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**THE ISOLATION AND CHARACTERIZATION OF *RHIZOBIUM LOTI*  
EXOPOLYSACCHARIDE MUTANTS**

A Thesis presented in partial fulfilment of  
the requirements for the degree of  
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at Massey University, Palmerston North  
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## ABSTRACT

PN184, a streptomycin resistant derivative of the broad host range *Rhizobium loti* strain NZP2037, was shown to be Calcofluor-bright due to the production of a Calcofluor-binding exopolysaccharide (EPS) with both high and low molecular weight forms, the low molecular weight form being predominant.

Eight Calcofluor-dark EPS mutants (three smooth and five rough) were generated by Tn5 mutagenesis of PN184. Each mutant was shown to carry a single, independent Tn5 insertion. Cosmids that complemented the mutation carried by each of the rough, PN184-derived EPS mutants were isolated from a pLAFR1 gene library to NZP2037 by complementation of the Calcofluor-dark phenotype. The genetic regions identified were shown to be located on the chromosome, and were not closely linked. The mutants were divided into three (complementation) groups.

While the rough, PN184-derived EPS mutants failed to synthesize EPS, the smooth, PN184-derived EPS mutants were found to synthesize an EPS which failed to bind Calcofluor, and which was shown, by  $^1\text{H-NMR}$  spectroscopy, to be significantly less acetylated than the EPS produced by PN184. Furthermore, PN1177, one of the smooth, PN184-derived EPS mutants, was shown to produce only a small amount of high molecular weight EPS compared to PN184.

All the PN184-derived EPS mutants induced the formation of fully effective ( $\text{Nod}^+\text{Fix}^+$ ) nodules on *Lotus pedunculatus*, a determinate nodulating host legume, but, in contrast, induced the formation of ineffective ( $\text{Nod}^+\text{Fix}^-$ ) nodules on *Leucaena leucocephala*, an indeterminate nodulating host legume. Each rough, PN184-derived EPS mutant, carrying its complementing cosmid, was fully effective on *L. leucocephala*.

PN4115, a streptomycin resistant derivative of the restricted, effective host range *R. loti* strain NZP2213, was shown to be Calcofluor-dark. PN4115 was shown to produce an EPS, which fails to bind Calcofluor, that is acetylated to approximately the same extent as the EPS produced by PN184. Like PN1177, PN4115 was shown to produce only a small amount of high molecular weight EPS. Examination of  $^1\text{H-NMR}$  spectra of EPS from PN4115 and the smooth, PN184-derived EPS mutants suggests that these strains produce an EPS of similar structure, with the exception of the degree of *O*-acetylation.

Three non-mucoid, Calcofluor-bright, EPS mutants were generated by Tn5 mutagenesis of PN4115. Each mutant was shown to carry a single, independent Tn5 insertion. Cosmids could not be isolated which stably complemented the mutation carried by each mutant. None of the mutants produced EPS, but all three mutants produce a Calcofluor-binding EPS, possibly cellulose.

All three PN4115-derived EPS mutants induced the formation of fully effective nodules on *Lotus corniculatus*, a determinate nodulating host legume. On *L. leucocephala*, PN4115 induced the formation of both small, ineffective, nodular swellings and large, ineffective, tumour-like structures. Occasionally, a low level of nitrogen fixation was observed. In contrast, the PN4115-derived EPS mutants all induced the formation of only small, ineffective, nodular swellings.

These results, obtained in isogenic *Rhizobium* backgrounds, support suggestions that EPS is required for effective nodulation of indeterminate nodulating legumes, but is not required for effective nodulation of determinate nodulating legumes.

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## TABLE OF CONTENTS

	PAGE
ABSTRACT	ii
ACKNOWLEDGEMENTS	iv
TABLE OF CONTENTS	v
LIST OF TABLES	xi
LIST OF FIGURES	xii
 Chapter 1. INTRODUCTION	 1
1.1 BIOLOGICAL NITROGEN FIXATION	1
1.2 <i>LOTUS</i> spp. IN NEW ZEALAND AGRICULTURE	2
1.3 <i>RHIZOBIUM LOTI</i>	2
1.4 NODULE STRUCTURE AND DEVELOPMENT	3
1.4.1 Common Features of Nodule Development	3
1.4.2 Nodule Formation in <i>Lotus</i> and <i>Leucaena leucocephala</i>	11
1.5 MOLECULAR BIOLOGY OF THE <i>RHIZOBIUM</i> -LEGUME SYMBIOSIS	13
1.5.1 The Molecular Approach to Understanding the Symbiosis	13
1.5.2 <i>Rhizobium</i> Polysaccharides in Nodulation and Infection	15
1.5.2.1 Lipo-oligosaccharides	15
1.5.2.2 Extracellular Polysaccharides	21
1.5.2.2.1 Lipopolysaccharide	22
1.5.2.2.2 $\beta$ -1,2 Glucan	22
1.5.2.2.3 Exopolysaccharide	24
1.5.3 The Involvement of EPS in Infection	25
1.5.4 The Genetics of EPS Synthesis	28
1.6 NODULINS	33
1.7 BACKGROUND AND AIMS OF THIS INVESTIGATION	36
 Chapter 2. MATERIALS AND METHODS	 38
2.1 BACTERIAL STRAINS, PLASMIDS, COSMIDS AND BACTERIOPHAGE	38
2.2 GROWTH OF BACTERIA	38
2.3 LONG TERM MAINTENANCE OF BACTERIA	42
2.4 NODULATION TESTS	42

2.4.1	Inoculation and Growth of Plants	42
2.4.2	Determination of Nitrogen Fixation	43
2.4.2.1	Acetylene Reduction	43
2.4.3	Reisolation of Bacteria From Nodules	43
2.4.4	Light and Electron Microscopy	44
2.4.4.1	Sections Stained with Toluidine Blue for Light Microscopy, and Thin Sections for Electron Microscopy	44
2.4.4.2	Sections Stained with Calcofluor White and Acridine Orange	45
2.5	MEDIA	45
2.5.1	LB Medium	45
2.5.2	TY Medium	46
2.5.3	YM Medium	46
2.5.4	S20 Medium	46
2.5.5	Thornton's Medium	46
2.5.6	Hoagland's Medium	47
2.6	BUFFERS AND SOLUTIONS	48
2.6.1	TBE Buffer	48
2.6.2	STET Buffer	48
2.6.3	<i>Hae</i> III (Universal) Buffer	48
2.6.4	SDS Loading Buffer	48
2.6.5	TE Buffer	48
2.6.6	TEC Buffer	48
2.6.7	Hybridization Buffer	48
2.6.8	Tris-Equilibrated Phenol	48
2.6.9	5 x Ligation Buffer	49
2.6.10	20 x SSC	49
2.6.11	2 x SSC	49
2.6.12	TES (10/1/100)	49
2.6.13	Phage Dilution Buffer	49
2.6.14	Phosphate Buffered Saline	49
2.7	DNA ISOLATION	49
2.7.1	Isolation of Total DNA from <i>Rhizobium</i>	49
2.7.2	Plasmid Isolation by the Rapid Boiling Method	50
2.7.3	Plasmid Isolation by the Alkaline Lysis Method : Miniprep Cosmid Isolation	51
2.8	PURIFICATION OF DNA BY PHENOL/CHLOROFORM EXTRACTION	52

2.9	PRECIPITATION OF DNA WITH ETHANOL OR ISOPROPANOL	52
2.10	DNA QUANTIFICATION	52
2.10.1	Spectrophotometric Determination of DNA Concentration	53
2.10.2	Minigel Method for Determination of DNA Concentration	53
2.11	RESTRICTION ENDONUCLEASE DIGESTION OF DNA	53
2.12	AGAROSE GEL ELECTROPHORESIS OF DNA	54
2.13	RECOVERY OF DNA FROM AGAROSE GELS	54
2.14	DNA LIGATIONS	55
2.14.1	CAP-Treating of Vector DNA	55
2.14.2	Ligation	55
2.15	PREPARATION AND TRANSFORMATION OF COMPETENT CELLS	55
2.16	SOUTHERN BLOT TECHNIQUE	56
2.17	HYBRIDIZATION	57
2.17.1	Preparation of $^{32}\text{P}$ -Labelled Probe DNA	57
2.17.2	Probe DNA/Free Nucleotide Separation by Conventional Column Chromatography	58
2.17.3	Probe DNA/Free Nucleotide Separation by Minispin Column Chromatography	58
2.17.4	Hybridization of Probe DNA to Southern Blots	59
2.18	BACTERIAL CROSSES	59
2.18.1	Tn5 Mutagenesis - Isolation of EPS Mutants	59
2.18.2	Isolation of Complementing Cosmids	60
2.18.2.1	Cosmids Complementing the Mutation Carried by Each of the PN184-Derived EPS Mutants	60
2.18.2.2	Cosmid Cross Complementation	61
2.18.2.3	Cosmids Complementing the Mutation Carried by Each of the PN4115-Derived EPS Mutants	61
2.18.3	Tn5 Mutagenesis - Isolation of LPS Mutants	62
2.19	DETECTION, ISOLATION AND ANALYSIS OF EXOPOLYSACCHARIDE	62
2.19.1	Detection of EPS Production on Solid Media	62
2.19.2	EPS Isolation from Broth Culture	63
2.19.2.1	Cetrimide Precipitation of EPS	63
2.19.2.2	Ethanol Precipitation of EPS	64
2.19.3	Examination of EPS by $^1\text{H}$ Nuclear Magnetic Resonance	65



Spectroscopy	
2.19.4 Paper Chromatography of EPS	66
2.19.5 Column Chromatography of EPS	66
2.19.6 Determination of Glucose Equivalents by the Anthrone Method	67
2.20 ISOLATION AND ANALYSIS OF LIPOPOLYSACCHARIDE	68
2.20.1 LPS Isolation	68
2.20.2 LPS Analysis	69
2.20.2.1 Periodic Acid-Silver Staining	69
2.20.2.2 Periodic Acid-Schiff Staining	70
2.21 ISOLATION OF CAPSULAR POLYSACCHARIDE	70
2.22 ANALYSIS OF TOTAL PROTEIN BY SDS-PAGE	71
2.22.1 Coomassie Brilliant Blue Staining	71
2.23 SODIUM DODECYL SULPHATE (SDS)-POLYACRYLAMIDE GELS	72
2.24 BACTERIOPHAGE $\Phi$ 2037/1 SENSITIVITY TESTS	72
2.24.1 Purification of Bacteriophage $\Phi$ 2037/1	72
2.24.2 Preparation of a Liquid Lysate	75
2.24.3 Sensitivity of the PN184-Derived EPS Mutants to $\Phi$ 2037/1	75
2.24.3.1 Plaque Formation	75
2.24.3.2 Spot Tests	75
Chapter 3. RESULTS	76
3.1 FLUORESCENCE OF <i>R. LOTI</i> STRAINS ON CALCOFLUOR AGAR	76
3.2 THE ISOLATION AND CHARACTERIZATION OF EPS MUTANTS DERIVED FROM NZP2037	81
3.2.1 The Isolation of EPS Mutants by Tn5 Mutagenesis of PN184	81
3.2.2 Colony Characteristics of the PN184-Derived EPS Mutants	84
3.2.3 Resistance of the PN184-Derived EPS Mutants to $\Phi$ 2037/1	89
3.2.4 Confirmation of the Presence of Tn5 Sequences	89
3.2.4.1 Confirmation of a Single Tn5 Insertion	89
3.2.4.2 Confirmation that Each Mutation arose from an Independent Tn5 Insertion	95
3.2.5 The Isolation and Characterization of Cosmids Restoring the Calcofluor Bright Phenotype of PN184	95
3.2.5.1 Cosmid Isolation	95
3.2.5.2 The <i>Eco</i> RI Digestion Profiles of Complementing Cosmids	98

3.2.5.3	Cosmid Cross-Complementation	101
3.2.5.4	Complementation Tests with <i>R. meliloti</i> Cosmids	101
3.2.6	Cloning of the Wild Type Fragments Predicted to Carry a Tn5 Insertion in Each Rough PN184-Derived EPS Mutant	103
3.2.7	Confirmation that the Cloned Wild Type <i>EcoRI</i> Fragment Corresponds to the <i>EcoRI</i> Fragment in Each Rough Mutant Carrying a Tn5 Insertion	103
3.2.7.1	The 0.8 kb Fragment Present in Cosmids pPN33 and pPN34	104
3.2.7.2	The 4.1 kb Fragment Present in Cosmids pPN31, pPN32, and pPN35	105
3.2.8	Identification of the Location of the Wild Type DNA Sequences	108
3.2.9	Exopolysaccharide Isolation and Characterization	111
3.2.9.1	EPS Isolation	111
3.2.9.2	<sup>1</sup> H-NMR Spectroscopy of PN184 EPS	111
3.2.9.3	<sup>1</sup> H-NMR Spectroscopy of EPS from the PN184-Derived EPS Mutants PN1177, PN1178, and PN1179	115
3.2.9.4	<sup>1</sup> H-NMR Spectroscopy of EPS from the <i>L. pedunculatus</i> Infection Mutants PN1018, PN1019, and PN1027	118
3.2.9.5	Analysis of PN184 EPS by Paper Chromatography	118
3.2.9.6	Analysis of Cetrимide Precipitated EPS for Fluorescence on Calcofluor agar	121
3.2.9.7	Column Fractionation of Cetrимide Precipitated EPS	126
3.2.9.7.1	Column Fractionation of PN184 EPS	126
3.2.9.7.2	Column Fractionation of PN1177 EPS	131
3.2.10	Lipopolysaccharide Analysis	134
3.2.11	Examination of Capsular Polysaccharide for Calcofluor Fluorescence	137
3.2.12	Nodulation Tests	138
3.3	THE ISOLATION OF LPS MUTANTS DERIVED FROM PN184	148
3.4	THE ISOLATION AND CHARACTERIZATION OF EPS MUTANTS DERIVED FROM NZP2213	155
3.4.1	The Isolation of EPS Mutants by Tn5 Mutagenesis of PN4115	155
3.4.2	Colony Characteristics of the PN4115-Derived EPS Mutants	156
3.4.3	Confirmation of the Presence of Tn5 Sequences	156
3.4.3.1	Confirmation of a Single Tn5 Insertion	156
3.4.3.2	Confirmation that Each Mutation Arose From an Independent Tn5 Insertion	161

