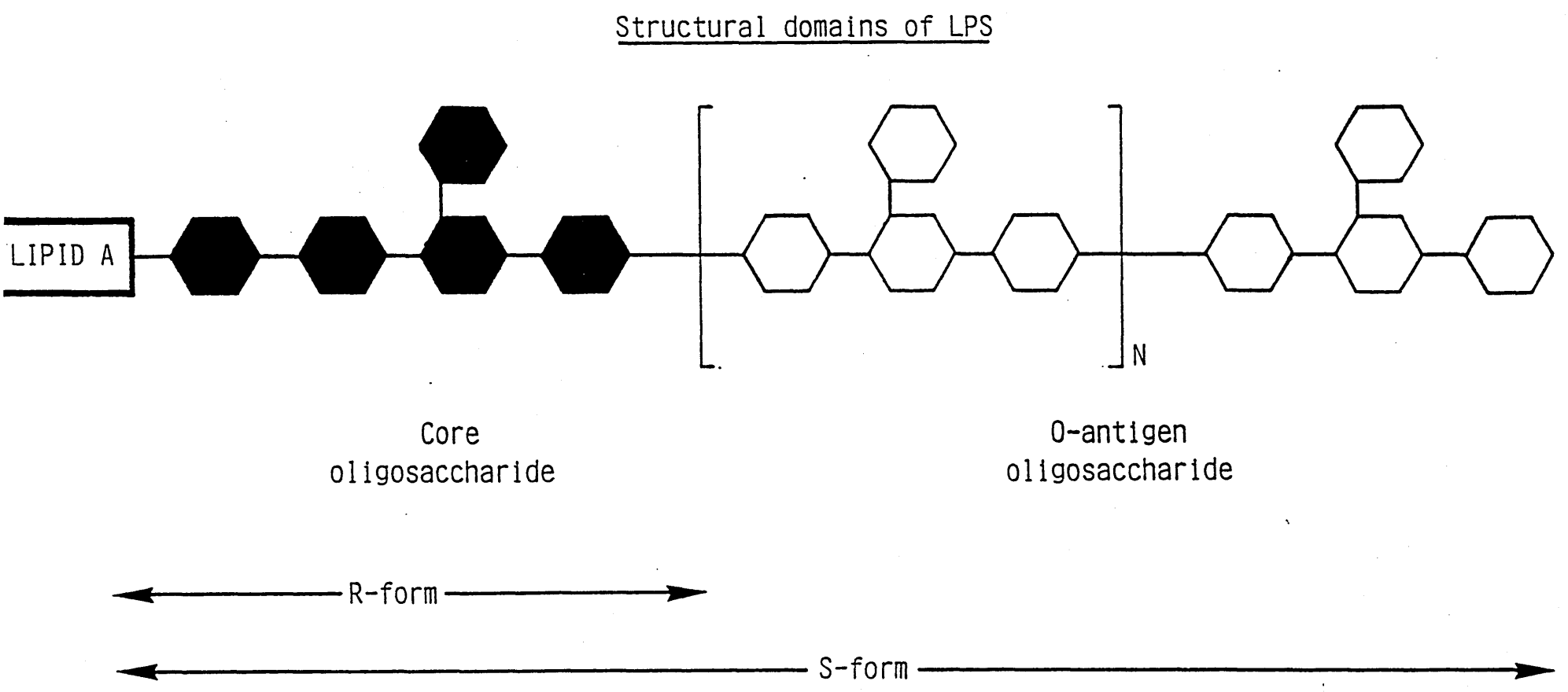

Structural domains of LPS
LIPID A
N
Core
oligosaccharide
O-antigen
oligosaccharide
R-form
S-form

Figure 1.3.

structure is generally referred to as the diglucosamine backbone of the lipid A molecule (Figure 1.4a). Long chain fatty acids are bound to the diglucosamine backbone through ester and amide linkages to the hydroxyl and amino groups of the backbone. Typically, the types of fatty acid found are myristate, palmitate and laurate (for review see Takayama and Qureshi, 1986). The major fatty acids are characteristically D-3-hydroxy fatty acids and they enter into linkage with the backbone through the amino groups exclusively (see review Rietschel et al., 1977). They are not normally found in the other lipids present in gram-negative cell walls (Rietschel, 1976). However, studies of the lipid A component from E. coli (Van Alphen et al., 1979) and Salmonella (Wollenweber et al., 1983) have found the fatty acid composition to be dependent on the temperature of growth.

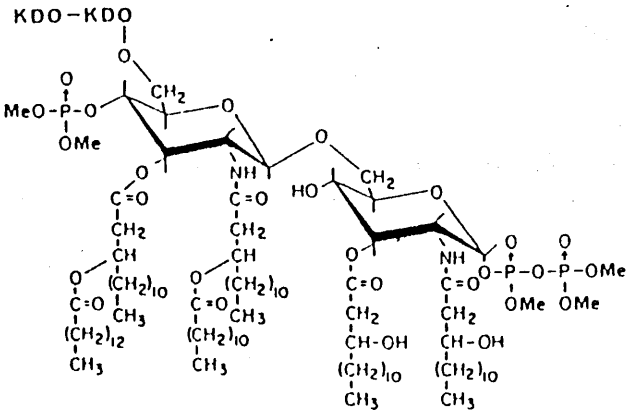
The third component of the enteric lipid A molecule is phosphate. C-1 and C-4' of the diglucosamine backbone are substituted with phosphate groups. Rosner et al. (1979c) identified two populations of lipopolysaccharide molecules in one preparation of lipopolysaccharide from an E. coli K12 heptoseless mutant. The two populations differed in phosphate substitution at the C-1 position of the diglucosamine backbone as one population was substituted with a monophosphate group and the other with a pyrophosphate

Figure 1.4

a. Structure of the lipid A moiety of lipopoly-saccharide extracted from the Re mutant of *E. coli* (Cotter *et al.*, 1987).

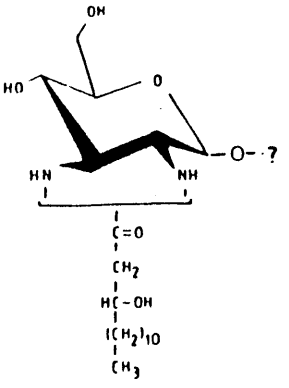
b. Different types of lipid A with 2,3-diamino-2,3-dideoxy-D-glucose replacing the backbone sugar D-glucosamine (Weckesser and Mayer, 1988).

a.

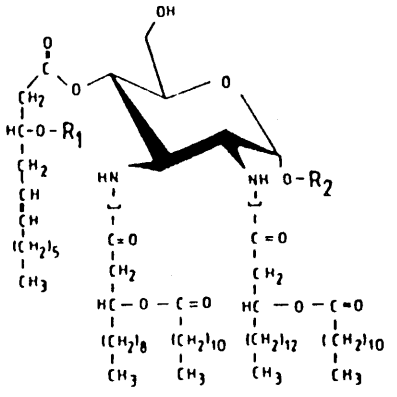


b.

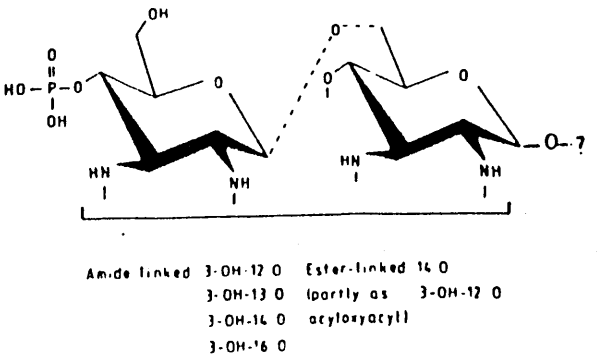
1.



2.



3.



Amide linked 3-OH-12 0 Ester-linked 14 0
3-OH-13 0 (partly as 3-OH-12 0
3-OH-14 0 acyloxyacyl)
3-OH-16 0

Figure 1.4.

group. The ratio of one population to the other was found to be dependent upon the phosphate concentration in the growth medium. It was previously thought that the lipid A molecules in the outer membrane were linked through their phosphate groups by phosphodiester bonds (Nowotny, 1961) but Rosner et al. (1979b) have shown there to be no phosphodiester linkages and that lipopolysaccharide molecules exist as monomeric entities. The phosphate groups themselves can also be further substituted. Some strains of Salmonella are found to have their phosphate groups substituted with the sugar, 4-amino-arabinose (Vaara et al., 1981). Apart from substitution of the phosphate groups, all other components of the enteric lipid A molecule appear to be the same (Rosner et al., 1979a).

The lipid A as described for the Enterobacteriaceae (Figure 1.4a) is only one form of lipid A. There is another form. In this lipid A the diglucosamine backbone of enteric lipid A is replaced by 2,3-diamino-2,3-dideoxy-D-glucose (Roppel et al., 1975) of which there are several types (Figure 1.4b). Some species of bacteria e.g. Bacillus and Thiobacillus, are found to contain both types of lipid A. As yet it still has to be determined whether these two types of lipid A exist as two distinct forms in a lipopolysaccharide population or as hybrids of D-glucosamine and 2,3-diamino-2,2-dideoxyglucose.

As the lipid A moiety would appear to be highly conserved within families of bacteria, it is of taxonomic interest and has been shown to clarify classification by 16S rRNA cataloguing (see review by Weckesser and Mayer, 1988). Another consequence of its conserved structure is the possible use of it or derivatives of it (Ribi et al., 1986) as a vaccine against gram-negative bacterial infections (Galanos et al., 1977). However, it is the lipid A moiety that is responsible for the toxic properties of lipopolysaccharide. It is called endotoxin and has a variety of biological activities. It is a pyrogen, activates B-lymphocytes, macrophages and can cause tumour necrosis (Milner et al., 1971). It was determined to be the toxic component of lipopoly-saccharide by comparing the activity of natural lipid A from E. coli and synthetic forms (Galanos et al., 1985). The activities of the two forms were identical.

4.2.3.1.1.2 Core region

The core region of the lipopolysaccharide molecule (Figure 1.3) is linked to the lipid A moiety through linkage with the sugar, 2-deoxy-3-manno-octulosonic acid (KDO). KDO is a rare, eight carbon sugar which is linked to the lipid A moiety through glycosidic linkage with the C-6' of the diglucosamine backbone of Enterobacteriaceae (Cotter et al., 1987). The core

region can be divided into two sub-regions, inner and outer, of which KDO is one of two sugars characteristic of the inner core. The other sugar is L-glycero-D-mannoheptose. Early studies of the presence of KDO in Salmonella and E. coli lipopolysaccharide revealed that there were three residues per lipopolysaccharide molecule. Two of the KDO residues were in the main sugar chain linked to the lipid A. The other residue was a branch on the main chain KDO residue linked to the lipid A (Prehm et al., 1975). More recent structural studies do not agree with this arrangement of the KDO residues. Only one KDO residue is now considered to be in the main sugar chain linked to heptose and lipid A. The other two KDO residues are branched from the main chain KDO residue (Brade and Galanos, 1983a).

L-glycero-D-mannoheptose is the other sugar constituent of the inner core region of enteric bacteria. As there are three residues of heptose in this region it is also referred to as the heptose region (Hammerling et al., 1973). Two of the residues are main chain sugars with the third residue branched from the heptose residue which links the inner core to the outer core. This inner core region consisting of KDO and heptose is like the lipid A region in that it is a highly conserved structure in enteric organisms (Jansson et al., 1981). As was observed for lipid A, the inner

core region can exhibit a certain degree of heterogeneity. This observed heterogeneity is the result of substitution of the heptose and KDO residues which branch from the main chain with phosphorylethanolamine. Such substitution imparts charge to the inner core region (Lehmann , 1971) and was previously believed to crosslink lipopolysaccharide molecules.

Brade and Galanos (1983b) have identified an antigen in the inner core region of lipopolysaccharide from various families of gram-negative bacteria against which many mammals, including humans, have natural antibodies. The antigen was determined to be composed of KDO and a neutral sugar, Brade and Galanos (1983a). Such a conserved antigen present in various different families of bacteria makes it a candidate for vaccine production for the treatment of gram-negative bacterial infections. The precise nature of the linkage of the neutral sugar to the KDO residue was found to be essential in determining its antigenicity (Brade et al., 1987).

The outer core region is slightly more variable in structure than the inner core region. The outer core region is also termed the hexose region as the commonly found sugars are glucose, galactose and glucosamine in enteric organisms (Luderitz et al., 1971). Studies of the outer cores of Salmonella and E. coli have shown them to generally be composed of five hexose residues.

The regions are very similar, differing only in the order and/or linkage of the residues (Jansson et al., 1981). However, Sandulache and Prehm (1985) showed the hexose region of a E. chrysanthemi strain to consist of only two glucose units. This result would suggest that the expected core region of E. carotovora may be similar to this and may well be different from those found in E. coli and Salmonella.

4.2.3.1.1.3 O-Chain

The O-chain region of the lipopolysaccharide molecule is attached to the core-lipid A moiety through glycosidic linkage with the terminal sugar residue of the outer core region (Figure 1.3). The O-chain consists of identical repeating units of oligosaccharides. It has been shown for E. coli and Salmonella that the repeating units generally consist of pentasaccharides (Luderitz et al., 1971, Orskov and Orskov, 1984). One notable exception is the 141 O-antigen of E. coli which has a decasaccharide repeating unit (Jann et al., 1966). Structural studies by Ray et al. (1987) of the O-chain of E. amylovora determined the repeating unit to be a trisaccharide consisting of fucose, glucose and galactose.

O-chain is found to be present only in smooth strains of enteric bacteria (Luderitz et al., 1966) and is observed to exhibit a heterogeneity of chain length

within one lipopolysaccharide population (Goldman and Leive, 1980; Jann et al., 1975). This heterogeneity of O-chain length was demonstrated by subjecting purified lipopolysaccharide to sodium dodecyl sulphate - polyacrylamide gel electrophoresis. The broad specificity of the translocase enzyme which catalyses the transfer of O-chain onto the core-lipid A moiety is believed to be responsible for the heterogeneity of O-chain length (Munford et al., 1980). A study of the lipopolysaccharide of Salmonella by Labischinski et al. (1985) using X-ray diffraction found the O-chain to fold back on itself masking the core region (Figure 1.5).

All three regions of the lipopolysaccharide molecule have been shown to be immunogenic (Galanos, et al., 1971) but it is the O-chain that is commonly regarded as the antigenic part of the molecule. It is therefore also referred to as the O-antigen and it is on this structure that the major serotyping systems for E. coli (Orskov and Orskov, 1984) and Salmonella (Kauffmann, 1966) are based. More variability of sugar composition is found for the O-chain region as compared to the conserved core region of enteric lipopolysaccharide. In addition to glucose and galactose, mannose, rhamnose, fucose and galactosamine are common constituents. Unusual sugars are less commonly found. These are 3,6-dideoxyhexose sugars of which five had been identified to date: abequose, tyvelose, ascarylose,

Figure 1.5

Schematic, geometrical model of a Salmonella lipopolysaccharide molecule showing the O-chain folding back over itself and masking the core region. (Labischinski et al, 1985)

PS : polysaccharide
KDO : 2-keto-3-mannooctulosonic acid
HR : hydrophilic region
LR : lipophilic region

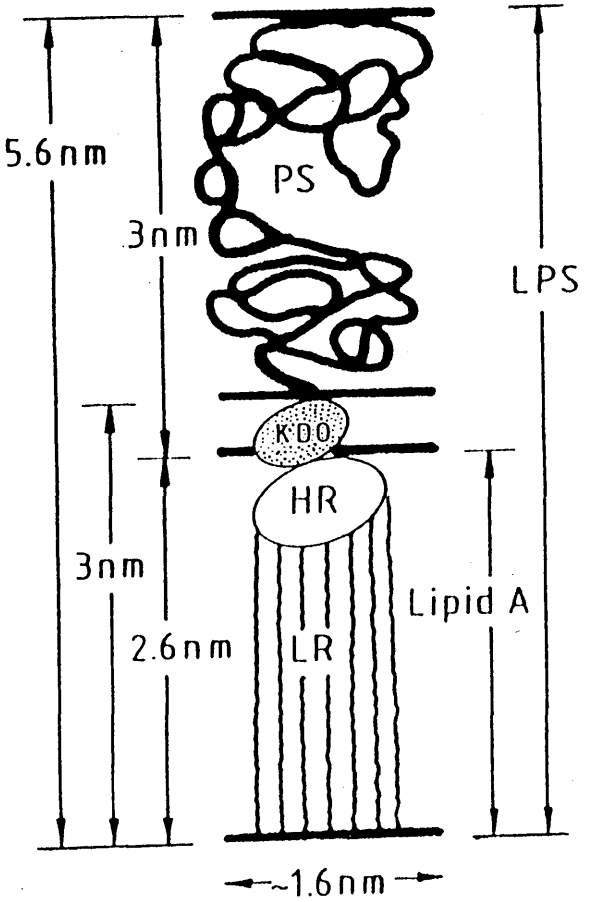


Figure 1.5.

colitose and paratose (Ashwell and Hickman, 1971). The diversity observed for the different serotypes of E. coli and Salmonella is not due exclusively to the presence of unusual sugars but to the sequence of the sugars in the repeating unit, the configuration of the sugars, and the linkage between the residues. Some of the residues may be substituted with a sugar or an acetyl group. Different combinations of these factors contribute to the large number of O-chain structures observed for E. coli and Salmonella. The structures of approximately 60 different O-units are known for Salmonella and E. coli strains (Luderitz et al., 1971).

Heterogeneity of O-chain length is not always observed for the smooth lipopolysaccharide of gram-negative organisms analysed by SDS-PAGE as typified by the Enterobacteriaceae. Lipopolysaccharide from strains of Aeromonas salmonicida (Chart et al., 1984) and Aeromonas hydrophila (Dooley et al., 1985) was observed to possess a homogeneity of chain length when analysed by SDS-PAGE. Unlike the ladder-pattern produced by the SDS-PAGE of lipopolysaccharide from enterobacteria, the lipopolysaccharide profile of A. salmonicida consisted of a very fast migrating band equivalent to the lipid A-core band, and one slower migrating, higher Mr band. Lipopolysaccharide from A. hydrophila produced a similar profile but with the addition of a few intermediate migrating bands. The

O-chain lengths for the two species were different but the O-chain length was the same for different strains of the same species (Chart et al., 1984; Dooley et al. (1985). Dooley et al., (1985) suggested that this observed constancy of O-chain length was important to the virulence of the pathogens. Further, O-chain is not universally present among wild type strains of gram-negative organisms. O-chain has been found to be generally absent from the lipopolysaccharide of nonenteric organisms e.g. Neisseria and Bordetella species, as revealed from SDS-PAGE analyses (Hitchcock et al., 1986). However to compensate for the lack of O-chain, the core region of these lipopolysaccharide molecules would appear to possess a heterogeneity of structure analogous to that of the O-chain of enteric organisms (Tsai et al., 1983).

4.2.3.1.2 Lipopolysaccharide Biosynthesis

Biosynthesis of the core and O-chain regions of the lipopolysaccharide molecule are reasonably well understood in comparison to the lipid A moiety because of the availability of appropriate mutants defective at different stages in their biosynthesis (Luderitz et al., 1982). Very little is known about the actual biosynthesis of lipid A as a defect in the synthesis of lipid A appears to be lethal to the cell. However, Lehman (1977) isolated a temperature-sensitive mutant of

Salmonella that produced an incomplete lipid A moiety above the permissive temperature and still survived. This incomplete lipid A precursor was found to possess a similar structure to lipid A but lacked the hydroxylated fatty acids of the complete molecule (see Figure 1.4a). More recently, Takayama et al. (1983) identified an early precursor of the diglucosamine backbone, lipid X, from E. coli. The lipid X was determined to consist of one glucosamine residue phosphorylated at C-1 and acylated with 3-hydroxymyristic acid residues through amine linkage at C-2 and ester linkage at C-3.

The synthesis of the core region and the O-chain is believed to occur in and on the cytoplasmic membrane. The two regions are synthesised separately and when complete they are covalently joined together (Figure 1.6). Biosynthesis of the core region occurs by the sequential addition of the appropriate monosaccharides from nucleotide sugar precursors on to the lipid A-KDO moiety. The lipid A-KDO moiety is anchored in the hydrophobic environment of the cytoplasmic membrane (Osborn and Rothfield, 1971). Assembly of the O-chain region does not occur in the same manner as that of the core region. The monosaccharides of the O-chain are donated from nucleotide sugar precursors but they are not donated sequentially to form the O-chain. Rather the sugars are first assembled into a monomeric repeat unit linked via a pyrophosphate bridge to a

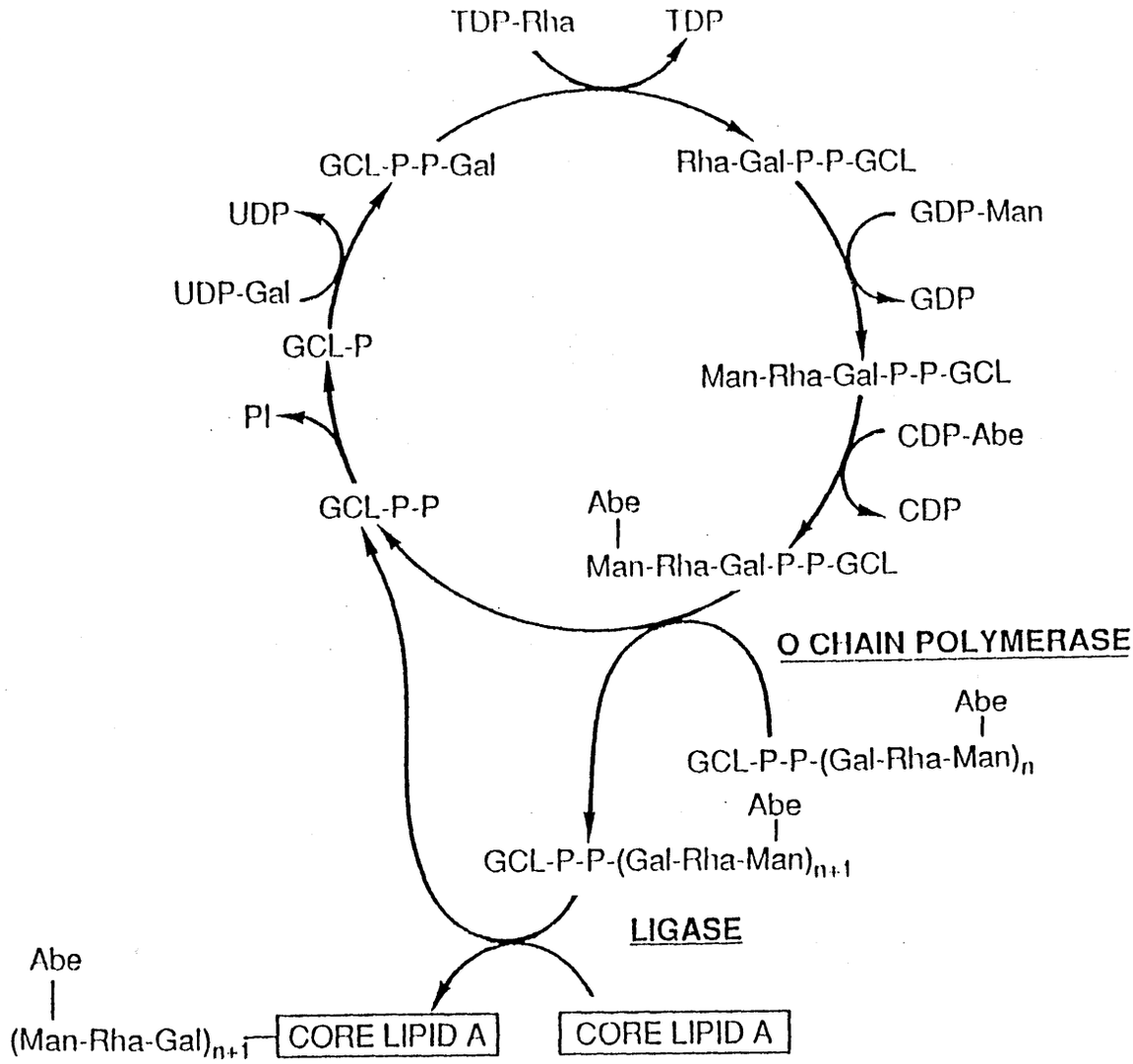


Figure 1.6. Lipopolysaccharide O-chain biosynthesis. Sugars: abe(abequose), gal(galactose), glc(glucose), man(mannose), rha(rhamnose). Nucleotides: CDP(cytidine diphosphate), GDP(guanine diphosphate), TDP(thymidine diphosphate), UDP(uridine diphosphate). GLC: membrane-bound carrier lipid. P: phosphate.

C_{55} polyisoprenoid alcohol in the cytoplasmic membrane. This lipid is referred to as the antigen carrier lipid. Chain elongation is achieved by the transfer of the monomeric repeat unit onto another lipid-linked repeat unit. A similar mechanism of chain elongation is observed in fatty acid and protein biosynthesis. The repeat units in the O-chain are polymerised together and a translocase enzyme catalyses the transfer of the O-chain onto the core-lipid A moiety regenerating the antigen carrier lipid (Robbins and Wright, 1971). All the enzymes involved in these syntheses are present in the cytoplasmic membrane. Rough strains of gram-negative bacteria do not synthesise O-chain. This is due to a mutation in the genes responsible for O-chain biosynthesis (Luderitz et al., 1971).

Exactly how lipopolysaccharide synthesised at the cytoplasmic membrane is transported to the outer membrane is not yet known. Zones of adhesion between the cytoplasmic and outer membranes have been implicated as sites of transport by several researchers (Bayer et al., 1982; Mulhradt and Golecki, 1975). Two models have however been suggested. One model postulates that the newly synthesised lipopolysaccharide is blebbed off from the cytoplasmic membrane in a vesicle which travels through the periplasm and through gaps in the peptidoglycan to fuse with the outer membrane. The second model proposes that there are temporary fusions

between the cytoplasmic and outer membranes and through these temporary fusions lipopolysaccharide travels from the cytoplasmic membrane to the outer membrane by a 'flip-flop' mechanism. The 'flip-flop' mechanism would correlate with the observed zones of adhesion (Lugtenberg and Van Alphen, 1983). Once localised at the surface of the outer membrane, the newly synthesised lipopolysaccharide remains at the site of insertion for one generation. It then diffuses across the membrane (Mulhradt et al., 1973) and organises itself into domains of the same O-chain length (Leive, 1977).

4.2.3.1.3 Lipopolysaccharide function

As a surface structure, smooth lipopolysaccharide gives stability to the outer membrane and acts as a hydrophilic barrier to hydrophobic compounds toxic to the cell (Havekes et al., 1976). Both smooth and rough lipopolysaccharide function as receptors for attachment of certain bacteriophages (Rapin and Kalckar, 1971). It has been suggested that lipopolysaccharide may be involved in the attachment of bacteria to plant cells by binding to plant lectins (Duvick and Sequeira, 1984). In a study of the attachment of Rhizobium leguminosarum cells to pea root hairs, lipopolysaccharide was shown not to be directly involved (Smit et al., 1989). Rather a surface protein was identified which was believed to initiate the attachment of the bacterial cells to the

pea root hairs. The O-chain of the lipopolysaccharide molecule however, was found to be necessary for the establishment of normal nodule development. Lipopolysaccharide mutants of R. leguminosarum lacking the O-chain part of the molecule were found to produce small nodules on the roots of the legume which were unable to fix nitrogen (de Maagd et al., 1989).

4.2.3.2 Outer membrane protein antigens

There are three different groups of major proteins in the outer membrane of gram-negative bacteria (DiRenzo et al., 1978). They are Braun's lipoprotein, Omp A and the porins. Braun's lipoprotein (Braun and Rehn, 1969) is the most abundant of all the outer membrane proteins. About one third of this lipoprotein is covalently bound to the peptidoglycan, the other two thirds are present in the outer membrane in an unbound form (Inouye et al., 1972). Since the identification of Braun's lipoprotein in 1969 several other forms of lipoprotein have been identified in E. coli which are immunologically distinct (Mizuno, 1979; Ichihara et al., 1981).

Omp A is one of the major outer membrane proteins (omp) of gram-negative bacteria. Initially it was believed that there was only one major outer membrane protein (Schnaitman, 1970) but with the development of improved gel systems possessing greater resolution, this one protein was found to consist of several proteins

(Lugtenberg et al., 1975). Omp A is a heat-modifiable protein, a characteristic not possessed by the other outer membrane proteins. It has been shown to be important for the maintenance of the integrity of the outer membrane (Sonntag et al., 1978) and bacterial conjugation (Schweizer and Henning, 1977). There are two types of porin molecule (for review see Hancock, 1987). The major class of porin is a general diffusion pore. The outer membrane proteins Omp C and Omp F belong to this class. It is an aqueous pore which spans the outer membrane lipid bilayer and is noncovalently associated with the peptidoglycan and Braun's lipoprotein (Direnzo et al., 1978). General diffusion pores are oligomers consisting of trimers of porin proteins of which there appears to be some variation in the arrangement of the channels. The OmpF porin from E. coli was shown to consist of a trimer possessing three channels to the exterior of the cell which fused to produce one channel exposed to the interior of the cell (Engel et al., 1985). Similarly the Omp F from Ps. aeruginosa consists of a trimer with three channels but which are separate and continuous from the exterior to the interior of the cell (Woodruff et al., 1986). General diffusion pores are permeable to a variety of different solutes simply differentiating between solutes on the basis of the size of the molecule. Nakae and Nikaido (1975) determined the exclusion limit to be

about 600 molecular mass. One characteristic of a general diffusion pore is that the porin and solute do not interact with each other. Porin and solute interaction is a characteristic of the minor class of porin molecule. These porins function for the transport of specific solutes. In E. coli, the Lam B protein is specific for the transfer of maltose across the outer membrane (Luckey and Nikaido, 1980) and the Tsx protein is specific for the uptake of nucleosides (Maier et al., 1988)

As well as the three major classes of outer membrane protein there are also ten to twenty minor proteins present. Many of these proteins have been identified as receptors for phages and colicins, and many are involved in the uptake of nutrients into the cell (Direnzo et al., 1978).

The outer membrane proteins are highly immunogenic and constitute the second major group of gram-negative surface antigens (Smyth, 1985). Within the family Enterobacteriaceae there is considerable cross-reactivity of the outer membrane proteins (Hofstra and Dankert, 1979). This observed cross-reactivity did not appear to be influenced by the different molecular weights of the outer membrane proteins (Benz and Bauer, 1988) from strain to strain (Hofstra and Dankert, 1980). The exception to this was Braun's lipoprotein which was found to be a conserved antigen amongst the

Enterobacteriaceae (Braun et al., 1976).

Biosynthesis of the outer membrane proteins occurs in the cytoplasm (Halegoua and Inouye, 1979) where they are synthesised as precursor proteins with an attached polypeptide leader sequence. The leader sequence is removed as the protein is translocated across the cytoplasmic membrane, periplasm and peptidoglycan to the outer membrane (for review see Randall et al, 1987). The exact mechanism of their translocation is not known but it has been suggested that the proteins may be co-translocated with newly synthesised lipopolysaccharide (Datta et al., 1977) as these proteins are found complexed with lipopolysaccharide in the outer membrane. Also, Gmeiner and Schlecht (1979) and Smit et al. (1975) found that bacterial mutants defective in their lipopolysaccharide were found to have reduced amounts of protein in their outer membrane.

4.2.3.3 Enterobacterial common antigen

The enterobacterial common antigen (ECA) is found to be present in all species of gram-negative bacteria belonging to the family Enterobacteriaceae (Kunin et al., 1962) with the exception of E. chrysanthemi. As its name implies, ECA is unique to the Enterobacteriaceae. Its structure is the same in all species and it shows general structural features similar to those of lipopolysaccharide. There is a lipid

moiety, a glycerophospholipid, which anchors the antigen in the outer leaflet of the lipid bilayer of the outer membrane. There is a sugar chain which is attached to the lipid component through the ester-linked phosphate group on the C-3 of the glycerol backbone. The sugar chain is made up of repeating units of a trisaccharide of amino sugars (Lugowski et al., 1983) which projects outwards from the surface of the bacterium. A heterogeneity of chain lengths is observed (Kuhn et al., 1987) similar to that of smooth lipopolysaccharide (see Figure 1.2a). As ECA and lipopolysaccharide have similar structures it is to be expected that they would share a similar biosynthetic pathway (Kuhn et al., 1988). One consequence of this similar biosynthesis is that the ECA sugar chain can be found substituted onto core stubs in populations of rough lipopolysaccharide (Kiss et al., 1978).

The immunogenicity of ECA is found to depend on whether or not it is complexed with other membrane antigens. Only when ECA is complexed with either rough lipopolysaccharide or outer membrane proteins is it immunogenic (Whang et al., 1972; Kuhn et al., 1981). This relative non-immunogenicity of uncomplexed ECA is thought to be a consequence of its small relative molecular mass.

5. Serology of E. Carotovora

Unlike the extensive literature on the serology of Salmonella (Kauffmann, 1966; Lindberg and Le Minor, 1984) and E. coli (Orskov et al., 1977) very little is known about the serology of E. carotovora. An early study by Lazar (1971) examined strains belonging to ten different species of Erwinia and found that serologically all the species were quite similar. Graham (1963) raised antisera against whole cells of E. carotovora subsp. atroseptica that could be used in slide agglutination tests to diagnose blackleg in the potato plant and tuber. In the last ten years, a body of knowledge on the serology of E. carotovora has been amassed more or less exclusively by Dr. S.H. De Boer and co-workers. Using Ouchterlony's (1958) technique of immunodiffusion, De Boer developed a system for the identification of serogroups of E. carotovora strains (De Boer et al., 1979). Specifically, the technique involves cutting six peripheral wells and a central well out of agar in petri dishes. Whole, live bacterial cells are added to the peripheral wells and to each is added a drop of aqueous phenol. Serogroup specific antiserum is added to the central well and the plates incubated at room temperature for 24 to 48 hours. The plates are then examined for lines of precipitation. From the presence or absence of precipitation the

serogroups of an unknown strain can be identified. Further, total, partial or non-serological identity between different strains can be identified.

In the initial study of the serogroups of E. carotovora by De Boer (1979), eighteen serogroups were identified and the serogroups were designated one to eighteen using Roman numerals e.g. I, XVIII. The number of serogroups has since increased to about forty (De Boer and Sasser, 1986). The nature of the reactive immunogen in this system of immunodiffusion was found to be heat-stable and extractable with 45% aqueous phenol (De Boer et al., 1979). De Boer et al. (1985) subsequently confirmed the immunogen to be lipopolysaccharide from ELISA tests: lipopoly-saccharide reacted with antiserum specific for the serogroup of the strain from which the lipopolysaccharide was purified. By analogy with the serogroups of E. coli and Salmonella, it is probable that the diversity of O-chain structure is responsible for the large number of E. carotovora serogroups. Monoclonal antibodies against lipopolysaccharide from E. carotovora subsp. atroseptica belonging to serogroup I, were observed to be specific for lipopolysaccharide O-chain (De Boer and McNaughton, 1987). However, these monoclonal antibodies also cross-reacted with O-chain of serogroup XXII lipopolysaccharide indicating a similarity of O-chain structure. The titre of the

monoclonal antibodies was lower against the serogroup XXII lipopolysaccharide than the homologous serogroup I lipopolysaccharide possibly due to the lower incidence of the particular epitope in serogroup XXII lipopoly-saccharide O-chain.

Analysis of the distribution of E. carotovora subsp. atroseptica strains and E. carotovora subsp. carotovora strains within the serogroup showed subsp. atroseptica strains to type into only a few serogroups whereas subsp. carotovora strains were serologically more diverse. Serogroup I was the major serogroup for subsp. atroseptica strains. In British Columbia, De Boer determined 96% of atroseptica strains to type into serogroup I (De Boer and McNaughton, 1987). The other 4% of atroseptica strains typed into serogroups XVIII, XX and XXII (De Boer and Sasser, 1986). Apart from serogroup XVIII, serogroups I, XX and XXII were generally found to be the groups into which atroseptica strains only, typed. Subsp. carotovora strains were found to type into serogroup XVIII, and the other thirty six serogroups.

Not only will this system of serotyping become more complex as new serogroups of E. carotovora are identified but some strains have already been found to type into more than one serogroup (De Boer et al., 1987). This problem highlights one of the major problems of using whole cells as the antigen in

immunodiffusion: there can be more than one precipitable antigen present and reacting in this system.

Project Aims

The overall aim of this project was to study the major surface antigens, the outer membrane proteins and lipopolysaccharide, of *E. carotovora* for the purpose of increasing our knowledge of the surface of gram-negative bacteria. Further, to provide suitable antigens for the raising of novel antisera which may ultimately be used for the detection of pathogenic and non-pathogenic strains of *E. carotovora* in Scotland.

Using techniques successfully applied to other enteric organisms, the surface antigens were to be extracted and purified then analysed using the easy and relatively inexpensive technique of SDS-PAGE. The antigenicity of these surface antigens, separately and as an integral part of the cell wall, was to be investigated using available diagnostic antisera. Further, to investigate the nature of these important antigens with reference to the serotyping system being developed for *E. carotovora*, particularly the lipopolysaccharide component as this had been identified as a reactive antigen in this system. The sugar composition of the lipopolysaccharide antigens would be determined by GLC analysis.

As results concerning the lipopolysaccharide antigens obtained in the initial stages of this project proved highly interesting and worth greater

consideration, the subsequent research concentrated solely on the lipopolysaccharide antigens. This study is therefore of the lipopolysaccharide surface antigens of E. carotovora: by comparison to other enteric bacterial lipopolysaccharides, relevance to the serotyping system for E. carotovora, structure, immunogenicity, and sugar composition.

MATERIALS AND METHODS

1. Materials

All reagents were the best grade commercially available and where this is AR grade it is indicated. With the exceptions of the materials listed below, reagents were obtained from BDH Chemicals Ltd., Poole, Dorset, BH12 4NN.

Perseitol; 3-phenylphenol - Aldrich Chemical Co. Ltd., The Old Brickyard, New Road, Gillingham, Dorset, SP8 4JL.

4-chloro-1-naphthol - Bio-Rad Laboratories Ltd., Watford, Hertfordshire.

Dithiothreitol; peroxidase, type II (Horseradish peroxidase); Boehringer Corp. Ltd., Boehringer Mannheim House, Bell Lane, Lewes, East Sussex, BN7 1LG.

Freund's Incomplete Adjuvant - Difco Laboratories, Detroit, Michigan 48201, USA.

Boric acid AR; glucose AR; phenol AR; sodium chloride AR; sucrose AR - Formachem, 80 Kirk Street, Strathaven, ML10 6BA.

Acrylamide; NN'-methylene bisacrylamide; sodium dodecyl sulphate - FSA Laboratory Supplies, Bishop Meadow Road, Loughborough, LE11 0RG.

Pyronin Y - George T. Gurr Ltd., London, SW6.

Silver nitrate - Johnson Matthey Materials Technology U.K., Orchard Road, Royston, Herts, SG8 5HE.

Raffinose - Koch-Light Laboratories Ltd., Colnbrook,

Bucks.

Citric Acid AR - May and Baker Ltd., Dagenham, England.

Nutrient broth (CMI); Nutrient agar (CM3) - Oxoid Ltd., Basingstoke, Hants.

Dextran Blue 2000; Ribonuclease A; Sephadex G50 (fine) - Pharmacia LKB Biotechnology, S-751 82, Uppsala, Sweden.

Normal donkey serum; HRP - donkey anti-rabbit IgG - Scottish Antibody Production Unit, Glasgow and West of Scotland Blood Transfusion Service, Law Hospital, Carluke ML8 5ES.

Agarose, type II; bacterial alkaline phosphatase (E. coli), type III-S, 10 units/mg protein; Coomassie Brilliant Blue G250; cysteine-HCL; proteinase K, protease type XI (fungal), 10-20 units/mg protein; galactose oxidase, type V (Dactylium dendroides); mannoheptulose; ribonuclease A, type I-A (bovine pancreas, minimum 60% ribonuclease A); sodium azide; stachyose; Triton X-100; Tween 20 - Sigma Chemical Co. Ltd., Poole, Dorset, BH17 7NH.

Arabinose; galactose; ribose; xylose - Thomas Kerfoot and Co. Ltd., Vale of Bardsley, Lancashire.

2. Methods

2.1 Microbiological Techniques

2.1.1 Microorganisms

Erwinia carotovora strains used in this study are listed in Table 2.1. Type strains for some of the serogroups (De Boer et al, 1979) were obtained from Dr. S.H. De Boer (Agriculture - Canada, Vancouver, Canada) and listed in Table 2.1 as de Boer. Other strains were from the personal collections of Dr. D.C. Graham (Agriculture Scientific Services, Department of Agriculture and Fisheries for Scotland, East Craigs, Edinburgh, EH8) and Dr. M.C.M. Perombelon (Scottish Crop Research Institute, Invergowrie, Dundee). These strains were listed in Table 2.1 as ECR and SCRI, respectively. Salmonella minnesota strains R5, R7, R60, R345 were kindly given by Dr. R. Parton, Department of Microbiology, University of Glasgow.
Erwinia amylovora EA3 and Erwinia chrysanthemi NCPPB 402 were from the culture collection of Dr. D.C. Graham.
Erwinia salicis strain 152 was à gift from Mr. M. de Kam, Dorschkamp Research Institute for Forestry and Landscape Planning, Wageningen, Bosrandweg, The Netherlands.
Pseudomonas putida NCIB 10553, Pseudomonas aeruginosa NCIB 10548 and Klebsiella aerogenes NCIB 418 were

TABLE 2.1 **E. carotovora strains used in this study.**

Subspecies and strain	Source	Serogroup
atroseptica		
31	de Boer*	I
436	NCPPB**	I
509	de Boer	I
549	NCPPB	I
1001	SCRI+	I
1002	SCRI	I
1034	SCRI	I
1039	SCRI	I
1042	NCPPB	I
1043	SCRI	I
1061	SCRI	I
1062	SCRI	I
1063	SCRI	I
1064	SCRI	I
1065	SCRI	I
1066	SCRI	I
1067	SCRI	I
1068	SCRI	I
1069	SCRI	I
1070	SCRI	I
G398	ECR++	I
6	de Boer	XVIII
DB6	SCRI	XVIII
11	de Boer	XVIII
DB39	SCRI	XVIII
DB58	SCRI	XX
196	de Boer	XX
DB199	SCRI	XX
DB1056	SCRI	XX
DB1058	SCRI	XX
G331	ECR	XX
DB198	SCRI	XXII
DB420	SCRI	XXII
A46	ECR	XXII
A139	ECR	XXII
G222	ECR	XXII
432	NCPPB	XXXV

carotovora

327	de Boer	I
547	NCPPB	I
1745	NCPPB	I
G275	ECR	IV
G298	ECR	XII
192	de Boer	XVIII
G301	ECR	XVIII
G303	ECR	XVIII
P1	ECR	XXV
312	SCRI	XXIX
193	SCRI	XXXIII
438	NCPPB	XXXIV
553	de Boer	XXII/XXXV
556	de Boer	XX/XLI
557	de Boer	XXII/XLI
559	de Boer	XX/XL

betavasculorum

545	de Boer	XL/XLIII
546	de Boer	XXXV
548	de Boer	XXXV
551	de Boer	XXXV

de Boer* - cultures received from Dr. S.H. de Boer

NCPPB** - cultures received from the National Collection of Plant Pathogenic Bacteria, Hatching Green, Harpenden, Herfordshire, AL5 2JN.

SCRI+ - cultures received from Dr. M.C.M. Perombelon

ECR++ - cultures received from Dr. D.C. Graham.

strains in routine use in the Biochemistry Department, University of Glasgow and had been obtained from the National Collection of Industrial Bacteria, Torry Research Station, P.O. Box 31, 135 Abbey Road, Aberdeen, AB9 8DG.

2.1.2 Storage of the Bacteria

For short term maintenance, cultures in Oxoid nutrent broth (CMI) were stored at 4°C. For long term maintenance, either cultures on Oxoid nutrient agar (CM3) slants were stored at room temperature or an equal volume of sterile glycerol was added to fully grown nutrient broth cultures and the cultures stored at -20°C.

2.2 Media

2.2.1 Nutrient broth media

Oxoid nutrient broth medium (CMI) was prepared at both the normal concentration ($13g.l^{-1}$) described by the manufacturer and at double recommended strength. Prepared nutrient broth was dispensed (10ml) into universals for routine use for inocula preparation and culture storage while the double strength medium was dispensed (500ml) into 2l Erlenmeyer flasks.

2.2.2 Minimal media

Minimal media were prepared as described by Hamilton and Holms (1970) except that the initial pH was 7.5.

The media were prepared as four components which were sterilised separately.

a) Phosphate buffer was prepared containing 15.54g KH_2PO_4 per litre and made to pH 7.5 with sodium hydroxide.

b) 160mM glucose

c) Iron solution containing 800μM $FeSO_4$ solution was adjusted to pH 2.0 with HCl before sterilisation.

d) MgNS which contains 40mM $MgSO_4$ and 800mM $(NH_4)_2SO_4$

Prior to inoculation, the $FeSO_4$ solution, MgNS (both at $12.5ml.l^{-1}$), and glucose to the required final concentration, were added to the phosphate buffer.

2.2.3 Detergent containing media

Minimal glucose salts containing medium was prepared as described in Section 2.2.2 except the pH was adjusted to 7.0. To the medium, sterile deoxycholate was added to give final concentrations of 0.05, 0.1, 0.2 and 0.5% (w/v). After addition of detergent the pH of the medium was unaltered.

2.2.4 Nutrient agar

Nutrient agar (Oxoid CM3) was prepared at $28g.l^{-1}$ as described by the manufacturer. After sterilisation,

the sterile molten medium was kept at 55°C and poured into petri dishes (9cm diameter - Sterilin Ltd., Teddington, Middlesex) and left to set. Plates were left inverted for 24h at 30°C to dry and then stored at 4°C.

2.3 Sterilisation

2.3.1 Moist heat

All media were autoclaved at 120°C and 5 psi, depending on their volume for the times established by Fewson (unpublished results). A Bastian and Allen (Harrow, England) autoclave and boiler was used. Each sterilisation run was verified by using a Browne's tube (Type 3, A. Browne Ltd., Chancery Street, Leicester).

2.3.2 Dry heat

Glass pipettes were heated at 160°C for 1.75h either wrapped in Kraft paper or placed in metal canisters. Each sterilisation was checked by including a Browne's tube type 1.

2.4 Growth of Bacteria

2.4.1 Small scale growth

Small scale growth was carried out using 500ml of double strength nutrient broth media in 2l Erlenmeyer

flasks stoppered with polystyrene foam bungs. The cultures were inoculated with 10ml of a fully grown nutrient broth culture of the required organism and grown on a rotary shaker (M.K.V. Orbital Shaker, L.H. Engineering Co. Ltd., Stoke Pages) at 120 r.p.m.

The temperature and duration of growth depended on the organism being grown. E. carotovora were grown at 23°C; E. amylovora, E. chrysanthemi, S. minnesota, Ps. putida, Ps. aeruginosa and K. aerogenes were all grown at 30°C.

Erwinia cultures were also grown on nutrient agar plates at 23°C for 48h.

2.4.2 Large scale growth

Large scale growth of erwinias was carried out in 4l of minimal salts medium containing 20mM glucose in 10L flat bottomed flasks each closed with a plug of non-absorbent cotton wool bound with muslin through which passed a cotton wool-plugged glass pipette (10ml). The cultures were grown at 23°C on the apparatus described by Harvey et al (1968). Air (400ml.min^{-1}) was passed through the glass pipette into the flask.

Inocula were prepared by adding 0.1ml of a nutrient broth stock culture to approximately 100ml of 10mM glucose minimal salts medium, pH 7.0 (Methods 2.2.3) and grown for about 24h on a rotary shaker at 23°C. 10ml of this culture was added aseptically to 4l of 20mM glucose

minimal salts medium (Methods 2.2.2).

2.4.3 Growth on deoxycholate

100ml of detergent containing minimal medium (Methods 2.2.3) in 250ml Erlenmeyer flask was inoculated with 5ml of a 10ml nutrient broth culture and grown on a rotary shaker at 120 r.p.m. and 23°C. Growth of the cultures was measured on addition of the inocula and at hourly intervals thereafter.

2.5 Harvesting and Storage of Bacteria

Bacterial cultures were routinely harvested when cultures were in late log phase or early stationary phase (A_{500} = approximately 5). Growth curves for typical E. carotovora cultures can be seen in Figure 2.1.

Both small and large scale growth cultures were harvested by centrifugation of 500ml volumes in 750ml plastic centrifuge bottles (MSE Ltd., Buckingham Gate, London) at 7,000g for 20 min at 4°C in an MSE Mistral 6L centrifuge. Each pellet was resuspended in 100ml of ice-cold 10mM Tris-HCl buffer, pH 7.4 and recentrifuged in the same 750ml plastic bottles at 7,000g for 20 min at 4°C. The pellets were washed again in 50ml of ice-cold 10mM Tris-HCl, pH 7.4 and centrifuged in 100ml polypropylene centrifuge tubes at 3,000g for 20 min at 4°C in an MSE Highspeed 18 centrifuge. The supernatants

Figure 2.1

Growth curves of typical E. carotovora subsp. atroseptica strains

Strains were grown on 20mM glucose minimal salts medium, pH 7.5 (Methods 2.2.2) at 23°C as described in Methods 2.4.2. Absorbance values were read at 500nm as described in Methods 2.5.

Symbols (□), (■), (●) represent three different Erwinia strains.

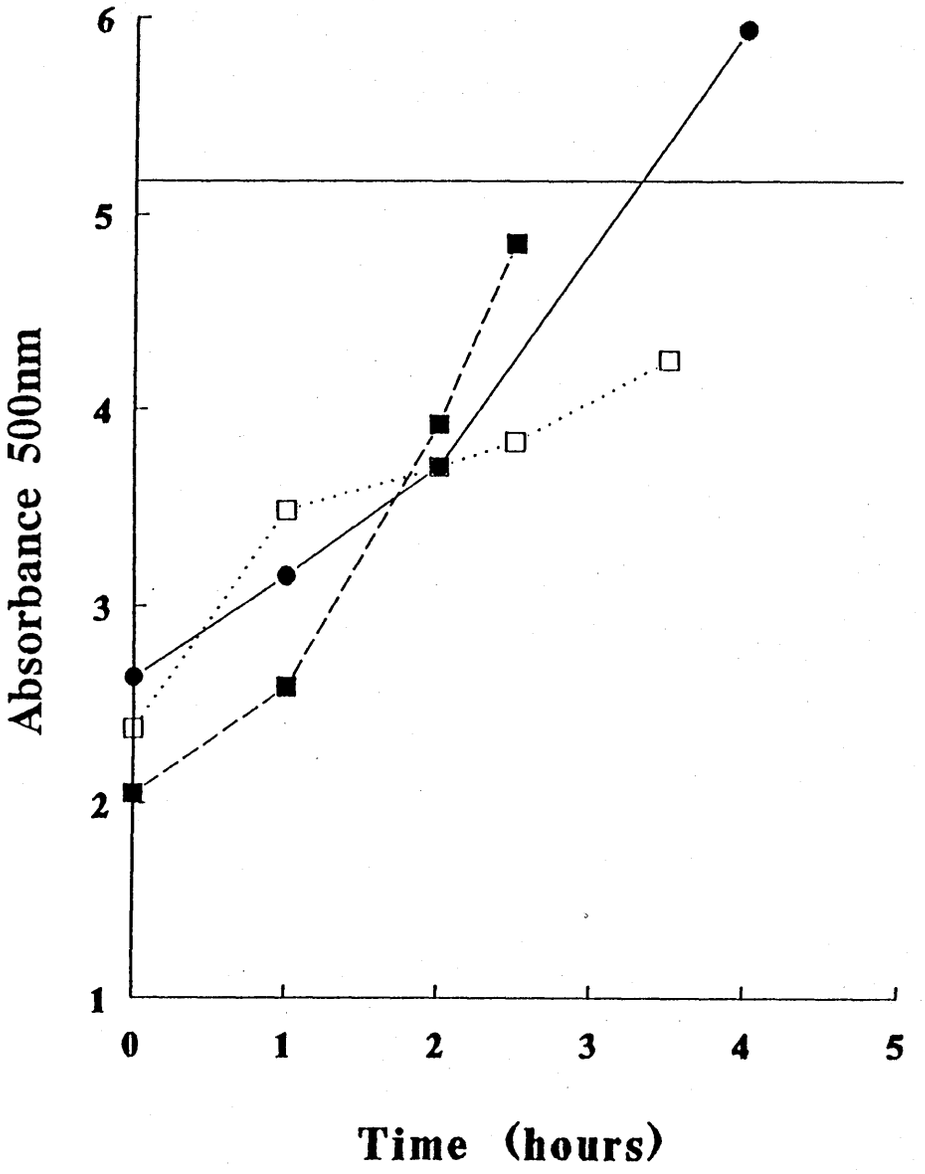


Figure 2.1.

were carefully decanted, the bacterial pellets were combined and then freeze-dried. The freeze-dried pellets were either used immediately or stored at -20°C until used.

2.6 Measurement of Bacterial Growth

Growth of the bacteria was followed by measurement of the turbidity of the culture. Turbidity was measured by determining the absorbance at 500nm using a Pye Unicam SP30 spectrophotometer (Pye Unicam Instruments Ltd., 576 West Harbour Road, Granton, Edinburgh EH5 1PP).

2.7 Lipopolysaccharide Extraction and Purification

2.7.1 Phenol/water extraction

Using the hot aqueous phenol method of Westphal and Jann (1965), lipopolysaccharide was extracted from the freeze-dried bacterial pellets (see Methods 2.9).

A known weight of freeze-dried bacteria was suspended in distilled water at a concentration of 1g dried cells to 17.5ml distilled water in a 100ml polypropylene centrifuge tube or 50ml capped polypropylene tube (Alpha Laboratories, 40 Parham Drive, Eastleigh, Hants SO5 4NU). The suspension was heated at 68°C in a water bath and then an equal volume of 90% liquid phenol (90g phenol: 11ml distilled water)

pre-heated to 68°C was added. The mixture was stirred continuously with a glass rod for 10 min at 68°C. The mixture was then cooled in an ice-water bath for 10 min to give partial separation of the phenol-rich and water-rich phases prior to centrifuging. The mixture was then centrifuged for 45 min at 3,000g at 10°C. The upper aqueous phase produced as a result of centrifugation at the lower temperature was removed and retained: this contained the lipopolysaccharide. The remaining bacterial mass in the lower phenol-rich layer was subjected to this extraction procedure twice more using the same volume of distilled water as used for the initial extraction. The 3 aqueous fractions were pooled and dialysed against 4l of distilled water for 4 days. The distilled water was changed twice each day. Freeze-drying of the dialysate gave a crude preparation of lipopolysaccharide.

To purify the lipopolysaccharide from contaminating nucleic acids, a weighed amount of lipopolysaccharide was resuspended in distilled water using a hand-held glass homogeniser (Jencons, England) to give a 3% (w/v) solution. The solution was centrifuged in polycarbonate tubes in a Ti50 rotor (Beckman Instruments Inc., Palo Alto, California) for 8h at 125,000g max. in a Beckman preparative ultracentrifuge (model L2-65B) at 10°C. If large volumes were involved, Ti60 tubes and a Ti60 rotor were used. The resulting pellet was resuspended in 5ml

distilled water and centrifuged at 150,000g max for 3h in the ultracentrifuge at 10°C. This step was repeated. The final pellet was freeze-dried and stored as described in Methods 2.8.

2.7.2 Phenol/chloroform/petroleum spirit extraction

The method of Galanos et al. (1969) was used with slight modifications to account for the small amount of material used.

To obtain a fine cell suspension, 0.4g of freeze-dried bacterial cells were mixed with 5ml of the extraction mixture in a 10ml graduated glass tube. The extraction mixture consisted of 90% (w/v) liquid phenol, petroleum spirit A.R. (bp. 40-60°C) and chloroform A.R. (2:5:8, by volume). The mixture was centrifuged in a Beckman bench-top centrifuge (model TJ-6) at 1,200g for 20 min. The supernatant was removed and retained. Two further extractions were performed on the bacterial residue using the same volume of extraction mixture. The 3 supernatants were pooled. The petroleum spirit and chloroform were evaporated at 40°C under a stream of nitrogen gas. Sufficient distilled water was then added dropwise until the lipopolysaccharide was seen to precipitate out. To bring down the precipitate, the lipopolysaccharide was centrifuged at 1,200g for 10 min in the bench top centrifuge. The precipitate was washed by centrifugation, three times with 5ml 80% (w/v) phenol

and three times with 5ml diethyl ether. It was then dried in a desiccator under vacuum over calcium chloride for at least 3 hours. The dried lipopolysaccharide was taken up in a small volume of distilled water, warmed to 45°C for 10 min and then vacuum carefully applied to remove traces of diethyl ether and phenol for 2 min. The solution was then vortexed and centrifuged in a Beckman ultracentrifuge at 150,000g for 4h at 10°C. The final pellet was resuspended in a minimum volume of distilled water and freeze-dried.

2.7.3 Proteinase K digestion

The method as described by Hitchcock and Brown (1983) was used.

Erwinia cultures were grown on nutrient agar as described in Methods 2.4.1. The whole bacterial growth on a plate was harvested and suspended in 1ml distilled water. Using a Unicam SP8/100 spectrophotometer (Pye Unicam Instruments Ltd.), absorbance of the bacterial suspension was measured at 420nm as compared to 500nm for growth of bacteria in liquid culture (Methods 2.6). The suspension was subsequently diluted with distilled water to give an absorbance of 0.4. 1.5ml was centrifuged for 1.5 min at 10,000g in an Eppendorf 3200 centrifuge in an Eppendorf tube. The supernatant was discarded and the cell pellet dissolved in 50µl of a lysing buffer at 100°C for 10 min. 1ml of the lysing

buffer contained 278mg sucrose, 52mg SDS, 20mg DTT and 3.5mg Pyronin Y. After cooling, the lysate was incubated for 1h at 60°C with another 10ul of the lysing buffer containing 25µg of proteinase K.

Modifications were made to this method to try to resolve more of the electrophoretic components of the lipopolysaccharide and reduce the high background smearing of the gel. Modifications included more extensive washing of the cell pellet obtained from the agar plate and heating the lipopolysaccharide containing extract with 10µg DNAse/RNAse to room temperature for 10 minutes.

2.7.4 Triton lipopolysaccharide extraction

This method was supplied by Dr M.C.M. Perombelon (SCRI) and based on the method used by Expert and Touissaint (1985).

