A RECOMBINANT DNA APPROACH TO THE DESIGN AND SYNTHESIS OF NOVEL POLYSACCHARIDES

by

DONALD DAVIDSON EASSON JR.

B.S., Chemical Engineering
University of South Carolina
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Submitted to the Department of Applied Biological Sciences
in Partial Fulfillment of the Requirements for the Degree of
DOCTOR OF SCIENCE
in Biochemical Engineering at the
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

August 1987

Signature of Author_____
Department of Applied Biological Sciences
August 31, 1987

Certified by_____
Anthony J. Sinskey
Professor of Applied Microbiology
Thesis Supervisor

Certified by__
Charles L. Cooney
Professor of Chemical and Biochemical Engineering
Committee Chairman

Accepted by_____
Professor Steven R. Tannenbaum
Chairman, Committee on Graduate Students

This doctoral thesis has been examined by a Committee of the Department of Applied Biological Sciences as follows:

Professor Charles L. Cooney — Chairman
Department of Applied Biological Sciences
Department of Chemical Engineering

Professor Anthony J. Sinskey — Thesis Supervisor
Department of Applied Biological Sciences

Professor ChoKyun Rha
Department of Applied Biological Sciences

Professor Graham C. Walker
Department of Biology

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ABSTRACT

The genetics of biosynthesis of exopolysaccharides produced by strains of Zoogloea ramigera was investigated for this thesis project. The objective of this work was to develop a system in which polysaccharide structure can be controlled through genetic manipulation of the producing organism. As a result, a system does now exist that will allow for the genetic control of exopolysaccharide (EPS) structure in Z. ramigera I-16-M and 115.

In Z. ramigera I-16-M, Tn5 insertion mutants deficient in EPS production were isolated by screening for the absence of fluorescence on plates containing the dye Cellufluor. Complementation of these mutations was achieved with a Z. ramigera I-16-M gene library constructed in a broad host range cosmid vector and introduced into the I-16-M mutants by conjugation. Four recombinant plasmids able to restore EPS production to all of these mutants were found to contain at least 14 kb of common insert DNA. Subcloning of the common region and restriction mapping the locations of Tn5 insertions has identified two complementation groups contained within a chromosomal segment of DNA that is bewteen 4.6 and 6.5 kb in size. Also, it has been clearly demonstrated that genetic instability in this region leads to spontaneous deletions and possibly rearrangements resulting in loss of EPS production.

Due to the capsular nature of the 115 EPS conjugation in this strain was not successful. To enable genetic manipulation in this species, a capsule nonforming derivative was constructed and designated Z. ramigera 115SL. This strain excretes its EPS as a slime layer which does not remain cell bound and dissolves away from the cells. Conjugation in 115SL was successful and was used to isolate EPS Tn5 mutants and to complement those mutants with a 115 gene library constructed in a broad host range cloning vector. Analysis of the mutants and complementing plasmids has resulted in the identification of two unlinked complementation groups involved in EPS biosynthesis.

A segment of Z. ramigera 115 DNA was identified that has sequence homology to the X. campestris pyruvyl transferase gene. This segment, cloned

on pEX0.9, was confirmed to have significant homology to the X. campestris gene and is presumed to contain the Z. ramigera EPS pyruvyl transferase gene.

Characterization of the EPS isolated from Z. ramigera 115 and 115SL demonstrated that it contains the monosaccharides glucose and galactose in an approximate molar ratio of 2:1, respectively. Also, the presence of pyruvyl substituents was confirmed, which are found in a higher proportion on the 115SL EPS. A simplified, less degradative EPS purification procedure for 115SL resulted in the identification of O-acetyl groups on the polysaccharide. These alkali labile substituents are presumed also to be present on the 115 EPS but have not previously been detected due to the use of an alkali treatment in its purification. Rheological studies show that the 115 EPS has pseudoplastic and viscoelastic properties.

Thesis Supervisor: Dr. Anthony J. Sinskey
Title: Professor of Applied Microbiology

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ABBREVIATIONS

Media and Antibiotics

LB - Luria-Bertani Media
TSB - Trypticase Soy Broth
Km - Kanamycin
Ap - Ampicillin
Nm - Neomycin
Cb - Carbenicillin
Sm - Streptomycin
Tc - Tetracycline
Cm - Chloramphenicol
Rf - Rifampicin

Chemicals, Mutagens and Enzymes

CF - Cellufluor
NTG - N-Methyl-N'-Nitro-N-Nitrosoguanidine
TMS - Tetramethylsilane
SDS - Sodium Dodecylsulfate
TFAA - Trifluoroacetic Acid
CIP - Calf Intestinal Alkaline Phosphatase
PEG - Polyethylene Glycol

Genetic Designations

EPS - Exopolysaccharide
Eps^- - EPS Deficient
Cel^- - Nonfluorescent on Cellufluor
Flc^- - Nonflocculating
exo - EPS Locus
kb - Kilobases of DNA

1. INTRODUCTION

This thesis report summarizes research studies on the extracellular polysaccharide (EPS) of Zoogloea ramigera, its production and the genes coding for its production.

- o The primary objective of this investigation is to develop a system in which polysaccharide structure and function can be controlled through genetic manipulation of the producing organism.

Biopolymers have found applications in many industries, including the food, cosmetic, chemical, biomedical, waste treatment and oil industries (Table 1). However, the potential for the development of new biopolymers with unique properties is still enormous. Biotechnology can help develop this potential and substantially increase the applicability and usage of biologically synthesized polymers. The availability of recombinant and classical DNA techniques to manipulate the genetics of an organism allows the development of strategies to alter the polymer structure, in vivo, and hence its function. The application of these technologies to the production of microbial polysaccharides will lead to the structural manipulation at the genetic level and the development of unique, well-defined polymers for specific functional applications. This research project is a study on a bacterial exopolysaccharide, produced by the organism Zoogloea ramigera, which has interesting rheological behavior and metal adsorption properties.

This report summarizes research in which the original objective has been realized and that has led to the development of a system that will be used to genetically engineer novel polysaccharides. In applying this system to Z. ramigera I-16-M and 115, EPS biosynthetic genes have been cloned and analyzed.

Table 1

Some Commercial and Industrial Uses of Hydrocolloids

Functions	Applications
Adhesive or Binding	Paper, pulp and textile products, construction uses.
Bulking or Carrier	Drugs, germicides, fungicides, insecticides, fertilizers.
Chelating	Metal recoveries, reaction controls, pollution controls, water purifications.
Coagulant	Protein precipitations or recoveries, industrial and food processing, waste treatment, clarification of beverages.
Dispersant, stabilizer or surface active agents	Cosmetic and personal care products, drugs, photographic products, paints, pigments, inks, food products.
Finishing	Textile, paper, and pulp products, surgical adjuncts.
Gellant	Food products, oil-field applications, cleaning agents, personal care products, cosmetics, drugs, lubricants.
Matrix	Separation or chromatographics, structure matrix for food or sustained release, immobilization of enzymes or cells, biomedical or surgical materials.
Membrane or Films	Separations or filtrations, edible films or packages.

2. LITERATURE SURVEY

2.1 Bacterial Exopolysaccharides

Biologically synthesized polymers, especially polysaccharides, have many commercial applications as was shown in Table 1. These products are derived from plants, bacteria, fungi and higher eukaryotes. Unique viscosifying, emulsion stabilizing and surface tension modifying properties make polysaccharides attractive for a wide variety of uses. Other advantages of biopolysaccharides include the fact that they have low toxicity, are environmentally safe, are naturally derived and are viable alternatives to petroleum based polymers. Bacterial polysaccharides represent a small fraction of the current biopolymer market but have one of the largest potentials for development of novel and improved products with the successful application of new genetic engineering technologies. Some bacterial polysaccharides that already have commercial significance include xanthan, alginate and dextrans.

2.1.1 Xanthan Gum

Xanthan gum is produced commercially by Xanthomonas campestris. Xanthan gum is a polyanionic exopolysaccharide composed of D-glucose, D-mannose and D-glucuronic acid in a molar ratio of 2:2:1, respectively (Jansson et al., 1975). The repeating unit is a pentasaccharide (Figure 1) consisting of a β(1→4) linked glucose backbone with branches occurring every other unit consisting of a mannose-β-1,4-glucuronic acid-β-1,2-mannose-α-1,3 linked trisaccharide. Pyruvic acid is ketally linked to the external mannose and the internal mannose is O-acetylated (Sutherland and Ellwood, 1979). These two moieties occur at varying proportions.

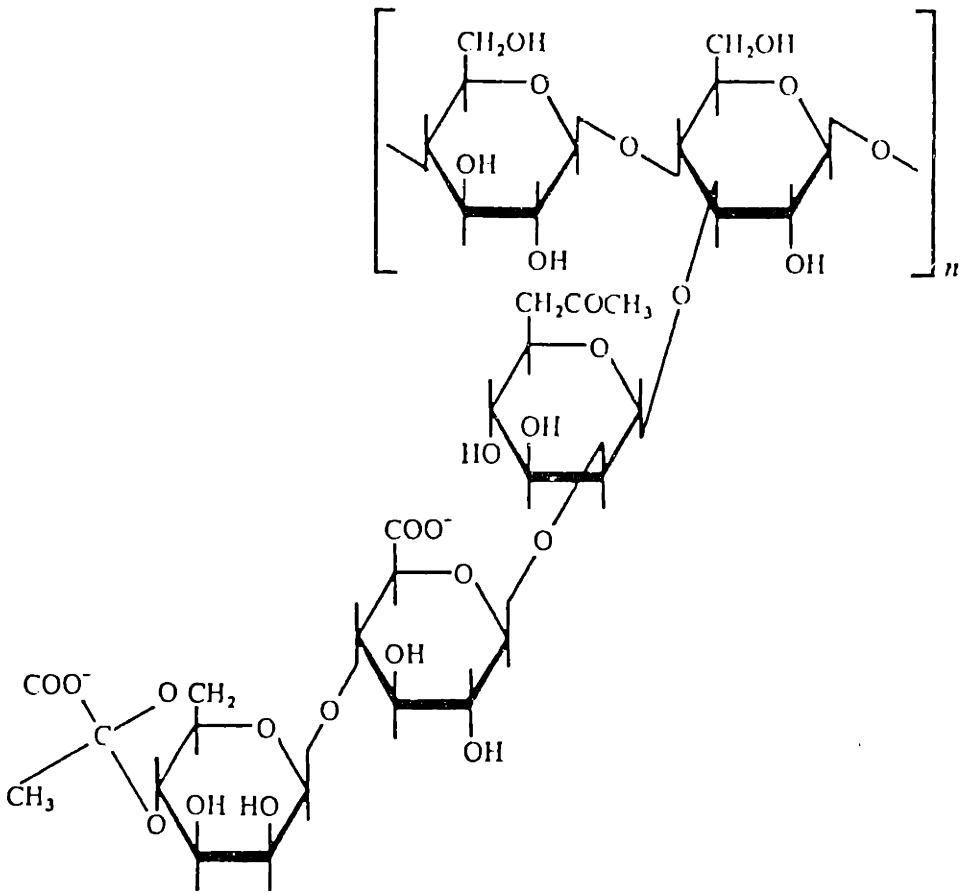


Figure 1. Structure of xanthan (Jansson et al., 1975).

Biosynthesis of xanthan begins when glucose is transferred from UDP-glucose to an isoprenoid lipid carrier in the membrane, followed by the addition of a second glucose from UDP-glucose, a mannose from GDP-mannose, a glucuronic acid from UDP-glucuronic acid and mannose from GDP-mannose (Ielpi et al., 1981a). This five sugar repeat unit is then acetylated on the internal mannose by an acetal transferase using acetyl coenzyme A as the donor (Ielpi et al., 1983). Next it is pyruvylated on the terminal mannose by a pyruvyl transferase from phosphoenolpyruvate (Ielpi et al., 1981b). Last it is polymerized, the lipid carrier is recycled and the growing polysaccharide is extruded from the cell. The pathway for xanthan biosynthesis is shown in Figure 2.

Reported titers of xanthan in batch culture generally exceed 25 g/liter with yields on carbon source ranging from 56% to 70% (Souw and Demain, 1979; Evans et al., 1979; Cadmus et al., 1978). Xanthan gum has found many commercial applications as film-forming agents, thickeners, emulsifiers, stabilizers and enhanced oil recovery agents (Lawson and Sutherland, 1978). The U.S. production of xanthan in 1983 was 11 million lb at a total value of $51 million. Despite its commercial success xanthan biosynthesis and its regulation, at the molecular level, are not well understood.

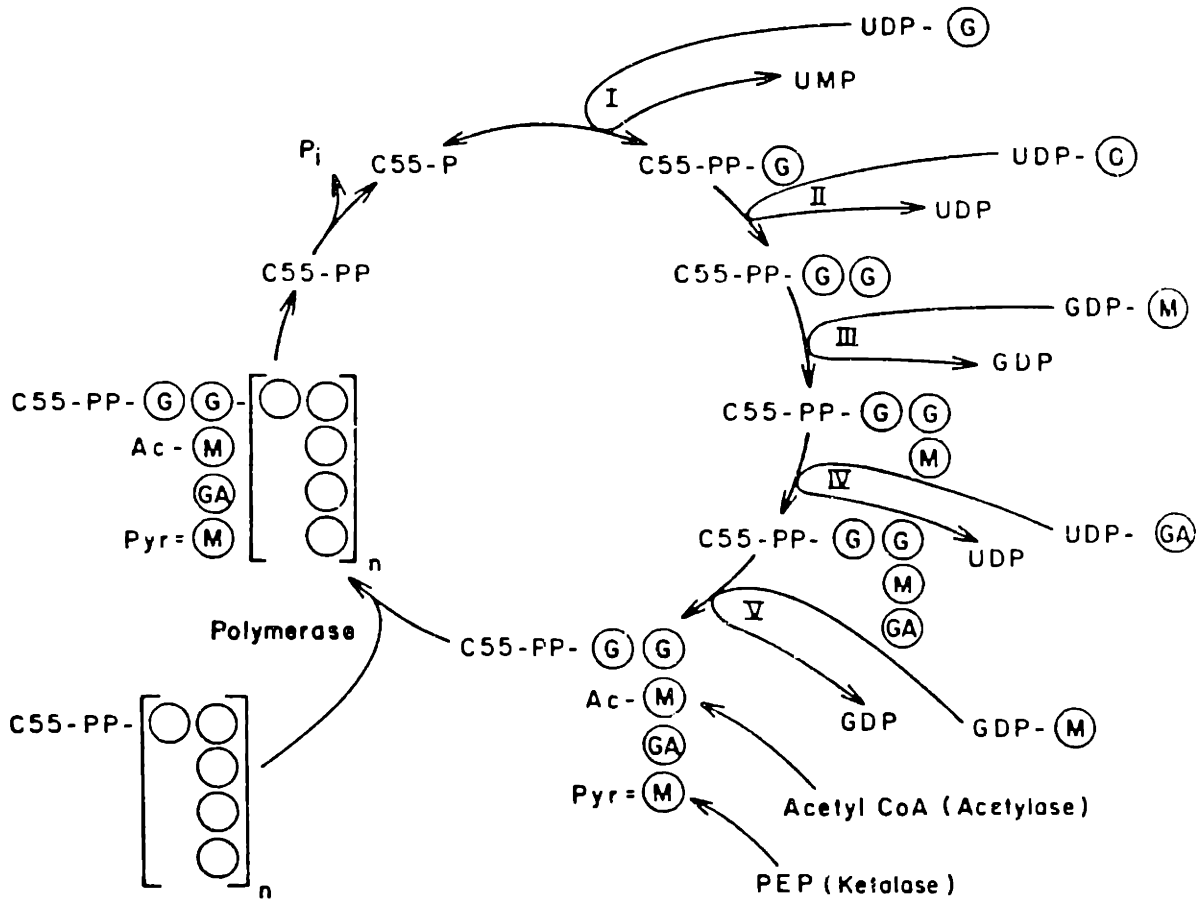


Figure 2. Pathway for xanthan biosynthesis. UDPG = uridine-5'-diphospho-glucose; GDPM = guanidine-5'-diphosphomannose; UDP-GA = uridine-5'-diphosphoglucuronic acid; GG = cellobiose; C55 = isoprenoid lipid carrier; PEP = phospho(enol)pyruvate; Acetyl CoA = acetyl coenzyme A; I-V = glycosyltransferases I-IV; Ac = acetate; and Pyr = pyruvate (Ielpi et al., 1981a,b; Ielpi et al., 1983; Troy et al., 1971; Betlach et al., in press).

2.1.2 Alginate

Alginates are heteropolysaccharides containing D-mannuronic acid and L-guluronic acid linked β(1→4). They are produced by brown seaweeds and several bacterium including Azotobacter vinelandii and Pseudomonus aeruginosa. The bacterial alginate differs from the algal alginate in that a fraction of the D-mannuronic acid residues are O-acetylated (Figure 3) (Davidson et al., 1977).

The proposed pathway for the synthesis of alginate in Azotobacter vinelandii (Pindar and Bucke, 1975) is shown in Figure 4. It has been proposed that this same pathway exists in P. aeruginosa in which phosphomannose isomerase, phosphomannomutase, GDP-mannose pyrophosphorylase and GDP-mannose dehydroenase activities have been detected (Deretic et al., 1987).

In batch culture of A. vinelandii, alginate production has been reported to be 5 to 7 g per g dry cell weight corresponding to a yield on carbon of approximately 25% (Deavin et al., 1977). Commercially, alginates are used extensively for their water holding, gelling, stabilizing and emulsifying properties (Kelco). Currently, large scale production is more economical for the algal alginate, however, production of microbial alginate would have the advantages of constant composition, constant yields and less pollution (Sutherland and Ellwood, 1979) and could become feasible if the process is optimized and the above advantages result in a more desirable product.

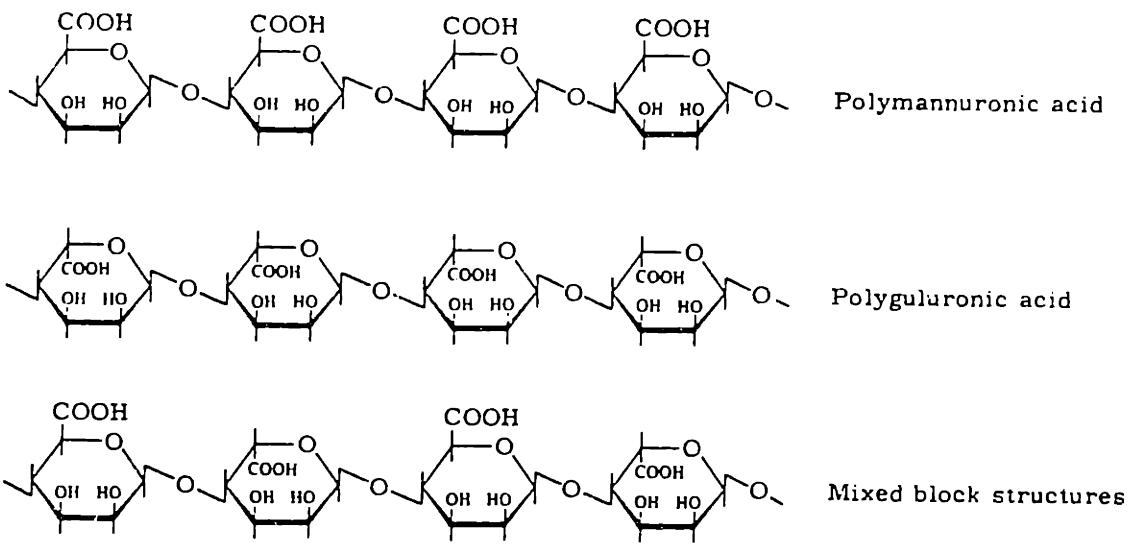


Figure 3. The three sequences of uronic acids which may be found in bacterial alginate (Sutherland, 1983).

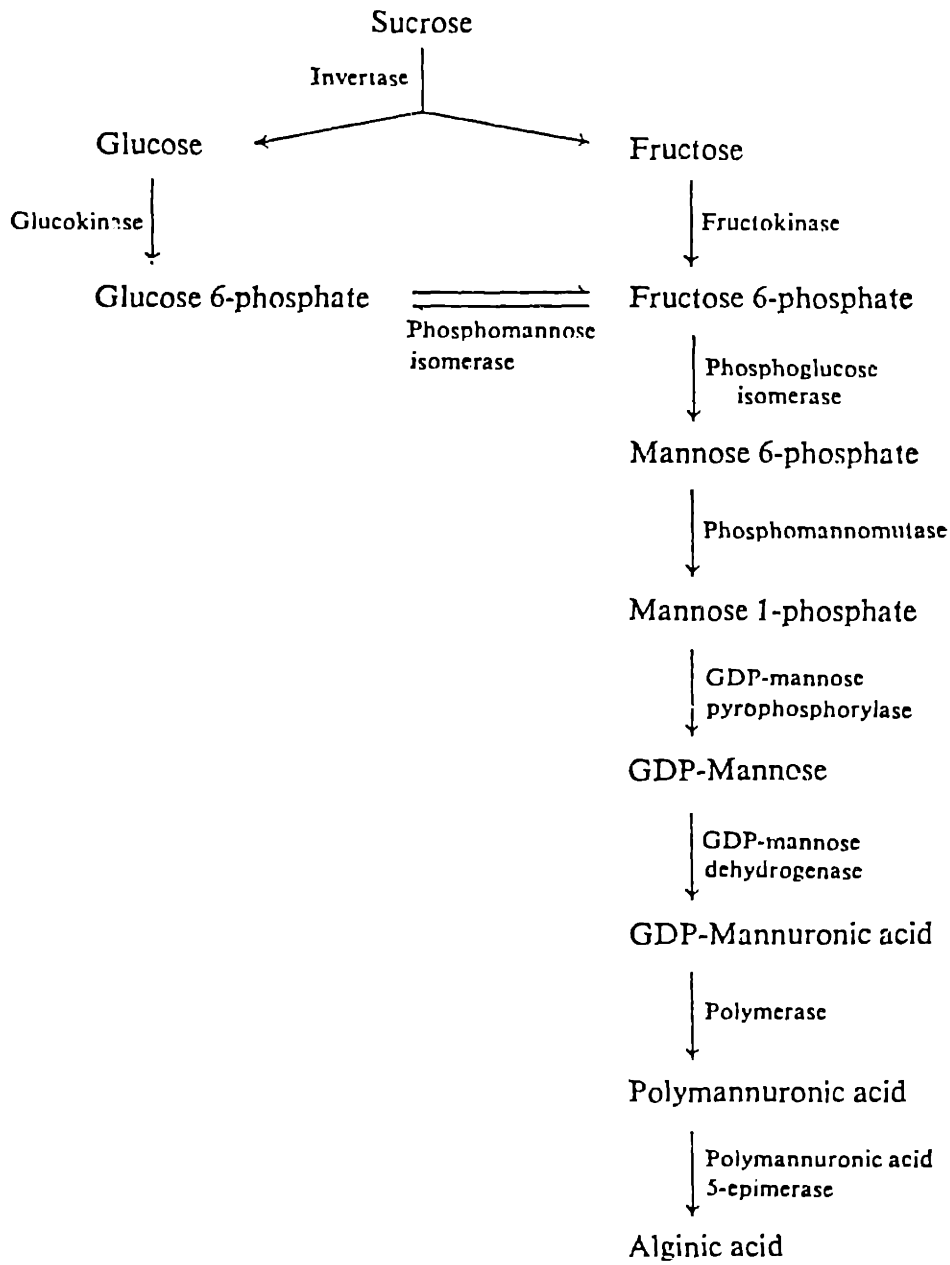


Figure 4. Biosynthesis of alginate in Azotobacter vinelandii (Pindar and Bucke, 1975).

2.1.3 Dextrans

Dextrans are glucose homopolysaccharides that have an β(1→6) linked main chain with α(1→3) linked branches (Figure 5). Dextrans are synthesized by Lactobacillus, Streptococcus, Leuconostoc and other bacterial species. Its biosynthesis is unique in that it does not require nucleotide bound precursors. Glucose for biosynthesis is provided by sucrose, which is extracellularly converted to dextran by the enzyme dextransucrase. Dextran and its derivatives are used in the pharmaceutical and chemical industries as plasma extenders, anticoagulants (sulfated dextrans) and adsorbents (DEAE dextran).

2.1.4 Curdlan

Curdlan is a linear β(1→3)-D-glucan produced by Alcaligenes faecalis. Yields of 50% on glucose have been reported (Harada, 1977). The commercial significance of curdlan is its ability to form a non-reversible gel upon heating of aqueous solutions. Proposed applications include use as a gelling agent, thickener or stabilizer (Sutherland, 1983).

2.1.5 Other Bacterial Exopolysaccharides

Many other bacterial species produce extracellular polysaccharides that are diverse in composition, structure and physical properties. Relatively few have been studied extensively, so there is still a great deal of potential that exists for the development of new commercial polysaccharides.

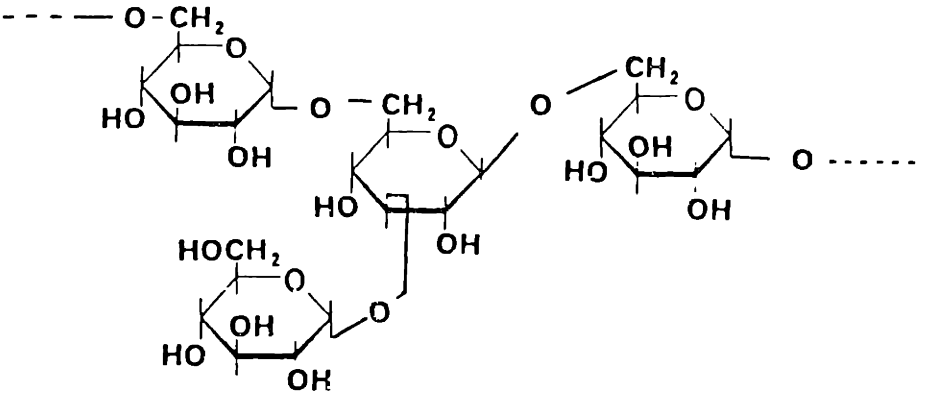


Figure 5. A portion of the structure of dextran (Sutherland, 1982).