3.4.4 The Attempted Isolation of Cosmids Restoring the Mucoid, Calcofluor Dark Phenotype of PN4115	167
3.4.5 Exopolysaccharide Isolation and Characterization	167
3.4.5.1 EPS Isolation	167
3.4.5.2 <sup>1</sup> H-NMR Spectroscopy of PN4115 EPS	167
3.4.5.3 Analysis of Cetrimide Precipitated PN4115 EPS for Fluorescence on Calcofluor Agar	171
3.4.5.4 Column Fractionation of PN4115 EPS	171
3.4.6 Nodulation Tests	171
Chapter 4. DISCUSSION	188
4.1 THE SYMBIOTIC EFFECTIVENESS OF <i>R. LOTI</i> EPS MUTANTS	189
4.2 COMPLEMENTATION OF THE MUTATIONS CARRIED BY THE PN184-DERIVED EPS MUTANTS	191
4.3 THE BIOCHEMICAL CHARACTERIZATION OF THE MUTATIONS	194
4.4 THE REQUIREMENTS FOR FUNCTIONAL EPS	202
4.5 LIPOPOLYSACCHARIDE ANALYSIS	206
Chapter 5. SUMMARY and CONCLUSIONS	210
REFERENCES	213
APPENDIX 1	246

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## LIST OF TABLES

TABLE		PAGE
1	Steps in the <i>Rhizobium</i> -legume symbiosis.	4
2	Bacterial strains, plasmids, cosmids, and bacteriophage.	39
3	Solutions used in the preparation of SDS-polyacrylamide gels.	73
4	<i>Eco</i> RI digestion fragments of the PN184-derived EPS mutants containing a Tn5 insertion.	94
5	Cosmid cross complementation.	102
6	Isolation of LPS from <i>R. loti</i> PN184 and from the putative PN184-derived LPS mutants.	154
7	<i>Eco</i> RI digestion fragments of the PN4115-derived EPS mutants containing a Tn5 insertion.	164

## LIST OF FIGURES

FIGURE		PAGE
1A	Stylized representation of a determinate root nodule.	9
1B	Stylized representation of an indeterminate root nodule.	9
2A	The arrangement of the <i>nod</i> genes of <i>R. meliloti</i> .	18
2B	The structure of the <i>R. meliloti</i> lipo-oligosaccharide NodRm-1.	18
3A	The structure of <i>R. meliloti</i> EPSI.	27
3B	The structure of <i>R. meliloti</i> EPSII.	27
3C	The structure of <i>Rhizobium</i> sp. NGR234 EPS.	27
4	Fluorescence of PN184, and non-fluorescence of PN4115 on YM-Calcofluor agar.	78
5	Fluorescence of PN184, and the PN184-derived <i>L. pedunculatus</i> infection mutants PN1018, PN1019, and PN1027 on YM-Calcofluor agar.	78
6	Mucoid, white, phenotype of PN184 and mucoid, cream, phenotype of PN4115 on YM-Calcofluor agar.	80
7	Mucoid phenotypes of PN184 and the PN184-derived <i>L. pedunculatus</i> infection mutants PN1018, PN1019, and PN1027 on YM-Calcofluor agar.	80
8	Fluorescence of PN184 and non-fluorescence of the PN184-derived EPS mutants on S20-Calcofluor agar.	83
9	Total protein profiles of PN184, NZP2213, and the PN184-derived <i>L. pedunculatus</i> infection mutants PN1018, PN1019, and PN1027.	86
10A	Smooth or rough phenotypes of PN184 and the PN184-derived EPS mutants on YM agar.	88

10B	Orange colouration of PN184, and the PN184-derived EPS mutants on YM Congo Red agar.	88
11	Resistance of the PN184-derived EPS mutants to $\Phi$ 2037/1.	91
12A	Confirmation of a single Tn5 insertion in each PN184-derived EPS mutant.	93
12B	The PN184-derived EPS mutant GH4 shows two <i>Eco</i> RI fragments hybridizing to $^{32}$ P-labelled pKan2 DNA.	93
13	Confirmation that each PN184-derived EPS mutant arose from an independent Tn5 insertion.	97
14	<i>Eco</i> RI digestion profiles of cosmids complementing the rough PN184-derived EPS mutants.	100
15	Insertion of Tn5 into a 0.8 kb wild type <i>Eco</i> RI fragment in the PN184-derived EPS mutants PN1182 and PN1183.	107
16	Insertion of Tn5 into a 4.1 kb wild type <i>Eco</i> RI fragment in the PN184-derived EPS mutants PN1180, PN1181, and PN1184.	110
17	$^1$ H-NMR spectra of EPS from PN184 and the smooth PN184-derived EPS mutants.	113
18	$^1$ H-NMR spectra of PN184 EPS, isolated from the supernatants of YM broth cultures at different growth stages.	117
19	$^1$ H-NMR spectra of EPS from PN184 and the PN184-derived <i>L. pedunculatus</i> infection mutants PN1018, PN1019, and PN1027.	120
20A	Paper chromatography of PN184 EPS.	123
20B	Fluorescence of sugars upon exposure to long wave UV light.	123
21	Calcofluor-induced fluorescence of PN184 EPS and non-fluorescence of PN1177 EPS.	125

22	DEAE-Sephacel column fractionation of PN184 EPS.	128
23	<sup>1</sup> H-NMR spectra of high and low molecular weight PN184 EPS fractions separated by DEAE-Sephacel column chromatography.	130
24	DEAE-Sephacel column chromatography of PN1177 EPS.	133
25A	SDS-PAGE profiles of LPS from PN184, and the PN184-derived EPS mutants.	136
25B	SDS-PAGE profiles of LPS from PN184, NZP2213, the PN184-derived EPS mutants, and PN184-derived <i>L. pedunculatus</i> infection mutants PN1018 and PN1027.	136
26	Electron micrograph of bacteroids in an infected cell of a <i>L. leucocephala</i> nodule after inoculation with PN184.	140
27A	Fluorescence of <i>L. leucocephala</i> cells containing PN184 bacteroids in Calcofluor-acridine orange stained nodule sections examined by epifluorescence illumination.	142
27B	Fluorescence of <i>L. leucocephala</i> cells containing PN184 bacteroids in Calcofluor-acridine orange stained nodule sections examined by epifluorescence illumination.	142
28	Ineffective nodular swellings formed on <i>L. leucocephala</i> after inoculation with the PN184-derived EPS mutant, PN1183.	145
29	Light micrograph of a transverse section through a nodule formed after inoculation of <i>L. leucocephala</i> with the PN184-derived EPS mutant, PN1183.	145
30	Effective nodule formed on <i>L. leucocephala</i> after inoculation with the PN184-derived EPS mutant PN1183 carrying its complementing cosmid pPN34.	147
31	Light micrograph of a transverse section through a nodule formed after inoculation of <i>L. leucocephala</i> with the PN184-	147

	derived EPS mutant PN1182, carrying its complementing cosmid pPN33.	
32	Electron micrograph of an infection thread formed on <i>L. leucocephala</i> after inoculation with the PN184-derived EPS mutant PN1184, carrying its complementing cosmid pPN35.	150
33	Electron micrograph of bacteroids in an infected cell of a <i>L. leucocephala</i> nodule formed after inoculation with the PN184-derived EPS mutant PN1183 carrying its complementing cosmid pPN34.	150
34	SDS-PAGE profiles of LPS from PN184 and the putative PN184-derived LPS mutants.	153
35	Mucoid phenotype of PN4115 and non-mucoid phenotypes of the PN4115-derived EPS mutants on YM agar.	158
36A	Fluorescence of the PN4115-derived EPS mutants on YM-Calcofluor agar.	160
36B	Red phenotype of the PN4115-derived EPS mutants and orange phenotype of PN4115 YM-Congo red agar.	160
37A	Confirmation of a single Tn5 insertion in the PN4115-derived EPS mutants PN1312, PN1313, and PN1314.	163
37B	The PN4115-derived EPS mutants GH106 and GH214 show multiple <i>Eco</i> RI fragments hybridizing to <sup>32</sup> P-labelled pKan2 DNA.	163
38	Confirmation that the PN4115-derived EPS mutants PN1312, PN1313 and PN1314, arose from independent Tn5 insertions.	166
39	<sup>1</sup> H-NMR spectra of EPS from PN184, PN4115, and PN1177.	169
40	DEAE-Sephacel column chromatography of PN4115 EPS.	173



41	Fluorescence of <i>L. corniculatus</i> cells containing PN4115 bacteroids in Calcofluor-acridine orange stained sections examined by epifluorescence illumination.	176
42	Electron micrograph of bacteroids in an invaded cell of a <i>L. corniculatus</i> nodule formed after inoculation with PN4115.	176
43	Electron micrograph of bacteroids in an invaded cell of a <i>L. corniculatus</i> nodule formed after inoculation with PN184.	178
44	Light micrographs of (A) a small, ineffective nodular swelling and (B and C) large, tumor-like nodular structures formed on <i>L. leucocephala</i> roots after inoculation with pN4115.	180
45	Irregular fluorescence of <i>L. leucocephala</i> cells invaded by PN4115 in a Calcofluor-acridine orange stained section of a tumour-like nodule examined by epifluorescence illumination.	182
46	Electron micrograph of bacteria and fibrous material in a <i>L. leucocephala</i> cell after invasion by PN4115.	182
47A	Electron micrograph of infection threads formed after inoculation of <i>L. leucocephala</i> with PN4115.	184
47B	Electron micrograph of a infection thread formed after inoculation of <i>L. leucocephala</i> with PN4115.	184
48	Electron micrograph of bacteria in an invaded <i>L. leucocephala</i> cell after inoculation with the PN4115-derived EPS mutant PN1312.	187

## Chapter 1. INTRODUCTION

### 1.1 BIOLOGICAL NITROGEN FIXATION

Symbiotic relationships between organisms are common in the biological world. Few, however, are likely to be as intimate as the nitrogen-fixing symbiosis between *Rhizobium*, *Bradyrhizobium*, or *Azorhizobium* bacteria and leguminous plants. Indeed, this relationship is likely to be a major factor in the ecological success of the Leguminosae. During the symbiosis, the bacteria induce their host plants to form novel developmental structures, root nodules, which they invade and inhabit, eventually establishing metabolic cooperation with the host plant. The bacteria reduce atmospheric nitrogen into ammonia, which is exported and is, therefore, available to the plant for assimilation. The bacteria benefit in turn by obtaining sugars and organic acids, produced by the host plant, which are translocated to the root nodules.

Nitrogen fixation in leguminous root nodules is the largest source of organic nitrogen in the global nitrogen cycle. In New Zealand, for example, it has been estimated (Mackinnon *et al.*, 1975) that approximately 97% of the nitrogen available annually to biological systems is fixed by rhizobia, with the *Rhizobium*-white clover (*Trifolium repens*) and *Rhizobium*-red clover (*Trifolium pratense*) associations being the most important. The *Rhizobium*-alfalfa (lucerne, *Medicago sativa*) association is of lesser importance.

It is therefore not surprising that the symbiosis has attracted considerable research attention. This reflects the ecological and economic importance of the symbiosis and the fundamental interest in the processes of plant-microbe recognition, communication and differentiation involved in the association, which may aid in the identification of indigenous plant systems for processes such as signal transduction, development and gene regulation.

This Introduction will concentrate specifically on those aspects of the symbiosis that are especially relevant to the research described in this thesis. Hence the developmental aspects of root nodule formation and the molecular interactions involved in the control of nodule development will be discussed, but the genetic and biochemical details of symbiotic nitrogen fixation will be discussed only peripherally.

## 1.2 *LOTUS* spp. IN NEW ZEALAND AGRICULTURE

This Thesis will describe the symbiotic and other characteristics of specific strains, either wild type or mutant, of *Rhizobium loti*, a species so named due to its symbiotic association with *Lotus* species.

There are five principal *Lotus* species of importance in New Zealand agriculture (Smetham, 1973). Two species, *Lotus angustissimus* and *Lotus subbiflorus*, are annuals, while the remaining three species, *Lotus pedunculatus* (Lotus major, big trefoil), *Lotus tenuis* (slender birdsfoot trefoil), and *Lotus corniculatus* (common birdsfoot trefoil), are perennials. *L. pedunculatus* and *L. corniculatus* are the most important species, with *L. pedunculatus* cv. "Grasslands Maku" being the most important *Lotus* cultivar sown. *Rhizobium-Lotus* symbioses are, however, of minor importance compared to the *Rhizobium*-clover and *Rhizobium*-alfalfa symbioses.

Nevertheless on acid hill country of low fertility (Nordmeyer and Davis, 1977) and on tussock grasslands (Lowther, 1977; Scott, 1979) "Grasslands Maku" gives better yields than white clover (Brock, 1973; Gibson *et al.*, 1975; Hart *et al.*, 1981), shows greater response to phosphate fertilizers due to its higher tolerance to aluminium toxicity (Davis, 1981a, b and c), and has a higher resistance to insect damage (Barratt and Johnstone, 1984). *L. corniculatus* has been recommended to aid revegetation of eroded hill country (Nordmeyer and Davis, 1977) and, in addition, is also useful in pastoral situations due to its non-bloating properties (Jones *et al.*, 1970) and resistance to grass grub damage (Kain and Atkinson, 1975). More recently Scott (1985) demonstrated that the relative productivity of *L. pedunculatus*, *L. tenuis*, and *L. corniculatus* in the South Island high country is dependent on the soil type.

## 1.3 *RHIZOBIUM LOTI*

*Rhizobium loti* (Jarvis *et al.*, 1982) is a group of broad host range (Jarvis *et al.*, 1982; Pankhurst, 1977; Pankhurst *et al.*, 1979; Pankhurst *et al.*, 1987), fast growing rhizobia subdivided into two broad groups differentiated by their capacity to form effective (Nod<sup>+</sup>Fix<sup>+</sup>) or ineffective (Nod<sup>+</sup>Fix<sup>-</sup>) nodules on different *Lotus* species (Greenwood and Pankhurst, 1977; Pankhurst *et al.*, 1979; Pankhurst and Jones, 1979). The first group, represented by "type strain" NZP2037, forms effective nodules on a wide range of *Lotus* species, while the second group, represented by

"type strain" NZP2213, forms effective nodules only on *L. tenuis* and *L. corniculatus* var. *cree*, and ineffective nodules on other *Lotus* species examined (Pankhurst *et al.*, 1987).