10ml of a 100ml 10mM glucose minimal salts culture (Methods 2.2.3) was harvested when the culture entered stationary phase (A_{500} = approximately 2.5). The pellet was resuspended in 1ml of 0.025 M Tris/0.25mM EDTA, pH 7.5 buffer, transferred to an Eppendorf tube and sonicated 10 times, using 80 watts of power, for 3 sec each time with a 5 sec break in between each sonication, using the microtip probe of the Dawe Soniprobe (Type 7332A; Dawe Instruments Ltd., London). The sonicate was centrifuged in the Eppendorf 3200 centrifuge for 30 sec

at 10,000g and the supernatant transferred to a Ti50 tube. 100µl of 20% (v/v) Triton X-100 was added to the supernatant which was then ultracentrifuged for 10 min at 21,400g max and 4°C. The supernatant was discarded and the tube was left to stand inverted for 10 min at 4°C to dry. 100µl of tracking dye mix (Methods 2.10.1.5)/0.2M DTT (1:2, v/v) containing 5µg proteinase K was used to suspend the pellet and the tube incubated at 37°C for 120 min. The tube was covered with parafilm and then left overnight at 4°C. The digested sample was vortexed, transferred to an Eppendorf tube and centrifuged for 30 sec. The resulting pellet was retained and heated at 100°C for 10 min with another 60µl of the tracking dye mix/0.2 M DTT (1:2, v/v). Proteinase K was again added to give a final concentration of 100µg ml^{-1} and incubated at 37°C for 1h. The preparation was then ready for electrophoresis.

2.8 Storage of Lipopolysaccharide

Unless otherwise stated all freeze-dried purified lipopolysaccharide preparations were dissolved in distilled water to a final concentration of 10mg ml^{-1} and stored at -20°C.

2.9 Freeze-drying of Samples

Bacterial cells and lipopolysaccharide preparations were all shell-frozen with a mixture of methanol and

dry-ice prior to freeze-drying in a Lyolab A laboratory freeze-drier (Lyolab, Life Sciences Laboratory Ltd., Sedgewick Road, Luton, Bedfordshire).

2.10 Polyacrylamide Gel Electrophoresis

2.10.1 Tris/Glycine SDS-PAGE

The method used was based on the discontinuous SDS-PAGE system described by Laemmli (1970).

2.10.1.1 Stock solutions

Solution A - separating gel buffer

Solution A was prepared containing 36.6g Tris and the pH adjusted to 8.8 with concentrated HCl. 0.23ml of TEMED was added and the volume made up to 100ml with distilled water.

Solution B - electrophoresis buffer

Electrophoresis buffer contained 3g Tris, 14.4g glycine and 1g SDS made up to 1 litre with distilled water.

Solution C - acrylamide

100ml of acrylamide solution contained 28g acrylamide and 0.735g NN'-methylene-bisacrylamide

Solution D - stacking gel buffer

19.2ml of 0.1M Tris/HCl, pH 6.0, 0.8ml 20% (w/w) SDS and 0.5ml TEMED were made up to 100ml with distilled water.

All these solutions were stored at room temperature.

Solution E - SDS

20% (w/v) SDS solution was stored at 37°C.

2.10.1.2 Gel plates

Glass plates (dimensions 9.5 x 20cm and 10 x 20cm) were cleaned routinely by soaking in Decon 75, scouring with powder and rinsing liberally with tap water then distilled water. The plates were assembled with 1.5mm Teflon spacers and silicon grease (RS Components, Corby, Northants) prior to being placed in a gel-making manifold suitable for making 4 gels at a time.

2.10.1.3 Preparation of 12.5% (w/v) acrylamide separating gel

The following volumes of the stock solutions were mixed:

Solution A - 25ml

Solution C - 89.3ml

Solution E - 1ml

plus 82.2ml distilled water.

The solution was then degassed for approximately 2 min., 150mg ammonium persulphate was added and mixed, and the solutions were degassed for a few seconds. It was then poured into the gel-making manifold and a 1ml overlay of propan-2-ol was pipetted on top of each gel.

Once the gels had polymerised (about 30 min), the propan-2-ol was washed off first with tap water several times then distilled water several times. The top of the gels were then blotted dry with Whatman chromatography paper.

2.10.1.4 Preparation of 5.2% (w/v) acrylamide stacking gel

The following volumes of the stock solutions were mixed:

Solution D - 10ml

Solution C - 17.5ml

plus 55ml distilled water

The solution was then degassed for approximately 2 min. 150mg of ammonium persulphate was added and mixed, and the solution was degassed for a few seconds. The gel mixture was poured on top of the separating gel and 4 x 18-track teflon combs inserted. The gels were left to polymerise for about 15 min, stored at 4°C and used always within 7 days of preparation. Experience showed that gels prepared in advance gave poorer resolution and higher background staining than freshly prepared gels.

2.10.1.5 Sample preparation and loading

Lipopolysaccharide was made up in a tracking dye solution consisting of 1.5g SDS, 8g sucrose and 100µg Pyronin Y in 10ml of 0.5M Tris/HCL, pH 8.8. Each

lipopolysaccharide sample was made up in the following proportions: 10 µl LPS (10mg. ml^{-1}); 34 µl distilled water; 36µl 0.2M DTT; and 20 µl of the tracking dye solution. Prior to loading, the samples were routinely boiled at 100°C for 2 min. Once the electrophoresis apparatus had been assembled and both reservoirs had been filled with electrophoresis buffer (solution B), 20µl of sample was loaded per track using an automatic pipette (Gilson Medical Electronics, France).

2.10.2 Electrophoresis conditions and sample running

The electrophoresis was carried out with a current of 90 mA until the tracking dye was seen to reach the bottom of the gel (about 50 min). During electrophoresis the gel was cooled by pumping ice-water from a tank through a cooling plate in the electrophoresis apparatus.

2.10.3 Tris/glycine PAGE

The non-SDS-PAGE system used was as described by De Boer and McNaughton (1987) and adapted to the system described in Methods 2.10.1.

2.10.3.1 Stock solutions

Solution A (separating gel buffer) and solution C (acrylamide) were as described in Methods 2.10.1.

Solution B - electrophoresis buffer (SDS free)

Electrophoresis buffer (SDS free) contained 3g Tris and 14.4g glycine made up to 1l with distilled water.

Solution D - stacking gel buffer (SDS free)

19.2ml of 0.1M Tris/HCl, pH 6.0 and 0.5ml TEMED were made up to 100ml with distilled water.

2.10.3.2 Preparation of 12.5% (w/v) acrylamide separating gel

The following volumes of the stock solutions were mixed: Solution A - 25ml

Solution C - 89.3ml

plus 82.2ml distilled water.

Rest of preparation of separating gel was as described in Methods 2.10.1.3.

2.10.3.3 Preparation of 5.2% (w/v) acrylamide stacking gel

The following volumes of the stock solutions were mixed:

Solution D - 10ml

Solution C - 17.5ml

plus 55ml distilled water.

Rest of preparation of stacking gel was as described in Methods 2.10.1.4

2.10.3.4 Sample preparation and loading

Sample preparation and loading was exactly the same as described for SDS-PAGE (Methods 2.10.1.5) except that the 100µg of Pyronin Y in the tracking dye solution was replaced by 100µg of Bromophenol Blue.

Preparation of gel plates, electrophoresis conditions and sample running were as previously described in Methods 2.10.1 and 2.10.2.

2.10.4 Tris/borate SDS-PAGE

The method used was that described by Rainer, (1971) and modified to include SDS.

2.10.4.1 Stock solutions

Solution C (acrylamide), solution D (stacking gel buffer) and solution E (SDS) were the same as described in Methods 2.10.1.

Solution A - separating gel buffer containing citric acid

Separating gel buffer contained 7ml of 1M citric acid, 9.1g Tris and 0.24ml TEMED made up to 100ml with distilled water.

Solution B - electrophoresis buffer containing boric acid

1 litre electrophoresis buffer contained 78.6g Tris, 19.52g boric acid and 1g SDS at pH 9.0.

Solution C - acrylamide

32g of acrylamide and 0.8g of NN' -methylene-bisacrylamide were made up to 100ml with distilled water.

Solution F - ammonium persulphate solution

Ammonium persulphate solution contained 0.105g made up to 100ml with distilled water.

2.10.4.2 Gel plates

Glass plates (dimensions 8.5 x 8cm and 8.1 x 8.1cm) were cleaned as described in Methods 2.10.1.2. One gel was assembled at a time using 1.5mm Teflon spacers, silicon grease and spring clips.

2.10.4.3 Preparation of 12.5% (w/v) acrylamide separating gel

The following volumes of the stock solutions were mixed:

Solution A - 8ml

Solution C - 8ml

Solution E - 0.32ml

The mixture was degassed for about 2 min. 16ml of the ammonium persulphate solution (F) was added and mixed and the solution was degassed for a few seconds. Using a pasteur pipette, the liquid gel was pipetted between the plates, an overlay of propan-2-ol was added, and the gel left to polymerize for about 30 min. The

propan-2-ol was poured off, the gel top washed several times with tap water and distilled water then blotted dry.

2.10.4.4 Preparation of 5.2% (w/v) acrylamide stacking gel

The following volumes of the stock solutions were mixed:

Solution D - 1ml

Solution C - 1.75ml

plus 5.5ml distilled water.

The solution was degassed, 15mg ammonium persulphate added, the solution mixed and degassed again. It was then poured on top of the main gel and an 8-track Teflon comb inserted. The gel was left to polymerize for about 15 min, stored at 4°C and used within 7 days.

2.10.4.5 Sample preparation and loading

The tracking dye solution used consisted of 5g sucrose, 1.5g SDS, 67mg citric acid, 0.45g Tris and 100µg Pyronin Y made up to 10ml with distilled water. Sample was prepared and loaded as described in Methods 2.10.1.5.

2.10.4.6 Electrophoresis conditions and sample running

The electrophoresis was carried out without cooling until the tracking dye was seen to reach the bottom of the gel (about 30 min).

2.10.5 2-Dimensional SDS-PAGE

2.10.5.1 Electrophoresis in the first dimension

Electrophoresis in the first dimension was as described in Methods 2.10.1 and 2.10.2.

2.10.5.2 Electrophoresis in the second dimension

Stock solutions, preparation of gel plates and preparation of the 12.5% (w/v) acrylamide stacking gel were as previously described in Methods 2.10.1.

2.10.5.2.1 Preparation of 5.2% (w/v) acrylamide stacking gel

The following volumes of the stock solutions were mixed:

Solution D - 10ml

Solution C - 17.5ml

plus 55ml distilled water.

The solution was degassed, 150mg ammonium persulphate added, mixed and degassed again. It was poured on top of the four separating gels in the

gel-making manifold and single track teflon combs inserted. Gels were left to polymerize for about 15 min, stored at 4°C and used within 7 days.

2.10.5.2.2 Sample preparation and loading

A track was cut from a previously run lipopolysaccharide SDS-PAGE gel and placed horizontally in the single track of the stacking gel. It was secured in place with sufficient 1% (w/v) hot liquid agarose and the agarose allowed to cool and set to seal the gel track from the ID gel onto the stacking gel.

2.10.5.2.3 Electrophoresis and sample running

The electrophoresis was carried out with a current of 90mA with cooling until the tracking dye was about two thirds of the way down the gel.

2.10.6 Gel scans

Stained gels were scanned using an LKB 2202 Ultroscan Laser Densitometer. A typical gel scan of *E. carotovora* lipopolysaccharide is shown in Results 3.2.1, Figure 3.6.

2.10.7 Photography

After staining gels were photographed by members of staff at the Medical Illustration Unit, University of Glasgow.

2.11 Different Conditions for the Preparation of Lipopolysaccharide Prior to SDS-PAGE

Standard conditions for the preparation of lipopolysaccharide samples for SDS-PAGE were as previously described in Methods 2.10.1.5.

2.11.1 Boiling of samples for different periods of time

Lipopolysaccharide samples were boiled at 100°C for 2, 5, 10 and 20 minutes prior to loading.

2.11.2 Mixing of two different lipopolysaccharide preparations

5µl of each lipopolysaccharide preparation ($10mg.ml^{-1}$) were mixed, 36µl 0.2M DTT and 20µl tracking dye added and then made to 100µl with distilled water. Samples were boiled for 2 min. and loaded as described in Methods 2.10.1.5.

2.11.3 Incubation with bacterial alkaline phosphatase

The method used was that described by Rosner *et al* (1979a).

100µl of lipopolysaccharide ($10mg.ml^{-1}$) was incubated at 58°C in an Eppendorf tube with 9.3 units of bacterial alkaline phosphatase (*E. coli*), type 111-S. A control was set up with 100µl of distilled water, replacing the lipopolysaccharide. At 0 min, 1h, 2h, 3h,

and 4h, 10µl samples were removed, mixed with 34µl distilled water, 36µl 0.2M DTT and 20µl tracking dye then boiled for 2 min. and loaded as described in Methods 2.10.1.5.

2.12 Stains for Gels

2.12.1 Coomassie blue stain for protein

The method of Lumsden and Coggins (1977) was used. Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue G250 in methanol/acetic acid/distilled water (50:10:40, by volume) for 1h at 60°C. The gels were destained with methanol/acetic acid/distilled water (10:10:80, by volume) at 60°C.

2.12.2 Silver stain for lipopolysaccharide

The silver stain method of Tsai and Frasch (1982) was used.

2.12.2.1 Stock solutions

Solution A

200ml of oxidising solution contained 1.4g of periodic acid in 40% ethanol/5% acetic acid (v/v) made up in distilled water. This was prepared as required.

Solution B

150ml staining reagent was prepared by adding 2ml

of 14.8 M NH_3 to 28ml of 0.1M NaOH. To this was added 5ml of 20% (w/v) silver nitrate solution with mixing. 115ml of distilled water was added 1 min. later. The staining reagent was prepared as required.

Solution C

1L of developing solution contained 50mg citric acid and 0.5ml formaldehyde (40% w/v) made up in distilled water. This was stored at room temperature.

2.12.2.2 Staining of the gel

All steps of the protocol were performed on an orbital shaker with gentle agitation at 23°C. Each step was performed in a clean glass dish and gels were only handled wearing rubber gloves.

After electrophoresis the gel was soaked in 200ml of 40% ethanol/5% acetic acid solution overnight. The gel was then oxidised for 5 min in 200ml of oxidising solution A. It was then washed three times in 500ml of distilled water with 15 min for each wash. The gel was then stained with 150ml of staining reagent solution B for 10 min. Another three washes were performed with 500ml of distilled water for 10 min each wash before development of the gel in 200ml of the developing solution C. The gel was only allowed to develop until the first band was seen to appear (about 20 sec) then quickly removed and washed several times with 500ml of distilled water (Figure 2.2, track 1). If the gel was

Figure 2.2

SDS-PAGE of E. carotovora subsp. atroseptica 1034 lipopolysaccharide developed for different periods of time during silver staining.

Each track contained 20μg of lipopolysaccharide and run on a 12.5 % acrylamide gel. After staining with silver reagent (b) pieces of the gel were developed for different periods of time in the developing solution (c) as described in Methods 2.12.2.

Tracks: (1) 20s development
(2) 60s development
(3) 90s development

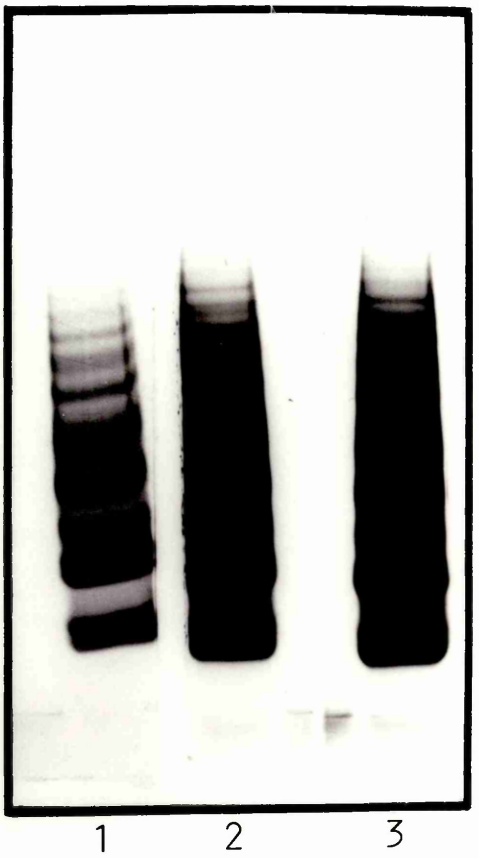


Figure 2.2.

left in the developer for a longer period of time than about 20 sec, the bands of the ladder pattern became darker and the background staining increased until very little detail could be observed (Figure 2.2, tracks 2 and 3). This overdevelopment is not obvious when the gel is still in the developer but becomes apparent later as the gel continues to develop when being washed with the distilled water. It was therefore found that the best time to remove the gel from the developer was when the first few bands were beginning to appear. Development of the rest of the ladder occurred in the distilled water washes.

Destaining of the gel was attempted with Acufix photographic fixer (Paterson Products Ltd., 301-311 Rainham Road South, Dagenham, Essex, England), at a 1:5 dilution with distilled water. The over-staining was reduced but the bands were not as clearly defined as when developed for the correct period of time.

Gels were stored flat in self-sealing plastic bags.

2.12.3 Silver stain for protein

The method described by Wray et al. (1981) was used.

2.12.3.1 Stock solutions

The same stock solutions B and C as described in Methods 2.12.2.1, were used.

2.12.3.2 Staining of the gel

All steps of the protocol were performed on an orbital shaker with gentle agitation at 23°C. As before each step was performed in a clean glass dish and gels were only handled wearing rubber gloves.

After electrophoresis the gel was soaked in 50% (v/v) methanol for at least one hour. It was then stained for 15 min with 150ml of staining reagent solution B. After staining, the gel was washed with 3 changes of 500ml of distilled water for 10 min each change. The gel was then placed in 200ml of developing solution C for approximately 10 to 15 min when the bands began to appear. To stop the development, the gel was transferred to 10% (v/v) methanol and kept.

2.13 Polyclonal Antiserum

2.13.1 Introduction

Two of the antisera used in the immunological methods were gifts from Dr. D.C. Graham (DAFS). The antisera were raised against glutaraldehyde treated whole cells of subsp. atroseptica strains 436 and 1042. Both strains typed into serogroup I.

2.13.2 Raising of polyclonal antisera

The other rabbit polyclonal antisera used in this study were raised against whole, live bacterial cells (Table 2.2). The animal procedures were carried out by

TABLE 2.2 **E. carotovora strains used for the raising of polyclonal antisera**

Strain	Serogroup
1034	I
G301	XVIII (*Ecc*)
6	XVIII (*Eca*)
1056	XX
198	XXII

Dr. I.D. Hamilton. The immunisation protocol used was as described by de Boer et al. (1979).

2.13.2.1 Preparation of cells

Erwinia stock cultures were streaked out on nutrient agar plates and grown as described in Methods 2.4.1. Cells were suspended in 5ml sterile distilled water and the absorbance measured at 420nm. At this wavelength, an absorbance of 1 is produced by a cell density of 1.48×10^8 cells per ml. The cell suspension was washed and made up in sterile distilled water to give a final cell concentration of 10^{10} cells.ml^{-1}.

2.13.2.2 Injection of rabbit and preparation of serum

0.5ml of cells were emulsified with an equal volume of Freund's incomplete adjuvant (Difco Laboratories). A New Zealand white rabbit weighing about 4 Kg, was injected subcutaneously at several different sites with the whole volume (0.1ml/site). After a 3 week interval 0.3ml, 0.5ml, 1ml and 2ml of bacterial suspension (2.5×10^9 cells ml^{-1}) without adjuvant were injected at 3 day intervals. One week after the final injection, a test bleed of about 20ml was collected from the rabbit ear's marginal vein. Blood samples were allowed to clot at room temperature and left overnight at 4°C. The serum was pipetted off and centrifuged at 1,200g for 25 min in a bench top centrifuge. One ml samples were aliquoted

into Eppendorf tubes and the aliquots were stored at -20°C.

2.13.3 Titre of polyclonal rabbit antiserum

2.13.3.1 Immunoblotting

In immunoblots of lipopolysaccharide purified from the inoculating strains, antisera raised against whole cells from serogroup I, XVIII (Ecc), XX and XXII could be used at the normal dilution of 1:50 (Methods 2.14.2). Antiserum raised against serogroup XVIII (Eca) had a lower titre against its own lipopolysaccharide and was used at a 1:20 dilution.

2.13.3.2 Agglutination

Titres for the polyclonal antisera raised against whole cells from serogroup I, XVIII (Ecc), XVIII (Eca), XX and XXII were determined using the Widal agglutination method (Methods 2.14.3) and are summarised in Table 2.3.

2.14 Immunological Methods

2.14.1 Immunoblotting method

The method used was based on that originally described by Towbin et al (1979) as modified by Nimmo et al (1986).

TABLES 2.3 **Titres of polyclonal antisera raised against whole cells of E. carotovora from the important potato pathogenic serogroups**

Antiserum raised against			Serogroup of cells			
Strain	serogroup	I	XVIII(*Ecc*)	XVIII(*Eca*)	XX	XXII
1034	I	1:512	1:64	1:8192	-	1:8192
G301	XVIII(*Ecc*)	-	1:2048	1:8192	-	1:8192
6	XVIII(*Eca*)	-	1:8192	1:8192	-	1:8192
1056	XX	-	-	1:128	1:8192	-
198	XXII	1:256	-	1:256	-	1:8192

2.14.1.1 Stock solutions

Solution A - transfer buffer

Transfer buffer contained 0.19M glycine, 0.025M Tris, 0.02% (w/v) SDS and 20% (v/v) methanol. This was prepared as required.

Solution B - blocking buffer

Blocking buffer consisted of 0.02M Tris buffer, pH 7.2 containing 0.15M NaCl and 0.5% (v/v) Tween 20. It was stored at room temperature.

Solution C - first incubation buffer

First incubation buffer consisted of 0.02M Tris buffer, pH 7.2 containing 0.15M NaCl, 0.5% (v/v) Tween 20 and 20% (v/v) normal donkey serum. This was prepared as required.

Solution D - second incubation buffer

Second incubation buffer consisted of 0.02M Tris buffer, pH 7.2 ccntaining 0.15M NaCl and 20% (v/v) normal donkey serum. This was prepared as required.

Solution E - 4-chloro-1-naphthol stain

A solution of 30mg 4-chloro-1-naphthol in 10ml methanol, 50ml 10mM Tris buffer, pH 7.5 and 150µl 4% (w/v hydrogen peroxide AR (1ml 30% (w/v) hydrogen peroxide AR: 6.5ml distilled water) were mixed immediately before use. It was found that preparation of the stain immediately before use gave the best staining results of the immunoblots.

2.14.1.2 Immunoblotting conditions

SDS-PAGE was carried out as described in Methods 2.10. After electrophoresis, the lipopolysaccharide was transferred from the gel to 0.45 μ nitrocellulose paper (Anderman and Co., Kingston upon Thames, Surrey, KT2 6NH) using a Bio-Rad Trans-blot cell containing transfer buffer (solution A). The transfer was run at 300mA with cooling for 4h after which the nitrocellulose paper was placed in blocking buffer (solution B) and incubated at 4°C for at least 16h. The nitrocellulose paper was then transferred to the first incubation buffer (solution C) to which the antibody of interest was added to give a final dilution of 1:50 (see Methods 2.13.3.1). This was incubated at 23°C for 90 min after which the nitrocellulose paper was washed with four changes of 100ml of 0.02M Tris buffer, pH 7.2 containing 0.15M NaCl and 0.5% (v/v) Tween 20 for 12 min each change. It was then washed for 12 min with 100ml of 0.02M Tris buffer, pH 7.2 containing 0.15M NaCl. The nitrocellulose paper was placed in the second incubation buffer (solution D) to which horseradish peroxidase conjugated donkey anti-rabbit IgG was added to give a final dilution of 1:500 and the paper incubated at 23°C for 90 min. The nitrocellulose paper was then washed with 5 changes of 100ml of 0.02M Tris buffer, pH 7.2, containing 0.15M NaCl, for 12 min each change. The paper was then placed in the 4-chloro-1-naphthol stain (solution E) for about

2 min at 23°C when the staining was clearly defined and then washed with several changes of distilled water. The nitrocellulose paper was then dried and stored.

The concentrations of serum, antiserum and conjugated antiserum used were determined to be the optimum for the development of lipopolysaccharide immunoblots. A concentration of 20% (v/v) normal donkey serum was determined to be the optimum from testing 5%, 10%, and 20% (v/v) normal donkey serum. A dilution of 1:50 anti-Erwinia antiserum was determined to be the optimum from testing 1:200, 1:100, and 1:50 dilutions of antiserum. A 1:500 dilution of HRP-conjugated donkey anti-rabbit IgG was determined to be the optimum from testing 1:2000, 1:1000, and 1:500 dilutions of conjugated antiserum.

2.14.2 Double immunodiffusion

The method used was as described by Zamze et al. (1986).

Double immunodiffusion was performed in small plastic petri dishes (5cm diameter; Sterilin Ltd). 0.65% (w/v) agarose type II, was prepared by boiling the agarose in 5mM Na H_2PO4/140mM NaCl, pH 7.2 (PBS) containing 0.02% (w/v) NaN_3. 5ml of hot agarose solution was added to each plate and allowed to set. The wells were cut out of the agarose. Wells (0.7cm diameter) were arranged 0.7cm apart in a hexagonal

pattern around a central well. The central well was filled with 20µl of undiluted antiserum. To 30µl of lipopolysaccharide solution (1mg ml^{-1} in distilled water) was added 20µl of PBS containing 0.02% (w/v) NaN_3 and 0.2% (w/v) SDS. This 50µl of lipopolysaccharide solution was added to the surrounding wells. The plates were incubated at room temperature for a week before precipitation lines could be observed.

2.14.3 Widal agglutination

Widal agglutination tests were carried out on whole bacteria using the method described by Mackie and McCartney (1956).

2.14.3.1 Stock solution

Solution A - sodium chloride solution

Sodium chloride was made to a final concentration of 0.85% (w/v) with distiled water. To this was added sodium azide to a final concentration of 0.02% (w/v). The sodium chloride solution was stored at room temperature.

2.14.3.2 Preparation of cells

Growth of cells was in 10mM glucose minimal salts medium, pH 7.0 as described in Methods 2.2.1. Cells were harvested and washed with solution A then resuspended with solution A to give an absorbance of 5.0 at 500nm.

2.14.3.3 Agglutination assay

Agglutination assays were performed in 96-well plastic plates (Becton Dickinson Laboratories, Becton Dickinson and Co., 1950 Williams Drive, Oxnard, California). The antibody preparation was initially diluted to 1:16 (v/v) final concentration with solution A. 50µl of this initial antibody preparation was added to each well of row 1 of the plate. 50µl of solution A was then added to all the wells of the plate including the wells of row 1. By serial dilution 50µl of antibody solution (1:32 (v/v) concentration) was then transferred down the plate using an octapipette from the wells of row 1 to the wells of row 8. The final excess 50µl of antibody solution from the wells of row 8 was discarded. 50µl of resuspended cells (Methods 2.14.3.2) was added to each well of the plate. This gave a final antibody dilution of 1.64 (v/v) to 1:8192 (v/v) down the plate from row 1 to row 8, respectively. The plate was incubated at 23°C for 2h and then checked for agglutination using a light microscope at x4 magnification.

2.15 Absorption of Antisera

2.15.1 Absorption of antisera with lipopolysaccharide

20µl lipopolysaccharide solution (10mg ml^{-1}) was added to 1ml of undiluted antiserum in an Eppendorf

tube, vortexed for 30s then incubated at room temperature for 1h. It was then centrifuged in an Eppendorf 3200 centrifuge at 10,000g for 2.5 min to spin down the antibody-antigen complexes. The supernatant was retained and the procedure was repeated another three times with 20µl of lipopolysaccharide solution (10mg ml^{-1}). The resulting supernatant was incubated with a further 50µl lipopolysaccharide solution (10mg.ml^{-1}) for 2h at room temperature, centrifuged and the supernatant retained. 100µl lipopolysaccharide solution (10mg.ml^{-1}) was added to this penultimate supernatant and incubated at room temperature for about 16h after centrifugation. The final supernatant was retained and stored at -20°C.

2.15.2 Absorption of antiserum with bacterial cells subjected to mild acid hydrolysis

2.15.2.1 Preparation of cells

Cells were harvested from 10ml of nutrient broth grown at 23°C for 48h. After washing with 10mM Tris/HCl, pH 7.4, the cell pellet was resuspended in 1ml 10mM Tris/HCl, pH 7.4 and centrifuged in a Eppendorf 3200 centrifuge at 10,000g for 2.5 min.

2.15.2.2 Mild acid hydrolysis

The conditions normally applied to the removal of

the lipid A moiety from lipopolysaccharide were used (Methods 2.17.1.1). The cell pellet was resuspended in 1ml of 1% (v/v) glacial acetic acid and incubated at 100°C for 90 min. The suspensions were centrifuged in an Eppendorf 3200 centrifuge for 2.5 min at 10,000g, washed twice with 10mM Tris/HCl, pH 7.4 and the pellets resuspended in 1ml distilled water.

2.15.2.3 Absorption of Antisera

100µl of cell suspension of the hydrolysed cells was added to 500µl of undiluted antiserum, vortexed for 30s then incubated at room temperature for 1h. It was then centrifuged in an Eppendorf 3200 centrifuge for 2.5 min at 10,000g. The supernatant was retained and subjected to the same procedure another three times with 100µl of cell suspension each time. The resulting supernatant was incubated with a further 200µl of cell suspension for 2h at room temperature, centrifuged and the supernatant retained. 400µl of cell suspension was added to the penultimate supernatant and incubated at room temperature for about 16h. After centrifugation the final supernatant was retained and stored at -20°C.

2.16 Incubation of Lipopolysaccharide with Chymotrypsin

10µl of lipopolysaccharide solution (10mg.ml^{-1}) was incubated overnight with 10µl chymotrypsin solution

($5mg.ml^{-1}$) at 23°C, prior to electrophoresis. The sample was subsequently made up with 24µl distilled water, 36µl 0.2M DTT and 20 µl tracking dye for loading onto gelsas described in Methods 2.10.1.5.

2.17 GLC of Sugars from the Lipopolysaccharide O-Chain

2.17.1 Routine GLC of Alditol Acetates

The monosaccharides from the lipopolysaccharide O-chain were routinely analysed as their respective alditol acetates by capillary column glc using a Perkin-Elmer 8420 gas chromatograph (Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, England) fitted with a flame ionisation detector. The column used was anSGE (Scientific Glass Engineering Pty Ltd., 7 Argent Place, Ringwood, Victoria, 3134, Australia) bonded phase (0.1µm film thickness) capillary column, BP-225, bonded to a vitreous silica column (25m x 0.22mm). BP225 is a stationary phase equivalent to OV-225 (SGE catalogue). The analyses were carried out isothermally at 240°C. Injection port and detector temperatureswere 300°C. Nitrogen was the carrier gas at a flow rate of 0.7 $ml.min^{-1}$.

2.17.1.1 Production of Alditol Acetates

The method of Ray et al. (1986) was used to

hydrolyse the lipid A moiety from the lipopolysaccharide and the subsequent derivatisation was as described by Albersheim et al. (1967).

To remove the lipid A moiety, 1ml of lipopolysaccharide solution (10mg.ml^{-1}) was hydrolysed with 1% (v/v) glacial acetic acid final concentration in acid washed, teflon-lined screw cap tubes at 100°C for 90 min on a heating block (Tecam Dri-Block DB3). After cooling, the lipid A was removed by 4 extractions with an equal volume of diethyl ether. Any excess diethyl ether in the remaining polysaccharide fraction was blown off under a stream of nitrogen gas. The polysaccharides produced were hydrolysed in 1M trifluoroacetic acid at 120°C for 1h to give their constituent monosaccharides. This hydrolysate was then dried under a stream of nitrogen gas at 70°C. 10mg of sodium borohydride in 1ml of 0.5M NH_3 was added to the residue and the mixture incubated at room temperature for at least 1h to allow reduction of sugars to alditols. 1 ml of dry methanol was added to the alditol mixture and the mixture evaporated to dryness at 70°C under a stream of nitrogen gas. This was repeated another 5 times with 1ml volumes of dry methanol. 1ml of an acetic anhydride/dry pyridine (4:1, v/v) mixture was then added to the residue and the solution heated at 120°C for 3h. The sample was then evaporated to dryness at 70°C under a stream of nitrogen gas and then made up in 0.5ml

dichloroethane prior to injecting onto the column. 1µl of sample was injected using either a 1µl or 5µl Hamilton syringe (Hamilton Company, Reno, Nevada).

2.17.1.2 Response Factor Determination for Standard Sugars

Preliminary experiments showed that lipopolysaccharide did not contain sugars which gave an alditol acetate with a similar retention time to 2-deoxyglucose. 2-deoxyglucose was then used as the internal standard. It was necessary to determine the Response Factor (R) for each standard sugar relative to the internal standard for quantitation as the detector may respond differently to each sugar derivative. This was done by injecting alditol acetate solutions of exactly 5.0mg of 2-deoxyglucose in combination with exactly 5.0 mg of one of the following standard sugars: erythrose; rhamnose; fucose; ribose; xylose; mannose; galactose; and glucose. The Response Factor for alditol acetate x was determined as:

$$Rx = \frac{\text{Peak Area of 2-deoxyglucose}}{\text{Peak Area of Standard Sugar}_x}$$

2.17.1.3 Determination of Molar Ratios

Molar ratios were calculated relative to glucose as preliminary experiments showed that lipopolysaccharide

from several different serogroups all contained glucose. The Response Factor (R) determined above was used to calculate the amount (mg) of each identifiable sugar present in the sample using the equation:

$$\text{mg sugar} = \frac{\text{Area sugar x R x Amount 2-deoxyglucose}}{\text{Area 2-deoxyglucose}}$$

This figure was converted to moles and then the molar ratio for that sugar, relative to glucose, calculated.

2.17.2 GLC - Mass Spectrometry

GLC-MS of E. carotovora subsp. atroseptica 1034 lipopolysaccharide was performed by Dr. C. Griffiths (Chemistry Dept., DAFS).

2.17.2.1 GLC - MS of Alditol Acetates

Sugar alditol acetates were prepared as described (Methods 2.17.1.1) but were made up in 100ml chloroform prior to injection. Sample volume was 1µl. Mass spectra were obtained with a Finnigan-MAT 4500 quadrupole mass spectrometer, interfaced to a Finnigan-Mat 9610 gas chromatograph (Finnigan-Mat, 355 River Oaks Parkway, San Jose, California, USA). An on-column syringe (OC1-2, SGE, Australia) was used on the gas chromatograph, enabling 1µl aliquots to be injected directly into a Quadrex (Quadrex Corporation,

PO Box 3881, New Haven Conneticut, U.S.A.) bonded phase (0.25μm film thickness) capillary column, OV-225, bonded to a fused silica column (25m x 0.25mm). Helium was the carrier gas and was used at 27cm sec^{-1}. The oven temperature was programmed to increase from 60°C (held for 1 min) to 200°C at 25°C.min^{-1} then increased to 250°C at 10°C min^{-1}. The column was connected directly to the ion source which was held at about 230°C. The filament emission current was operated at 0.2mA and the electron energy was 70eV. The quadrupole analyser gave unit resolution to 1000 a.m.u. (approx) and the scale was calibrated using the mass spectrum produced from perfluorotributylamine. The analyser was programmed to scan from 50-500 a.m.u. in a 1 second interval. The electron multiplier was set at 1800 volts providing full scan spectra from a few nanograms per component. Data were acquired on a Data General Nova 3/12 computer and stored on a 96 M byte module drive system (Newbury Data Recording Ltd., Hawthorne Road, Staines, Middlesex, TW18 3BJ). Computer control and scanning parameters were manipulated via a Microcolour Graphics terminal M 2250-T (Microcolour Graphics Ltd., Futures Way, Bolling Road, Bradford, BD4 7TU).

2.17.2.2 Interpretation of Mass Spectra

Mass spectrometry, as a general rule, provides final and conclusive evidence for identification of an

unknown substance provided that a high resolution spectrum is obtained. The mass spectrum of a compound consists of a characteristic pattern of peaks representing molecular fragments with different mass to charge (m/e) ratios produced by bombardment of the compound with electrons in the ionisation chamber. By chemical ionisation with a reagent gas a pseudomolecular ion is produced which provides the value of the molecular weight of the ion (Harrison, 1983). D-glucitol hexaacetate derived in this study (Figure 2.3) produced the expected molecular ion fragments of 73, 145, 217, 289 and 361 a.m.u. for D-glucitol hexaacetate as revealed by other studies (Bjorndal et al, 1970). However, the molecular ion (M+) for D-glucose hexaacetate (433 a.m.u.) is not obviously apparent in this mass spectrum.

2.17.3 Thin Layer Chromatography

The method used was as described by Komagata and Suzuki (1987).

10cm x 4cm cellulose t.l.c. plates (ART5577; E. Merck, Darmstadt, FRG) were used. Samples were applied as spots with a glass micropipette 1cm from the bottom of the plate.

2.17.3.1 Development of the Chromatogram

All thin layer chromatograms were run at room

Figure 2.3

Mass spectral fragmentation pattern of D-glucitol hexaacetate

The alditol acetate of D-glucose was prepared as described in Methods 2.17.1.1. It was separated on a Quadrex bonded phase (0.25μm film thickness) capillary column, OV-225, bonded to a fused silica column (25m x 0.25mm) attached to a mass spectrometer as described in Methods 2.17.2.

Annotations

*: Common molecular ion fragment characteristic of a sugar alditol acetate produced under the conditions described in Methods 2.17.2.

M+: parent ion.

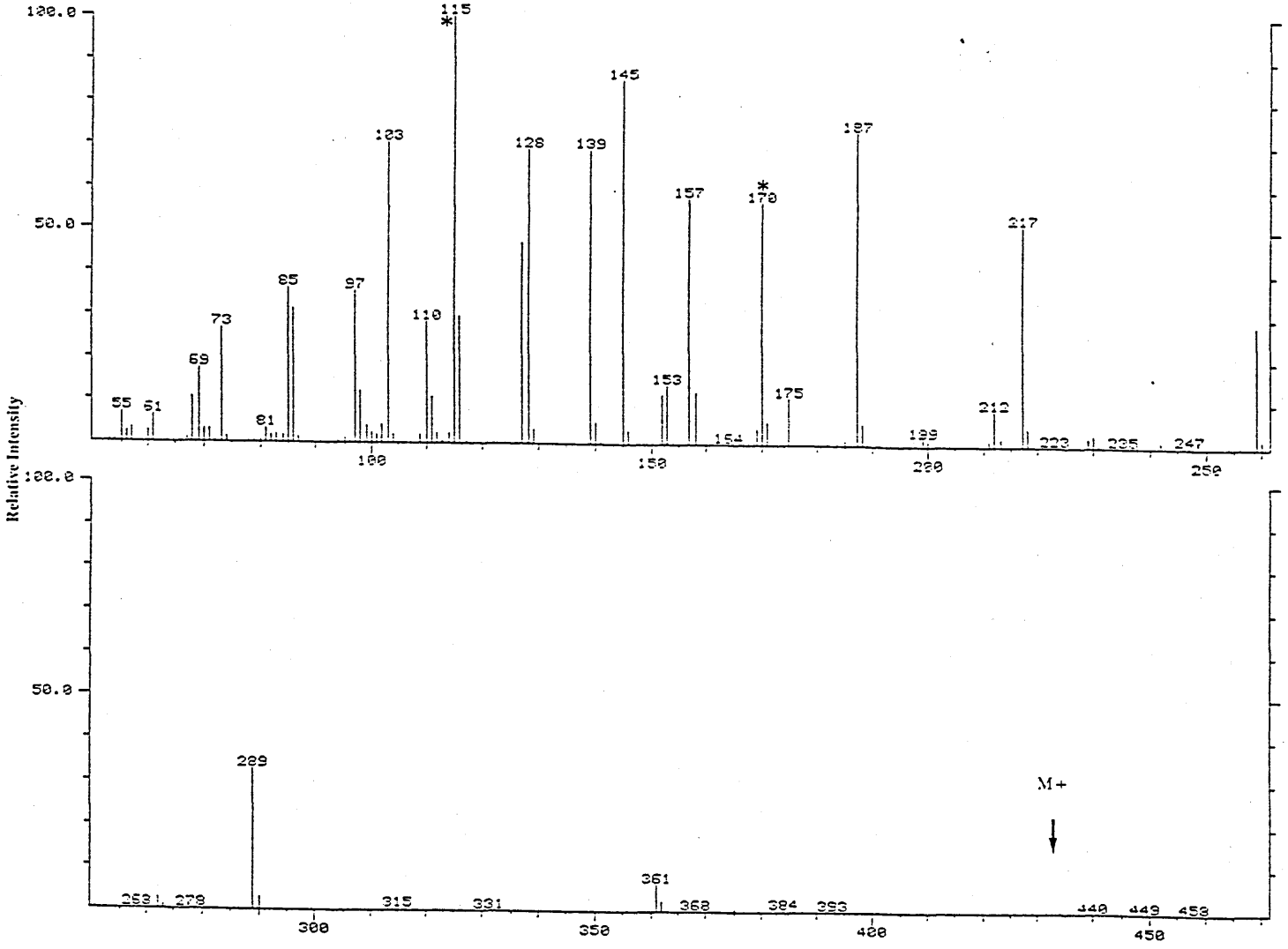


Figure 2.3. D-glucose

temperature in an ascending direction in an Eastman 'Chromatogram' Developing Apparatus Model 104 (Kodak Ltd., London). The solvent system used was n-butanol/water/pyridine/toluene (10:6:6:1, by volume) with 10ml of this solvent used in the trough. A glass beaker was placed over the apparatus to help maintain a solvent saturated atmosphere.

2.17.3.2 Preparation of Standards

3 standard sugar mixtures were prepared containing 0.1% (w/v) of each sugar in distilled water:

a) Galactose, arabinose and xylose;

b) Rhamnose, glucose and ribose; and

c) Fucose, glucose and ribose.

2.17.3.3 Preparation of Sample

The monosaccharide fraction of the lipopolysaccharide used was prepared as described for the preparation of lipopolysaccharide sugars for the glc alditol method (Methods 2.17.1.1).

2.17.3.4 Loading and Development of Chromatogram

0.5µl of each standard sugar solution was applied and optimally 15µl of the monosaccharide fraction. The chromatogram was run for about 1h.

2.17.3.5 Visualisation of Sugars

The plate was allowed to dry in the fume cupboard then sprayed with a solution of 100ml water saturated n-butanol, 2ml aniline and 3.25g phthalic acid (Smith and Feinberg, 1965). The sprayed plate was heated at 100°C for 4 min. Maroon coloured spots were observed for the sugars.

2.18.1 Fractionation of the Polysaccharide Fraction of Lipopolysaccharide by Gel Filtration

2.18.1.1 Sample Preparation and Loading

The method of Ray et al.(1986) was used.

The polysaccharide fraction released after hydrolysis of 1ml of lipopolysaccharide solution (10mg ml^{-1}) as described (Methods 2.17.1.1) was freeze-dried and resuspended in 1ml of 0.05M pyridine/0.05M acetic acid buffer, pH 5.4. Buffer was pumped through the column at 50ml.h^{-1} and fractions collected from the column at 12 minute intervals. Fractions were assayed for carbohydrate at A_{480} using the phenol-sulphuric acid assay (Methods 2.20.3.).

2.18.1.2 Calibration of the Column

The void volume (Vo) was determined by loading 5ml of Dextran Blue 2000 at a concentration of 1mg.ml^{-1} in 0.05M pyridine/0.05M acetic acid buffer, pH 5.4 onto the

column. Buffer was pumped through the column at 50ml h^{-1} and the elution of Dextran Blue monitored at A_{280}. Under the same conditions, 4ml of Ribonuclease A (Mr 13,700) at a concentration of 10mg.ml^{-1} in 0.05M pyridine/0.05M acetic acid, pH 5.4 was loaded onto and eluted from the column.

2.19 Modification of Lipopolysaccharide Sugar Residues

2.19.1 Galactose Oxidase Treatment of Whole Lipopolysaccharide

The method used was based upon those described by Avigad et al (1962) and Hugget and Nixon (1957).

2.19.1.1 Stock Solutions

Solution A - peroxidase chromogen buffer

Peroxidase-chromogen buffer contained 5mg dianisidine/HCL and 5mg peroxidase made up in 100ml of 0.04M KH_2PO_4, pH 7.0 and stored at 4°C.

Solution B - galactose standard solution

Galactose was made up in distilled water to a final concentration of 1mM and stored at 4°C.

Solution C - galactose oxidase

150 units of galactose oxidase type V were made up in 400µl of 0.04M KH_2PO_4, pH 7.0 and stored at 4°C.

2.19.1.2 Reaction Assay

To 500µl of solution A and 490µl of solution B or 100µl of lipopolysacharide solution (10mg.ml^{-1}) made to 490µl with distilled water was added 10µl of solution C. After mixing the mixture was incubated at 30°C and absorbance readings were taken every 10 min at 223nm in a LKB Ultraspec II spectrophotometer (LKB Instruments Ltd., L.H. Engineering Co. Ltd., Stoke Poges). Absorbance readings were made against a distilled water blank and two controls were set up to correct for background due to absorbance of solutions A and B.

2.19.2 Periodate Oxidation of Whole Lipopoly-saccharide

The method used was as described by Dixon and Lipkin (1954).

2.19.2.1 Stock Solutions

Solution A - glucose standard solution

Glucose was made up in distilled water to a final concentration of 40mM and stored at 4°C.

Solution B - so dium metaperiodate solution

Sodium metaperiodate was made up in distilled water to a final cocentration of 240mM. This concentration was calculated to give a 20% excess of periodate with respect to glucose as recommended in the method based on the fact that five moles of periodate oxidise one mole

of glucose (Percival, 1962). The solution was stored at room temperature.

2.19.2.2 Reaction Assay

To 500µl of solution A or 20µl of lipopolysaccharide solution (10mg.ml^{-1}) made to 500µl with distilled water, 500µl of solution B was added. After mixing oxidation was assayed by measuring the change in absorbance at 223nm in a LKB Ultraspec II spectrophotometer against a distilled water blank. Controls contained either sugar/lipopolysaccharide solution or periodate solution. Problems were encountered with precipitation of the lipopolysaccharide in the assay mixture which made assaying for the oxidation of the lipopolysaccharide difficult. To overcome this, 1ml of lipopolysaccharide assay mixture was taken at 10 minute intervals and centrifuged in an Eppendorf 3200 centrifuge for 2.5 min at 10,000g and the supernatant assayed for oxidation. But despite centrifugation to remove precipitated material oxidation was still difficult to detect.

2.20 Assays of Whole Lipopolysacchride

2.20.1 Protein Estimation

The method of Lowry et al. (1951) was used to estimate the protein content of lipopolysaccharide

preparations using bovine serum albumin as standard (0-200 µg BSA).

2.20.2 Nucleic Acid Estimation

The method of Maniatis et al. (1982) was used to estimate the nucleic acid content of lipopolysaccharide preparations. The absorbance of the lipopolysaccharide solution was measured at 260nm. At this wavelength, an absorbance of 1 is produced by nucleic acids at a concentration of 50µg.ml^{-1}.

2.20.3 Total Carbohydrate Assay

The method used was that described by Ashwell (1966).

2.20.3.1 Stock Solutions

Solution A - glucose standard solutions

0, 10, 20, 50 and 70µg of glucose standards were each made up in 2ml of distilled water and stored at 4°C.

Solution B - phenol solution

20ml of distilled water was added to 80g of phenol to give a final concentration of 80% (w/v) phenol solution. The solution was stored at room temperature.

2.20.3.2 Reaction Assay

To 2ml of each of the standard sugar solutions A or

10µl of lipopolysacharide solution (10mg.ml^{-1}) made up to 2ml with distilled water was added 50µl of phenol solution B and vortexed. All samples were prepared in triplicate. As quickly as possible (about 5s), 5ml of concentrated sulphuric acid was added to a sample from a fast-flowing pipette and vortexed. This was done for all samples. After standing at room temperature for 30 min the absorbance values were read at 480nm in a LKB Ultraspec II spectrophotometer against a distilled water blank.

2.20.4 Total Heptose Estimation

The method used was that described originally by Dische (1953) and modified by Osborn (1963).

2.20.4.1 Stock Solutions

Solution A - mannoheptulose standard solution

0, 25, 50, 75 and 100µg of mannoheptulose standards were each made up in 500µl of distilled water and stored at 4°C.

Solution B - sulphuric acid solution

Sulphuric acid: 6 volumes of concentrated H_2SO_4 were added to 1 volume of distilled water and stored at room temperature.

Solution C - cysteine-HCL solution

Cysteine-HCl: cysteine-HCl was made up in distilled water to a final concentration of 3% (w/v) and stored at 4°C.

2.20.4.2 Reaction Assay

500µl of each of the mannoheptulose standard solutions A and 50µl of lipopolysaccharide solution ($10mg.ml^{-1}$) made up to 500µl with distilled water were prepared in duplicate and incubated in an ice water bath for 10 min. 2.25ml of solution B was slowly added to each sample and vortexed fore 1 min. After 3 min, samples were tranferred to a water bath at 20°C and incubated a further 3 min. Samples were then boiled in a boiling water bath for exactly 10 min. After cooling, 50µl of solution C was added to only one sample of each duplicate; the other sample which served as a blank had 50µl of distilled water added. Absorbance values were read at 505nm and 545nm exactly 2h after the addition of solution C in a LKB UltraspecII spectrophotometer. To determine the total heptose content, the absorbance value at 656nm was subtracted from the absorbance value at 505nm.

2.20.5 Total Uronic Acid Estimation

The method used was as described by Blumenkrantz and Absoe-Hansen (1973).

2.20.5.1 Stock Solutions

Solution A - galacturonic acid standard solutions

0, 0.5, 1, 5, 10 and 20µg of galacturonic acid standards were each made up in 200µl distilled water,

and stored at 4°C.

Solution B - tetraborate solution

Sodium tetraborate was made up to a final concentration of 0.0125M in concentrated H_2SO_4, and stored at room temperature.

Solution C - 3-phenylphenol solution

3-phenylphenol was made up to a final concentration of 0.15% (w/v) in 0.5% (w/v) NaOH, and stored at 4°C.

Solution D - sodium hydroxide solution

Sodium hydroxide was made up to a final concentration of 0.5% (w/v) in distilled water, and stored at room temperature.

2.20.5.2 Reaction Assay

To duplicate samples of 200µl of each of the galacturonic standard solution S and 20µl of lipopolysacharide solution (10mg.ml^{-1}) made up to 200µl with distilled water was added 1.2ml of solution B. Samples were refrigerated for 10 min. on ice, vortexed, boiled for 5 min and then cooled in an ice-water bath. 20µl of solution C was added to only one sample of each duplicate. 20µl of solution D was added to the other tube which served as a blank. Samples were vortexed and values were read at 520nm within 5 min of adding solution C in a PU8670 VIS/NIR spectrophotometer (Pye Unicam Ltd., York Street, Cambridge, CB1 2PX)

2.21 pH Measurement

The pH values of all solutions were determined using a direct reading pH meter (Model 7010, E.I.L. Ltd., Cumbernauld, Glasgow G67 1AG) connected to a combined glass electrode (224; Probion Ltd., Glenrothes, Fife, KY6 3AE).

2.22 Glassware

2.22.1 General Glassware

All growth flasks were cleaned before use by autoclaving in 1% (w/v) Haemosol solution (Meinecke and Co., Baltimore, U.S.A.). The glassware was thoroughly rinsed with tap water and then distilled water before drying in an oven. Other glassware was washed in Haemosol, rinsed and dried.

2.22.2 Pipettes

All pipettes were cleaned by soaking in 'Kirbychlor' disinfectant solution (H. and T. Kirby and Co. Ltd., Meldenhall, Suffolk) then in Haemosol solution followed by thorough rinsing in tap water and deionised water and drying in an electrically heated pipette drier. All pipettes were plugged with non-absorbent cotton wool.

2.23 Safety

Bacterial cultures were killed by autoclaving before disposal. Any bacterial spillage was swabbed with 10% (v/v) propan-1-ol. All other precautions taken in the interest of safety were as described in the University of Glasgow Safety Handbook.

RESULTS

1. Extraction and Purification of E. carotovora Lipopolysaccharide

1.1 Extraction of Lipopolysaccharide under Different Conditions

Lipopolysaccharide was extracted from E. carotovora subsp. atroseptica strain 1034 by the phenol/water method of Westphal and Jann (1965) and the phenol/chloroform/petroleum spirit method of Galanos et al (1969) to observe the effect of these extraction methods on both the yield of lipopolysaccharide obtained (as g dry weight lipopolysaccharide/g dry weight of bacteria) and on the electrophoretic pattern produced on SDS-PAGE gels (Table 3.1, Figure 3.1). Strain 1034 was chosen as the standard strain for serogroup I as a result of consultation with research staff at DAFS and SCRI. It was used as the standard strain in all comparative analyses as 80% of all subsp. atroseptica strains type into serogroup I in Scotland.

A 100% increase in the yield of lipopolysaccharide was obtained by performing three phenol/water extractions of the cellular material (Methods 2.7.1) instead of the two extractions recommended by the Westphal and Jann (1965) method (Table 3.1).

Figure 3.1, Table 3.1

Extraction of Lipopolysaccharide under Different Conditions

Lipopolysaccharide was extracted from E. carotovora subsp. atroseptica strain 1034 under different conditions to determine the effect of the following on lipopolysaccharide yield and electrophoretic pattern:

a) Growth of cells on 20mM glucose minimal salts medium (Methods 2.2.2) and double strength nutrient broth (2 x NB) (Methods 2.2.1);

b) Phenol/water (PW) extraction (Methods 2.7.1) or phenol/chloroform/petroleum spirit (PCP) extraction (Methods 2.7.2);

c) Two or three extractions of cellular material (Methods 2.7.1);

d) Late log/early stationary phase cells (A_{500} = 5) or early log phase cells (A_{500} = 0.6) as described in Methods 2.5.

TABLE 3.1 The effect on the yield of lipopolysacharide extracted under different conditions

Track No.	Growth Medium	Extraction Method	No. of Extractions	Growth Phase	Yield %(w/w)
1	2 x NB	PCP	-	late log	0.35
2	Glucose	PW	3	late log	3.14 $\pm$ 0.2
3	2 x NB	PW	3	late log	3.56
4	2 x NB	PW	2	late log	1.16
5	2 x NB	PW	3	early log	1.25

Figure 3.1

SDS-PAGE of lipopolysaccharide extracted from E. carotovora subsp. atroseptica strain 1034 in a number of different ways.

Each track contained 20μg of lipopolysaccharide and run on a 12.5% acrylamide gel and stained as described in Methods 2.12.2. These are the optimal conditions used for electrophoresis of E. carotovora lipopolysaccharide as described in Methods 2.10.1. Track numbers correspond to those in Table 3.1.

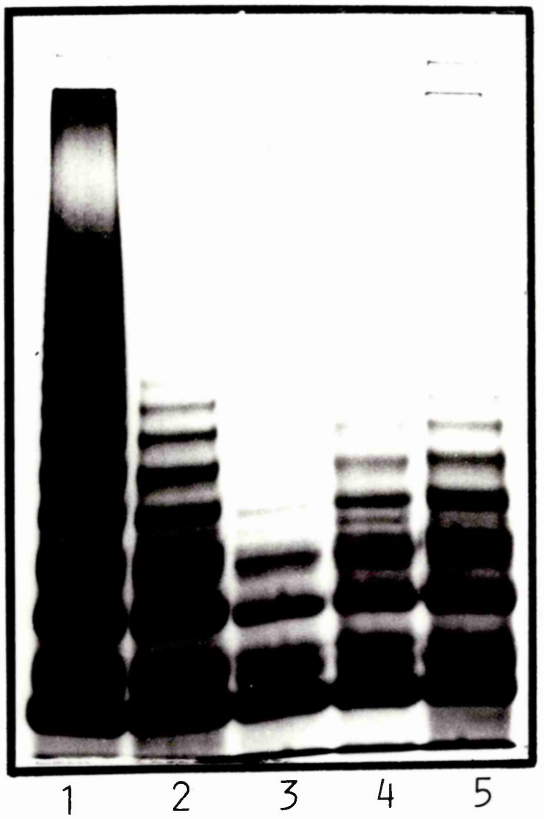


Figure 3.1.

Lipopolysaccharide extracted by the phenol/chloroform/petroleum spirit method (Methods 2.7.2) gave a yield which was only about 10% (w/w) of that obtained for lipopolysaccharide extracted by the phenol/water method (Table 3.1).

Similar yields (Table 3.1) were obtained for lipopolysaccharide extracted by the phenol/water method from cells grown on 20mM glucose minimal salts medium (Methods 2.2.2) and double strength nutrient broth (Methods 2.2.1). Phenol/water extracted lipopolysaccharide from early log phase cells gave a yield about one third of that obtained for lipopolysaccharide similarly extracted form late log/early stationary phase cells (Table 3.1). Although the yields varied for lipopolysaccharide extracted by these different methods and from the different stages of bacterial cell growth, very similar electrophoretic patterns were produced by the lipopolysaccharides (Figure 3.1) extracted by the different procedures summarised in Table 3.1.

1.2 Protein Content of Lipopolysaccharide Preparations.

Four of the five different lipopolysaccharide preparations (Figure 3.1., tracks 2 - 5) were run on another SDS-PAGE gel but stained specifically for protein (Methods 2.12.3). Nanogram quantities of

protein are detected by this silver stain. The different lipopolysaccharide preparations appeared to be free of protein as there were no obvious protein bands observed on the gel (Figure 3.2). Surprisingly, the protein silver stain stained the lipopolysaccharide producing the expected lipopolysaccharide banding pattern.

A Lowry protein assay (Methods 2.20.1) estimated the protein content of one lipopolysaccharide preparation from strain 1034 to be less than 3% (w/w).

1.3 Nucleic Acid Content of Lipopolysaccharide Preparations.

One lipopolysaccharide preparation from E. carotovora subsp. atroseptica strain 1034 was estimated from its absorbance to contain about 4% (w/w) nucleic acid (Methods 2.20.2).

1.4 Stability of Lipopolysaccharide Preparations.

In contrast to observations with lipopolysaccharide from Escherichia coli (Goldman & Leive, 1980), storing of lipopolysacchairde at -20°C over a period of two years did not produce any change in electrophoretic behaviour on SDS-PAGE. A shift in staining intensity from the lower band of the doublet to the upper band as described by Goldman & Leive (1980) was not observed (Figure 3.3). The lipopolysaccharide

Figure 3.2

SDS-PAGE of four different lipopolysaccharide preparations from E. carotovora subsp. atroseptica strain 1034 and stained specifically for protein.

20μg samples were run on a 12.5% acrylamide gel (Methods 2.10.1) and stained as described in Methods 2.12.3. Tracks 1 to 4 correspond to tracks 2 to 5 of Figure 3.1. Track 5 contained protein molecular weight markers.