2.2 Biosynthesis of Bacterial Exopolysaccharides

The synthesis of bacterial exopolysaccharides, with the exception of dextrans, levans and mutan, involves activated sugars in the form of nucleotide diphosphate intermediates. Monosaccharides are then transferred from their sugar nucleotide derivatives to the growing polysaccharide chain usually via isoprenoid lipid intermediates. A possible biosynthetic pathway for a hypothetical glucose and galactose polysaccharide from glucose is shown in Figure 6. Glucose is transported into the cell as glucose-6-phosphate, which is then converted to glucose-1-phosphate by phosphoglucomutase (Metzler, 1977). A sugar nucleotide diphosphoate is formed by a nucleotidyl transfer to the sugar phosphate, forming, in this case, UDP-glucose. At this stage, the activated sugar can be interconverted into a variety of sugar nucleotides. Reactions that accomplish these interconversions include epimerization, dehydration and decarboxylation. Almost without exception, the presence of a particular monosaccharide in a polysaccharide requires the existence of that particular monosaccharide nucleotide diphosphate as a precursor. In this example, UDP-glucose is epimerized to UDP-galactose. The reactions leading to polymerization of the sugar nucleotide are varied and not completely understood, however, there is strong evidence supporting the presence of lipid intermediates in some systems (Sutherland, 1982). Troy et al. (1971) proposed the transfer of sugar-1-phosphate to isoprenoid lipid phosphate, followed by the addition of sugars from sugar nucleotides to form the lipid bound oligosaccharide repeat units. Multiples of the repeat unit are then formed by transfer to the reducing end of the growing polysaccharide. A system such as this has been confirmed to carry out xanthan biosynthesis (Ielpi et al., 1981a).

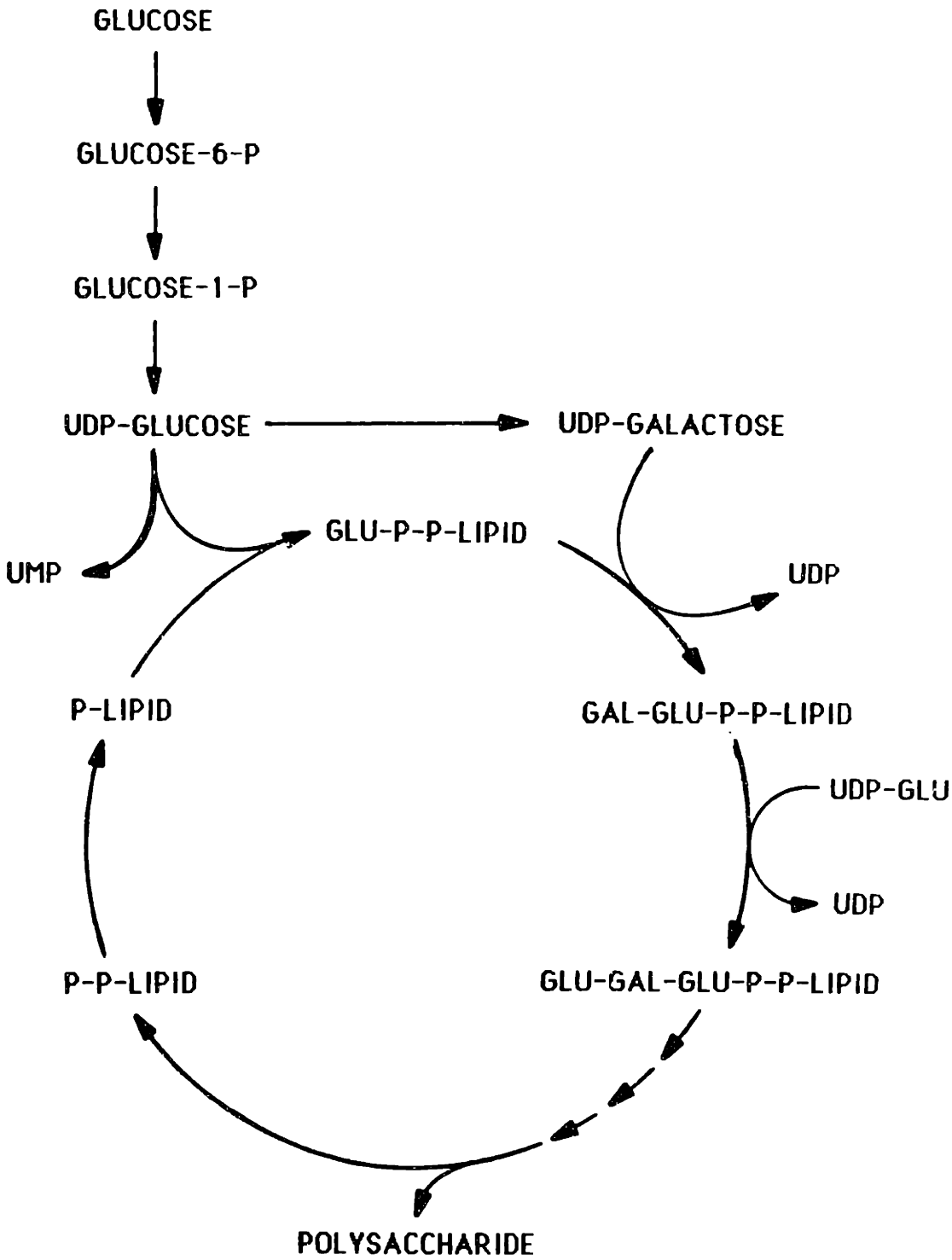


Figure 6. Possible biosynthetic pathway for a glucose and galactose exopolysaccharide.

Subsequently, the exopolysaccharide must be released so that the isoprenoid lipid can be returned to the pool, and then it must pass through the hydrophobic outer membrane. The probable mechanism involves the adhesion sites of Gram-negative bacteria, known as Bayer sites in which the outer and inner membranes are associated with each other (Sutherland, 1982). It is proposed that exopolysaccharides are exported through these channels (Bayer and Thurow, 1977) in a way that is analogous to the import of some substrates through porins (Smit and Nikaido, 1978).

It must be emphasized that the above scheme is a compilation of proposed pathways for several expolysaccharide producing organisms much of which remains to be proven. One variation on the scheme has been documented in the production of alginate by Azotobacter vinelandii in which there is no evidence supporting the involvement of lipid intermediates. In this pathway, GDP-mannuronic acid is polymerized to polymannuronic acid by alginic acid polymerase followed by epimerization to alginic acid (Pindar and Bucke, 1975).

2.3 Zoogloea ramigera

Zoogloea ramigera has been isolated from sewage and aerobic waste treatment facilities and is generally thought to be the major contributor to flocculation in activated sludge. Cell flocs of Z. ramigera also have the ability to accumulate heavy metal ions. The flocculation of the cells and the adsorption of heavy metal ions to the cell flocs is caused by the presence of extracellular polysaccharides. Z. ramigera is also known to accumulate poly-β-hydroxy butyric acid (PHB) granules intracellularly. Previously, Z. ramigera strains have been studied for their role in aerobic waste treatment, the adsorption of heavy metals, the production of PHB, and

the production of extracellular polysaccharides. In addition, purified polysaccharide has been characterized, to some extent, both chemically and physically.

Z. ramigera is a gram-negative, rod-shaped, floc-forming, obligate aerobe that is motile and does not form spores (Buchanan and Gibbons, 1974). They are distinguished from other gram-negative pseudomonads by the presence of an exocellular polysaccharide which causes flocculation and occurs, in some strains, as a zoogloeal or capsule-like matrix (Dugan, 1981). In nature, the polysaccharide is thought to function to concentrate nutrients around the cell flocs enabling them to grow in nutrient deficient environments (Dugan, 1975). Several isolates of Z. ramigera have been reported and, presently, three strains are contained in the ATCC (American Type Culture Collection). They are Z. ramigera strains I-16-M, 106 and 115. All three strains flocculate and all three strains produce different extracellular polysaccharides.

Isolate I-16-M produces a non-capsular exopolysaccharide that contains glucose, galactose and mannose (Dugan, 1981). Friedman and Dugan (1968) concluded that this strain is either a non-zoogloeal matrix-producing Zoogloea or a Pseudomonas species.

Isolate 106 produces an extracellular polysaccharide that contains amino sugars (Farrah and Unz, 1976).

Z. ramigera isolate 115 is a zoogloeal matrix forming strain which, when grown in a nitrogen limiting medium, converts 60% (w/w) of the available glucose substrate into exopolymer (Norberg and Enfors, 1982) where yields of more than 15 g/l have been reported in batch culture (Norberg and Enfors, 1982; Stauffer and Leeder, 1978). The polysaccharide is initially produced during logarithmic growth and continues after maximum

growth has been reached. It remains attached to the cells until late stationary phase when it is partially released (Norberg and Enfors, 1982). The accumulation of polysaccharide occurs after the available carbon source has been depleted and is concurrent with a decrease in the accumulated PHB (Figure 7) (Parsons and Dugan, 1971). No degradation of the polysaccharide was observed when the viscosity of a culture remained unchanged while holding at 26°C for 100 hours after the viscosity had reached its maximum (Norberg and Enfors, 1982).

The exopolysaccharide is formed when Z. ramigera 115 is grown on a wide variety of carbon sources including glucose, galactose, fructose, lactose, mannose, soluble starch, hydrolyzed whey and many others (Parsons and Dugan, 1971; Stauffer and Leeder, 1978). The carbon source used has no effect on the polysaccharide composition (Parsons and Dugan, 1971). The carbon/ nitrogen (C/N) ratio of the cutlure medium can partially regulate polysaccharide synthesis. A decrease in the C/N ratio below 38 has a negative effect on polysaccharide production (Norberg and Enfors, 1982). Under optimum conditions in fed-batch cultivation the polysaccharide accumulation reaches 38 g/liter (Norberg and Enfors, 1982).

The polymer/cell flocs are able to adsorb Cu^{++} up to 34% of their weight and 25% of their weight of Co^{++}. They also have a high affinity for Fe^{++}, Zn^{++}, Ni^{++}, Cd^{++} and U^{++} (Norberg and Persson, 1984; Friedman and Dugan, 1968). To utilize these metal binding properties, a reversible process for the recovery of metals from water, using Z. ramigera 115 biomass as an adsorbant was proposed by Norberg and Persson (1984).

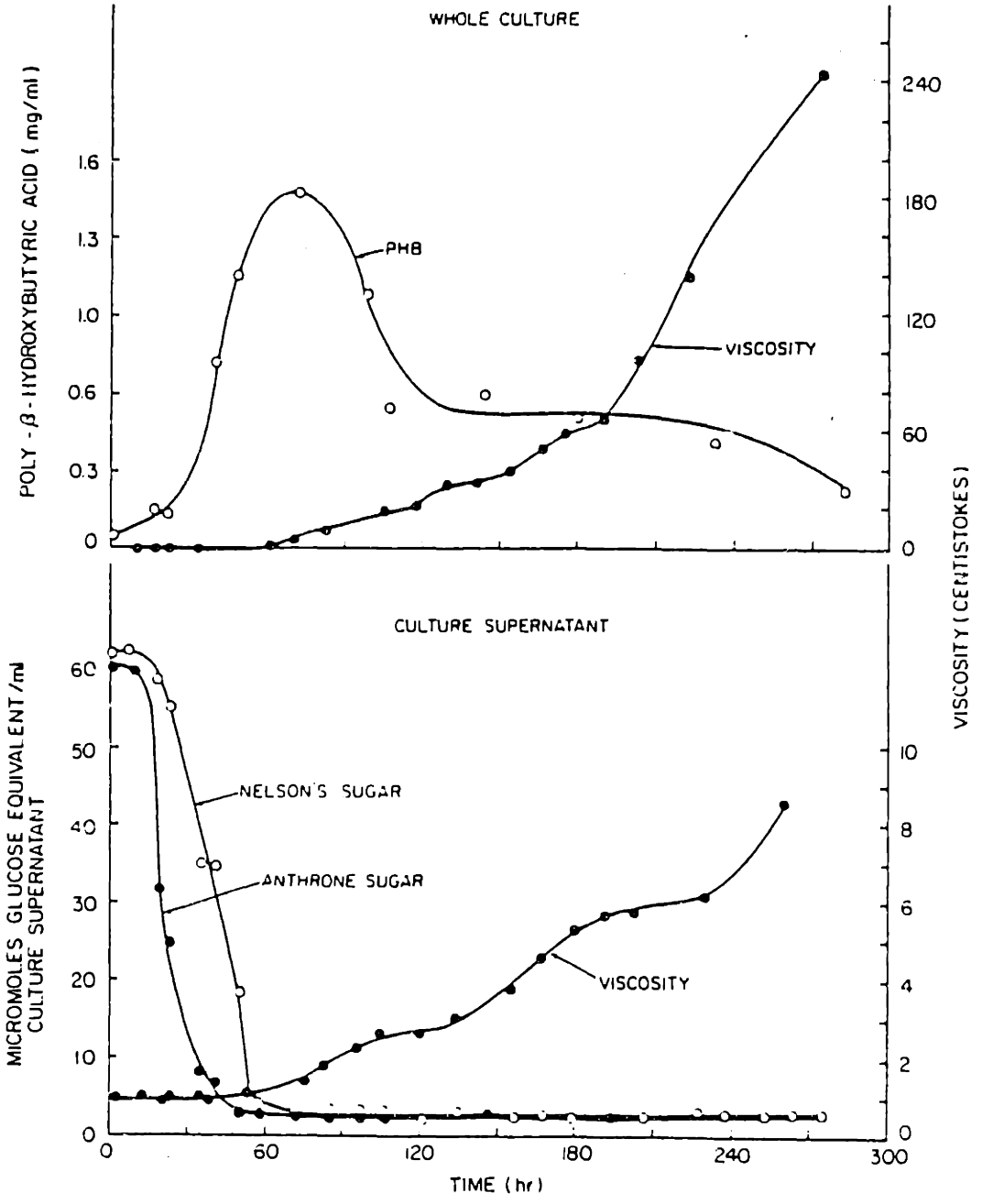
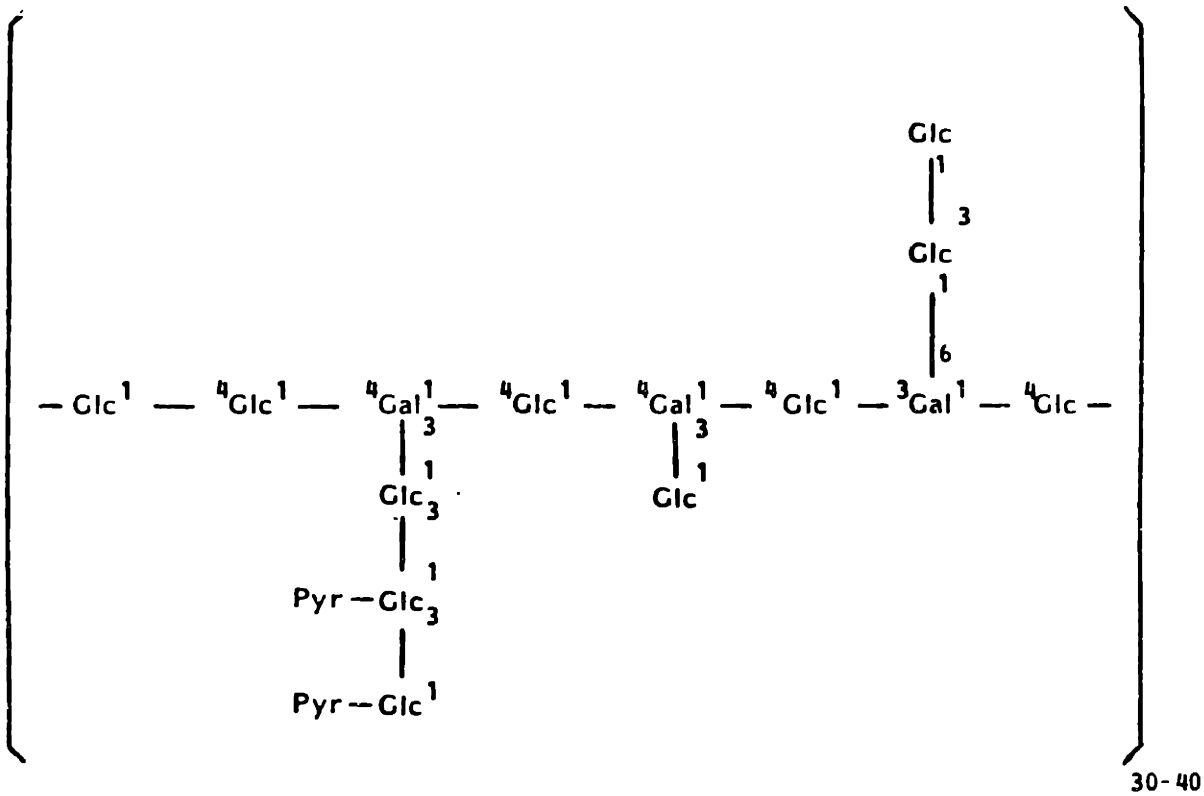


Figure 7. Curves showing viscosity of both whole culture and supernatant, sugar available in medium measured by both Nelson's and anthrone tests and PHB accumulation in Z. ramigera 115 culture vs. time. Growth in 10 L batch fermentor at 24°C ± 2°C (Dugan, 1975).

There have been two structural investigations of the biopolymer produced by Z. ramigera 115. Ikeda et al. (1982) reported that it is a highly branched heteropolysaccharide composed of glucose, galactose and pyruvate in a molar ratio of 11:3:1.5 with a molecular weight of approximately 10^5 (Figure 8). The proposed polysaccharide structure has a glucose and galactose main chain linked predominantly $\beta(1\rightarrow4)$. Branching occurs at the galactose units and consists of short $\beta(1\rightarrow4)$ linked glucose chains, some of which contain pyruvate moieties. The negatively charged carboxyl groups of the pyruvate are thought to be primarily responsible for the biopolymer's high affinity for heavy metal ions (Ikeda et al., 1982). A more recent investigation contradicts this data on several points. Franzen and Norberg (1984) found that the polysaccharide contains glucose and galactose in the ratio of approximately 2:1 and a pyruvic acid acetal accounting for 3.6% of the total polysaccharide. Although a complete structure was not proposed, several linkages were determined on the basis of methylation analysis. Some of these linkages are not in agreement with the data of Ikeda et al. (1982) and at least one α linkage was proposed. Franzen and Norberg (1984) postulated that the discrepancies in data were due to differences in the strain or growth conditions of the organism.



Glc: Gal:Pyr = 11:3:1.5

All linkages are β

M.W. = 10^5

Figure 8. Possible repeating structure of Z. ramigera 115 polysaccharide (Ikeda et al., 1982).

The rheological properties of the Z. ramigera 115 polysaccharide in aqueous solutions have been partially characterized by Stauffer et al. (1980). They report that at polymer concentrations of less than 0.2%, Newtonian behavior is observed. Between concentrations of 0.2-0.4% the solution displays Bingham plastic or mixed flow and above 0.4% has a mixed type flow which results in a solution having viscoelastic properties. The apparent viscosity of the polysaccharide solution as a function of concentration is shown in Figure 9 (Stauffer et al., 1980). It was also shown that the apparent viscosity of the polysaccharide solution was stable to a pH range of 3.0-10.0 and temperature cycling between -20°C and 90°C. Also, polymer solutions held at 40°C for 14 days containing either 1% acetic acid and 5% NaCl or 1% citric acid were considered to be acid-stable when compared to identical preparations of xanthan and tragacanth. The Z. ramigera polysaccharide was found to be compatible with guar, locust bean, arabic, karaya, xanthan and tragacanth gums and also has the ability to lower the surface tension of water (Stauffer et al., 1980).

2.4 Genetics of Exopolysaccharide Production

To date no literature has been published on genetic manipulations of Z. ramigera. However, genetic manipulations and the isolation of EPS genes in related, exopolymer producing organisms such as Xanthamonas, Pseudomonas and Rhizobium, have been reported utilizing broad host range cloning vectors.

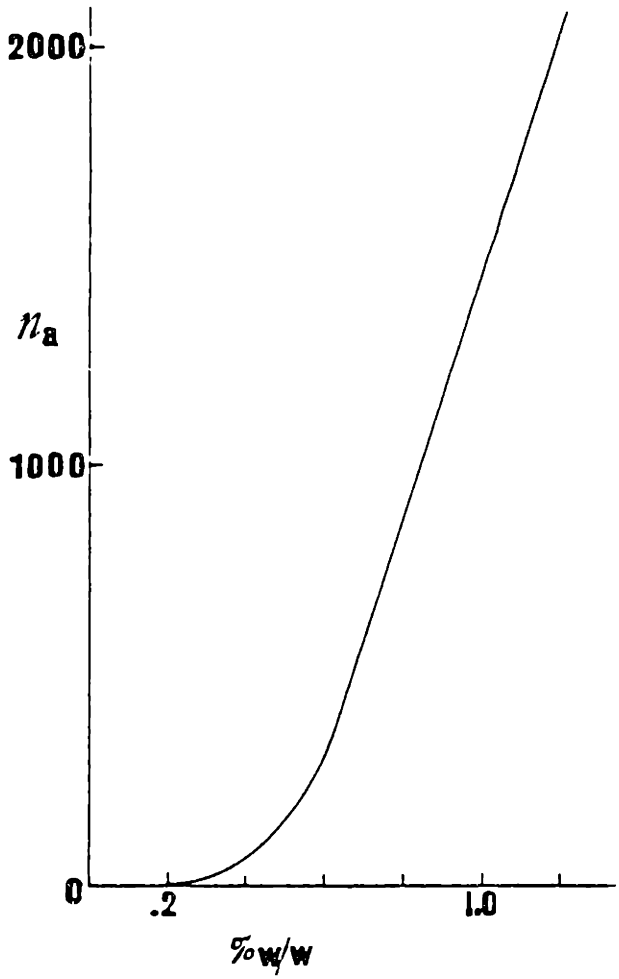


Figure 9. Apparent viscosity as a function of concentration of Z. ramigera 115 polyslaccharide at 3.84 sec^{-1} (Stauffer et al., 1980).

Isolation of genes involved in xanthan biosynthesis have been reported by Barrere et al. (1986), Harding et al. (1987) and Thorne et al. (1987). These studies have indicated the clustering of xanthan genes in several unlinked regions. One 13.5 kb region contains at least 5 complementation groups and has the effect of increasing xanthan production by 10% when cloned into wild-type X. campestris on pCHC3. This plasmid is also able to restore pyruvylation function to a mutant which produces pyruvate-free xanthan gum and increases the xanthan pyruvate content by about 45% when cloned into wild-type (Harding et al., 1987). This indicates the isolation of the xanthan pyruvyl transferase gene on pCHC3, but this has yet to be confirmed.

Goldberg and Ohman (1984 and 1987) have reported the cloning and expression in P. aeruginosa of a gene involved in alginate production. Similarly, Chakrabarty and coworkers have reported the cloning of several alginate biosynthetic genes (Darzins and Chakrabarty, 1984; Darzins et al., 1985a; Darzins et al., 1985b; Gill et al., 1986; Deretic et al., 1987; Sa-Correia et al., 1987). Their work has demonstrated the presence of 7 complementation groups involved in alginate biosynthesis (Darzins et al., 1985b). All of these groups except one, corresponding to the algR gene, are clustered within a 20 kb region closely linked to the argF locus at 45 minutes on the P. aeruginosa chromosome map (Darzins et al., 1985b). Two genes from this area have been identified: algA coding for phosphomannose isomerase (Darzins et al., 1985a) and algD coding for GDP-mannose dehydrogenase (Deretic et al., 1987a). Preliminary transcription regulation studies have led to the proposal of a simple regulatory model which indicates that the algR gene positively controls transcription of

algD whose gene product in turn regulates the flow of intermediates into the alginate biosynthetic pathway (Deretic et al., 1987b).

Exopolysaccharide genes from Rhizobium meliloti have been isolated and reported by Finan et al. (1985), Leigh et al. (1985) and Finan et al. (1986). Six complementation groups have been identified containing genes involved in EPS biosynthesis, at least three of which appear to be genetically linked (Leigh et al., 1985).

The bacterial species described above, X. campestris, P. aeruginosa and R. meliloti, represent the most studied of the EPS producing Gram-negative aerobes. The genera Pseudomonas, Xanthamonas and Zoogloea are closely related and make up most of the Pseudomonadaceae family. Common characteristics, relevant to this thesis project, of the EPS producing, Gram-negative aerobes are that broad host range cloning systems and Tn5 mutagenesis have been successful, EPS genes have been isolated and commonly are found in clusters. As is discussed herein these genetic techniques are also successful (after modification) in Z. ramigera and genetic analysis of EPS biosynthesis has revealed similarities between these related organisms.

3. MATERIALS AND METHODS

3.1 Bacterial Strains and Plasmids

The strains and plasmids used in this study are shown in Table 2.

3.2 Media and Culture Conditions

Z. ramigera cultures are stored frozen at -70°C in trypticase soy broth (TSB) containing 7% (v/v) dimethyl sulfoxide (DMSO) and 15% (v/v) glycerol. TSB and a defined medium as described by Norberg and Enfors (1982) were used for routine cultivation of the various Z. ramigera strains. The defined medium had the following composition: glucose, 25 g; K_2HPO_4, 2 g; KH_2PO_4, 1 g; NH_4Cl, 1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; yeast extract (Difco Laboratories), 0.01 g in 1 liter distilled water. Glucose, $MgSO_4 \cdot 7H_2O$, yeast extract and salts were autoclaved separately.

100 ml cultures of Z. ramigera for EPS isolation were grown on a rotary shaker (200 rpm) at 30°C in 500 ml baffled shake flasks for three days (I-16-M) or 1-2 weeks (115). Cell growth was measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories) after cells were lysed by heating at 90°C for 10 min in 1 N NaOH (Gerhardt, 1981).