In addition to *Lotus* species, which form determinate nodules, *R. loti* can also nodulate legumes such as *Leucaena leucocephala* (NZP2037 effective, NZP2213 ineffective), which form indeterminate nodules (Chen *et al.*, 1985). The structure of determinate and indeterminate nodules is described in Section 1.4.1.

## 1.4 NODULE STRUCTURE AND DEVELOPMENT

### 1.4.1 Common Features of Nodule Development

Nodules normally arise within the cortex of young roots following infection of a root hair by a compatible strain of *Rhizobium* and (generally) within two weeks appear as lateral, dome-shaped outgrowths of the root surface. This process involves the expression of both plant genes (including nodulins, Section 1.6) and rhizobial genes (for example *nod*, *exo*, *lps*, *ndv*, Sections 1.5.2 to 1.5.4) and (host) specific interactions between plant and rhizobia (Vincent, 1974; Newcomb, 1981). These interactions are potentially manifested at a number of stages leading to nodule formation.

Vincent (1980) distinguishes four stages in nodule development: preinfection (stage 1), infection and nodule formation (stage 2), nodule function (stage 3), and nodule senescence (stage 4). These stages are further subdivided into more specific steps leading to the development of an effective nodule (Table 1). Recent research has, however, demonstrated that these stages do not constitute a linear sequence in that, for example, nodule-like structures form on plants inoculated with infection (Inf) mutants (see, for example, Finan *et al.*, 1985; Leigh *et al.*, 1985; Chua *et al.*, 1985; Noel *et al.*, 1986).

During preinfection (stage 1) compatible rhizobia are initially chemotactic toward plant roots, specifically towards the flavonoid able to induce *nod* gene expression (Bergman *et al.*, 1988; Caetano-Anolles *et al.*, 1988). Host specificity may therefore be first mediated in the rhizosphere. Subsequently the compatible rhizobia recognize and attach to host root hairs or root cells developmentally predisposed to become root hairs (Bauer, 1981; Calvert *et al.*, 1984), although only

Table 1. Steps in the *Rhizobium*-legume symbiosis

Stage	Event	Phenotypic code
1. Preinfection		
	a) Root colonization	Roc
	b) Root adhesion	Roa
	c) Hair branching	Hab
	d) Hair curling	Hac
2. Infection and nodule formation		
	e) Infection	Inf
	f) Nodule initiation	Noi
	g) Bacterial release	Bar
	h) Bacterial development	Bad
3. Nodule function		
	i) Nitrogen fixation	Nif
	j) Complementary function	Cof
	k) Nodule persistence	Nop
4. Nodule senescence		
From Vincent (1980).		

a small percentage of root hairs become infected (Dart, 1974). As a result, most nodules are found on roots formed after or immediately prior to inoculation, although in *Arachis hypogaea* (peanut), for which the crack entry mechanism operates, the nodules form in the axils of the lateral roots (Chandler, 1978). Several cases have also been reported in which nodule development occurs on the aerial portions of the plant (Yatazawa and Yoshida, 1979; Subba and Yatazawa, 1984).

The molecular mechanism(s) whereby rhizobia recognize and attach to the surface of the host plant have received considerable attention over recent years. Albersheim and Anderson-Prouty (1975) proposed the hypothesis that host plant lectins interact selectively with polysaccharides on the surface of compatible *Rhizobium* species. This hypothesis proposes that legumes produce specific lectins which may bind bacteria by joining a specific polysaccharide on the root hair to antigenically similar polysaccharides on the bacterial surface. This might allow an efficient exchange of signals between *Rhizobium* and host.

Today an extensive body of literature exists providing experimental evidence both for and against the lectin hypothesis (see, for example, Bohlool and Schmidt, 1974; Albersheim and Anderson-Prouty, 1975; Broughton, 1978; Schmidt, 1979; Dazzo and Truchet, 1983; Halverston and Stacey, 1986).

In the most convincing evidence to date, Diaz *et al.* (1989) cloned the gene (*psl*) encoding a pea lectin and transferred this into white clover using the *Agrobacterium rhizogenes* Ri plasmid as the vector. They found that *R. leguminosarum* bv. *viciae*, a *Rhizobium* species able to nodulate pea, but not normally able to nodulate clover (although it is able to induce root hair curling, but not infection thread formation), was able to induce a low level of delayed nodulation. This suggests that pea lectin may have a role in host specific infection thread formation.

More recently Smit *et al.* (1987, 1989) have presented evidence that both cellulose and a  $\text{Ca}^{2+}$ -dependent adhesin (rhicadhesin) are involved in attachment.

Following attachment, the infective rhizobia induce root hair deformation and/or curling and the bacteria become trapped in the curled root hair, or between a root hair and another cell, and proliferate (Yao and Vincent, 1969). In the *Rhizobium*

*meliloti*-alfalfa symbiosis, root hair deformation is elicited by a sulphated, acylated glucosamine tetrasaccharide (lipo-oligosaccharide, NodRm-1) produced by the bacterium and likely requiring the functioning of the products of most *nod* genes (Lerouge *et al.*, 1990; Truchet *et al.*, 1991). Molecules with similar structure are also produced by *Rhizobium* sp. NGR234 (Broughton *et al.*, 1991) and *R. leguminosarum* bv. *viciae* (Spaink *et al.*, 1991). A *R. meliloti* *nodH* mutant is unable to elicit root hair deformation on alfalfa, but is able to induce root hair deformation on vetch. This mutant produces a lipo-oligosaccharide signal molecule (NodRm-2) lacking the sulphate group present on NodRm-1, demonstrating that specific structural features are required for each host (Roche *et al.*, 1990). Root hair deformation is, therefore, also a host specific step in nodulation. Lerouge *et al.* (1990) have proposed that the lipo-oligosaccharide receptor may be a lectin.

During infection and nodule formation (stage 2) degradation of the existing plant cell wall occurs at the site of infection thread formation (Callaham and Torrey, 1981). The bacteria may produce hydrolytic enzymes themselves or may induce the localized production of endogenous plant hydrolytic enzymes (Nap and Bisseling, 1990). In any case, the physical presence of the rhizobia is apparently required, both for infection thread formation to occur, and to proceed (Nap and Bisseling, 1990). The host cell deposits new cell wall material internally to the point at which the rhizobia are trapped by the curled root hair. The plasmalemma invaginates and the newly deposited wall material is used to form the tubular infection thread. Rhizobia are carried into the infection thread which grows towards the base of the root hair. Development of the infection thread is usually found to be related to the position of the nucleus which enlarges and stays close to the tip of the infection thread during its growth towards the base of the root hair (Dart, 1977). At the same time the rhizobia divide within the infection thread mucopolysaccharide matrix. It is likely that both plant and bacterium contribute to this matrix. VandenBosch *et al.* (1989) have identified a plant glycoprotein which is present, both in infection threads, and in intercellular spaces. Cellulose microfibrils, produced by rhizobia within the infection thread, may also contribute to infection thread formation (Napoli *et al.*, 1975).

Alternatively, bacteria may enter the host plant via "crack entry" through the root epidermis and/or the root cortex by way of intercellular spaces or the middle lamella. In the non-legume *Parasponia*, the rhizobia enter the root through a break

in the epidermis at the site of *Rhizobium*-induced cortical cell proliferation (Lancelle and Torrey, 1984). Similarly, intercellular infection is also the primary route for rhizobial invasion in more primitive legume associations. In *Arachis* (peanut), infection occurs at the point of lateral root emergence (Chandler, 1978).

VandenBosch *et al.* (1989) have recently shown that a plant glycoprotein is present in both infection threads and intercellular spaces, suggesting that the infection thread, rather than being a unique structure, may be considered as a specialized anatomical modification of the intercellular space, particularly intercellular spaces at three way cell junctions. This hypothesis reconciles the two different mechanisms of *Rhizobium* entry.

Morphological changes are observed in cortical cells in advance of penetrating infection threads. Microtubules rearrange, the nucleus migrates towards the centre of the cell, and an additional cell wall is formed. These changes resemble those observed in the initial preparatory stages of cell division, and it has been suggested that the cortical cells may be effectively arrested in prophase (Nap and Bisseling, 1990).

Concomitant with the invasion process, the fully differentiated cells within the root cortex are induced to form meristematic tissue (the nodule primordium), from which the cells of the nodule differentiate. The establishment of a meristematic zone in advance of the invading rhizobia suggests that meristem formation is induced by (a) diffusible signal molecule(s). Recent work has shown that nodule meristem formation is induced by the lipo-oligosaccharide signal molecules (Truchet *et al.*, 1991; Spaink *et al.*, 1991).

The cortical tissue within which the nodule primordium is established depends upon the type of nodule, determinate (Fig. 1A), or indeterminate (Fig. 1B).

In determinate nodulating legumes (most tropical legumes, including soybean, *Phaseolus* bean, and *Lotus* species) the nodule primordium originates in the root outer cortex (Newcomb *et al.*, 1979). Within the developing nodule, rhizobia are released into the outer cortical cells from the invading infection threads, which arise in the root hairs. The rhizobia are spread by the division of pre-infected cells before they cease to divide and swell. Although the infection threads do branch and ramify to some extent in the outer root cortex, this is not extensive compared



Figure 1A:

Stylized representation of a determinate root nodule.

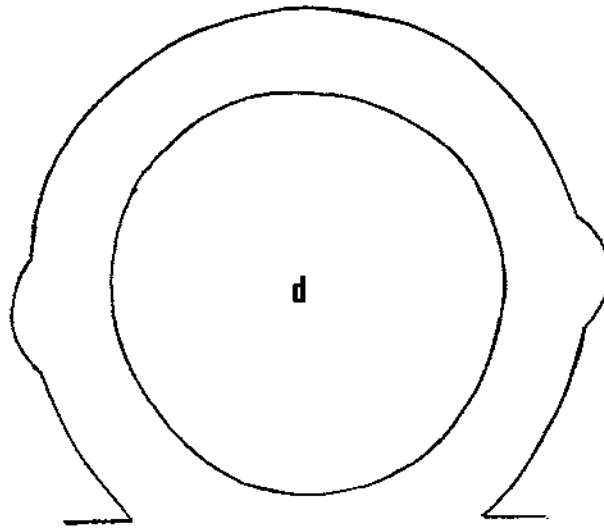
Figure 1B:

Stylized representation of an indeterminate root nodule.

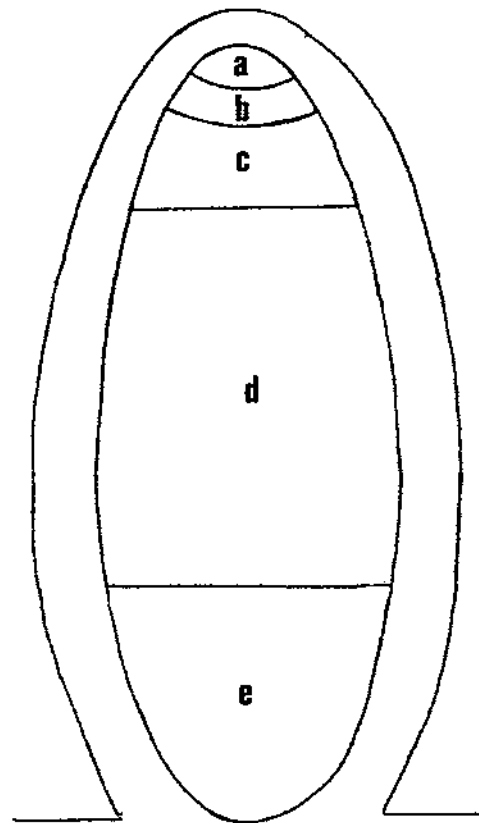
Key

- a - meristematic zone
- b - invasion zone
- c - early symbiotic zone
- d - symbiotic zone
- e - senescent zone

1A



1B



to infection thread ramification in indeterminate nodules. Since determinate nodules lack a persistent meristem all cells within the central tissue of the nodule are progressing through the same developmental stage.

In indeterminate nodulating legumes (most temperate legumes, including pea, vetch, clover, alfalfa and *Leucaena*), the nodule primordium originates in the root inner cortex, generally opposite a xylem pole of the root vascular system (Newcomb *et al.*, 1979). Nap and Bisseling (1990) have suggested that xylem derived factors may also be involved in triggering cell division and that an opposing gradient of the xylem factor(s) and the lipo-oligosaccharide signal molecule (or a plant factor induced by the lipo-oligosaccharide) may determine whether cortical cells divide to form the nodule primordium or prepare themselves for infection thread penetration. Within the developing nodule, rhizobia are released into new cells from the infection threads, which arise in the root hair, pass through the outer cortical cells, then ramify in the inner cortex. The cells, initially in the nodule primordium in the inner cortex, remain meristematic, forming a persistent apical meristem ahead of the advancing infection thread, which reverses its original direction of passage. The direction of infection thread growth therefore appears to be correlated to the position of mitotically active cells. Cells that are invaded by the advancing infection threads cease to divide and enlarge and swell. In this way five separate developmental zones can be recognized in the indeterminate nodule. In the invasion zone, situated immediately behind the meristem, bacterial release from advancing infection threads continues to establish newly infected cells. The early symbiotic zone exists immediately behind the invasion zone and is characterized by elongating plant cells and bacterial proliferation. In the (late) symbiotic zone, which follows the early symbiotic zone, infected cells are filled with bacteria which have completed differentiation into bacteroids, which are able to fix nitrogen. A fifth zone, the senescent zone, in which both plant and bacterial cells degenerate, is found in older nodules. As a result of their development, indeterminate nodules are club shaped.

In most legume species the inner cells of the nodule meristem contain double the numbers of chromosomes present in root tip cells, suggesting that a cortical endoreduplication cycle is stimulated in the region of nodule meristem formation prior to the first mitosis (Sutton, 1983).