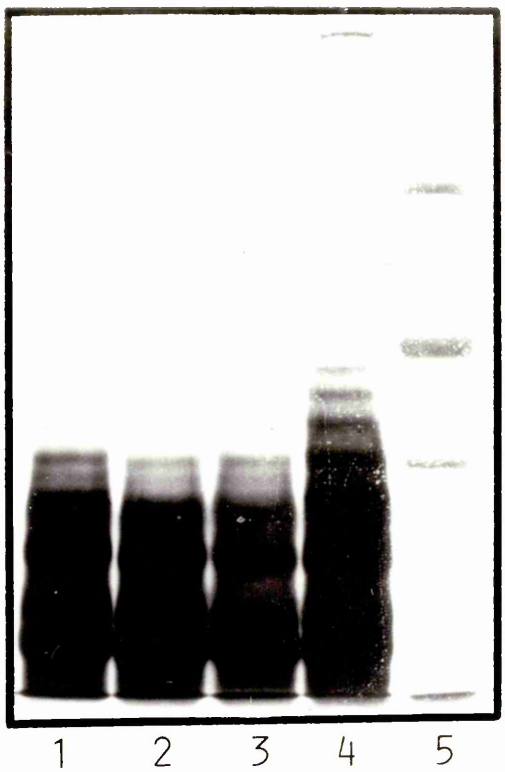


Figure 3.2.

Figure 3.3

SDS-PAGE of four different preparations of phenol/water extracted lipopolysaccharide from E. carotovora subsp. atroseptica strain 1034.

Each track contained 20μg of lipopolysaccharide run on a 12.5% acrylamide gel (Methods 2.10.1) and stained as described in Methods 2.12.2. The age of each preparation was

Track (1) 24 months, approx.
(2) 18 months
(3) 18 months
(4) freshly prepared.

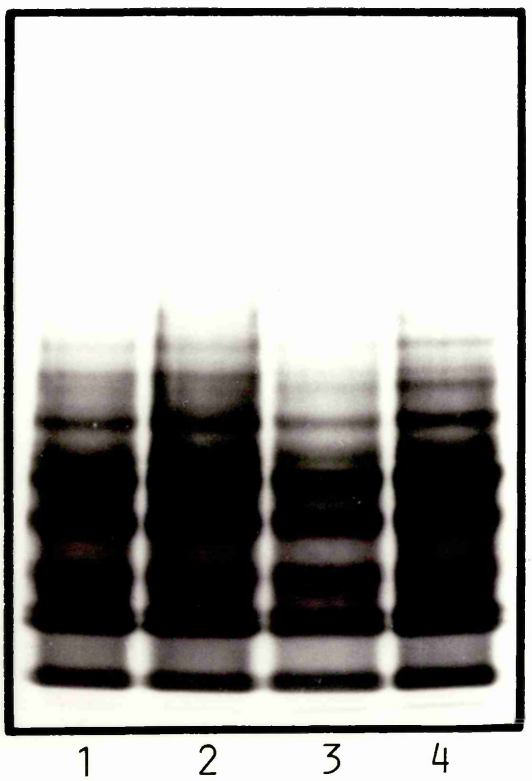


Figure 3.3.

preparation in track 1 has been continually frozen and thawed many times over a two year period and run on at least 50 gels. Lipopolysaccharide preparations in tracks 2 and 3 had been prepared in the intervening period and stored frozen. They were thawed several times. Lipopolysaccharide in track 4 was freshly prepared.

1.5 Alternative Methods of Extraction.

Two rapid methods of lipopolysaccharide extraction were attempted but both proved unsatisfactory. Lipopolysaccharide extracted by the proteinase K method (Methods 2.7.3) as described by Hitchcock & Brown (1983) was observed to contain only the fastest migrating bands of the expected electrophoretic pattern and produced smearing of the track (Figure 3.4). Modifications made to this method as described in Methods 2.7.3 to try to extract more of the lipopolysaccharide components and decrease the background staining produced only a slight increase in the number of the bands of the ladder observed but the electrophoretic mobility of the bands appeared to be different from the phenol/water extracted lipopolysaccharide (Figure 3.5). Similar results were obtained for the Triton method (Methods 2.7.4).

Figure 3.4

SDS-PAGE of proteinase K extracted lipopolysaccharide

Samples were run on a 12.5% acrylamide gel (Methods 2.10.1) and stained as described in Methods 2.12.2. 20µl of proteinase K extracted samples (Methods 2.7.1) were run and 20µg of phenol/water extracted lipopolysaccharide (Methods 2.7.3).

Tracks
(1) Eca 1034, phenol/water
(2) Eca 1034, proteinase K
(3) Eca 1034, proteinase K
(4) Eca 1034, phenol/water
(5) Eca 1043, phenol/water
(6) Eca 1043, proteinase K
(7) Eca 1043, proteinase K (from 20 colonies)
(8) Eca 1043, phenol/water
(9) Eca 549, phenol/water
(10) Eca 549, proteinase K
(11) Eca G398, phenol/water
(12) Eca G398, proteinase K.

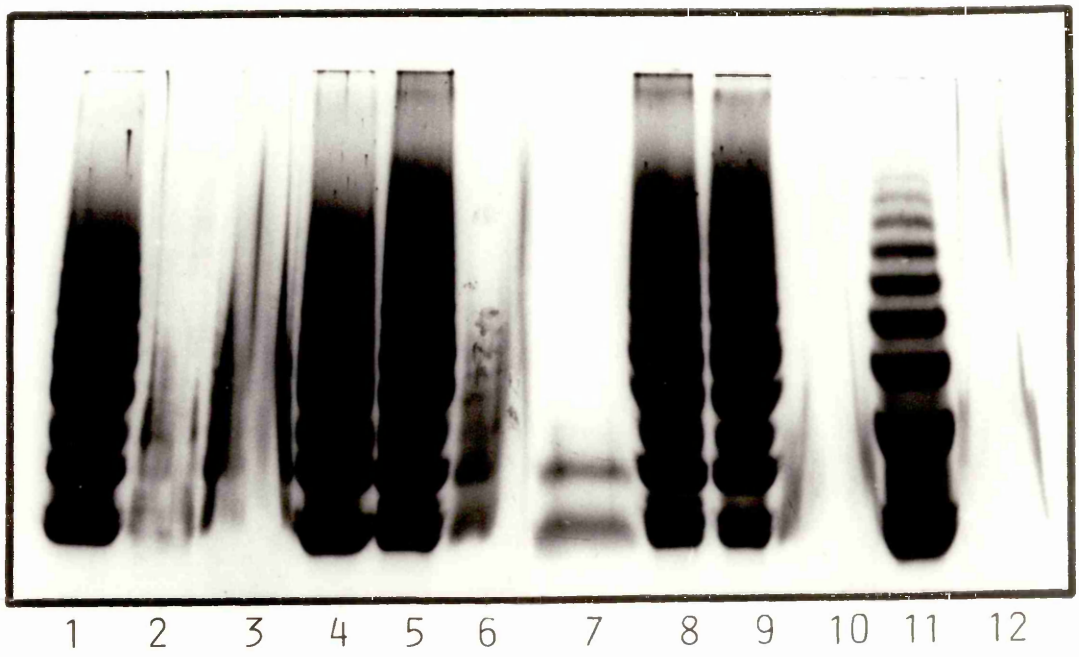


Figure 3.4.

Figure 3.5

SDS-PAGE of lipopolysaccharide extracted using the modified method of proteinase K extraction.

Samples were run on a 12.5% acrylamide gel as described in Methods 2.10.1. 20µg of phenol/water extracted (Methods 2.7.1) lipopolysaccharide from *E. carotovora* subsp. *atroseptica* strain 1034 was run. Gel was stained as described in Methods 2.12.2.

Tracks (1) *Eca* 1034, phenol/water.
(2) 5µl *Eca* 1034, proteinase K.
(3) 10µl *Eca* 1034, proteinase K.
(4) 20µl *Eca* 1034, proteinase K.

Tracks (5) to (8) and (9) to (12) were repeats of tracks (1) to (4).

Samples (2) to (4) were incubated with 10µg of RNAse/DNAse after boiling in lysing buffer as described in Methods 2.7.3.

Samples (6) to (8) were washed with 10mM Tris/HCl, pH 7.4 prior to lysis as described in Methods 2.7.3.

Samples (10) to (12) were washed with 10mM Tris/HCl, pH 7.4 prior to lysis and incubated with 10µg RNAse/DNAse after lysis as described in Methods 2.7.3.

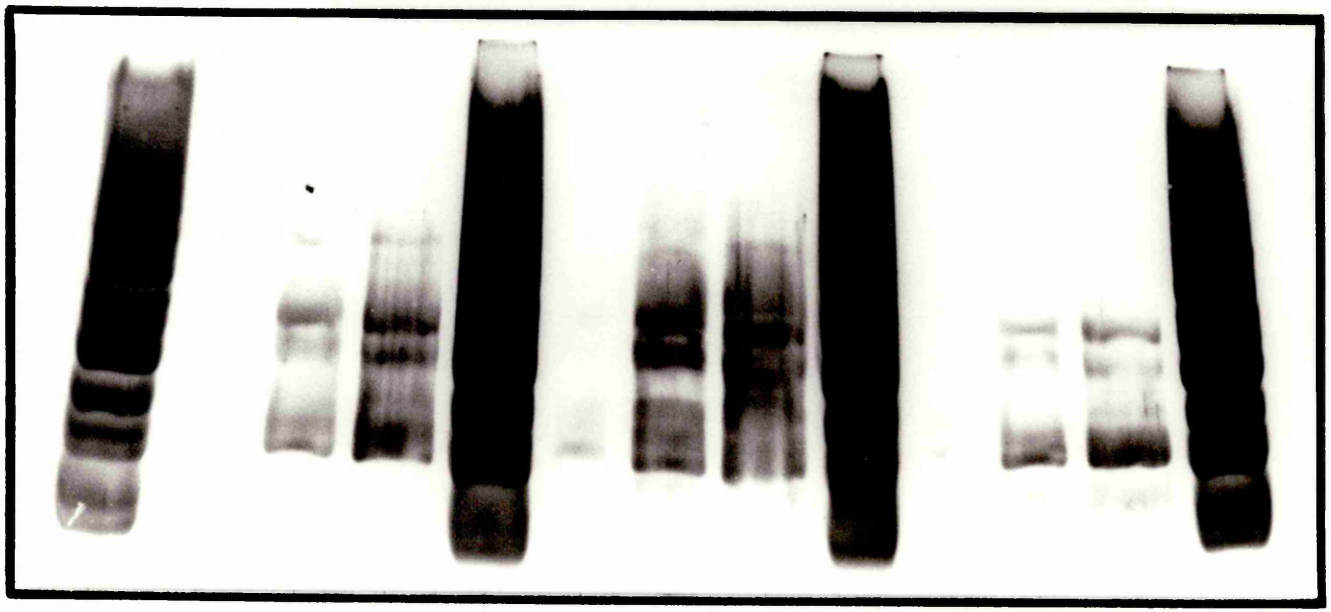


Figure 3.5.

2. SDS-PAGE Characteristics of E. carotovora Lipopolysaccharide.

2.1 Electrophoresis of E. carotovora Lipopolysaccharide.

It was found that lipopolysaccharide from E. carotovora strains can be resolved into up to 30 components upon SDS-PAGE electrophoresis (Methods 2.10.1., 2.10.2., Figure 3.6.). The stained gels (Methods 2.12.2.) had a characteristic ladder-like appearance. High mobility bands were often resolved into doublets, which could show different colours of staining (see Figure 3.11.). In terms of staining intensity, bands with highest mobility predominated.

2.2 Optimisation of Lipopolysaccharide Electrophoretic Conditions.

2.2.1. Different Durations of Boiling Lipopolysaccharide in SDS Buffer.

Increasing the sample boiling time (Methods 2.11.1.) from 2 minutes to 20 minutes did not effect the electrophoretic pattern produced (Figure 3.7.).

2.2.2. Effect of Different Amounts of Sample Loaded.

Increasing the sample loading from 10 to 50ug of lipopolysaccharide (Methods 2.10.1.5.) did have an effect on the ladder produced in terms of the amount of ladder and staining intensity of the bands (Figure

Figure 3.6

SDS-PAGE of lipopolysaccharide from E. carotovora subsp. atroseptica strain 1034.

The sample contained 20µg of lipopolysaccharide (Methods 2.10.1.5) and was run on a 10% acrylamide gel (Methods 2.10.1). The gel was stained as described in Methods 2.12.2 and scanned using a LKB 2202 Ultroscan Laser Densitometer.

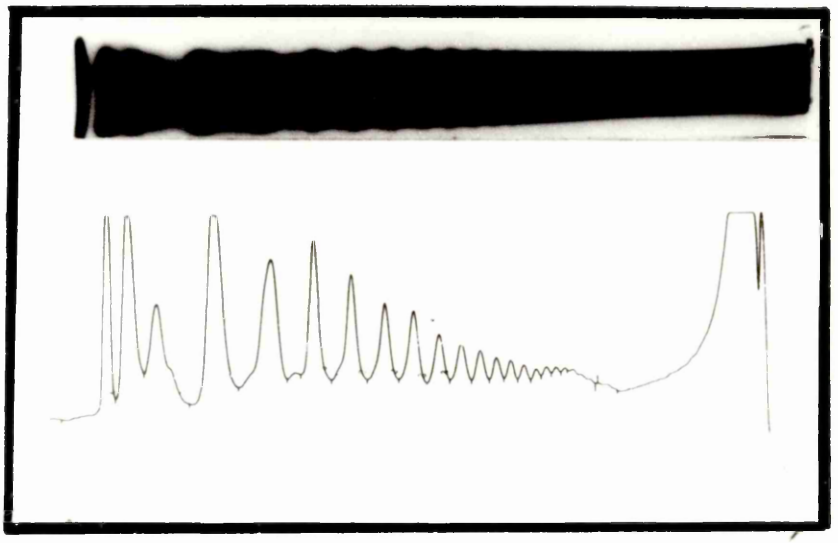


Figure 3.6.

Figure 3.7

SDS-PAGE of lipopolysaccharide from E. carotovora subsp. atroseptica strain 1034 subjected to boiling in SDS buffer for different durations (Methods 2.11.1)

Each track contained 20μg of lipopolysaccharide and was run on a 12.5% acrylamide gel as described in Methods 2.10.1. The gel was stained as described in Methods 2.12.2.

Tracks	(1)	2 min
	(2)	5 min
	(3)	10 min
	(4)	15 min
	(5)	20 min

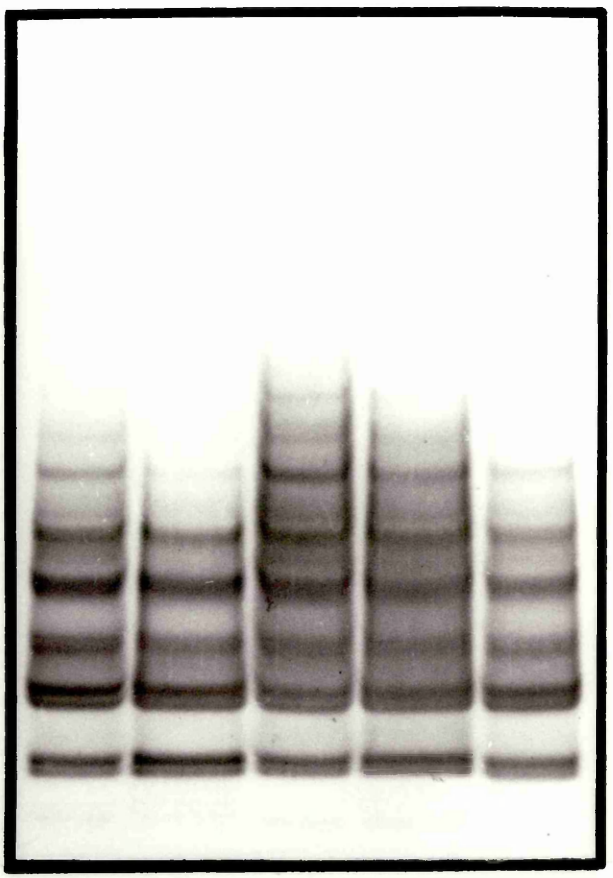


Figure 3.7.

3.8.). At loadings of 20μg and greater, more of the higher molecular weight components of the ladder were observed. However at the heavier loadings of 40 and 50μg the staining of the bands was too intense for purposes of clarity of detail. 20μg dry weight of lipopolysaccharide was therefore determined to be the optimum loading for electrophoresis.

2.2.3. Effect of Varying the SDS Concentration in the Gel System.

Optimal SDS concentrations for electrophoresis were determined to be 3% (w/v) SDS in the sample buffer and 0.1% (w/v) SDS in the separating gel, stacking gel and running buffer (Figure 3.9a.). Increasing the SDS concentration in the sample buffer from 1 to 6% (w/v), and from 0.1% to 0.4% (w/v) throughout the gel system (Methods 2.10.1.) had no effect on the electrophoretic separation obtained. Gels b) and c) of Figure 3.9 required to be left in the developer for a longer period of time than the standard gel (Figure 3.9a.) to detect all the components of the ladder but produced overstaining of the highest mobility components and higher background staining. The increased development time may have been due to the incomplete removal of SDS from the gels at the higher SDS concentrations. Removal of gels b) and c) of Figure 3.9 too soon from the developer stained only the highest mobility components.

Figure 3.8

Effect of the amount of lipopolysaccharide loaded on the SDS-PAGE of lipopolysaccharide from E. carotovora subsp. atroseptica strain 1034

Different amounts of lipopolysaccharide sample (Methods 2.10.1.5) were run on a 12.5% acrylamide gel as described in Methods 2.10.1. The gel was stained as described in Methods 2.12.2.

Tracks (1) 10µg lipopolysaccharide
(2) 20µg lipopolysaccharide
(3) 30µg lipopolysaccharide
(4) 40µg lipopolysaccharide
(5) 50µg lipopolysaccharide

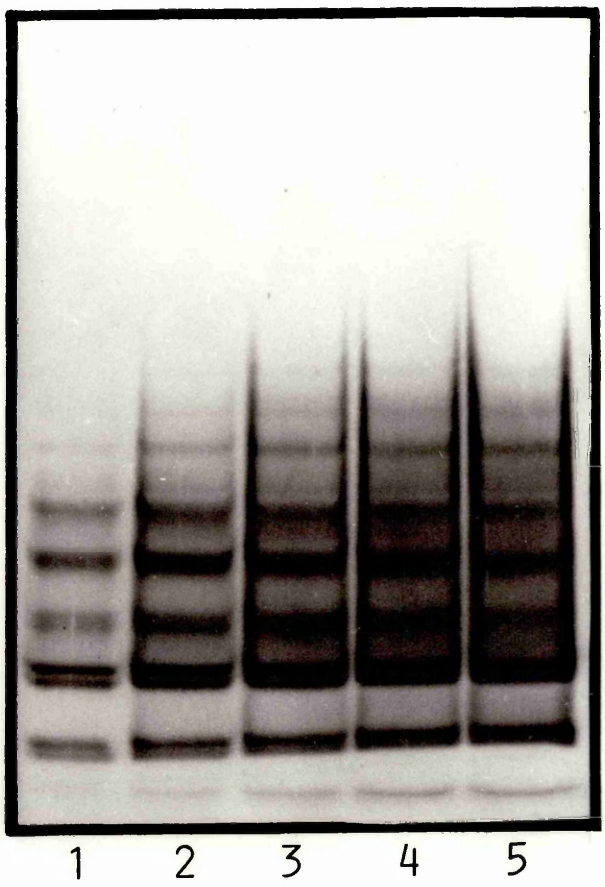


Figure 3.8.

Figure 3.9

Effect of varying the SDS concentration in the gel system on the SDS-PAGE of lipopolysaccharide from E. carotovora subsp. atroseptica strain 1034

20µg of lipopolysaccharide was run on a 12.5% acrylamide gel as described in Methods 2.10.1. SDS concentrations used in the gel system were a) 0.1%, b) 0.2% and c) 0.4% (w/v). The gel was stained as described in Methods 2.12.2.

Tracks (1) and (2) - 1% SDS in sample buffer
(3) and (4) - 3% SDS in sample buffer
(5) and (6) - 6% (w/v) SDS in sample buffer.

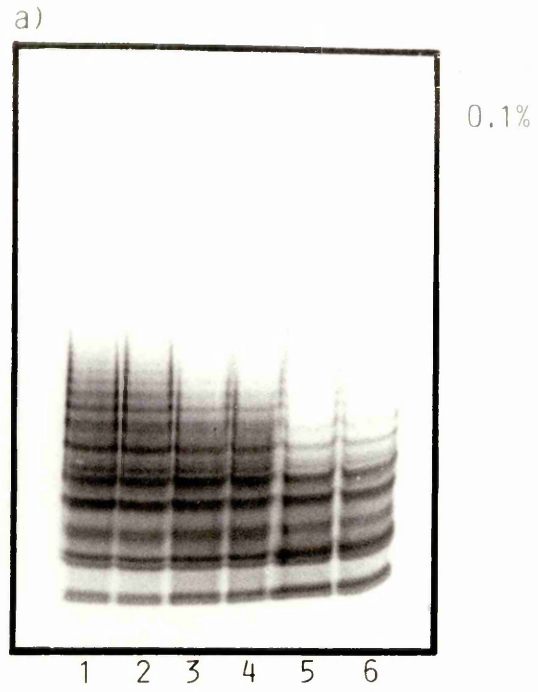


Figure 3.9.

2.3 Comparison of E. carotovora Lipopolysaccharide Electrophoretic Behaviour to Salmonella minnesota Mutants

E. carotovora lipopolysaccharide was compared to lipopolysaccharide from several S. minnesota mutant strains. The lipopolysaccharide of the mutant strains lacked O-chain and possessed differing degrees of core structure. It was observed that the mobility of the fastest migrating band of E. carotovora lipopolysaccharide was comparable to the fastest migrating band extracted from mutant strains R60, R345 and R5 (Figure 3.10).

2.4 Comparison of E. carotovora Lipopolysaccharide Electrophoretic Behaviour to Other Erwinia Species and Gram-Negative Soil Bacteria

The distribution of component mobilities of E. carotovora lipopolysaccharide (Figure 3.11, track 1) was different from those of E. amylovora and E. chrysanthemi (Figure 3.11, tracks 2 & 3). E. salicis had a similar distribution of component mobilities to that of E. carotovora (Figure 3.11, tracks 4 & 1) but the two profiles could be distinguished from each other. Pseudomonads and E. carotovora although commonly found together in diseased potato tissue, produced distinctly different electrophoretic patterns (Figure 3.11, tracks 6,7 and 1).

Figure 3.10

SDS-PAGE of lipopolysaccharide from E. carotovora subsp. atroseptica strain 1034 and rough lipopolysaccharide mutants of S. minnesota

Each track contained 20μg of lipopolysaccharide and was run on a 12.5% acrylamide gel (Methods 2.10.1) and stained as described in Methods 2.12.2.

Tracks (1) S. minnesota R60
(2) S. minnesota R345
(3) S. minnesota R5
(4) Eca 1034
(5) S. minnesota R7

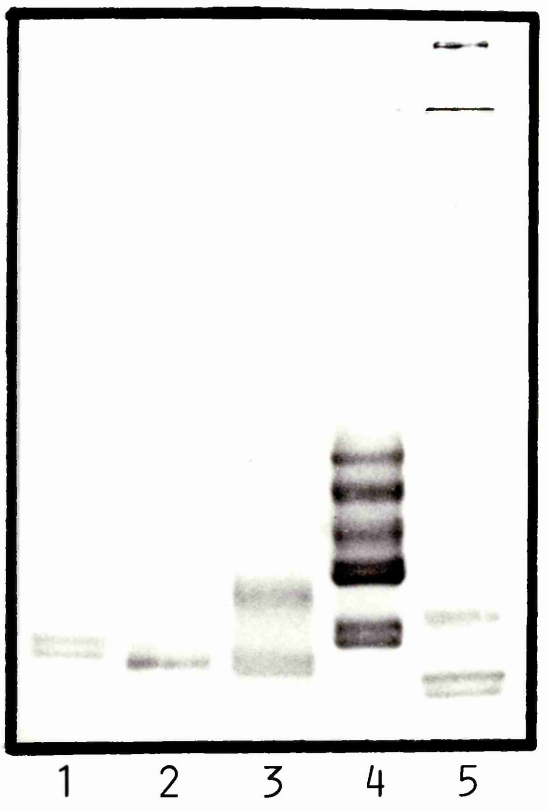


Figure 3.10.

Figure 3.11

SDS-PAGE of lipopolysaccharide from several Erwinia species and other gram-negative bacteria.

20µg samples were run on a 12.5% acrylamide gel (Methods 2.10.1). The gel was stained as described in Methods 2.12.2.

Tracks (1) *E. carotovora* subsp. *atroseptica*, NCPPB 1034.
(2) *E. amylovora*, EA3.
(3) *E. chrysanthemi*, NCPPB 402.
(4) *E. salicis*, strain 152.
(5) *K. aerogenes*, NCIB 418.
(6) *Ps. putida*, NCIB 10553.
(7) *Ps. aeruginosa*, NCIB 10548.

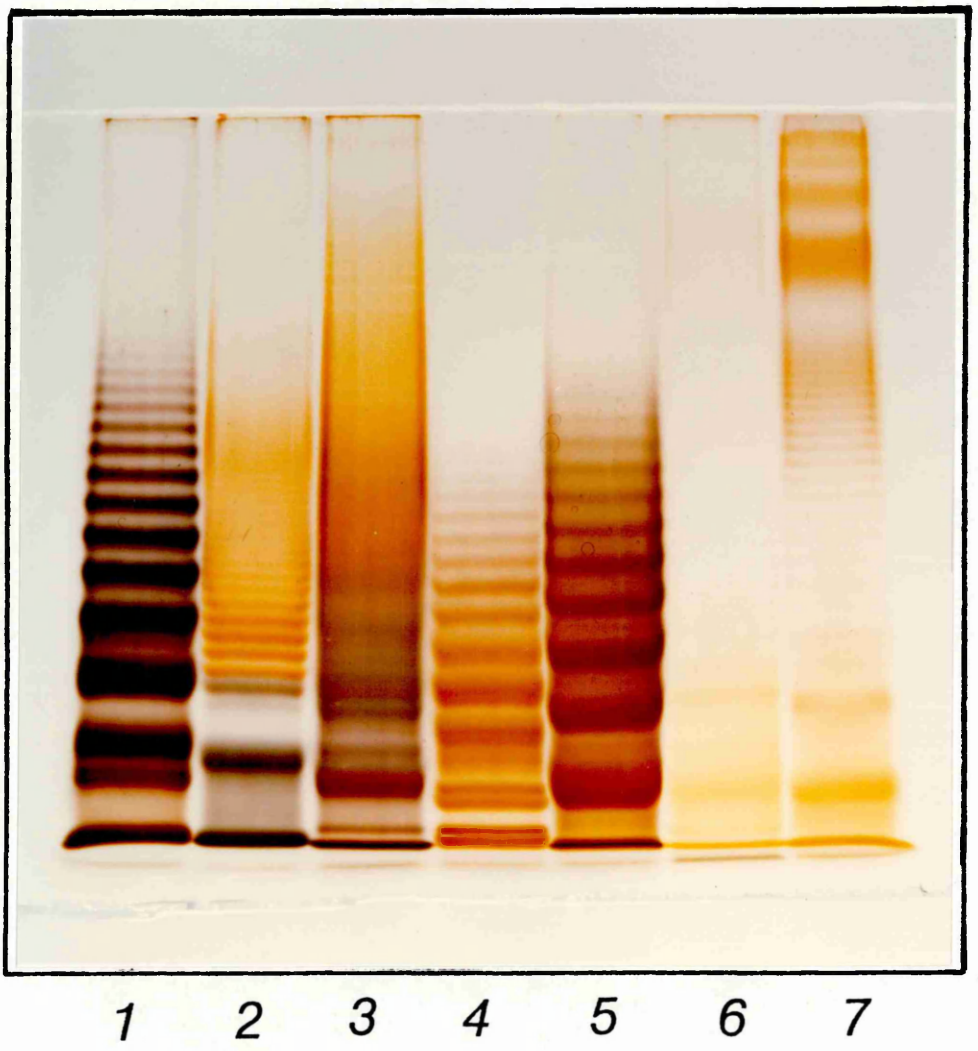


Figure 3.11.

The gel demonstrates the variety of colours that can be produced by the silver stain.

2.5 Tris/Borate SDS - PAGE.

E. carotovora lipopolysaccharide was electrophoresed on a Tris/borate SDS - containing gel with Tris/borate sample buffer and running buffer, at pH 9.0 (Methods 2.10.4.). The gel system did not produce any interpretable results (Figure 3.12.). The gel stained darkly producing zones of black colour with high background staining and smearing of the track.

2.6 Action of Bacterial Alkaline Phosphatase On E. carotovora Lipopolysaccharide Electrophoretic Behaviour.

Incubation of lipopolysaccharide from E. carotovora subsp. atroseptica strain 1034 with bacterial alkaline phosphatase prior to sample preparation (Methods 2.11.3.) did not alter its electrophoretic mobility (Figure 3.13.).

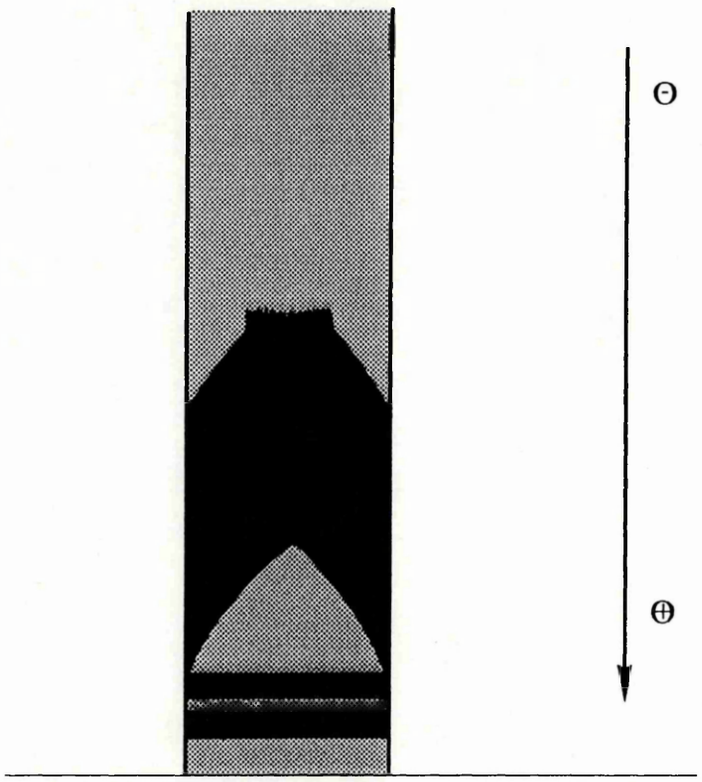


Figure 3.12. Tris/borate SDS-PAGE of Erwinia lipopolysaccharide.
Lower portion of gel.

Figure 3.13

Incubation of lipopolysaccharide from E. carotovora subsp. atroseptica strain 1034 with bacterial alkaline phosphatase for different durations prior to SDS-PAGE

20µg of sample was run on a 12.5% acrylamide gel (Methods 2.10.1). The gel was stained as described in Methods 2.12.2.

Tracks (1) Eca 1034 LPS + bacterial alkaline phosphatase.
(2) bacterial alkaline phosphatase control.

Tracks (3) and (4), (5) and (6), (7) and (8), (9) and (10) and (11) and (12) were repeats of tracks (1) and (2), respectively.

samples (1) and (2) incubated for 0 min.
(3) and (4) incubated for 30 min.
(5) and (6) incubated for 60 min.
(7) and (8) incubated for 120 min
(9) and (19) incubated for 180 min.
(11) and (12) incubated for 240 min.

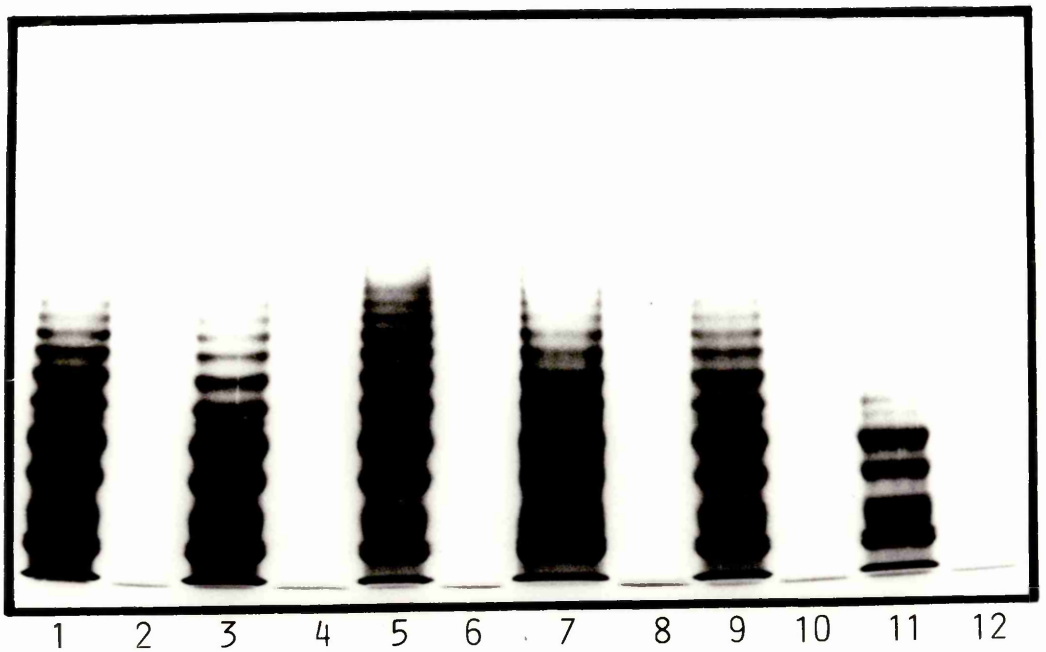


Figure 3.13.

3. Interaction of Lipopolysaccharide and Detergent.

Experiments in this section deal with the interaction between lipopolysaccharide and detergent when Erwinia cells are grown in the presence of deoxycholate, and in SDS-PAGE.

3.1 Growth of E. carotovora Cells in the Presence of Detergent.

Growth of E. carotovora subsp. atroseptica strain 1034 in the presence of 0.05% (w/v) and 0.1% (w.v) deoxycholate was possible (Methods 2.4.3.). At 0.05% (w/v) deoxycholate the growth was comparable to that of cells grown in the absence of detergent. At the higher deoxycholate concentrations of 0.2% (w/v) and 0.5% (w/v) problems were encountered with the detergent coming out of solution.

3.2 Two Dimensional - Gel Electrophoresis.

Two dimensional SDS-PAGE of E. carotovora subsp. atroseptica lipopolysaccharide from strain 1034 (Methods 2.10.5.) gave a pattern of bands on the expected diagonal. Those bands with the highest electrophoretic mobility in the first dimension additionally produced a partial ladder in the second dimension (Figure 3.14.a.).

Similar results were obtained with S. minnesota lipopolysaccharide from mutant strain R7 (Methods 2.10.5) which gave a pattern of three bands on the

Figure 3.14a

Two dimensional SDS-PAGE of lipopolysaccharide from E. carotovora subsp. atroseptica strain 1034.

20µg of lipopolysaccharide was run on a 12.5% acrylamide gel in the first dimension as described in Methods 2.10.1. Electrophoresis was performed on a SDS-containing 12.5% acrylamide gel in the second dimension (Methods 2.10.5). Gel was silver stained as described in Methods 2.12.2.

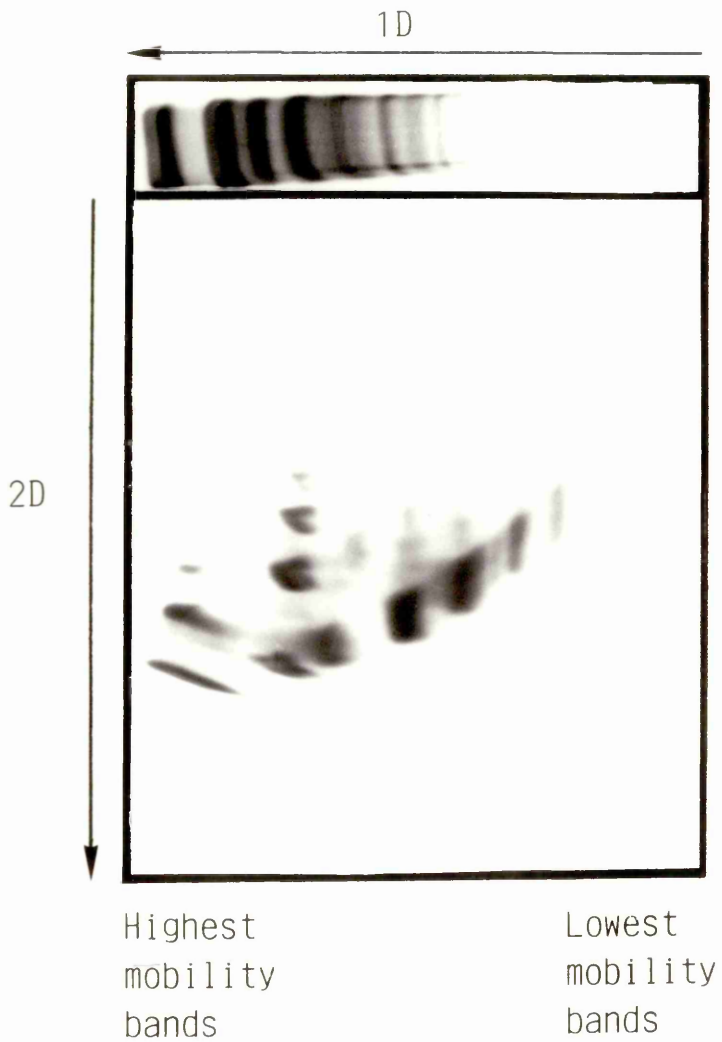


Figure 3.14a.

expected diagonal in the second dimension (Figure 3.14.b) and a partial ladder.

3.3 Mixed Lipopolysaccharide Preparations.

Mixtures of lipopolysaccharides from the same serogroup and from different serogroups were prepared as described in Methods 2.11.2. Mixtures of lipopolysaccharide from the same serogroup (see Results 4.2) gave the same pattern whereas mixtures of lipopolysaccharide from different serogroups (see Results 4.1) gave a composite pattern of the two different preparations (Figure 3.15).

3.4 Log Molecular Size vs Mobility

For three different preparations of lipopolysaccharide from three serogroups of E. carotovora subsp. atroseptica the $\log_{10}$ band number was plotted against mobility of that band. Lipopolysaccharides from serogroup I, serogroup XVIII, and serogroup XXII were examined. A proportional relationship was observed (Figure 3.16.).

3.5 Non - SDS PAGE

Lipopolysaccharide from E. carotovora subsp atroseptica strain 1034 was electrophoresed on a non-SDS PAGE system as described in Methods 2.10.3. The

Figure 3.14b

Two dimensional SDS-PAGE of S. minnesota lipopolysaccharide from mutant strain R7.

20μg of lipopolysaccharide was run on a 12.5% acrylamide gel in the first dimension as described in Methods 2.10.1. Electrophoresis was performed on a SDS-containing 12.5% acrylamide gel in the second dimension (Methods 2.10.5). Gel was silver stained as described in Methods 2.12.2.

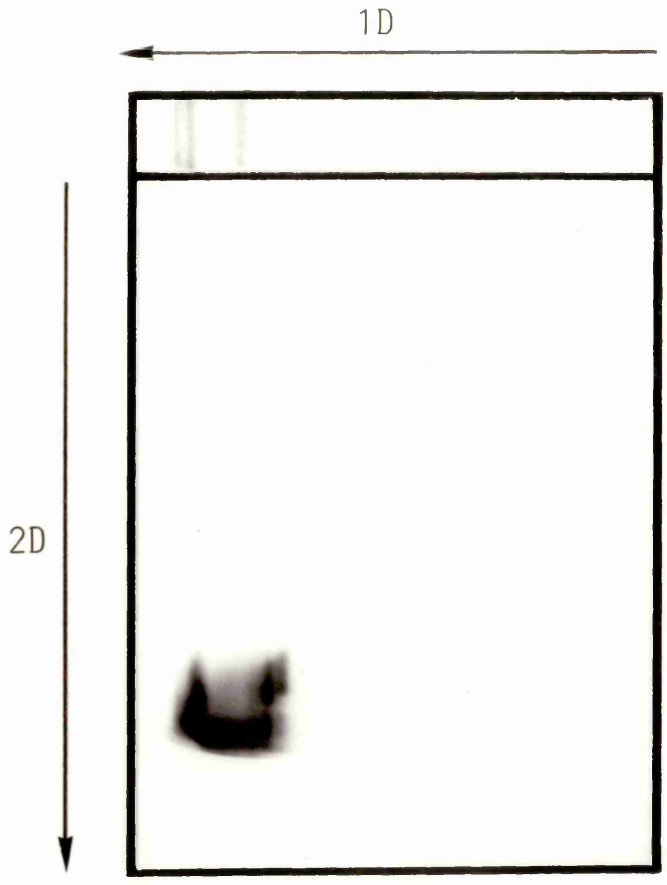


Figure 3.14b.

Figure 3.15

SDS-PAGE of mixtures of lipopolysaccharide preparations

20μg (total) of lipopolysaccharide was run on a 12.5% acrylamide gel as described in Methods 2.10.1.

Gel was stained as described in Methods 2.12.2.

Tracks
(1) Eca 1034, serogroup I lipopolysaccharide only.
(2) Eca 1034 and Eca 1002, mixture of serogroup I lipopolysaccharides.
(3) Eca 1002, serogroup I lipopolysaccharide only.
(4) Eca 1034, serogroup I lipopolysaccharide only.
(5) Eca 1034 and Ecc 312, mixture of serogroup I and serogroup XXVIII lipopolysaccharides.
(6) Ecc 312, serogroup XXVIII lipopolysaccharide only.

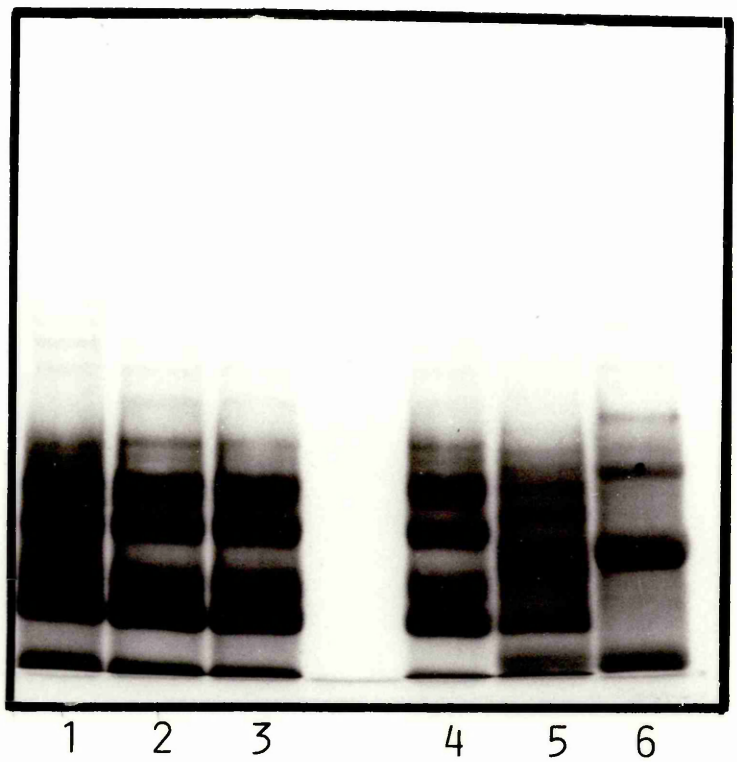


Figure 3.15.

Figure 3.16

Relationship between mobility and band number of lipopolysaccharide components.

The bands were numbered consecutively with the highest mobility band being 1. Mobilities were from samples run on 12.5% acrylamide gels as described in Methods 2.10.1. In cases where doublets were obvious mobility was measured to centre (gap) of doublet.

Symbols (O) serogroup I strain

(●) serogroup XVIII strain

(△) serogroup XXII strain

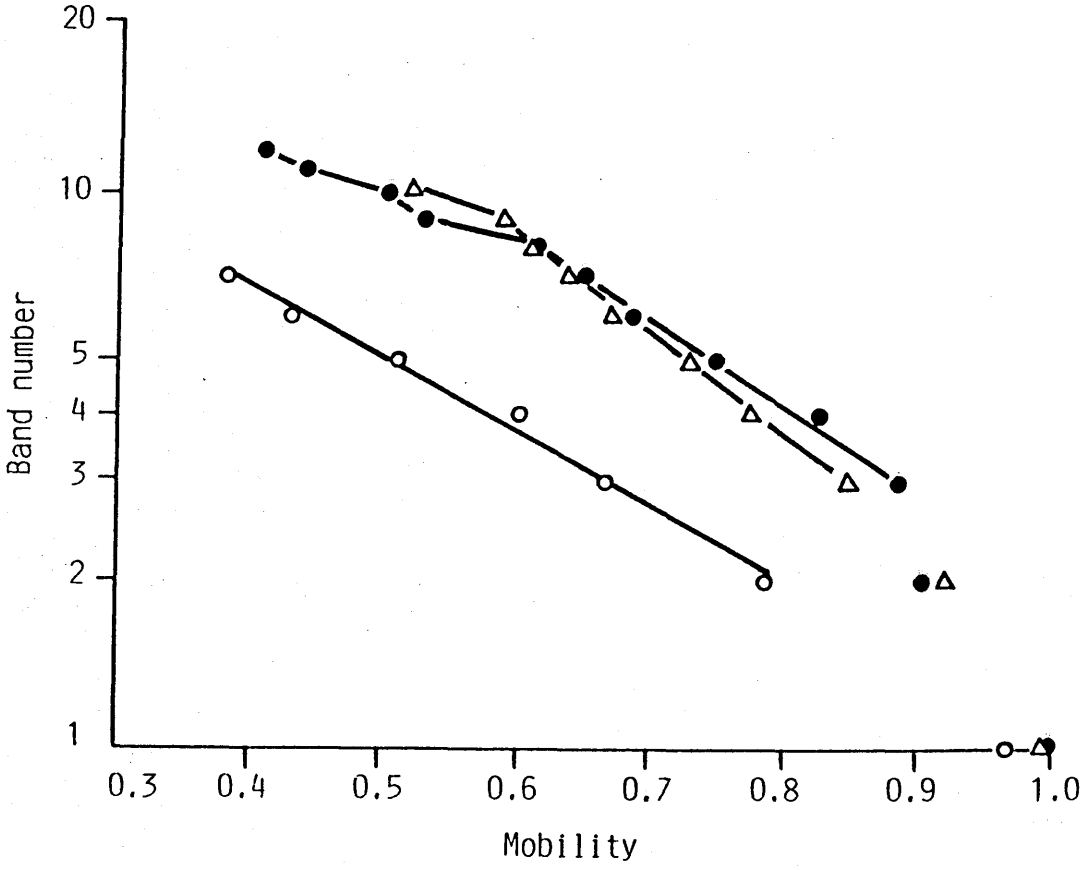


Figure 3.16.

majority of the lipopolysaccharide remained in the stacking gel with a few faint bands appearing at the top of the separating gel.

4. Relationship Between Lipopolysaccharide Electrophoretic Behaviour and Serogroup.

4.1 Variation of Lipopolysaccharide Pattern with Serogroup.

SDS-PAGE of lipopolysaccharide (Methods 2.10.1.) from representative strains of 12 different E. carotovora serogroups gave different electrophoretic patterns except for the subspecies atroseptica strains belonging to serogroups XVIII and XX which gave a very similar pattern (Figure 3.17., tracks 5 & 6). A similar observation was made for those atroseptica strains from serogroup XXII and XXXV (Figure 3.17., tracks 7 & 13). The subspecies carotovora strain belonging to serogroup XVIII gave a pattern which was different from the lipopolysaccharide of the serogroup XVIII atroseptica subspecies (Figure 3.17., tracks 4 & 5).

4.2 Constancy of Lipopolysaccharide Behaviour Within Serogroups.

Lipopolysaccharide from twenty serogroup I strains was examined by SDS-PAGE as described in Methods 2.10.1. (Figure 3.18.). Eighteen of the twenty strains gave an identical pattern and one which is identical to the pattern obtained from strain 31, the strain selected by De Boer as a type strain for serogroup I (De Boer et al, 1979; Figure 3.19). Among the serogroup I strains, only

Figure 3.17

SDS-PAGE of lipopolysaccharide of different E.carotovora subsp. atroseptica (Eca) and subsp. carotovora (Ecc) serogroups.

20µg samples were run on a 12.5% acrylamide gel (Methods 2.10.1). Gel was stained as described in Methods 2.12.2.

Tracks (1) Eca 1034, serogroup I
(2) Ecc G275, IV
(3) Ecc G298, XII
(4) Ecc G301, XVIII
(5) Eca 39 , XVIII
(6) Eca 1056, XX
(7) Eca 198 , XXII
(8) Ecc P1 , XXV
(9) Ecc 312 , XXVIII
(10) Ecc 133 , XXIX
(11) Ecc 193 , XXXIII
(12) Ecc 438 , XXXIV
(13) Eca 432 , XXXV

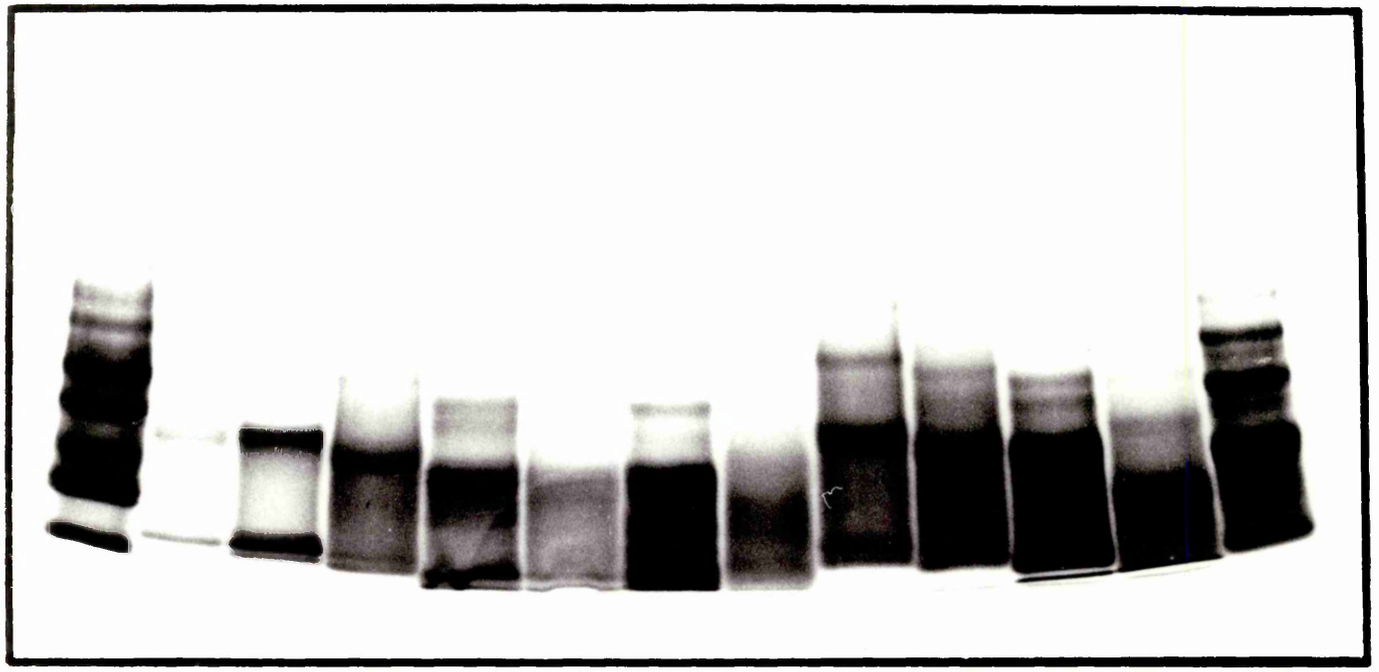


Figure 3.17.

Figure 3.18

SDS-PAGE of lipopolysaccharide from E. carotovora serogroup I strains.

20µg samples of lipopolysaccharide were run on 12.5% acrylamide gels (Methods 2.10.1) and stained as described in Methods 2.12.2.

Tracks
(1) Eca 1034
(2) Eca 1039
(3) Eca 1042
(4) Eca 1043
(5) Ecc 1745
(6) Ecc 547
(7) Eca G398
(8) Eca 436
(9) Eca 1001
(10) Eca 1002
(11) Eca 1061
(12) Eca 1062
(13) Eca 1063
(14) Eca 1064
(15) Eca 1065
(16) Eca 1066
(17) Eca 1067
(18) Eca 1068
(19) Eca 1069
(20) Eca 1070

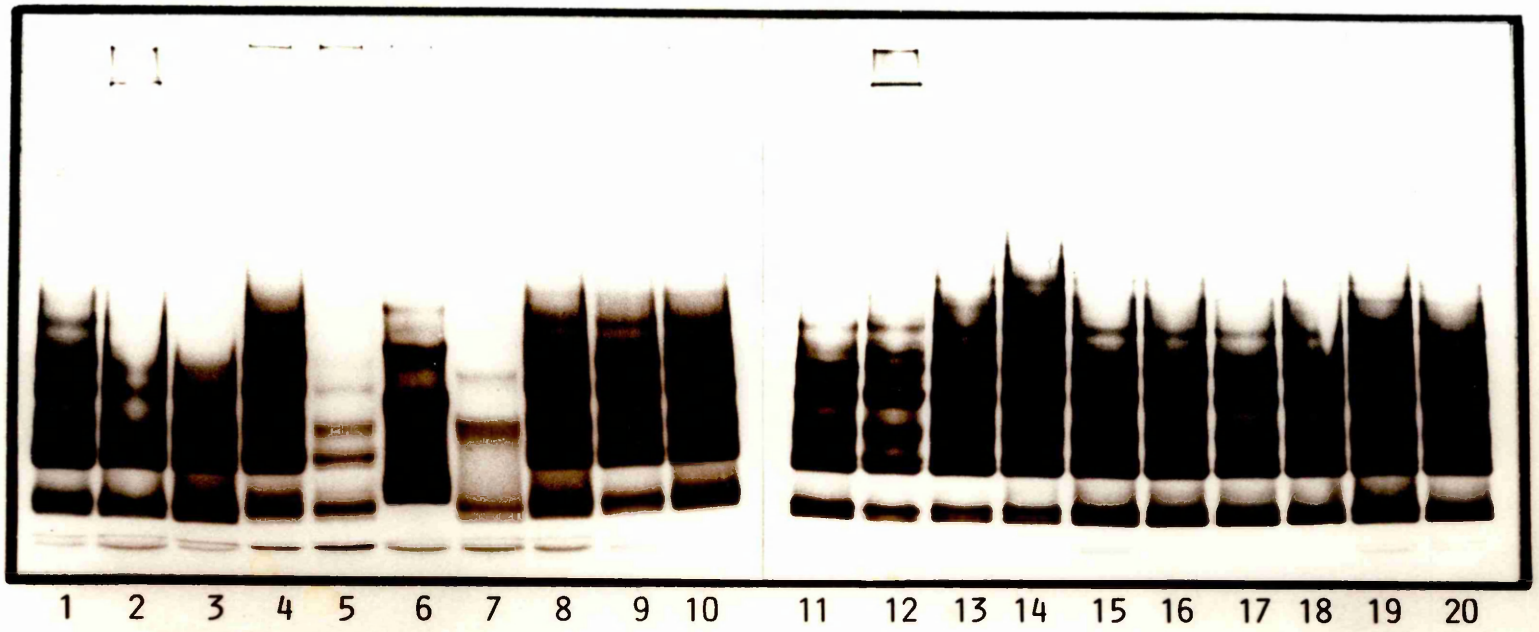


Figure 3.18.

Figure 3.19

SDS-PAGE of lipopolysaccharide from E. carotovora subsp. atroseptica strain 1034 for comparison with De Boer's type strain for serogroup I.

20µg samples of lipopolysaccharide were run on a 12.5% acrylamide gel (Methods 2.10.1) and stained as described in Methods 2.12.2.

Tracks (1) Eca 1034 , serogroup I
(2) Eca 31 , I (De Boer's serogroup I type strain)
(3) Ecc 327 , I

Figure 3.19.

strains 547 (Figure 3.18., track 6) and G398 (Figure 3.18., track 7) did not show the same electrophoretic pattern of lipopolysaccharide as the other eighteen serogroup I strains. Strain G398 produces an identical electrophoretic pattern to that obtained from a representative strain from serogroup XX (Figure 3.20.). Initial preparations of lipopolysaccharide from strains 1043 and 1745 (Figure 3.18., tracks 4 & 5) were found to produce the standard serogroup I electrophoretic pattern but with an extra band between the first and second doublets (Figure 3.21.a.). Upon recloning of these cultures the extra band disappeared (Figure 3.21.b.).

4.3 Important Potato Pathogenic Serogroups of E. carotovora subsp. atroseptica.

Pathogenic strains have been classified as belonging to serogroups I, XVIII, XX and XXII (De Boer and McNaughton, 1987). SDS - PAGE of lipopolysaccharide (Methods 2.10.1.) from five serogroup XXII strains (Figure 3.22., tracks 11 - 15) gave an identical pattern. Four of the five serogroup XX strains gave identical patterns (Figure 3.22., tracks 6 - 10). With serogroup XVIII strains, both subspecies carotovora and subspecies atroseptica strains were examined. The two carotovora subspecies (Figure 3.22., tracks 2 & 3) gave the same pattern while the two atroseptica strains gave a different pattern (Figure 3.22., tracks 4 & 5) but one

Figure 3.20

SDS-PAGE of lipopolysaccharide from E. carotovora subsp. atroseptica strain G398.

20µg of sample was run on a 12.5% acrylamide gel as described in Methods 2.10.1. Gel was stained as described in Methods 2.12.2.

Tracks (1) Eca 1034 , serogroup I
(3) Eca G398 , given as serogroup I
(3) Eca 1056 , serogroup XX

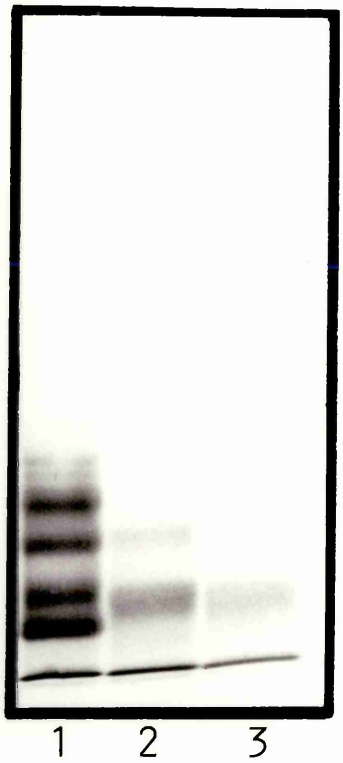


Figure 3.20.