E. coli strains were grown in LB (Luria-Bertani) medium, 1% (w/v) NaCl, 1% (w/v) tryptone (Difco) and 0.5% (w/v) yeast extract (Difco).

3.3 Microscopic Examination

The exopolymer capsular matrix and cell morphology was visualized with a light microscope using a 1% aqueous crystal violet stain.

Table 2

Bacterial Strains and Plasmids

Strain/Plasmid	Relevant Characteristics	Source or Reference
Z. ramigera		
I-16-M	Wild type, Cb^r, ATCC 19623	ATCC
S99	I-16-M, Δexo-99 (spontaneous)	This study
T18	I-16-MΩ118[chr::Tn*5*], Δexo-18	This study
T24	I-16-MΩ24[exo-24::Tn*5*]	This study
T25	I-16-MΩ125[chr::Tn*5*], Δexo-25	This study
T27	I-16-MΩ27[exo-27::Tn*5*]	This study
T28	I-16-MΩ28[exo-28::Tn*5*]	This study
T30	I-16-MΩ130[chr::Tn*5*], Δexo-30	This study
T33	I-16-MΩ33[exo-33::Tn*5*]	This study
T44	I-16-MΩ44[exo-44::Tn*5*]	This study
T48	I-16-MΩ148[chr::Tn*5*], Δexo-48	This study
115	wild type, ATCC 25935	ATCC
115SL	115, slime forming mutant (NTG)	This study
115SLR	115SL, Rf^r (spontaneous)	This study
M1	115SLRΩ1[chr::Tn*5*]	This study
M3	115SLRΩ3[chr::Tn*5*]	This study
M6	115SLRΩ6[chr::Tn*5*]	This study
E. coli		
HB101	*hsdS20*(r_B^- m_B^-), *recA13*, *proA2* *rpsL20*, (Sm^r), *supE44*,	Maniatis *et al.*, 1982
MM294A	*pro-82*, *thi-1*, *endA1*, *hsdR17*, *supE44*	Leigh *et al.*, 1985
DH5	*end A1*, *hsdR17* (r_k^-,m_k^+), *supE44*, *thi-1*, *recA1*, *gyrA96*, *relA1*	BRL
DH5α	*end A1*, *hsdR17* (r_k^-,m_k^+), *supE44*, *thi-1*, *recA1*, *gyrA96*, *relA1*, Δ(*argF-laczya*)U169, ϕ80d*lacz*ΔM15	BRL
Plasmids		
pUC8	Ap^r	Vieira and Messing, 1982
pRK2013	Nm^r	Figurski and Helinski, 1979
pLAFR3	Tc^r, cosmid vector	B. Staskawicz via G. Walker
pRK602	Cm^r, Nm^r pRK2013 nm::Tn*9* containing Tn*5*	Leigh *et al.*, 1985

3.4 Cellufluor Assay for Polysaccharide Production

Cellufluor (Polysciences Chemicals) is a fluorescent dye that binds specifically to β(1-3) and β(1-4) glycosyl linkages and fluoresces when exposed to UV light (Easson et al., 1986; Haigler et al., 1980). Cellufluor was added to agar plates at a concentration of 200 μg/ml (Leigh et al., 1985) and used to determine exopolysaccharide production by Z. ramigera colonies.

3.5 Exopolysaccharide Isolation

Exopolysaccharide from Z. ramigera I-16-M was isolated using a cellulose purification procedure (Deinema and Zevenhizen, 1971). Dried EPS was quantitated gravimetrically or in solution using the Phenol Reaction (Gerhardt, 1981).

Exopolysaccharide from Z. ramigera 115 was purified by the addition of concentrated NaOH to the cell culture to a final concentration of 0.2 M, followed by the addition of 3 volumes of ethanol to precipitate the polymer and other materials which were collected and redissolved in half the original volume of water. Protein was removed by extracting twice with phenol, followed by extraction with ether to remove excess phenol. The aqueous phase was dialyzed, lyophilized and ground to yield a fine white powder.

Exopolysaccharide from Z. ramigera 115 SL and its derivatives was purified by removing cells by centrifugation followed by precipitation with 2 volumes of ethanol. EPS precipitate was collected, redissolved, dialyzed, lyophilized and ground to yield a fluffy, white material.

3.6 Viscosity Measurements

Initial studies on the viscosity of the culture broth and polymer solutions were measured with a Brookfield Syncrho-Lectric Viscometer, model LVT, equipped with an Ultra-Low Adapter, at 21°C. The apparent viscosities were measured at varying shear rates from 0.3 to 60 rpm.

3.7 Carbohydrate Determination - Hexose Assay

Total carbohydrate concentration in culture broths and polymer solutions was determined by the Phenol reaction (Gerhardt, 1981). Glucose, galactose and xanthan gum (Sigma Chem. Co.) were used as standards.

3.8 Protein Determination

Total protein concentration in culture broths and polymer solutions was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, 1979). Lysozyme was used as the standard. Cellular protein was released by boiling in 0.2 N NaOH.

3.9 Ultraviolet Mutagenesis

Z. ramigera cultures, at a cell density of approximately 2×10^8 cells/ml, were exposed to UV light for a time determined to produce 1% survival and subsequently plated on trypticase soy agar medium. Potential mutants which could not grow on galactose were screened by replicating onto defined medium containing glucose or galactose as the carbon source.

3.10 Nitrosoguanidine Mutagenesis

Cells at a density of 2 x 10^8 cells/ml were exposed to 50 μg/ml NTG (Gerhardt, 1981) for varying times up to 60 minutes. Cells were plated out at a survival rate of 30-40% (~35 minute exposure) and screened.

3.11 Compositional Analysis of Polysaccharides

Purified polysaccharide was hydrolyzed in 1 M trifluoroacetic acid at 120°C for varying times between 1/2 hour and 2 hours. Monosaccharides in the polysaccharide hydrolysate were separated using a Waters HPLC equipped with a Brownlee Polypore PB, lead loaded cation exchange column, operated at 85°C, with water as the eluent. Detection was by refractive index using a Waters Model 401 Differential Refractometer.

3.12 Proton NMR Spectroscopy

The polysaccharide hydrolysate (~10mg) was dissolved in D_2O and analyzed using a 500 MHz proton NMR spectrometer. Experiments were performed at the NMR Facility for Biomolecular Research located at the Francis Bitter National Magnet Laboratory, MIT.

3.13 Infrared Spectroscopy

Infrared spectra of purified polysaccharide were obtained on a Perkin Elmer Model 283B Infrared Spectrophotometer. Samples were prepared by grinding 1-5 mg of polysaccharide with 100 mg dry KBr and pressing the mixture into a disk.

3.14 Size Exclusion Chromatography

Polysaccharide solutions were analyzed by size exclusion chromatography using a Waters HPLC equipped with BioRad Bio-Gel TSK-40 and TSK-60 columns connected in series. Distilled, deionized water containing 0.05% sodium azide was used as the solvent. Detection of polysaccharides was by differential refractive index (Waters, Model 401).

3.15 DNA Manipulations

Restriction endonucleases and T4 DNA ligase were purchased from IBI, New Haven, CT. Calf intestinal alkaline phosphatase (CIP) was obtained from Boehringer Mannheim (Indianapolis, IN). All enzymes were used under manufacturer's recommended conditions. DNA polymerase I was obtained from Amersham (Arlington Heights, IL).

Chromosomal DNA was isolated from Z. ramigera I-16-M as follows: 200 ml of mid log phase cells were harvested by centrifugation (8,000 rpm, GSA rotor, 10 min), washed in 80 ml of 20 mM Tris HCl, pH 8.2 and re-harvested as described above. Cell pellets were resuspended in 10 ml of 20 mM Tris. HCl, pH 8.2, and 10 ml of PEG 8000 (24% w/v) and 2 ml of lysozyme (25 mg/ml) added. The mixture was then incubated for 30 min at 37°C. Spheroplasts were then collected by centrifugation (10,000 rpm, 8x50 rotor, 10 min) resuspended in 5 ml of TE buffer (10 mM Tris HCl, pH 8.2, 1 mM EDTA), 300 μl of 10% (w/v) SDS added followed by a 10 min incubation at 55°C to lyse the cells. The solution was diluted by adding 10 ml of TE and treated with RNase A (150 μl of a 5 mg/ml stock solution) for 15 min at 37°C. Proteinase K, 50 μl of a 10 mg/ml solution, was added followed by a 1 h incubation at 45°C. The DNA was then purified by cesium chloride gradient centrifugation as follows: The solution was added to 40.8 g of CsCl and

the total volume made up to 36 ml with TE buffer and the density adjusted to 1.70; gradients were centrifuged at 46000 rpm for 18 h at room temperature in a VTi 50 rotor; the gradients were then fractionated, DNA containing fractions pooled, the CsCl diluted out with TE and DNA recovered by precipitation with 2 volumes of ethanol. The precipitated DNA was washed 3 times with 70% (v/v) ethanol, dried, resuspended in TE buffer at a final concentration of 1 μg/μl and stored at 4°C.

For large numbers of samples where only small amounts of DNA are required, 10 ml lots of cells were harvested by centrifugation (10,000 x g, 10 min), washed in 10 ml of 20 mM Tris HCl, pH 8.2 and re-harvested as described above. Cell pellets were resuspended in 1 ml of 20 mM Tris HCl, pH 8.2, and 1 ml of PEG 8000 (24% w/v) and 0.2 ml of lysozyme (25 mg/ml) added. The mixture was then incubated for 30 min at 37°C. Spheroplasts were collected by centrifugation (10,000 x g, 10 min) resuspended in 250 μl of TE buffer (10 mM Tris HCl, pH 8.2, 1 mM EDTA), 15 μl of 10% (w/v) sodium dodecylsulfate added followed by a 10 min incubation at 55°C to lyse the cells. The solution was diluted by adding 500 μl of TE and treated with RNase A (7.5 μl of a 5 mg/ml stock solution) for 15 min at 37°C. Proteinase K, 2.5 μl of a 10 mg/ml solution, was added followed by a 1 h incubation at 45°C. Following phenol extraction to remove proteins, the DNA was recovered by ethanol precipitation and resuspended in TE buffer at a final concentration of 1 μg/μl.

Plasmid DNA was prepared from E. coli using the methods of Birnboim and Doly (1979) as modified by Ish-Horowicz and Burke (1981).

3.16 Construction of Z. ramigera Gene Libraries

MboI partial digestion conditions for Z. ramigera DNA were determined to yield fragments in the size range 15-28 kb, as described by Maniatis et al. (1982). 40 μg lots of DNA were digested for 1 h with appropriate amounts of enzyme, followed by the addition of EDTA to 25 mM and incubation at 68°C for 10 min. After recovery by ethanol precipitation the DNA was treated with CIP and electrophoresed on a 0.75% (w/v) agarose gel. Agarose containing fragments in the size range 15-28 kb was excised and the DNA electroeluted, ethanol precipitated and resuspended in TE. pLAFR3 DNA was prepared as follows. Two aliquots (10 μg each) were digested to completion, one with HindIII and one with EcoRI, followed by CIP treatment. Samples were purified by phenol extraction, ethanol precipitation and resuspended in TE. Both aliquots were then completely cleaved with BamHI and purified by phenol extraction. The desired fragments were precipitated with 0.7 volumes isopropanol in the presence of 0.2 M sodium acetate and resuspended in TE at a concentration of 1 μg/μl. Ligation reactions contained 1 μg of HindIII/BamHI cut vector, 1 μg of EcoRI/BamHI cut vector and 2 μg of target DNA in a total volume of 10 μl and were incubated for 12-16 hours at 14°C. Ligated DNA was packaged using in vitro packaging extracts prepared from E. coli BHB2688 and BHB2690 as described by Ish-Horowicz and Burke (1981). Recombinant phage particles were transduced into E. coli DH5 as described by Maniatis et al. (1982) and plated on LB agar containing tetracycline (10 μg/ml).

3.17 Subcloning of the EPS Gene Region

The EPS gene region cloned in pPS27 and pPS48 was subcloned by either complete digestion with EcoRI or PstI or partial digestion with SauIIIA.

The EcoRI and PstI fragments from digests of pPS48 DNA were excised from an agarose gel after separation by electrophoresis. DNA within the gel pieces was electroeluted, ethanol precipitated and resuspended in TE. The fragments were then ligated into pLAFR3 which had been completely digested with EcoRI or PstI and treated with CIP. Ligated DNA was transformed into E. coli DH5α cells and plated on LB agar plates containing tetracycline (10 μg/ml) and Xgal (50 μg/ml). Clones containing recombinant plasmids were isolated by screening for white colonies and confirmed by restriction analysis of their plasmid DNA.

Fragments ranging in size from 4 to 7 kb were generated by partial digestion of pPS27 DNA with SauIIIA (Maniatis et al., 1982), ligated into BamHI cleaved, CIP treated pLAFR3, and transformed into DH5α competent cells. Approximately 10^3 colonies were pooled and used in conjugation experiments to complement the various exo mutations.

3.18 Conjugation in Z. ramigera

Transfer of pLAFR3 and pLAFR3 recombinant DNA molecules into Z. ramigera was accomplished using the conjugative plasmid pRK2013 as follows: E. coli MM294A (pRK2013) and E. coli DH5 containing pLAFR3 or a pLAFR3/Z. ramigera gene library were each grown up in LB broth containing neomycin (50 μg/ml for pRK2013) or tetracycline (10 μg/ml for pLAFR3) to a density of approximately 2 x 10^9 cells/ml. Equal amounts (0.5 ml) of each were mixed, after washing, with 0.5 ml of I-16-M parent (previously sonicated to disperse flocs) or mutant strains. The mixture was deposited dropwise onto the center of a single 100 mm TS agar plate, allowed to dry and incubated overnight at 30°C. Cells were resuspended in 1 ml TSB and dilutions were

plated on TS agar containing tetracycline (10 μg/ml) and carbenicillin (100 μg/ml) for I-16-M or rifampicin (50 μg/ml) for 115SLR.

Transposon Tn*5* mutagenesis was carried out using cultures of *Z. ramigera* grown up in TSB to a density of approximately $5x10^9$ cells/ml. In the case of I-16-M, 20 ml of the flocculated cell suspension was sonicated for 2 min to break up the cell flocs. Transfer of pRK602 into *Z. ramigera* was carried out as described above using *E. coli* MM294A (pRK602) as the only donor. Tn*5* insertions into the *Z. ramigera* chromosome were selected for by growth on neomycin (50 μg/ml) and carbenicillin (100 μg/ml) for I-16-M or rifampicin (50 μg/ml) for 115SLR.

3.19 DNA Blotting and Hybridization Analysis

DNA fragments, separated on 1% (w/v) agarose gels, were transferred to nitrocellulose filters by the sandwich blot method (Smith and Summers, 1980) based on the technique developed by Southern (1975). Filters were hybridized with DNA probes labelled to a high specific activity (0.1-1 x 10^8 cpm/μg of DNA) with [α-^{32}P]-dATP, by nick translation (Ribgy *et al.*, 1977). For the detection of Tn*5* insertions, probes were prepared using either the purified Tn*5* sequence on a 5.5 kb *Hpa*1 fragment from pRK602 or the entire pRK602 plasmid. Control experiments indicated no detectable homology between the plasmid and wild type *Z. ramigera* chromosomal DNA. Prehybridizations and hybridizations were carried out at 65°C in sealed polyethylene bags as previously described (Peoples *et al.*, 1987). Final wash conditions were 2 x SSC (20 x SSC: 3.0 M NaCl, 0.3 M sodium citrate), 0.1% (w/v) sodium dodecylsulfate at 65°C.

4. RESULTS

4.1 Morphology of Z. ramigera Strains

Cell flocs of Z. ramigera isolate 115 were visualized using 1% aqueous crystal violet stain and are shown in Figure 10. The cells are grouped together and are surrounded by capsules formed by the exopolysaccharide matrix. A similar preparation of isolate I-16-M is shown in Figure 11. No capsule is observed, although strain I-16-M does flocculate and produces extracellular polysaccharide.

Differences in the colony morphology of 115 and I-16-M were also observed. Isolate 115 colonies had an irregular form and a high dome shape (pulvinate) with a rough and bumpy surface. The morphology of the 115 colonies, except for size, did not change throughout development of the colony. Colonies were tough, not readily broken up, and entire colonies were easily lifted from plates. The colonies of isolate I-16-M were circular and glistening with a raised center and smooth edges.

4.2 Polysaccharide Staining with Cellufluor

The use of the fluorescent dye Cellfluor as a plate assay to detect the presence of bacterial EPS has been described by Leigh et al. (1985). To partially characterize the activity of this dye, its binding specificity was investigated using various polysaccharides with different glycosidic linkages. Glucose polysaccharide standards were chosen that contain the most prevalent types of glycosidic linkages with little or no branching. 20 μl of a 1% solution of each standard and several common heteropolysaccharides were deposited onto solidified agar containing 200 μg/ml Cellufluor, allowed to dry and examined for fluorescence under UV light. The results are shown in Table 3.

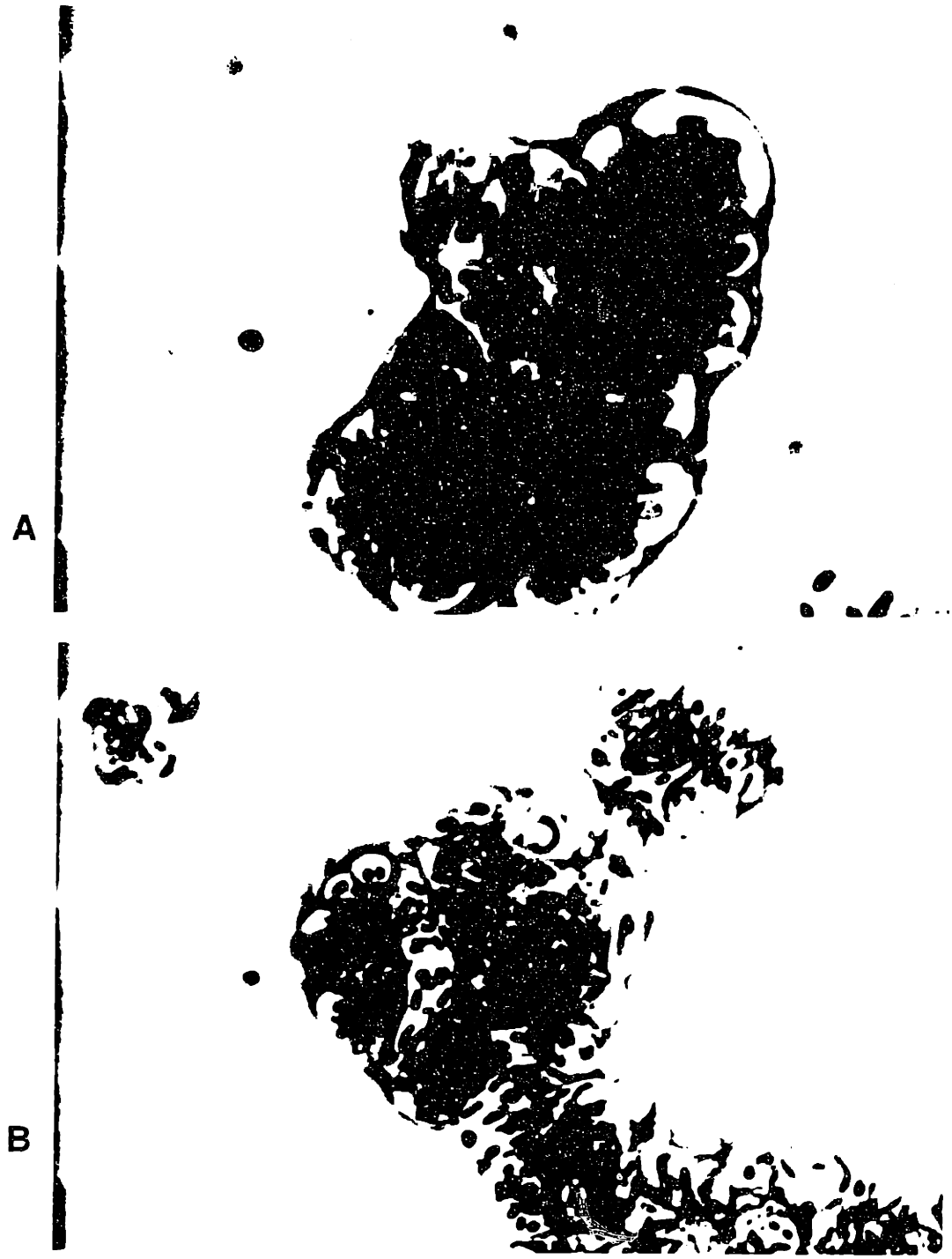


Figure 10. Z. ramigera 115 flocs stained with 1% aqueous crystal violet magnified 1000X (A) and 630X (B).

Figure 11. Z. ramigera I-16-M floc stained with 1% aqueous crystal violet magnified 1000X.

Table 3

Specificity of Cellufluor

Compound	Linkage	Components	Fluorescence
Glucose	-	Glucose	-
Galactose	-	Galactose	-
Lactose	β(1-4)	Glucose, Galactose	-
Cellobiose	β(1-4)	Glucose	-
Laminarin	β(1-3)	Glucose	+
Methylcellulose	β(1-4)	Glucose	+
Pustulan	β(1-6)	Glucose (O-acetyl)	-
Amylose	α(1-4)	Glucose	-
Dextran	α(1-6), α(1-3)	Glucose	-
Zooglan 115	β(1-4)? β(1-3)?	Glucose, Galactose	+
Xanthan	β(1-4) main chain	Glucose, Mannose, Glucuronate	-
Alginate	β(1-4)	Mannuronate, Guluronate	-
Chitosan	β(1-4)	Glucosamine	+

Glucose monomers and β(1-4) linked dimers did not bind Cellufluor. Glucose polymers that were linked β(1-3) or β(1-4) did bind the dye and fluoresce, while β(1-6), α(1-4) and α(1-6) linked glucose polysaccharides showed no affinity for Cellufluor. As for the more complex heteropolysaccharides, xanthan and alginate show no affinity for Cellufluor while chitosan and the Z. ramigera 115 exopolysaccharide (Zooglan 115) were able to bind the dye and fluoresce when exposed to UV light.

4.3 Strategy for Isolating Exopolysaccharide Biosynthetic Genes

Control of a polysaccharide's structure by manipulation of the genes that code for its biosynthesis requires that those genes first be isolated and characterized. The strategy developed involves cloning the polysaccharide genes directly in Z. ramigera and is shown schematically in Figure 12. The requirements to accomplish this are 1) a method for introducing foreign DNA into Z. ramigera, 2) the construction of a Z. ramigera gene library, 3) the isolation of polysaccharide mutant strains, and 4) the subsequent complementation of those mutants using the Z. ramigera gene library. This procedure was carried out in strains I-16-M and 115.

4.4 Cloning of Z. ramigera I-16-M EPS Biosynthetic Genes

4.4.1 Genetic Transfer in Z. ramigera I-16-M

Several broad host range cloning vectors have been obtained and transferred into Z. ramigera I-16-M by conjugation. The procedure used was a triparental mating between two E. coli strains and the Z. ramigera recipient. The donor was E. coli HB101 harboring the broad host range vector, pLAFR3 (B. Staskawicz, personal communication). This cosmid

confers tetracycline resistance and is mobilizable but not self-transmissible. The mating requires a second E. coli strain which contains the "helper" plasmid pRK2013 carrying the RK2 transfer functions ligated to a ColE1 replicon which can mobilize pLAFR3 into Z. ramigera. The transconjugants were selected for by growth on appropriate medium. The transfer of pLAFR3 to Z. ramigera I-16-M occurred at a frequency of approximately 10^{-3} transconjugants/recipient.

4.4.2 Construction of Z. ramigera Gene Libraries

The cosmid pLAFR3, derived from RK2 via pRK290, was used to construct Z. ramigera I-16-M and 115 gene libraries as shown in Figure 13. This procedure increases the cloning efficiency by ensuring that only recombinant molecules can be packaged. A "right" and "left" arm are created which can only be packaged if they ligate to an insert molecule that is between 15 and 28 Kb, thus a high frequency of recombinants are obtained. The recombinant molecules were packaged in vitro and transduced into E. coli DH5. This procedure was carried out for both I-16-M and 115 and yielded approximately 10^5 recombinants/μg of insert DNA in each case.