Upon release from the infection thread into the cytoplasm of the host cortical cells,

the rhizobia are surrounded by a host derived peribacteroid membrane (Robertson *et al.*, 1978) and are thus not truly intracellular.

In stage 3 (nodule function), the rhizobia may divide within the peribacteroid membrane before enlarging and differentiating into bacteroids. The structure of mature bacteroids is dependent on both the host plant and the *Rhizobium* strain (Sutton, 1983). Mature bacteroids, via the activity of nitrogenase, are capable of reducing atmospheric nitrogen into ammonia which the host plant is able to assimilate for growth and development. Bacterial genes required for nitrogen fixation include *nif* and *fix*, some of which are the structural genes for nitrogenase. Nitrogenase is composed of two iron-sulphur proteins, dinitrogen reductase (the Fe-protein) and dinitrogenase (the MoFe-protein) both of which are irreversibly inactivated by oxygen (Postgate, 1982). A detailed discussion of the activity of nitrogenase and the genetics of nitrogen fixation is beyond the scope of this Introduction (for further information see Banfalvi *et al.*, 1981; Ruvkun *et al.*, 1982; Quinto *et al.*, 1985; Gussin *et al.*, 1986; Nixon *et al.*, 1986; Ditta *et al.*, 1987; Szeto *et al.*, 1987; David *et al.*, 1988; Batut *et al.*, 1989; Hertig *et al.*, 1989; and Hennecke, 1990).

Most of the ammonia excreted by the mature bacteroids is assimilated into either ureides or amides and is exported to other parts of the plant (Schubert, 1986). The onset of this process in developing nodules results in increased nitrogen levels in the nodule and plant sap (Scott *et al.*, 1976).

#### **1.4.2 Nodule Formation in *Lotus* and *Leucaena leucocephala***

Effective strains of *Rhizobium loti* enter the host plant cells via an infection thread and become enclosed by a peribacteroid membrane derived from the plant cell plasmalemma surrounding the infection thread at the time of bacterial release. The rhizobia divide once or twice before enlarging into bacteroids (Pankhurst *et al.*, 1979). The mature bacteroids are capable of reducing atmospheric nitrogen to ammonia which the host plant is able to assimilate for growth and development.

The effective nodules formed by NZP2037 on all *Lotus* species and by NZP2213 on *L. tenuis* and *L. corniculatus* var. *cree* have similar morphology, and are of the determinate type. Fully developed nodules are spherical, containing a central region of plant cells filled with small, rod shaped bacteroids surrounded by a cortex

8-10 cells thick containing vascular traces and flavolans (tannins) in its outer two or three cell layers (Pankhurst *et al.*, 1987).

On other *Lotus* species examined (Pankhurst *et al.*, 1979; Pankhurst *et al.*, 1987) NZP2213 is ineffective with small, nodular swellings or larger, tumour-like structures being formed.

The rhizobia may fail to initiate infection threads (*L. pedunculatus*, *L. angustissimus*) and are concentrated amongst the root hairs and epidermal cells covering the tumour. These bacteria are generally associated with a zone of meristematic cells two or three layers from the tumour surface (Pankhurst *et al.*, 1979; Pankhurst *et al.*, 1987). Pankhurst (1970) has shown that the percentage of "markedly curled" root hairs found on *L. pedunculatus* seedlings inoculated with NZP2213 is significantly less than the percentage of "markedly curled" root hairs found on *L. pedunculatus* seedlings inoculated with NZP2037. In contrast to the nodules formed on plants inoculated with NZP2037, flavolan-containing cells are found to be randomly distributed throughout the entire structure (Pankhurst *et al.*, 1979).

Alternatively, infection threads may be found within the meristematic cells (*L. subbiflorus*). However, the rhizobia are not readily released, although occasional cells are found showing evidence of bacterial release (Pankhurst *et al.*, 1987). Bacterial release, where it occurs, is abnormal, the rhizobia being contained within a loose membrane structure analogous to the infection droplet associated with normal bacterial release (Newcomb, 1981), rather than being encapsulated by the plasma membrane as individual units, as is the case for effective nodules (Pankhurst *et al.*, 1979).

The structures formed on *L. corniculatus* var. *hirsutus* inoculated with NZP2213 are anatomically similar to those formed on *L. corniculatus* var. *cree* (Pankhurst *et al.*, 1987). However, bacteria released into the plant cells are contained within a single, interconnected, infection droplet similar to that described for *L. subbiflorus* except that the bacteria multiply to occupy the entire lumen of the plant cell (Pankhurst *et al.*, 1987).

The effective root nodules formed on *Leucaena leucocephala* by NZP2037 are cylindrical and of the indeterminate type and contain a zone of bacteria-filled plant

cells surrounded by a cortex with a peripheral zone of flavolan-containing cells (Pankhurst *et al.*, 1987). Approximately four to eight bacteroids are found per peribacteroid membrane (Pankhurst *et al.*, 1987).

The ineffective root nodules formed on *L. leucocephala* by NZP2213 are irregular in size and shape, varying from small, spherical, nodular swellings to large, irregular, tumour-like structures (Pankhurst *et al.*, 1987). While cells containing infection threads are present, release of bacteria from infection threads, and their enclosure in a peribacteroid membrane does not occur, although infection droplets containing many rhizobia are observed (Pankhurst *et al.*, 1987).

In addition, Al-Mallah *et al.* (1989) have shown that, in the presence of polyethylene glycol, NZP2037 is able to induce a very low frequency of nodule-like structures on the roots of rice (*Oryza sativa*) plants treated with cell wall-degrading enzymes.

## 1.5 MOLECULAR BIOLOGY OF THE *RHIZOBIUM*-LEGUME SYMBIOSIS

### 1.5.1 The Molecular Approach to Understanding the Symbiosis

In the last decade, advances in molecular genetics have greatly enhanced our understanding of the symbiosis. Three principal approaches have been used to manipulate the *Rhizobium* genome for genetic study: loss of function, gain of function, and specific activation (or inhibition).

The loss of function approach involves large scale mutagenesis, plating for single colonies, and screening for the loss or alteration of symbiotic effectiveness. While random chemical mutagenesis has been employed (eg *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, Maier and Brill, 1976; Beringer *et al.*, 1977), it has several disadvantages, the most important being the lack of a suitable marker for mutated genes. As a result, transposon mutagenesis has become the method of choice. Transposon Tn5 has become the preferred mutagen, inserting generally at random (Beringer *et al.*, 1978; Meade *et al.*, 1982; Noel *et al.*, 1984), and leading to (generally) non-leaky polar mutations. In addition, both spontaneous reversion and secondary transposition are low (Beringer *et al.*, 1978; Meade *et al.*, 1982; Noel *et al.*, 1984).

Several plasmid vectors have been designed to enable transposon delivery. All are "suicide plasmids" (Beringer *et al.*, 1978) which can be transferred conjugatively between *Escherichia coli* and *Rhizobium* but which cannot replicate in *Rhizobium*. The transposon is therefore only rescued after insertion into the rhizobial DNA.

The initial plasmids used had phage Mu sequences inserted into RP4 (Beringer *et al.*, 1978). RP4 can transfer and replicate in both *E. coli* and *Rhizobium*, however, insertion of phage Mu sequences eliminates the ability of RP4 to replicate in *Rhizobium*. Two principal problems exist with RP4/Mu based plasmids, firstly, the simultaneous transposition of phage Mu and the resident transposon (Meade *et al.*, 1982; Forrai *et al.*, 1983; Noel *et al.*, 1984), and secondly that their suicide nature can occasionally be overcome (Noel *et al.*, 1984).

To overcome these problems the pSUP series of vectors was developed, carrying an origin of replication functional only in *E. coli* and a mobilisation sequence for conjugation (Simon *et al.*, 1983; Simon, 1984). Depending on the vector, a transfer function may also be carried, otherwise it must be provided *in trans*. For example, this Thesis will describe the use of pSUP1011 (Simon *et al.*, 1983), a pSUP series vector carrying an origin of replication, a *mob* function, and Tn5. The *tra* function is provided *in trans* by an integrated RP4 in the chromosome of the donor *E. coli* strain, SM10 (Simon *et al.*, 1983).

The gain of function approach generally involves the transfer of DNA, frequently symbiotic plasmids or defined regions of symbiotic plasmids, and the testing of the derived strain for gain or alteration of symbiotic effectiveness.

Most frequently, the generation of loss of function mutations is followed by a gain of function approach to isolate the parental (wild type) sequence into which Tn5 insertion has occurred. This is generally achieved by the construction of a genomic library to the parental strain in one of several broad host range mobilizable cosmids, such as pLAFR1 (Friedman *et al.*, 1982) that were derived from pRK290, and examination for complementation, following transfer of the library *en masse* into the mutant strain.

To isolate genes that respond to specific stimuli the selective activation or inhibition approach is used. Here promoters of the genes of interest are fused to reporter genes with readily assayable products, for example  $\beta$ -galactosidase

activity in *E. coli lacZ* fusions (eg Tn3-HoHo1, Stachel *et al.*, 1985) and glucuronidase activity in *E. coli uidA* fusions (Sharma and Signer, 1990).

In recent years these techniques have been applied to yield our current understanding of the molecular basis for the symbiosis, as discussed briefly in subsequent sections.

### 1.5.2 *Rhizobium* Polysaccharides in Nodulation and Infection

*Rhizobium* species synthesize several diverse polysaccharides ranging in size from relatively low molecular weight, such as the secreted lipo-oligosaccharide signal molecule synthesized by the Nod proteins (Lerouge *et al.*, 1990), to high molecular weight exopolysaccharides (Finan *et al.*, 1985; Leigh *et al.*, 1985).

#### 1.5.2.1 Lipo-oligosaccharides

The fast growing *Rhizobium* species *R. leguminosarum* bv. *viciae*, *R.l.* bv. *phaseoli*, *R. l.* bv. *trifolii*, and *R. meliloti*, all carry the genes for nodulation (and nitrogen fixation) on large indigenous plasmids (200-300 kb, *R. leguminosarum* biovars) or megaplasmids (1200-1500 kb, *R. meliloti*). See for reviews Denarie *et al.* (1981), and Prakash and Atherly (1986). See also Johnston *et al.* (1978), Zurkowski and Lorkiewicz (1979), Hooykaas *et al.* (1981), Rosenberg *et al.* (1981), Hombrecher *et al.* (1981), Lamb *et al.* (1982), and Scott and Ronson (1982).

In contrast, the nodulation and nitrogen fixation genes of *R. loti*, like those of *Bradyrhizobium* spp. are located on the chromosome (Pankhurst *et al.*, 1983; Chua *et al.*, 1985). Furthermore Pankhurst *et al.* (1986) have shown that plasmid cured derivatives of *R. loti*, strain NZP2037, are significantly more effective in nitrogen fixation and show an improved capacity to compete with other root nodule bacteria for nodulation of *L. pedunculatus* relative to the wild type strain.

Recent work has shown that the proteins encoded by the *R. meliloti nod* genes are required for the synthesis of a sulphated, acylated glucosamine tetrasaccharide (lipo-oligosaccharide) (Lerouge *et al.*, 1990; Fig. 2B), which is required for root hair curling (Rossen *et al.*, 1984; Lerouge *et al.*, 1990) and nodule meristem formation (Dudley *et al.*, 1987; Truchet *et al.*, 1991). Similarly Spaink *et al.* (1991) and Broughton *et al.* (1991) have described equivalent molecules which are



produced by *R. leguminosarum* bv. *viciae* and *Rhizobium* sp. NGR234, respectively.

A genetic map of the nodulation gene region of *R. meliloti* (Kondorosi, 1991), together with the structure of NodRm-1, the first lipo-oligosaccharide described (Lerouge *et al.*, 1990) is presented in Figure 2.

The *nod* genes can be divided into three groups. The first group of genes, the "common" *nod* genes, are structurally similar and functionally interchangeable, and determine, as their name suggests, basic properties of nodulation that are conserved between different *Rhizobium* species. Mutations in the common *nod* genes *nodA*, *B* or *C*, completely abolish the ability to nodulate. Fisher *et al.* (1985) have shown that these genes are functionally equivalent in different *Rhizobium* species. The *nodA* and *nodB* genes in *R. meliloti* and *R. leguminosarum* bv. *trifolii* may be translationally coupled (Schofield and Watson, 1986; Little *et al.*, 1989) suggesting that their protein products may operate as a complex (Normark *et al.*, 1983).

*NodI* and *J*, located downstream of *nodABC*, are similarly normally considered to be common *nod* genes. *NodI* shows sequence similarity to inner membrane ATP-dependent transport proteins of *E. coli* and *Salmonella typhimurium* (Evans and Downie, 1986). *NodJ* is hydrophobic suggesting that it might be membrane bound (Evans and Downie, 1986).

The second group of *nod* genes, the "host specificity" genes, are involved in determining host specificity of nodulation. Although the sequences of equivalent genes are often highly conserved, they are not functionally interchangeable. Bacteria carrying mutations in these genes (*nodFEGHQ*P [*R. meliloti*]; *nodFEL*, *nodMNT*, *nodO*, and *nodX* [*R. leguminosarum* bv. *viciae*], Long and Ehrhardt, 1989), elicit abnormal root hair reactions on their host plants and may also elicit root hair reactions on an extended range of plants (Debelle *et al.*, 1986; Djordjevic *et al.*, 1985). In more general terms nodulation may be delayed or reduced, or the host range altered.