Figure 3.21a

SDS-PAGE of initial preparations of lipopolysaccharide from E. carotovora subsp. atroseptica strain 1043 and E. carotovora subsp. carotovora strain 1745.

20µg samples were run on a 12.5% acrylamide gel (Methods 2.10.1) and stained as described in Methods 2.12.2.

Tracks (1) Eca 1034 , serogroup I
(2) Eca 1043 , I
(3) Ecc 1745 , I

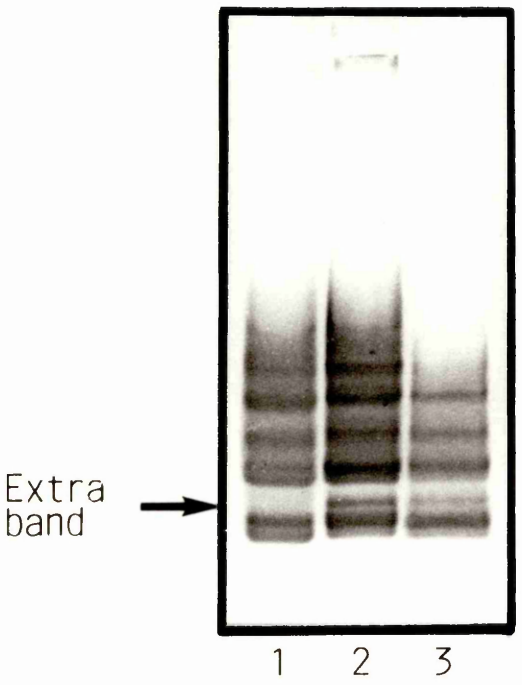
Figure 3.21b

SDS-PAGE of lipopolysaccharide from recloned strains of E. carotovora subsp. atroseptica strain 1043 and E. carotovora subsp. carotovora strain 1745 grown on double strength nutrient broth.

20µg samples were run on a 12.5% acrylamide gel (Methods 2.10.1) and stained as described in Methods 2.12.2.

Tracks (1) Eca 1043 , original preparation
(2) Eca 1043 , recloned
(3) Eca 1043 , recloned
(4) Eca 1043 , recloned
(5) Ecc 1745 , recloned
(6) Ecc 1745 , recloned
(7) Ecc 1745 , recloned
(8) Ecc 1745 , recloned

a)



b)

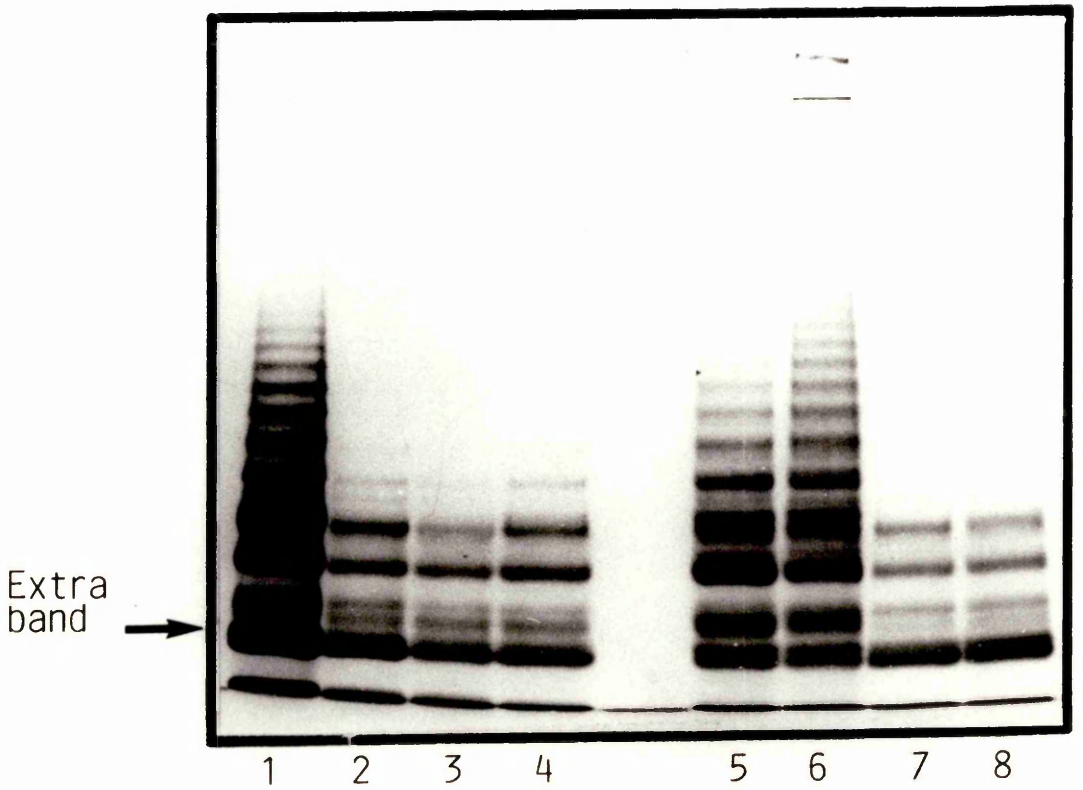


Figure 3.21.

Figure 3.22

SDS-PAGE of lipopolysaccharides from the important potato pathogenic serogroups of E. carotovora subsp. atroseptica.

The samples contained 20µg of lipopolysaccharide and were run on a 12.5% acrylamide gel (Methods 2.10.1). Gel was stained as described in methods 2.12.2.

Tracks	(1) Eca 1034	, serogroup I
	(2) Ecc G301	, XVIII
	(3) Ecc G303	, XVIII
	(4) Eca 39	, XVIII
	(5) Eca 6	, XVIII
	(6) Eca G331	, XX
	(7) Eca 1056	, XX
	(8) Eca 1058	, XX
	(9) Eca 58	, XX
	(10) Eca 199	, XX
	(11) Eca G222	, XXII
	(12) Eca A46	, XXII
	(13) Eca A139	, XXII
	(14) Eca 198	, XXII
	(15) Eca 420	, XXII
	(16) Eca 432	, XXXV

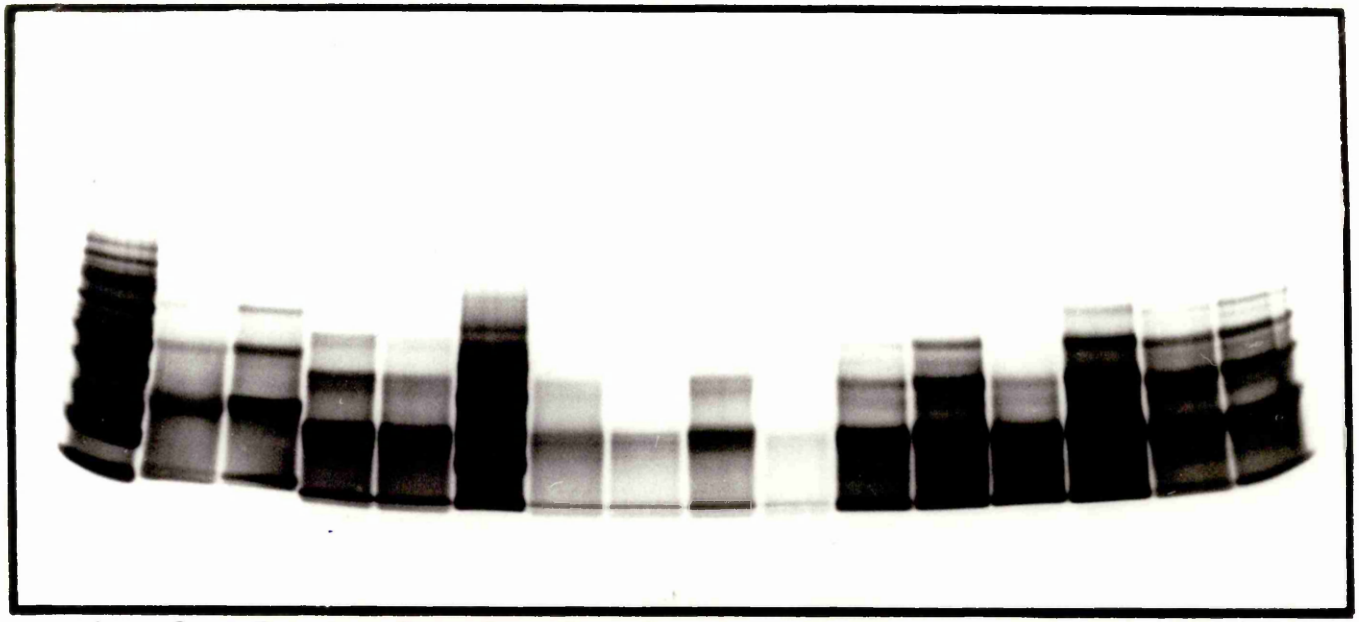


Figure 3.22.

which was identical to the pattern obtained with the four serogroup XX strains (Figure 3.22., tracks 7 - 10). The single atroseptica strain examined as an example of serogroup XXXV (Figure 3.22., track 16) gave a pattern identical to that obtained for the five serogroup XXII strains (Figure 3.22., tracks 11 - 15) and one which was quite different from the pattern observed for three authentic serogroup XXXV betavasculorum strains (Figure 3.23.). The single anomalous strain of serogroup XX, strain G331 (Figure 3.22., track 6) gave a serogroup I type lipopolysaccharide electrophoretic pattern (Figure 3.22., track 1).

4.4 Electrophoretic Behaviour of Lipopolysaccharide from Strains Typing into More Than One Serogroup

Lipopolysaccharide from five strains found to type into more than one serogroup was examined by SDS-PAGE (Methods 2.10.1). Each strain produced one identifiable electrophoretic pattern. Strains 553 (XXII, XXXV) and 557 (XXII, XLI) produced a pattern (Figure 3.24, tracks 7 and 8) identical to six other serogroup XXII strains (Figure 3.24, tracks 1 to 6). These six serogroup XXII strains included strain 432 shown to belong to serogroup XXII in this study (Results 4.3). Strains 556 (XX,XLI) and 559 (XX, XL, XLIII) gave a pattern identical to four other serogroup XX strains (Figure 3.25, tracks 5 & 6). Strain 545 (XLI, XLIII) gave a pattern identical to the three serogroup XXXV betavasculorum strains (Figure 3.23, track 2).

Figure 3.23

SDS-PAGE of lipopolysaccharide from E. carotovora subsp. atroseptica strain 432 for comparison with lipopolysaccharide from three authentic serogroup XXXV E. carotovora subsp. betavasculorum strains.

20µg of sample was run on a 12.5% acrylamide gel (Methods 2.10.1) and stained as described in Methods 2.12.2.

Tracks (1) Eca 432 , serogroup XXXV (?)
(2) Ecb 545 . XL/XLIII
(3) Ecb 546 , XXXV
(4) Ec? 548 , XXXV
(5) Ecb 551 , XXXV

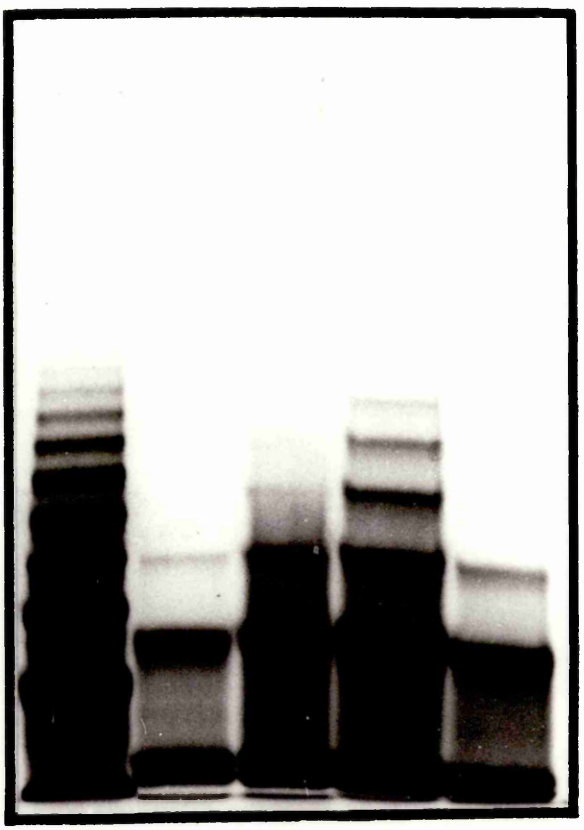


Figure 3.23.

Figure 3.24

SDS-PAGE of lipopolysaccharide from E. carotovora subsp. atroseptica strains observed to type into more than one serogroup and their comparison to lipopolysaccharide from E. carotovora subsp. atroseptica serogroup XXII strains.

20 µg samples were run on a 12.5% acrylamide gel (Methods 2.10.1) and stained as described in Methods 2.12.2.

Tracks (1) Eca G222 , serogroup XXII
(2) Eca A46 , XXII
(3) Eca A139 , XXII
(4) Eca 198 , XXII
(5) Eca 420 , XXII
(6) Eca 432 , XXII
(7) Eca 553 , XXII, XXXV
(8) Eca 557 , XXII, XLI

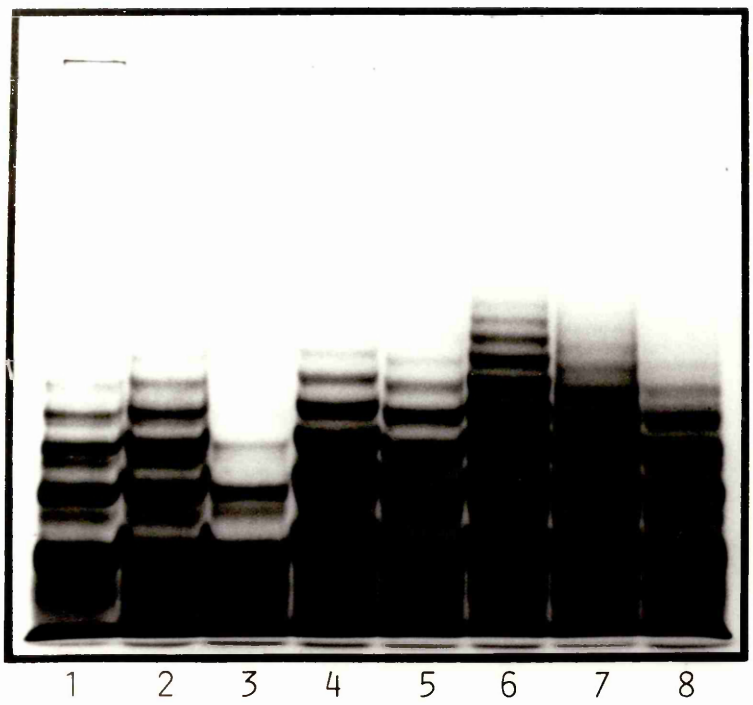


Figure 3.24.

Figure 3.25

SDS-PAGE of lipopolysaccharide from E. carotovora subsp. atroseptica strains observed to type into more than one serogroup and their comparison to lipopolysaccharides from E. carotovora subsp. atroseptica serogroup XX strains.

20µg samples were run on a 12.5% acrylamide gel (Methods 2.10.1) and stained as described in Methods 2.12.2.

Tracks (1) Eca 1056 , serogroup XX
(2) Eca 1058 , XX
(3) Eca 58 , XX
(4) Eca 199 , XX
(5) Eca 556 , XX, XLI
(6) Eca 559 , XX, XL, XLIII

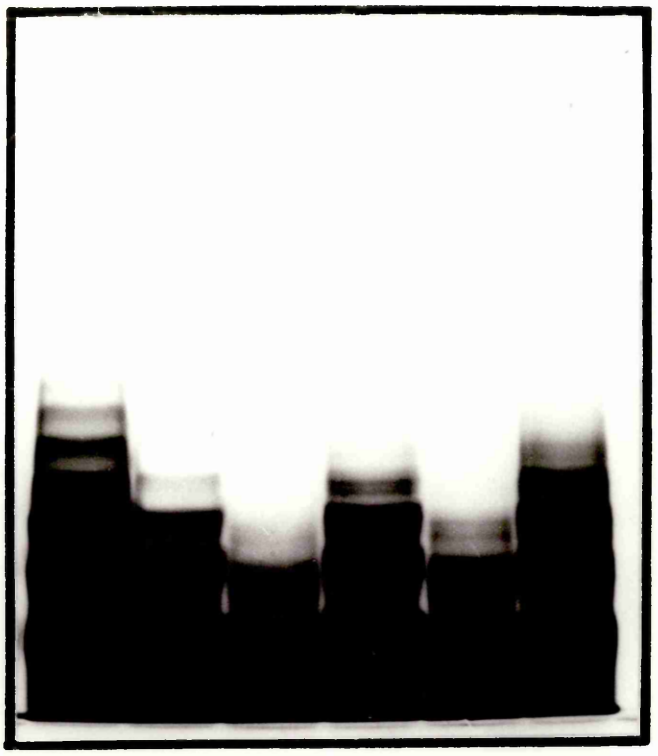


Figure 3.25.

5. Serological Reactivity of Lipopolysaccharide from Potato Pathogenic Serogroups of E. carotovora subsp. atroseptica

5.1 Serogroups with Common Epitopes

Antisera raised (Methods 2.13.2) against strains from the important potato pathogenic serogroups of E. carotovora subsp. atroseptica were reacted against lipopolysaccharide purified from these strains in a series of immunoblotting experiments as described in Methods 2.14.1 (Table 3.2). Cross-reactivity was observed between serogroup I lipopolysaccharide and serogroup XXII lipopolysaccharide, and serogroup XVIII (Eca) lipopolysaccharide and serogroup XX lipopolysaccharide. Serogroup XVIII (Ecc) lipopolysaccharide reacted only with its own homologous antiserum.

5.2 Antisera Absorption with Lipopolysaccharide

5.2.1 Serogroups I and XXII

Absorption of serogroup I antiserum (raised against whole live cells of Eca 1034) with its own homologous lipopolysaccharide (Methods 2.15.1) removed all the reacting antibodies against both serogroup I lipopolysaccharide and serogroup XXII lipopolysaccharide (Table 3.3). Whereas absorption of serogroup I

TABLE 3.2 **Serological reactivity of lipopolysaccharide from the potato pathogenic serogroups of E. carotovora subsp. atroseptica**

Serogroup of strain antibody raised against	Serogroup of LPS against which antibody reacts/crossreacts
I	I, XXII
Ecc XVIII	Ecc XVIII
Eca XVIII	Eca XVIII
XX	Eca XVIII, XX
XXII	I, XXII

TABLE 3.3 **Antibody absorptions of serogroup I antiserum and serogroup XXII antiserum with serogroup I lipopolysaccharide and serogroup XXII lipopolysaccharide.**

Antibody serogroup	Serogroup of absorbing LPS	Serogroup of LPS reacting after absorption
I	I	–
I	XXII	I
XXII	I	–
XXII	XXII	–

antiserum with serogroup XXII lipopolysaccharide left antibodies reactive with serogroup I lipopolysaccharide but not serogroup XX lipopolysaccharide (Table 3.3).

Both absorptions of serogroup XXII antiserum (raised against whole live cells of Eca 198) with serogroup I lipopolysaccharide and with serogroup XXII lipopolysaccharide removed all antibodies reactive against both serogroup I lipopolysaccharide and serogroup XXII lipopolysaccharide (Table 3.3).

5.2.2 Serogroups XVIII (Eca) and XX

Both absorptions of serogroup XVIII (Eca) antiserum (raised against whole, live cells of 6) with its own homologous lipopolysaccharide and with serogroup XX lipopolysaccharide (Methods 2.15.1) removed all reacting antibodies against both serogroup XVIII (Eca) lipopolysaccharide and serogroup XX lipopolysaccharide (Table 3.4). The same result was observed for both the absorptions of serogroup XX antiserum (raised against whole, live cells of Eca 1056) with serogroup XVIII (Eca) lipopolysaccharide and with serogroup XX lipopolysaccharide (Table 3.4).

5.3 Serological Reactivity of Lipopolysaccharide Producing Anomalous Electrophoretic Patterns

Lipopolysaccharide from the two anomalous serogroup I strains, Eca 547 and G398 (Figure 3.18, tracks 6 and

TABLE 3.4 **Antibody absorptions of serogroup XVIII (Eca) antiserum and serogroup XX antiserum with serogroup XVIII (Eca) lipopolysaccharide and serogroup XX lipopolysaccharide**

Antibody serogroup	Serogroup of absorbing LPS	Serogroup of LPS reacting after absorption
Eca XVIII	Eca XVIII	-
Eca XVIII	XX	-
XX	Eca XVIII	-
XX	XX	-

7) did not react in immunoblotting (Methods 2.14.1) or immunodiffusion (Methods 2.14.2) experiments with two different serogroup I antisera (raised against glutaraldehyde fixed cells of Eca 436 and Eca 1042). Lipopolysaccharide from strain G398 did react in immunoblotting experiments against serogroup XX antiserum (raised against whole, live cells of Eca 1056).

Strain G331 was the single anomalous strain from serogroup XX (Figure 3.22, track 6). Serogroup I antisera (raised against glutaraldehyde fixed cells of Eca 436 and Eca 1042) were reactive against lipopolysaccharide from strain G331 in immunoblotting experiments (Methods 2.14.1).

The lipopolysaccharide from strain 432, the single atroseptica strain examined as an example of serogroup XXXV (Figure 3.22, track 16) reacted with serogroup XXII antiserum (raised against whole, live cells of Eca 198) in immunoblotting experiments (Methods 2.14.1).

Lipopolysaccharide from strains 553 (XXII, XXXV) and 557 (XXII, XLI) cross-reacted with serogroup I antiserum (raised against glutaraldehyde, fixed cells of Eca 436) in immunoblotting experiments (Methods 2.14.1) as did lipopolysaccharide from the six strains belonging uniquely to serogroup XXII (Figure 3.24). The lipopolysaccharide from strains 556 (XX, XLI) and 559 (XX, XL, XLIII) did not cross-react with serogroup I

antiserum (raised against glutaraldehyde fixed cells of Eca 436) in immunoblotting experiments (Methods 2.14.1) nor did lipopolysaccharide from four uniquely serogroup XX strains (Figure 3.25).

5.4 A Possible Protein Antigen Common to Serogroup I and Serogroup XXXV Strains

Development of an immunoblot (Methods 2.14.1) of serogroup XXXV lipopolysaccharides with serogroup I antiserum (raised against glutaraldehyde fixed cells of Eca 436) appeared to detect antigens which were not lipopolysaccharide antigens (Figure 3.26.a). Incubation of the lipopolysaccharides with chymotrypsin (Methods 2.16) prior to electrophoresis and immunoblotting removed these antigens indicating their proteinaceous nature (Figure 3.26.b).

Four identical immunoblots of the serogroup XXXV lipopolysaccharides were developed with serogroup I antiserum (raised against glutaraldehyde fixed cells of Eca 436) absorbed with whole cells of the four serogroup XXXV strains after these had been subjected to a mild acid hydrolysis (Methods 2.15.1). In all four immunoblots the same result was obtained: the proteinaceous antigens were still detected and those antibodies specific for serogroup I lipopolysaccharide were unaffected and could still identify serogroup I lipopolysaccharide (Figure 3.27).

Figure 3.26

a) Immunoblot of lipopolysaccharide from serogroup XXXV E. carotovora subsp. betavasculorum strains developed with serogroup I antiserum.

20µg of sample was run on a 12.5% acrylamide gel (Methods 2.10.1) and immunoblotted as described in Methods 2.14.1.

Tracks (1) Ecb 551 , serogroup XXXV
(2) Ec? 548 , XXXV
(3) Ecb 546 , XXXV
(4) Ecb 545 , XL, XLIII
(5) Eca 1034 , I

b) Immunoblot of chymotrypsin digested lipopolysaccharide from serogroup XXXV E. carotovora subsp. betavasculorum strains developed with serogroup I antiserum.

20µg of sample was incubated with chymotrypsin (Methods 2.16) prior to electrophoresis (Methods 2.10.1) and immunoblotting (Methods 2.14.1).

Tracks (1) Control (chymotrypsin)
(2) Ecb 551 , serogroup XXXV
(3) Ec? 548 , XXXV
(4) Ecb 546 , XXXV
(5) Ecb 545 , XL, XLIII
(6) Eca 1034, I

c) Immunoblot of lipopolysaccharide from E. carotovora subsp. atroseptica strain 1034 developed with serogroup I antiserum.

20µg of sample was run on a 12.5% acrylamide gel (Methods 2.10.1) and immunoblotted as described in Methods 2.14.1.

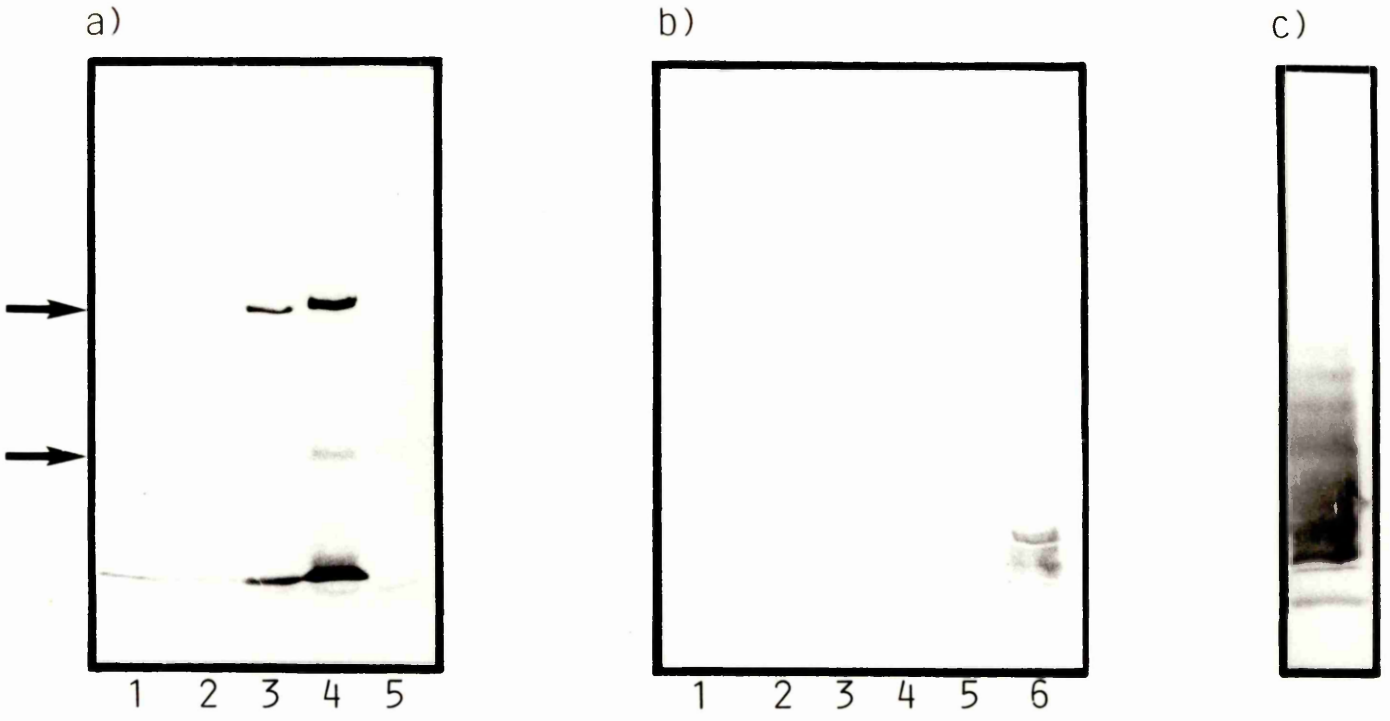


Figure 3.26.

Figure 3.27

Immunoblot of lipopolysaccharide from serogroup XXXV *E. carotovora* subsp. *betavasculorum* strains developed with serogroup I antiserum absorbed with serogroup XXXV cells.

20µg of sample was run on a 12.5% acrylamide gel (Methods 2.10.1) and immunoblotted as described in Methods 2.14.1. Immunoblot was developed with serogroup I antiserum absorbed with whole cells that had been subjected to a mild acid hydrolysis (Methods 2.15.1).

Tracks (1) *Ecb* 551 , serogroup XXXV
(2) *Ec?* 548 , XXXV
(3) *Ecb* 546 , XXXV
(4) *Ecb* 545 , XXXV
(5) *Eca* 1034 , I

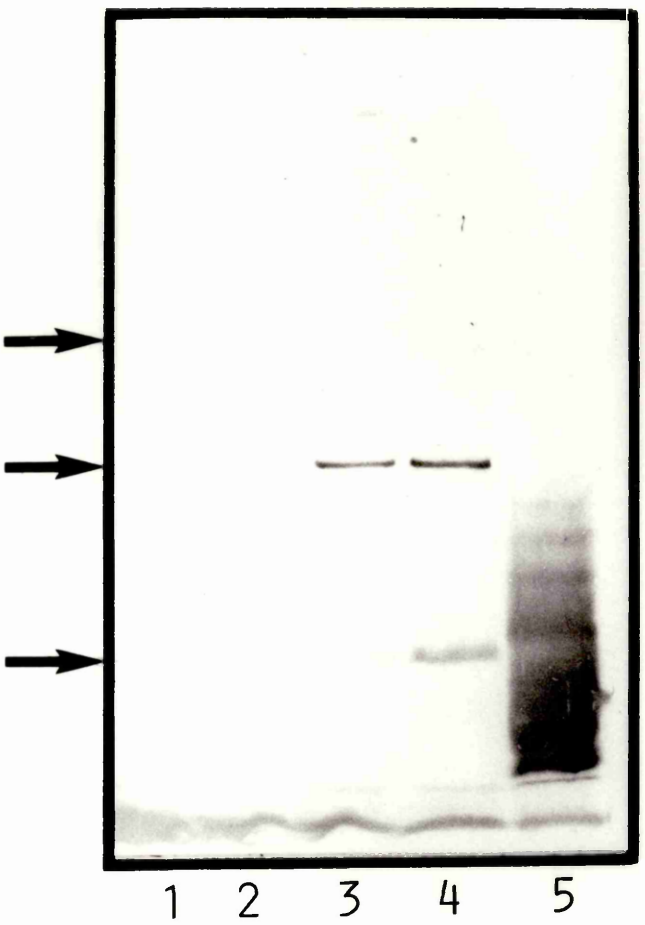


Figure 3.27.

5.5 Immunoblotting of Lipopolysaccharide from S. minnesota Mutant Strains and Development with E. carotovora Antiserum

Lipopolysaccharide from the S. minnesota smooth and rough mutant strains (Figure 3.10) was immunoblotted and developed with serogroup I antiserum (raised against glutaraldehyde fixed cells of Eca 436) as described in Methods 2.14.1. The E. carotovora antiserum reacted only with the control E. carotovora serogroup I lipopolysaccharide. The antiserum did not react with the Salmonella lipopolysaccharides.

6. Chromatography of the Lipopolysaccharide O-chain Fraction

6.1 Fractionation of E. Carotovora Polysaccharide Fraction

Gel filtration (Methods 2.18) of the polysaccharide fraction from hydrolysed E. carotovora lipopolysaccharide (Methods 2.17.1.1) gave a carbohydrate elution profile (Methods 2.20.3) consisting of three distinct peaks (Figure 3.28).

6.2 GLC and GLC-MS Combined Analyses

6.2.1 GLC Analysis of Alditol Acetates Produced From Standard Sugars

Alditol acetates of the available sugar standards (Methods 2.17.1.1) eluted off the column in order of increasing carbon number (Figure 3.29). For the two different columns used in this study, the elution order was the same. The retention times of the different sugar alditol acetates were determined to be very variable (Table 3.5) for the Perkin-Elmer apparatus described in Methods 2.17.1.

6.2.2 Response Factors of Alditol Acetates Produced From Standard Sugars Analysed by GLC

The response factors were determined for the

Figure 3.28

Fractionation of E. carotovora polysaccharide fraction by gel filtration.

The polysaccharide fraction of hydrolysed lipopolysaccharide (15mg) from E. carotovora subsp. atroseptica strain 1034 (Methods 2.17.1.1) was applied to a Sephadex G50 column (90cm long x 2.2cm diam.) which had been equilibrated with 0.05M pyridine/acetic acid, pH 5.4 (Methods 2.18). Carbohydrate was eluted at a flow rate of 50ml h^{-1} and the fractions collected assayed for carbohydrate at A_{480} as described in Methods 2.20.3.

Peak designations are according to Ray et al. (1986).

Peak I: O-chain + core
Peak II: core
Peak III: KDO, phosphate

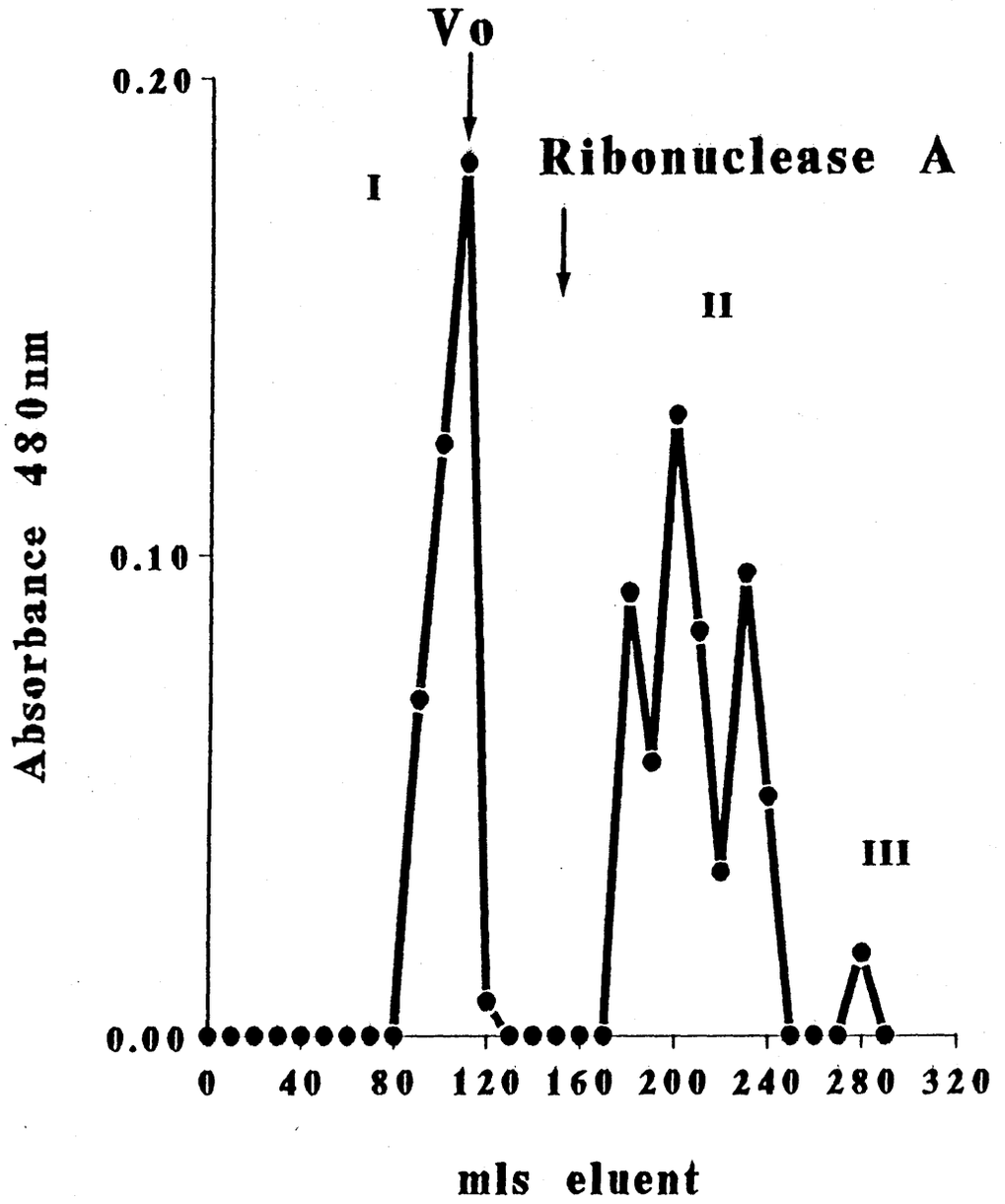

Vo
Ribonuclease A
I
II
III
0.20
0.10
0.00
Absorbance 480nm
0
40
80
120
160
200
240
280
320
mls eluent

Figure 3.28

Figure 3.29

GLC analysis of alditol acetates produced from standard sugars.

Alditol acetates were produced from standard sugars as described in Methods 2.17.1.1. They were separated on a SGE bonded phase (0.1μm film thickness) capillary column, BP-225, bonded to a vitreous silica column (25M x 0.22mm) attached to a flame ionisation detector as described in Methods 2.17.1.

Abbreviations

sp	:	solvent peak
eryth	:	erythrose
rh	:	rhamnose
fuc	:	fucose
rib	:	ribose
xyl	:	xylose
is	:	2-deoxyglucose (internal standard)
man	:	mannose
gal	:	galactose
glc	:	glucose

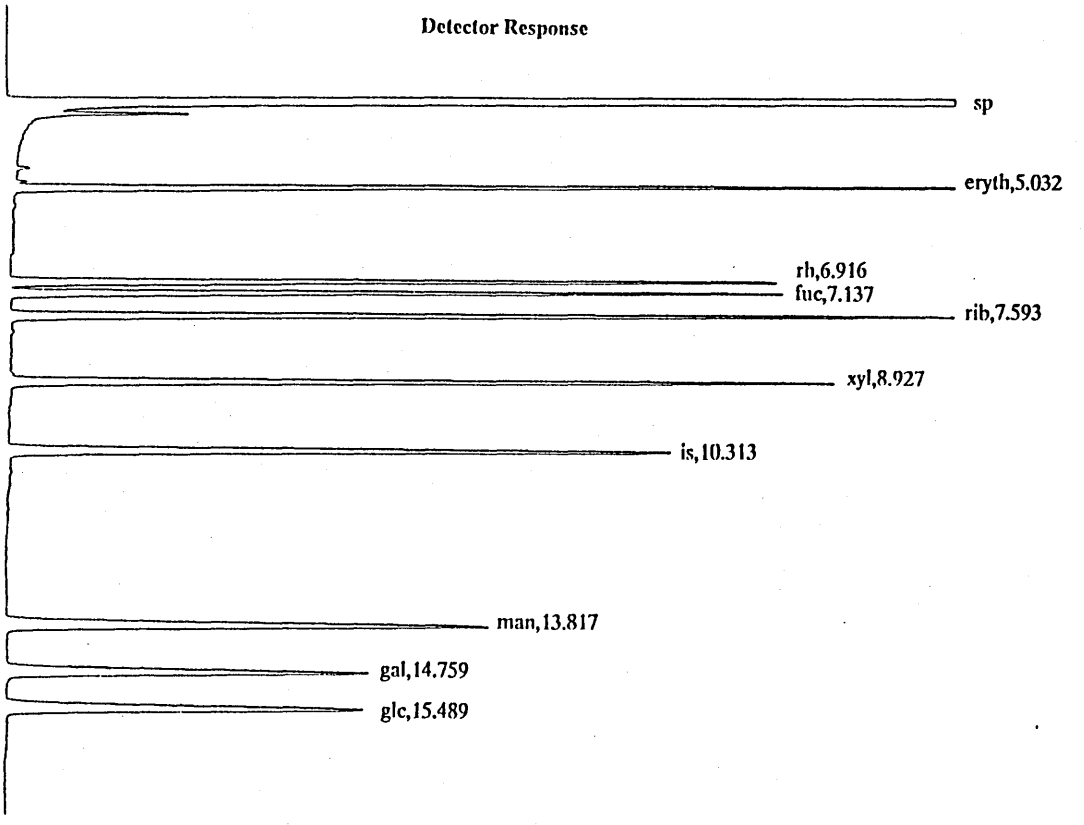

Detector Response
sp
eryth,5.032
rh,6.916
fuc,7.137
rib,7.593
xyl,8.927
is,10.313
man,13.817
gal,14.759
glc,15.489
Retention Time(min)

Figure 3.29.

TABLE 3.5 **Variability of the retention times of the alditol acetates of standard sugars analysed by GLC**

	Retention times	
Standard sugar	I	II
Erythrose	5.032	-
Rhamnose	6.916	5.733
Fucose	7.137	5.888
Ribose	7.593	6.147
Xylose	8.927	-
2-deoxyglucose	10.313	7.865
Mannose	13.817	10.029
Galactose	14.759	10.621
Glucose	15.489	11.004
Glyceromannoheptose	27.673	19.196

- : omitted from sugar mix

Retention times were determined for sugar alditol acetates on the Perkin-Elmer apparatus as described in Methods 2.17.1.

alditol acetates of the different sugar standards with respect to the alditol acetate of the internal standard 2-deoxyglucose as described in Methods 2.17.1.2. The results obtained are shown in Table 3.6.

6.2.3 Sugar Composition of Serogroup I Lipopolysaccharide O-chain Fraction

6.2.3.1 GLC-MS Combined Analysis

GLC-MS analysis (Methods 2.17.2.1) was performed on the alditol acetates of eleven selected serogroup I lipopolysaccharide preparations including strain Eca 31 De Boer's type strain for serogroup I. From GLC-MS analysis of the alditol acetates of the standard sugars (Figure 3.30) it was shown that under the conditions used, the apparatus would detect common molecular ion fragments at 115 a.m.u. and 170 a.m.u. These molecular ion fragments are not primary ion fragments produced by GLC-MS fragmentation of sugar alditol acetates (Figure 3.39) but are common to all standard sugar alditol acetates analysed. They were therefore used to pick out possible sugar peaks in the lipopolysaccharide GLC profiles. By comparison to the scan number and mass spectral fragmentation pattern for the standard sugar alditol acetates, all eleven serogroup I lipopolysaccharides were found to contain 6-deoxyhexose, probably rhamnose, ribose, mannose, galactose and

TABLE 3.6 **Response factors for the alditol acetates of standard sugars analysed by GLC**

Sugar	Response Factor
Erythrose	0.81
Rhamnose	1.13
Fucose	1.5
Ribose	0.88
Xylose	0.92
Mannose	1.08
Galactose	1.43
Glucose	1.36

$$\text{Response Factor} = \frac{\text{area of internal standard (5mg)}}{\text{area of standard sugar (5mg)}}$$

Response Factors were determined for sugar alditol acetates on the Perkin-Elmer apparatus as described in Methods 2.17.1.

Figure 3.30

Mass spectral fragmentation patterns of alditol acetates of standard sugars.

Alditol acetates were prepared from standard sugars as described in Methods 2.17.1.1. They were separated on a Quadrex bonded phase (0.25μm film thickness) capillary column, OV-225, bonded to a fused silica column (25m x 0.25mm) attached to a mass spectrometer as described in Methods 2.17.2.

Annotation

* : common molecular ion fragment characteristic of a sugar alditol acetate produced under the conditions described in Methods 2.17.2.

M+ : parent ion

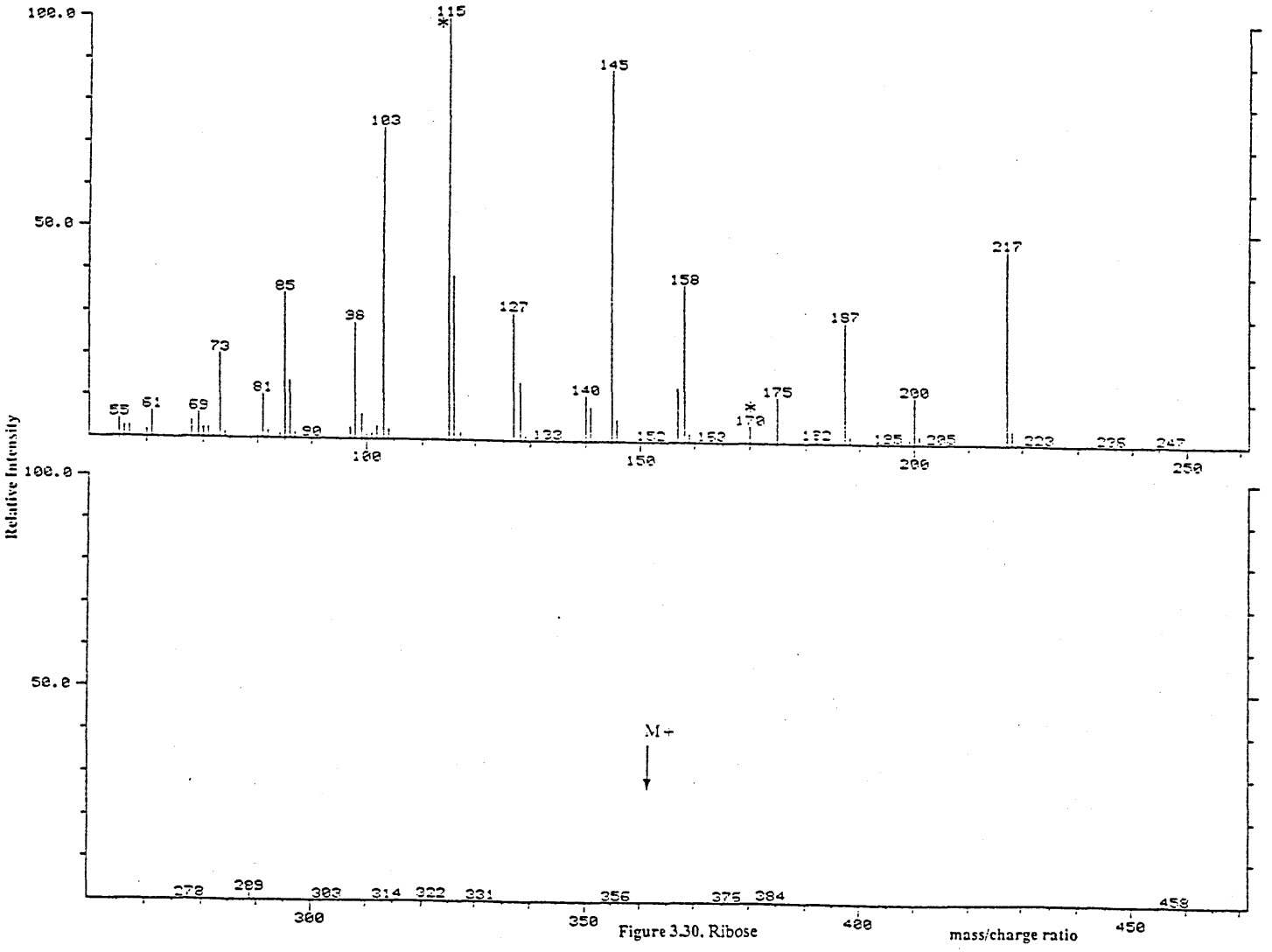


Figure 3.30. Ribose

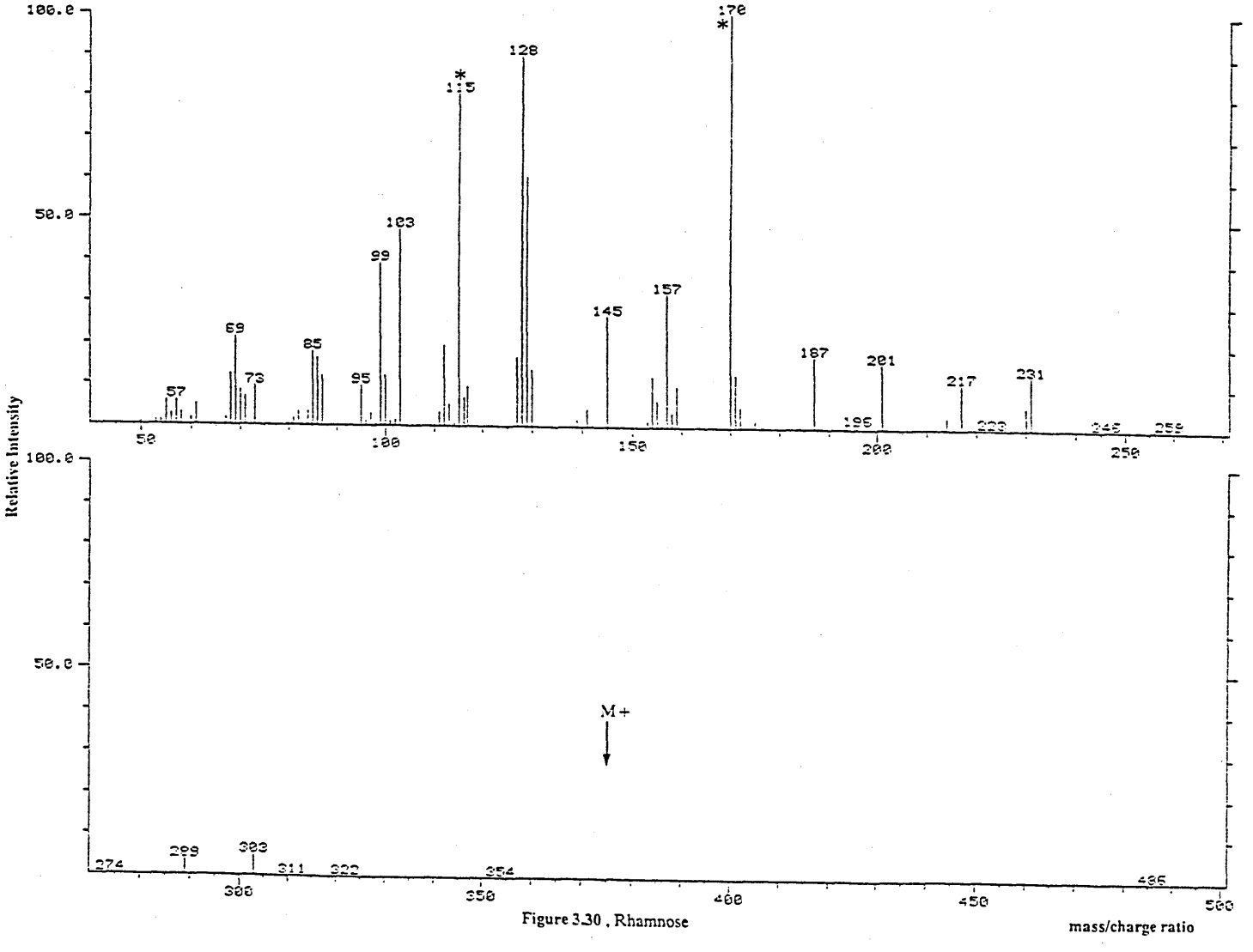


Figure 3.30 , Rhamnose

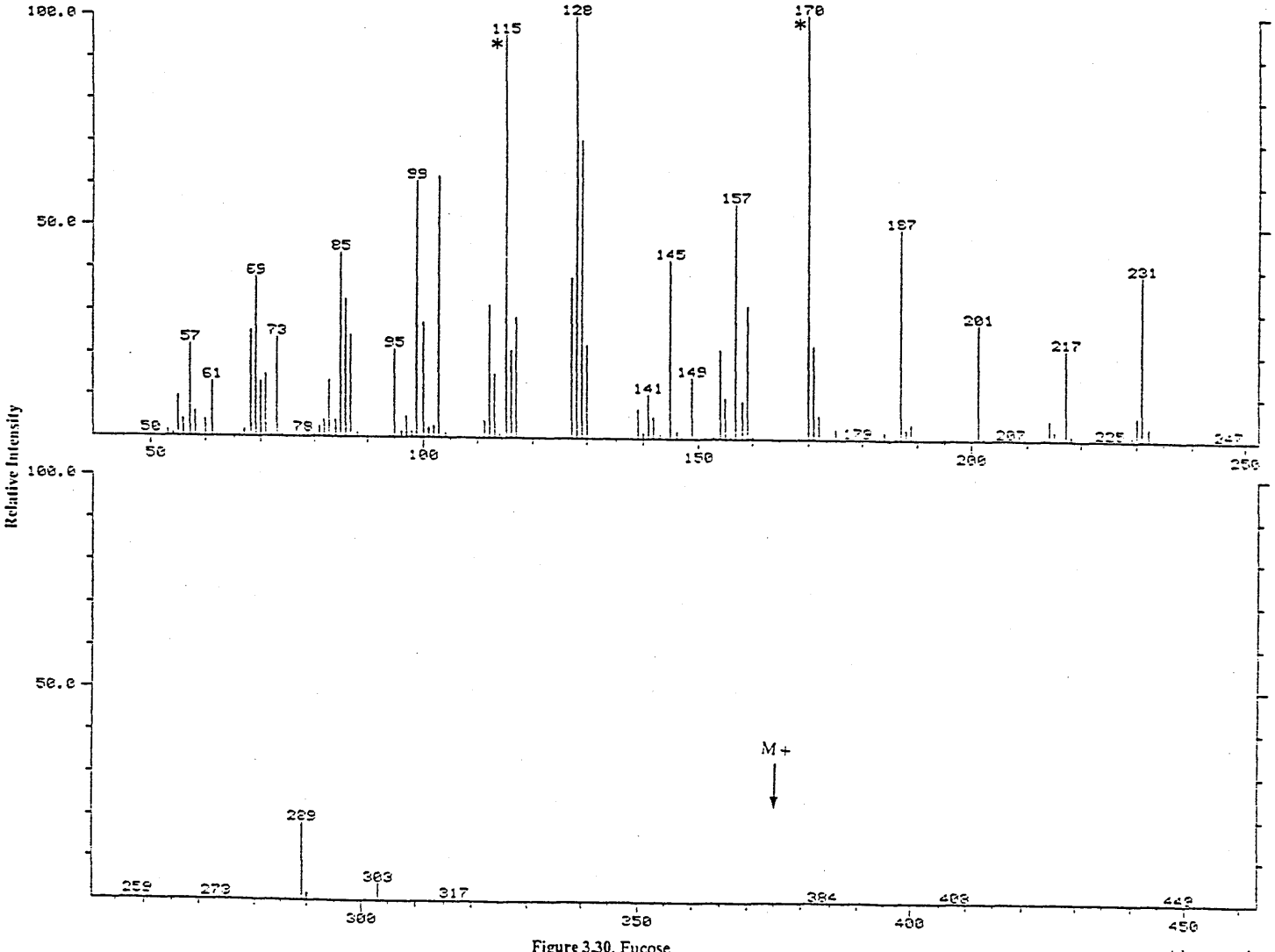


Figure 3.30, Fucose

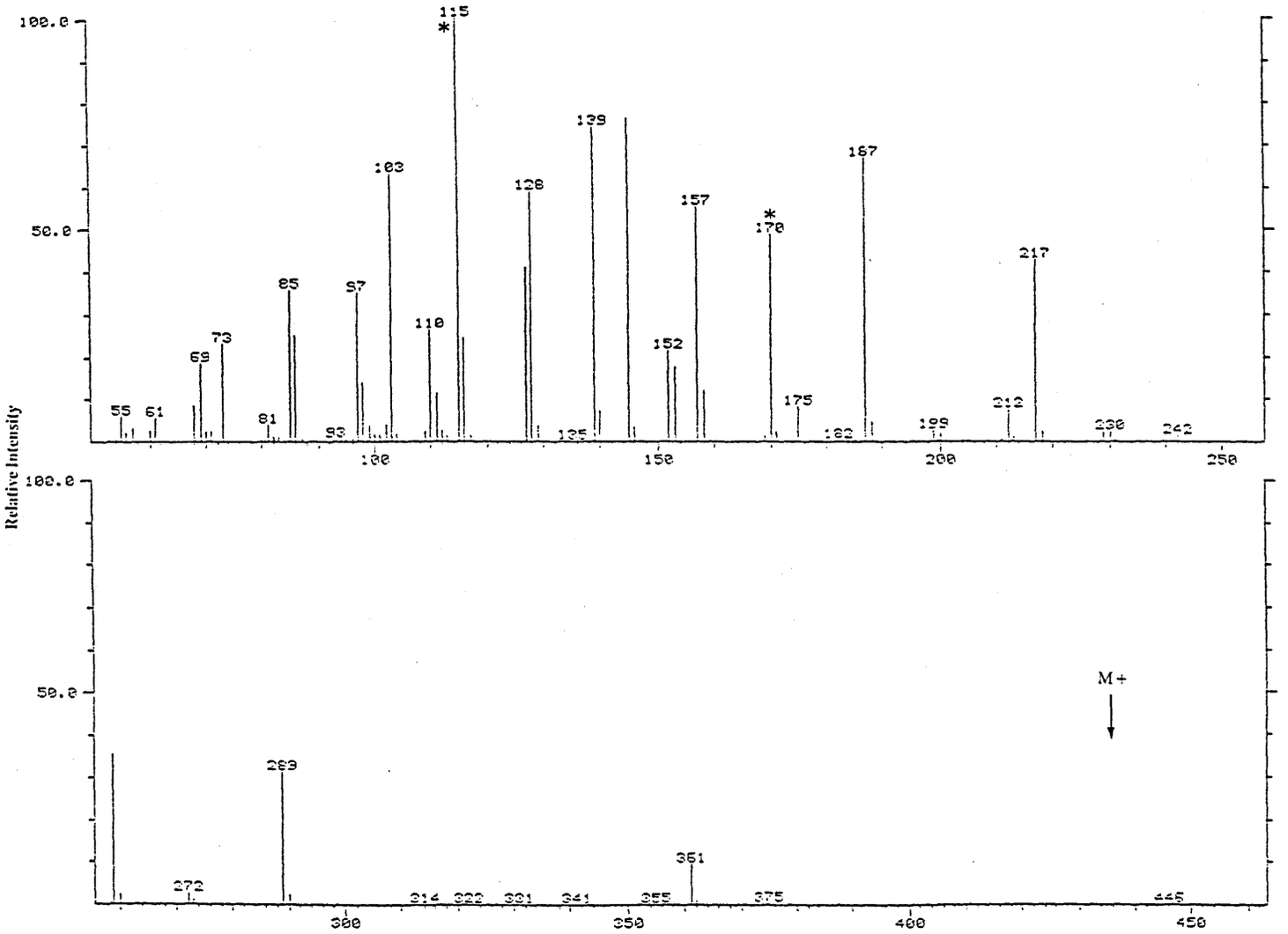


Figure 3.30, Mannose

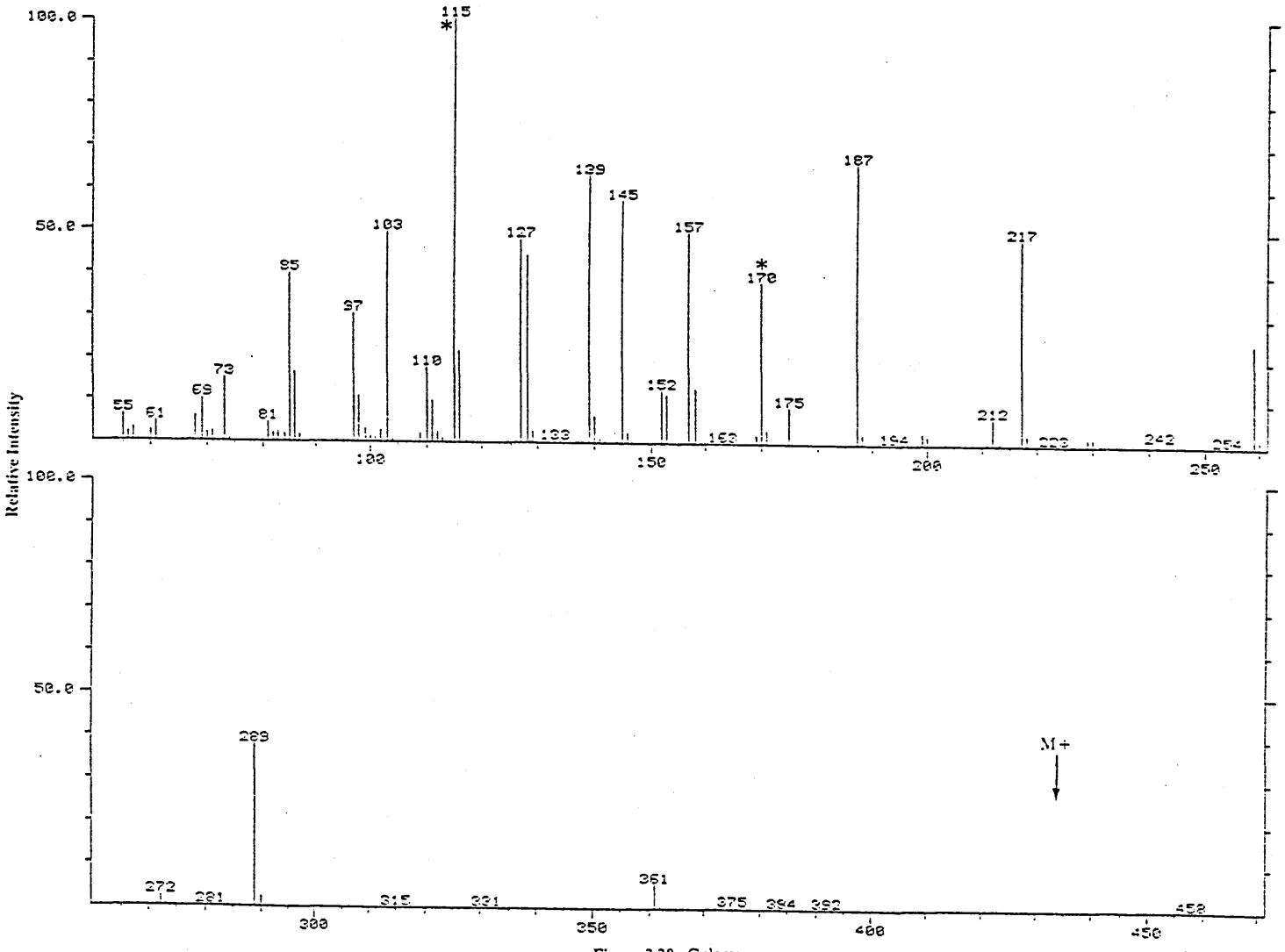


Figure 3.30, Galactose

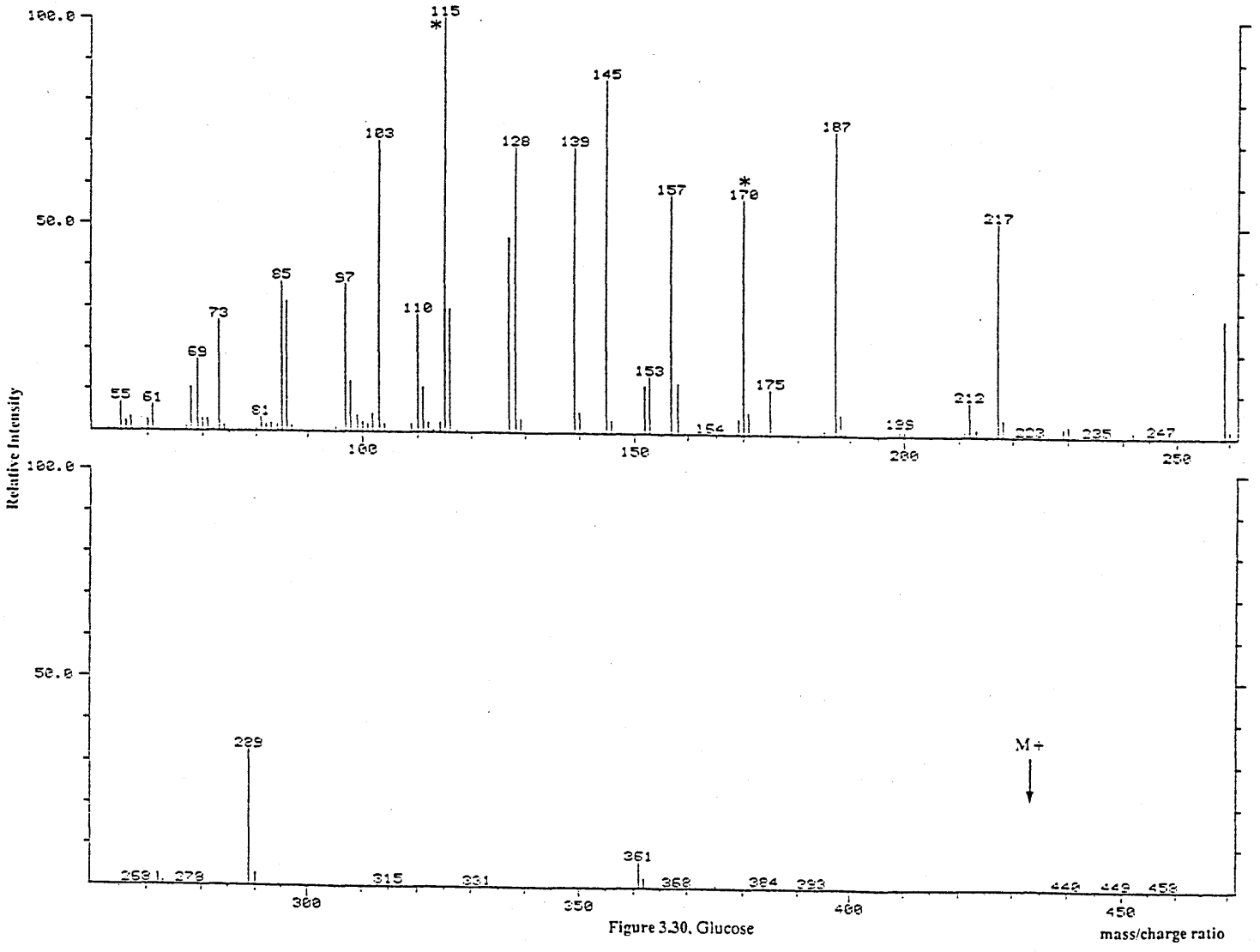


Figure 3.30. Glucose

glucose (Figure 3.31).