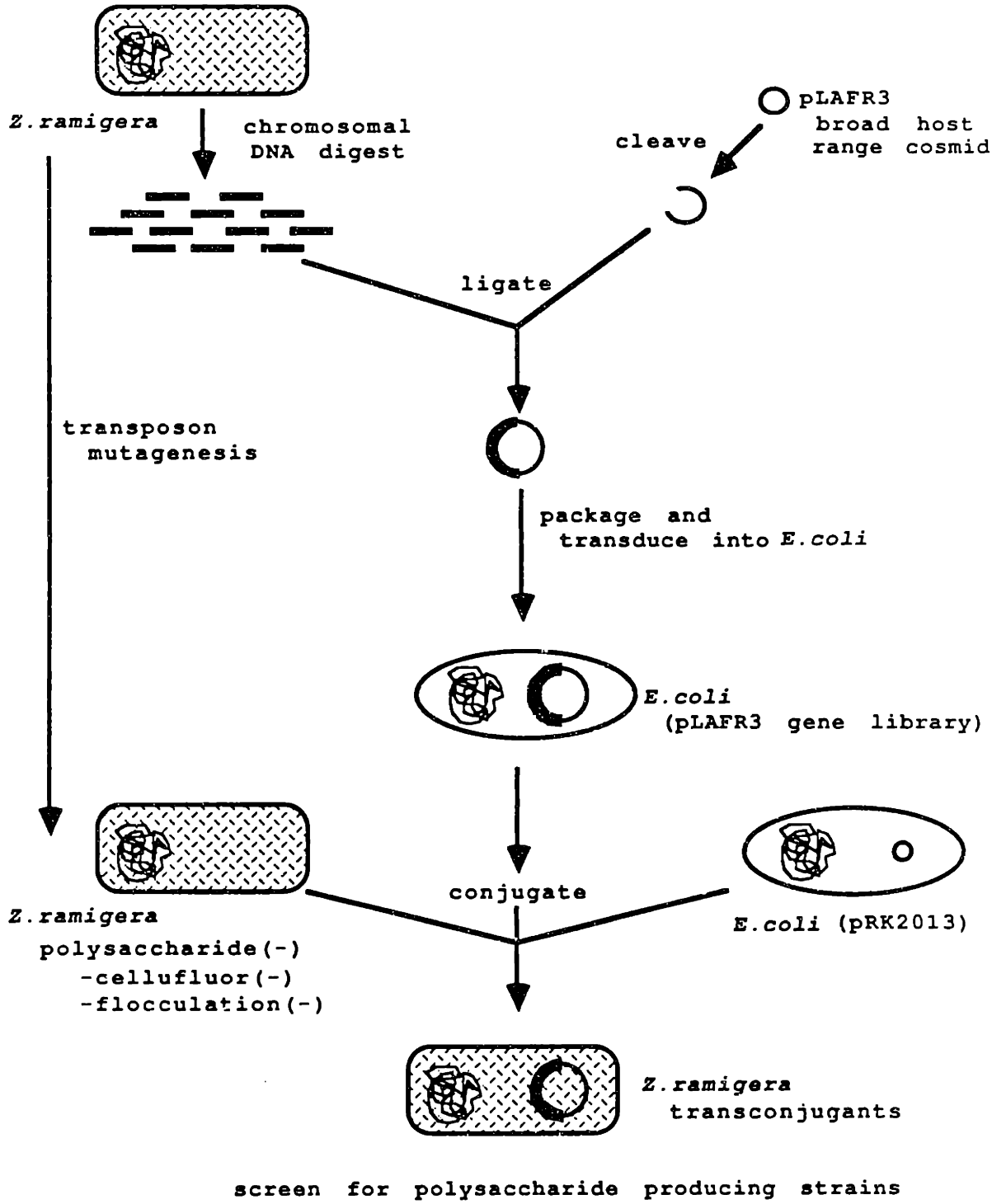


Figure 12. Strategy for the cloning of Z. ramigera polysaccharide genes.

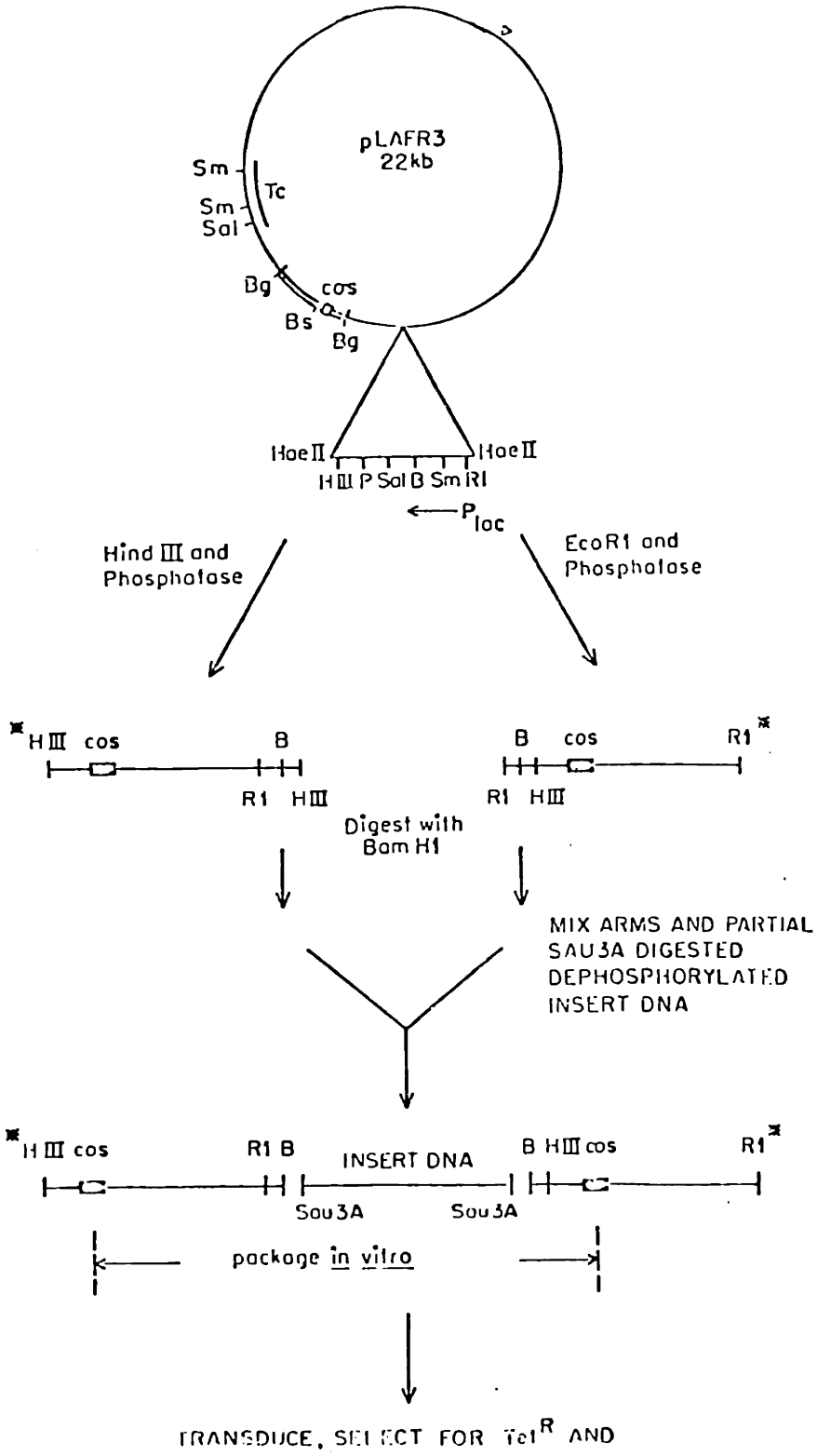


Figure 13. Method for clone bank construction in pLAFR3 (B. Staskawicz; personal communication).

4.4.3 Isolation and Characterization of I-16-M EPS Non-Producing Mutants

Selecting for mutants with altered, or devoid of, exopolysaccharides is difficult because this results in no significant effect on the growth of the organism. Therefore, potential mutants were screened using the fluorescent dye Cellufluor (Finan et al., 1985; Leigh et al., 1985). Cellufluor binds specifically to β(1-3) and β(1-4) glycosidic linkages (Section 4.2; Haigler et al., 1980) and thus is concentrated around cells or colonies producing β-linked polysaccharides. Colonies of the wild type Z. ramigera I-16-M fluoresce under UV light when grown on plates containing this dye. Mutants devoid of exopolysaccharide were obtained spontaneously and via transposon mutagenesis by screening colonies grown on Cellufluor plates for nonfluorescence. Spontaneous nonfluorescing mutants were obtained at a frequency of approximately 1 in 10^4.

Transposon insertions into the Z. ramigera I-16-M chromosome were made by introducing Tn5 on the delivery vector pRK602. Tn5 insertions were selected on plates containing neomycin and carbenicillin and obtained at a frequency of approximately 10^{-5} insertions/recipient. Nonfluorescing mutants were found at a frequency of about 1 in 10^2 colonies, of which nine (Table 2) were chosen at random for further analysis. It was subsequently determined that these nine mutants had Tn5 insertions at unique sites.

Both the spontaneous and the Tn5 derived mutants were tested for flocculation in liquid culture. All of the nonfluorescing mutant strains produced a turbid or nonflocculated culture as opposed to Z. ramigera wild-type strains that characteristically grow as large visible flocs which rapidly settle to the bottom of the tubes leaving a clear supernatant.

Cultures of two of the mutants (T25 and T27) were measured for exopolysaccharide content (Table 4, rows 2 and 3), the results of which showed no detectable cellulose-like material. Thus, we can conclude that loss of exopolysaccharide production (Eps^-) results in the inability of the cells to flocculate and the loss of fluorescence on plates containing Cellufluor. Conversely, and more importantly, nonfluorescing colonies are Eps^-.

A series of Southern hybridization experiments were performed to determine the chromosomal location of the Tn5 insertion in each mutant strain. The results for the nine unique Tn5 insertion mutants are described below. Figure 14 shows the result of one such experiment where the chromosomal DNA from each of the nine mutant strains was digested with PstI and hybridized with ^{32}P-labelled Tn5 DNA. It is clear that for these nine mutants Tn5 has inserted into different locations since in each lane a different hybridization pattern is seen for the two junction fragments which contain both Tn5 and I-16-M DNA. The three bands appearing in every lane are internal PstI fragments of Tn5. In the case of T33 (Figure 14A, lane 4) the second junction fragment comigrates with the 1.1 kb internal Tn5 fragment. The sizes of the wild-type PstI fragments which contain Tn5 in these mutants were calculated by summing the fragments in each lane minus 5.7 kb (size of Tn5) and are shown in Table 5. In another experiment chromosomal DNA from each mutant was digested with EcoRI and hybridized with ^{32}P-labelled Tn5 DNA (Figure 15). Since Tn5 contains no EcoRI sites a single hybridization band appears in each lane corresponding to the Tn5-containing EcoR1 fragment.

Table 4

Quantitation of Exopolysaccharide (EPS) Levels in *Z. ramigera* Cultures

Strain	Cells (g/liter)[a]	EPS (mg/liter)	EPS/Cells (mg/g)
I-16-M	1.92	40.0	20.8
I-16-M-T25	2.36	<1.0[b]	<0.4
I-16-M-T27	2.36	<1.0[b]	<0.4
I-16-M-T25/pPS25	2.40	31.5	13.1
I-16-M-T27/pPS27	0.76	10.5	13.8
I-16-M-T48/pPS48	1.08	47.5	22.0

[a] Based on protein content assuming protein accounts for 50% of dry cell weight.
[b] Not detected by method used.

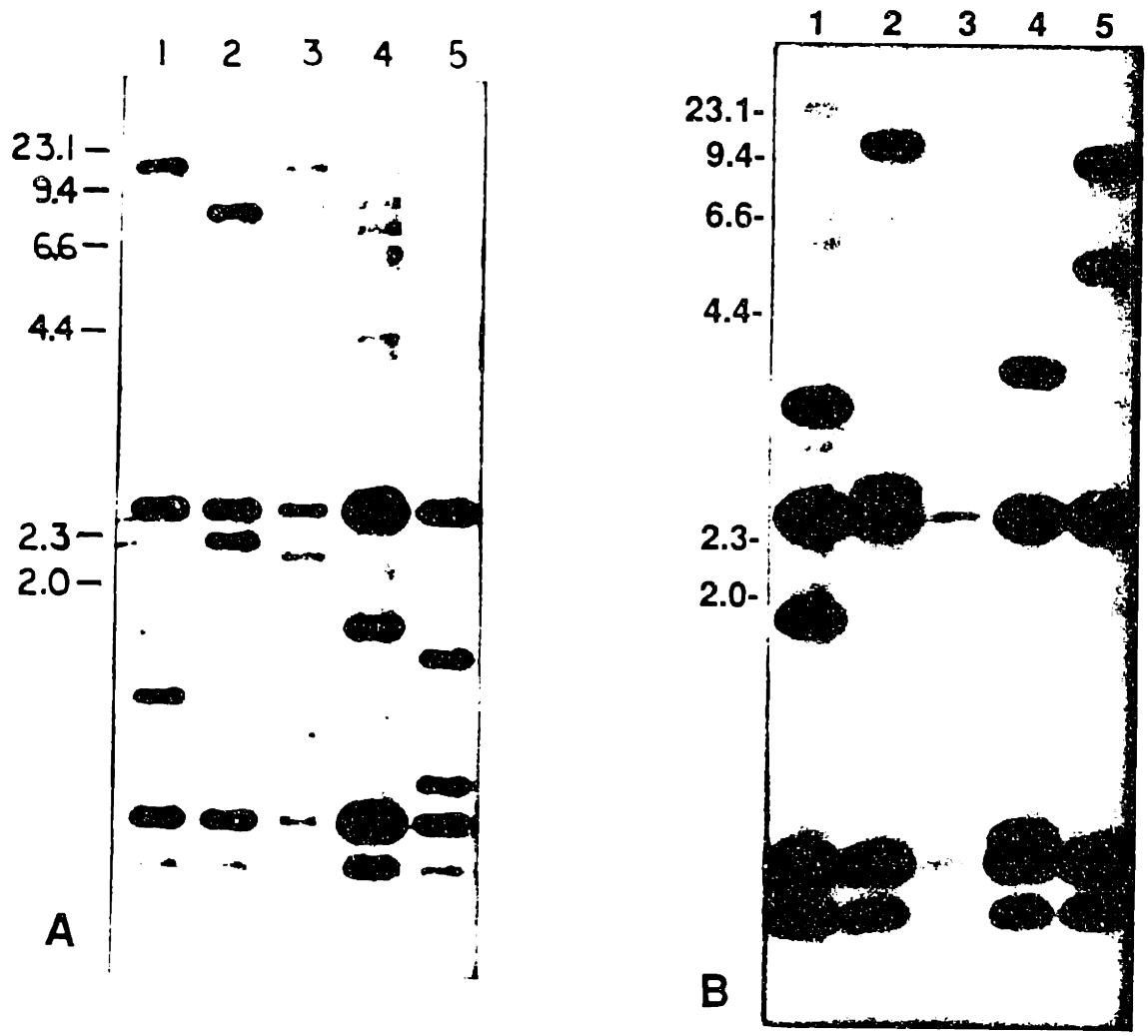


Figure 14. Autoradiograms of ^{32}P-labelled Tn5 DNA hybridized to a Southern blot of PstI digested chromosomal DNA (~1-3 μg/lane) from mutants T24, T27, T28, T33, T44 (A, lanes 1-5, respectively) and T18, T25, T27, T30, T48 (B, lanes 1-5, respectively). The 2.5, 1.1 and 0.9 kb fragments in all lanes are internal Tn5 restriction fragments. Size markers shown are in kb.

Table 5

Restriction Fragments Containing Tn5 in Eps⁻ Mutants

Strain	Size of EcoRI Fragment with Tn5 Insertion (kb)	Size of PstI Fragment with Tn5 Insertion (kb)	Distance of Tn5 From Nearest PstI Site (kb)
T18	4.3	4.1	1.3
T24	4.0	17.1	0.8
T25	5.0	15.0	2.1
T27	7.6	8.6	1.6
T28	4.0	17.1	1.5
T30	2.2	3.8	0.6
T33	7.6	1.5	0.3
T44	7.6	1.5	0.5
T48	23.0	15.4	5.0

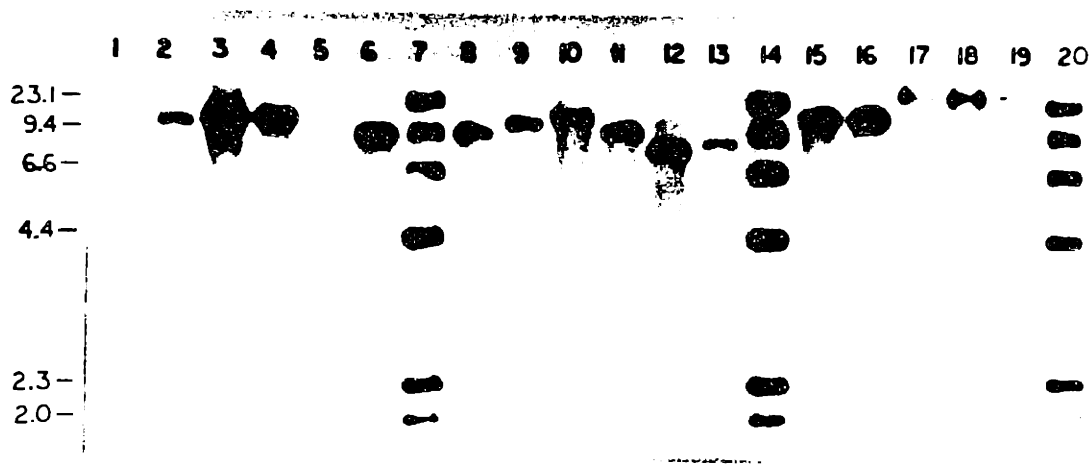


Figure 15. Autoradiogram of ^{32}P-labelled Tn5 DNA hybridized to a Southern blot of EcoRI digested chromosomal DNA from 15 Eps$^-$ mutants. Lanes 2-6, 8-13, and 15-18 contain DNA from mutant strains T7, T9, T12, T18, T24, T25, T27, T28, T30, T32, T33, T44, T48, and T49, respectively. Lane 19 contains EcoRI cut pRK602 and lanes 1, 7, 14, and 20 contain size markers.

The size of the wild-type EcoRI fragment was determined for each mutant by subtracting 5.7 kb from the calculated size of the hybridization signal in each lane. Data from these experiments are summarized in Table 5.

Six of the fifteen mutants shown in Figure 15 were later determined to have insertions at the same site as one of the remaining nine. These identical insertion mutants probably are siblings and not discussed further.

4.4.4 Complementation of Eps$^-$ Mutants

An I-16-M/pLAFR3 gene library was initially mated from E. coli DH5 into four of the nine I-16-M Tn5 Eps$^-$ mutants (T25, T27, T30 and T48). More than 5000 transconjugants for each mutant strain were screened on Celïufluor plates and fluorescent candidates identified at a frequency of approximately 1 in 100-200 colonies. For each mutant strain, a single fluorescent colony was picked and cultured for further analysis. The plasmids responsible for the complementation were designated pPS25, pPS27, pPS30 and pPS48 with respect to the individual mutant strain complemented.

Two additional experiments confirmed the restoration of the Eps$^+$ phenotype. First, complemented strains were grown in liquid media to test for flocculation. Figure 16 shows cultures of wild-type I-16-M (left tubes), mutant T27 (center tubes) and complemented mutant T27 containing plasmid pPS27 (right tubes). The top picture shows the tubes just after shaking to disperse the flocs while the bottom picture shows the same tubes after the flocs were allowed to settle.

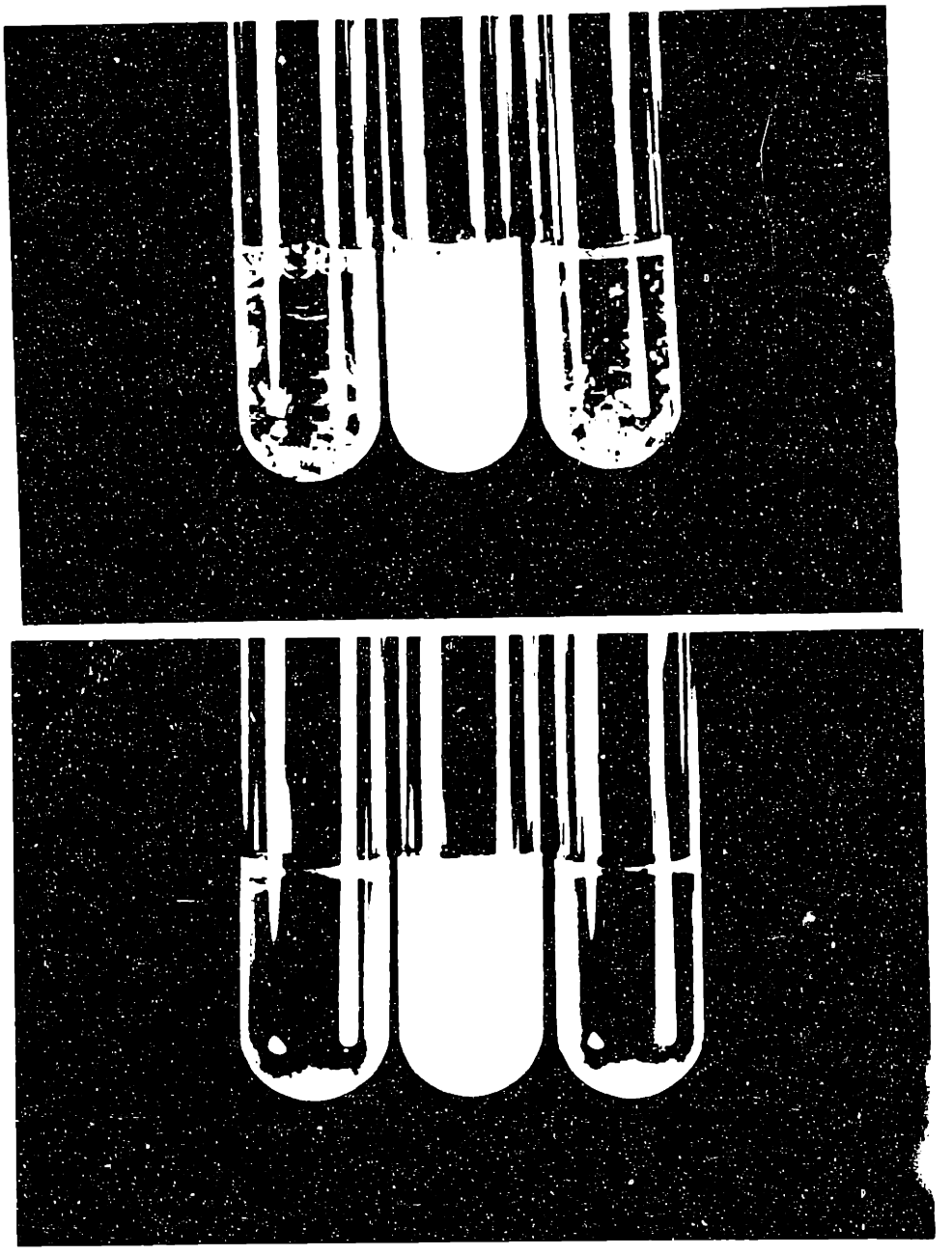


Figure 16. Pictures of Z. ramigera I-16-M cultures. The top picture shows the tubes just after shaking to disperse the cell flocs. The bottom picture shows these same tubes after the flocs are allowed to settle. The left tubes are wild-type I-16-M, the center tubes are mutant T27 and the right tubes are mutant T27 complemented with plasmid pPS27.

In both pictures it is clear that the mutant strain forms a turbid culture while both the wild-type and complemented mutant form cell flocs. Second, EPS production was measured for a number of strains. From the results shown in Table 4, it is clear that the mutants are not producing any detectable cellulose-like EPS, while these same mutants containing recombinant plasmids have restored EPS production at levels comparable with that of the wild-type I-16-M.

To determine any linkage of the mutations in the Eps$^-$ strains, the four plasmids able to restore EPS production to mutants, T25, T27, T30, and T48, were isolated, packaged *in vitro* and transfected into *E. coli* DH5. The resulting DH5/pPS strains were used to introduce each of the plasmids pPS25, pPS27, pPS30 and pPS48 into each of the Eps$^-$ mutants (Table 1) to test for cross complementation of the different mutations. It was found that plasmids pPS25, pPS27, pPS30 and pPS48 restored EPS biosynthesis to all of the mutant strains.

Restriction analysis of purified pPS25, pPS27, pPS30 and pPS48 DNA demonstrated that they all contain at least 14 kb of common insert DNA. Figure 17 illustrates the *EcoRI* restriction map of the four plasmid inserts and the composite region covered by these plasmids. It can be concluded from both the cross complementation and restriction mapping data that the cloned genes involved in EPS biosynthesis are located in the region common to all four plasmids (Figure 17). A consequence of this is that the *Tn5* insertions causing the Eps$^-$ phenotype should be located in this region. Since all four plasmids can complement each Eps$^-$ mutant and contain similar inserts, subsequent work was carried out predominantly with pPS27 and pPS48.

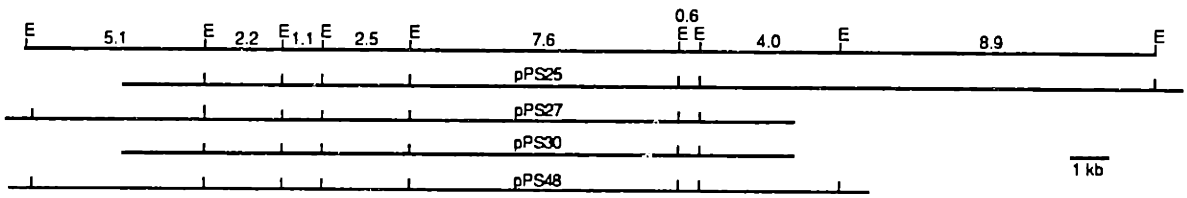


Figure 17. EcoRI restriction maps of pPS25, pPS27, pPS30, pPS48 inserts and the composite region (above). End points of the plasmid inserts have not been defined beyond the outermost EcoRI sites. Sizes of each fragment are shown in kb.

4.4.5 Location of the exo Mutations within the Region Cloned in pPS27

Southern hybridization experiments were carried out to map the location of the Tn5 insertions in the chromosomal fragment cloned in pPS27. ^{32}P-labelled pPS27 DNA was hybridized to EcoRI digests of chromosomal DNA from the nine mutants (Figure 18A, lanes 2-6; Figure 18B, lanes 3-7). As controls chromosomal DNA from I-16-M (Figure 18A, lane 1; Figure 18B, lane 1) and a spontaneous Eps$^-$ mutant (S99; Figure 18B, lane 2) were also included.

The expected result of this experiment is to obtain identical hybridization patterns between the wild-type I-16-M and each Tn5 mutant except for a 5.7 kb increase in size of the fragment that contains Tn5. This is the result that can be seen when comparing the hybridization pattern of the wild-type digest (Figure 18A, lane 1) to those of the mutants T24, T27, T28, T33 and T44 (Figure 18A lanes 2-6, respectively). T24 (Figure 18A,lane 2) and T28 (Figure 18A,lane 4) contain Tn5 insertions in the 4.0 kb EcoR1 fragment creating a 9.7 kb band. T27 (Figure 18A,lane 3), T33 (Figure 18A,lane 5) and T44 (Figure 18A,lane 6), have Tn5 insertions in the 7.6 kb EcoRI fragment yielding a 13.3 kb signal which comigrates with another similarly sized wild-type fragment hybridized by pPS27.