*NodG* shows sequence similarity to ribitol dehydrogenase from *Klebsiella aerogenes* (Debelle and Sharma, 1986). *NodE* shows sequence similarity to  $\beta$ -ketoacyl synthase and *nodF* with acyl carrier proteins (Shearman *et al.*, 1986). *NodEF* (and *G*) belong to the same operon in *R. meliloti* and may be translationally

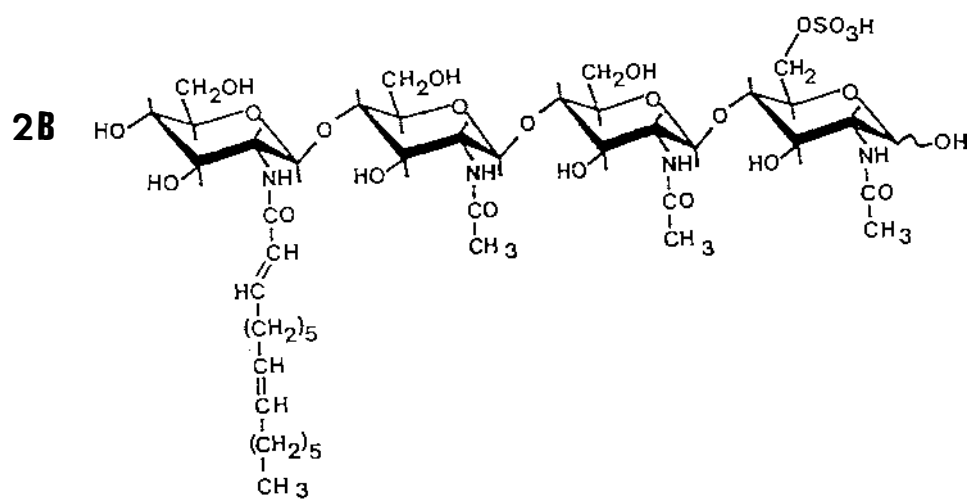
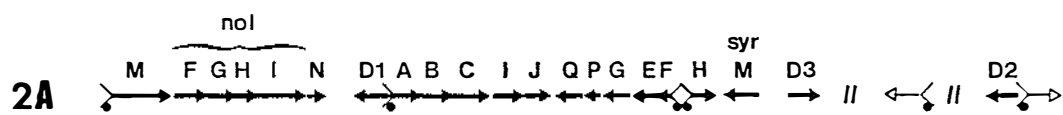
Figure 2A:

The arrangement of the *nod* genes of *R. meliloti*.

- ← Gene
- ⇐ Open reading frame
- ↙ Nod box/direction

Figure 2B:

The structure of the *R. meliloti* lipo-oligosaccharide NodRm-1.



coupled. In *R. leguminosarum* bv. *viciae*, the NodE protein is required for the production of the highly unsaturated fatty acid moiety present on the lipo-oligosaccharide signal molecule (Spaink *et al.*, 1991).

*NodH* inhibits nodulation in heterologous hosts (Debelle *et al.*, 1986) and may be involved in either modifying the lipo-oligosaccharide signal molecule or a substrate intermediate in its formation (Lerouge *et al.*, 1990). Roche *et al.* (1991) have subsequently shown that mutations in *R. meliloti nodH* lead to the production of a lipo-oligosaccharide signal lacking the sulphate group, which elicits root hair deformation on vetch but not on alfalfa.

*NodL* shows sequence similarity to the *lacA* and *cysE* acetyl transferases of *E. coli* (Downie, 1989) and encodes a protein (NodL) which is involved in acetylation of the lipo-oligosaccharide signal molecule (Truchet *et al.*, 1991; Spaink *et al.*, 1991).

*NodP* and *nodQ* are homologous to *cysD* and *cysN* of *E. coli* (Schwedock and Long, 1989, 1990). Together CysD and CysN have ATP sulphurylase activity (Leyh *et al.*, 1988). Based upon this similarity and the observation that NodP and NodQ also have ATP sulphurylase activity, Schwedock and Long (1990) have proposed that NodP and NodQ synthesize an activated sulphate that is an intermediate in the formation of the alfalfa specific lipo-oligosaccharide signal.

NodO is a  $\text{Ca}^{2+}$ -binding protein which contains a multiple tandem repeat of a nine amino acid domain (Economou *et al.*, 1990). This domain has sequence similarity to repeated sequences in several exported bacterial proteins including haemolysin (HlyA) and cyclolysin (CyaA) which are secreted by a mechanism not involving cleavage of an amino-terminal transit sequence. Secretion of HlyA and CyaA requires the gene products HlyB and CyaB respectively, both of which have homology to NodI (Higgins *et al.*, 1986; Glaser *et al.*, 1988). However, neither mutation of *nodI* nor elimination of the symbiotic plasmid (strain carrying only *nodO* and *nodD*) affected secretion of the NodO protein, suggesting that genes located elsewhere in the genome are required for, or can substitute for, secretion (Economou *et al.*, 1990). Economou *et al.* (1990) have suggested that the NodO protein may play a role in nodule development by direct interaction with the root hair cells in a  $\text{Ca}^{2+}$ -dependent manner.

*NodX* extends the host range of *R. leguminosarum* bv. *viciae* to include Afghanistan

peas (Gotz *et al.*, 1985).

The third "group" comprises the regulatory, and constitutively expressed (in both the free living and symbiotic states), *nodD* gene(s) which, depending on the *Rhizobium* species involved, display characteristics of both common and host specific *nod* genes. Flavonoid compounds exuded by the host plant can act as inducers of *nod* genes due to their interaction with NodD, which regulates the expression of other *nod* genes (Innes *et al.*, 1985; Mulligan and Long, 1985; Rossen *et al.*, 1985; Peters *et al.*, 1986; Redmond *et al.*, 1986; Zaat *et al.*, 1987; Fisher *et al.*, 1988) via its interaction, as a positive regulator, with a conserved sequence upstream of each *nod* operon, the "nod box" (Rostas *et al.*, 1986). In *R. leguminosarum* bv. *viciae* *nodD* also autogenously regulates its own expression (Rossen *et al.*, 1985). The deduced amino acid sequence of NodD has shown that it is a member of the LysR family of positive gene activator proteins (Henikoff *et al.*, 1988).

It has been found that different flavonoid compounds act as inducers or inhibitors of *nod* gene expression in different *Rhizobium* species (Firman *et al.*, 1986; Zaat *et al.*, 1987) and that this is linked to the presence of a particular *nodD* gene (Spaink *et al.*, 1987; Hovarth *et al.*, 1987). In several species, multiple *nodD* genes are present (Applebaum *et al.*, 1988; Gottfert *et al.*, 1986; Honma and Ausubel, 1987) and it has been proposed that this confers broad responsiveness to various host plants (Gyorgypal *et al.*, 1988; Honma and Ausubel, 1987).

Nodulation genes may also be involved in the regulation of EPS synthesis. *NodD2* from *R. fredii* (Appelbaum *et al.*, 1988) does not regulate the *nodABC* operon, but instead is found to inhibit EPS production in strains of *R. fredii* and *Rhizobium* sp. NGR234 carrying extra plasmid encoded copies. Since the *exoY* promoter of *Rhizobium* sp. NGR234 has *nod*-box-like sequences it is possible that the *nodD2* gene product may bind to this promoter, repressing its transcription, thereby inhibiting EPS synthesis in a similar manner to that proposed for the absence of *exoY* (Gray and Rolfe, 1990).

An additional gene of *R. meliloti*, *syrM*, which shows deduced amino acid sequence similarity to NodD proteins and to other proteins in the LysR family, can affect the expression of both *nod* and *exo* genes (Mulligan and Long, 1989). In conjunction with *nodD3*, *syrM* increases transcription of the *nod* genes (without

added plant inducer), while in conjunction with *syrA* it increases the transcription of *exo* genes. However mutations in *syrM* do not affect *in vivo* transcription of *nod* genes (Sharma and Signer, 1990).

Co-inoculation experiments whereby an  $\text{Exo}^- \text{Nod}^+$  strain is assisted to form an effective nodule by a  $\text{Exo}^+ \text{Nod}^-$  strain have been performed by several groups (Rolfe *et al.*, 1980; Chen and Rolfe, 1987; Borthakur *et al.*, 1988; Klein *et al.*, 1988; Muller *et al.*, 1988). These experiments have shown that intercellular complementation can occur between an  $\text{Exo}^- \text{Nod}^+ \text{Fix}^+$  mutant and a  $\text{Exo}^+ \text{Nod}^- \text{Fix}^-$  mutant, but not with a  $\text{Nod}^+$  helper or the wild type strain. That is, one of the coinoculants must provide the  $\text{Exo}^-$  phenotype and the other the  $\text{Nod}^-$  phenotype.

### 1.5.2.2 Extracellular Polysaccharides

Extracellular bacterial polysaccharides comprise the capsules and slimes produced by many bacteria including *Rhizobium* species. They are structurally, chemically and functionally diverse, and they constitute a complex macromolecular interface between the bacterial cell and its environment (Morris and Miles, 1986).

The extracellular polysaccharides synthesised by *Rhizobium* species fall into three primary classes: lipopolysaccharide (LPS), (acidic) exopolysaccharide (EPS), and cyclic  $\beta$ -1,2 glucan. LPS is a component of the rhizobial outer membrane, EPS is loosely attached to the outer membrane surface, while  $\beta$ -1,2 glucan is located in the periplasm, but is also secreted from the cell.

Recent work has shown that several extracellular polysaccharides are involved in the interactions leading to the establishment of an effective *Rhizobium*-legume symbiosis. These include the acidic Calcofluor-binding EPS (succinoglycan) of *R. meliloti* (wild type colonies are fluorescent in the presence of Calcofluor [Calcofluor-bright] while mutants that produce no succinoglycan are non-fluorescent [Calcofluor-dark], Finan *et al.*, 1985; Leigh *et al.*, 1985), acidic exopolysaccharides or capsular polysaccharides from other *Rhizobium* species (Borthakur *et al.*, 1986; Chakravorty *et al.*, 1982; Chen *et al.*, 1985; Diebold and Noel, 1989), a second exopolysaccharide synthesized by *R. meliloti* (Glazebrook and Walker, 1989; Zhan *et al.*, 1989), lipopolysaccharide (Noel *et al.*, 1986), cyclic  $\beta$ -1,2 glucan (Geremia *et al.*, 1987), and a peptidoglycan-bound polysaccharide (Jones *et al.*, 1987).

### 1.5.2.2.1 Lipopolysaccharide

Rhizobial LPS resembles enterobacterial LPS, consisting of a complex phosphorylated heteropolysaccharide linked to a glucosamine-containing lipid (lipid A) through 2-keto-3-deoxyoctanoic acid (Carlson *et al.*, 1978). The polysaccharide portion can be divided into two regions, an internal core region and the peripheral O-antigen. The core region may be further subdivided into an inner core and an outer core region. The O-antigen constitutes the immunodominant portion of the molecule.

Noel *et al.* (1986) have isolated LPS mutants of *R. leguminosarum* bv. *phaseoli* which lack the O-antigen (Carlson *et al.*, 1989) and are ineffective on *Phaseolus vulgaris* (bean) due to arrested development of infection threads, usually within root hairs. Subsequently, Cava *et al.* (1989) have isolated LPS mutations in three genetic regions of *R. leguminosarum* bv. *phaseoli* and have further identified nine complementation groups affecting LPS synthesis on one of these regions, a chromosomal region contained on the recombinant cosmid pCOS109.11 (Cava *et al.*, 1990).

Priefer (1989) has identified a cluster of three genes located on the *R. leguminosarum* bv. *viciae* chromosome which are required for synthesis of the O-antigen and for effective nodulation of *Vicia hirsuta*. de Maagd *et al.* (1989) have similarly described LPS mutants of *R. leguminosarum* bv. *viciae* which are ineffective on both *V. hirsuta* and *Vicia sativa*.

In contrast, Clover *et al.* (1989) have isolated lipopolysaccharide mutants of *R. meliloti* that are not defective in symbiosis. In addition, Williams *et al.* (1990) and Putnoky *et al.* (1990) have shown that *R. meliloti*, strain RM41, carries a gene (*lpsZ*<sup>+</sup>) that alters the composition and structure of the LPS such that it can substitute for EPS on alfalfa.

### 1.5.2.2.2 $\beta$ -1,2 Glucan

The cyclic  $\beta$ -1,2 glucan produced by bacteria in the *Rhizobiaceae*, including both *Rhizobium* and *Agrobacterium* species is a cyclic homopolymer consisting of 17 to 24  $\beta$ -1,2 linked glucose residues (York *et al.*, 1980; Zevenhuizen and Scholten-Koerselman, 1979) that can be modified with anionic moieties (Batley *et al.*, 1987;

Hisamatsu *et al.*, 1987; Miller *et al.*, 1987). In *R. meliloti* as much as 90% of the periplasmic cyclic  $\beta$ -1,2 glucan can be substituted, principally by phosphoglycerol (Miller *et al.*, 1987). *Bradyrhizobium* species also produce cyclic  $\beta$ -glucans (Miller *et al.*, 1990), however these are structurally distinct from the cyclic  $\beta$ -1,2 glucans produced by *Rhizobium* and *Agrobacterium* species in that they are smaller (10 to 13 glucose residues) and, in addition, are linked primarily by  $\beta$ -1,3 and  $\beta$ -1,6 glycosidic bonds.

Puvanesarajah *et al.* (1985) have described avirulent mutants of *A. tumefaciens* that fail to produce  $\beta$ -1,2 glucan. These mutants map to the chromosomal virulence locus *chvB* (Douglas *et al.*, 1985) and fail to form a 235kD inner membrane protein intermediate required for the synthesis of  $\beta$ -1,2 glucan (Zorreguieta and Ugalde, 1986). This 235kD protein is also present on the inner membrane of *R. meliloti* (Zorreguieta and Ugalde, 1986).

Dylan *et al.* (1986) have isolated genes of *R. meliloti* that are structurally and functionally related to the *chvA* and *chvB* genes of *A. tumefaciens*. Homologous sequences were also found to exist in the genomes of *R. leguminosarum* bv. *trifolii*, *R. leguminosarum* bv. *viciae* and *R. leguminosarum* bv. *phaseoli* (Dylan *et al.*, 1986). Since the mutations in the *R. meliloti* *chv*-like genes lead to abnormal nodule development they were designated *ndv* genes (Dylan *et al.*, 1986).

Mutants at the *ndv* loci induce nodules that contain neither bacteroids nor infection threads but do induce root hair curling (Dylan *et al.*, 1986) and are thus similar to a *R. meliloti* mutant, GRT21s, isolated by Geremia *et al.* (1987), that is ineffective and also fails to synthesize  $\beta$ -1,2 glucan.

More recently Stanfield *et al.* (1988) have shown that an *ndvA* mutant (Dylan *et al.*, 1986) of *R. meliloti* is defective in the production of  $\beta$ -1,2 glucan, even though the 235kD protein intermediate is present and active in vitro. Sequence analysis revealed that the *ndvA* locus encodes a 67kD protein with sequence similarity to a number of bacterial ATP-binding transport proteins, most notably *E. coli* HlyB, a protein involved in the export of hemolysin (Stanfield *et al.*, 1988), suggesting that NdvA may be involved in the export of  $\beta$ -1,2 glucan from the cell.