6.2.3.1.1 Confirmation and Identity of the 6-deoxyhexose

Confirmatory analyses revealed the 6-deoxyhexose to be rhamnose. By comparison to GLC analysis of the lipopolysaccharide alditol acetate mixture, addition of rhamnitol acetate to this mixture produced an increased peak area for the rhamnitol acetate upon subsequent GLC analysis. Similar addition of fucitol acetate produced an extra peak in the GLC profile (Figure 3.32) which did not correspond to any other peak.

Thin layer chromatography (Methods 2.17.3) of the monosaccharide fraction from a serogroup I lipopoly-saccharide preparation confirmed the presence of rhamnose, ribose, mannose, galactose and glucose in serogroup I lipopolysaccharide. A very fast migrating spot was also observed in the lipopolysaccharide sample run.

6.2.3.2 GLC Analysis

Alditol acetates were produced from the constituent sugars of seven selected serogroup I lipopolysaccharide preparations as described in Methods 2.17.1.1 and analysed by GLC (Methods 2.17.1). Two preparations had previously been analysed by combined GLC-MS.
Constituent sugars were identified as rhamnose, ribose,

Figure 3.31

GLC-MS combined analysis of the alditol acetates produced from serogroup I lipopolysaccharide.

Alditol acetates were produced from lipopolysaccharide as described in Methods 2.17.1.1. They were separated on a Quadrex bonded phase (0.25µm film thickness) capillary column, OV-225, bonded to a fused silica column (25m x 0.25mm) attached to a mass spectrometer as described in Methods 2.17.2.

Abbreviations

rh	:	rhamnose
rib	:	ribose
is	:	2-deoxyglucose (internal standard)
man	:	mannose
gal	:	galactose
glc	:	glucose

1 : GLC profile.

2 & 3 : Mass / charge ratios of 170 & 115 a.m.u.'s, respectively. Peaks from (1) possessing these fragments have been picked out as they are characteristic of sugar alditol acetates produced under conditions used in Methods 2.17.2. (see Figure 3.30.).

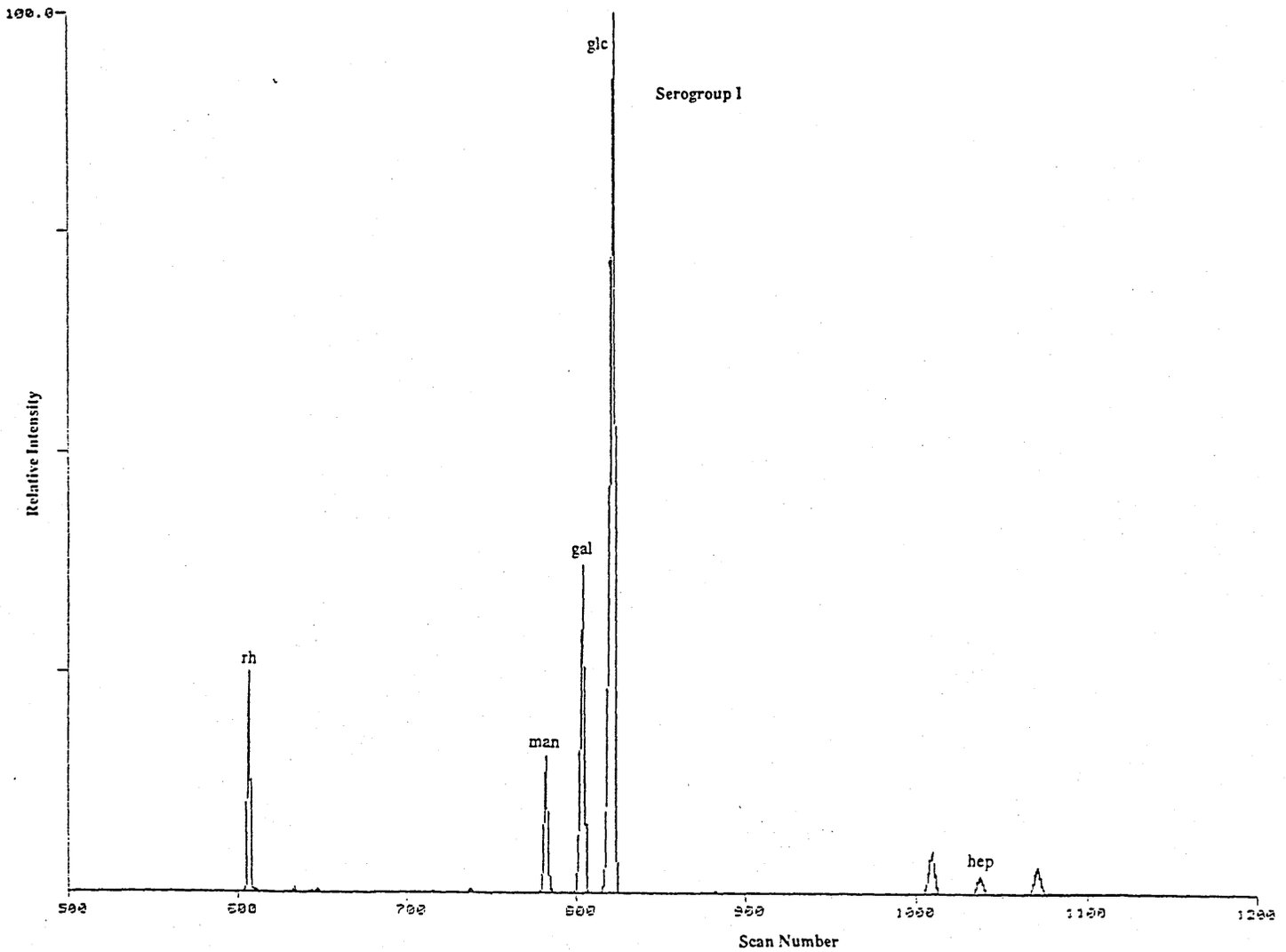

100.0
Relative Intensity
glc
Serogroup I
gal
rh
man
hep
500
600
700
800
900
1000
1100
1200
Scan Number

Figure 3.31.

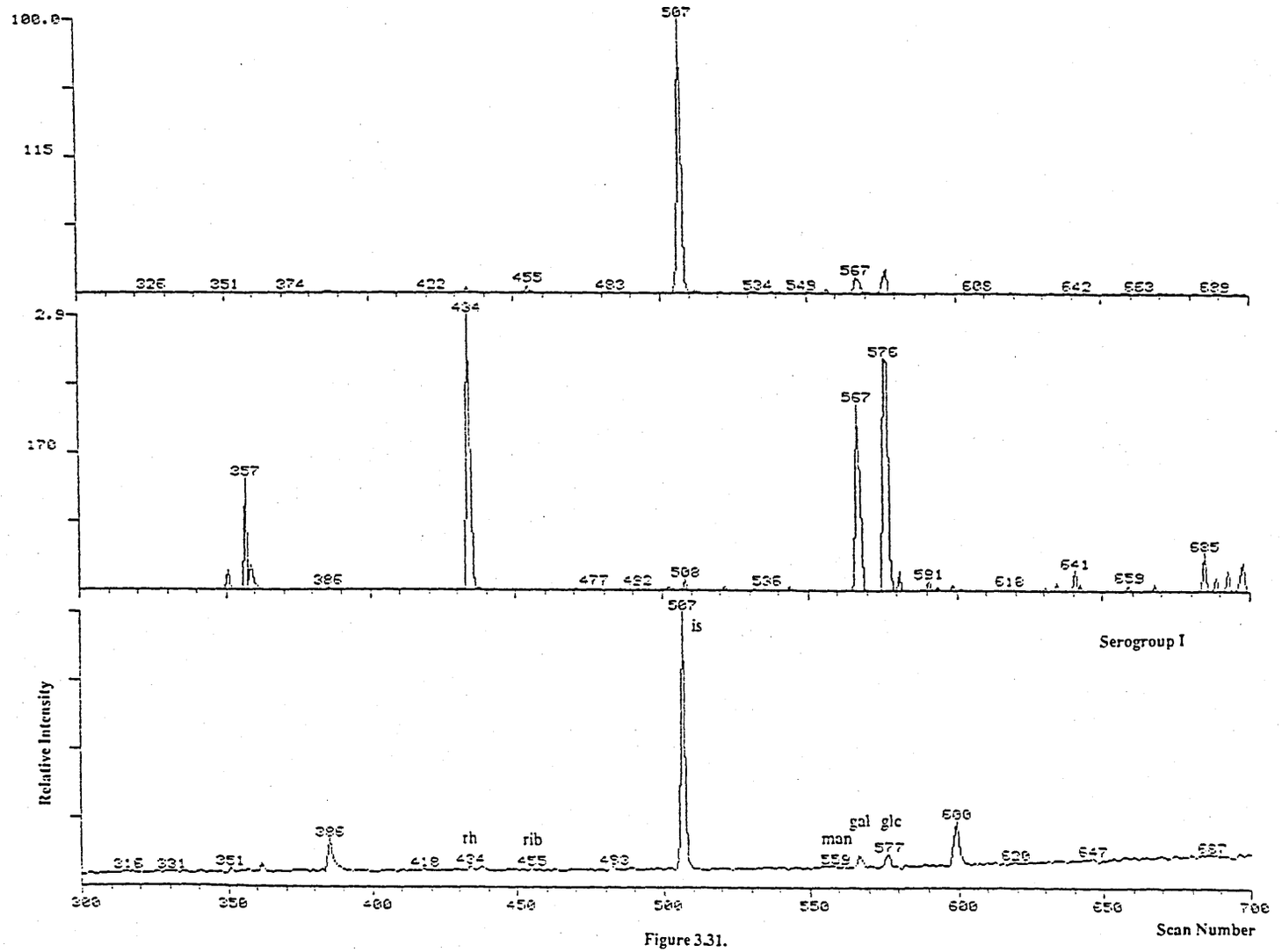

100.0
115
507
326
351
374
422
455
483
534
549
567
608
642
663
699
3
2.9
170
434
357
576
567
386
477
492
508
536
591
618
641
659
685
2
Serogroup I
507
is
Relative Intensity
395
rh
rib
gal
glc
man
577
600
316
331
351
418
434
455
493
559
620
647
687
1
300
350
400
450
500
550
600
650
700
Scan Number

Figure 3.31.

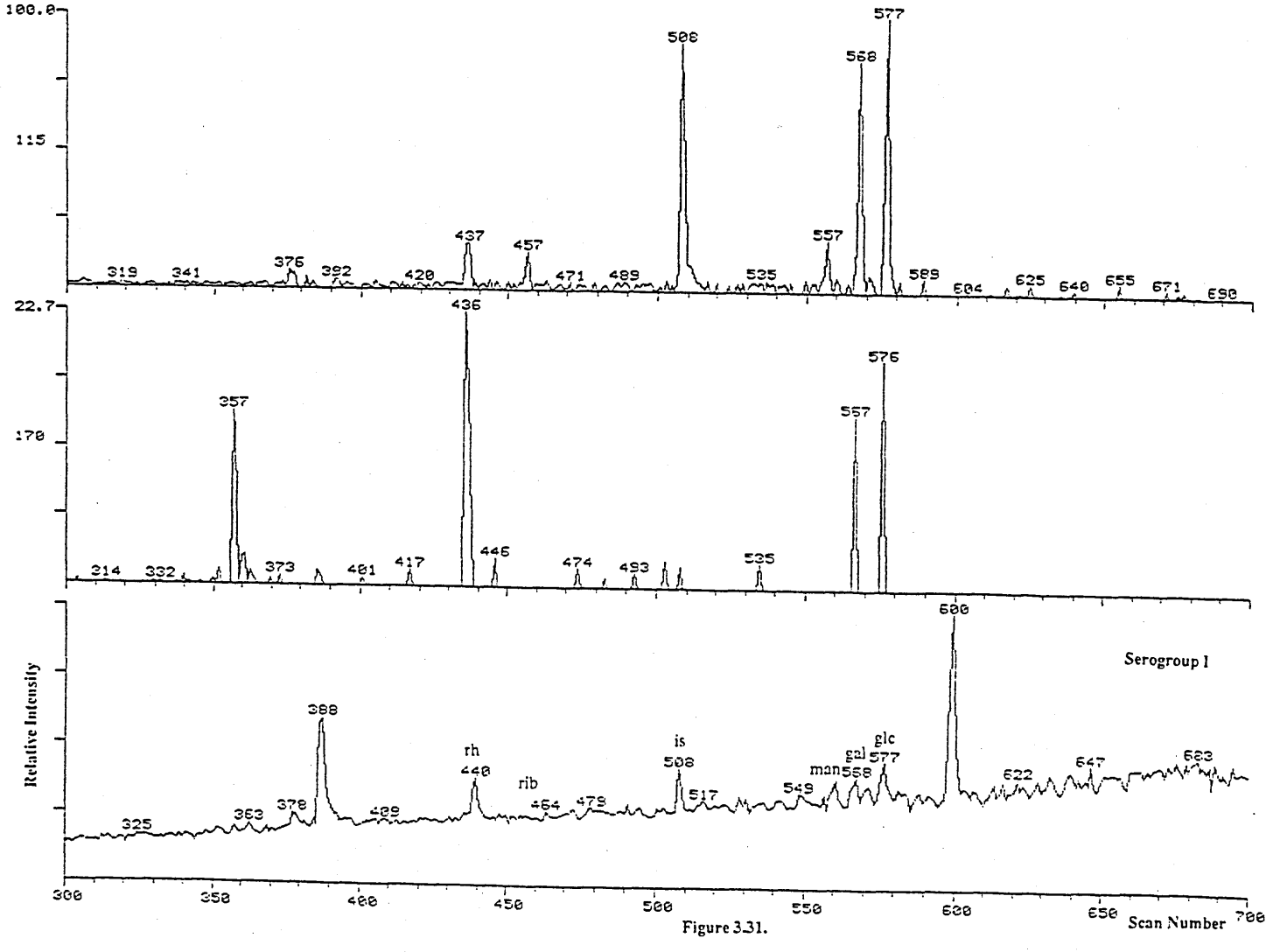

100.0
115
22.7
170
Relative Intensity
Serogroup 1
3
2
1
rh
rib
is
gal
man
glc
Scan Number

Figure 3.31.

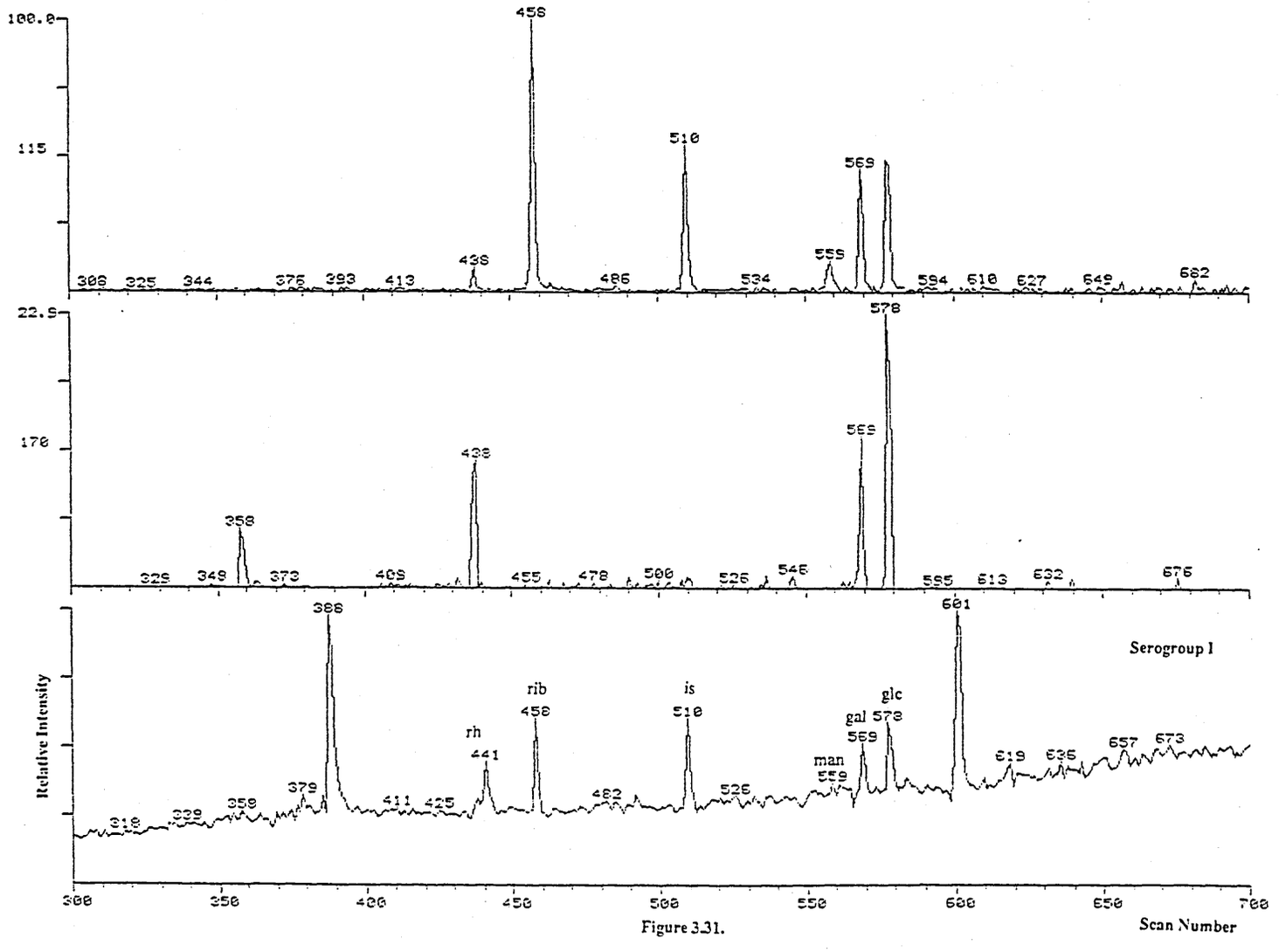

100.0
115
458
510
569
438
555
308 325 344 376 393 413 438 486 534 555 569 594 610 627 649 682
3
22.9
170
578
569
438
358
329 348 373 409 455 478 500 526 545 585 613 632 676
2
388
601
Serogroup I
rib
458
is
510
glc
gal 578
569
rh
441
man
559
318 339 359 379 411 425 482 526 619 635 657 673
1
Relative Intensity
300 350 400 450 500 550 600 650 700
Scan Number

Figure 3.31.

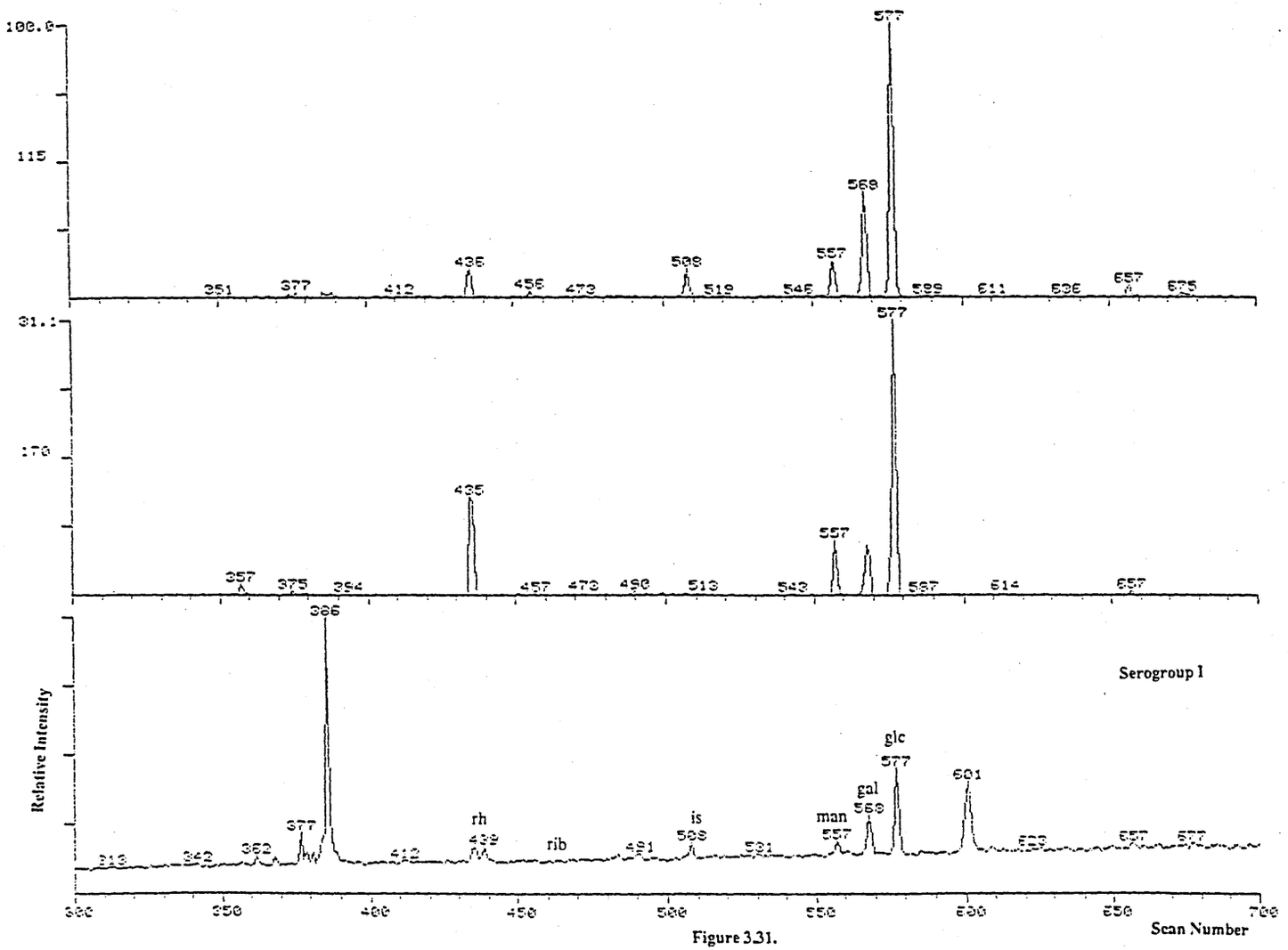

3
2
1
Serogroup I
Relative Intensity
Scan Number
glc
gal
man
rh
rib
is

Figure 3.31.

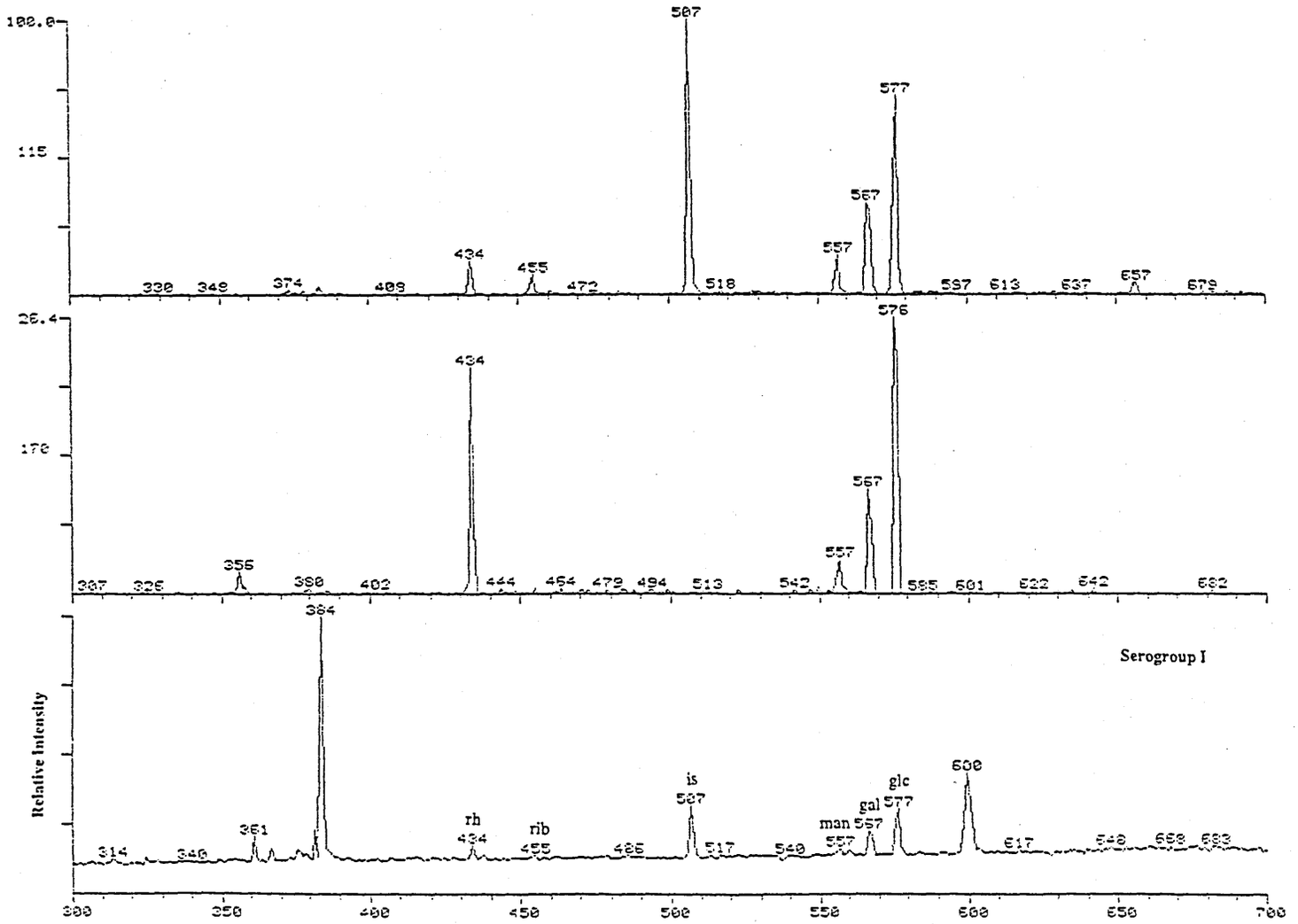
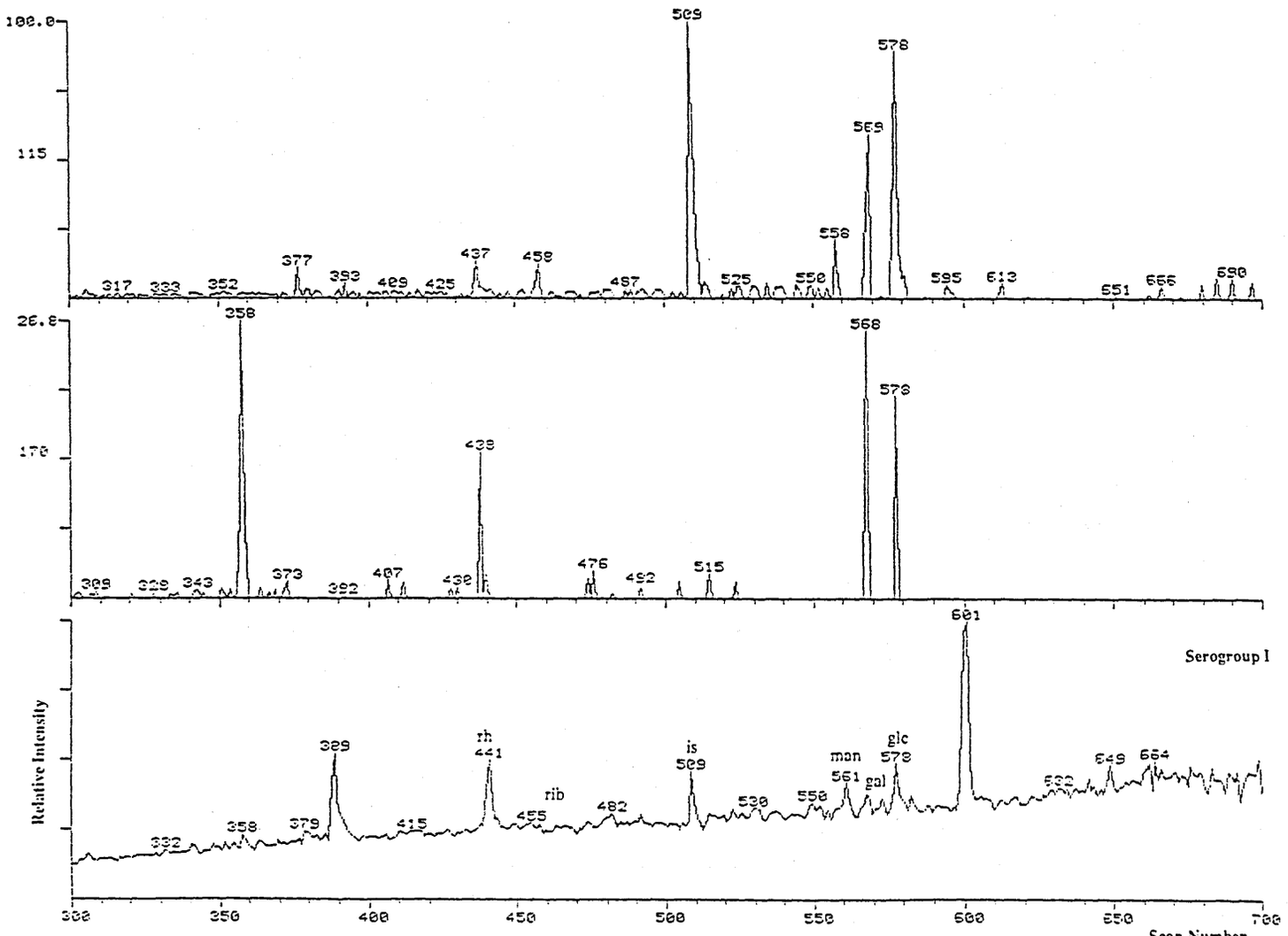

100.0
115
507
577
567
557
434
455
330 348 374 409 472 518 597 613 637 657 679
3
26.4
178
576
434
567
557
355
307 326 380 402 444 464 479 494 513 542 585 601 622 642 682
2
Serogroup I
384
Relative Intensity
600
is
507
glc
gal 577
rh
434
rib
455
man 557
361
314 340 485 517 540 557 617 648 668 683
1
300 350 400 450 500 550 600 650 700
Scan Number

Figure 3.31.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115
509
578
569
558
377
393
409
425
437
458
487
525
550
595
613
551
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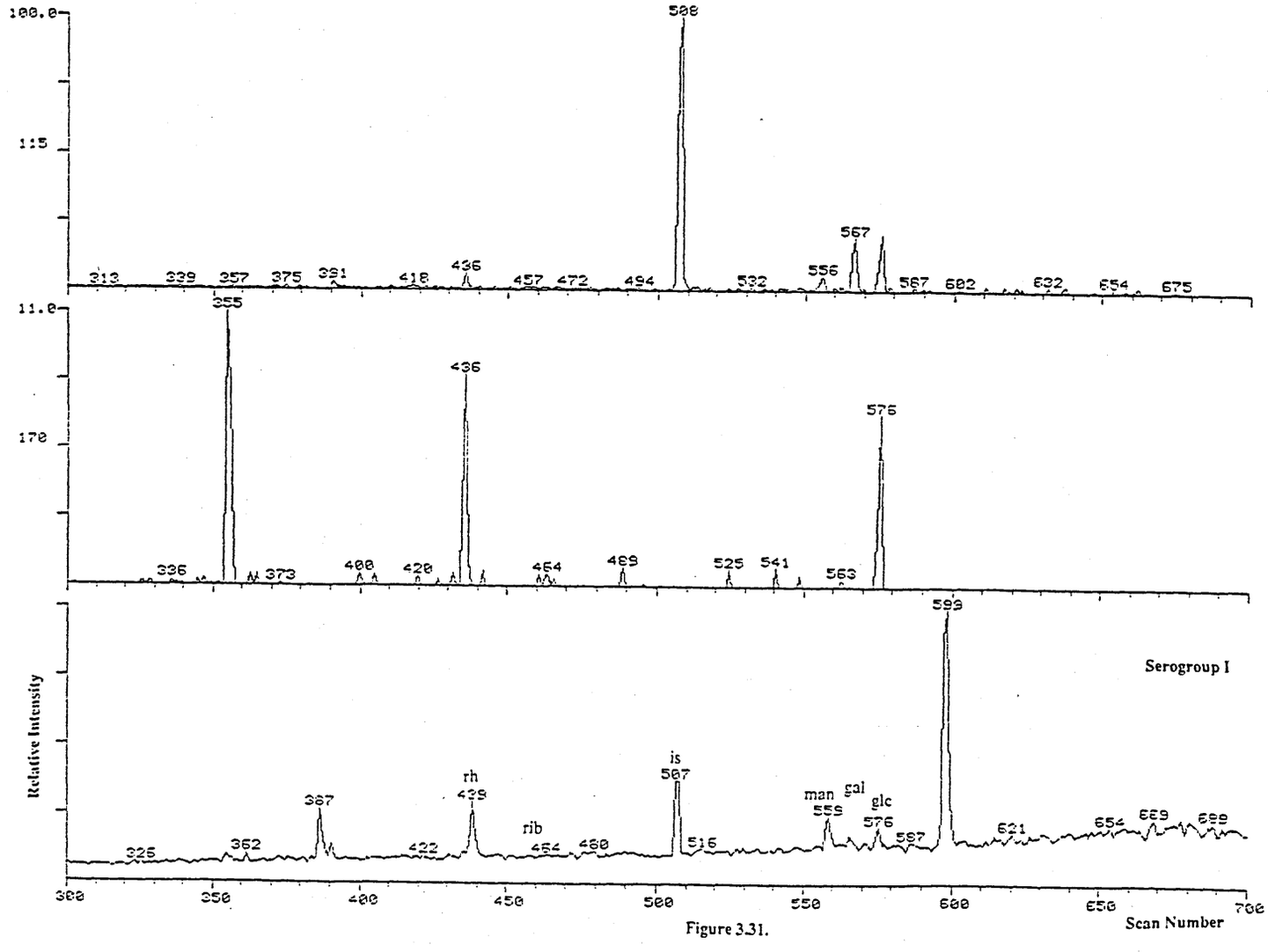
690
317
333
352
26.8
170
358
568
578
438
309
329
343
373
392
407
430
476
492
515
601
Serogroup I
Relative Intensity
rh
441
is
509
man
561
glc
578
gal
rib
455
482
530
550
532
649
664
332
358
379
389
415
300
350
400
450
500
550
600
650
700
Scan Number
3
2
1

Figure 3.31.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115
313
339
357
375
391
418
436
457
472
494
508
532
556
567
587
602
632
654
675
3
11.0
170
355
336
373
400
420
436
464
489
525
541
563
576
2
Relative Intensity
325
362
387
422
rh
439
rib
464
480
is
507
515
man
559
gal
glc
576
597
599
621
654
669
699
1
Serogroup I
300
350
400
450
500
550
600
650
700
Scan Number

Figure 3.31.

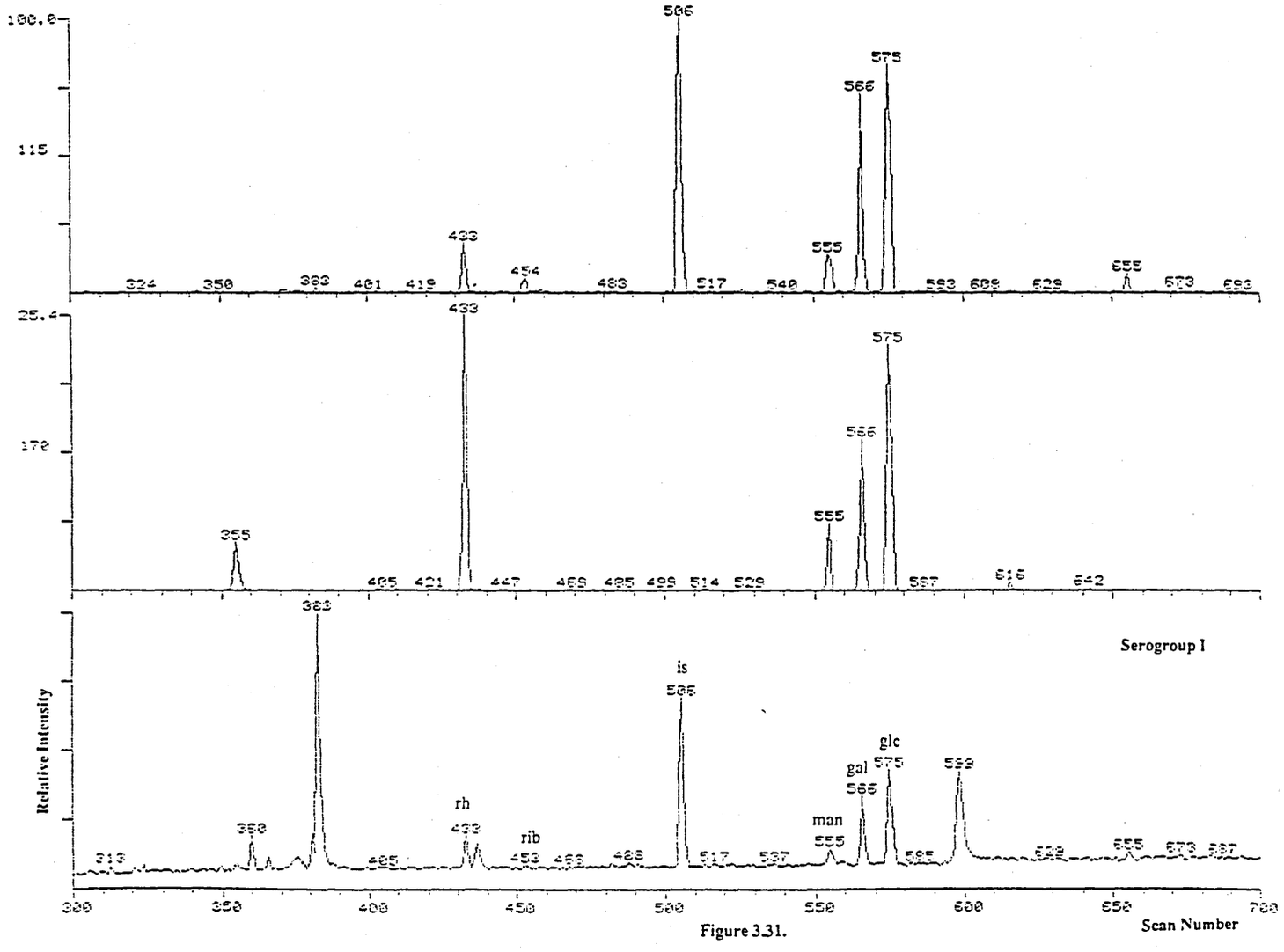
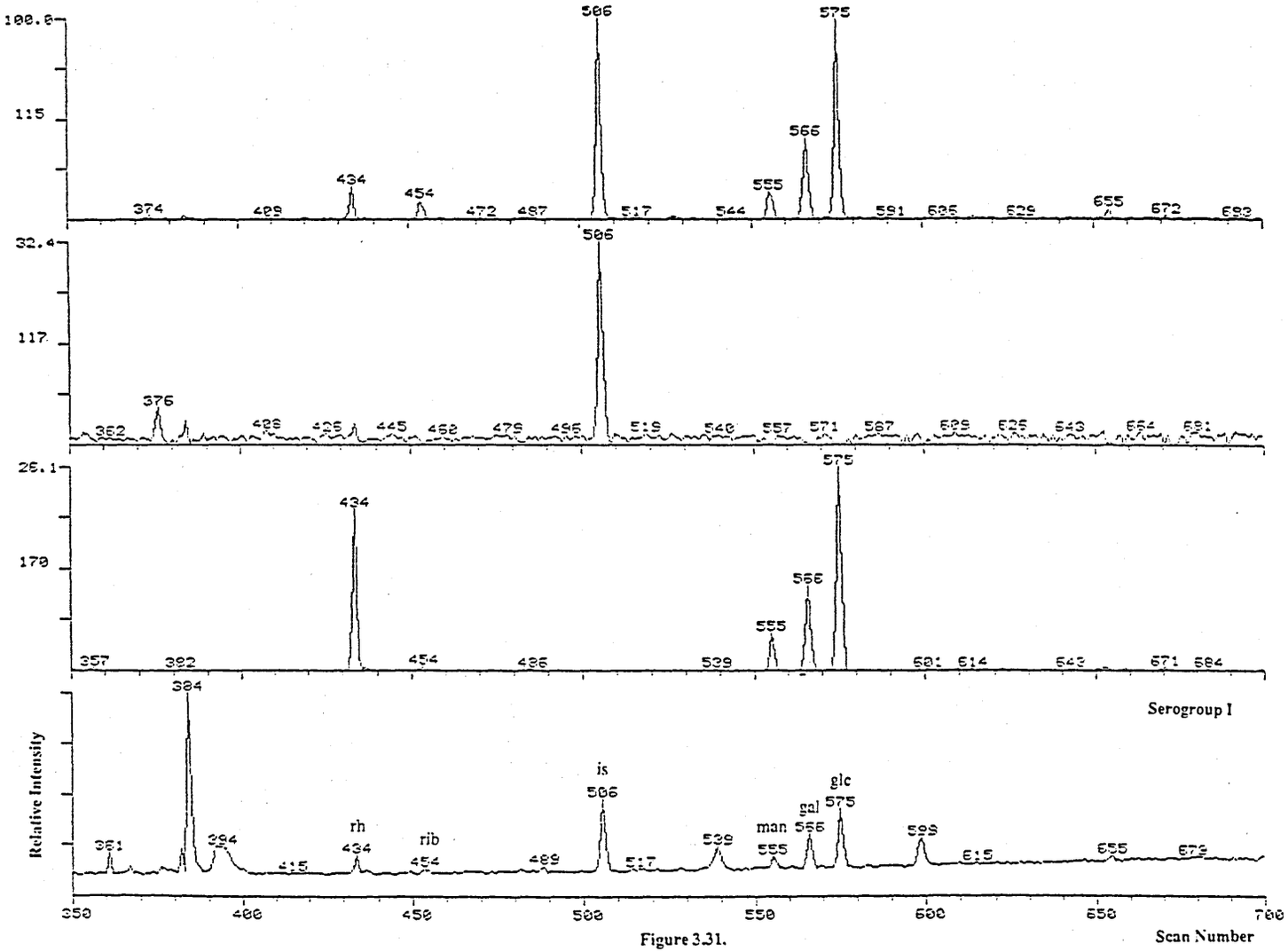

100.0
115
506
575
566
555
433
454
324 350 383 401 419 483 517 540 593 609 629 655 673 693
3
25.4
170
433
575
566
555
355
405 421 447 469 485 499 514 529 587 616 642
2
383
Serogroup I
is
506
gal
566
glc
575
599
rh
433
rib
453
man
555
313 360 485 517 537 585 629 655 673 687
1
Relative Intensity
300 350 400 450 500 550 600 650 700
Scan Number

Figure 3.31.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115
506
575
566
434
454
555
374
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472
487
517
544
591
605
629
655
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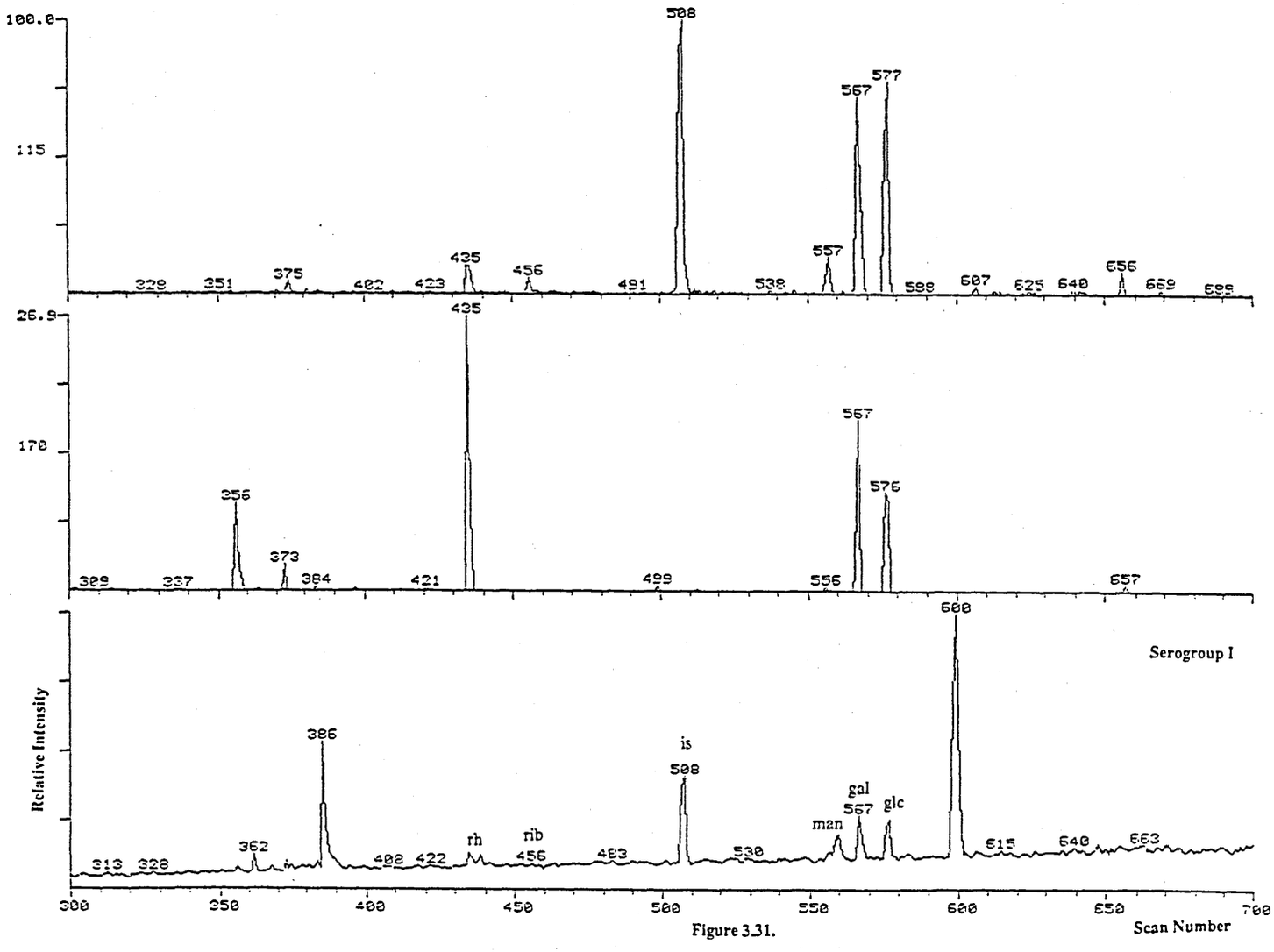
692
3
32.4
117
506
376
362
488
425
445
462
479
496
519
540
557
571
587
609
625
643
664
681
26.1
170
575
434
566
555
357
382
454
486
539
601
614
643
671
684
2
Serogroup I
Relative Intensity
384
is
506
glc
gal
575
rh
434
rib
454
man
555
566
361
394
415
489
517
539
593
615
655
679
1
350
400
450
500
550
600
650
700
Scan Number

Figure 3.31.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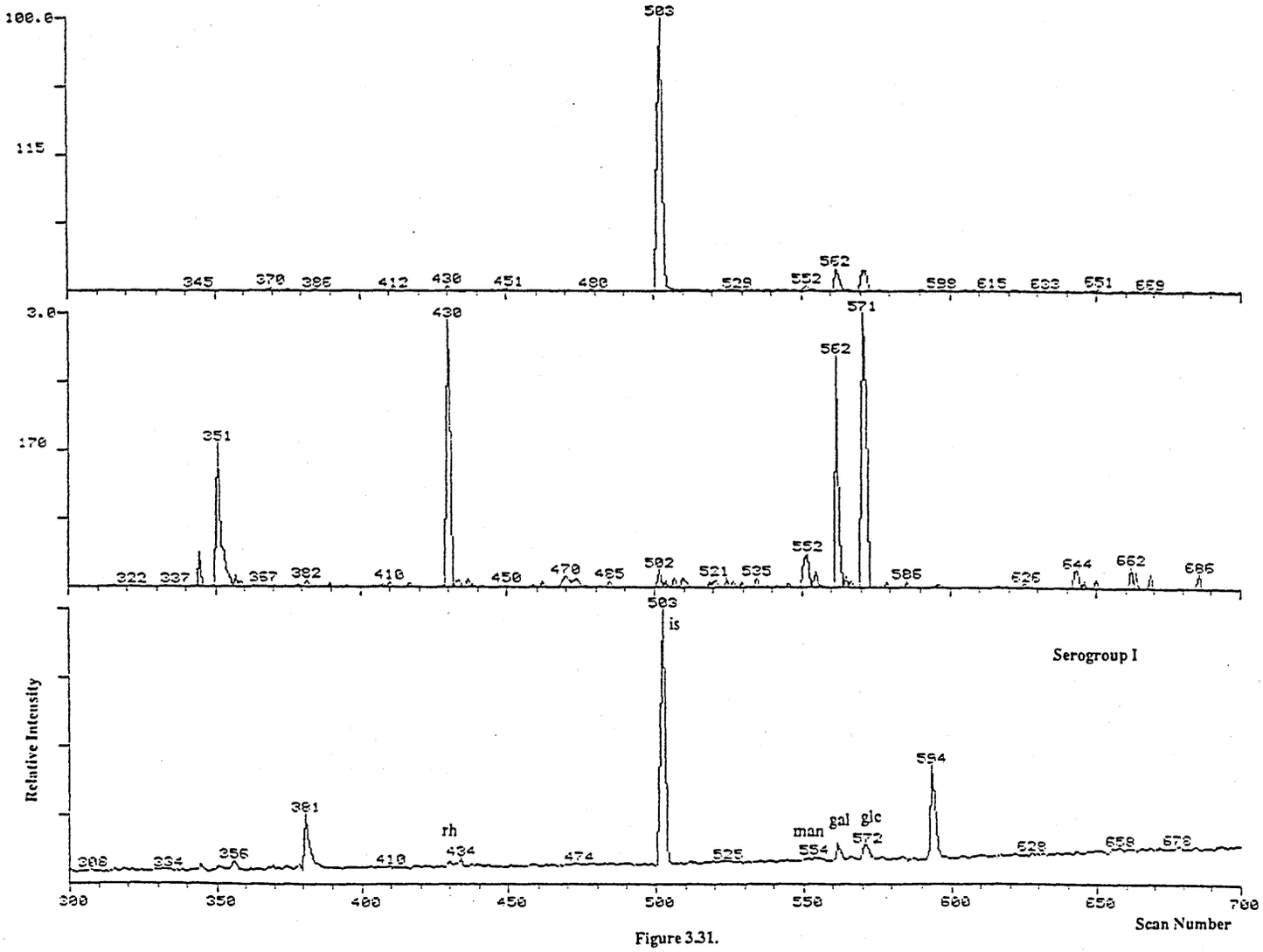
508
567
577
557
435
456
375
351
328
402
423
491
538
588
607
625
640
656
669
695
3
26.9
170
435
567
576
356
373
309
337
384
421
499
556
657
2
600
Serogroup I
Relative Intensity
386
is
508
gal
567
glc
man
rh
rib
362
313
328
408
422
456
483
530
615
640
663
1
300
350
400
450
500
550
600
650
700
Scan Number

Figure 3.31.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115
503
345
370
386
412
430
451
490
529
552
562
588
615
633
651
669
3
3.0
170
430
571
562
351
322
337
357
382
410
450
470
485
502
521
535
552
586
626
644
662
686
2
503
is
Serogroup I
1
554
381
rh
434
man
gal
glc
572
308
334
356
410
474
525
554
628
658
678
Relative Intensity
300
350
400
450
500
550
600
650
700
Scan Number

Figure 3.31.

Figure 3.32

Identification of the 6-deoxyhexose present in E. carotovora lipopolysaccharide preparations.

a) Alditol acetates were prepared from a serogroup I lipopolysaccharide preparation as described in Methods 2.17.1.1. They were separated on a Quadrex bonded phase (0.25μm film thickness) capillary column, OV-225, bonded to a fused silica column (25m x 0.25mm) attached to a mass spectrometer as described in Methods 2.17.2.

b) Fucitol pentaacetate was added to the alditol acetate mixture prepared from the serogroup I lipopolysaccharide described in a) and analysed under the same conditions as described above.