An unexpected result was obtained for mutants T18, T25, T30, T48 and S99 as shown in Figure 18B. A comparison of the hybridization pattern of the wild type digest (Figure 18B, lane 1) to those of the mutants S99, T18, T25, T30 and T48 (Figure 18B, lanes 2-4 and 6-7, respectively) reveals significant differences in the banding patterns.

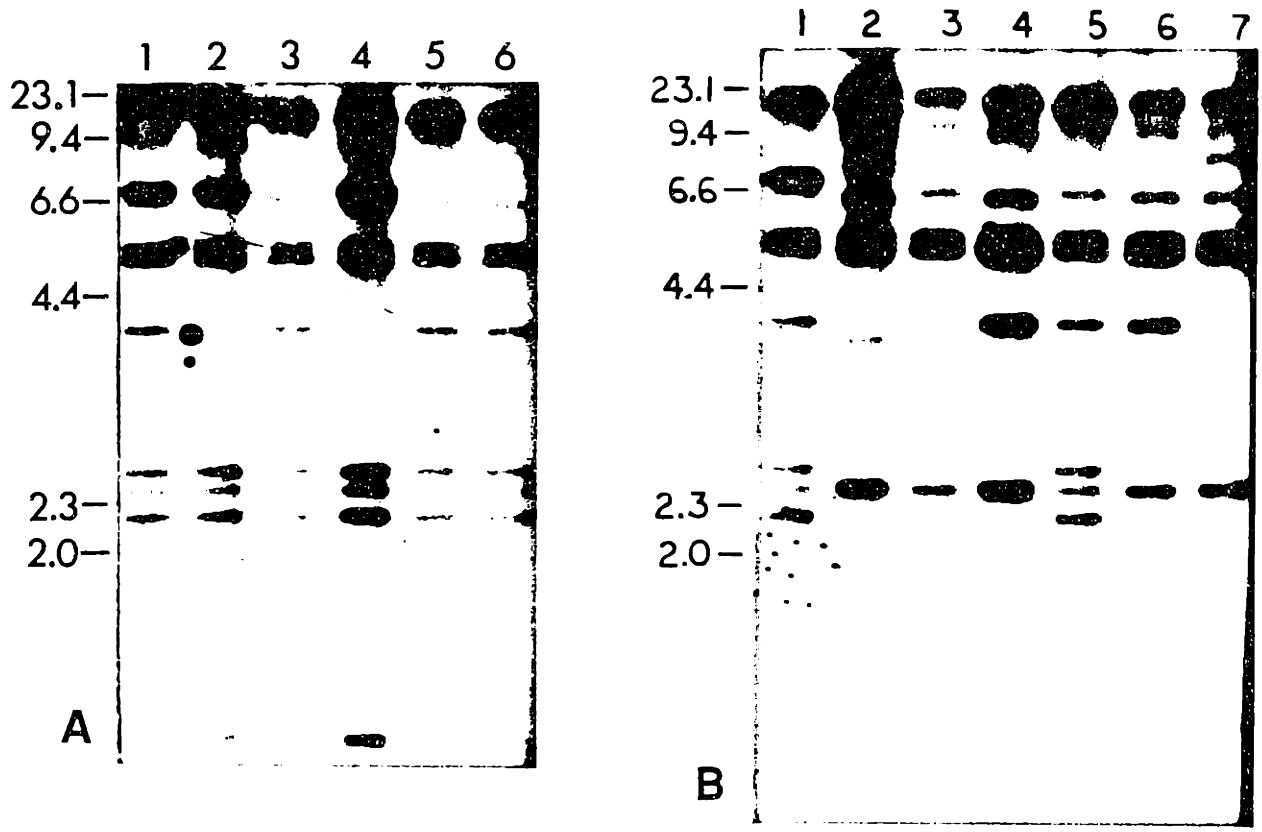


Figure 18. Autoradiograms of ^{32}P-labelled pPS27 DNA hybridized to Southern blots of EcoRI digested chromosomal DNA (~1-2 μg/lane) from wild-type I-16-M and Eps$^-$ mutants. (A) lanes: 1, I-16-M; 2, T24; 3, T27; 4, T28; 5, T33; 6, T44. (B) lanes: 1, I-16-M; 2, S99; 3, T18; 4, T25; 5, T27; 6, T30; 7, T48. Size markers shown are in kb.

The most significant difference observed for Tn5 mutants, T18, T25, T30 and T48, is the absence of a number of hybridization signals (Figure 18B, lanes 3, 4, 6 and 7, respectively). All of these mutants are missing the 1.1, 2.2, 2.5 and 7.6 kb bands. T18 and T48 are also missing the 4.0 kb signal (Figure 18B, lanes 3 and 7). Similarly, the spontaneous Eps^- mutant S99 is missing these same 5 fragments but differs from the Tn5 mutants in that additional strongly hybridizing bands of greater than 8 kb are present (Figure 18B, lane 2). Figure 19 illustrates the extent of the chromosomal deletions in these five mutant strains. The faint bands of hybridization, present in the lanes containing chromosomal DNA from the Eps^- mutants (excluding T27; Figure 18B, lanes 2, 3, 4, 6, and 7), are probably due to the presence of residual fragments from the ends of the deleted regions.

From this data and the complementation results it appears that the loss of EPS production in T18, T25, T30 and T48 was due to the occurrence of a deletion and possibly rearrangement event and not to the insertion of Tn5. To confirm this the Tn5 containing genomic EcoRI fragments from mutants T25, T27 (as a control containing a genuine Tn5 insertion in the region cloned in pPS27), T30 and T48 were subcloned into pUC8 (designated pCLT25, pCLT27, pCLT30, pCLT48, Figure 20) and hybridized to EcoRI digests of pPS27, pPS30 and pPS48 DNA (Figure 21). Genomic fragments containing Tn5 from mutants T25 (A, pCLT25), T30 (C, pCLT30) and T48 (D, pCLT48) show no homology to the pPS plasmids. Only in the case of the genomic fragment from T27 (B, pCLT27) was the expected result obtained, i.e., a 7.6 kb band of hybridization in EcoRI digests of pPS27, pPS30 and pPS48.

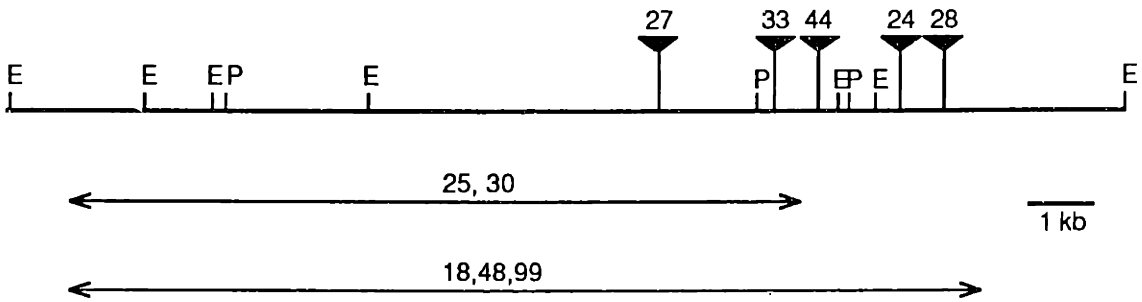


Figure 19. Partial restriction map of the EPS biosynthetic gene region. Shown are the approximate locations of the deletions/rearrangements occurring in mutants S99, T18, T48, T25 and T30 and the positions of Tn5 insertions in mutants T24, T27, T28, T33, and T44. Abbreviations: E, EcoRI; P, PstI.

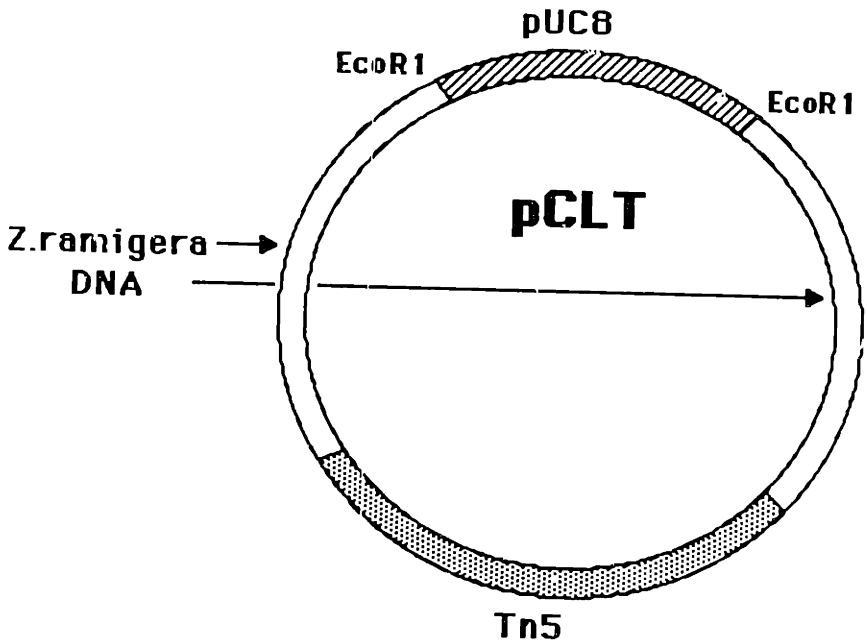


Figure 20. Configuration of recombinant pCLT plasmids.

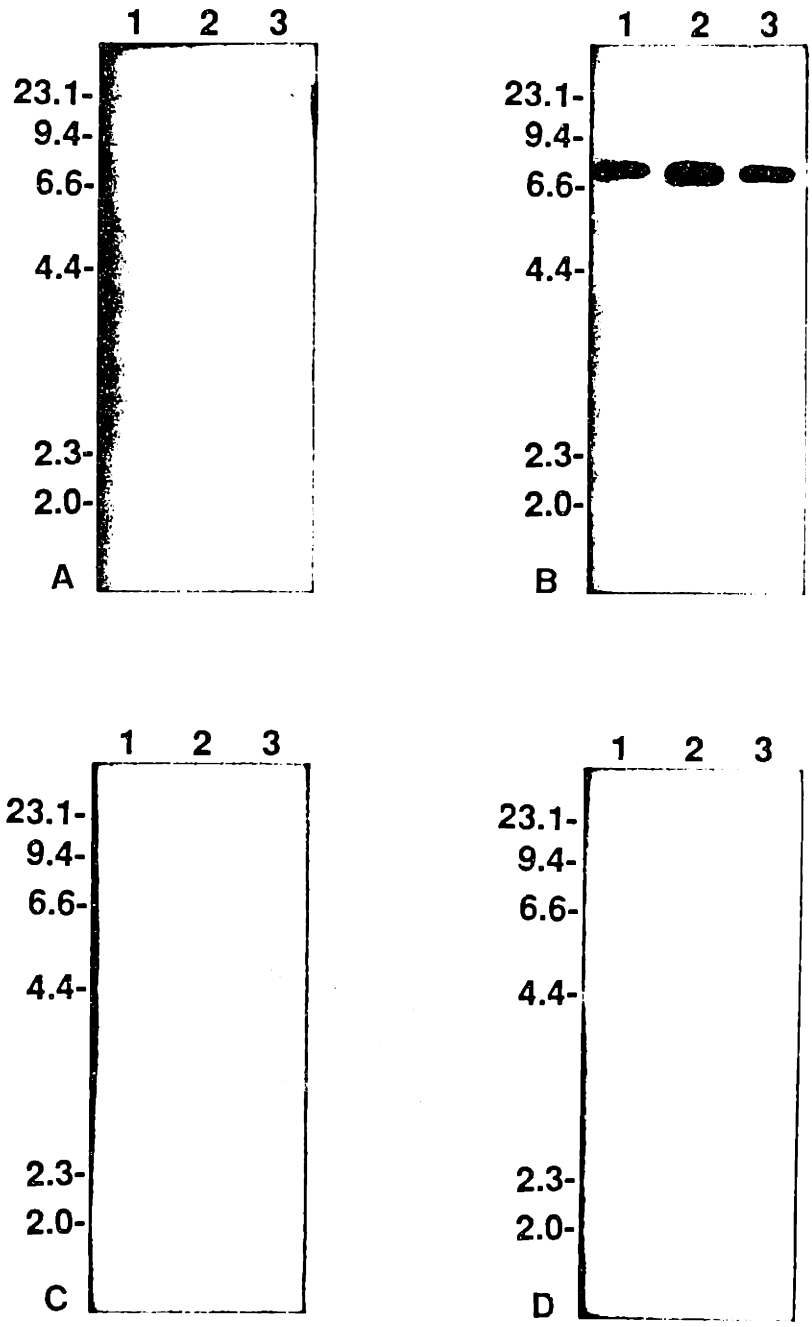


Figure 21. Autoradiograms of ^{32}P-labelled pCLT25 DNA(A), pCLT27 DNA(B), pCLT30 DNA(C) and pCLT48 DNA(D) hybridized to Southern blots of EcoRI digested pPS27, pPS30 and pPS48 DNAs (lanes 1-3, respectively).

Thus it is clear that two classes of Eps$^-$ mutants were obtained. One class, T24, T27, T28, T33 and T44, contain Tn*5* insertions in the region of DNA cloned in pPS27 (also pPS25, pPS30 and pPS48) and a second, T18, T25, T30, T48, have deletions and/or rearrangements in this same region. In the case of the deletion mutants the Tn*5* has inserted elsewhere in the chromosome seemingly having no relation to the loss of EPS production since these same deletions were also observed in a spontaneously derived Eps$^-$ mutant (S99).

The positions of the five insertions within the region cloned in pPS27 were determined by hybridizing Tn*5* DNA to *Pst*I digests of chromosomal DNA from these mutants (Figure 14A). The position of the insertions relative to the wild-type *Pst*I sites could be calculated since we know that Tn*5* contains *Pst*I sites 0.6 kb in from each end. Thus, 0.6 kb subtracted from each end of the junction fragment yields the precise spot of insertion. The nearest *Pst*I site to the point of insertion in each mutant is shown in Table 5. Figure 19 shows the approximate locations of these insertions, thereby identifying at least 4.6 kb of DNA which contains one or more genes involved in EPS biosynthesis.

4.4.6 Subcloning of the EPS Gene Region

Subcloning of the region of DNA cloned in pPS27 and pPS48 and complementation of Tn*5* insertions by subcloned fragments has demonstrated the presence of at least two complementation groups. Representative fragments subcloned into pLAFR3 are shown in Figure 22 with the mutations they complement immediately above.

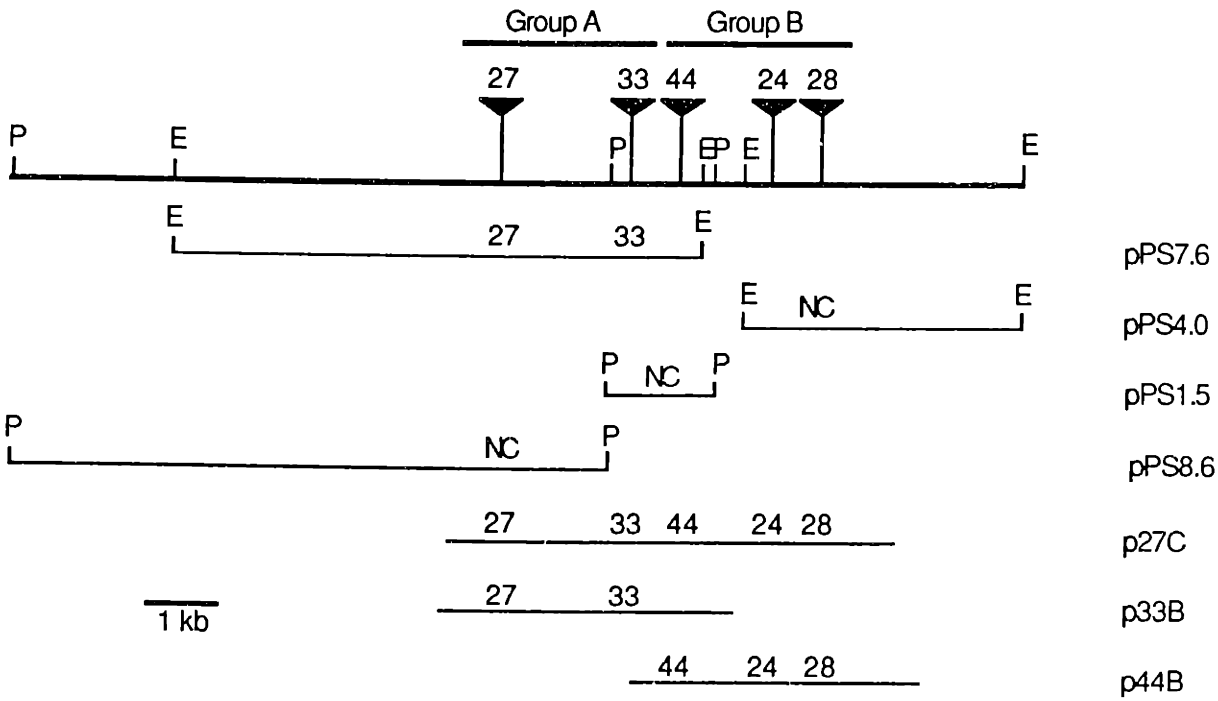


Figure 22. EPS biosynthetic gene region showing the approximate locations of the Tn5 insertions in mutants T24, T27, T28, T33 and T44. Also shown are subcloned fragments and the mutations they complement. Abbreviations: E, EcoRI; P, PstI; NC, fragment not able to complement any of the Tn5 insertions.

EcoRI and PstI fragments were isolated from pPS48, subcloned into pLAFR3 and tested for the ability to complement the five Tn5 insertions. Plasmid pPS7.6 (contains 7.6 kb EcoRI fragment of pPS48) is able to complement the exo-27 and exo-33 insertions. Plasmids pPS4.0 (4.0 kb EcoRI fragment insert), pPS1.5 (1.5 kb PstI fragment insert) and pPS8.6 (8.6 kb PstI fragment insert) cannot complement any of the Tn5 insertions.

The inserts cloned in p27C, p33B and p44B were obtained by a partial digestion of pPS27 DNA with SauIIIA and ligated into BamHI cleaved pLAFR3. Plasmid p27C has a 6.5 kb insert and is able to complement all five of the Tn5 insertions. Plasmid p33B has a 4.2 kb insert and is able to complement the exo-27 and exo-33 insertions while p44B has a 4.1 kb insert and can complement the insertions exo-44, exo-24 and exo-28.

In no case could any of the insertions be complemented individually. That is, any plasmid able to complement exo-27 could also complement exo-33 and vice-versa. Similarly, any plasmid able to complement exo-44, exo-24 or exo-28 could also complement the other two. This indicates the presence of two complementation groups whose approximate locations are shown above the restriction map in Figure 22. Group A is represented by insertion mutations exo-27 and exo-33 while group B is represented by insertion mutations exo-44, exo-24 and exo-28.

4.5 Cloning of EPS Biosynthetic Genes in Z. ramigera 115

4.5.1 Conjugation in Z. ramigera 115

The conjugation system described for I-16-M is unsuccessful in Z. ramigera 115 probably due to the thick capsule layer of polysaccharide surrounding the 115 cells. The gel-like matrix could act as a physical

barrier to intercellular transfer of genetic material. Methods to remove this capsule by sonication and incubation with a number of glucanases proved unsuccessful in aiding the conjugal transfer of DNA. To solve this problem a capsule nonforming, i.e., slime producing mutant, was derived from the parent by nitrosoguanidine (NTG) mutagenesis (Figure 23).

Cells exposed to NTG at a survival rate of 30-40% were plated and screened for changes in colony morphology. Z. ramigera 115 displays a unique morphology characterized by a rugose surface, crenated edges and high dome shape. A morphological mutant was isolated after screening 5000 colonies that has a smooth colony surface with a mucoid appearance. When touched with an inoculating loop a colony of this mutant strain can be strung out from the plate into a long polymer-like strand.

This strain, designated 115SL, was confirmed to be a derivative of strain 115 by hybridizing ^{32}P-labelled pHP48 DNA (a recombinant plasmid from the pLAFR3/115 gene library able to cause a colony morphology change in the I-16-M Eps$^-$ mutants) to chromosomal digests of the 115 parent strain DNA and the mutant strain 115SL DNA. From the autoradiogram shown in Figure 24, it can be seen that 115 (lanes 1-3) and 115SL (lane 4) have identical hybridization patterns demonstrating that the mutant strain 115SL is a derivative of the wild type 115.

The mutant is a capsule nonforming, floc nonforming strain producing an exopolysaccharide that does not remain cell bound and is released into the culture broth. Genetic transfer into strain 115SL was shown to be successful by introducing Tn5 on pRK602 via conjugation. Expression of the Tn5 encoded antibiotic resistances was used to select for and demonstrate its presence. The EPS produced by 115SL was shown to contain the same components as that of the parent (see section "Characterization of the 115SL EPS").

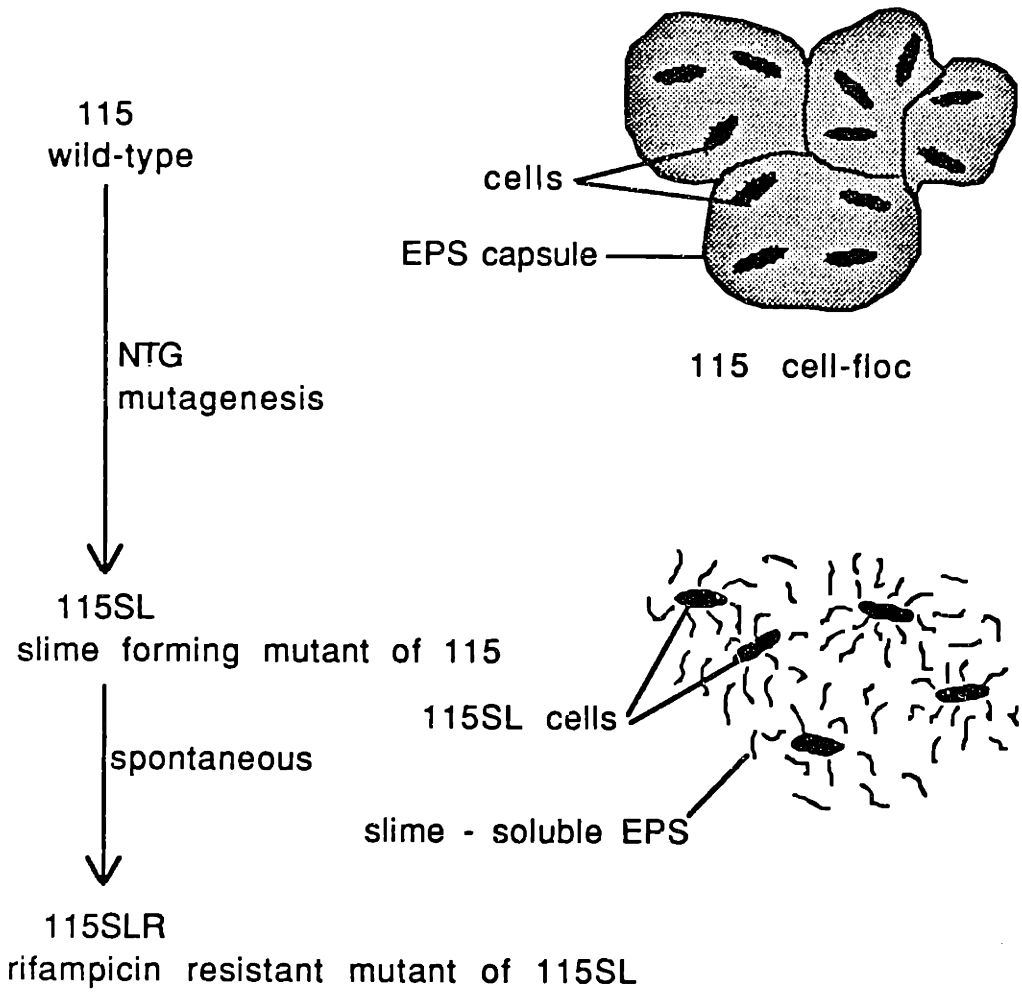


Figure 23. Diagram showing derivation of strains 115SL and 115SLR and schematic respresentation of the nature of the EPS produced by strains 115 and 115SL.

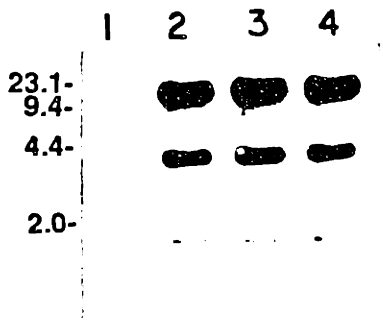


Figure 24. Autoradiogram of ^{32}P-labelled pHP48 DNA hybridized to a Southern blot of EcoRI digested chromosomal DNA (~1-3 μg/lane) from wild-type 115 (lanes 1-3) and 115SL (lane 4). Size markers shown are in kb.

To facilitate further genetic manipulations, a spontaneous rifampicin resistant strain was derived from 115SL and designated 115SLR. The advantage to this strain over 115SL is that it provides a means (rifampicin resistance) to select against the donor strains in experiments involving the conjugal transfer of plasmids and plasmid gene libraries. The frequency of transfer in strain 115SLR is approximately 10^{-4} transconjugants/recipient.

4.5.2 Isolation and Characterization of EPS Mutants

Mutants with altered Cellufluor binding properties were obtained by Tn*5* mutagenesis. Nonfluorescent strains were found at a frequency of 1 in 2×10^3, and some of these still appeared to be producing EPS (as opposed to I-16-M mutans which were deficient in EPS) since they formed slimy, stringy colonies. This was confirmed and EPS from several of these mutants was isolated and given to Dr. Per Foss for analysis (in Professor C.K. Rha's laboratory) to determine what compositional or structural changes have led to the inability to adsorb Cellufluor. Three nonfluorescent strains M1, M3 and M6 were isolated for further study.