The cyclic  $\beta$ -1,2 glucans produced by both *Rhizobium* and *Agrobacterium* species resemble a class of anionic, periplasmic oligosaccharides within *E. coli*, the



membrane derived oligosaccharides (MDO's), in their periplasmic localization, intermediate size, and  $\beta$ -D-1,2 glucan backbones (Miller *et al.*, 1986). MDO synthesis in *E. coli* is regulated in response to the osmolarity of the surrounding medium (Miller *et al.*, 1986). Miller *et al.* (1986) have shown that the synthesis of cyclic  $\beta$ -1,2 glucan by *A. tumefaciens* is osmoregulated in a similar manner to that of MDO in *E. coli*. This suggests that periplasmic oligosaccharides may have a general role in minimizing osmotic differential across the cytoplasmic membrane in Gram-negative bacteria, and that they may function in this role in the *Rhizobium*-legume symbiosis. Subsequently, Dylan *et al.* (1990) have shown that the *R. meliloti*  $\beta$ -1,2 glucan may also be involved in osmotic adaptation. It is thus possible that cyclic  $\beta$ -1,2 glucan has the same (passive) role in both *Rhizobium*-plant and *Agrobacterium*-plant interactions.

### 1.5.2.2.3 Exopolysaccharide

Rhizobial exopolysaccharides are complex, (generally) high molecular weight, heteropolysaccharide polymers, with oligosaccharide repeat units of seven, eight, or nine sugar (generally hexose) residues linked by  $\alpha$ - and  $\beta$ -anomeric glycosidic bonds, either in a linear order or with branched side chains. The sugars may be modified with succinyl, pyruvyl, acetyl, methyl, and hydroxybutanoyl residues. The acidic nature of the EPS's derives from uronic acids and/or from succinyl and pyruvyl residues. Generally, rhizobia produce only one major EPS at a time, although EPS structure varies between *Rhizobium* species.

EPS synthesis involves the polymerization of lipid-linked oligosaccharide repeats, however, while the synthesis of the oligosaccharide repeats has been studied, and is thought to occur at the inner-membrane surface (Sutherland, 1985), the steps leading to oligosaccharide polymerization have not been elucidated.

In general terms, however, it is thought that monosaccharide substrates are transported into the cell where they are activated by conversion, first, into the monosaccharide phosphate, and then into the sugar diphosphonucleotide. In subsequent steps membrane bound enzymes transfer the sugar-1-phosphate to an isoprenoid lipid phosphate acceptor to generate a lipid-pyrophosphate-sugar while releasing uridine monophosphate. The lipid acceptor appears to be identical to that utilized for peptidoglycan and lipopolysaccharide synthesis. Subsequently, further sugars are thought to be transferred from sugar diphosphonucleotides to form the

oligosaccharide repeat. Polymerisation of the oligosaccharide repeats then results in the generation of the high molecular weight EPS (Sutherland, 1979; Tolmasky *et al.*, 1982; Sutherland, 1985). The involvement of EPS in infection and the genetics of EPS synthesis are discussed in sections 1.5.3 and 1.5.4.

The structures of the acidic-Calcofluor-binding exopolysaccharide of *R. meliloti*, a second acidic exopolysaccharide synthesised by *R. meliloti*, and the acidic exopolysaccharide of *Rhizobium* sp. strain NGR234 are shown in Figs. 3A, 3B, and 3C, respectively (Gray and Rolfe, 1990).

### 1.5.3 The Involvement of EPS in Infection

The relationship between polysaccharide requirement and effective nodulation is complex. This is exemplified by current evidence which suggests that (acidic) EPS is required for the establishment of effective nodules on indeterminate nodulating legumes but is not required for the establishment of effective nodules on (most) determinate nodulating legumes. Hence it has been shown that EPS is required for the following indeterminate type symbioses: *R. meliloti* and alfalfa (Finan *et al.*, 1985; Leigh *et al.*, 1985); *R. leguminosarum* bv. *viciae* and peas (Borthakur *et al.*, 1986); *R. leguminosarum* bv. *trifolii* and clover (Chakravorty *et al.*, 1982); and *Rhizobium* sp. NGR234 and *Leucaena leucocephala* (Chen *et al.*, 1985). In contrast, EPS is not required for the following determinate type symbioses: *R. fredii* and soybeans (Kim *et al.*, 1989; Ko and Gayda, 1990); and *R. leguminosarum* bv. *phaseoli* and *Phaseolus* bean (Borthakur *et al.*, 1986; Diebold and Noel, 1989). However, EPS is apparently required for the effective nodulation of siratro (*Macropitium atropurpureum*) by *Rhizobium* sp. NGR234 (Chen *et al.*, 1985; Djordjevic *et al.*, 1987) and appears to have some role, although it may not be absolutely required, in the nodulation of soybean by *Bradyrhizobium japonicum* (Law *et al.*, 1982). Both siratro and soybean are determinate nodulating legumes.

The most persuasive approaches used in previous studies demonstrating a nodule type specific requirement for EPS have involved either introducing EPS (*pss*) mutations by marker exchange into two strains derived from *R. leguminosarum* bv. *phaseoli* differing only in their symbiotic plasmids and hence their host plants (Borthakur *et al.*, 1986), or utilizing haploid recombination to transfer EPS mutations between *R. leguminosarum* bv. *phaseoli*, and either *R. leguminosarum*

Figure 3A:

The structure of *R. meliloti* EPSI.

Figure 3B:

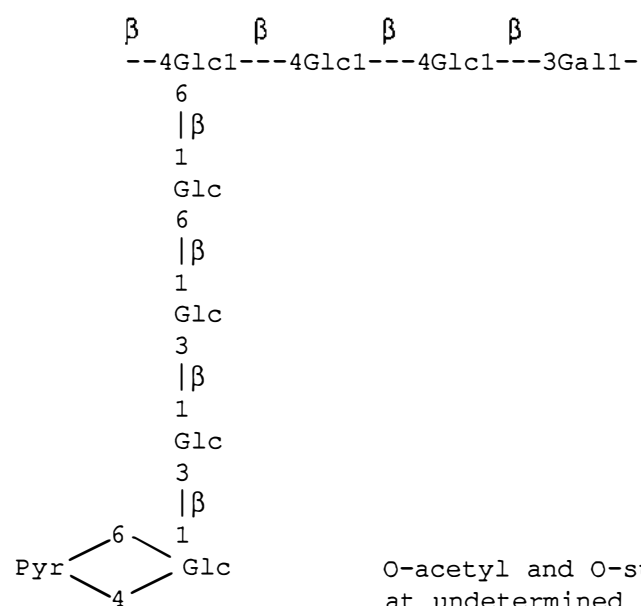
The structure of *R. meliloti* EPSII.

Figure 3C:

The structure of *Rhizobium* sp. NGR234 EPS.

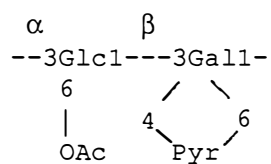
Glc (glucose), Gal (galactose), GlcA (glucuronic acid)

A

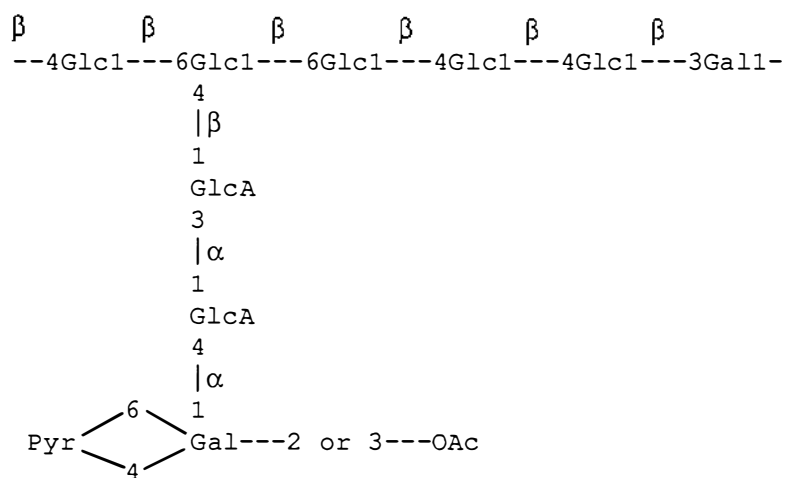


O-acetyl and O-succinyl groups present  
at undetermined sites

B



C



bv. *viciae* or *R. leguminosarum* bv. *trifolii* (Diebold and Noel, 1989).

In the first case, Borthakur *et al.* (1986) isolated a non-mucoid EPS mutant derived from *R. leguminosarum* bv. *phaseoli*, strain 8002, which induced effective nodules on *Phaseolus* bean. Two mutations in this gene (*pss*) were transferred by marker exchange into two near-isogenic *Rhizobium* strains, which differ in the identity of their symbiotic plasmids, strain 8002 that nodulates *Phaseolus* bean and strain 8401pRL1JI that nodulates peas. Strains resulting from the transfer of *pss* mutations were non-mucoid. The *pss*::Tn5 derivatives induced effective nodules on *Phaseolus* bean, but failed to nodulate peas, although they could curl pea root hairs (Borthakur *et al.*, 1986).

In the second case, Diebold and Noel (1989) isolated a series of EPS mutants derived from *R. leguminosarum* bv. *phaseoli*, which formed effective nodules on *Phaseolus* bean. The *exo*::Tn5 alleles from these mutants were transferred to *R. leguminosarum* bv. *viciae* (host pea) and *R. leguminosarum* bv. *trifolii* (host clover) with all EPS-deficient transconjugants isolated being symbiotically deficient, inducing no nodules and ineffective nodules, respectively, on each host.

Chen *et al.* (1985) have isolated ninety mutants with altered EPS synthesis from *Rhizobium* sp. NGR234 which is capable of nodulating hosts which form both determinate and indeterminate nodules. While the exopolysaccharide-defective mutants showed altered host range there was no consistent relationship between altered host range and nodule type. For example, the EPS<sup>-</sup> (rough) mutants induced the formation of callus-like structures on *L. leucocephala*. However, less extensive inhibition of effective nodulation was also observed for some mutants on siratro. In addition, coinoculation of EPS with an EPS<sup>-</sup> mutant restored this mutant to effective nodulation on both *L. leucocephala* and siratro (Djordjevic *et al.*, 1987).

#### 1.5.4 The Genetics of EPS Synthesis

The genetics of EPS synthesis has been most studied in *R. meliloti*, *R. leguminosarum* bv. *phaseoli*, and *Rhizobium* sp. NGR234. Synthesis of the acidic-Calcofluor-binding EPS (EPSI or EPSa) of *R. meliloti* SU47, which has both high and low molecular weight forms, requires the products of a cluster of genes located on the second megaplasmid, pRmeSU47b, together with several genes located on

the chromosome. Mutations in *exoA*, *exoB*, *exoF*, *exoL*, *exoM*, *exoP*, *exoQ*, and *exoT* completely abolish production of EPSI and result in mutants that induce the formation of ineffective nodules on alfalfa (Finan *et al.*, 1985; Leigh *et al.*, 1985; Long *et al.*, 1988; Reuber *et al.*, 1991). Buendia *et al.* (1991) have recently shown that *exoB* encodes a UDP-glucose 4-epimerase, interconverting UDP-glucose and UDP-galactose. This explains the observations that *exoB* is required for synthesis of EPSI, EPSII (Glazebrook and Walker, 1989; Zhan *et al.*, 1989; discussed later in this section), and LPS (Leigh and Lee, 1988). Examination of Tn*phoA* translational fusions to *exoF* and *exoP* suggested that their gene products are membrane borne (Long *et al.*, 1988). In addition, by staining nodule sections for the *in situ* localization of alkaline phosphatase activity, Reuber *et al.* (1991) have used Tn*phoA* fusions to show that the *exoA*, *exoF* and *exoP* gene products are actively expressed almost exclusively in the invasion zone of the nodule, suggesting that EPSI is not required in later stages of nodulation.

Mutations in *exoG* and *exoJ* diminish the production of Calcofluor-binding material, *exoG* mutants producing no high molecular weight material, and result in mutants which form effective nodules, but with reduced efficiency (Long *et al.*, 1988). Mutations in *exoN* result in diminished production of EPSI but do not affect the symbiotic phenotype (Long *et al.*, 1988). Similarly, mutations in *exoK* cause a decrease in EPSI production and, in addition, a delay in the appearance of a fluorescent halo on Calcofluor plates, but do not affect the symbiotic phenotype. Mutations in *exoH* result in the synthesis of EPSI which is not succinylated and mutants that fail to form a fluorescent halo on Calcofluor plates (Leigh *et al.*, 1987). More recently, Leigh and Lee (1988) have shown that *exoH* mutants produce little low molecular weight EPSI. For the *exoH* mutants, root hair curling is significantly delayed and infection threads abort in the nodule cortex, leading to the formation of ineffective nodules (Leigh *et al.*, 1987). Similarly, Muller *et al.* (1988) have isolated mutants which synthesize EPSI lacking the terminal pyruvate residue on the side chain of the repeating octasaccharide, which also induce the formation of ineffective nodules lacking infection threads. This mutation was located on the chromosome (Muller *et al.*, 1988) and when considered alongside *exoH* mutants, shows that non-carbohydrate modifications to the octasaccharide repeating unit are required for the function(s) of EPSI in the symbiosis.