Abbreviations

rh	:	rhamnose
fuc	:	fucose
is	:	2-deoxyglucose (internal standard)
man	:	mannose
gal	:	galactose
glc	:	glucose

1 : GLC profile.

2 & 3 : Mass / charge ratios of 170 & 115 a.m.u.'s, respectively. Peaks from (1) possessing these fragments have been picked out as they are characteristic of sugar alditol acetates produced under conditions used in Methods 2.17.2. (see Figure 3.30.).

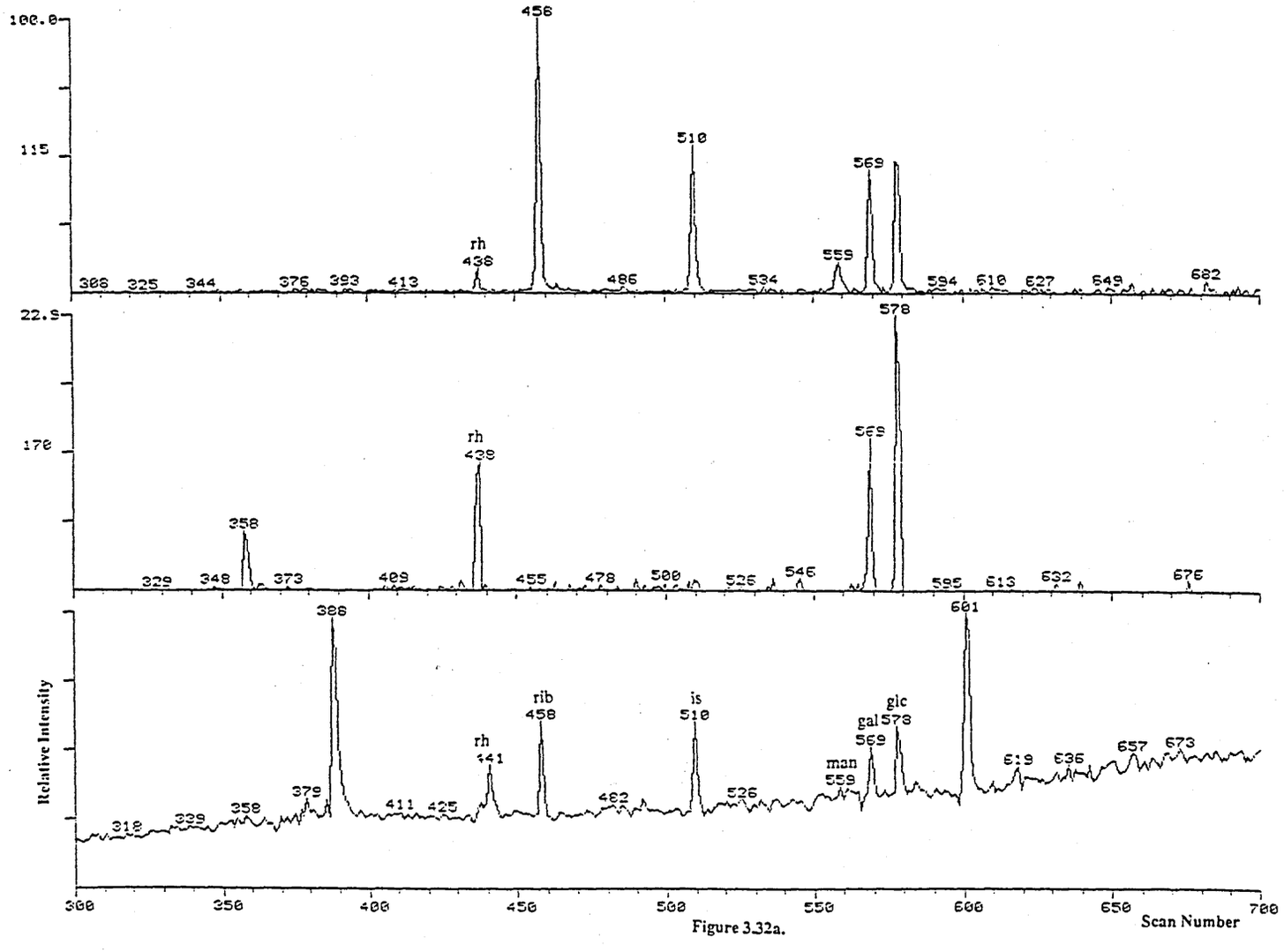
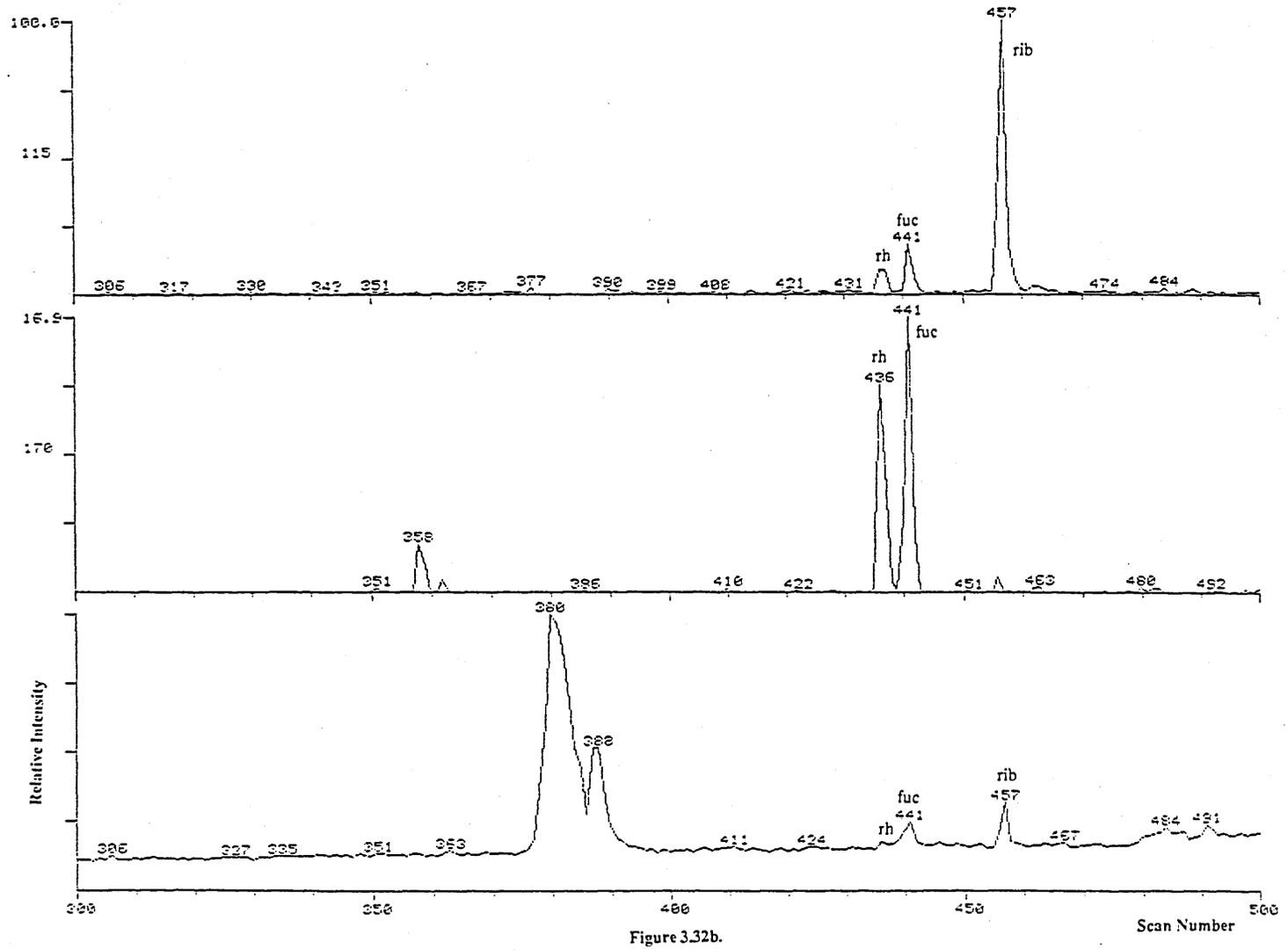

100.0
115
456
510
569
rh
438
559
308 325 344 376 393 413 486 534 594 610 627 649 682
3
22.9
178
578
569
rh
438
358
329 348 373 409 455 478 500 525 546 595 613 632 676
2
388
601
rib
458
is
510
glc
578
gal
569
rh
441
man
559
379
358
318 339 411 425 462 526 619 636 657 673
1
Relative Intensity
300 350 400 450 500 550 600 650 700
Scan Number

Figure 3.32a.



3

2

1

Figure 3.32b.

mannose, galactose and glucose (Figure 3.33). Molar ratios were calculated relative to glucose (Methods 2.17.1.3) only for those sugars identifiable from the available standard sugars (Table 3.7). The ratio of the sugars differed from strain to strain but was unaltered by growth conditions with strains grown on 20mM glucose minimal salts medium (Methods 2.2.2) or double strength nutrient broth (Methods 2.2.1) giving similar results (Table 3.9). Small amounts of heptose were detected in most chromatograms by comparison to the retention of glyceromannoheptitol acetate (Table 3.5). The presence of heptose was assayed for in whole serogroup I lipopolysaccharide (Methods 2.20.4) and estimated to be about 6% (w/w). In some chromatograms one or two small peaks were also observed with retention times similar to heptose (Figure 3.34).

6.2.3.2.1 Reproducibility of the GLC Method

6.2.3.2.1.1 GLC Analysis of the Alditol Acetates of Stachyose

Stachyose has the structure α-D-galactosyl-α-D-galactosyl-α-D-glucosyl-β-D-fructose. Fructose is a ketose and on reduction (Methods 2.17.1.1) of two moles of fructose yields one mole of glucose and one mole of mannose. After hydrolysis and reduction the expected molar ratio for the sugar alditol acetates of stachyose

Figure 3.33

GLC analysis of the alditol acetates produced from serogroup I lipopolysaccharide.

Alditol acetates were produced from a serogroup I lipopolysaccharide preparation as described in Methods 2.17.1.1. They were separated on a SGE bonded phase (0.1μm film thickness) capillary column, BP-225, bonded to a vitreous silica column (25m x 0.22mm) attached to a flame ionisation detector as described in Methods 2.17.1.

Abbreviations

sp	:	solvent peak
rh	:	rhamnose
rib	:	ribose
*	:	unidentified sugar
man	:	mannose
gal	:	galactose
glc	:	glucose
hep	:	heptose

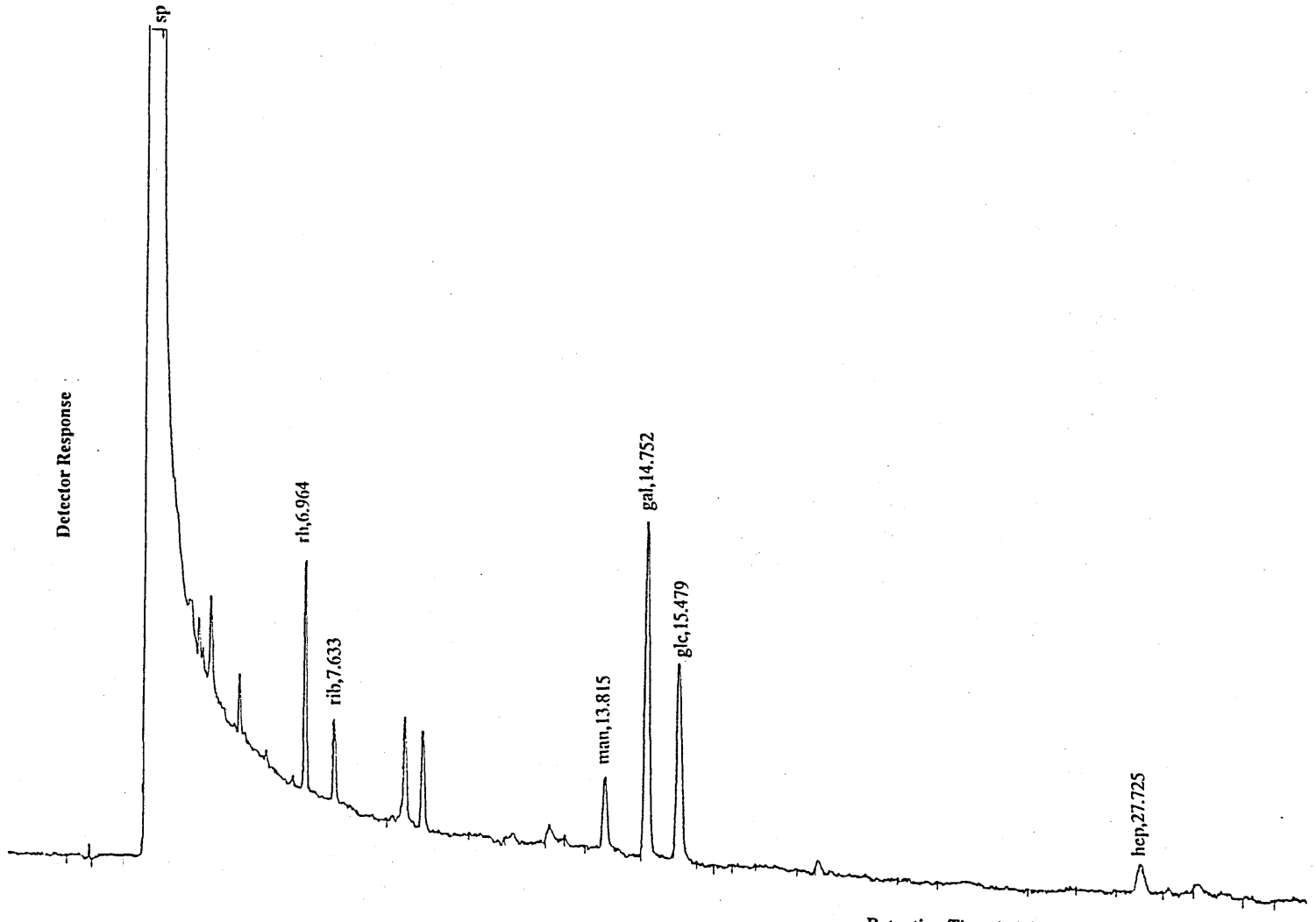


Figure 3.33. Serogroup I LPS

TABLE 3.7 **Sugar composition of serogroup I lipopolysaccharides by GLC analysis of the alditol acetates produced.**

		Molar Ratios					
Strain	Medium	Rh	Fuc	Rib	Man	Gal	Glc
1034	glucose	0.93	-	0.71	0.2	1.16	1
1001	glucose	0.93	-	0.2	0.21	0.52	1
1061	glucose	0.51	-	0.16	0.39	0.97	1
1063	glucose	1.25	-	1.19	0.21	0.83	1
G331	glucose	1.2	-	0.94	0.44	1.64	1
1745	2XNB	0.24	-	ND	0.22	0.65	1
1043	2XNB	1.2	-	0.79	0.32	1.23	1

glucose : 20mM glucose minimal salts medium (Methods 2.2.2)
2XNB : double strength nutrient broth (Methods 2.2.1)

Rh : Rhamnose
Fuc : Fucose
Rib : Ribose
Man : Mannose
Gal : Galactose
Glc : Glucose
ND : Not determined due to instrument failure

Molar ratios calculated relative to glucose (Methods 2.17.1.3)

Figure 3.34

Presence of heptose in E. carotovora lipopolysaccharide as analysed by GLC-MS of the alditol acetates produced.

Alditol acetates were produced from serogroup I lipopolysaccharide as described in Methods 2.17.1.1. They were separated on a Quadrex bonded phase (0.25µm film thickness) capillary column, OV-225, bonded to a fused silica column (25m x 0.25mm) attached to a mass spectrometer as described in Methods 2.17.2.

Abbreviations

rh	:	rhamnose
rib	:	ribose
man	:	mannose
gal	:	galactose
glc	:	glucose
hep	:	heptose
*	:	unidentified sugars with retention times similar to heptose.

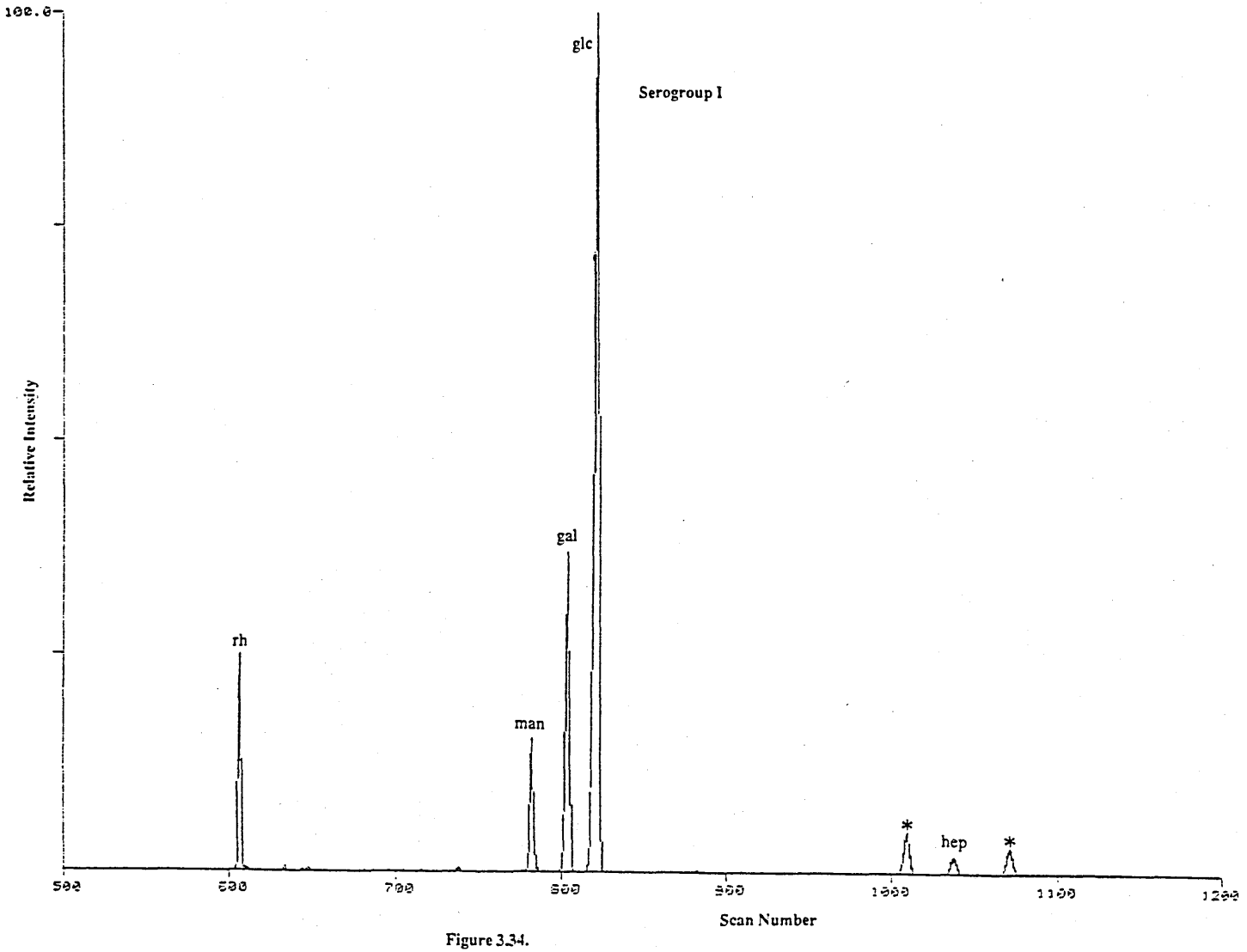

100.0
Relative Intensity
glc
Serogroup I
gal
rh
man
*
hep
*
500
600
700
800
900
1000
1100
1200
Scan Number

Figure 3.34.

would therefore be 4 moles galactose, 3 moles glucose and 1 mole mannose. The actual molar ratio obtained for the galactose, glucose and mannose alditol acetates of stachyose by GLC analysis was 4.1:3:1, respectively (Figure 3.35).

6.2.3.2.1.2 Injection of Alditol Acetates Produced From Lipopolysaccharide Sample

Variability was observed between two consecutive injections of the same alditol acetate mixture (Table 3.8). Greater variability was observed with the more volatile sugars. The standard deviation for rhamnose and ribose was $\pm$ 0.48 and $\pm$ 0.49, respectively. The standard deviation for mannose and galactose was $\pm$ 0.15 and $\pm$ 0.1, respectively.

6.2.3.2.1.3 Composition and Stability of Alditol Acetate Mixture From Lipopolysaccharide Sample

Although the same sugar composition was obtained for a strain grown on either 20mM glucose minimal salts medium (Methods 2.2.2) or double strength nutrient broth (Methods 2.2.1), their respective sugar ratios were different (Table 3.9). The differences, with the exception of rhamnose, were not due to sample preparation because two different alditol acetate preparations, produced from the same lipopolysaccharide

Figure 3.35

GLC analysis of the alditol acetates of stachyose, a tetrasaccharide.

Alditol acetates were produced from stachyose as described in Methods 2.17.1.1. They were separated on a SGE bonded phase (0.1µm film thickness) capillary column, BP-225, bonded to a vitreous silica column (25m x 0.22mm) attached to a flame ionisation detector as described in Methods 2.17.1.

sp : solvent peak
man : mannose
gal : galactose
glc : glucose

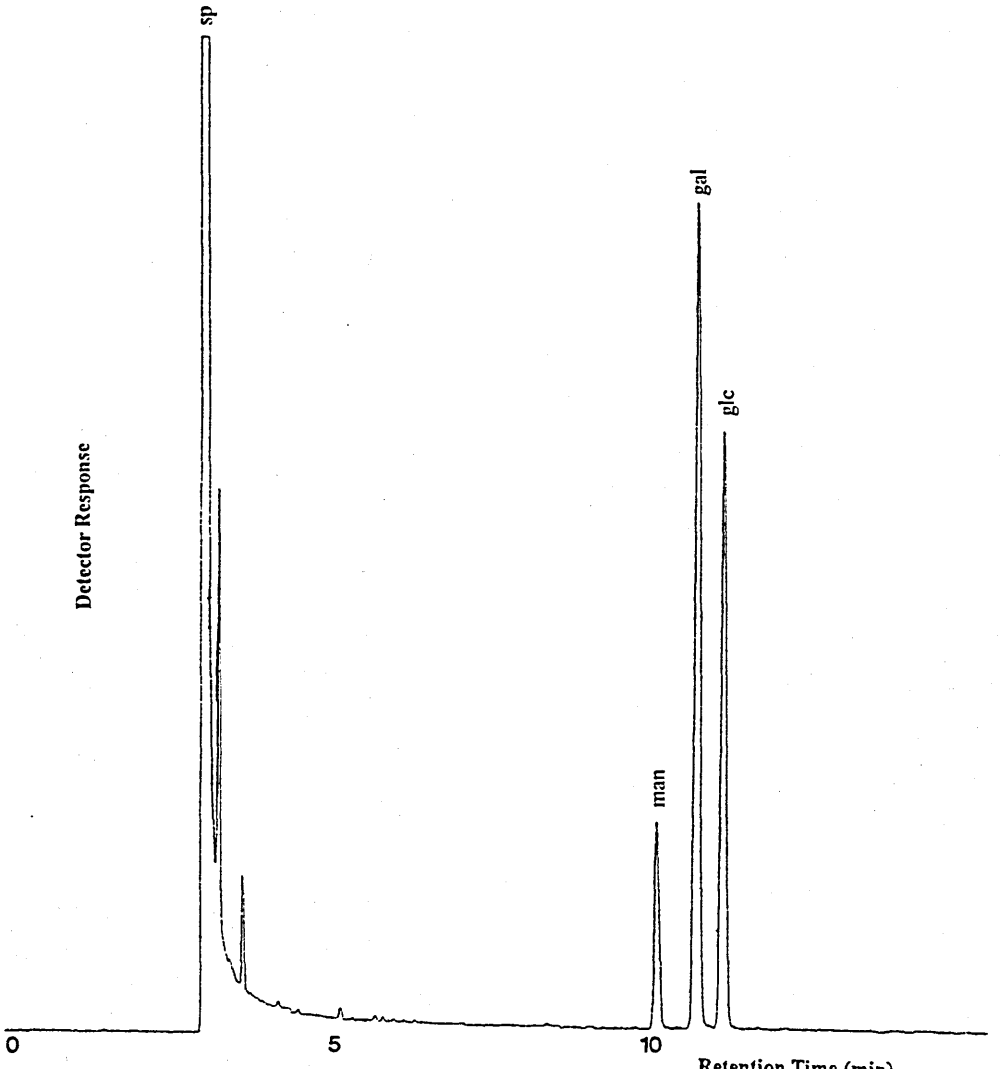


Figure 3.35.

TABLE 3.8 **Injection of alditol acetates produced from lipopolysaccharide sample**

	Molar Ratios					
Injection No.	Rh	Fuc	Rib	Man	Gal	Glc
1	1.2	-	0.94	0.44	1.64	1
2	0.52	-	0.24	0.23	1.5	1

TABLE 3.9 **Composition and stability of alditol acetates produced from lipopolysaccharide sample**

		Molar Ratios					
Strain	Medium	Rh	Fuc	Rib	Man	Gal	Glc
1001	glucose	0.93	-	0.2	0.21	0.52	1
1001	2XNB	0.5	-	ND	0.34	0.97	1
1001	2XNB i)	0.99	-	0.83	0.25	1.14	1
	ii)	0.69	-	0.78	0.34	0.91	1

glucose: 20mM glucose minimal salts medium (Methods 2.2.2)
2XNB : double strength nutrient broth (Methods 2.2.1)
2XNB i): injection of alditol acetate mixture freshly made up in dichloroethane running solvent.
2XNBii): injection of mixture i) twenty four hours later.

Rh: Rhamnose, Fuc: Fucose, Rib: Ribose, Man: Mannose, Gal: Galactose, Glc: Glucose.

Molar ratios calculated relative to glucose (Methods 2.17.1.3).

sample, gave quite similar sugar ratios (Table 3.9). In addition, with the exception of rhamnose, alditol acetate preparations made up in running solvent were quite stable over a twenty four hour period (Table 3.9).

6.2.4 Sugar Composition of Lipopolysaccharide O-chain Fraction From the Potato Pathogenic Serogroups of E. Carotovora subsp. Atroseptica

6.2.4.1 GLC-MS Combined Analysis

One strain was selected from serogroup XVIII (Ecc), serogroup XVIII (Eca), serogroup XX, and serogroup XXII, and its alditol acetates analysed by GLC-MS as described in Methods 2.17.2.1. The serogroup XVIII (Ecc) lipopolysaccharide preparation contained rhamnose, ribose, mannose, galactose and glucose (Figure 3.36a). Both serogroup XVIII (Eca) and serogroup XX lipopolysaccharide preparations contained rhamnose, ribose, galactose and glucose (Figure 3.36, b and c). Serogroup XXII lipopolysaccharide preparation contained only rhamnose, ribose and glucose (Figure 3.36.d).

6.2.4.2 GLC Analysis

At least one more strain from each of the potato pathogenic serogroups of E. carotovora subsp. atroseptica was selected and the alditol acetates of its lipopolysaccharide analysed by GLC (Methods 2.17.1).

Figure 3.36

GLC-MS combined analysis of the alditol acetates of lipopolysacharide from the potato pathogenic serogroups of E. carotovora subsp. atroseptica.

a. Serogroup XVIII (Ecc) lipopolysaccharide
b. Serogroup XVIII (Eca) lipopolysaccharide
c. Serogroup XX lipopolysaccharide
d. Serogroup XXII lipopolysaccharide

Alditol acetates were produced from the different lipopolysaccharide preparations as described in Methods 2.17.1.1. They were separated on a Quadrex bonded phase (0.25µm film thickness) capillary column, OV-225, bonded to a fused silica column (25m x 0.25mm) attached to a mass spectrometer as described in Methods 2.17.2.

Abbreviations

rh	:	rhamnose
rib	:	ribose
is	:	2-deoxyglucose (internal standard)
man	:	mannose
gal	:	galactose
glc	:	glucose

1 : GLC profile.

2 & 3 : Mass / charge ratios of 170 & 115 a.m.u.'s, respectively. Peaks from (1) possessing these fragments have been picked out as they are characteristic of sugar alditol acetates produced under conditions used in Methods 2.17.2. (see Figure 3.30.).

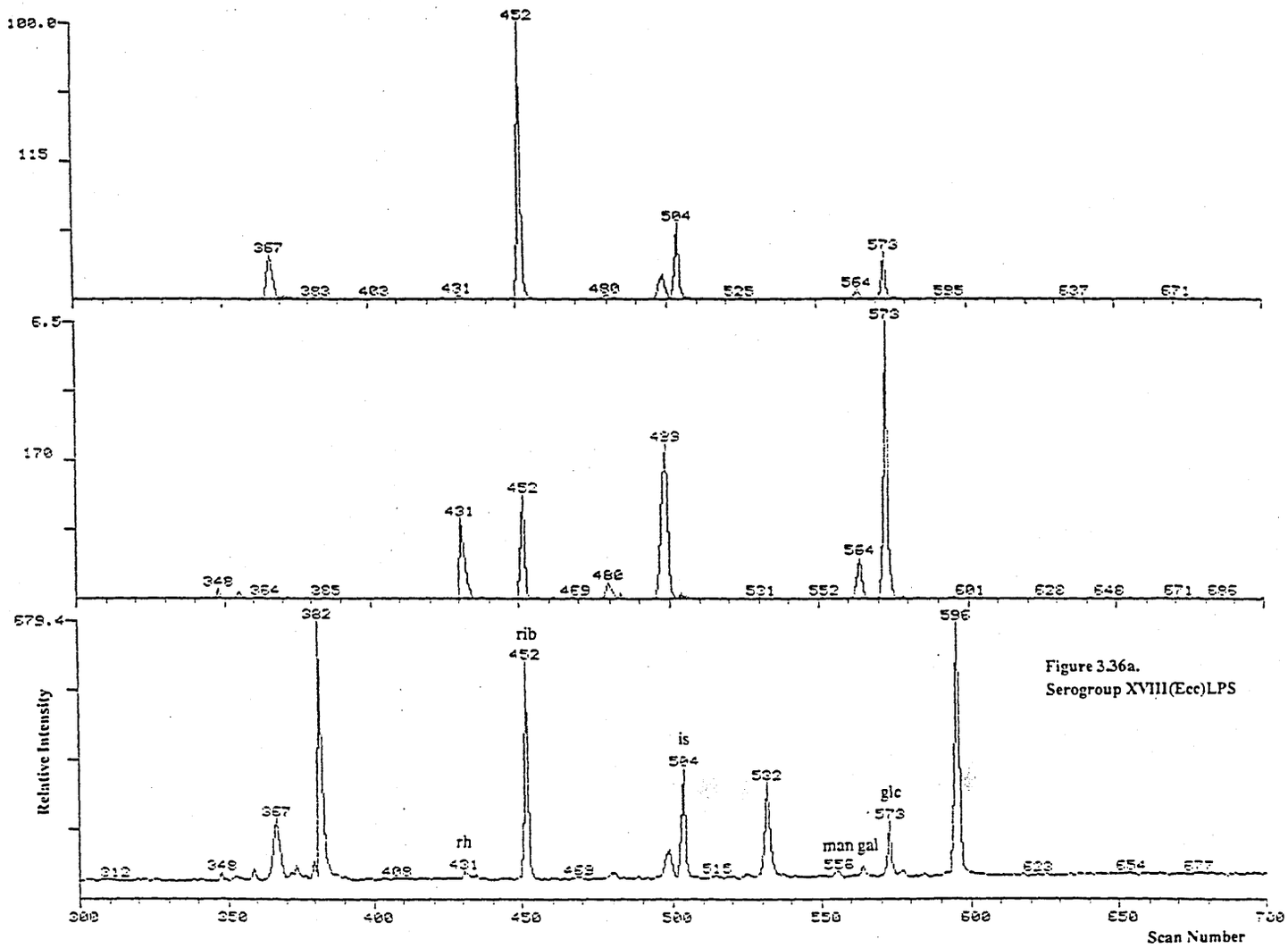


Figure 3.36a.
Serogroup XVIII(Ecc)LPS

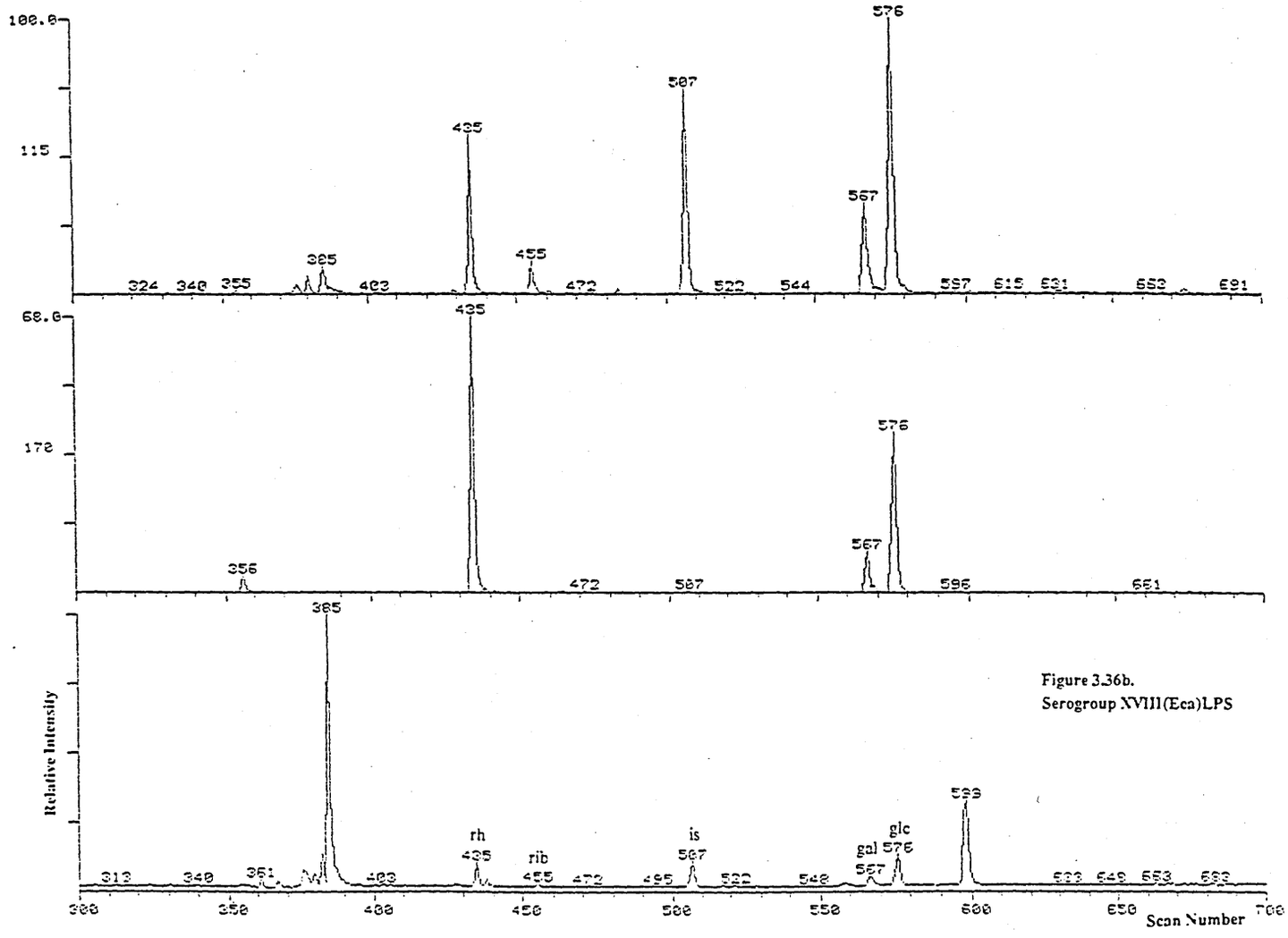


Figure 3.36b.
Serogroup XVIII(Eca)LPS

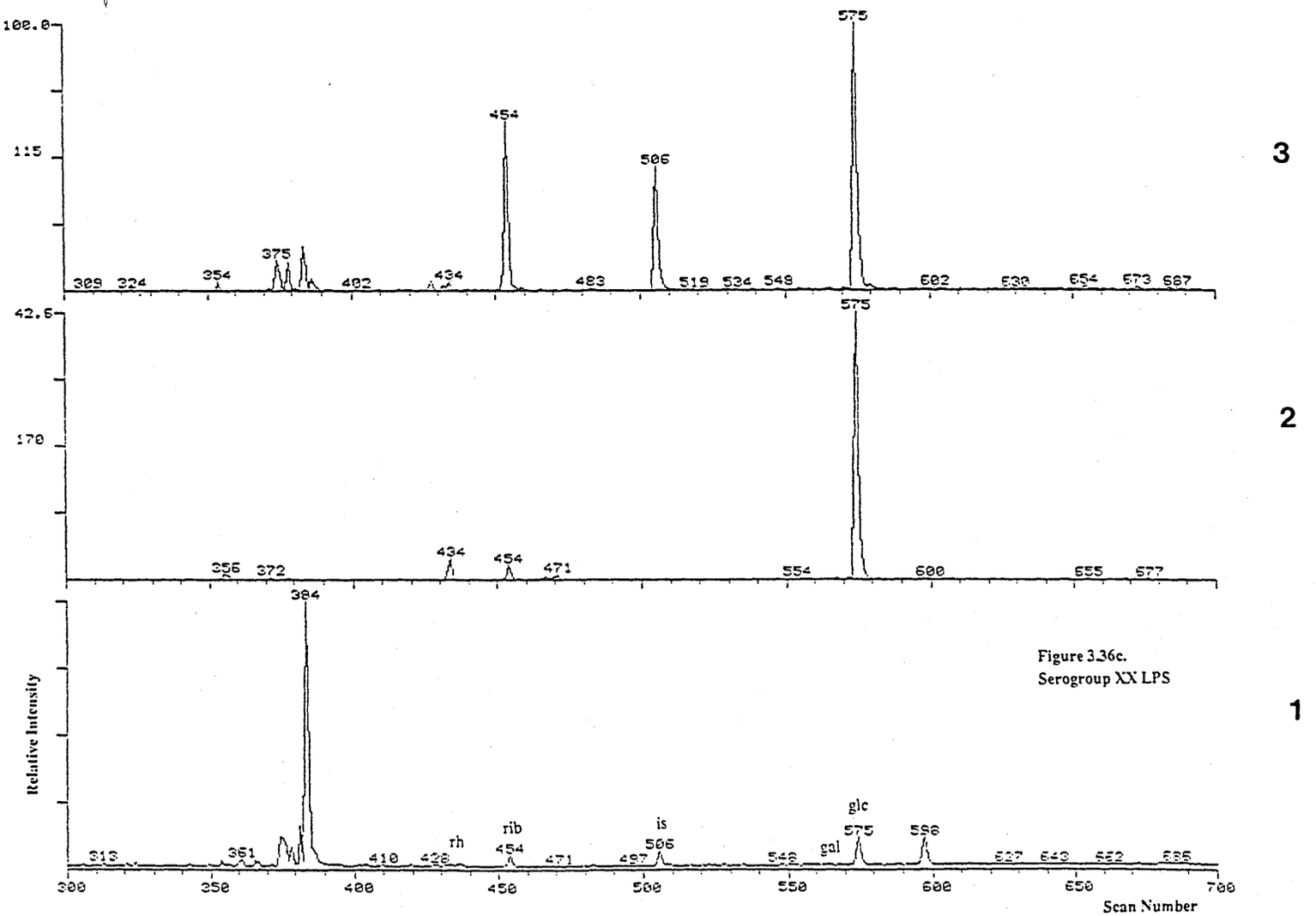


Figure 3.36c.
Serogroup XX LPS

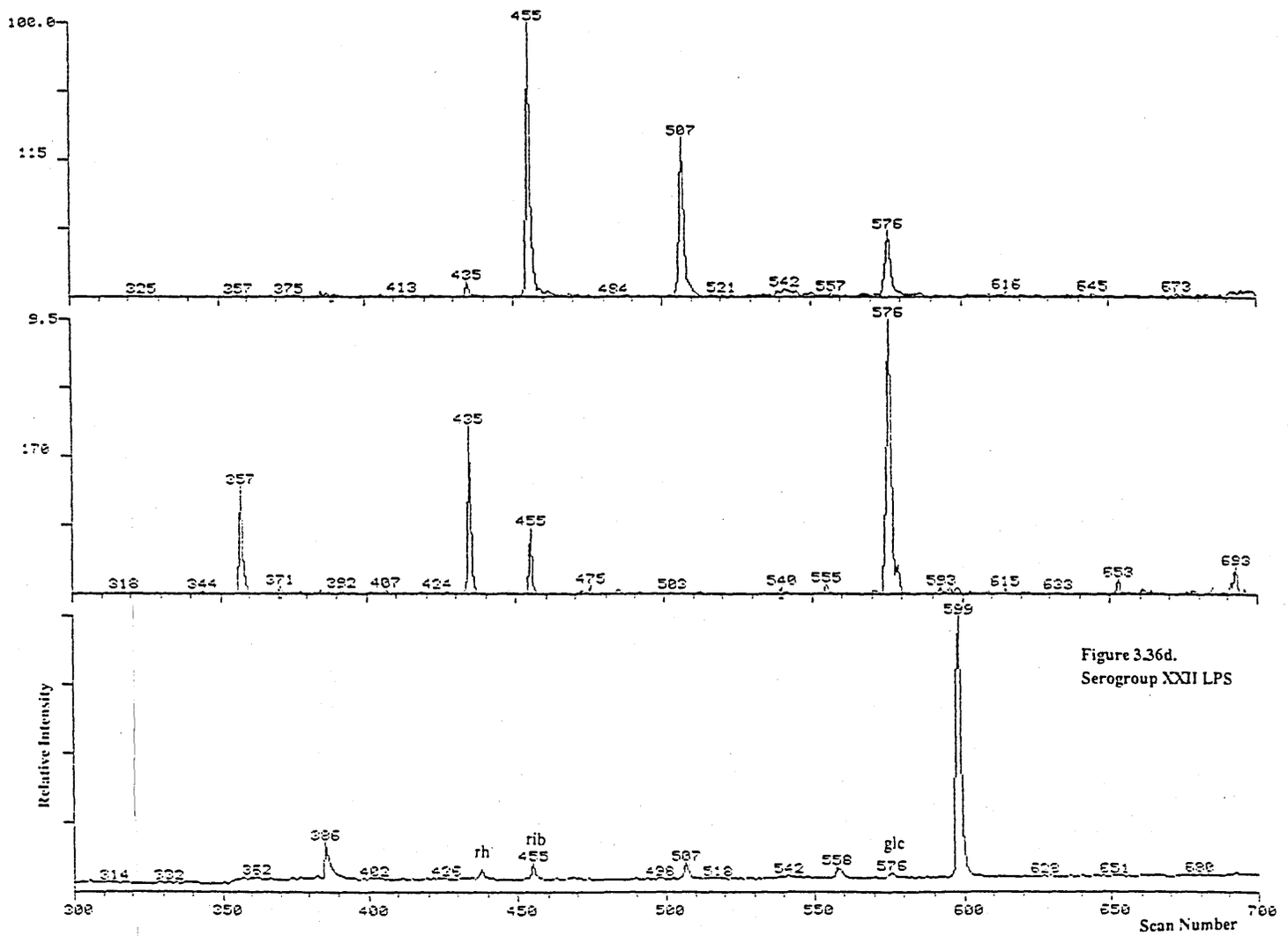


Figure 3.36d.
Serogroup XXII LPS

Two E. carotovora subsp. betavasculorum strains from serogroup XXXV were also included in this analysis (Table 3.10). The sugar composition of the different serogroups was the same as determined for those strains analysed by GLC-MS (Results 6.2.4.1) with one exception. One of the two serogroup XXII strains analysed by GLC contained a small amount of galactose, the other strain did not (Table 3.10). Previously, a serogroup XXII strain analysed by GLC-MS was not found to contain galactose (Results 6.2.4.2, Figure 3.36.d). However, the anomalous galactose-containing strain was the atroseptica strain 432 given as belonging to serogroup XXXV but found to give a serogroup XXII lipopolysaccharide SDS-PAGE pattern (Results 4.3). The sugar composition for the lipopolysaccharide of the two E. carotovora subsp. betavasculorum strains was the same (Table 3.10). They contained rhamnose, ribose, galactose and glucose.

6.2.5 Unidentified Peaks

Several other peaks were always observed in the gas chromatograms (Perkin-Elmer) but it was not possible to identify them from available standard sugars. The derivatisation of KDO (Methods 2.17.1.1) was determined not to be responsible for any of these peaks. Irrespective of serogroup all lipopolysaccharide preparations produced peaks with scan numbers of about

TABLE 3.10 Sugar composition of lipopolysaccharide from the potato pathogenic serogroups of E. carotovora subsp. atroseptica by GLC analysis of the alditol acetates produced.

		Molar Ratios					
Serogroup	Medium	Rh	Fuc	Rib	Man	Gal	Glc
XVIII(Ecc)	glucose	1.05	-	0.59	0.64	0.17	1
XVIII(Eca)	glucose	0.2	-	0.76	-	0.56	1
XX	glucose	4.47	-	0.63	-	0.53	1
XXII	glucose	4	-	2.1	-	-	1
XXII	glucose	0.15	-	0.13	-	0.03	1
XXXV(Ecb)	2XNB	2.52	-	1.7	-	0.37	1
XXXV	2XNB	4.7	-	6.46	-	0.4	1

glucose: 20mM glucose minimal salts medium (Methods 2.2.2)
2XNB: double strength nutrient broth (Methods 2.2.1)

Rh: Rhamnose, Fuc: Fucose, Rib: Ribose, Gal: Galactose, Glc: Glucose.

Molar ratios calculated relative to glucose (Methods 2.17.1.3).

361 and 384 (Figure 3.37). Mass spectral analysis determined that these peaks were not produced by sugars. Peaks observed with scan numbers of 394, 539 and 599 were artefacts produced by phthalate which is a contaminant (Figure 3.37) picked up by contact with plastic or the rubber septum. It is generally detected when using a temperature programme.

In most lipopolysaccharide preparations, particularly serogroup I, two peaks were observed which eluted between ribose and the 2-deoxyhexose. These peaks were more apparent on the Perkin-Elmer chromatograms than those produced on the Finnigan GLC-MS (Figure 3.38). Mass spectral analysis revealed that only one of these peaks with a scan number of about 484 possessed both molecular ions characteristic of a sugar (115 a.m.u. and 170 a.m.u.) for all but three of eleven serogroup I lipopolysaccharides (Figure 3.31, Table 3.11). From possible fragmentation patterns of known sugar alditol acetates, the sugars could not be identified (Figure 3.39). One of the three other lipopolysaccharides (1063) produced two peaks at scan times of 473 and 489 which possessed the molecular ion fragments of 115 a.m.u. and 170 a.m.u. (Table 3.11). Serogroup XVIII (Ecc) lipopolysaccharide produced two sugar peaks with scan numbers 480 and 499 (Table 3.11). Their mass spectral fragmentation patterns were virtually identical differing only in the relative

Figure 3.37

GLC-MS combined analysis of the other components detected in lipopolysaccharide alditol acetate preparations.

Alditol acetates were produced from lipopolysaccharide as described in Methods 2.17.1.1. They were separated on a Quadrex bonded phase (0.25µm film thickness) capillary column, OV-225, bonded to a fused silica column (25m x 0.25mm) attached to a mass spectrometer as described in Methods 2.17.2.

Abbreviations

NS	:	not sugars.	scan numbers 361, 384.
phth	:	phthalate.	scan numbers 394, 539, 599
rh	:	rhamnose	
rib	:	ribose	
man	:	mannose	
gal	:	galactose	
glc	:	glucose	