Mutants M1 and M6 are similar in colony morphology and texture, however, M3 is quite different from these two. M3 colonies are much less slimy and stringy producing little or perhaps a different EPS. Polysaccharide isolated from the supernatant of mutant cultures indicates that M1 and M6 produce EPS at levels similar to the parent while M3 produces much less.

Purification of EPS using the cationic detergent cetrimide (Leigh *et al.*, 1985), which can be used to precipitate anionic polysaccharides,

demonstrates a striking difference between the EPS produced by M3 and that of M1, M6 and the parent 115SLR. Addition of cetrimide to the supernant of 115SLR, M1 and M6 cultures result in a fine EPS precipitate that must be collected by centrifugation while M3 cultures yield a fibrous precipitate that can be spooled on an inoculating needle and removed from the supernatant all at once.

4.5.3 Complementation of EPS Mutants

A pLAFR3/115 gene library was constructed and introduced into the nonfluorescent mutants and the resulting transconjugants were screened for recovery of fluorescence on plates containing Cellufluor. Fluorescent colonies were isolated for mutants M1, M3 and M6 at a frequency of approximately 2 in 10^3. Fluorescent colonies of M3 also had a colony morphology very similar to the wild-type 115. These colonies were rugose and dome shaped as opposed to the slimy, viscous colonies of 115SLR and complemented mutants M1 and M6. This indicates the complementation of both EPS related mutations of mutant M3, i.e., one NTG induced mutation resulting in the loss of ability to form a capsule, and a second Tn*5* mutation leading to loss of fluorescence on plates containing Cellufluor and the production of EPS which yields a fibrous cetrimide precipitate.

Plasmids restoring fluorescence to M1 (pEX1F) and M6 (pEX6F) were isolated for further characterization as was plasmid pEX3B causing fluorescence and the 115-type morphology in M3.

Rugose, dome-shaped colonies were then screened for in the M1 and M6 recombinants and found at a frequency of approximately 3 in 10^3. In all cases these 115-type colonies were nonfluorescent. The plasmids cloned in

these strains were isolated and designated pEX1D (from M1) and pEX6F (from M6).

Restriction analysis of pEX1F, pEX3B, pEX1D and pEX6F (low yields of pEX6A prevented analysis) revealed that pEX3B, pEX1D and pEX6F contain several fragments of the same size indicating that they contain similar inserts, while pEX1F appears different. Thus, only pEX3B and pEX1F were characterized further. Hybridization analysis showed no homology between these two plasmids.

Cross complementation of the EPS mutants with plasmids pEX1F and pEX3B is summarized in Table 6. These results show that plasmid pEX1F can restore fluorescence to mutants M1 and M6, but not M3, and produces no change in the colony morphology in any of the mutants. Plasmid pEX3B is able to restore fluorescence to mutant M3, but not M1 nor M6, and is also able to restore the wild-type colony morphology to all of the mutants including 115SLR, the immediate parent of M1, M3 and M6, which carries a single mutation leading to the loss of capsule forming ability.

Table 6

Complementation of EPS Mutants with pEX1F and pEX3B

	No Plasmid		pEX1F		pEX3B	
	Fluorescence[a]	Morphology[b]	Fluorescence[a]	Morphology[b]	Fluorescence[a]	Morphology[b]
115[c]	+	+	ND	ND	ND	ND
115SLR	+	–	NA	–	NA	+
M1	–	–	+	–	–	+
M3	–	–	–	–	+	+
M6	–	–	+	–	–	+

NA – not applicable
ND – not determined
[a] ability to fluoresce on Cellufluor plates
[b] recovery of wild-type morphology
[c] wild-type; gene library constructed from this strain

4.5.4 Isolation of the 115 Pyruvyl Transferase Gene

The pyruvyl transferase (pyv) gene of Xanthamonas campestris was obtained from Kelco (Harding et al., 1987) on plasmid pNH232. The pyv gene was subcloned into pUC8 for use as a hybridization probe to detect and identify homologous sequences in Z. ramigera 115 DNA. A restriction enzyme could not be found that excised the pyv gene intact so it was subcloned in two PstI fragments (Figure 25). The pyv gene is known to span the BamHI site and probably the internal PstI site, so both the 1.4 kb and 2.2 kb PstI fragments were cloned into pUC8. These subclones were designated pPYV1.4 and pPYV2.2. Figure 26A shows ^{32}P-labelled pPYV1.4 DNA hybridized to plasmid DNA of pHP27, pEX1F and pEX3B digested with EcoRI (lanes 1-3) and PstI (lanes 4-6). From Figure 26A, lane 6 it can be seen that pEX3B contains a 0.9 kb PstI fragment that is hybridized by pPYV1.4. This 0.9 kb PstI fragment was isolated and subcloned into pUC8 yielding the plasmid pEX0.9 which was labelled with ^{32}P and hybridized to the same Southern blot of pHP27, pEX1F and pEX3B DNAs after the filter was boiled to remove the first probe (Figure 26B). From Figure 26B, lane 6 it can be seen that pEX0.9 hybridizes to the same sized PstI fragment that was hybridized by pPYV1.4.

Additional evidence indicating that this segment of Z. ramigera DNA has homology to the X. campestris pyv gene was obtained by hybridizing both pPYV1.4 DNA and pEX0.9 DNA to Z. ramigera 115 chromosomal DNA. Figure 27 shows a Southern blot of 115 chromosomal DNA digested with BamHI, EcoRI, PstI and SalI (lanes 1-4, respectively) probed with ^{32}P-labelled pPYV1.4 DNA (Figure 27A) and probed with ^{32}P-labelled pEX0.9 DNA (Figure 27B) after boiling to remove the first probe.

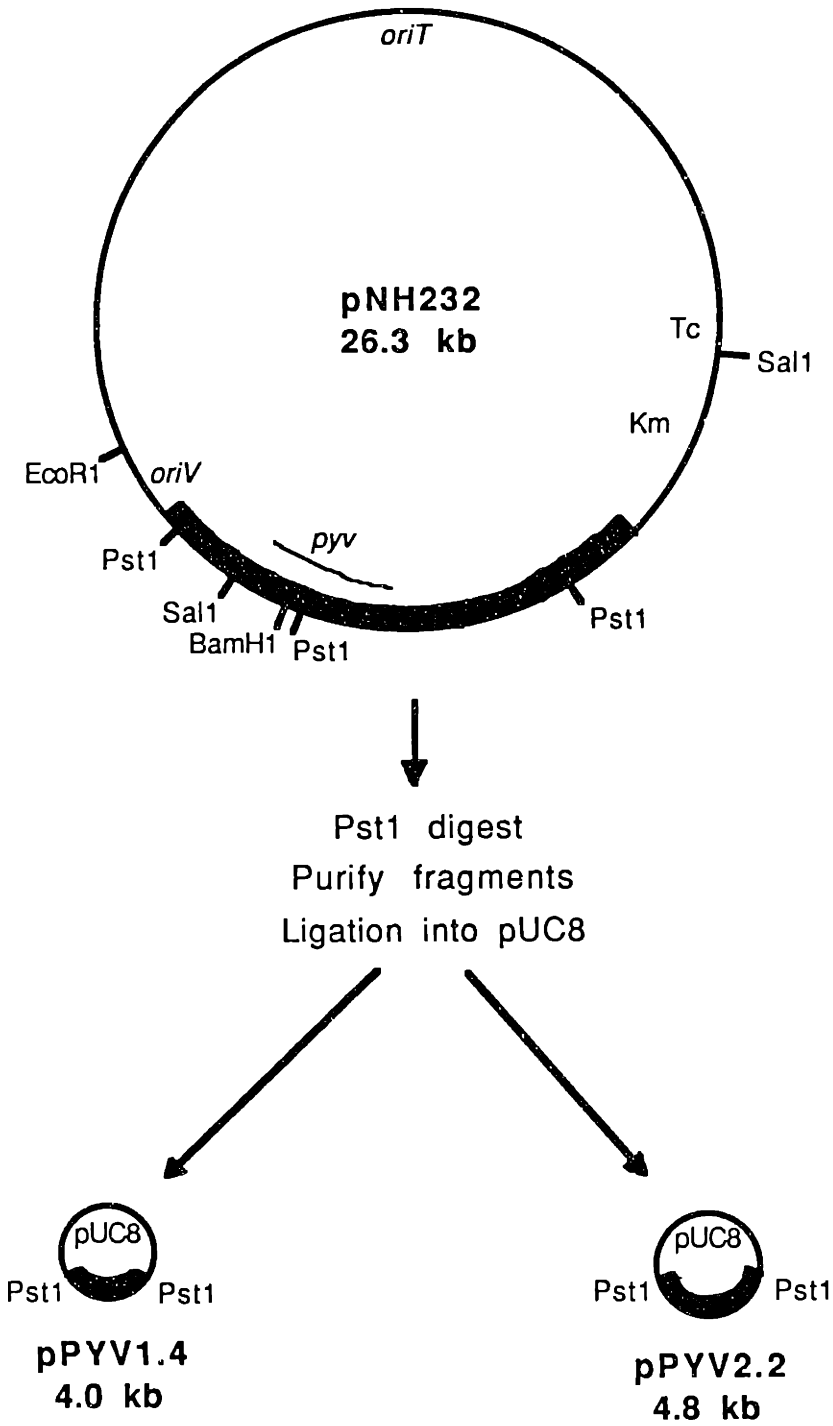


Figure 25. Subcloning of pyv gene from pNH232 into pUC8. Recombinants were designated pPYV1.4 and pPYV2.2.

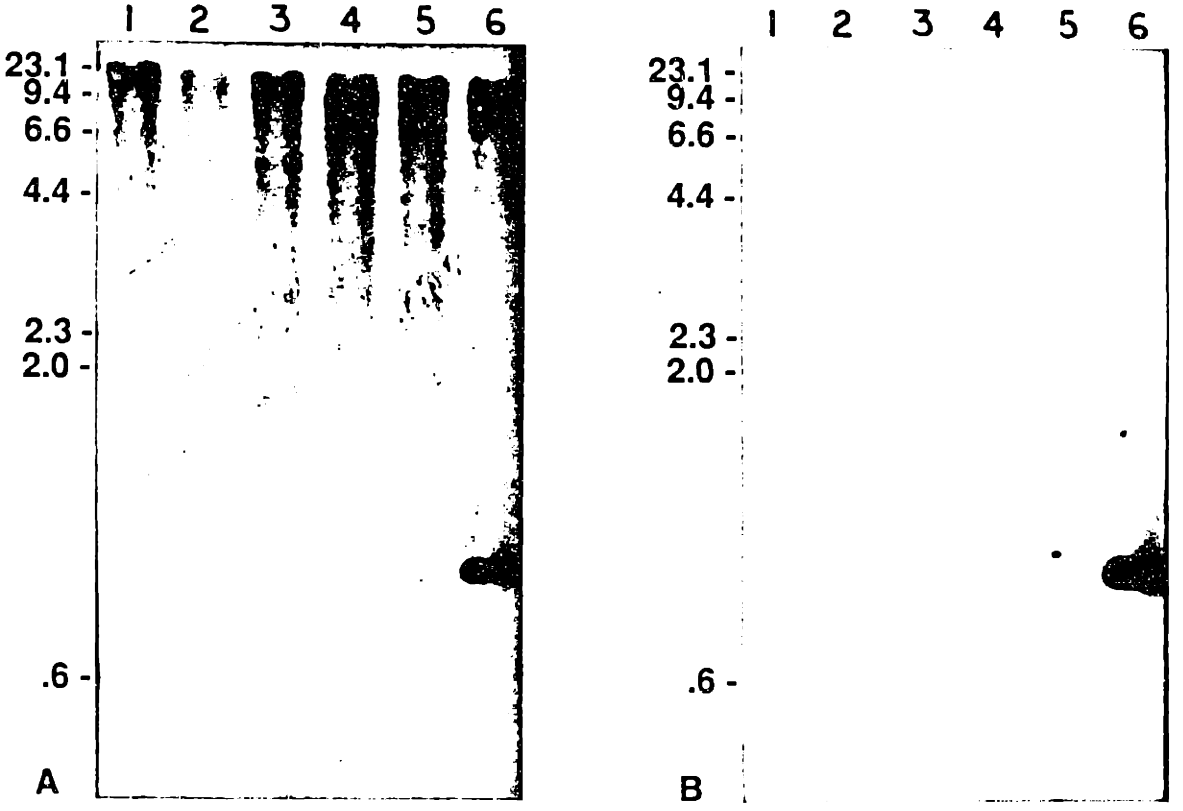


Figure 26. Autoradiograms of ^{32}P-labelled pPYV1.4 DNA(A) and pEX0.9 DNA(B) hybridized to Southern blots of plasmids pHP27, pEX1F and pEX3B digested with EcoRI (lanes 1-3, respectively) and PstI (lanes 4-6, respectively). Size markers are shown in kb.

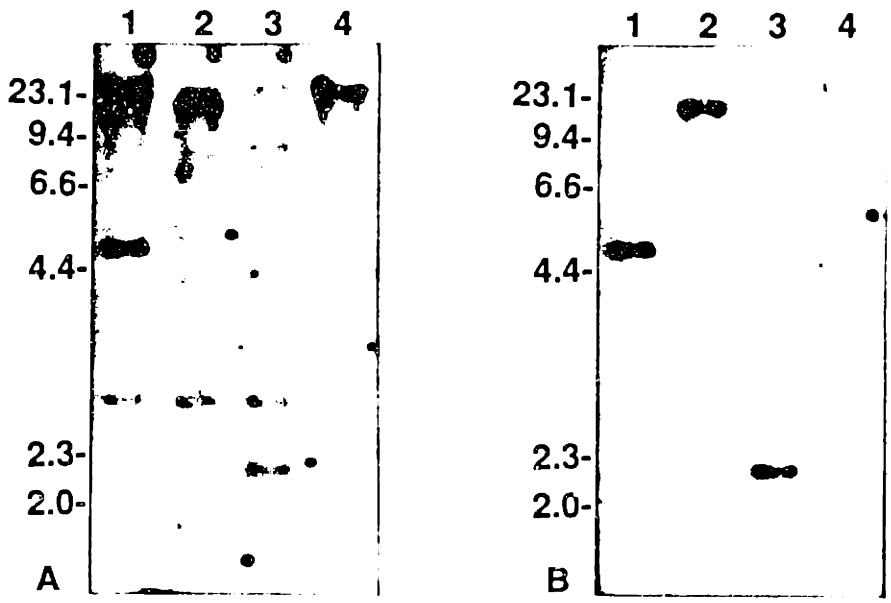


Figure 27. Autoradiograms of ^{32}P-labelled pPYV1.4 DNA(A) and pEX0.9 DNA(B) hybridized to Southern blots of 115 chromosomal DNA digested with BamHI, EcoRI, PstI and SalI (lanes 1-4, respectively). Size markers shown are in kb.

Comparing both cases, it can be seen that the two probes hybridize to the same size fragments in each of the four digests. From these results it can be concluded that the cloned segment of Z. ramigera 115 DNA has sequence homology to the 1.4 kb PstI fragment which contains part of the X. campestris pyv gene.

4.6 Exopolysaccharide Isolation from Z. ramigera 115

An extraction procedure for purifying 115 polymer has been developed and is described in the Materials and Methods section. The hexose sugars content of the purified polymer using different standards is shown in Table 7. Xanthan gum was used as a standard because it is a microbial polysaccharide that is largely hexose sugars and is commercially available in a purified form. The structure of xanthan (Sutherland, 1983) is shown in Figure 1. Samples of 115 polymer gave approximately the same response to the hexose assay as xanthan. Protein contamination in the purified polymer was approximately 3% (Table 8). Thus, it was concluded that the polysaccharide purity is about 97%. The yield of purified polysaccharide was approximately 1 gram/liter culture.

Table 7

Hexose Content of 115 Polysaccharide Using Hexose Assay

Sample (100 μg/ml)	OD(488)	% Hexose Relative to Glucose	% Hexose Relative to Xanthan
115 polymer	0.775	74	103
Xanthan	0.760	72	100
Galactose	0.895	85	-

Table 8

Protein Content in 115 Polysaccharide Using Biorad Assay

Sample	OD(595)	Protein μg/ml	Protein % of Polymer
0.1% 115	0.03	30	3
0.1% Xanthan	0.02	20	2

4.7 EPS Production in Z. ramigera 115 and 115SL

EPS production was compared in strains 115 and 115SL the results of which are shown in Figures 28 and 29. Duplicate flasks for each strain were grown in a high glucose, low nitrogen medium to promote EPS production. Samples were taken throughout the time course and assayed for released EPS and total EPS. Released EPS is defined as EPS in the supernatant after removal of cells, cell flocs and cell-bound EPS. Figure 28 shows the results for the wild-type strain 115 in which it can be seen that total EPS levels off at 2.8 g/liter and released EPS levels off at 0.9 g/liter.

In Figure 29 the results for strain 115SL shows that the curves for both released and total EPS are nearly identical leveling off at 4.5 g/liter. Clearly all of the EPS is being released in the 115SL flasks while less than 1/3 of the total is released in for 115. Also, a 50% higher increase in EPS titer is observed for strain 115SL.

4.8 Chemical Characterization of 115 Exopolysaccharides

4.8.1 EPS of Z. ramigera 115

The monosaccharide composition of the polysaccharide was determined by hydrolysis and identification of the resulting monosaccharides. The optimum conditions that led to complete hydrolysis to monosaccharides without further degradation was treatment with 1 M trifluoroacetic acid at 120°C for between 60 and 75 minutes. The hydrolysate was separated using HPLC and the two major peaks detected were identified as glucose and galactose on the basis of their retention times. Figure 30 shows a 60 minute hydrolysate in which a small amount of unhydrolyzed polysaccharide is still present and is eluted before the glucose and galactose.

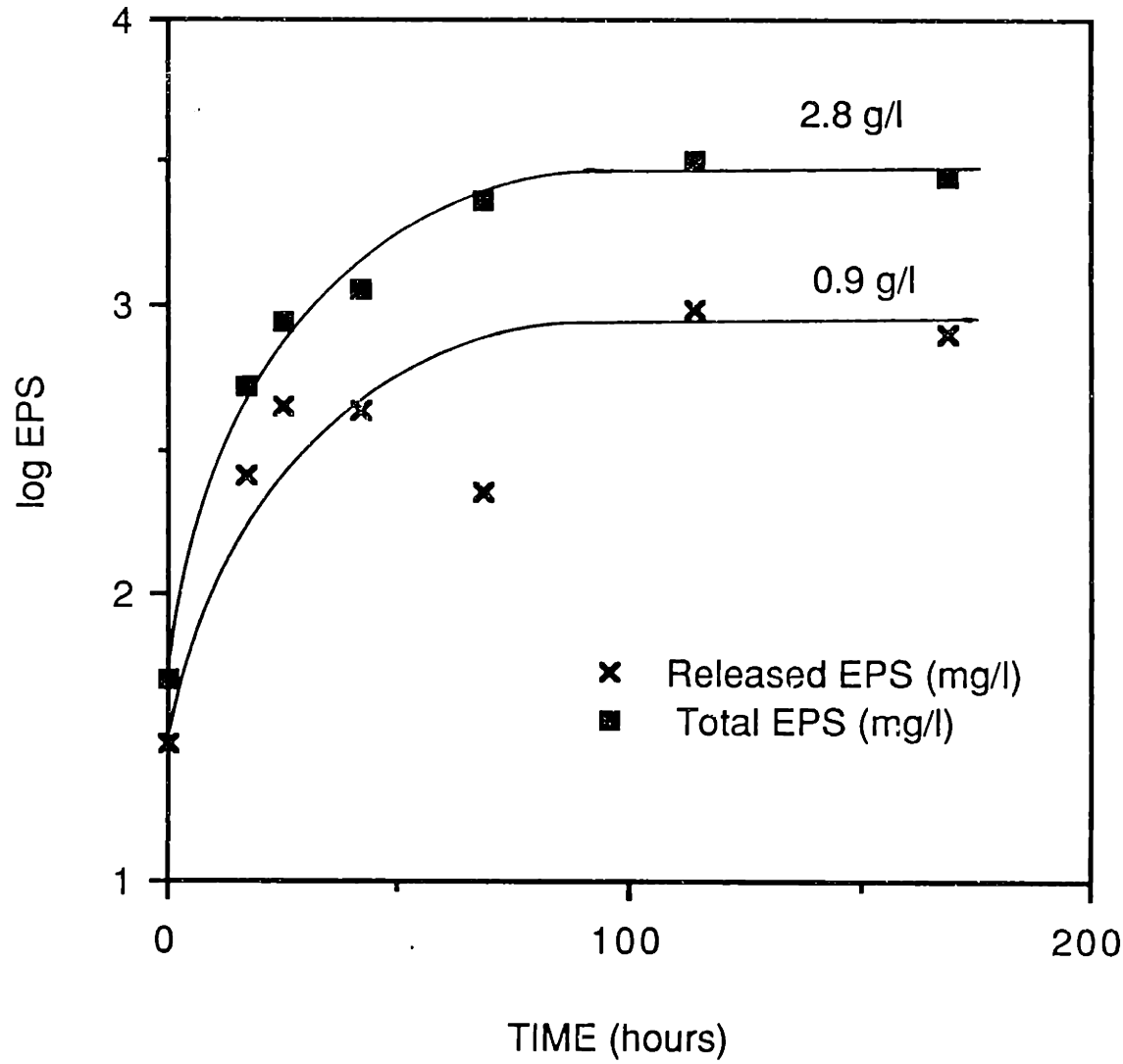


Figure 28. Time course of released EPS and total EPS in shake flask cultures of Z. ramigera 115.

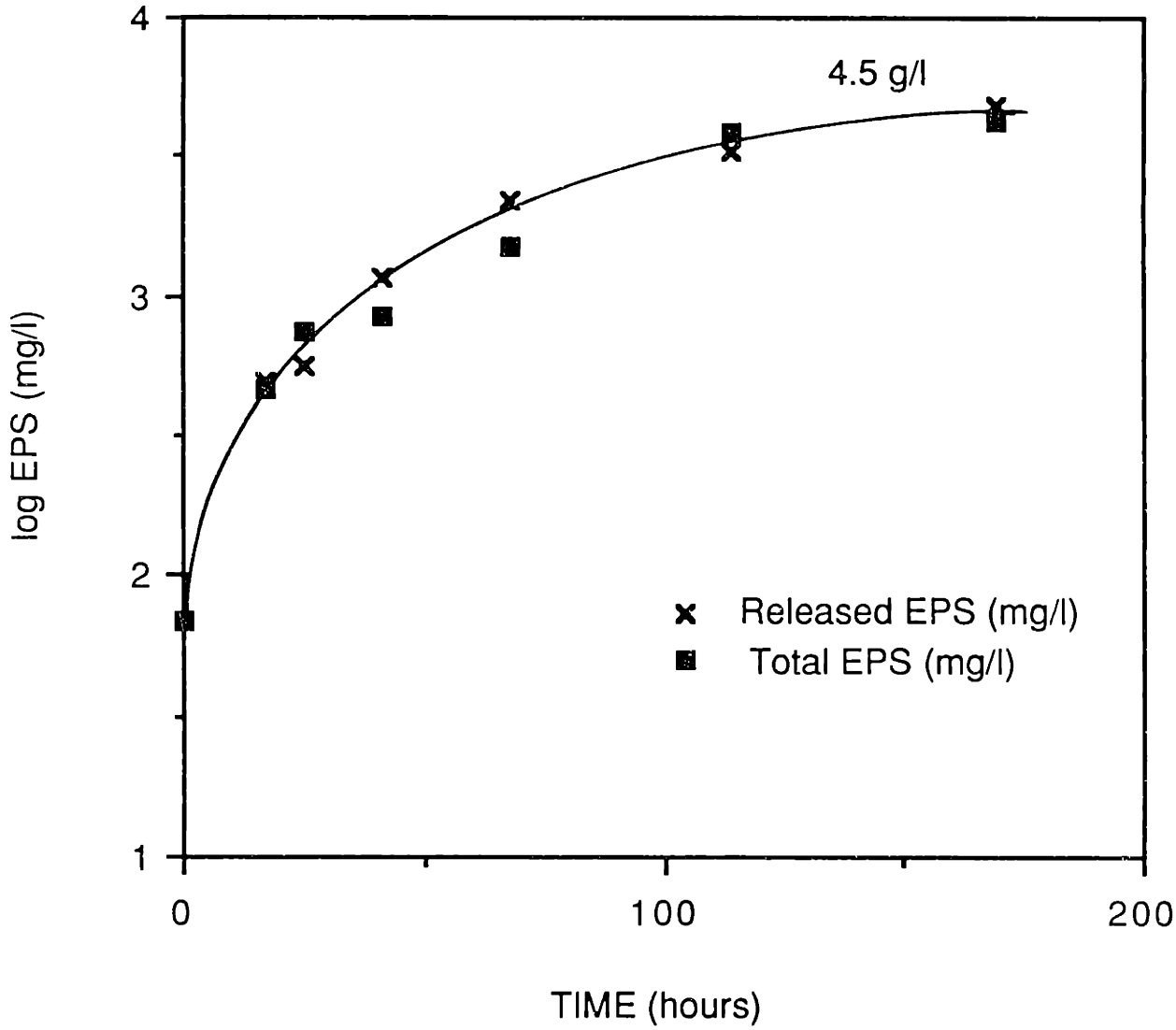


Figure 29. Time course of released EPS and total EPS in shake flask cultures of Z. ramigera 115SL.