Additional chromosomal loci, including *exoC* and *exoD* also affect the synthesis of EPSI (Finan *et al.*, 1986; Leigh *et al.*, 1985). Furthermore, two recently identified,

unlinked, chromosomal loci, *exoR* and *exoS*, play a negative role in regulating EPSI synthesis (Doherty *et al.*, 1988). Mutants in *exoR* and *exoS* produce more EPSI and are more Calcofluor-bright than the wild type strain (Doherty *et al.*, 1988). While *exoS* mutants induce normal nodules, *exoR* mutants induce ineffective nodules, suggesting that at least some regulation of EPSI synthesis is required for effective nodulation. In addition, *exoA*, *exoF*, *exoP*, *exoQ* and *exoT* are coordinately regulated by the products of *exoR* and *exoS*. This regulation probably acts at the level of transcription or mRNA stability as mRNA levels increase in an *exoR* background (Doherty *et al.*, 1988; Reuber *et al.*, 1991). The *exoR* gene product may, therefore, be involved in suppressing EPSI synthesis in the nodule (Reuber *et al.*, 1991). In addition, the *exoR* gene product may be involved in sensing the level of ammonia (Doherty *et al.*, 1988).

Similarly *Rhizobium* sp. NGR234 has a cluster of at least ten *exo* genes involved in the synthesis of its acidic EPS, most of which are functionally homologous to the cluster of *R. meliloti* *exo* genes (Zhan *et al.*, 1990).

A series of genes, *psi*, *pss*, and *psr* in the *R. leguminosarum* bv. *phaseoli* genome have been identified which are involved in EPS inhibition, EPS synthesis, and EPS restoration respectively (Borthakur *et al.*, 1985; Borthakur *et al.*, 1986; Borthakur and Johnston, 1987; Borthakur *et al.*, 1988). The *psi* and *psr* genes are located on the symbiotic plasmid close to the *nod* and *nif* genes (Borthakur and Johnston, 1987), while the *pss* gene(s) are presumably located on the chromosome (Borthakur *et al.*, 1986).

Genes homologous to *psi* have been isolated from several other bacterial species: *psdA* in *Agrobacterium tumefaciens* (Kamoun *et al.*, 1989); *exoX* in *Rhizobium* sp. NGR234 (Gray *et al.*, 1990); and *exoX* in *R. meliloti* (Zhan and Leigh, 1990). When these genes were cloned on multicopy plasmids and transferred into their wild type strains the transconjugants produced no, or greatly reduced amounts of EPS, while mutants with Tn5 inserts within the genomic copies of the genes produced normal quantities of EPS, except *R. meliloti* *exoX* mutants, which produced increased levels of EPS. *R. leguminosarum* bv. *phaseoli* *psi::Tn5* mutants are ineffective on *Phaseolus* bean, while the presence of *psi* on multicopy plasmids appears to inhibit nodulation (Borthakur *et al.*, 1985). In contrast, *R. meliloti* *exoX* mutants are fully effective on alfalfa while strains carrying multicopy *exoX* are ineffective (Zhan and Leigh, 1990).

The inhibitory effect of the *psi* and equivalent genes on EPS synthesis when cloned on multicopy plasmids is opposed when *pss* of *R. leguminosarum* bv. *phaseoli* (Borthakur *et al.*, 1988), *exoY* of *Rhizobium* sp. NGR234 (Gray *et al.*, 1990), or *exoF* of *R. meliloti* (Zhan and Leigh, 1990) are expressed to the same copy number. Mutations in these genes abolish production of EPS and the respective strains are unable to effectively nodulate their indeterminate hosts, except for *pss* mutants which fail to nodulate pea. However, *pss::Tn5* mutants are fully effective on the determinate nodulating *Phaseolus* bean (Borthakur *et al.*, 1986), suggesting that EPS is not required for effective nodulation of *Phaseolus* bean but that EPS repression (by *psi*) may be required in the bacteroid state (Borthakur *et al.*, 1985). Recently, Latchford *et al.* (1991) have indeed shown that *psi* is expressed in bacteroids. The presence of *exoX* of *R. meliloti* in multiple copies, however, has little or no effect on *exo* gene fusions (Zhan *et al.*, 1990) suggesting that *exoX* is unlikely to be involved in the regulation of EPSI synthesis in bacteroids.

*Psr* is known only from *R. leguminosarum* bv. *phaseoli* and when cloned on multicopy plasmids overcomes the EPS<sup>-</sup> defect in strains carrying multicopy *psi* and was shown to repress the transcription of *psi* (Borthakur and Johnston, 1987). Strains carrying multicopy *psr* are EPS<sup>+</sup>, but induce ineffective nodules on *Phaseolus* bean, while multicopy *psr* overcame both the EPS and nodulation defects of multicopy *psi* (Borthakur and Johnston, 1987). *Psr::Tn5* mutants showed reduced EPS synthesis but had no symbiotic effect (Borthakur and Johnston, 1987).

Based upon sequence similarity between *pss2*, *exoY* and *exoF*, and between *psi* and *exoX*, and considering that all the putative proteins have significant stretches of hydrophobic amino acids, Gray and Rolfe (1990), and Zhan and Leigh (1990) have proposed that the proteins form a membrane associated complex involved in EPS production.

Recently, Glazebrook and Walker (1989) and Zhan *et al.* (1989) have shown that *R. meliloti* can synthesize a second non-Calcofluor-binding EPS (EPSII or EPSb). Glazebrook and Walker (1989) have demonstrated that EPSII synthesis requires the functions of *exoB*, which encodes a UDP-glucose 4-epimerase (Buendia *et al.*, 1991), and at least six *exp* loci, clustered on megaplasmid pRmeSU47b. An additional mutation, *exp101*, located on the chromosome slightly counterclockwise from *trp33* is responsible for increased transcription of the megaplasmid-borne *exp*



genes (Glazebrook and Walker, 1989). Zhan *et al.* (1989) have identified a further chromosomal locus, *mucR*, located slightly counterclockwise from *pyr49* which is involved in repression of EPSII synthesis and stimulation of EPSI synthesis. In addition, Zhan *et al.* (1989) have shown that EPSII synthesis requires the functions of both *exoB* and *exoC*, and the functions of a cluster of genes on megaplasmid pRmeSU47b. Interestingly Glazebrook and Walker (1989) and Zhan *et al.* (1989) have shown that EPSII can substitute for EPSI on some hosts, for example alfalfa, but not on other hosts.

Based upon the common structural feature (see Figs 3A and 3B) of *R. meliloti* EPSI and EPSII (a Glu- $\beta$ (1,3)-Gal linkage) Glazebrook and Walker (1989) have proposed that the modification of this linkage in EPSII (and potential modification in EPSI) with *O*-acetyl and acidic substituents may protect the linkage from cleavage by  $\beta$ -1,3 glucanases. This hypothesis does not, however, explain the requirement for exopolysaccharides and the observation that EPSII can substitute for EPSI only on some hosts. Glazebrook *et al.* (1989) have proposed that if oligosaccharide fragments of EPSI and EPSII function as signal molecules, then plants which require EPSI production recognize a EPSI specific signal, while those plants that require either EPSI or EPSII recognize a common signal, perhaps a Glu- $\beta$ (1,3)-Gal dimer or modified dimer.

Cangelosi *et al.* (1987) have isolated mutants of *Agrobacterium tumefaciens* that fail to synthesize acidic EPS. Complementation analysis has shown that the *R. meliloti* complementation groups identified by Leigh *et al.* (1985) are represented among these mutants (Cangelosi *et al.*, 1987). *Agrobacterium* genes which complemented EPS defects in three *R. meliloti* *exo* mutants were cloned and in two of these cases the symbiotic defects were also complemented. All of the *Agrobacterium* EPS mutants, except *exoC*, formed normal crown gall tumours on four different host plants (Cangelosi *et al.*, 1987).

Thomashow *et al.* (1987) have identified an *Agrobacterium* gene *pscA* that affects polysaccharide composition and plant cell attachment. Mutants in the *pscA* locus are nonfluorescent on agar plates containing Calcofluor, do not produce cellulose fibrils, and produce little, if any, of the four polysaccharide species synthesized by the wild type strain (Thomashow *et al.*, 1987). Thus the *pscA* locus appears to have pleiotropic effects on polysaccharide synthesis in *Agrobacterium*. Furthermore, *pscA* was found to be related to *exoC* of *R. meliloti* (Leigh *et al.*,

1985; Thomashow *et al.*, 1987).

## 1.6 NODULINS

In addition to the requirement for the expression of specific *Rhizobium* genes, host specific nodule proteins or nodulins (Legocki and Verma, 1980) are also induced at each stage during the establishment of an effective symbiosis. The expression of nodulin genes has been established by comparison of *in vitro* translation products from nodules and root mRNA or by the isolation of nodule specific cDNA clones. By definition, nodulins include only plant genes expressed exclusively in root nodules. However, it is now clear that, while the nodulins are either specifically synthesized or present in higher concentration during nodule development (Legocki and Verma, 1980; Bisseling *et al.*, 1983; Fuller *et al.*, 1983; Govers *et al.*, 1987), at least some are expressed in other organs (Bennett *et al.*, 1989; Forde *et al.*, 1989; Scheres *et al.*, 1990a), and may also have nonsymbiotic counterparts in non-legumes (Bogusz *et al.*, 1988).

Until recently, studies on the expression of nodulin genes have generally been confined to the final stages of root nodule development. However, the formation of effective root nodules is developmentally complex and major decisions determining root nodule development and eventual morphology are made in the stages preceeding the final establishment of an effective nodule (Franssen *et al.*, 1987). Gloudemans *et al.*, (1987) have divided nodulins of soybean (*Glycine max*) into two classes according to their time of expression during nodule development.

Early (Class A) nodulins are expressed during the preinfection, and infection and nodulation stages (stages 1 and 2) while late (Class B) nodulins are expressed during the nodule function stage (stage 3), that is, around the onset of nitrogen fixation (Scheres *et al.*, 1990b). However Scheres *et al.* (1990b) have recently shown that the division of nodulins into two classes is arbitrary and does not actually reflect the existence of two main timepoints at which the invading *Rhizobium* strain induces expression of plant genes.

The late nodulins are involved in creating the physiological conditions required in the nodule for nitrogen fixation, ammonia assimilation, and transport. The late nodulins leghaemoglobin (Appleby, 1984), uricase (Bergman *et al.*, 1983; Nguyen *et al.*, 1985) and glutamine synthetase (Cullimore *et al.*, 1984) are among the best

characterized nodulins.

While late nodulins are essential for final nodule function, they are not required during the morphological development of the root nodule. Nodule development (including infection) requires instead, the expression of early nodulin genes, which are now also being intensively studied. cDNA clones representing early nodulins (ENOD2, ENOD13, and ENOD55) involved in nodule morphogenesis but not the infection process have been isolated from soybean (Franssen *et al.*, 1987, 1988), alfalfa (Dickstein *et al.*, 1988), and pea (van de Wiel *et al.*, 1990a). The best characterised of these is ENOD2, which is a hydroxyproline-rich protein likely to be a cell wall component (Franssen *et al.*, 1987) that is involved in the formation of the nodule parenchyma surrounding the central nodule tissue (van de Wiel *et al.*, 1990a). The nodule parenchyma contains relatively few and small intercellular spaces (through which oxygen is likely to diffuse into the nodule) compared to the nodule cortex and central nodule tissue, which have relatively large intercellular spaces. Hence, the nodule parenchyma may form an oxygen diffusion barrier (the concentration of free oxygen in the nodule declines rapidly across the nodule parenchyma), a necessity to protect nitrogenase (Tjepkema and Yocum, 1974, Witty *et al.*, 1986). Hence van de Wiel *et al.* (1990a) have proposed that the ENOD2 protein is a factor involved in cell wall differentiation leading to the specific tissue morphology of the nodule parenchyma.

An additional nodulin, Nms-30 from alfalfa, is also associated with nodule morphogenesis, but is only known as an *in vitro* translation product (Van de Wiel *et al.*, 1990b).

Transcripts for Nms-30 and ENOD2 are found in the ineffective nodules formed after inoculation of alfalfa by *R. meliloti* EPS (*exo*) mutants, *Agrobacterium tumefaciens* transconjugants carrying cloned nodulation genes, and after spot inoculation with compounds acting as auxin transport inhibitors (ATI s, N-[1-naphthyl] phthalamic acid, or 2,3,3-triiodobenzoic acid) (Van de Wiel *et al.*, 1990b; Hirsch *et al.*, 1989). The observation that auxin transport inhibitors induce the normal pattern of ENOD2 gene expression and that this expression is nodule specific suggests that ENOD2 gene expression and nodule cell divisions are related to the hormonal status of the plant and that the ATI s mimic endogenous factors responsible for nodule development, that is, rhizobia are capable of controlling the plants developmental programming (Van de Wiel *et al.*, 1990b; Hirsch *et al.*, 1989).

The ATI s are therefore able to substitute for the activity of compounds synthesized or activated following the induction of nodulation genes. Induction of the nodulation genes results in the synthesis of a lipo-oligosaccharide signal molecule (Lerouge *et al.*, 1990; Truchet *et al.*, 1991; Spaink *et al.*, 1991), however, this signal molecule is structurally quite different from the ATI s suggesting that they are unlikely to act at the same site. Hence, the lipo-oligosaccharide signal molecule may function by stimulating the production of an endogenous plant factor that directly or indirectly interacts with the same site as the ATI s (Van de Wiel *et al.*, 1990b), therefore interfering with auxin transport. Alternatively, the lipo-oligosaccharide signal molecule may stimulate the production of compounds that interfere with auxin synthesis or action.

It may be, however, that the substitution by ATI s for compounds synthesized or activated following nodulation gene activation is not the result of their role as ATI s directly, but rather due to perturbation (increase) of the endogenous cytokinin/auxin ratio (Hirsch *et al.*, 1989). An increase in the cytokinin/auxin ratio could also be achieved if the mode of action of the lipo-oligosaccharide signal molecule is to directly or indirectly influence cytokinin secretion. Interestingly, spot inoculation of alfalfa roots with a *nodA* mutant of *R. meliloti*, carrying the *tzs* (*trans*-zeatin synthesis or secretion) gene of *A. tumefaciens*, results in the formation of empty nodules, although it is not known whether ENOD2 and Nms-30 are expressed in these nodules (reported in Hirsch *et al.*, 1989).