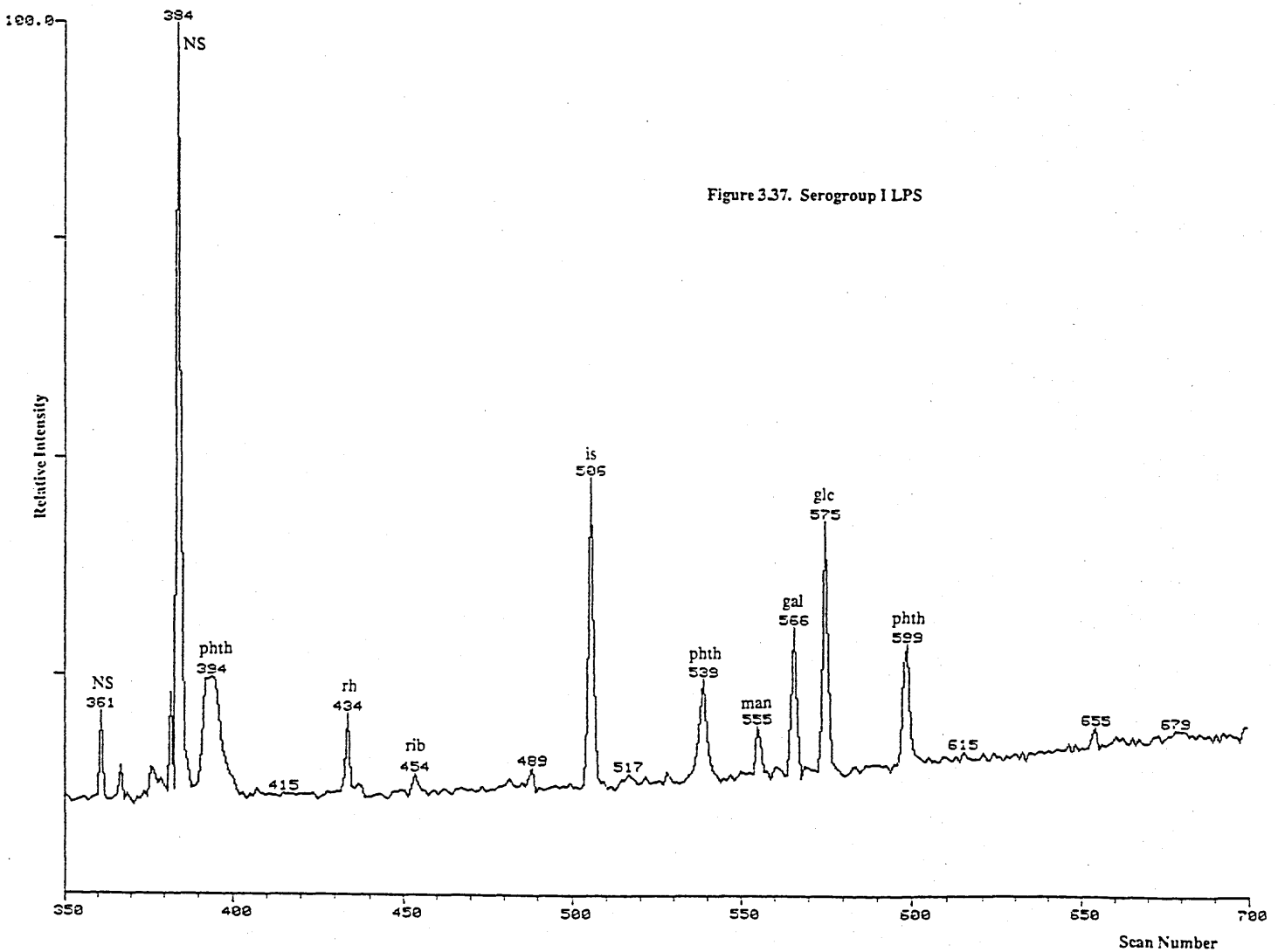


Figure 3.37. Serogroup I LPS

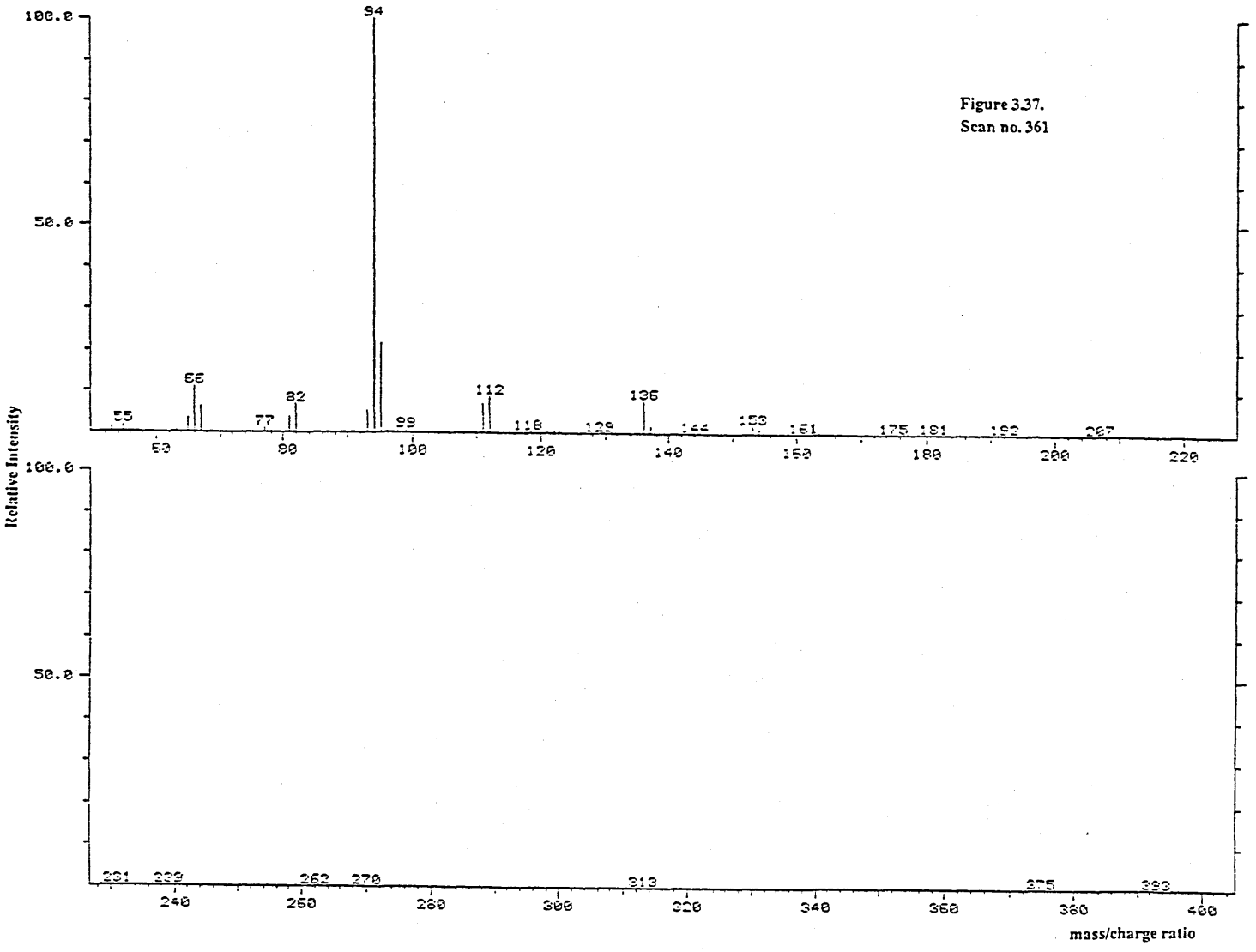


Figure 3.37.
Scan no. 361

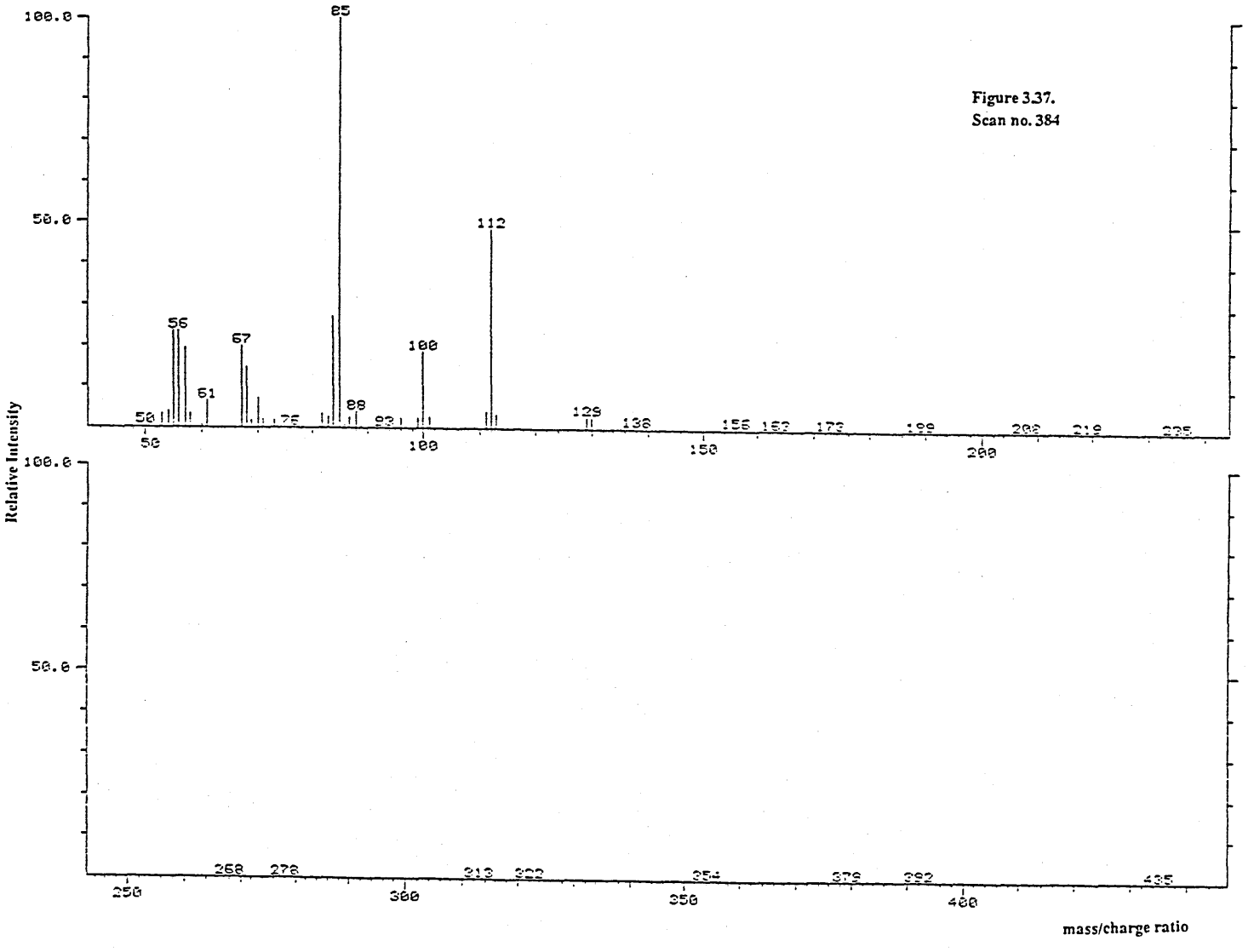


Figure 3.37.
Scan no. 384

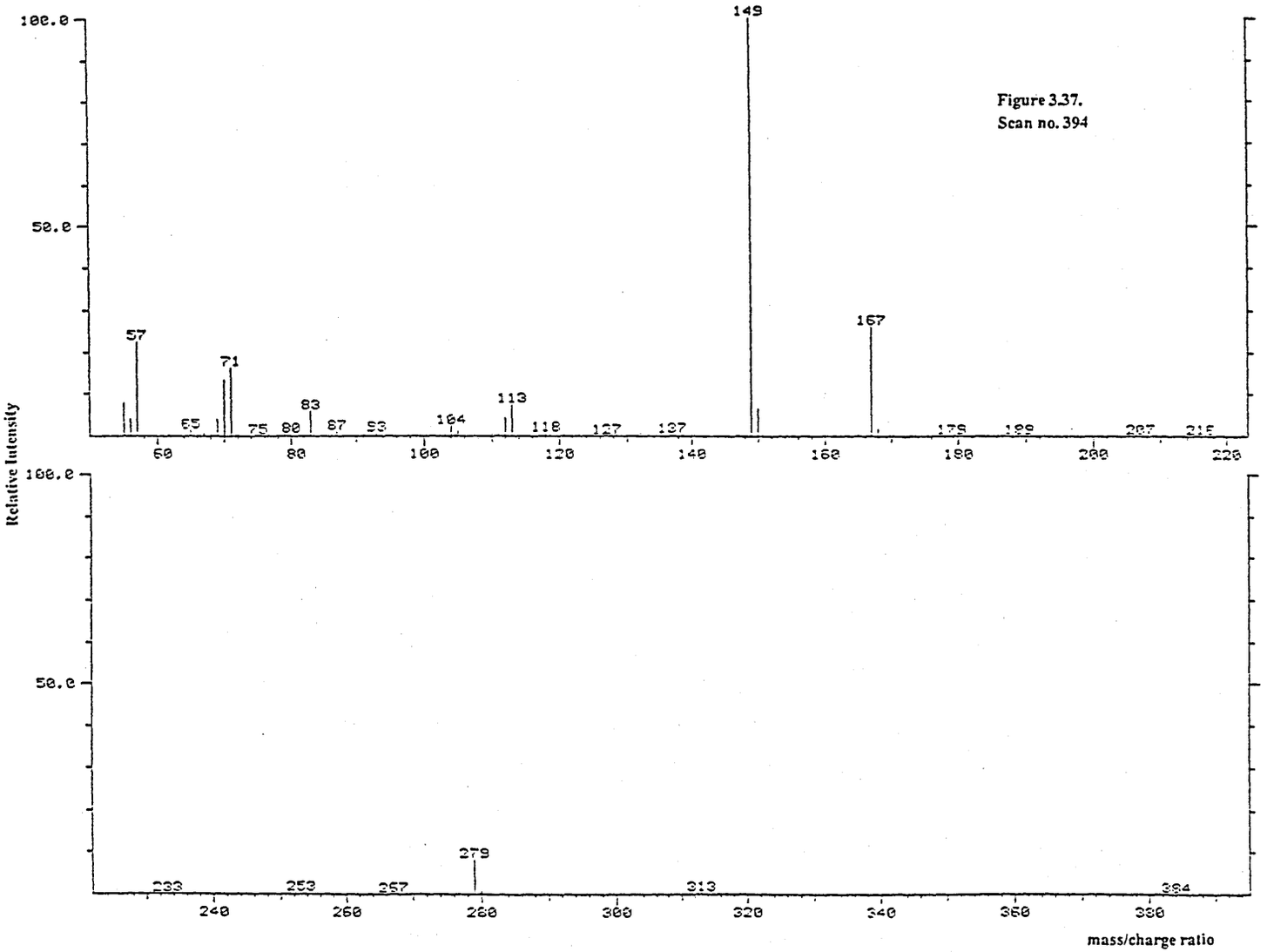


Figure 3.37.
Scan no. 394

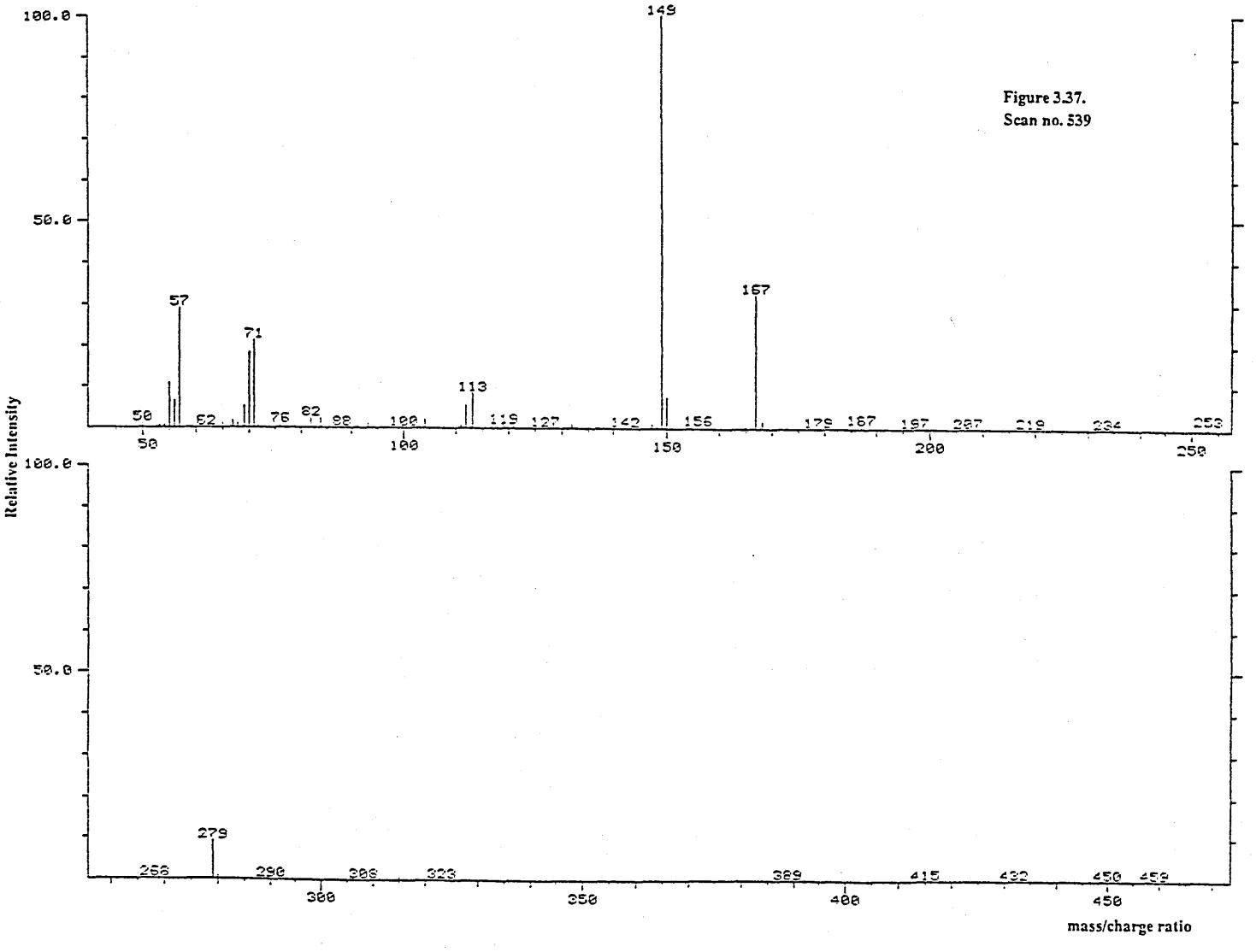


Figure 3.37.
Scan no. 539

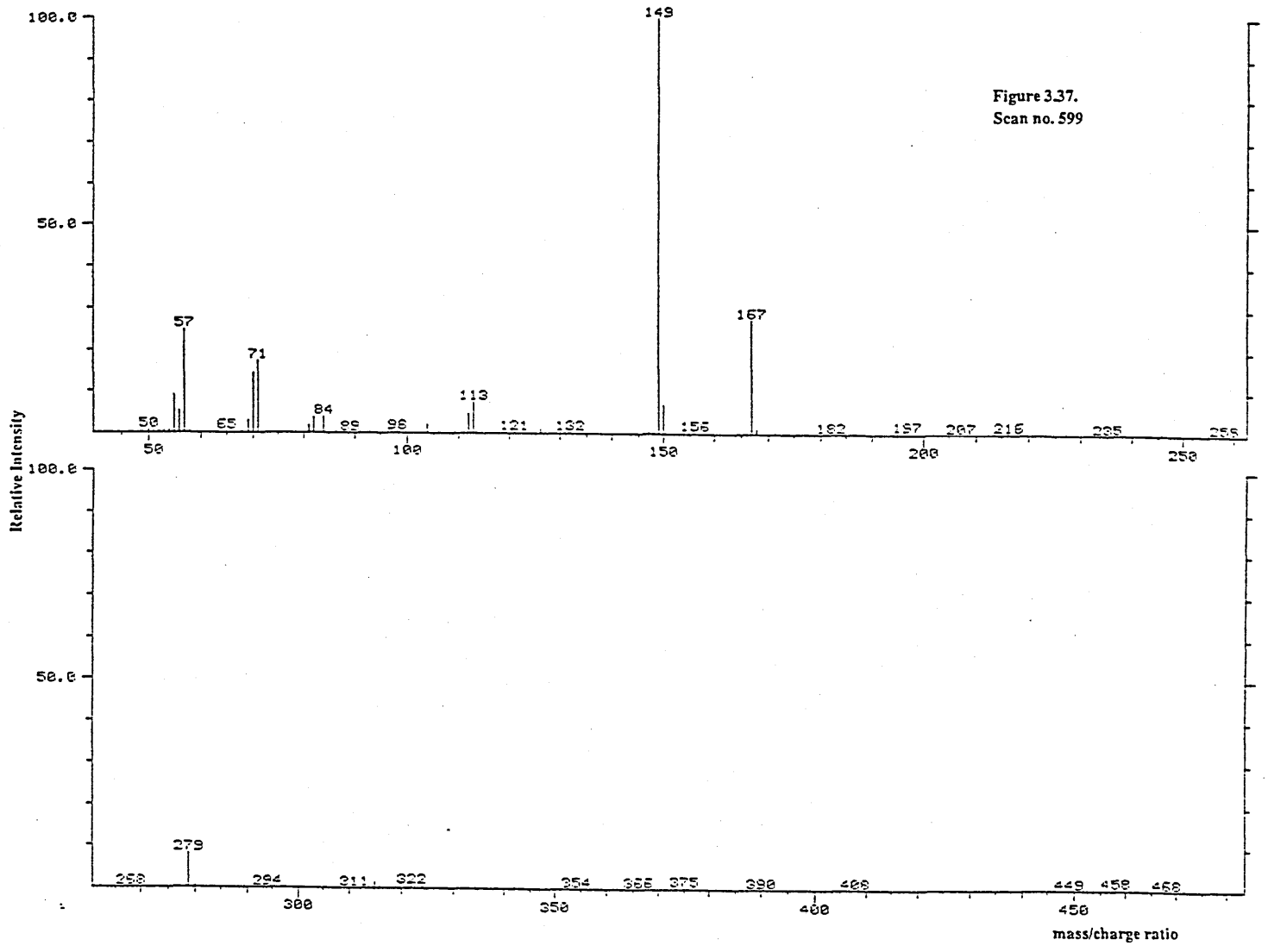


Figure 3.37.
Scan no. 599

Figure 3.38

Detection of alditol acetates of unidentified sugars by GLC and GLC-MS.

Alditol acetates were produced from lipopolysaccharide as described in Methods 2.17.1.1. They were separated on:

a. a SGE bonded phase (0.1μm film thickness) capillary column, BP-225, bonded to a vitreous silica column (25m x 0.22mm) attached to a flame ionisation detector as described in Methods 2.17.1.

b. a Quadrex bonded phase (0.25μm film thickness) capillary column, OV-225, bonded to a fused silica column (25m x 0.25mm) attached to a mass spectrometer as described in Methods 2.17.1.

Abbreviations

sp	:	solvent peak
rh	:	rhamnose
rib	:	ribose
*	:	unidentified sugars
is	:	2-deoxyglucose (internal standard)
man	:	mannose
gal	:	galactose
glc	:	glucose

1 : GLC profile.
2 & 3 : Mass / charge ratios of 170 & 115 a.m.u.'s, respectively. Peaks from (1) possessing these fragments have been picked out as they are characteristic of sugar alditol acetates produced under conditions used in Methods 2.17.2. (see Figure 3.30.).

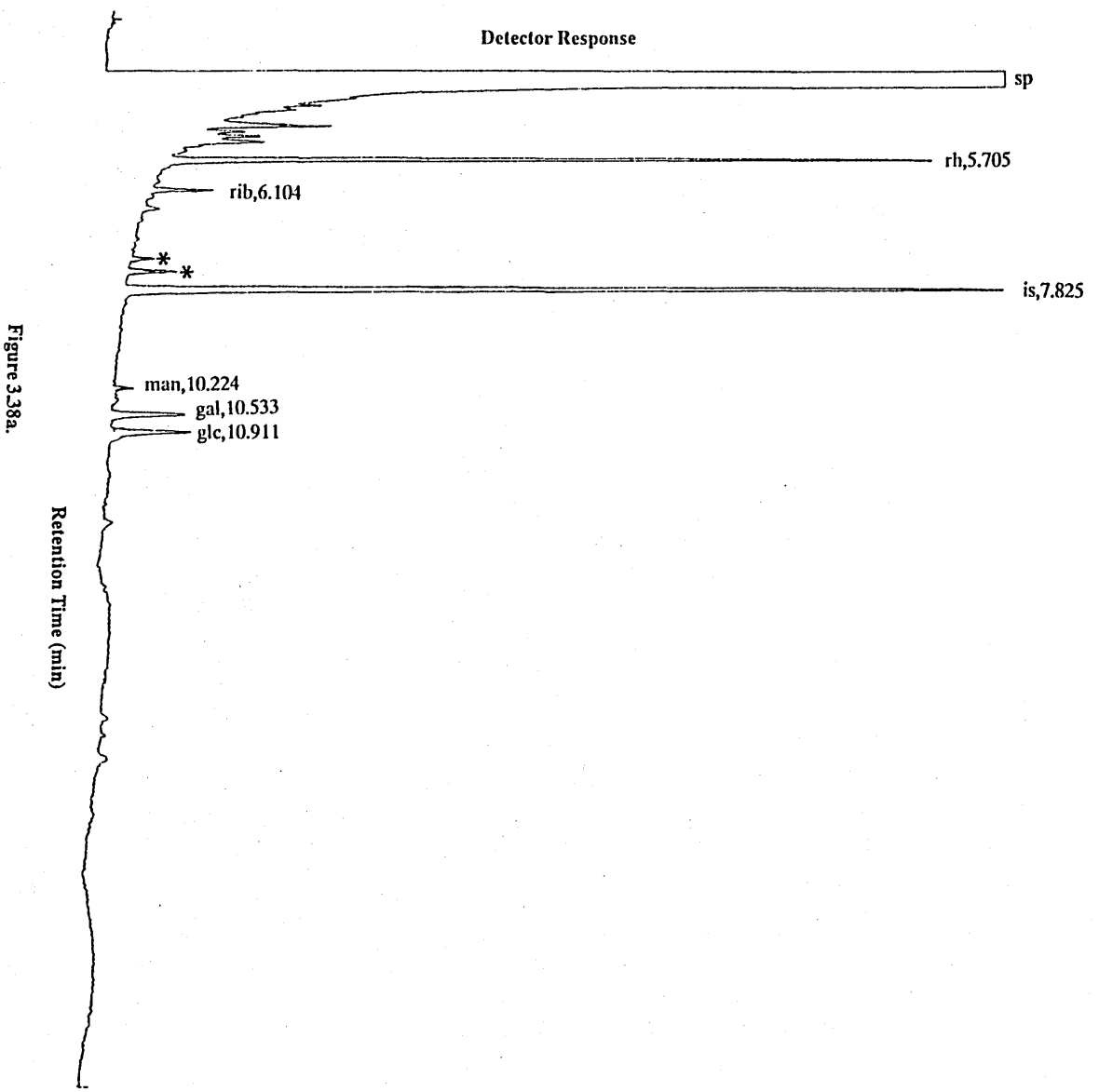

Detector Response
sp
rh,5.705
rib,6.104
*
*
is,7.825
man,10.224
gal,10.533
glc,10.911
Retention Time (min)

Figure 3.38a.

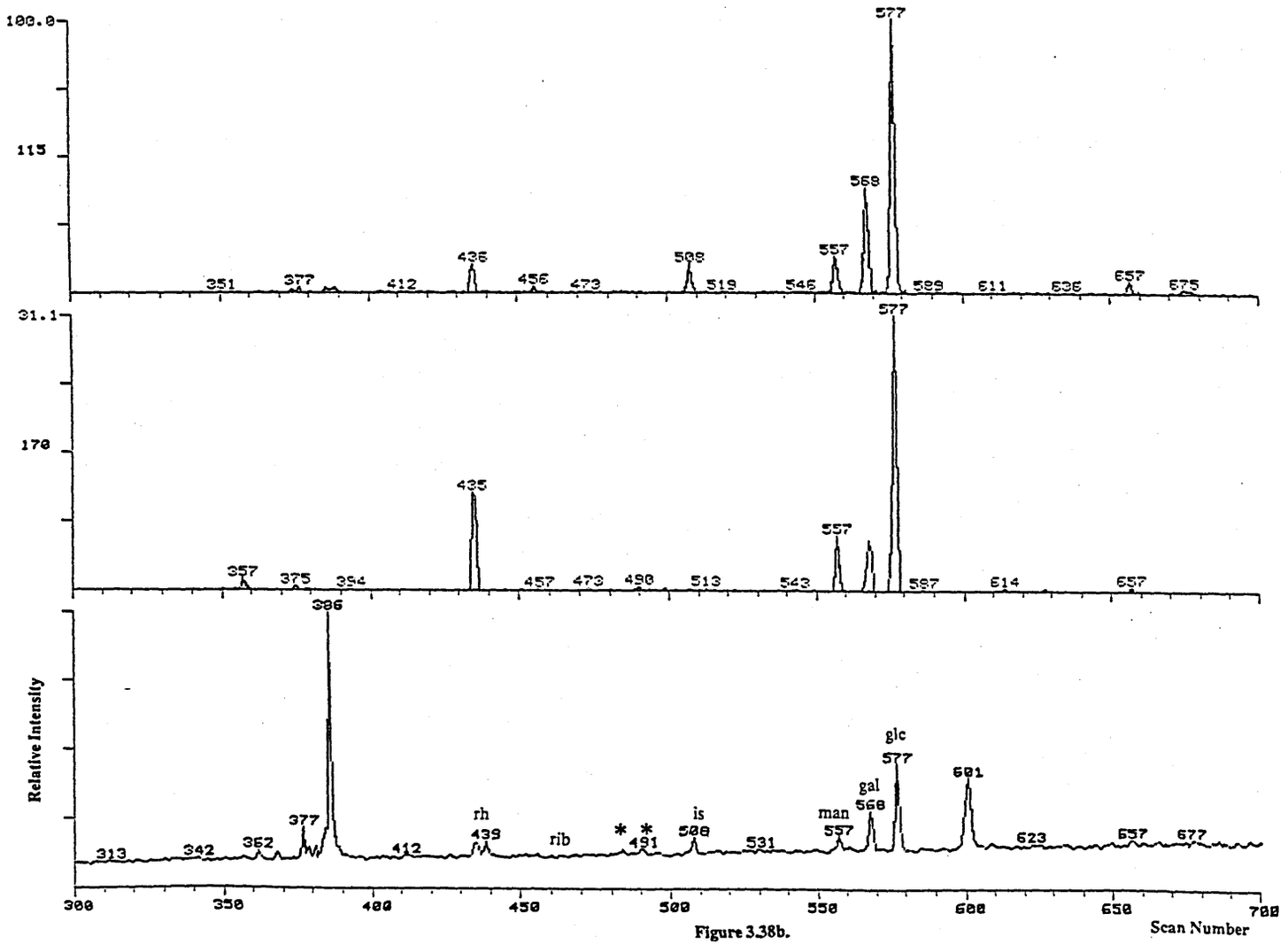

100.0
115
577
568
557
436
508
351
377
412
456
473
519
546
588
611
636
657
675
3
31.1
170
577
435
557
357
375
394
457
473
490
513
543
587
614
657
2
386
Relative Intensity
glc
577
601
gal
568
man
557
rh
439
rib
is
508
377
313
342
352
412
491
531
623
557
677
1
300
350
400
450
500
550
600
650
700
Scan Number

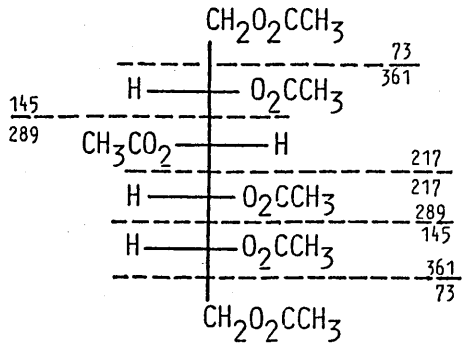
Figure 3.38b.

TABLE 3.11 **Scan numbers of unidentified components possessing the two molecular ion fragments (115 a.m.u. and 170 a.m.u) characteristic of sugars in the GLC profiles of E. carotovora lipopolysaccharide.**

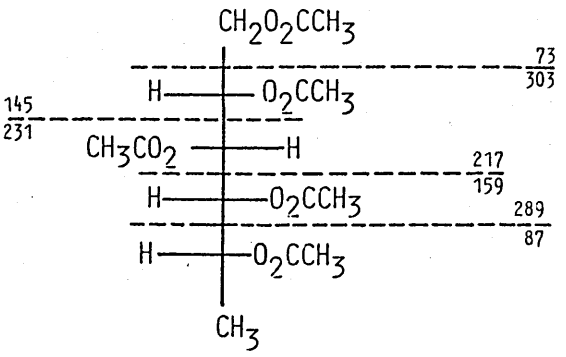
Strain	Serogroup	Scan number
1061	I	486
1062	I	485
1063	I	473, 489
1064	I	485
1065	I	-
1066	I	486
1067	I	483
1068	I	483
1069	I	-
1070	I	483
31	I	483
192	XVIII (*Ecc*)	480, 499
11	XVIII (*Eca*)	472
196	XX	471
A46	XXII	475, 484

Scan number : sample was scanned once a second as it eluted from the column on the GLC-MS system as described in Methods 2.17.2. The scan number corresponds to the time (seconds) from injection of sample to elution of alditol acetate from column.

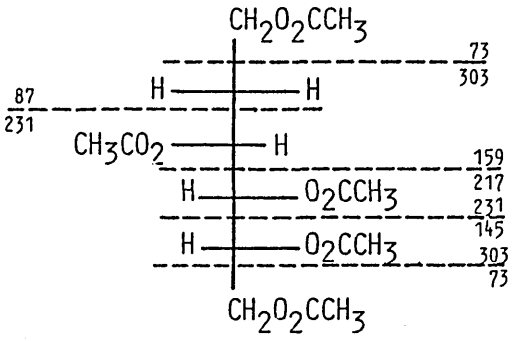
abundance of the different molecular ions (Figure 3.40). By reference to the expected fragmentation pattern for the alditol acetates of uronic acids (Figure 3.39) they could be uronic acids but there was a discrepancy of two a.m.u. between the values found and the literature values. From the assay of ten different lipopolysaccharide preparations the uronic acid content was estimated to be about 3% (w/w) (Methods 2.20.5). Serogroup XVIII (Eca) lipopolysaccharide produced one sugar peak with a scan number of 472 (Figure 3.41, Table 3.11). The fragmentation pattern for this peak was different from those observed for serogroup XVIII (Ecc) lipopolysaccharide and could not be identified from the fragmentation patterns of possible sugars. But it did contain fragments of 115 a.m.u. and 170 a.m.u. which are characteristic of sugar alditol acetates. After fragmentation, the serogroup XX lipopolysaccharide produced one peak with scan number 471 which possessed the common molecular ion fragments (Figure 3.42, Table 3.11). The sugar was not identifiable. GLC-MS analysis of serogroup XXII lipopolysaccharide produced two peaks with scan numbers 475 and 484 which appeared to be sugars (Figure 3.43, Table 3.11). The fragmentation patterns of the two sugars were different and not identifiable.



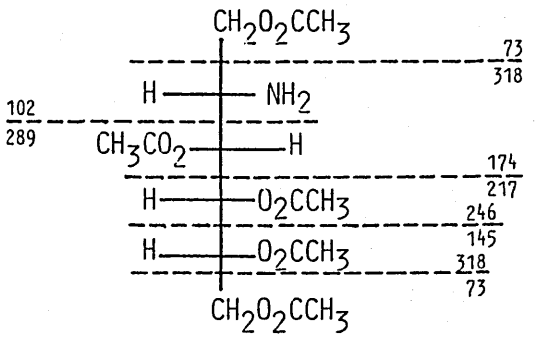
Hexose



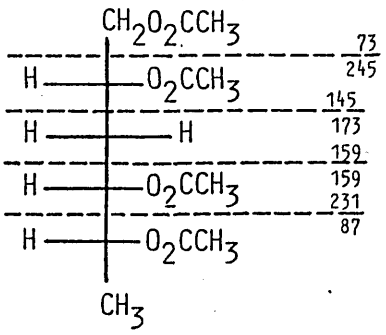
6-deoxyhexose



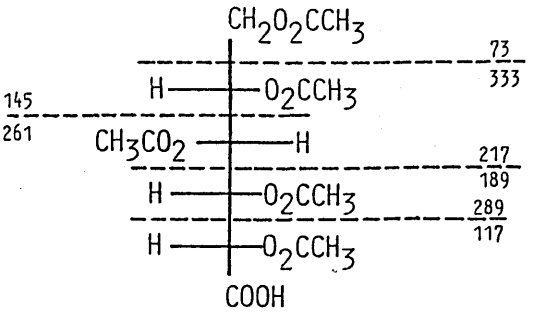
2-deoxyhexose



2-aminohexose



3,6-dideoxyhexose



Uronic acid

Figure 3.39 Molecular ion fragments of various sugar alditol acetates.

Figure 3.40

Mass spectral fragmentation patterns of unidentified sugars in serogroup XVIII (Ecc) lipopolysaccharide with scan numbers 380 and 499

Alditol acetates were produced from serogroup XVIII (Ecc) lipopolysaccharide as described in Methods 2.17.1.1. They were separated on a Quadrex bonded phase (0.25μm film thickness) capillary column, OV-225, bonded to a fused silica column (25m x 0.25mm) attached to a mass spectrometer as described in Methods 2.17.2.

Annotation

* : common molecular ion fragments characteristic of a sugar alditol acetate produced under the conditions described in Methods 2.17.2.

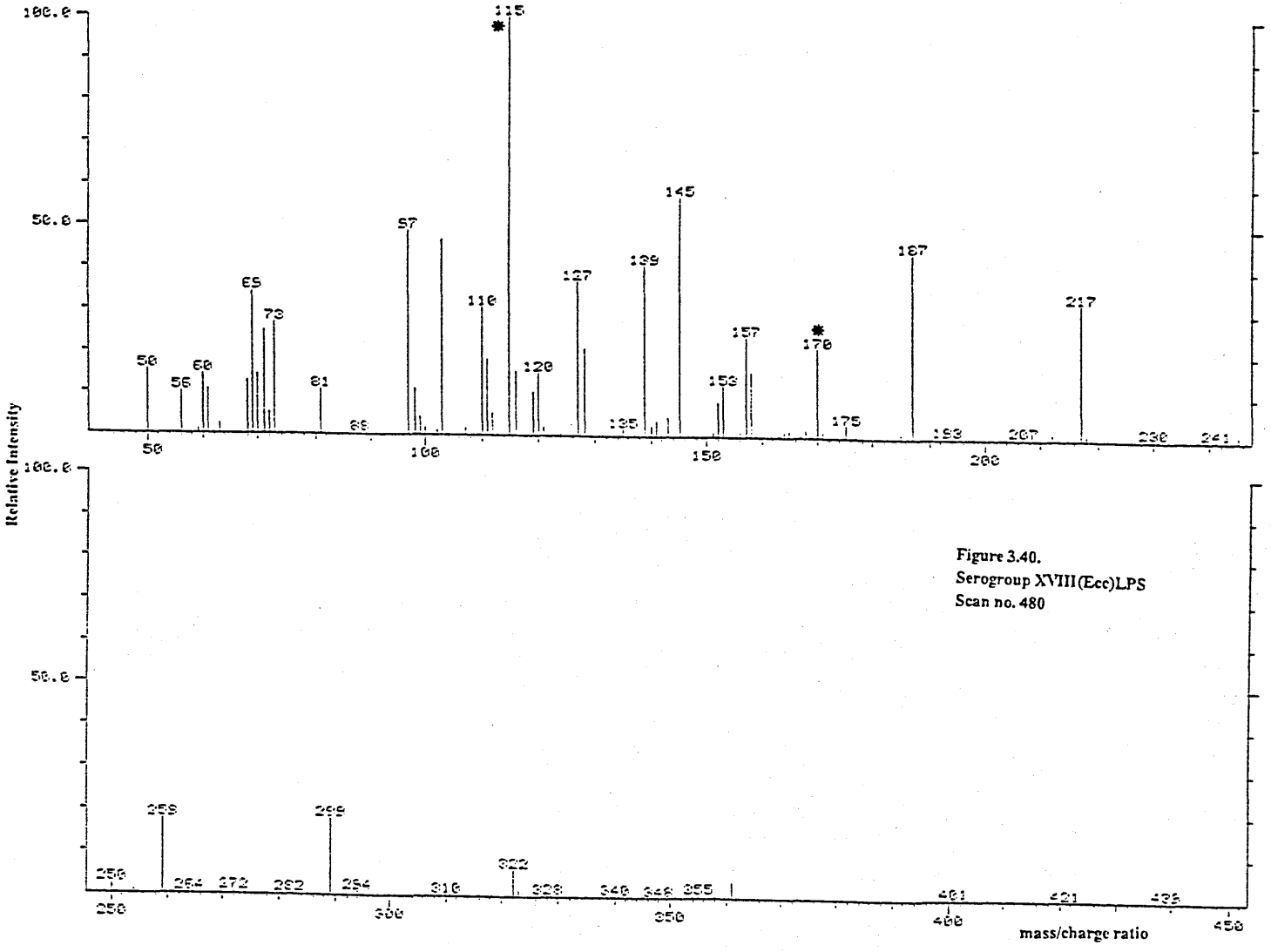


Figure 3.40.
Serogroup XVIII(Ecc)LPS
Scan no. 480

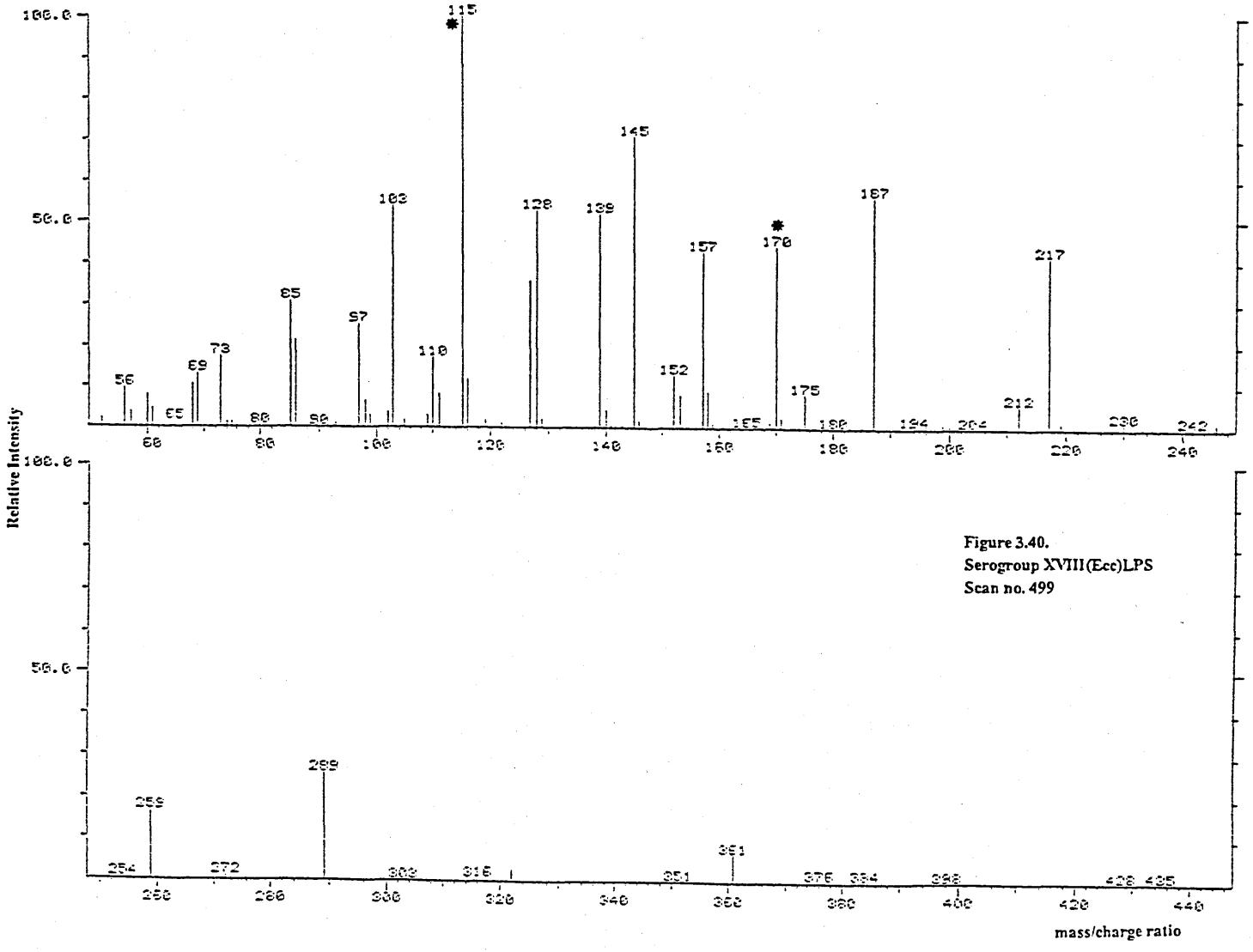


Figure 3.40.
Serogroup XVIII(Ecc)LPS
Scan no. 499

Figure 3.41

Mass spectral fragmentation pattern of an unidentified sugar in serogroup XVIII (Eca) lipopolysaccharide with scan number 472.

Alditol acetates were produced from serogroup XVIII (Eca) lipopolysaccharide as described in Methods 2.17.1.1. They were separated on a Quadrex bonded phase (0.25µm film thickness) capillary column, OV-225, bonded to a fused silica column (25m x 0.25mm) attached to a mass spectrometer as described in methods 2.17.2.

Annotation

* : common molecular ion fragment characteristic of a sugar alditol acetate produced under the conditions described in Methods 2.17.2

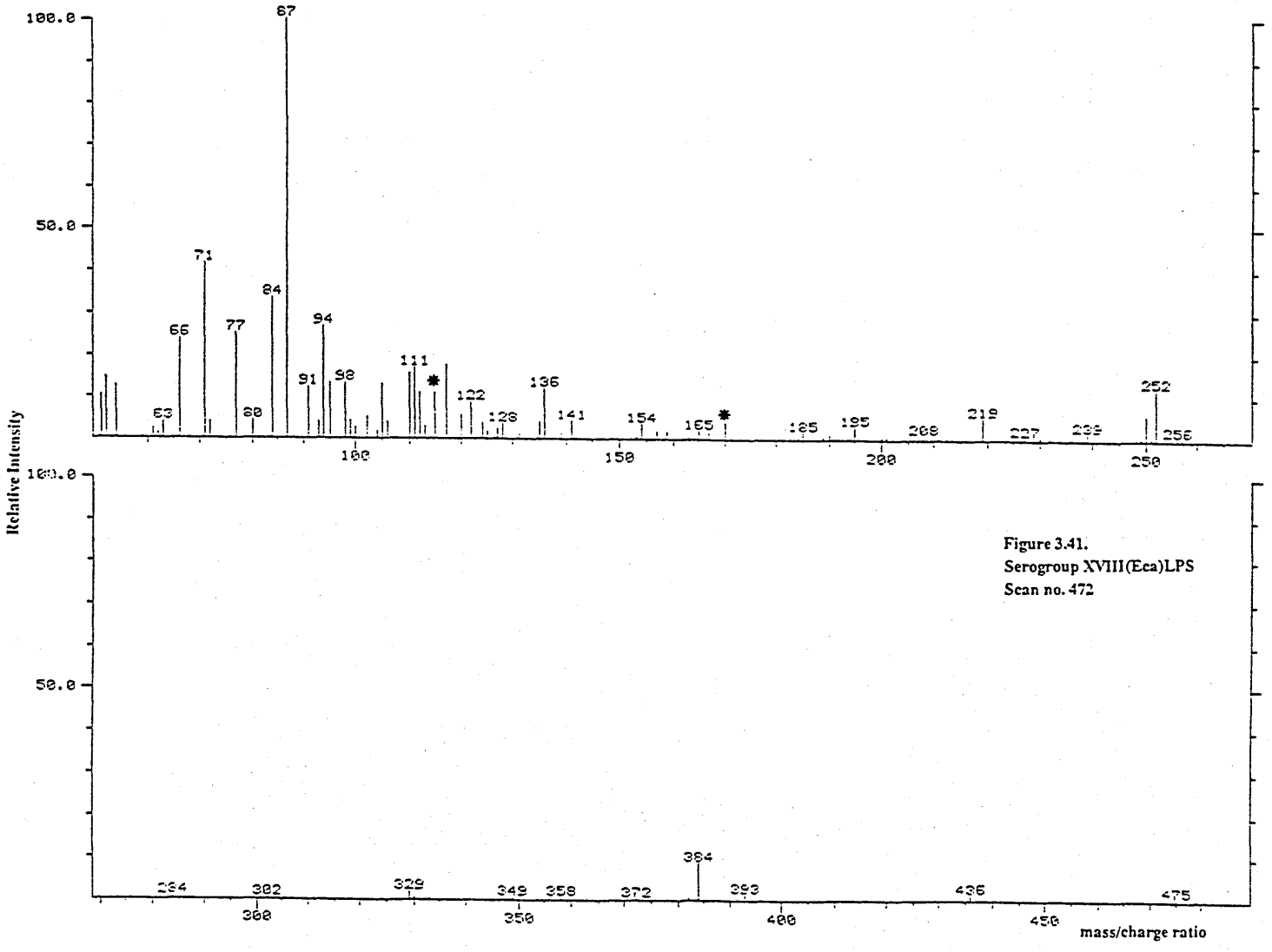


Figure 3.41.
Serogroup XVIII(Eca)LPS
Scan no. 472

Figure 3.42

Mass spectral fragmentation pattern of an unidentified sugar in serogroup XX lipopolysaccharide with scan number 471.

Alditol acetates were produced from serogroup XX lipopolysaccharide as described in Methods 2.17.1.1. They were separated on a Quadrex bonded phase (0.25μm film thickness) capillary column, OV-225, bonded to a fused silica column (25m x 0.25mm) attached to a mass spectrometer as described in methods 2.17.2.

Annotation

*	:	common molecular ion fragment characteristic of a sugar alditol acetate produced under the conditions described in Methods 2.17.2

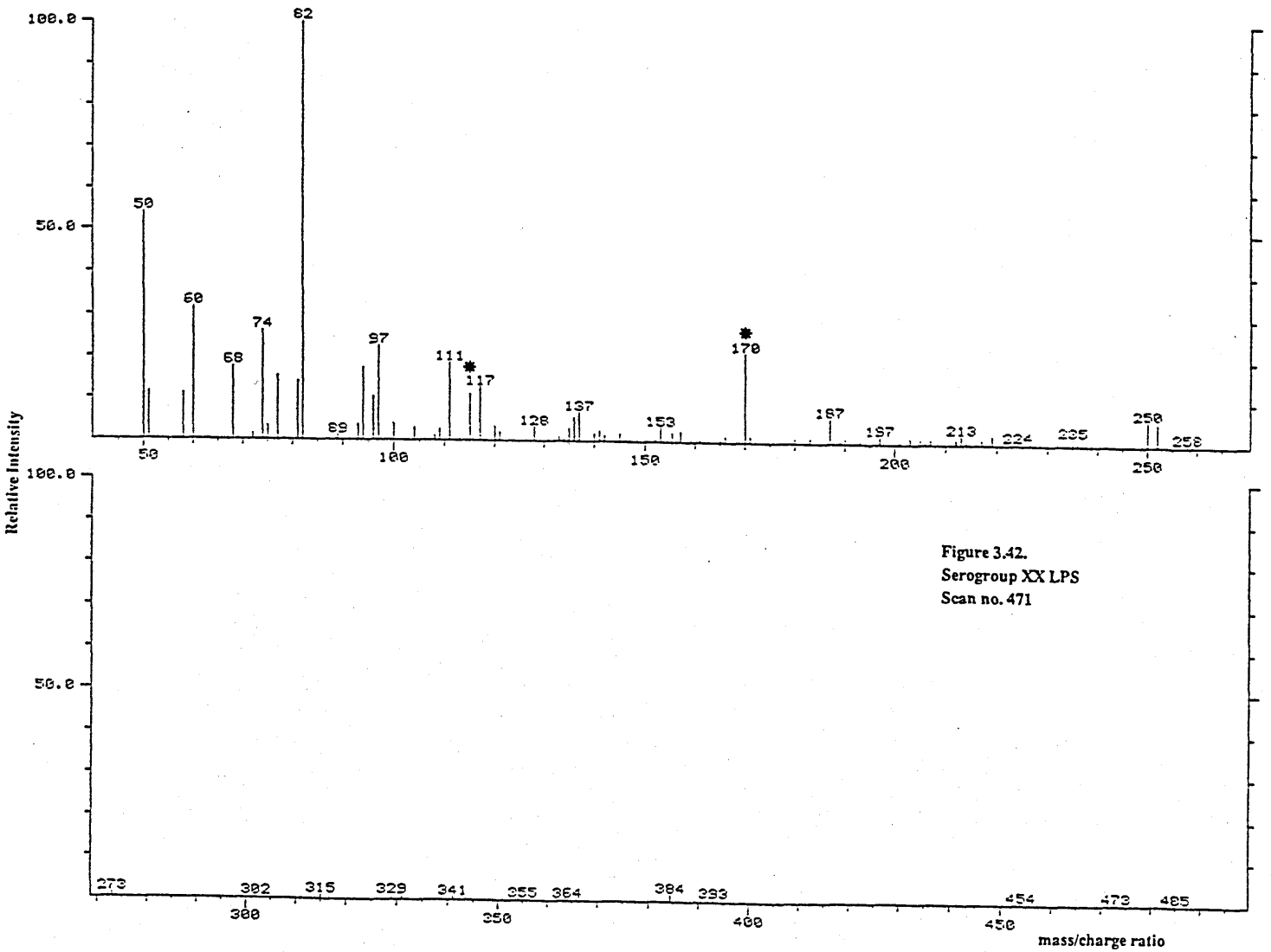


Figure 3.42.
Serogroup XX LPS
Scan no. 471

Figure 3.43

Mass spectral fragmentation patterns of unidentified sugars in serogroup XXII lipopolysaccharide with scan numbers 475 and 484.

Alditol acetates were produced from serogroup XXII lipopolysaccharide as described in Methods 2.17.1.1. They were separated on a Quadrex bonded phase (0.25µm film thickness) capillary column, OV-225, bonded to a fused silica column (25m x 0.25mm) attached to a mass spectrometer as described in methods 2.17.2.

Annotation

* : common molecular ion fragment characteristic of a sugar alditol acetate produced under the conditions described in Methods 2.17.2

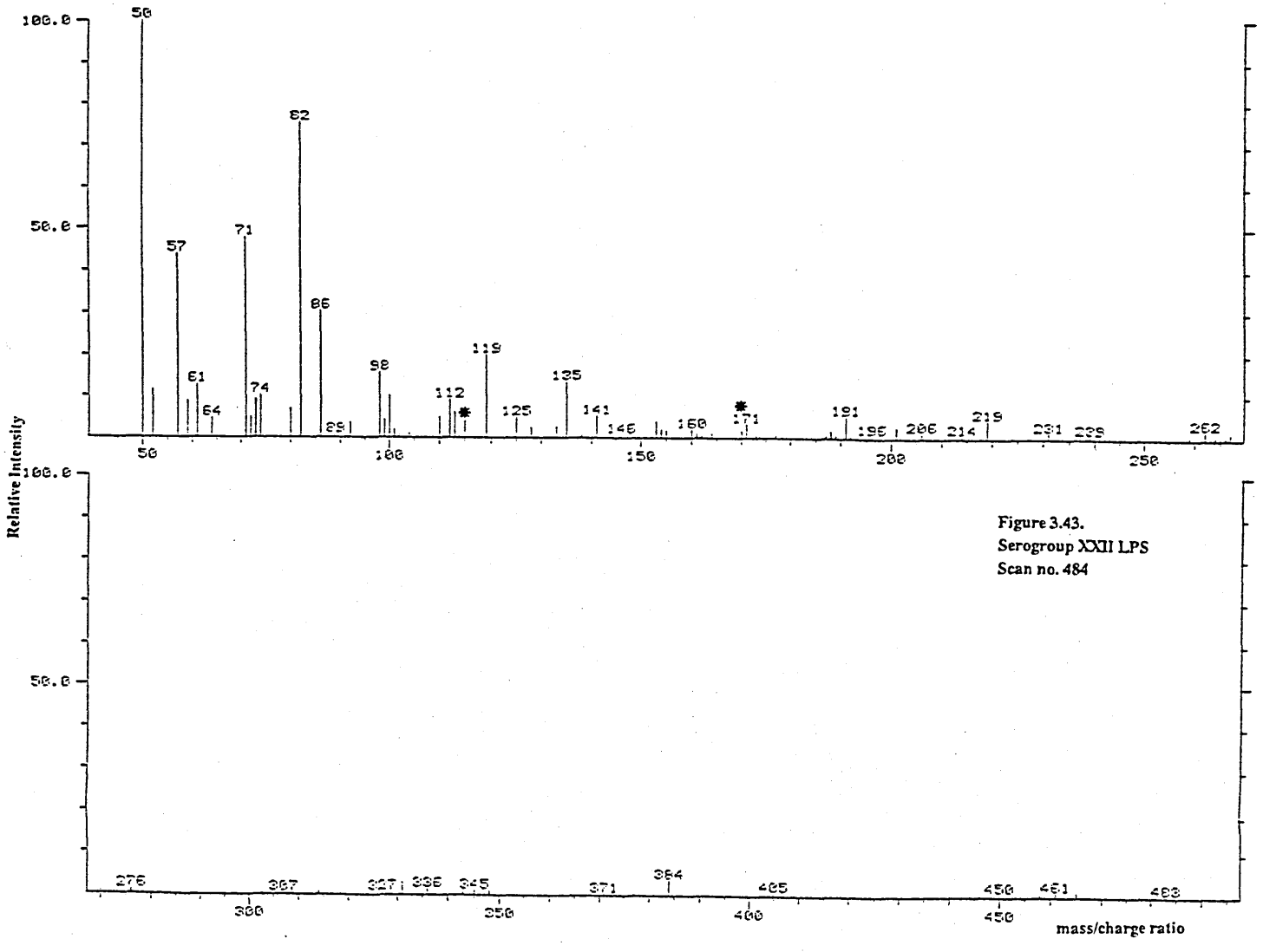


Figure 3.43.
Serogroup XXII LPS
Scan no. 484

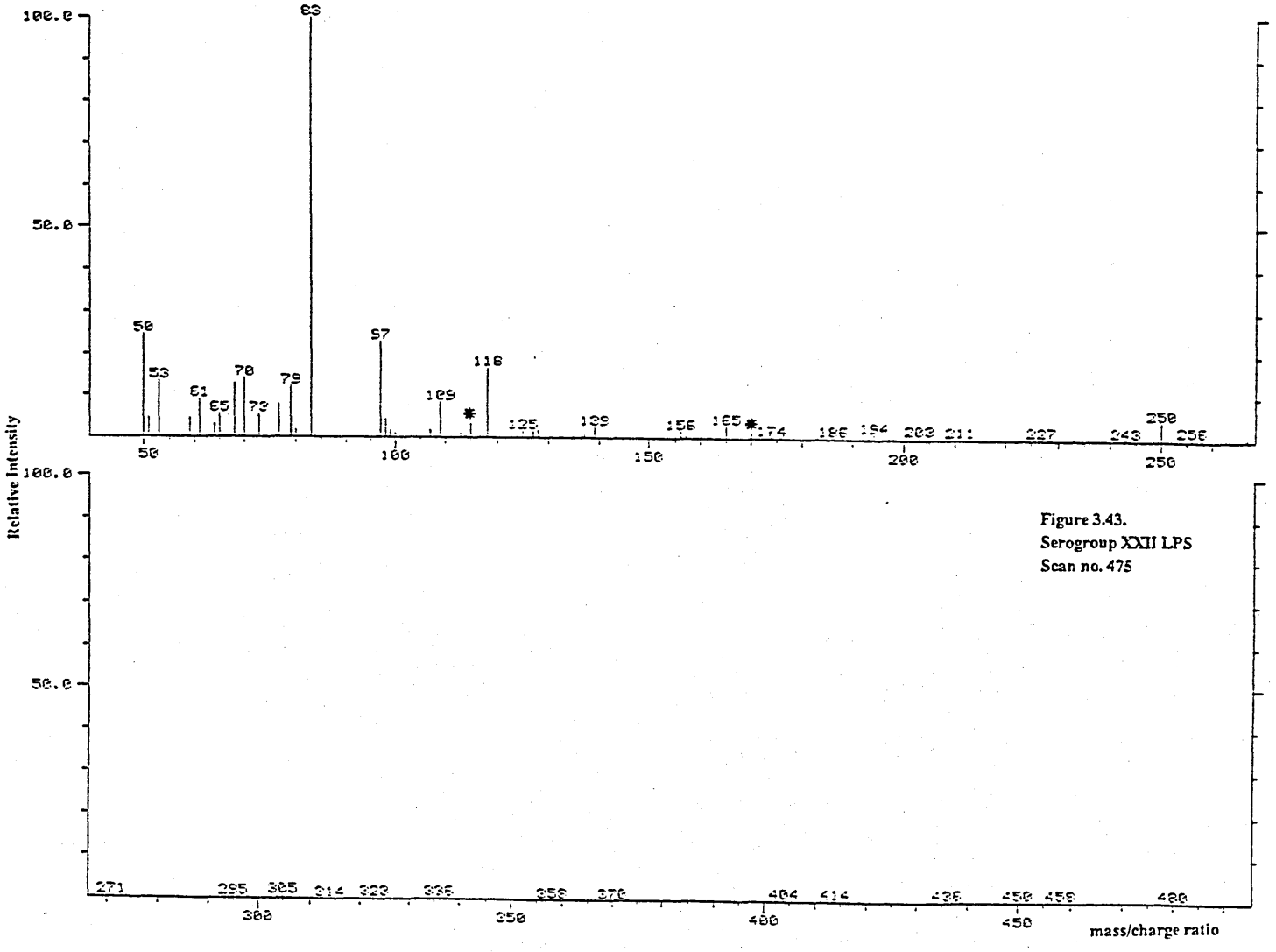


Figure 3.43.
Serogroup XXII LPS
Scan no. 475

7. Chemical Modification of the Sugar Residues of E. carotovora Lipopolysaccharide

7.1 Galactose oxidation of lipopolysaccharide

Intact lipopolysaccharide was incubated with galactose oxidase as described in Methods 2.19.1. The lipopolysaccharide precipitated out of solution and neither the precipitate nor the solution were coloured. The galactose was not considered to be in the correct conformation for oxidation. Galactose oxidase was observed to oxidise galactose and raffinose, a galactose containing trisaccharide.

7.2 Periodate oxidation of lipopolysaccharide

Intact lipopolysaccharide was incubated with periodate as described in Methods 2.19.2. It was difficult to monitor the oxidation of the lipopolysaccharide as problems were encountered with lipopolysaccharide preciptiation. Periodate was observed to oxidise a standard (glucose) sugar in solution.

DISCUSSION

DISCUSSION

1. Extraction of E. carotovora Lipopolysaccharide for Characterisation of Serogroups

As a variety of different protocols exist for the extraction of lipopolysaccharide from the cell walls of gram-negative bacteria, it was important to try these different methods to assess their suitability and reproducibility for the extraction of lipopolysaccharide from E. carotovora. Methods developed for the extraction of lipopolysaccharide from E. coli and Salmonella may not necessarily be suitable for the extraction of lipopolysaccharide from E. carotovora. The phenol/water method of Westphal and Jann (1965) is routinely used for the extraction of smooth lipopolysaccharide but is also capable of extracting rough lipopolysaccharide in low yield. The phenol/chloroform/petroleum spirit method of Galanos et al (1969) is considered to be specific for the extraction of rough lipopolysaccharide. However, Goldman and Leive (1980) and Ray et al (1986) found phenol/ chloroform/petroleum spirit extraction capable of extracting lipopolysaccharide from smooth bacteria. Also initially, the choice of extraction procedure was confounded by the difficulty in differentiating between supposedly smooth strains of E. carotovora and rough

strains of S. minnesota used in this study just by looking at their colony morphologies. Further, some extraction protocols do not differentiate between rough or smooth lipopolysaccharide, being capable of extracting both forms (Leive, 1965; Darveau and Hancock, 1983) and other protocols co-extract associated membrane components along with the lipopolysaccharide (Hitchcock and Brown, 1983). Bearing all these points in mind, the choice of lipopolysaccharide extraction procedure was not only required to be reproducible but also fast and simple so that the procedure could be used routinely in an agricultural establishment.

Phenol/water extraction was found to extract E. carotovora lipopolysaccharide in high yields reproducibly with low levels of contaminants (Results 1.1) Low yields of lipopolysaccharide were obtained by the phenol/chloroform/petroleum spirit extraction method (Results 1.1). The "quick proteinase K method" is not a quantitative method and problems were encountered with the electrophoresis of lipopolysaccharide extracted by this method (Results 1.5, see later in Discussion 4.1). For these reasons the phenol/water extraction method was the chosen method of extraction for E. carotovora lipopolysaccharide for these studies.

Both the phenol/water method and the phenol/ chloroform/petroleum spirit method appeared to extract exactly the same lipopolysaccharide components as

evidenced by their identical electrophoretic patterns (Figure 3.1). A similar effect was observed for lipopolysaccharide extracted from E. amylovora by Ray et al (1986). The authors reasoned that as the lipopolysaccharide possessed short sugar chains, containing a 6-deoxyhexose, this could potentially confer partial lipid solubility on the lipopolysaccharide making it amenable to extraction by the phenol/chloroform/petroleum spirit method. As the lipopolysaccharide was only partially lipid soluble, the extraction would produce low yields of lipopolysaccharide. The lipopolysaccharide would still be relatively polar making extraction by phenol/water possible as well. From comparisons of E. amylovora and E. carotovora lipopolysaccharide patterns (Figure 3.11) it can be seen that they do not possess the very high molecular weight components as observed for Pseudomonads. Further, in all the important potato pathogenic serogroups of E. carotovora subsp. atroseptica, GLC and GLC-MS analyses (Results 6.2.3, 6.2.4) of the lipopolysaccharide sugar composition revealed the presence of a 6-deoxyhexose which seemed to constitute a large proportion of the sugar residues. The 6-deoxyhexose was identified as rhamnose (Results 6.2.3.1.1). From the structure of the core region of E. chrysanthemi (Figure 4.3) which contains only glucose in the outer core region (Sandulache and Prehm, 1985) and

from the knowledge that the core region of other lipopolysaccharides is relatively conserved within a genus and across the family of Enterobacteriaceae, rhamnose is possibly a constituent of the O-chain. This could mean that E. carotovora subsp. atroseptica lipopolysaccharide will have similar properties to E. amylovora lipopolysaccharide.

Lipopolysaccharide extracted from other species of enteric bacteria by the phenol/chloroform/petroleum spirit method has also been observed to produce a ladder pattern, typical of smooth-type lipopoly-saccharide, upon SDS-PAGE. Goldman and Leive (1980) observed this effect for lipopolysaccharide from E. coli CL99. They proposed similar reasons to those of Ray et al (1986) explaining that the lipopolysaccharide could be overall more hydrophobic in nature than other E. coli lipopolysaccharides. Substitution of the sugar hydroxyl groups of the O-chain sugars by acetyl groups or similar small acyl groups would make the lipopolysaccharide molecule less polar. It would appear that the phenol/chloroform/petroleum spirit method is not totally specific for rough lipopolysaccharide and factors such as the number of sugar residues in the O-chain and core, sugar composition and substitution of sugars may be involved in determining extractibility of smooth lipopolysaccharide. Although not directly relevant to problems of extracting lipopolysaccharides of Erwinia

which may be more hydrophobic than others, it has been noted that lipopolysaccharide from strains of Acinetobacter is not easily extracted by the phenol/water method because this method gives complete solubilisation of cells making purification of lipopolysaccharide difficult (Brade and Galanos, 1982).

Although successfully applied to S. minnesota, the proteinase K method of Hitchcock and Brown (1983) did not extract lipopolysaccharide from E. carotovora to give the same components upon electrophoresis as phenol/water extracted lipopolysaccharide (Results 1.5, Figures 3.4, 3.5). However, unlike S. minnesota, E. carotovora do not possess lipopolysaccharides with very long O-chains. From the electrophoretic pattern (Results 2.1) and gel filtration profile (Results 6.1) of E. carotovora lipopolysaccharide they would appear to be a mixture of long and short O-chains. Micelles containing mixtures of long and short O-chain lipopolysaccharide have been shown by Peterson et al (1986) to form a more closely packed lamellar arrangement than long or short O-chain lipopolysaccharide by themselves. They suggest that such an arrangement may reflect a more tightly packed, rigid arrangement in membranes. As a result of this steric difference, the outer membrane proteins may not be as exposed on the surface of the E. carotovora cell as in organisms such as E. coli and Salmonella. Therefore,

all quick methods e.g. the proteinase K method (Methods 2.7.3) and the Triton method (Methods 2.7.4), are not suitable for the extraction of lipopolysacharide from E. carotovora. However, some material is extracted from E. carotovora cells by the proteinase K method which stains with silver after electrophoresis (Results 1.5, Figure 3.5). It is possible that these are protein-lipopolysaccharide complexes which would account for the few, thick bands observed. Similar electrophoretic patterns were obtained by a Finnish group (Pirhonnen et al, 1988) for proteinase K extracted E. carotovora lipopolysaccharide.