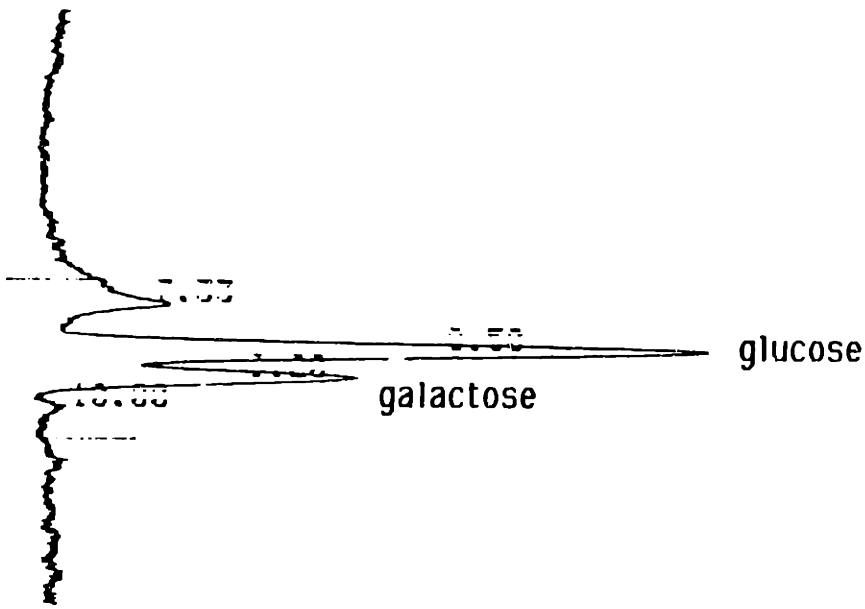


Figure 30. HPLC separation of Z. ramigera 115 polysaccharide hydrolyzed in 1 M TFAA at 120°C for 60 minutes.

Using standard calibration curves for glucose and galactose the relative concentrations of these two components in the polysaccharide was determined to be approximately 2:1, respectively. Acid treatment for 75 minutes achieved complete hydrolysis of the polysaccharide but also resulted in a significant amount of monosaccharide degradation products. However, the glucose to galactose ratio was still approximately 2:1.

These results were confirmed using proton-NMR. Figure 31 shows a partial 500 MHz spectrum of the hydrolyzed 115 polysaccharide and the assignments made to the peaks. The chemical shifts of the anomeric protons were assigned relative to TMS as follows; α-glucose at 5.25 ppm, α-galactose at 5.28 ppm, β-glucose at 4.66 ppm, and β-galactose at 4.60 ppm. Integration of the peaks confirms a glucose to galactose ratio of approximately 2:1, respectively.

An infrared scan of the 115 polysaccharide is shown in Figure 32 and is nearly identical to that published by Friedman et al. (1968). Functional group assignments to the peaks were made as follows: OH, 2.93 μm; C-H, 3.43 μm; C=0 of an ionized carboxyl, 6.15 μm and 7.15 μm; tertiary CH-OH, 8.65 μm; saccharide ring, 9.5 μm. The reported presence of pyruvatic acid in the polysaccharide (Ikeda et al., 1982; Frazen and Norberg, 1984) allowed the assignment of the peak at 6.15 μm and 7.15 μm to the C=0 of the ionized carboxyl group of this moiety.

Evidence that Z. ramigera 115 produces a single water soluble exopolysaccharide is shown in Figure 33 in which HPSEC chromatograms of culture supernatant and purified polysaccharide are compared. Figure 33A shows a chromatogram of the supernatant of a 6 day culture that was sonicated to disperse cell flocs and Figure 33B shows a chromatogram of the purified polysaccharide. Both samples yield a single large molecular weight peak of similar shape and retention time.

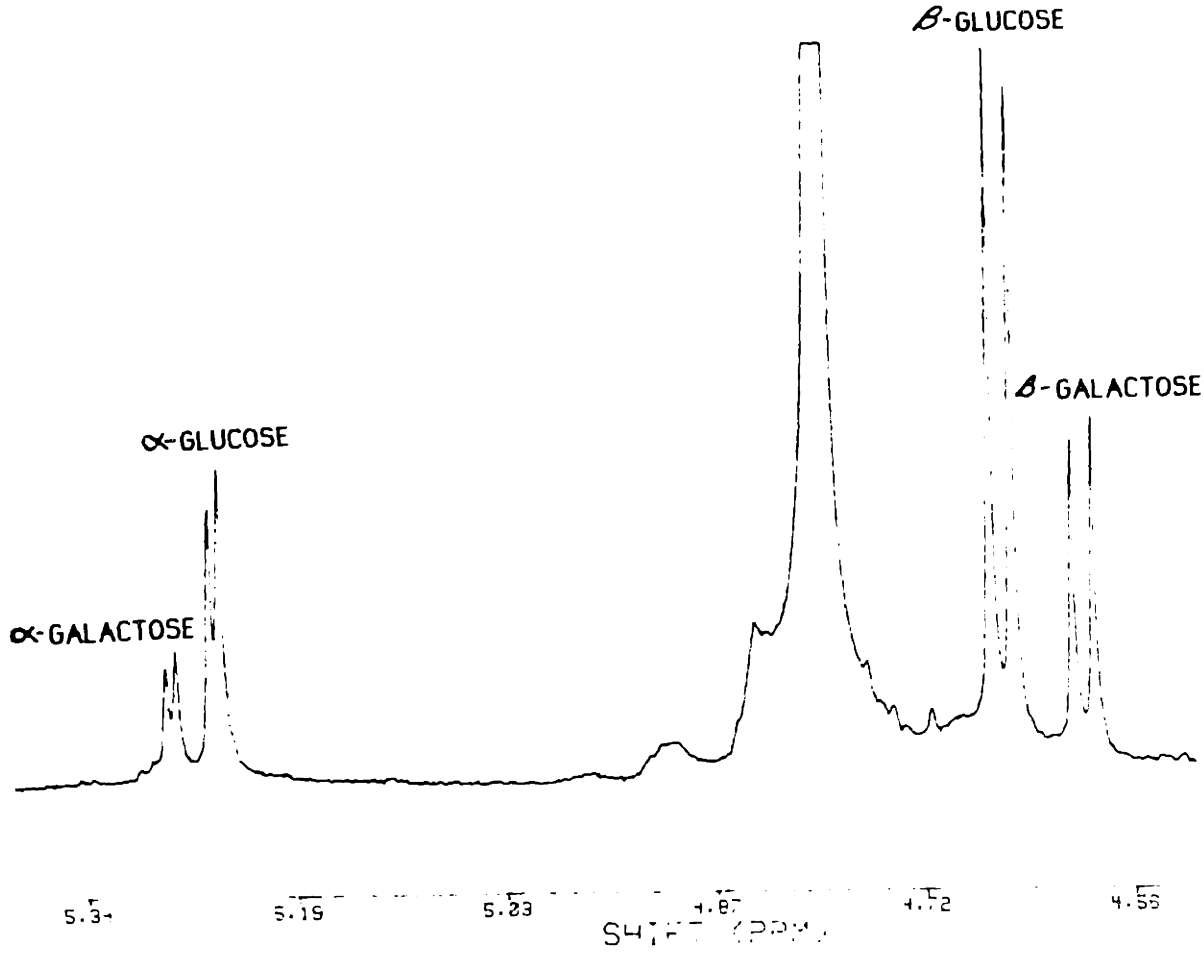


Figure 31. Partial 500 MHz ^{1}H-NMR spectrum of Z. ramigera 115 exopolysaccharide hydrolysate.

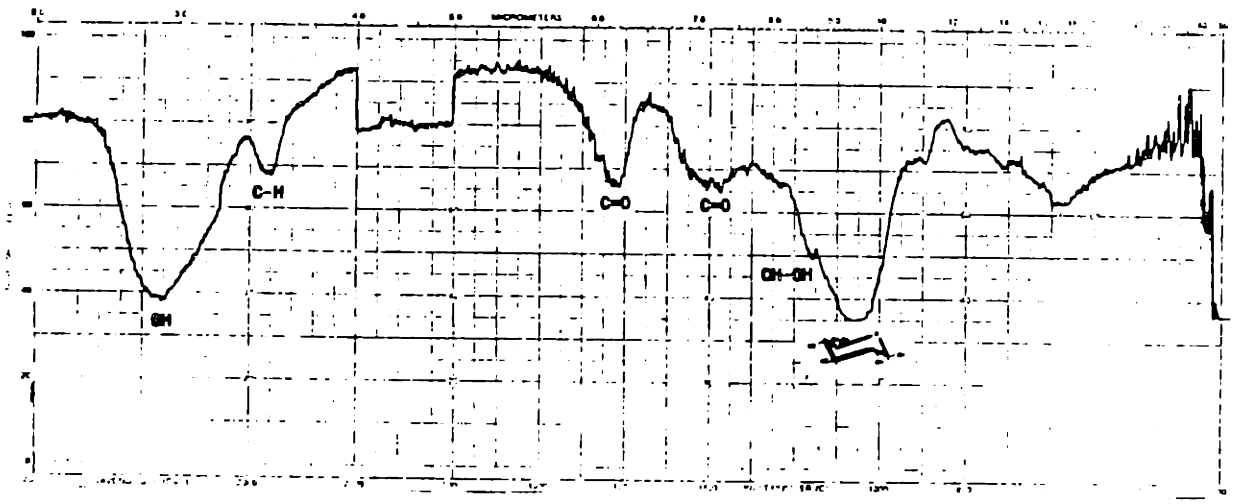


Figure 32. Infrared spectrum of Z. ramigera 115 exopolysaccharide.

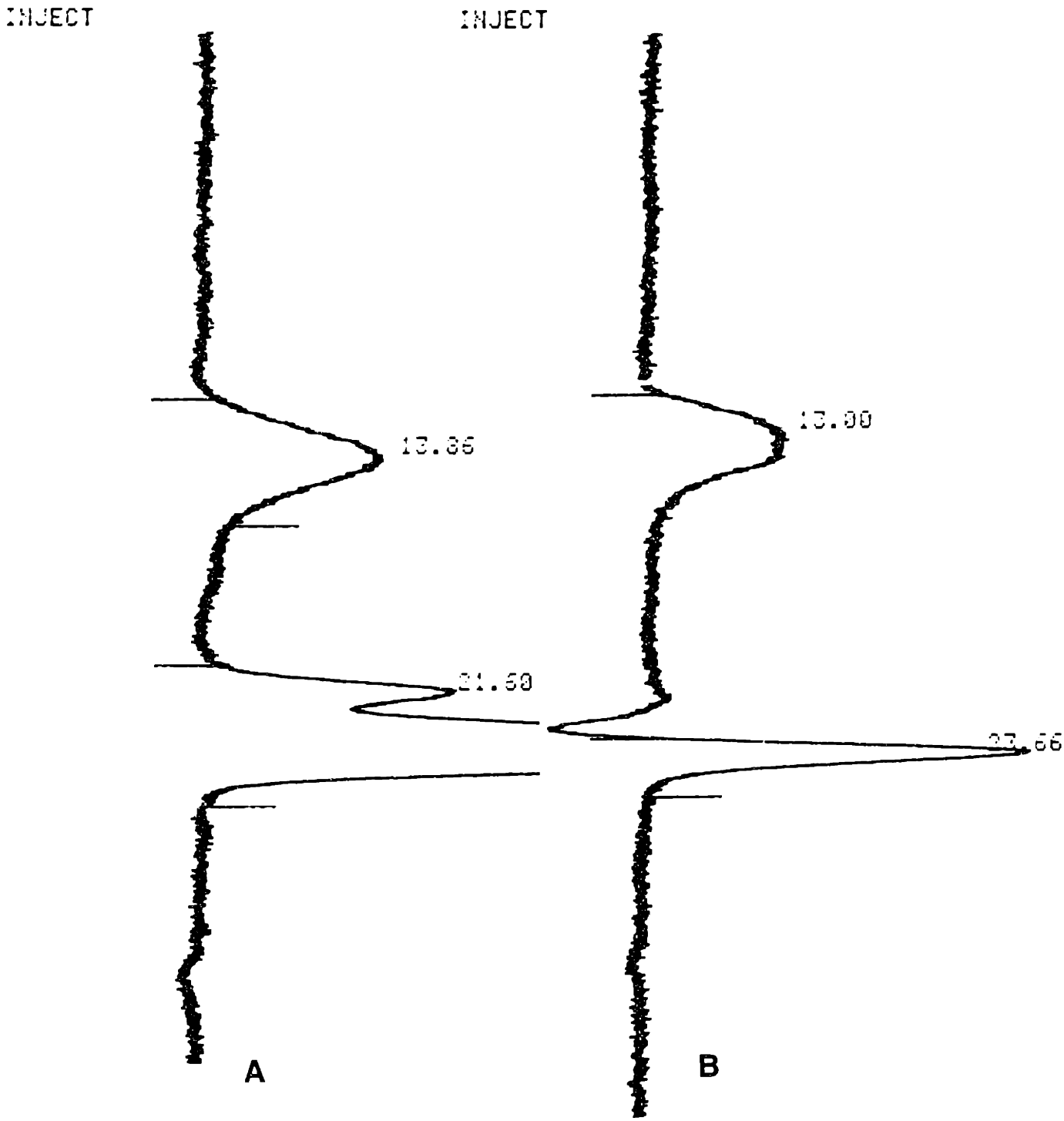


Figure 33. HPSEC chromatograms of Z. ramigera 115 culture supernatant after sonication to disperse cell flocs and release the exopolysaccharide (A) and the purified exopolysaccharide (B).

4.8.2 EPS of Z. ramigera 115SL

HPLC monosaccharide analysis showed that the EPS isolated from 115SL has the same glucose:galactose ratio as that of the parent 115, i.e., 2:1 glucose:galactose. It was also shown that the 115SL EPS contains pyruvate but at a higher relative percentage of the total EPS. Pyruvate was quantitated by enzymatic assay and ^{1}H-NMR by Dr. Per Foss (Professor Rha's laboratory). The pyruvate content was found to be 25 to 60% higher in the 115SL EPS than in the 115 EPS. A very important result from the ^{1}H-NMR analysis of the 115SL EPS was the identification of O-acetyl groups which have not previously been reported for the 115 EPS. Additionally, high pressure size exclusion chromatography of the 115SL EPS revealed a single high molecular weight peak of similar shape and retention time, as compared to the 115 EPS (Figure 34).

4.9 Rheological Characterization of the 115 EPS

The rheological properties of a polymer must be studied to characterize it completely. Viscosity studies have been performed on the polymer of isolate 115. The apparent viscosity of the 115 polymer as a function of concentration is shown in Figure 35. Apparent viscosity was measured at a fixed shear rate of 1.5 rpm on a Brookfield LVT viscometer using a UL adapter. A plot of apparent viscosity as a function of shear rate for a 0.3% solution of the 115 polymer indicates shear thinning or pseudoplastic behavior (Figure 36). Similar curves are obtained for other 115 polymer solutions at concentrations between 0.1 and 0.5%.

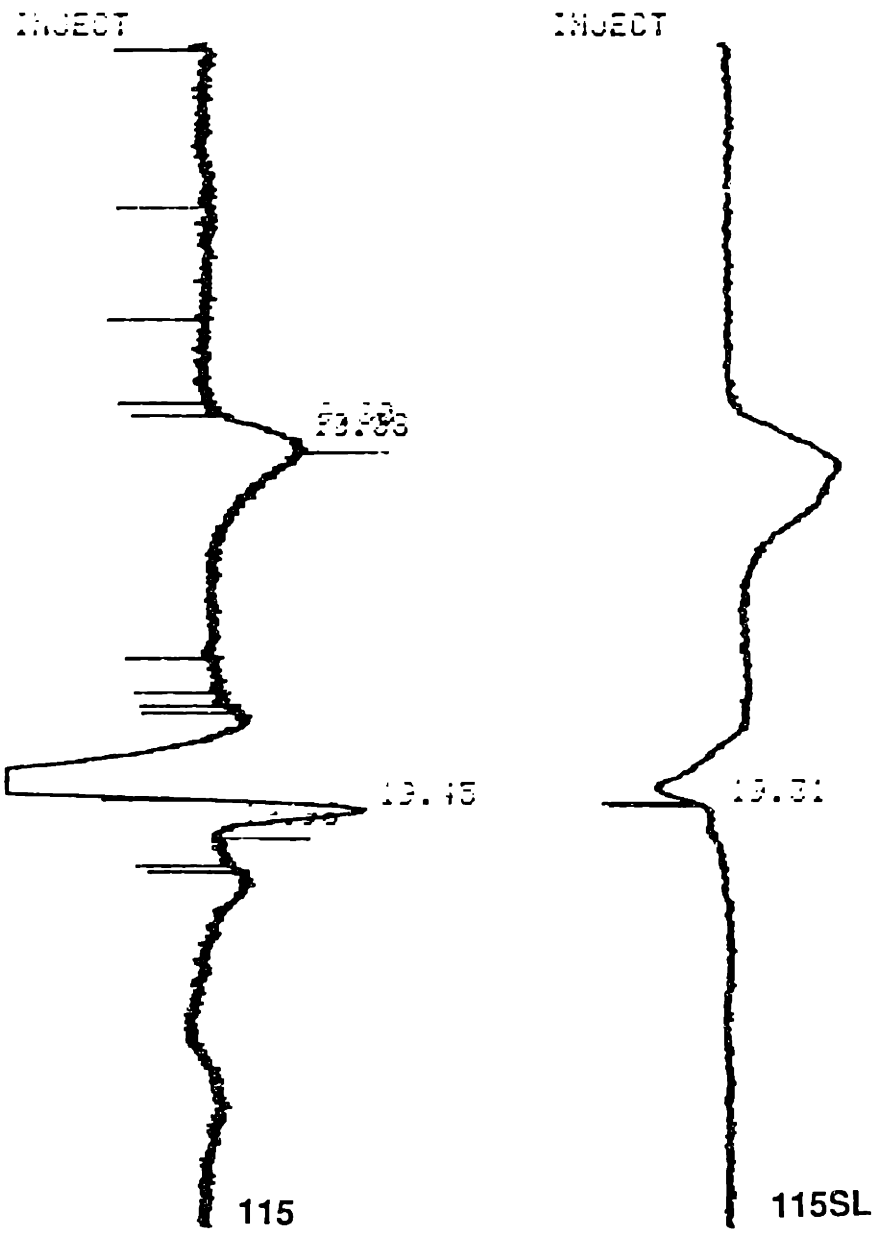


Figure 34. HPSEC chromatogram of purified exopolysaccharide of Z. ramigera 115 (A) and 115SL (B).

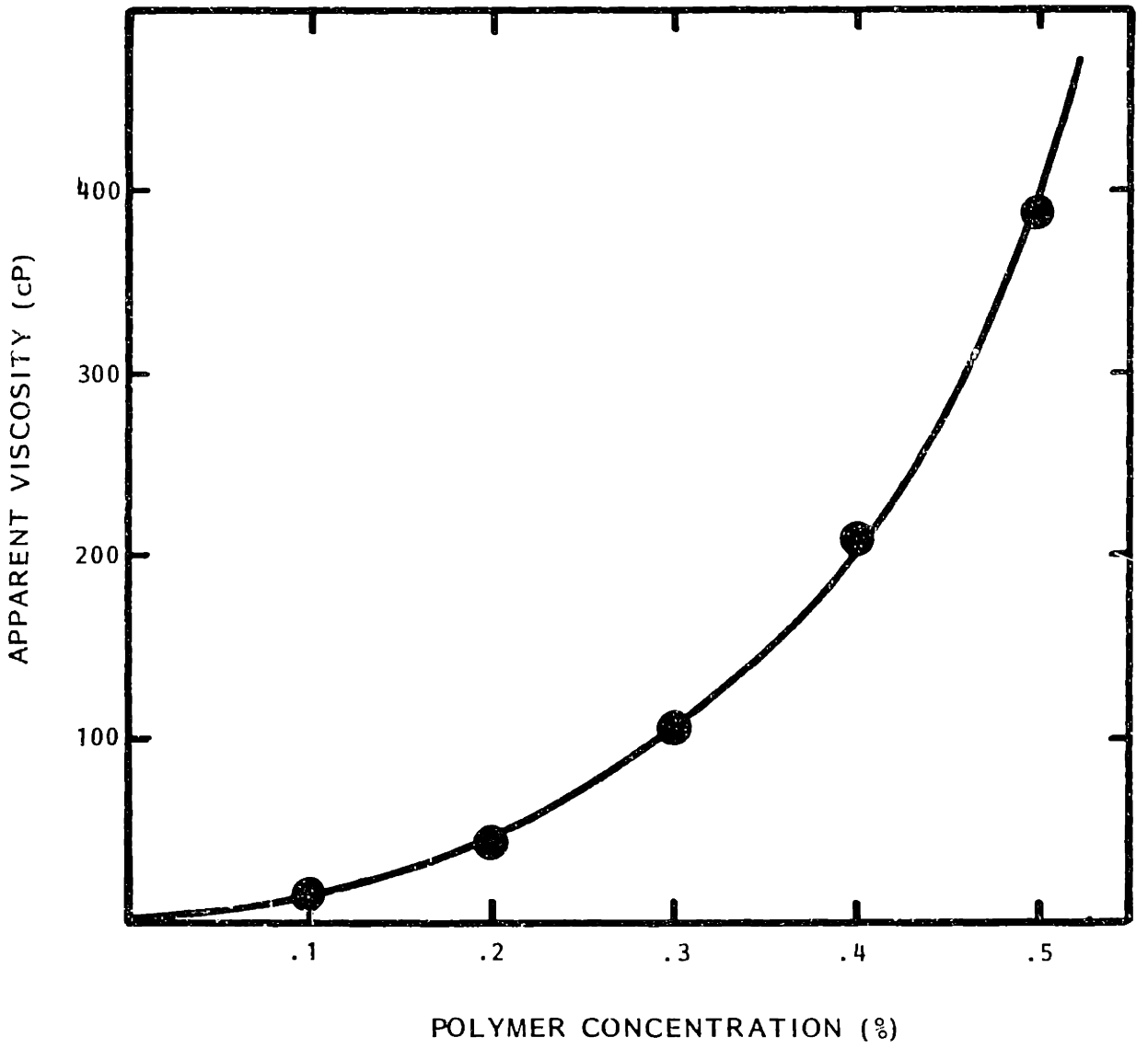


Figure 35. Apparent viscosity as a function of Z. ramigera 115 exopolysaccharide concentration. Measured using a Brookfield LVT viscometer with UL adapter at 1.5 rpm (46).

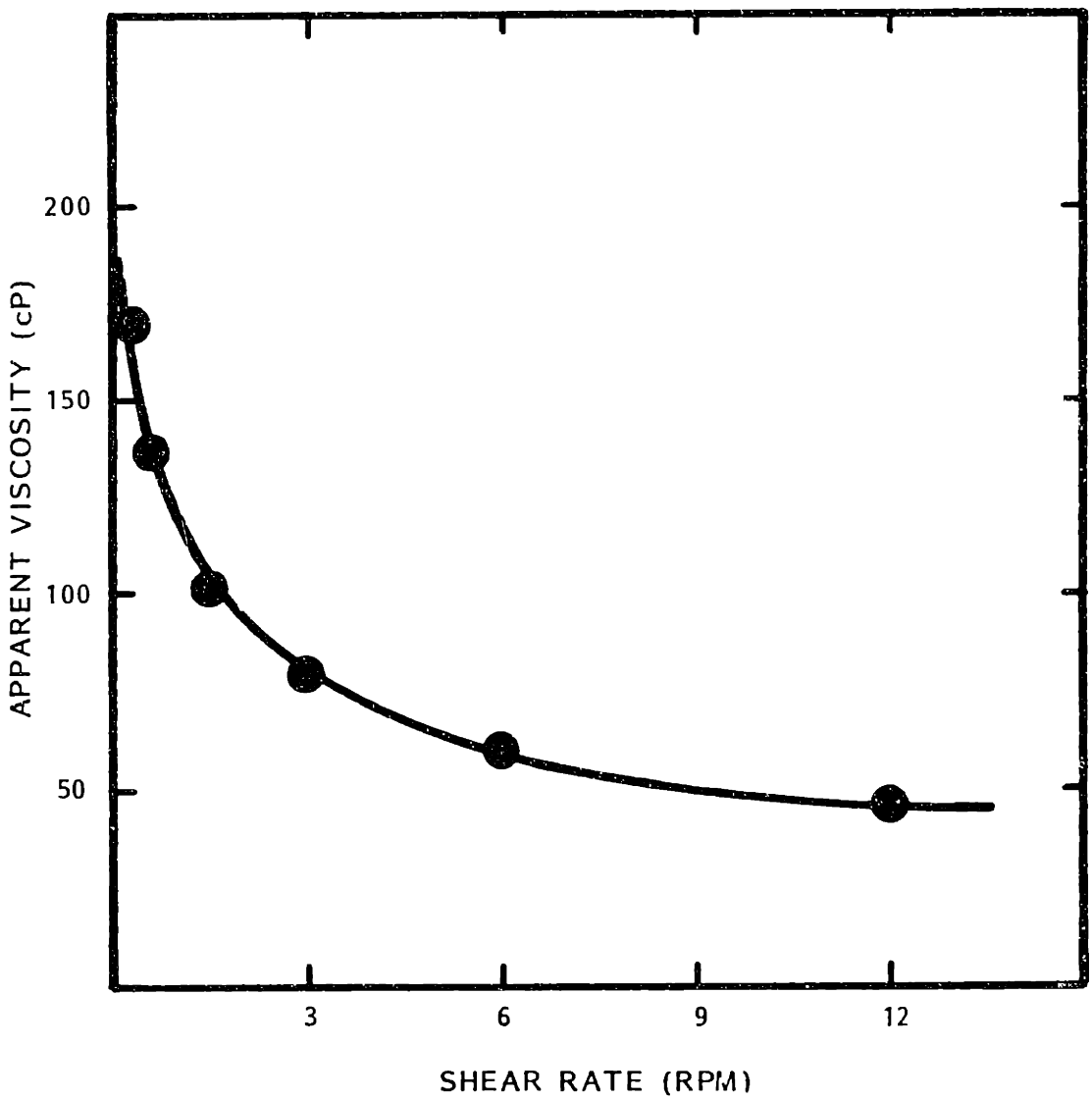


Figure 36. Apparent viscosity of a 0.3% Z. ramigera 115 polysaccharide solution as a function of shear rate. Measured using a Brookfield LVT viscometer with UL adapter (46).

When the polymer concentration is 1% or above, the solutions will gel at low shear rates and exhibit plastic flow behavior with a certain yield stress point at which they become fluid. Also, 1% polysaccharide solutions showed viscoelastic behavior by rising around the axis of a rotating stir-bar. This phenomenon, which denotes viscoelasticity, is known as the Weissenberg effect and is caused by an unbalanced stress action in the direction normal to the plane of flow and (Stauffer et al., 1980).

5. DISCUSSION

Bacterial exopolysaccharides make up a very important, diverse group of biopolymers found throughout nature. Despite this widespread existence and the integral, but varied, roles that these polymers play in the survival of many species of bacteria, relatively few have been investigated in much detail. Only recently has the isolation and characterization of exopolysaccharide biosynthetic genes been a focus of research, however, significant progress has been made in several exopolysaccharide producing bacteria. Most notable are investigations into alginate production by Pseudomonas aeruginosa for its role in cystic fibrosis (Darzins and Chakrabarty, 1984; Darzins et al., 1985a; Darzins et al., 1985b; Goldberg and Ohman, 1984), xanthan production by X. campestris to increase yields and improve the quality of xanthan (Harding et al., 1987), and the production of exopolysaccharides by R. melilotti for their part in the symbiotic nitrogen fixation process (Finan et al., 1985; Leigh et al., 1985). Summarized in this report has been work towards a long term objective in which it is planned to isolate and identify the genes responsible for exopolysaccharide biosynthesis in Z. ramigera. This will lead to the determination of the biosynthetic pathway, any control mechanisms it might be subject to and ultimately to the genetic engineering of novel polysaccharide structures.

5.1 Cellufluor as a Polysaccharide Stain

Cellufluor was shown to bind specifically to β(1-3) and β(1-4) linked glucose polymers. This dye does not bind to α(1-4) or α(1-6) linked glucose polymers nor to a β(1-6) linked acetyl glucose (pustulan). Cellufluor was also able to bind some complex polysaccharides containing

β(1-4) and β(1-3) linkages (Zooglan 115 and chitosan) but did not bind to others (xanthan and alginate). From this study it is clear that the presence of β(1-3) or β(1-4) linkages is required for the dye binding capability of a particular polysaccharide, however, the presence of these bonds does not always provide that Cellufluor will bind. Monosaccharides other than glucose, the presence of functional groups, or a high degree of branching could interfere with binding. These effects on binding capability could enable the use of Cellufluor to detect compositional or structural changes in a polysaccharide on the basis of its dye binding properties.

5.2 Genetic Manipulations in Z. ramigera

A strategy for isolating the Z. ramigera polysaccharide biosynthetic genes was devised and successfully carried out in strains I-16-M and 115. In doing so, some powerful genetic techniques were successfully applied to this genetically unstudied organism. A genetic transfer system was utilized which requires the conjugal properties of pRK2013 and a broad host range cloning vector derived from RK2, yielding a frequency of transfer of approximately 10^{-3} transconjugants/recipient for I-16-M and 10^{-4} transconjugants/recipient for 115SL. Table 9 compares this with conjugation frequencies from other Gram-negative organisms using vectors also derived from RK2. The transfer of genetic material by conjugation enabled the application of a transposon mutagenesis procedure using Tn5. Table 10 shows a comparison of transposition frequencies for Z. ramigera and R. meliloti. The Tables illustrate the successful application of these techniques to Z. ramigera at frequencies comparable to those found in the literature.

Table 9

Conjugation in Gram-Negative Bacteria

Strain	Plasmid	Frequency	Reference
Escherichia coli	pRK290	0.4	Ditta *et al.*, 1980
Pseudomonas aeruginosa	pRK290	0.3	Ditta *et al.*, 1980
Xanthomonas campestris	pLAFR1	10^{-4}	Daniels *et al.*, 1984
Rhizobium meliloti	pRK290 pLAFR1	0.05 0.9	Ditta *et al.*, 1980
Acinetobacter calcoaceticus	pRK290	10^{-3}	Ditta *et al.*, 1980
Zoogloea ramigera I-16-M	pLAFR3	10^{-3}	Easson *et al.*, 1987b
Zoogloea ramigera 115SL	pLAFR3	10^{-5}	Easson *et al.*, in press

Table 10

Tn5 Delivery in Gram-Negative Bacteria

Strain	Vector	Frequency	Reference
Rhizobium meliloti	pJB4JI (pPH1::Mu::Tn5)	10^{-5}	Meade *et al.*, 1982
Z. ramigera I-16-M	pRK602 (Tn5 donor)	10^{-5}	Easson *et al.* 1987b
Z. ramigera 115SL	pRK602 (Tn5 donor)	10^{-6}	Easson *et al.*, in press

5.3 Cloning of Z. ramigera I-16-M EPS Biosynthetic Genes

5.3.1 Identification of exo Complementation Groups

Tn5 insertions in five Eps$^-$ mutants (T24, T27, T28, T33, T44; Table 1) were located within two closely linked EcoRI fragments of size 4.0 kb and 7.6 kb (Figure 22). The five insertions span approximately 5 kb and comprise at least two complementation groups, which indicates the clustering of genes involved in EPS biosynthesis within this region. Clustering of exopolysaccharide genes appears to be a common trait and has been reported in P. aeruginosa (Darzins et al., 1985b), X. campestris (Harding et al., 1987) and R. melilotti (Leigh et al., 1985). At this point the number of genes located here has yet to be determined.

5.3.2 Genetic Instability in the exo Loci

Within the EPS gene region in Z. ramigera I-16-M there appears to be some inherent instability that leads to the loss of EPS production. What form this instability takes is not known, but it is clear that I-16-M chromosomal DNA sequences in this region spontaneously delete causing the loss of function of one or more EPS genes. The presence of Tn5 increases the frequency of chromosomal deletions in the EPS gene region from 1 in 10^4 for spontaneous deletions to 1 in 2×10^2 for deletions in Tn5-containing strains. It is possible in these cases that Tn5 originally inserted into or near the EPS gene region causing greater instability and deletions in adjacent sequences. Alternatively the Tn5 inserted elsewhere in the chromosome may provide some activating function that increases the frequency of the spontaneous event.

Instability in EPS production has been reported for several organisms including P. aeruginosa (Darzins and Chakrabarty, 1984; Goldberg and Ohman, 1984)), X. campestris (Sutherland, 1983) and Z. ramigera (Farrah and Unz, 1976). This appears to be a trait commonly found in pseudomonads, the genetic cause of which could be a general phenomenon. If instability exists as a regulatory mechanism then the organism could have some control over the expression of the EPS pathway and perhaps give the population a selective advantage under certain environmental conditions. Such types of regulation by genetic rearrangement, although rare, have been reported. For example, in Neisseria gonorrheae, pilus expression involves chromosomal rearrangement (Meyer et al., 1982); genetic rearrangements in a symbiotic plasmid of Rhizobium phaseoli were found in strains that had lost the symbiotic phenotype (Sobern-Chavez et al., 1986); and genes involved in isopropylbenzene metabolism in Pseudomonas putida RE204 are lost by spontaneous deletion of a 20 kb region (Eaton and Timmis, 1986). In fact, a control mechanism involving genetic rearrangement was implied to occur with alginate production in P. aeruginosa (Darzins and Chakrabarty, 1984). An explanation for this instability in Z. ramigera I-16-M may be found by characterizing the sequences at the termini of the deletions to determine if a large insertion element is responsible.

5.4 Cloning of Z. ramigera 115 EPS Biosynthetic Genes

5.4.1 Isolation of Z. ramigera 115SL

To achieve conjugation in strain 115 a capsule non-forming strain was isolated via NTG mutagenesis. This strain, designated 115SL, produces a novel EPS (increased pyruvate content) and has several desirable characteristics when compared to the parent strain. These include:

decreased lag phase; increased growth rate; more consistent growth and EPS production properties; increased polysaccharide titer; improved EPS separation; and the ability to receive foreign DNA via conjugation.

5.4.2 Conjugation in 115SL and Cloning of EPS Genes

A very important characteristic of strain 115SL is that it is able to receive foreign DNA via conjugation, unlike strain 115 in which this has been unsuccessful. To facilitate this conjugation and provide a means of selection for Z. ramigera, a rifampicin resistant mutant was isolated and designated 115SLR. Tn5 mutagenesis of 115SLR yielded several mutants which do not fluoresce on Cellufluor plates. These mutants may produce an exopolysaccharide that has an altered structure or produce an altered ratio of a family of exopolysaccharides resulting in the inability to adsorb Cellufluor. EPS from mutant strains was provided to Professor C.K. Rha's laboratory so that these theories could be investigated.

Mutant M3, in addition to having lost the ability to fluoresce on Cellufluor, produces much less EPS than 115SLR. This EPS also behaves much differently when treated with cetrimide. Addition of cetrimide to supernatants of 115SL, 115SLR, M1 and M6 cultures results in a fine dispersed EPS precipitate but yields a fibrous precipitate when added to a M3 culture supernatant. Also, ^{1}H-NMR analysis (performed by Per Foss) of the M3 EPS gives a quite different spectrum when compared to that of the 115SL EPS. Such drastic differences between parent EPS and mutant EPS are probably not due to a mutation but may be explained by the production of two or more EPS structures by the same strain. Under this hypothesis mutant M3 presumably has lost the ability to produce the Cellufluor binding EPS, which represents the major fraction, but continues to make a less significant fraction

(in terms of relative amounts) that exhibits different cetrimide precipitant properties and ^{1}H-NMR spectrum when compared to the parent EPS.

Complementation of these EPS mutants with a 115 gene library has resulted in the isolation of several genes involved in EPS biosynthesis, some of which appear to be closely linked as we have already seen in Z. ramigera I-16-M. The results of restriction, hybridization and complementation studies of pEX1F and pEX3B lead to the hypothesis that mutations in at least two complementation groups have occurred. One complementation group represented by Tn5 insertions in M1 and M6 which are either in the same complementation group or two closely linked complementation groups. The present data do not indicate mutations in two complementation groups since they have the same phenotype with respect to one another when either no plasmid, pEX1F or pEX3B is present.

Another complementation group, or perhaps two closely linked complementation groups, is represented by the Tn5 insertion in M3 and the NTG mutation in all of the strains (except wild-type 115). One possibility is that both mutations lie in the same complementation group. Strains with only the NTG mutation may produce an altered protein or enzyme resulting in loss of capsule forming ability. This single mutation can be complemented with pEX3B. Under this hypothesis strain M3, in addition to the NTG mutation, would also carry a Tn5 insertion in the same complementation group thereby inactivating a single gene or series of genes within that complementation group, possibly resulting in the loss of production of the major EPS fraction. This insertion can also be complemented by pEX3B restoring wild-type function to this complementation group.

A second possibility is that the NTG mutation and the Tn5 insertion lie in different, but linked, complementation groups. This could be

confirmed, one way or the other, subcloning pEX3B and attempting to complement each mutation individually.

5.4.3 Isolation of the Z. ramigera pyv Gene

Hybridization experiments with the X. campestris pyv gene has resulted in the isolation of a segment of Z. ramigera 115 DNA with homologous sequences. The cloning of this region (presumably containing the Z. ramigera 115 pyv gene) onto pLAFR3 and its introduction into 115 strains may result in an increased pyruvate content of the polysaccharide. It would also be useful to clone this segment into an E. coli expression vector and assay for pyruvyl transferase activity.

5.5 Z. ramigera 115 Exopolysaccharide Characterization

Isolation of purified polysaccharide from strain 115 has been achieved, the purity defined and the monosaccharide composition determined. The results indicate that glucose and galactose are present in a ratio of approximately 2:1 confirming the data of Franzen and Norberg (1984) but somewhat different from the 11:3 glucose to galactose ratio reported by Ikeda et al. (1982). Franzen and Norberg (1984) hypothesized that this discrepancy was due to differences in the growth conditions used or a difference in the Z. ramigera strain.

Infrared analysis indicated that the polymer is a polysaccharide that contains ionized carboxyl groups and resulted in a "fingerprint" scan of the 115 polysaccharide.

The monosaccharide composition data and the IR "fingerprint" scan of the polysaccharide can be used to compare the composition and IR scans of

polysaccharides from mutant or genetically manipulated strains to detect changes in composition and structure.

HPSEC of the Z. ramigera 115 exopolysaccharide reveals a single large molecular weight fraction that gives a similar peak shape and retention time in both culture supernatant samples and purified preparations. This indicates that a single water soluble exopolysaccharide species is being produced, or more accurately stated, does not give any indication that more than one EPS is present. This does not preclude the possibility that other exopolysaccharides may be present. For example, water insoluble polysaccharides, such as cellulose, would not be detected using HPSEC and would not be present in the purified preparations which use water solubility as a basis for separation from other components. Also, exopolysaccharides that are produced in low amounts relative to the total may go undetected, or that are similar in structure may be unresolved by analytical techniques.

5.6 Z. ramigera 115SL Exopolysaccharide Characterization

Compositional analyses of the exopolysaccharide produced by 115SL demonstrate that there is no difference between its EPS and the EPS of strain 115 with respect to monosaccharide composition. However, results indicate an increase in the pyruvate content in the EPS from 115SL, and for the first time the presence of O-acetyl groups have been detected in this EPS. This has not been previously reported for the Z. ramigera 115 EPS probably due to the fact that all previous investigators used an alkali treatment in their isolation procedures. Since O-acetyl groups are alkali labile its presence has gone undetected. A second, but highly unlikely, explanation is that the O-acetyl groups are a result of the mutation causing loss of capsule forming ability. Presumably this is not the case.

5.7 Growth and EPS Production in 115SL

The new strain, 115SL, has improved culture properties as compared to the parent 115. A characteristic of strains 115SL is that they do not flocculate during growth, unlike 115 which forms large cell flocs during the growth phase. This gives it the advantage of having more consistent and reproducible growth cycles and increases growth rate. This is probably due to the fact that cells are not trapped within a floc where they may grow at a reduced rate or die because of nutrient starvation. From this it follows that polysaccharide production should be more consistent, and it was shown that EPS titers are increased in 115SL.

Another characteristic of strain 115SL is that it does not produce an EPS capsule layer like that of strain 115. Instead the EPS is excreted as a slime layer and is not confined to the immediate area surrounding the cells. Because the EPS does not remain cell bound in 115SL, separation of the EPS from the cells is much easier than has previously been experienced with 115. The fact that 115SL produces a cell-nonbound and does not flocculate has resulted in a simplified EPS isolation procedure. Isolation of EPS from 115 first requires that it be dissociated or solubilized away from the cell-floc-capsule matrix. This is accomplished by an alkali treatment that also results in cell lysis and protein contamination of the EPS. With strain 115SL the cells can be removed from the fermentation broth by centrifugation eliminating the alkali treatment and protein removal steps. This shorter and faster procedure results in a higher EPS recovery and purity. In a production facility this will result in lower separation costs and a more economical EPS product.

5.8 Rheological Characterization of the 115 EPS

The exocellular polysaccharide of Z. ramigera 115 exhibits some interesting rheological properties. At low concentration (0.1 to 0.5%) the polymer solutions show pseudoplastic behavior. The polymer exhibits shear thinning when increasing stress is applied and its viscosity returns when the stress is removed. This property is advantageous when pumping solutions is required. At concentrations greater than 1.0%, strain 115 polymer solutions show viscoelastic behavior. These solutions demonstrate the Weissenberg effect and possess a shear stress yield value. Other researchers have shown this polymer is heat stable, pH stable, salt compatible and lowers the surface tension of water (Stauffer et al., 1980).

6. SUMMARY AND CONCLUSIONS

The principal findings and accomplishments of this thesis project are summarized below.

6.1 Z. ramigera I-16-M

1. Exopolysaccharide deficient (Eps^-) mutants were isolated and found to be floc nonforming.
2. A broad host range cloning system and transposon mutagenesis procedure were successfully modified for use in Z. ramigera I-16-M, enabling the introduction of foreign DNA by conjugation. This represented the first published use of these genetic techniques in any Zoogloea species.
3. Eps^- mutants were complemented with a pLAFR3/I-16-M gene library. Complemented mutants regained the ability to flocculate providing a strong correlation between the presence of EPS and flocculation in Z. ramigera I-16-M.
4. Genes involved in EPS biosynthesis were found to be located in two complementation groups contained within a 6.5 kb region on the I-16-M chromosome.
5. Genetic instability in the EPS gene region causes spontaneous deletions resulting in loss of EPS biosynthesis.

6.2 Z. ramigera 115

1. An EPS isolation procedure was developed for Z. ramigera 115.
2. Rheological studies on solutions of the 115 EPS have demonstrated pseudoplastic and viscoelastic behavior.

3. Monosaccharide composition analysis of the 115 EPS revealed the presence of glucose and galactose in a molar ratio of approximately 2:1.
4. Conjugation was not successful in 115 but was successful in a capsule nonforming, nonflocculating, slime producing derivative, 115SL. This was the first published use of conjugation in Z. ramigera 115 or its derivatives.
5. The 115SL EPS has the same 2:1 glucose:galactose ratio as that of the parent but has a higher pyruvate content.
6. A simpler and less degradative EPS isolation procedure for 115SL has enabled the identification of an O-acetyl component which is presumed also to be present in the parent 115 EPS.
7. 115SL has advantageous growth and EPS production characteristic when compared with the parent and was shown to produce approximately 50% more EPS than the parent in shake flask experiments.
8. Mutants producing EPS with altered Cellufluor binding properties were isolated and complemented with a pLAFR3/115 gene library.
9. Four mutations affecting EPS biosynthesis were found, identifying at least two complementation groups.
10. A segment of 115 DNA which has sequence homology to the X. campestris pyruvyl transferase gene was isolated and cloned.

7. SUGGESTIONS FOR FUTURE RESEARCH

The application of recombinant DNA technology to the production of bacterial exopolysaccharides represents a challenging and complex problem for genetic engineering. The goals here are not just to increase production but to exert some control over the exopolysaccharide biosynthetic pathway so as to manipulate the composition and structure of the product. In this thesis project, significant progress has been made towards this objective in Z. ramigera, demonstrating its ultimate feasibility. To bring the goals of the overall project to fruition, the following experiments are recommended.

7.1 Z. ramigera I-16-M

7.1.1 RNA Transcripts

Northern hybridization analysis of mRNA isolated from I-16-M would define the number of transcripts from the cloned exo genes. First, use of ^{32}P-labelled p27C DNA as a hybridization probe of I-16-M RNA would show the number of mRNAs transcribed by the wild-type exo genes that make up complementation groups A and B. Next, hybridization of p27C DNA to RNA from each of the exo mutants would determine which transcript results from each mutant gene.

7.1.2 Transcription Initiation

Nuclease S1 mapping using I-16-M RNA and p27C DNA would define the sites of transcription initiation and promoter regions for each of the transcripts read off the cloned exo genes. Once the approximate location of the promoters have been identified it would then be possible to regulate

the expression of any of the genes or operons by replacing the wild-type promoter with an inducible promoter.

7.1.3 Proteins of the exo Region

The number of proteins encoded in the exo region can be determined using an in vivo gene expression system. Expression of each DNA sequence that transcribes a single mRNA in the presence of ^{35}S-labelled methionine and subsequent analysis of the ^{35}S-labelled protein products by SDS-PAGE would identify the number and sizes of the exo gene products.

7.1.4 Regulation of EPS Biosynthesis

The ability to regulate the expression of one or more of the exo genes would enable the induction of EPS biosynthesis at any time in the growth cycle of Z. ramigera I-16-M cultures. A question with much practical importance could then be answered. Does the induction of EPS biosynthesis at late log phase, early stationary or late stationary phase lead to flocculation of the I-16-M culture? If yes, then some important industrial applications may follow.

For example, in a large scale process one can conceive of a two stage system in which cells are first grown to a high density with the polymer genes off, then in the second stage the genes are induced, and polysaccharide is produced resulting in the flocculation of the high density culture. Utilizing the EPS genes to control flocculation in Z. ramigera or perhaps inserting these genes into different host strains may lead to improved purification procedures for bacterial products. For example, bacteria that produce intracellular products could be flocculated by induction of the polysaccharide genes at the end of their production cycle

providing an easier separation of the cell flocs from the culture broth. Similarly, if the desired product is excreted into the broth, cells induced to flocculate at the end of the production stage can then be removed from the supernatant by sedimentation or in a settler as opposed to costly centrifugation or filtration.

7.2 Z. ramigera 115

7.2.1 Isolation of Pyruvyl Transferase Gene

The pyruvyl transferase (pyv) gene from X. campestris was obtained and used as a hybridization probe to identify the Z. ramigera pyv gene. A homologous segment has been cloned and can be overexpressed and its gene product assayed for pyruvyl transferase activity.

7.2.2 Mutants Producing Pyruvate Free EPS

Once the pyv gene is isolated it can be inactivated and reintroduced into the 115SL chromosome by recombination. The resulting strain would be deficient in pyruvyl transferase and presumably produce a pyruvate-free EPS. This polysaccharide would have no net charge and may have some unique physical properties. A neutral EPS would also enable rheological characterization of the uncharged chain. Important information about the secondary and tertiary behavior of this molecule could then be determined, helping to define its structure-function relationships.

7.2.3 Regulated Expression of the pyv Gene

Identification of the transcription initiation site of the pyv gene will allow the replacement of the wild-type promoter with a regulatable, high level expression promoter. Levels of pyruvyl transferase can then be

varied to possibly produce exopolysaccharides with varying degrees of pyruvate substitution and varying physical properties.

Pyruvyl groups give the polysaccharide a negative charge which plays a large role in determining its functional properties. Increased pyruvate content in xanthan gum has been shown to increase its viscosity profile. Control of pyruvylation could allow the production of polysaccharides with varying charge densities to give a range of viscosity profiles.

7.2.4 Determination of the EPS Biosynthetic Pathway

In vitro biosynthesis of the 115 EPS can be carried out as described for xanthan gum (Ielpli et al., 1981a). UDP-glucose, UDP-galactose, phosphoenol pyruvate and acetyl-CoA would be substrates for an enzyme preparation consisting of EDTA-treated cells (Ielpi et al., 1981a). The use of ^{14}C-labelled substrates and analysis of the resulting intermediates and final products will elucidate the EPS biosynthetic pathway. Analysis of xanthan intermediates has been described by Ielpi et al. (1981a; 1981b), Ielpi et al. (1983) and Betlach et al. (in press).

7.2.5 Location of Mutations in EPS Pathway

Using the above described in vitro synthesis system it will be possible to identify the step in which a particular EPS mutant is blocked. The accumulation of a particular intermediate indicates a defect in the enzyme that carries out the next reaction or the production of a final product lacking a certain substituent would indicate a defect in the enzyme responsible for the addition of that component.

7.2.6 Identification of *exo* Genes

Complementation of mutants characterized as described above would lead to the identification of genes for each enzyme in which a defect had occurred. Using this procedure it would be possible to identify all of the structural genes involved in EPS biosynthesis provided all the necessary mutants were isolated.

7.2.7 Manipulation of EPS Composition and Structure

Another enzyme (besides pyruvyl transferase) that would be useful to manipulate and control is the *Z. ramigera* EPS polymerase. In xanthan biosynthesis the polymerase adds five sugar repeat units to the growing polysaccharide chain. It has been shown that this enzyme accepts a three sugar repeat unit creating a modified xanthan (Ielpi *et al.*, 1981a; Betlach *et al.*, in press). This demonstrates a degree of nonspecificity in what the polymerase will accept as substrates. If the same is true for the *Z. ramigera* 115 polymerase it would be possible to produce a variety of novel polysaccharides. Once the biosynthetic pathway has been determined, key genes can be manipulated to produce an altered repeat unit which would then be polymerized into polysaccharide. Using such a system it would be possible to design novel polymer structures for specific functional applications.

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BIOGRAPHICAL NOTE

Donald Davidson Easson Jr. was born on December 15, 1961 in Wilmington, Delaware, where he lived until he was 15 years old. In March 1977 a relocation of David's family meant a move to Greenville, South Carolina where he finished high school and attended the University of South Carolina at Columbia. At South Carolina David was enrolled as a pre-med student majoring in chemical engineering and received several awards and honors including being named to the Dean's List, being a semi-finalist in the Carolina Scholars competition, a National semi-finalist in a Polystyrene Society design competition and scholarship awards from Carolina Eastman and the Daniel Foundation.

Summer employment during the four years at the University of South Carolina greatly contributed to David's experience. In the summer of 1980, he worked as a Process Engineering assistant at Daniel Engineering, Greenville, SC. In 1981, he spent the summer at 3M Corporation, Greenville, SC as a Chemical Engineering assistant in Plant Technical Services. The summer of 1982 found David in Cambridge, MA working in the Biochemical Engineering Laboratory of Professor Charles L. Cooney at M.I.T. It was during this time that David decided to pursue a graduate degree at M.I.T. rather than medical school and was accepted into the Biochemical Engineering program. Before beginning that, he worked at SmithKline and French Laboratories, Philadelphia, PA, in the summer of 1983, in the Chemical Engineering Research and Development Division.

After graduation from the University of South Carolina with a degree in Chemical Engineering, David entered M.I.T. in the fall of 1983. The next 4 years were spent working towards a Sc.D. degree in the biochemical engineering program in the Department of Applied Biological Sciences. His

thesis project entitled "A Recombinant DNA Approach to the Design and Synthesis of Novel Biopolymers" was under the direction of Professor Anthony J. Sinskey. During that time David coauthored 5 papers, 4 patent applications (see below) and presented his work at 3 conferences. David finished his Sc.D. thesis in August 1987 and received his degree September 16, 1987.

Life outside of M.I.T. focused around David's longtime girl friend, Lisa Passamano. On May 16, 1987, after 6 years of dating, David and Lisa were finally married.

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Patent Applications

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