2. The Comparative Behaviour of E. carotovora Lipopolysaccharide upon SDS-PAGE

E. carotovora, E. amylovora, E. chrysanthemi and E. salicis are all species of plant pathogenic bacteria belonging to the genus Erwinia (Slade and Tiffin, 1984). They are pathogens of different hosts producing different disease symptoms. Lipopolysaccharide from E. carotovora and E. salicis were observed to produce a more continuous distribution of components (Figure 3.11). The lipopolysaccharide of E. amylovora appeared to possess molecules with longer O-chains. These apparent differences in lipopolysaccharide structure may be related to the different environments in which the pathogens exist as suggested by de Maagd et al (1989). The function of the O-chain might be to interact with a plant receptor, such as a plant lectin. Work by Wolpert and Albersheim (1976) supports this suggestion as they found that lipopolysaccharide would bind to a lectin which had been isolated from the legume with which the Rhizobium was capable of entering into a symbiotic relationship but not with a lectin isolated from a legume with which the Rhizobium could not form a symbiotic relationship.

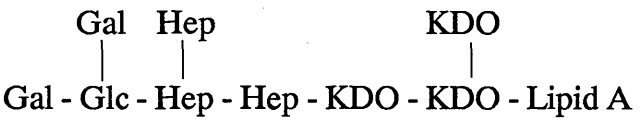
Unlike proteins, appropriate glycolipid molecular weight standards are not available for molecular weight determinations of lipopolysaccharide. Comparisons and estimations can be made however from known

lipopolysaccharide structures. The highest mobility component of E. carotovora lipopolysaccharide was observed to have a similar electrophoretic mobility to lipopolysaccharide from several S. minnesota mutant strains (Figure 3.10). These mutants represent rough chemotypes Ra (R60), Rb (R345), Rc (R5) and Rd1 (R7) (Figure 4.1), and this makes it probable that the highest mobility band from E. carotovora has a lipid A-core oligosaccharide structure. This similarity would be anticipated due to the relatively conserved nature of the lipid A moiety and core region between different genera of enteric bacteria (Figure 4.2). However, due to the lack of available molecular weight markers this is still a hypothesis.

If E. carotovora antisera had cross-reacted with S. minnesota lipopolysaccharide from these mutant strains it would have confirmed their homology but such a cross-reaction was not found (Results 5.5). One possible explanation for this lack of cross-reactive antibodies is that only core-specific antibodies of a low titre are generated as a result of the O-chain folding back on itself and masking the core region as an immunogen (Labischinski et al, 1985). These core-specific antibodies, although possibly capable of reacting with the core region of S. minnesota lipopolysaccharides were below detectable limits in the immunoblotting experiment. But, as the E. carotovora antiserum reacted

R60 (complete core)

R345



R5

R7

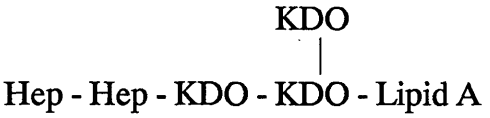


Figure 4.1: Rough chemotypes of *S. minnesota* mutant strains (Hitchcock & Brown, 1983).

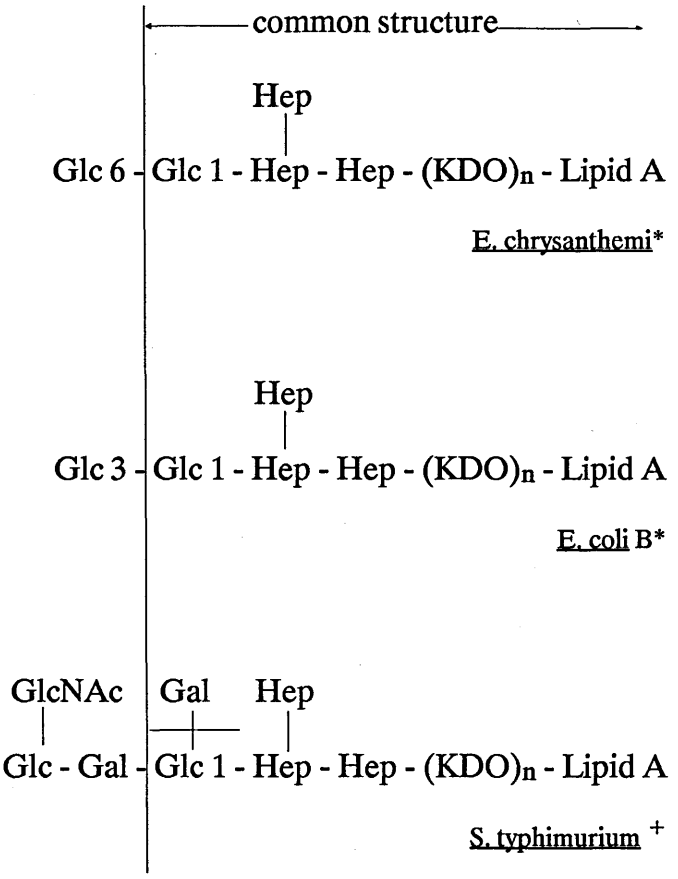


Figure 4.2. Core structures of lipopolysaccharide from different species of enterobacteria. (*: Sandaluche & Prehm, 1985; $^{+}$: Hammond et al, 1984).

with the lipid A-core band of its own lipopolysaccharide, this is not possible. Further, by analogy with O-chain structures, a single change in sugar composition, stereochemistry or conformation could produce a totally different immunological identity in the Erwinia core structure compared to the S. minnesota structure. However, this is based on the assumption that the fastest migrating component of E. carotovora lipopolysaccharide consists of only lipid A and core. But if one O-chain were attached to the lipid A-core moiety, and we have no evidence to show otherwise, then that could account for the reactivity of the E. carotovora lipopolysaccharide with its own antiserum. Therefore, the possibility still remains that the cores of E. carotovora and Salmonella are similar and detailed studies on the actual core structures are needed.

Those components in a lipopolysaccharide electrophoretic pattern which migrate after the highest mobility band are considered to represent lipopolysaccharide molecules with increasing numbers of attached O-chain units (Hitchock and Brown, 1980; Palva and Makela, 1980) and the data obtained (Figure 3.6) would indicate that the E. carotovora strains used in this study are smooth type. Growth of certain E. carotovora strains in the presence of 0.05% (w/v) and 0.01 (w/v) deoxycholate support this conclusion (Results 3.1). Lipopolysaccharide O-chain acts as a hydrophilic

barrier shielding the outer membrane from harmful hydrophobic detergents, dyes and antibiotics which would otherwise be soluble in the hydrophobic environment of the outer membrane. At the concentration of detergents used (Results 3.1) bacterial growth is only possible if lipopolysaccharide molecules possess O-chain (Rosner et al, 1979).

3. The Influence of Growth Conditions on the Electrophoretic Behaviour of E. carotovora Lipopolysaccharide

The electrophoretic patterns of lipopolysaccharide extracted from E. carotovora cells grown on 20mM glucose minimal salts medium and double strength nutrient broth were very similar but not identical (Figure 3.1, tracks 2 and 3). The intensity of staining of some of the doublet bands was different. Doublet bands are observed for most smooth lipopolysaccharides analysed by SDS-PAGE and are thought to be produced by some form of heterogeneity (Goldman and Leive, 1980; Hitchcock and Brown, 1980). The leading band of each doublet stained more intensely in lipopolysaccharide extracted from cells grown on 20mM glucose minimal salts medium by comparison to lipopolysaccharide extracted from cells grown on double strength nutrient broth. Similar observations were made by Palva and Makela (1979). They found that by comparison of lipopolysaccharide extracted from S. typhimurium cells grown on minimal and complex media, the upper band of each doublet band stained more intensely in lipopolysaccharide grown on complex medium compared with lipopolysaccharide grown on minimal medium.

Similar differences in intensity of staining of the doublet bands were observed with lipopolysaccharide extracted from late log/early stationary phase cells and

from early log phase cells (Figure 3.1, tracks 4 and 5). Palva and Makela (1975) also observed a similar difference in staining intensity of the doublet bands for lipopolysaccharide extracted from log and stationary phase cells of S. typhimurium.

It is suggested that these doublet bands are indicative of another level of heterogeneity i.e. microheterogeneity. The first level of heterogeneity is regarded as the result of different O-chain lengths within a lipopolysaccharide population. Differences in phosphate substitution at the level of the lipid A moiety have been proposed as a basis for the micro-heterogeneity which gives doublets (Goldman and Leive, 1980). Rosner et al (1979c) working with a heptoseless mutant of E. coli K-12 identified two lipopolysaccharide populations differing in the phosphate substitution of the lipid A moiety. One population of lipopolysaccharide molecules was substituted at C-1 of the non-reducing glucosamine residue with a monophosphate group (see Figure 1.4a). The other population of lipopolysaccharide molecules was substituted with a pyrophosphate group at the same C-1 position. The ratio of the two populations to one another was found to be dependent on the phosphate concentration in the growth medium. E. carotovora lipopolysaccharide was incubated with bacterial alkaline phosphatase in an attempt to investigate the effect of

phosphate substitution at the level of the lipid A moiety on doublet formation. The electrophoretic behaviour of the lipopolysaccharide remains unchanged after treatment with phosphatase (Results 2.6, Figure 3.13). According to Rosner et al 1979c, only the phosphate group at C-4' of the reducing glucosamine residue of the lipid A moiety tends to be hydrolysed as hydrolysis at the C-1 position is very slow due to steric factors. If there are similar phosphate groups in the lipid A moiety of E. carotovora lipopolysaccharide, they would appear not to be important to lipopolysaccharide electrophoretic behaviour. It was therefore not possible by this method to show that phosphate substitution at the C-1 position plays a role in producing microheterogeneity of Erwinia lipopolysaccharide.

Substitution of a phosphate group for a sugar residue at the level of the lipid A moiety has also been implicated in doublet formation. Lipopolysaccharide extracted from a S. typhimurium wild type strain and a mutant strain possessing increased resistance to polymyxin were observed to produce similar electrophoretic patterns but with different staining intensities of the bands of the doublets (Vaara et al, 1981). The lower band of each doublet in the lipopolysaccharide profile of the wild type strain stained more intensely than the upper band of the

doublet. Conversely, the upper band of each doublet in the lipopolysaccharide from the polymyxin resistant mutant strain stained more intensely than the lower band of the doublet. Vaara et al (1981) suggested that a possible reason for this observed difference was due to different amounts of the sugar, 4-amino-2-deoxy-L-arabinose. They found that the mutant strain possessed increased amounts of this sugar as compared to the wild type strain and that the sugar substituted for an acidic phosphate group at the level of the lipid A moiety. Such a substitution would produce a change in overall charge of the lipopolysaccharide molecule and account for the increased intensity of the upper band of each doublet.

Even though there is evidence that specific groups play a role in determining the microheterogeneity of specific lipopolysaccharides, the observed intensity of doublets can vary during storage at low temperatures which should prevent degradation of lipopolysaccharide. Goldman and Leive (1980) found that the staining intensity of the bands of the doublets of E. coli O111 lipopolysaccharide changed after storage at -20°C for thirty days; the initially more intense lower band became less intensely stained, making both bands as equally intense. After storage of these lipopolysacccharide preparations at -80°C for three months, the upper bands of the doublets stained more

intensely than the lower bands. E. carotovora lipopolysaccharides were stable over two years of storage at -20°C (Results 1.4, Figure 3.3). Long term stability of lipopolysaccharide preparations has been observed for S. minnesota lipopolysaccharide stored at 4°C and -20°C (Hitchcock and Brown, 1983). It is not understood why there are these observed differences in the stability of lipopolysaccharide preparations. It may be a consequence of there being different lipopolysaccharides from different species, the different methods of extraction used, the different gel systems or a combination of these factors. Further, it may even be the silver stain used (Tsai and Frasch, 1982) as the exact chemistry of this stain is not understood and as Hitchcock and Brown (1983) so aptly put it, "it is biochemically obscure". However, using the phenol/water extraction method of Westphal and Jann (1965), the SDS-PAGE system of Laemmli (1970), and the silver stain of Tsai and Frasch (1982), E. carotovora lipopolysaccharide preparations were found to be stable in this study.

In summary, the observed doublets (Goldman and Leive, 1980; Hitchcock and Brown, 1983; Vaara et al, 198) have been suggested in two instances (Rosner et al, 1978; Vaara et al, 1981) to be the result of some form of microheterogeneity of substituents on the lipid A region of lipopolysaccharide based on studies with

other organisms. The nature of this substitution in E. carotovora types is not known but it is stable. It would be interesting to find out what this substituent is in E. carotovora lipopolysaccharide.

4. Interpretation of the Electrophoretic Behaviour of E. carotovora Lipopolysaccharide

Many studies (Jann et al, 1975; Russell and Johnson, 1975; Goldman and Leive, 1980) have shown that as with proteins, molecular size is the major parameter responsible for the electrophoretic separation of lipopolysaccharide in the SDS-PAGE system of Laemmli (1970). Charge heterogeneity of lipopolysaccharide molecules was discounted by Goldman and Leive (1980) because isoelectric focusing of E. coli lipopolysaccharide produced only one band. A recent study suggested that charge may contribute to this separation (Duchesne et al, 1988) because at the lower pH of 6.8, lipopolysaccharide from Ps aeruginosa, S. typhimurium and E. coli was found to migrate faster than at the conventional SDS-PAGE pH of 8.8. Duschenne et al (1988) suggested that the increased migration rate was the consequence of the increased affinity of the lipopolysaccharide for SDS at the lower pH. It is however possible that this pH effect may be specific for lipopolysaccharide from certain organisms or be caused by the co-migration of protein with the lipopolysaccharide because the proteinase K method (Hitchcock and Brown, 1983) of extraction which was used in this study does not necessarily give a completely protein free preparation (see Discussion 1). Similar studies at low pH have not been done with Erwinia

lipopolysaccharide but increasing pH causes loss of resolution. Lipopolysaccharide was electrophoresed in Tris/borate, pH 9.0 SDS-PAGE (Results 2.5) to investigate the effect of borate on the electrophoretic separation of lipopolysaccharide because cis- and vic-glycols complex with borate to give different charged species in a solution of boric acid (Percival, 1962). The charged species produced by the interaction of borate with the glycols of the lipopolysaccharide molecules would appear to have interfered with the normal resolution process observed on the Tris/glycine system of Laemmli (1970).

Although it is considered that lipopolysaccharide does resolve in SDS-PAGE due to differences in molecular size, controversy exists as to the molecular nature of the species being separated. There are two schools of thought. One hypothesis postulates that single lipopolysaccharide molecules form micelles with molecules of SDS (Goldman and Leive, 1980; Hitchcock and Brown, 1983). These lipopolysaccharide - SDS micelles separate out on the gel according to the number of attached O-chain units with the lipid A-core molecules migrating the fastest (Figure 4.3a). Each band therefore represents a single size of lipopolysaccharide molecule with attached O-chain. The other hypothesis advocates the formation of micellar aggregates (Dooley et al, 1985; Peterson and McGroarty 1985) which consist

Figure 4.3

Two different hypotheses for the electrophoretic separation of lipopolysaccharide - SDS micelles:

a) sequential addition of one O-chain unit;

b) molecular aggregates.

Abbreviations

A : lipid A
C : core sugars
O : O-chain
* : SDS molecule

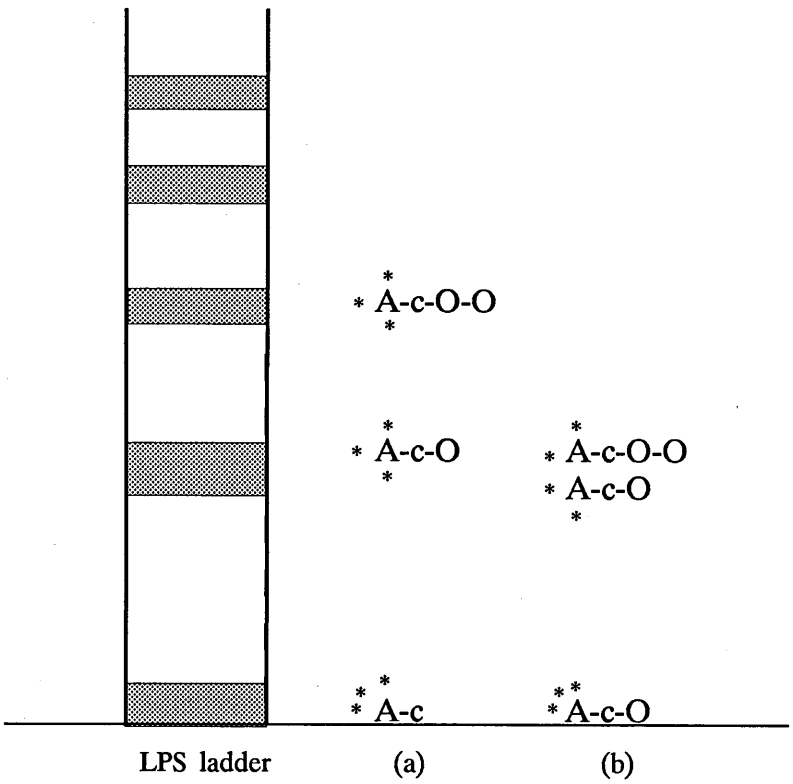


Figure 4.3.

of several molecules of SDS and one or several molecules of lipopolysaccharide; the lipopolysaccharide molecules can possess different numbers of attached O-chain units. Each band therefore represents different sizes of lipopolysaccharide molecule with different numbers of attached O-chain units. Those micelles with the least number of lipopolysaccharide molecules and attached O-chain units will therefore constitute the fastest migrating band observed in the lipopolysaccharide pattern (Figure 4.3b).

The presence of detergent is essential (see Results 3.5) to break up the large aggregates formed by the intrinsic nature of lipopolysaccharide molecules to autoaggregate in an aqueous environment (Ribi et al, 1966; Olins and Warner, 1967) so that they may migrate through the gel matrix under electrophoresis. The theoretical consideration of the amount of detergent required to produce mixed micelles of lipopolysaccharide and detergent has received much attention from workers as the exact interaction of lipopolysaccharide and detergent may help explain the electrophoretic pattern produced resolving the present confusion. The concentrations of sodium dodecyl sulphate (SDS) in the SDS-PAGE system of Laemmli (1970) used in this study were: 3% (w/v) SDS in the sample buffer, 0.1% (w/v) SDS in the gels, and 0.1% (w/v) SDS in the running buffer (Methods 2.10.1). These concentrations of SDS are

expected to be above the critical micellar concentration (CMC) for SDS by comparison to those values calculated by other researchers e.g. 0.015% (w/v) SDS in 0.5M NaCl (for review see Helenius and Simmons, 1975). By definition the CMC is "the narrow concentration range of surfactant below which no micelles are detected and above which virtually all surfactant forms micelles" (Lichtenberg et al, 1983). Mixed micelles of lipopolysaccharide and SDS would therefore be expected to form at the concentrations of SDS (3% and 0.1% (w/v)) used in electrophoresis. Hitchcock (1983) found that electrophoretic resolution of lipopolysaccharide was possible with SDS present only in the sample and the running buffers. All SDS-PAGE analyses of E. carotovora lipopolysaccharides performed in this study were with SDS also present in the gels. Electrophoretic resolution was not possible when SDS was omitted from the gel (Results 3.5). It seems probable that SDS is required in the running buffer to prevent the lipopolysaccharide-SDS micelles from dissociating into their constituents and the lipopolysaccharide molecules then forming large molecular aggregates which would be too large to migrate through the gel matrix. From the consistently reproducible SDS-PAGE patterns obtained for E. carotovora lipopolysaccharide, it appeared that this particular lipopolysaccharide resolves itself as monomers possessing the same O-chain length (see Figure

4.4a). Experiments were undertaken in an attempt to confirm this. Two-dimensional gel electrophoresis of E. carotovora lipopolysaccharide (Results 3.2) produced a pattern of single bands along the expected diagonal but the highest mobility bands from the first dimension produced several bands above the diagonal in the second dimension (Figure 3.14a). A similar pattern was obtained for lipopolysaccharide from S. minnesota mutant strain R7 except that only three bands were produced on the expected diagonal (Figure 3.14b). Hitchcock (1983) made a similar observation for the two-dimensional gel electrophoresis of S. typhimurium lipopolysaccharide. However, the extra bands were below the diagonal in her analysis. Hitchcock also found that pre-electrophoresis of the gel removed these bands therefore she suggested that dissociation of SDS from the lipopolysaccharide was their cause. The results obtained for E. carotovora are consistent with aggregation of low molecular weight material due to loss of SDS. The low molecular weight material, possessing less O-chain/core would be most susceptible to a drop in SDS concentration. However, Hitchcock (1983) found that reheating the gel slice from the 1D gel with 2% SDS lysing buffer before 2D-electrophoresis had no effect on the silver-stained pattern nor did the inclusion of 0.1% SDS in the 2D gel.

To confuse the situation further, Peterson and

McGroarty (1985) also observed extra bands produced by the two-dimensional electophoresis of S. minnesota, S. typhimurium and E. coli lipopolysaccharides but the pattern was different both from that of E. carotovora and S. minnesota lipopolysaccharide observed in this study. Further, the pattern obtained for S. typhimurium lipopolysaccharide by Peterson and McGroarty (1985) was different from that obtained by Hitchcock (1983) for S. typhimurium. Although the phenol/water extraction method was used by both groups, the Salmonella strains used were not specified making comparisons difficult. To explain the anomalous bands, Peterson and McGroarty (1985) suggested that they were produced by very high molecular weight aggregates of lipopolysaccharide. The aggregates were formed as the result of low SDS concentrations and large amounts of lipopolysaccharide. This explanation would not appear to be applicable to E. carotovora lipopolysaccharide as they do not possess very high molecular weight molecules. Further, these anomalous bands were not observed with 20 μg of E. carotovora lipopolysaccharide which is about ten times the amount used in most analyses of E. coli and Salmonella lipopolysaccharides. The amount of lipopolysaccharide for E. carotovora may appear large by comparison but it was found to be the optimum loading in terms of ladder produced and staining intensity (Results 2.2.2) under the conditions used in this study. It is

possible that the reason for such a large loading of lipopolysaccharide is due to the continuous size distribution of Erwinia lipopolysaccharide molecules which give a ladder pattern up the gel. This contrasts with the lipopolysaccharide molecules of E. coli and Salmonella which possess only short and long O-chains and therefore less material requires to be run on a gel to be visualised. Also, increasing the SDS concentration in the gel system from 0.1% (w/v) SDS to 0.4% (w/v) SDS and in the sample buffer from 1% (w/v) SDS to 6% (w/v) SDS did not effect the electrophoretic separation of E. carotovora lipopolysaccharide (Results 2.2.3, Figure 3.9). The concentrations of SDS used by Peterson and McGroarty (1985) in their gel system were also well above the CMC for SDS: 0.1% (w/v) SDS in the gels and running buffer, and 4% (w/v) SDS in the sample buffer. The data therefore suggest that under the conditions used Erwinia lipopolysaccharide molecules resolve themselves in SDS-PAGE as monomers possessing the same numbers of attached O-chain units.

This conclusion is supported by electrophoresis of mixtures of lipopolysaccharide preparations. Electrophoresis of a mixture of two lipopolysaccharide preparations from serogroup I gave only one lipopolysaccharide SDS-PAGE pattern, the standard serogroup I pattern (Results 3.3, Figure 3.15). More importantly, a mixture of two lipopolysaccharide

preparations from two different serogroups gave a mixed ladder pattern (Results 3.3, Figure 3.15). The mixtures were made by mixing the two lipopolysaccharide preparations together prior to the addition of SDS to give mixed lipopolysaccharide-SDS micelles. The pattern produced was clearly discernible as being a composite of the two different patterns; there were no mixed bands. The result suggests that some ordered process of separation is occurring upon electrophoresis rather than some aggregation of SDS and lipopolysaccharide of various O-chain lengths or structure. It favours the hypothesis that lipopolysaccharides of the same O-chain length migrate at the same rate and that the bands represent one type of lipopolysaccharide molecule.

The relationship between band number and mobility in the SDS-PAGE of E. carotovora lipopolysaccharide was observed to be logarithmic (Results 3.4, Figure 3.16). An increase in band number represents a proportional increase in molecular size. As the apparent increase in molecular size was logarithmic, small regular increases in molecular size were thought to be occurring. This would be anticipated for the sequential addition of O-chain units of the same molecular size. Further, this increase in molecular size was discounted as being the result of a doubling of micelles or micellar aggregation by Goldman and Leive (1980). They determined the galactose to phosphate ratio for each band in the

SDS-PAGE profile of lipopolysacharide from E. coli O111. Their logic was that as phosphate substitution is restricted to the core region and does not vary with chain length, and as galactose was present in the O-chain repeat unit, then the galactose to phosphate ratio should increase with increasing molecular weight. This was what they found.

Clearly this situation has not been resolved but the results presented for E. carotovora lipopolysaccharide would appear to be separation due to differences in O-chain length.

5. An Alternative System for the Serotyping of E. carotovora Strains

As a result of studies with sixteen strains belonging to six serogroups, De Boer et al 1985 proposed that the serogrouping of Erwinia carotovora was based on lipopolysaccharide O-antigens. Extending this work, a direct relationship was observed in this study between serogroup and lipopolysaccharide SDS-PAGE pattern. Lipopolysaccharide was extracted from a total of fifty nine E. carotovora strains belonging to twelve different serogroups (see Table 4.1). Twelve different lipopolysaccharide SDS-PAGE patterns were produced: I, IV, XII, XVIII, XX, XXII, XXV, XXVIII, XXIX, XXXIII, XXXIV, XXXV (Results 4.1, Figure 3.17).

Similar correlations have been observed in other studies using gel electrophoresis. From the SDS-PAGE analysis of outer membrane proteins from two hundred and thirty four strains of E. coli, Achtman et al (1983) observed some correlation between membrane pattern and O:K serotype. The correlation was not total as some membrane patterns were associated with more than one O:K serotype. The outer membrane protein patterns also gave indications as to the clonal descent of the respective strains and six general groupings within the E. coli strain were identified. Continuing this line of research, Moll et al (1986) used SDS-PAGE to analyse lipopolysaccharide from strains belonging to the O1

TABLE 4.1 Serogroups of E. carotovora strains (see Table 2.1)

Subspecies and strain	Serotype: As given	Serotype: by LPS pattern	Serotype: serological confirmation
atroseptica			
31	I	I	-
436	I	I	I (Glasgow)
509	I	I	-
549	I	I	I (Glasgow)
1001	I	I	-
1002	I	I	-
1034	I	I	I (Glasgow)
1039	I	I	I (Glasgow)
1042	I	I	I (Glasgow)
1043	I	I	I (de Boer; Glasgow)
1061	I	I	I (Glasgow)
1062	I	I	I (Glasgow)
1063	I	I	I (Glasgow)
1064	I	I	I (Glasgow)
1065	I	I	I (Glasgow)
1066	I	I	I (Glasgow)
1067	I	I	I (Glasgow)
1068	I	I	I (Glasgow)
1069	I	I	I (Glasgow)
1070	I	I	I (Glasgow)
G398	I	XX	I&XX(de Boer; XX Glasgow)
6	XVIII	XVIII/XX	-
DB6	XVIII	XVIII/XX	XVIII(de Boer
11	XVIII	XVIII/XX	-
DB39	XVIII	XVIII/XX	XVIII(de Boer)
DB58	XX	XVIII/XX	-
196	XX	XVIII/XX	-
DB199	XX	XVIII/XX	-
DB1056	XX	XVIII/XX	-
DB1058	XX	XVIII/XX	-
G331	XX	I	I (de Boer; Glasgow)
DB198	XXII	XXII	XXII (Glasgow)
DB420	XXII	XXII	XXII(Glasgow)
A46	XXII	XXII	XXII(Glasgow)
A139	XXII	XXII	XXII(Glasgow)
G222	XXII	XXII	XXII(Glasgow)
432	XXXV	XXII	XXII(Glasgow)

carotovora

327	I	I	-
547	I	unknown	not I(de Boer; Glasgow)
1745	I	I	I(de Boer; Glasgow
G275	IV	IV	-
G298	XII	XII	-
192	XVIII	XVIII*	-
G301	XVIII	XVIII*	-
G303	XVIII	XVIII*	-
P1	XXV	XXV	-
312	XXVIII	XXVIII	-
133	XXIX	XXIX	-
193	XXXIII	XXXIII	-
438	XXXIV	XXXIV	-
553	XXII/XXXV	XXII	-
556	XX/XLI	XX	-
557	XXII/XLI	XXII	-
559	XX/XL	XX	-

betavasculorum

545	XL/XLIII	XXXV	-
546	XXXV	XXXV	-
548	XXXV	XXXV	-
551	XXXV	XXXV	-

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de Boer - confirmatory immunological analyses undertaken by Dr. S.J. De Boer, Vancouver, Canada.

Glasgow - immunological analyses undertaken in this study.

* - pattern characteristic of EccXVIII strains only.

serogroup of E. coli. Their analyses revealed the existence of five lipopolysaccharide types within the O1 serogroup and only certain membrane protein patterns (Achtman et al, 1983) were associated with certain of these lipopolysaccharide types. Similarly, lipopolysaccharide electrophoretic patterns of purple non-sulphur bacteria were found to correlate with their respective chemoserotype and chemical composition (Krauss et al, 1988).

On the basis of the given serogroup of the strains used in this study, different serogroups gave different patterns. In cases where there was not a correlation between serogroup and LPS-PAGE pattern, it was shown on the basis of immunological tests that the strain had been misassigned to a serogroup (Results 5.3). Cultures of the anomalous strains 547 (Figure 3.18, track 6), G398 (Figure 3.18, track 7) and G331 (Figure 3.22, track 6) were sent to De Boer to confirm the identity of the serogroups of these strains. De Boer's subsequent analyses confirmed that the strains had been assigned to incorrect serogroups (Table 4.1) and that the serogroups proposed by lipopolysaccharide analyses were correct. De Boer confirmed strain 547 not to belong to serogroup I (Figure 3.18, track 6) but could not determine its actual serogroup. Strain G331 was confirmed not to be a serogroup XX strain and belonged to serogroup I as suggested by lipopolysaccharide analyses (Figure 3.2.2,

track 6). From its LPS-PAGE pattern and immunological tests G398 was found to be a serogroup XX strain (Results 5.8). However, De Boer found strain G398 to type into both serogroup I and serogroup XX. The data therefore indicate the potential of this system for the serotyping of E. carotovora strains as an alternative to immunodiffusion.

Although the knowledge of the serology of E. carotovora is not comparable to that of either E. coli or Salmonella, it is likely that the different serogroups of E. carotovora reflect differences in O-antigen structure and/or core structures. De Boer et al (1985) have proposed that the serogroups of E. carotovora are based on lipopolysaccharide antigens. Further, it is known that the O-antigens of different O-serotypes of E. coli have different structures (Orskov et al, 1984). Thus SDS-PAGE would appear to be able to detect quite subtle changes in the chemical structures of E. carotovora lipopolysaccharides as well as gross changes due to the addition of increasing numbers of O-antigen units.

The impetus of this study was the requirement for methods of identifying strains of E. carotovora subsp. atroseptica causing blackleg disease of potatoes. Previous work had shown that subsp. atroseptica strains of serogroups I, XVIII, XX and XXII constitute the bulk of erwinias isolated from diseased potato stems in

cooler climates and in fact, serogroup I strains constitute about 96% of all such isolates (De Boer and McNaughton, 1987). This study has shown that the SDS-PAGE of Erwinia lipopolysaccharide gives a considerable amount of information about their structure and allows the identification of erwinias assigned to serogroup I, XVIII, XX and XXII, the major potato pathogenic serogroups, independently of the availability of serogroup specific antisera (Figure 4.4). Compared with the analysis of only three serogroup I lipopolysaccharide preparations (De Boer et al, 1985), lipopolysaccharides from twenty-three serogroup I strains were analysed in this study. Consistent results were obtained with twenty-one of the twenty-three strains giving the same pattern. Twenty of these twenty one strains can be seen in Figures 3.18 and 3.19. Five serogroup XXII strains gave an identical pattern (Figure 3.22). Four serogroup XVIII strains of E. carotovora subsp. atroseptica and five serogroup XX strains gave identical lipopolysacharide SDS-PAGE patterns (Figure 3.22). This pattern was different from that of three serogroup XVIII strains of E. carotovora subsp. carotovora.

The bulk of these results would support the conclusion that De Boer's serogroups are based on lipopolysaccharide antigens as LPS-PAGE pattern correlates with serogroup. However, there is something

Figure 4.4

SDS-PAGE of lipopolysaccharide from representative strains of the potato pathogenic serogroups of *E. carotovora* subsp. *atroseptica*.

20µg of sample was loaded per track and run on a 12.5% acrylamide gel (Methods 2.10.1). The gel was stained as described in Methods 2.12.2.

Tracks (1) *Eca* 1034 , serogroup I lipopolysaccharide
(2) *Ecc* G301 , XVIII
(3) *Eca* 6 , XVIII
(4) *Eca* 1056 , XX
(5) *Eca* 198 , XXII

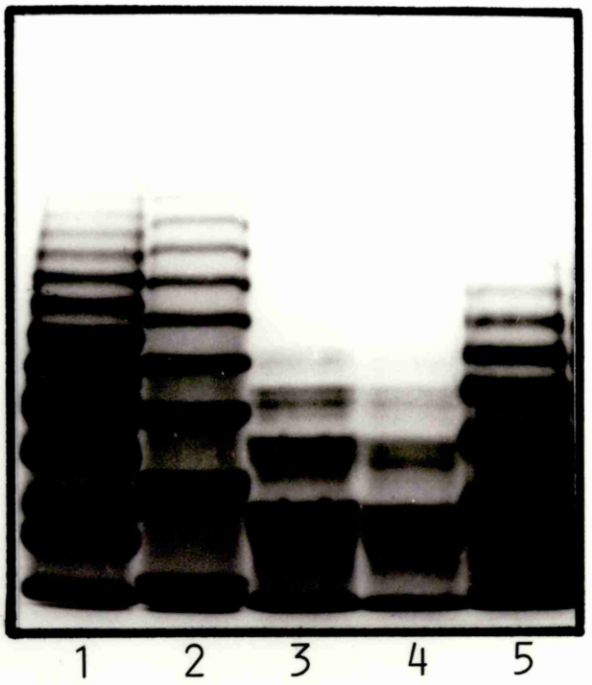

Figure 4.4.

peculiar about the serogroup XVIII strains. The lipopolysaccharides from serogroup XVIII strains of carotovora and atroseptica are different (Figure 3.22) but are confirmed as belonging to the same serogroup. It follows that antigens other than lipopolysaccharide epitopes are also important in this serogroup. Results obtained from agglutination and immunoblotting experiments confirm that a common epitope exists between these two subspecies.

From agglutination experiments (Methods 2.16.2) it was found that serogroup XVIII (Ecc) antiserum had a titre of 1:2048 against serogroup XVIII (Ecc) whole cells and a titre of 1:8192 against serogroup XVIII (Eca) whole cells (Table 2.3). Serogroup XVIII (Eca antiserum had a titre of 1:8192 against both serogroup XVIII Eca and Ecc whole cells (Table 2.3). Therefore, although serogroup XVIII subsp. atroseptica and carotovora have different lipopolysaccharide structures, their respective antisera appear to recognise some surface structure equally well.

A second possible protein antigen(s), common to serogroup I and serogroup XXXV, was detected by serogroup I antiserum in preparations of lipopolysaccharide from serogroup XXXV strains of E. carotovora subsp. betavasculorum (Results 5.4). In an attempt to find the cellular location of this protein antigen(s), whole cells of the serogroup XXXV strains

were subjected to a mild acid hydrolysis specifically for the removal of lipopolysaccharide O-chain. The hydrolysed cells were then used to absorb serogroup I antiserum (Methods 2.15.2). The resulting absorbed antisera still reacted with the proteinaceous antigen(s) present in the serogroup XXXV lipopolysaccharide preparations and with serogroup I lipopolysaccharide. Therefore, the protein(s) was not present on the surface of the cell. To confirm that O-chain had been hydrolysed from the lipopolysaccharide molecules, exposing protein normally masked by the sugar chain, lipopolysaccharide could have been subsequently extracted from the hydrolysed cells. Surface labelling of the cells would show conclusively whether this protein(s) was present on the surface of the cell or not.

If such an antigen were an outer membrane protein then it could account not only for serogroup XVIII strains but also for those strains found to type into more than one serogroup but have been shown in this study to produce only one recognisable LPS-PAGE pattern (Figures 3.24, 3.25) i.e. cross-reaction is due to protein. Another possibility is that the antisera used to type these strains could have originally been raised against a mixed culture. Two strains 1043 and 1745 demonstrated the presence of two populations of lipopolysaccharide molecules differing in the

presence/absence of one band in their electrophoretic patterns (Figure 3.21a, 3.21b).

It would appear that the method of lipopolysaccharide SDS-PAGE analysis developed in this study can identify the important blackleg strains (Figure 4.4, Table 4.1). If enough lipopolysaccharide could be isolated from diseased plant tissue, the fact that E. carotovora lipopolysaccharide patterns are distinct from those of other erwinia species and from pseudomonads could make the method directly applicable to diseased tissue where heterogeneous populations of bacteria exist.

The advantages of this system for the identification of the blackleg pathogen are several. It avoids the time required for and expense of raising a battery of antisera. As with immunodiffusion, SDS-PAGE of lipopolysaccharide has the same advantage over the Kauffmann-White approach to serotyping in that prior knowledge of all the antigenic groups is not required (Kauffmann, 1966). Additionally, it has the advantage of always giving a positive result as it does not produce false negative results due to lipopolysaccharide autoaggregation, a problem inherent to immunodiffusion. If lipopolysaccharide is present, a pattern is obtained. Further, in terms of lipopolysaccharide SDS-PAGE pattern, it simplifies the ever increasing complexity of De Boer's immunodiffusion system (De Boer et al, 1979)

as those strains found to type into more than one serogroup produce only one recognisable lipopolysaccharide pattern. All these advantages emphasise how potentially useful this method could be for monitoring levels of the blackleg organism in potato seed stocks. However, the system at its present state of development requires to be refined further to enable the rapid and routine analysis of lipopolysaccharide from many samples.

6. Relevance of Results to the Development of a Dipstick Test for the Detection of Blackleg

Lipopolysaccharide was identified (De Boer *et al*, 1985) as the major reactive antigen in De Boer's system for the serotyping of *E. carotovora* strains, and it was found that only four serogroups (I, XVIII, XX and XXII) were associated with potato pathogenic strains of *atroseptica* in temperate climates (De Boer and Sasser, 1986). This study however has shown that only three different lipopolysaccharide SDS-PAGE patterns are produced by lipopolysaccharide extracted from *atroseptica* strains belonging to these four serogroups (Figure 3.22). Further, only three serological groups were observed for *atroseptica* strains from reciprocal lipopolysaccharide immunoblots of the four potato pathogenic serogroups (Results 5.1). These three serological groups correlated with the three lipopolysaccharide SDS-PAGE patterns (I, XX and XXII). As a consequence of these two results, in terms of the lipopolysaccharide component only, serogroup XVIII can be discounted as a serogroup associated with potato pathogenic strains of *atroseptica*. Only serogroups I, XX and XXII were considered from these results to be associated with potato pathogenic strains of *atroseptica*. Serogroup XX was considered to include those *atroseptica* strains previously assigned to

serogroup XVIII. This does not mean that the atroseptica strains belonging to serogroups XVIII and XX are the same, rather they are detected by the same lipopolysaccharide antibody(s).

Clearly, it is conceivable from these results that a diagnostic kit could be constructed which would have the potential for detecting all potato pathogenic serogroups of E. carotovora subsp. atroseptica in Scotland. For ease of use in field testing tuber sap, a dipstick test would ideally be the most suitable (Dewey et al, 1989). Development of the dipstick could occur at a later stage in the laboratory. The dipstick would consist of a nitrocellulose strip to which two monoclonal antibodies were bound and together would be capable of detecting serogroups I, XX and XXII. One monoclonal antibody would be specific for those epitopes common to serogroup I and serogroup XXII (Results 5.2.1). The other monoclonal antibody would be specific for serogroup XVIII (Eca)/XX lipopolysaccharide (Results 5.2.2, Figure 4.5).

It is not known why these particular serogroups are pathogenic to potato. Possibly there is some involvement of the lipopolysaccharide molecule in the mechanism of pathogenicity of the blackleg organism. However, it would not be anticipated that the lipopolysaccharide molecules play a direct role in the pathogenic mechanism as it is known that it is the

Figure 4.5

Dipstick test for potato pathogenic erwinias in Scotland.

Abbreviations

E : enzyme
S : substrate
McAb : monoclonal antibody
McAb+E : enzyme conjugated monoclonal antibody

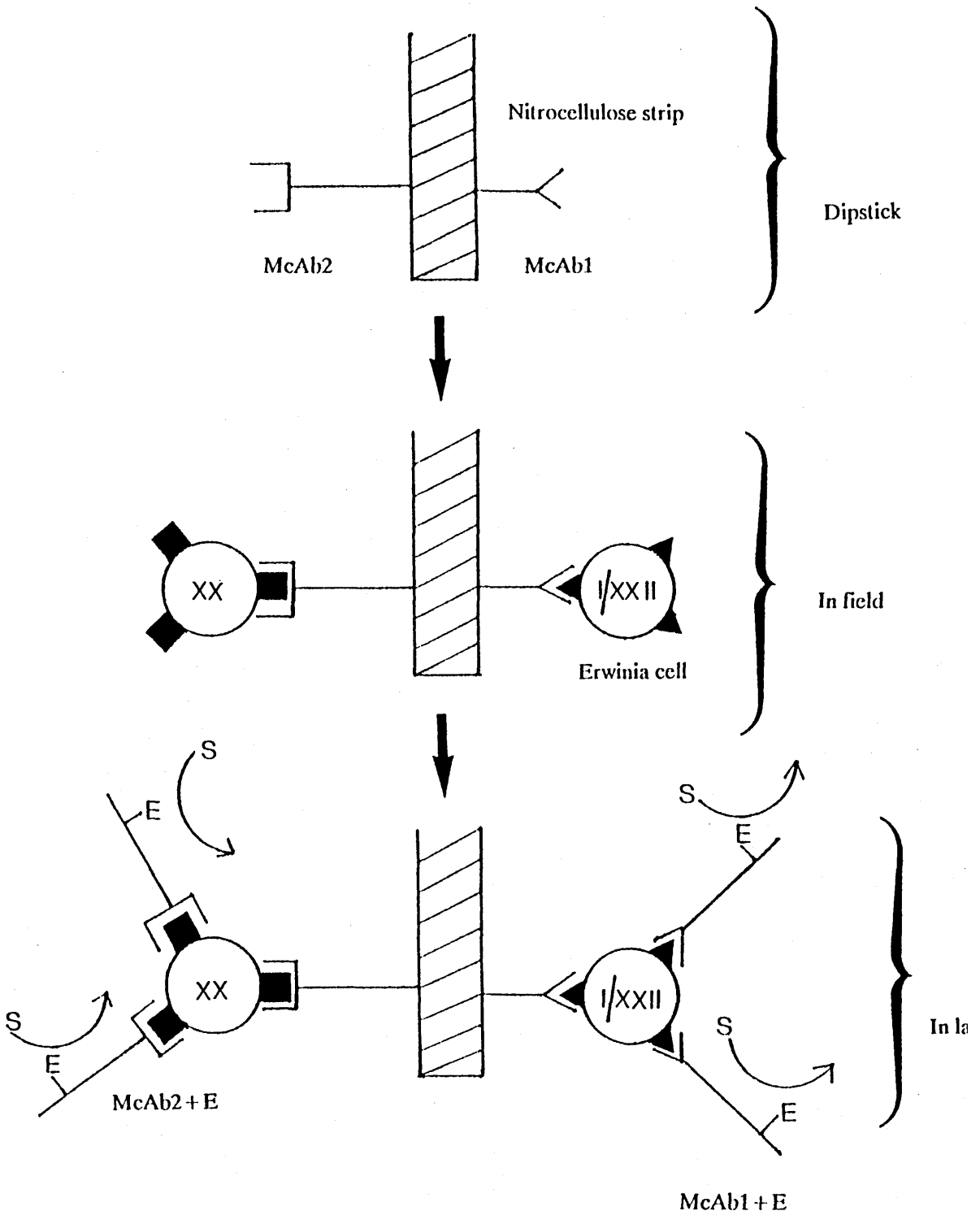
Nitrocellulose strip
McAb2
McAb1
Dipstick
XX
I/XX II
Erwinia cell
In field
S
E
XX
S
E
McAb2 + E
S
E
I/XXII
S
E
McAb1 + E
In la

Figure 4.5.

pectic enzymes of the blackleg organism that are responsible for maceration of the plant tissue leading to tuber rot and plant death (Basham and Bateman, 1975). It is possible that the lipopolysaccharides of these particular pathogenic serogroups are involved in the attachment of the blackleg organism to potato lectins thereby establishing the pathogen in the potato plant (Sequeira, 1978). Or, alternatively that lipopolysaccharide molecules may lack those structures recognised by the potato lectins and so the bacteria thereby evade a possible plant defence mechanism and are then able to move freely through the plant causing their effects. Drigues et al (1985) postulated a similar situation whereby extracellular polysaccharide from virulent strains of Ps. solanacearum preferentially bound the plant lectins instead of the lectins binding to the lipopolysaccharide, preventing the bacteria from being immobilised.

7. The Relationship of Lipopolysaccharide Sugar Composition and Serogroup

Studies of sugar compositions of lipopolysaccharide O-chain from Enterobacteriaceae have shown that the large number of serogroups determined for organisms such as E. coli and Salmonella are based on only a few sugars. Galactose, glucose, mannose, rhamnose and fucose were the commonly found neutral sugars (Orskov et al, 1977; Luderitz et al, 1971). The diversity of O-chain structures was produced by different arrangements of the sugars, different configurations, linkages and substitutions (see Introduction 4.2.3.1.3). The production of alditol acetates and their subsequent analysis by GLC is a widely used method for identifying the constituent neutral sugars of the lipopolysaccharide O-chain, irrespective of the organism (Kropinski et al, 1982; Bradshaw-Rouse et al, 1988; Romanowska et al, 1988). Using this approach, De Boer et al (1985) analysed lipopolysaccharide from sixteen strains of E. carotovora belonging to six serogroups to determine their sugar composition. The different serogroups were found to be composed of various combinations of galactose, glucose, mannose, rhamnose and fucose. There was no apparent correlation between the sugar composition of any strain's lipopolysaccharide and its serogroup.

In this study of the sugar composition of lipopolysaccharide from E. carotovora serogroups I, XVIII, XX, XXII and XXXV, no correlation was observed between serogroup and lipopolysaccharide sugar composition as analysed by GLC and GLC-MS (Results 6.2.3, 6.2.4).Serogroup I lipopolysaccharide and serogroup XVIII (Ecc) lipopolysaccharide possessed the same sugar composition as did lipopolysaccharide from serogroups XX and XXXV (Tables 3.7 and 3.10). However, the sugar compositions of lipopolysaccharides of the three potato pathogenic serogroups (I, XX and XXII) were different, correlating with their different lipopolysaccharide SDS-PAGE patterns (Figure 3.22) and their different immunogenicities (Results 5.1).

SDS-PAGE electrophoresis detected difference in the structure of lipopolysaccharide from serogroup XVIII subsp. atroseptica and subsp. carotovora strains and this was reflected in the difference in sugar composition found for their respective lipopolysaccharides (Results 6.24, Table 3.10).

Serogroup I lipopolysaccharide and serogroup XXII lipopolysaccharide were shown to have epitopes in common (Results 5.1, Table 3.2) and additionally, serogroup I lipopolysaccharide has one or more epitopes not common to serogroup XXII lipopolysaccharide (Results 5.2.1, Table 3.3). It is therefore possible that these epitopes unique to serogroup I lipopolysaccharide

contain mannose and galactose as these sugars were found to be present only in serogroup I lipopolysaccharide and not serogroup XXII lipopolysaccharide (Results 6.2.4, Table 3.10). However, one serogroup XXII strain was found to have a trace amount of galactose present in its lipopolysaccharide (Results 6.2.4, Table 3.10). This strain however, was the anomalous serogroup XXXV atroseptica strain which gave the standard serogroup XXII LPS-PAGE pattern and reacted with both serogroup XXII and serogroup I antisera (Results 4.3, 5.3). The slightly different sugar composition detected for this strain may account for its anomalous behaviour in electrophoresis and immunoblotting. To confirm the presence of mannose and galactose in those epitopes unique to serogroup I lipopolysaccharide an ELISA competition assay could be performed. Bound serogroup I lipopolysaccharide could compete with added mannose and galactose derivatives for serogroup I antiserum preabsorbed with serogroup XXII lipopolysaccharide. Conformationally, the galactose components of serogroup I lipopolysaccharide and lipopolysaccharide from the other potato pathogenic serogroups were not amenable to oxidation by galactose oxidase (Results 7.1). No evidence was found to show that E. carotovora lipopolysaccharide was amenable to oxidation by periodate either (Results 7.2). Problems were encountered with the lipopolysaccharide precipitating

out of solution. Using the same method of periodate oxidation (Dixon and Lipkin, 1954), Ray et al (1986) oxidised the polysaccharide side-chain liberated from the mild acid hydrolysis of E. amylovora lipopolysaccharide. It is therefore possible that this method of oxidation is not suitable for whole lipopolysaccharide.

In this study, a total of sixteen serogroup I lipopolysaccharide preparations were analysed by either GLC or combined GLC-MS (Results 6.2.3). In all the lipopolysaccharide preparations a 6-deoxyhexose sugar was detected. By the chromatographic techniques of TLC, GLC and GLC-MS (Results 6.2.3.1, 6.2.3.1.1) the 6-deoxyhexose was identified as rhamnose. De Boer's type strain for serogroup I was one of the sixteen serogroup I strains analysed. This finding is in contrast with the sugar composition reported for the lipopolysaccharide from this strain by De Boer et al (1985). In De Boer's study the sugar composition of only three serogroup I lipopolysaccharides were examined of which one of the three strains was De Boer's type strain for serogroup I. A 6-deoxyhexose was not identified in the lipopolysaccharide from any of the three strains. Whatever the reason for the disparity between the reported sugar compositions of De Boer et al (1985) and those found in this study, the same neutral sugars were found to compose the different serogroups of

E. carotovora (i.e. rhamnose, fucose, mannose, galactose and glucose). The same neutral sugars were also found to be present in the lipopolysaccharides of other species of Erwinia: E. amylovora (Ray et al, 1986), E. chrysanthemi (Bradshaw-Rouse et al, 1988) and E. stewartii (Bradshaw-Rouse et al, 1981). However, SDS-PAGE analysis of lipopolysaccharide from the different species of Erwinia reveals them to possess different structures (Figure 3.17). As the core region of the lipopolysaccharide is relatively conserved within a genus, the differences in structure must be as a consequence of the O-chain. Further, as they possess virtually the same neutral sugars, the differences in lipopolysaccharide structure must be produced by different arrangements of these sugars in the repeating unit e.g. the presence or absence of branches, substituents on the sugar residues e.g. acetyl groups, and differences in the nature of the linkage between the repeating units (Luderitz et al, 1971).

The unidentified peaks in the chromatograms of E. carotovora lipopolysaccharide were also observed by De Boer et al (1985) for E. carotovora lipopolysaccharide and by Bradshaw-Rouse et al (1988) for E. chrysanthemi lipopolysaccharide. Combined GLC-MS was helpful in determining whether or not these peaks were produced by sugars or were artefacts (Results 6.2.5). The GLC-MS fragmentation patterns of these peaks suggested that

they were sugars as they were found to possess common molecular ions of 115 a.m.u. and 170 a.m.u. which were determined to be characteristic of sugars under the conditions used in this system (Results 6.2.3.1, Figure 3.30). The sugars could not be identified by comparison of their fragmentation patterns to those expected for the alditol acetates of various standard sugars (Figure 3.39). Chemical ionisation of the sample in GLC-MS would have enabled accurate molecular weight determinations of these sugars to be made. The sugars may then have been identified by reference to known carbohydrate structures possibly by access to an appropriate data base library of carbohydrate structures.

The hydrolysis conditions employed in the derivatisation method for the production of alditol acetates of lipopolysaccharide were checked for possible incomplete hydrolysis of lipid A or degradation of KDO as possible sources of unknown carbohydrate components. The fractionation of the polysaccharide fraction from hydrolysed lipopolysaccharide on Sephadex-G50 (Results 6.1) gave the typical fragmentation pattern produced by other enteric organisms (Muller-Seitz et al, 1968). However, the pattern was slightly more complex in region II which is considered to be equivalent to the core region (Ray et al, 1986, see Figure 3.28). KDO, a very acid labile, eight-carbon sugar was subjected to the

derivatisation procedure for the production of alditol acetates but was not detected by GLC-MS (Results 6.2.5). Possible contaminating sugars from the enterobacterial common antigen (ECA) in lipopolysaccharide preparations were also discounted. Phenol/water preparations of lipopolysaccharide are known to be contaminated with the ECA (Mannel and Mayer, 1978) but Bradshaw-Rouse et al (1988) observed these unidentified sugar peaks in the GLC profiles of lipopolysaccharide from E. chrysanthemi, the only member of the Enterobacteriaceae known to lack the ECA (Kuhn et al, 1988).

Uronic acid is not a commonly found constituent of lipopolysaccharide but tends to be present in preparations as a result of contaminating capsular and extracellular polysaccharide. Most strains of E. carotovora possess capsules or microcapsules (Slade and Tiffin, 1984). There are a few examples where the uronic acid is covalently attached to lipopolysaccharide (Ashwell and Hickman, 1971). More recently, Ray et al (1986) found uronic acid to be associated with the core region of E. amylovora lipopolysaccharide, and uronic acid has also been found associated with the core region of rhizobial lipopolysaccharide (Carlson et al, 1978). E. carotovora lipopolysaccharide preparations were found to contain about 3% (w/w) uronic acid (Results 6.2.5). This is approximately one third of the content of uronic acid found in the lipopolysaccharide of E. amylovora

(Ray et al, 1986). By analogy, it is possible that the uronic acid of E. carotovora is core-associated but it could be due to contaminating capsular material. The lipopolysaccharide of E. carotovora was not subjected to any purification procedure for the removal of contaminating sugar as carried out for E. amylovora lipopolysaccharide.

Quantitatively, problems were encountered in determining the exact molar ratios of the sugar alditol acetates analysed by GLC (Results 6.2.3.2.1). In their respective studies of the alditol acetates of E. carotovora and E. chrysanthemi lipopolysaccharides, De Boer and McNaughton (1986) and Bradshaw-Rouse et al (1988), did not mention encountering problems with the quantitation of the sugar molar ratios. In this study, it was found that there was variability between injections of the same sample and this variability was more apparent with the most volatile sugars (Results 6.2.3.2.1.2). Although alditol acetate preparations from lipopolysaccharide were found to be quite stable in the injection solvent for twenty four hours, there was loss of the most volatile 6-deoxyhexose sugar (Results 6.2.3.2.1.3). It may have been an idea to try different solvents as a better solvent may exist (Holme and Peck, 1983). Incomplete removal of the borate after reduction which would inhibit the subsequent acetylation of the alditols (Burke et al, 1987) is another possibility to

account for the quantitative inconsistencies. This latter suggestion seems unlikely as six methanol evaporations were routinely performed for the removal of excess borate while Albersheim et al (1967) reported that five methanol evaporations was the optimum number to remove borate completely.

Consequently, conclusions could not be made as to the molar ratios of certain sugars within a serogroup (Table 3.7) and between serogroups (Table 3.10), or whether or not the amounts of the sugars were influenced by the growth media (Table 3.9). Even more importantly, the molar ratios and sugar compositions of alditol acetates should be interpreted with caution as the derivatisation procedure produces two different sugar alditol acetates from ketoses (Results 6.2.3.2.1.1). Stachyose contains the ketose, fructose. Derivatisation of two moles of fructose yields one mole of mannitol hexaacetate and one mole of glucitol hexaacetate. To ensure the correct identity of the sugars analysed by GLC of the alditol acetates produced, confirmatory analyses should be performed. TLC of hydrolysed E. carotovora lipopolysaccharide (Results 6.2.3.1.1) identified all the sugars detected by GLC and GLC-MS. Ketose sugars did not appear to be present in the lipopolysaccharide of E. carotovora. Interestingly, a fast migrating spot was observed on TLC plates of acid hydrolysates of E. carotovora lipopolysaccharides.

Unknown fast-moving sugars were similarly observed in the acid hydrolysates of lipopolysaccharide isolated from gram-negative bacteria by Westphal in 1952 (see review by Ashwell and Hickman, 1971). These sugars were later identified as the rare 3,6-dideoxyhexose sugars.

High performance liquid chromatography may provide a suitable alternative method for the quantitative analysis of alditol acetates. HPLC possesses the favourable features of high resolution capability and does not require the derivatisation of the sugars avoiding some of the problems which may occur in producing alditol acetates.

CONCLUSIONS

CONCLUSIONS

This study illustrates that the electrophoretic behaviour of E. carotovora lipopolysaccharide is comparable to other well characterised enteric bacteria. It has extended De Boer's initial study (1979) of the lipopolysaccharides of E. carotovora from 16 strains belonging to 6 serogroups, to 59 strains from 12 serogroups. A correlation has been established between serogroup and lipopolysaccharide structure using the technique of SDS-PAGE. Using this system, 3 and not 4 important potato pathogenic serogroups of E. carotovora subsp. atroseptica were identified. This result was verified immunologically and correlated with sugar analyses. Further, suitable immunogens were identified for the raising of monoclonal antibodies which could be employed in a diagnostic assay which would have the potential for detecting all potato pathogenic strains of E. carotovora in Scotland.

FUTURE WORK

As with E. coli and Salmonella, the most studied models of enteric bacterial lipopolysaccharides, it follows that future work must entail more detailed structural analyses of the lipopolysaccharide component. Creation of lipopolysaccharide core mutants would facilitate the elucidation of the core region (Luderitz et al, 1982) and NMR spectroscopy combined with GLC-MS and optical rotation data (Ray et al, 1987) would determine the structure of the O-chain repeating unit. In turn, such analyses may reveal those sugar structures responsible for the different serotypes of E. carotovora. Those sugars which are important to the serogrouping system of E. carotovora could be determined by lectin competition assays. Lectin binding assays may also determine whether or not there was serogroup specificity between lectins from the potato plant and lipopolysaccharide from the potato pathogenic serogroups. Wolpert and Albersheim (1976) found high species specificity between Rhizobium lipopolysaccharide and host legume.

As to the increasing complexity and confusion of de Boer's system of immunodiffusion, the involvement of other Gram negative surface membrane structures with the lipopolysaccharide moiety should be explored. The outer membrane proteins as they are also highly antigenic

(Geyer *et al*, 1979) would be a particularly important area of study. Results presented in this study suggest that they may be involved in the serogrouping of *Erwinia carotovora*.

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