Recently, Scheres *et al.* (1990a) have identified an early nodulin (ENOD12) involved in infection which, like ENOD2, is a hydroxyproline-rich protein likely to be a cell wall component. Subsequently, Scheres *et al.* (1990b) have shown that early pea nodulin mRNAs vary in their time course of appearance during root nodule development and are also located in different developmental zones of the root nodule. ENOD12 transcripts were found to be present in every cell of the invasion zone, while ENOD5, ENOD3 and ENOD14 transcripts were restricted to infected cells in successive, but partially overlapping, zones in the central tissue of the nodule (Scheres *et al.*, 1990b). ENOD5 is an arabinogalactan-like protein involved in the infection process, which may be membrane bound (has hydrophobic domains) or may be an extracellular protein present in both the infection thread and the peribacteroid space (Scheres *et al.*, 1990b). ENOD3 and ENOD14 both have a cysteine cluster and may have a role in metal ion transport (Scheres *et al.*, 1990b). ENOD12 gene expression appears to be induced by a

diffusable compound acting over a large distance and requires the bacterial *nod* genes (Scheres *et al.*, 1990a) suggesting that induction involves the action of the lipo-oligosaccharide signal molecule (Lerouge *et al.*, 1990). In contrast, a factor acting over a short distance appears to be involved in activation of the ENOD5 gene (Scheres *et al.*, 1990b).

While the early nodulins described above have been cloned on the basis that they exhibit interesting patterns of differential gene expression in root nodule tissue vs root tissue, and also within root nodule tissue, their functions *per se* have not been elucidated. Their possible functions can be inferred from their patterns of gene expression and from the (presumptive) amino acid sequence of the translated message, however, demonstration of function will require perturbation of gene expression. Since the respective cDNA clones have been sequenced approaches utilizing targeted gene mutations, or homologous recombination following site directed mutagenesis of the cDNA clone, possibly requiring the utilization of a heterologous host (Stougaard *et al.*, 1986; Stougaard *et al.*, 1987a and b; Akit *et al.*, 1987), may eventually provide this information.

## 1.7 BACKGROUND AND AIMS OF THIS INVESTIGATION

Infection mutants of *R. loti* strain PN184 (NZP2037 *str-1*) which induce the formation of ineffective, tumour-like nodules on *Lotus pedunculatus* have been isolated (Chua *et al.*, 1985). While the gene corresponding to one of these mutants has been sequenced (Ward *et al.*, 1989), the biochemical block(s) in this mutant is (are) unknown. One of the infection mutants (PN1018), however, lacks a peptidoglycan-bound polysaccharide present in NZP2037 which is correlated with the resistance of NZP2037 to prodelphinidin-rich flavolans produced by the host plant (Pankhurst *et al.*, 1987). This establishes the possibility that specific polysaccharides are required for effective *R. loti*-legume symbioses.

Furthermore, certain *R. loti* strains, including PN184, have a broad host range and are capable of inducing the formation of effective nodules on both determinate nodulating legumes such as *L. pedunculatus* and *Lotus corniculatus* var. *cree*, and indeterminate nodulating legumes such as *Leucaena leucocephala*. Other strains, such as PN4115 (NZP2213 *str-1*), have a more restricted host range and, of the legumes mentioned above, induce the formation of effective nodules only on *L. corniculatus* var. *cree*. The ineffective nodules formed after inoculation of *L.*

*pedunculatus* and *L. leucocephala* with PN4115 resemble in several ways the ineffective nodules formed on, for example, alfalfa after inoculation with infection mutants (exopolysaccharide, *exo*;  $\beta$ -1,2 glucan, *ndv*) of *R. meliloti* (Finan *et al.*, 1985, Leigh *et al.*, 1985), and *Phaseolus* bean after inoculation with infection mutants (lipopolysaccharide) of *R. leguminosarum* bv. *phaseoli* (Noel *et al.*, 1986). This suggests that PN184 may synthesize extracellular polysaccharides, either not synthesized, or synthesized, but with structural differences, by PN4115, which extend the host range of PN184 (NZP2213 is the *R. loti* type species).

The primary aims of this investigation were therefore:

- 1) To evaluate the potential for using *R. loti* as a model *Rhizobium* species for investigating the role of EPS in effective nodulation by isolating and characterizing EPS mutants, primarily from *R. loti* strain PN184, and secondarily from *R. loti* strain PN4115. In particular, this strategy is expected to facilitate the study of the symbiotic effect of EPS mutations on effective nodulation of both determinate and indeterminate nodulating legumes since the *Rhizobium* background will be isogenic.
- 2) To determine if the correlations between the requirement for EPS production (including structural considerations) and effective nodulation of indeterminate hosts, and the non-requirement for EPS production for effective nodulation of determinate hosts (see for example Borthakur *et al.*, 1986; Chen *et al.*, 1985; Diebold and Noel, 1989; Finan *et al.*, 1985; Kim *et al.*, 1989; Ko and Gayda, 1990; Leigh *et al.*, 1985) is also found for *R. loti*-legume associations.
- 3) To determine if any structural, or other, differences exist between the extracellular polysaccharides, in particular EPS, synthesized by PN184 and PN4115, and, if differences are found, to determine if these are sufficient to account for the inability of PN4115 to induce the formation of effective nodules on the extended range of hosts exhibited by PN184, in particular, the indeterminate nodulating host legume, *L. leucocephala*.

## Chapter 2. MATERIALS AND METHODS

### 2.1 BACTERIAL STRAINS, PLASMIDS, COSMIDS AND BACTERIOPHAGE

Bacterial strains, plasmids, cosmids and bacteriophage used in this study are listed in Table 2.

### 2.2 GROWTH OF BACTERIA

*R. loti* cultures were grown at either 30°C or 25°C in either TY broth (Section 2.5.2; Beringer, 1974), S20 broth (Section 2.5.4; Chua *et al.*, 1985), nitrogen-free S20 broth (Section 2.5.4; S20 medium [Chua *et al.*, 1985] lacking ammonium chloride) or YM broth (Section 2.5.3; M<sup>+</sup> medium, Pankhurst, 1977). *R. loti* cultures were maintained at 4°C on YM agar (Section 2.5.3). Single colony purifications were performed on TY agar (Section 2.5.2) for PN184, PN4115, and the PN184 derived mutants, or on S20 agar (Section 2.5.4) for the PN4115 derived mutants). Antibiotics were supplemented as required at the following concentrations: neomycin (Nm), PN184 derived mutants 400 µg/ml, PN4115 derived mutants 100 µg/ml; streptomycin (Sm), PN184 and the PN184 derived mutants 200 µg/ml, PN4115 and the PN4115 derived mutants 100 µg/ml; tetracycline (Tc), 2 µg/ml.

Growth of rhizobia in broth cultures was determined, where required, by measurement of A<sub>600</sub> and/or by dilution plating.

*E. coli* cultures were grown at either 30°C or 37°C in LB broth (Section 2.5.1; Miller, 1972). *E. coli* cultures were maintained on LB agar (Section 2.5.1) at room temperature and were subcultured regularly (approximately every 4-6 weeks). Antibiotics were supplemented as required at the following concentrations: Sm, 200 µg/ml; Tc, 15 µg/ml; Ampicillin (Ap), 100 µg/ml; Kanamycin (Km), 200 µg/ml. Ampicillin was used at 100 µg/ml to reduce the incidence of satellite colonies.

Growth of bacteria in broth cultures was determined, where required, by measurement of A<sub>600</sub> and/or by dilution plating.

Table 2. Bacterial strains, plasmids, cosmids and bacteriophage.

Strain, Plasmid, Cosmid or Phage	Genotype or Relevant Characteristics	Source or Reference
<i>Rhizobium loti</i>		
NZP2037	Nod <sup>+</sup> Fix <sup>+</sup> ( <i>Lotus pedunculatus</i> , <i>Leucaena leucocephala</i> )	DSIR Culture Collection
PN184	NZP2037 <i>str</i> -1	Chua et al. (1985)
PN4010	NZP2037, plasmid cured	Pankhurst et al. (1986)
PN1018	PN184::Tn5 Nod <sup>+</sup> Fix <sup>-</sup> ( <i>Lotus pedunculatus</i> )	Chua et al. (1985)
PN1019	PN184::Tn5 Nod <sup>+</sup> Fix <sup>-</sup> ( <i>Lotus pedunculatus</i> )	Chua et al. (1985)
PN1027	PN184::Tn5 Nod <sup>+</sup> Fix <sup>-</sup> ( <i>Lotus pedunculatus</i> )	Chua et al. (1985)
PN1177	PN184exo1::Tn5	This study
PN1178	PN184exo2::Tn5	This study
PN1179	PN184exo3::Tn5	This study
PN1180	PN184exo5::Tn5	This study
PN1181	PN184exo6::Tn5	This study
PN1182	PN184exo7::Tn5	This study
PN1183	PN184exo8::Tn5	This study
PN1184	PN184exo9::Tn5	This study
GH4	PN184exo4::Tn5	This study
LPS1-LPS14	PN184::Tn5 (putative LPS mutants)	This study
NZP2213	Nod <sup>+</sup> Fix <sup>+</sup> ( <i>Lotus corniculatus</i> ) Nod <sup>+</sup> Fix <sup>-</sup> ( <i>Lotus pedunculatus</i> , <i>Leucaena leucocephala</i> ).	DSIR Culture Collection
PN4115	NZP2213 <i>str</i> -1	DSIR Culture Collection



PN1312	PN4115exo1::Tn5	This study
PN1313	PN4115exo2::Tn5	This study
PN1314	PN4115exo3::Tn5	This study
GH106	PN4115exo4::Tn5	This study
GH180	PN4115exo5::Tn5	This study
GH214	PN4115exo6::Tn5	This study
<i>Escherichia coli</i>		
HB101	<i>pro leu thi lacY recA strA</i> <i>hsdD hsdM</i>	Boyer and Roulland- Dussoix (1969)
MC1022	<i>araD</i> $\Delta$ ( <i>ara leu</i> ) $\Delta$ ( <i>lacZ</i> ) M15 <i>galU galK strA</i>	Casadaban and Cohen (1980)
SM10	<i>thi thr leu tonA supE recA</i> [RP4.2 Tc::Mu] Ap <sup>R</sup> Tc <sup>R</sup> Km <sup>R</sup> Mu2 <sup>+</sup> Tra <sup>+</sup>	Simon et al. (1983)
PN302	SM10	D B Scott
PN362	HB101/pRK2013	D B Scott
PN1240	HB101/pPN31	This study
PN1241	HB101/pPN32	This study
PN1242	HB101/pPN33	This study
PN1243	HB101/pPN34	This study
PN1244	HB101/pPN35	This study
PN1315	MC1022/pPN47	This study
PN1316	MC1022/pPN48	This study
Bacteriophage		
$\Phi$ 2037/1	Virulent to NZP2037 and NZP2213	Patel (1976)
Plasmids/Cosmids		
pUC118	Ap <sup>r</sup>	Vieria and Messing (1983)
pPK2013	Km <sup>r</sup>	Ditta et al. (1980)
pSUP1011	Cm <sup>r</sup> Km <sup>r</sup> <i>oriT</i> RP4	Simon et al. (1983)
pKan2	pBR322 carrying the 3.5 kb <i>HindIII</i> fragment of Tn5	Scott et al. (1982)

pLAFR1	$\lambda$ cos derivative of pRK290	Friedman et al. (1982)
pPN31	pLAFR1 cosmid from a NZP2037 gene library that complements PN1180	This study
pPN32	pLAFR1 cosmid from a NZP2037 gene library that complements PN1181	This study
pPN33	pLAFR1 cosmid from a NZP2037 gene library that complements PN1182	This study
pPN34	pLAFR1 cosmid from a NZP2037 gene library that complements PN1183	This study
pPN35	pLAFR1 cosmid from a NZP2037 gene library that complements PN1184	This study
pPN47	pUC118 carrying the 0.8 kb <i>EcoRI</i> fragment from pPN33	This study
pPN48	pUC118 carrying the 4.1 kb <i>EcoRI</i> fragment from pPN32	This study
pD34	pLAFR1 cosmid from a <i>R. meliloti</i> gene library that complements exoPNMALKH mutations	Leigh et al. (1985)
pD56	pLAFR1 cosmid from a <i>R. meliloti</i> gene library that complements exoJGFQB mutations	Long et al. (1988)
pEX312	pLAFR1 cosmid from <i>R. meliloti</i> gene library that complements exoPNMALKHJGFQB mutations	Long et al. (1988)

## 2.3 LONG TERM MAINTENANCE OF BACTERIA

Bacteria (both *Rhizobium* and *E. coli* strains) were maintained for long term storage in glycerol.

200 µl sterile glycerol was added and mixed with either 800 µl of an overnight (30°C) LB broth (Section 2.5.1) culture of the *E. coli* strain, or 800 µl of a 2 day (30°C) TY broth (Section 2.5.2) culture of the *Rhizobium* strain, as appropriate. The culture, in glycerol, was then stored at -20°C.

## 2.4 NODULATION TESTS

### 2.4.1 Inoculation and Growth of Plants

Seeds of *Lotus pedunculatus*, *Lotus corniculatus* and *Leucaena leucocephala* were surface sterilized by immersion for 3 min in a 1:1 (v/v) mixture of 30% hydrogen peroxide and 95% ethanol, drained, then washed for 1 min in 95% ethanol. The surface sterilized seeds were drained and washed 5 times with sterile MilliQ water, placed on 1% (w/v) agar plates (water agar) and germinated, inverted (except *L. leucocephala*), in the dark at 22°C. A small chip was aseptically removed from the testa of surface sterilized *L. leucocephala* seeds prior to placement on water agar for germination.

Following germination, *L. pedunculatus* seeds were transferred aseptically to 20 x 150 mm test tubes containing 6 ml Thornton's medium (Section 2.5.5; Thornton, 1930). *L. corniculatus* and *L. leucocephala* seeds were aseptically transferred to sterile 35 x 200 mm tubes containing sterile pumice moistened with sterile MilliQ water. All tubes were left in the dark for 24-48 h to promote root growth, then transferred to artificial illumination (12 h or 16 h photoperiod) at 22°C until emergence of the cotyledons at which point the seedlings were inoculated.

For inoculation, 2 loopfulls of a single colony purified, YM agar (Section 2.5.3) maintained, *Rhizobium* culture, were suspended in 3 ml sterile MilliQ water (*L. pedunculatus* inoculations) or in 6 ml sterile MilliQ water (*L. corniculatus* and *L. leucocephala* inoculations). Either 0.5 ml (*L. pedunculatus*) or 1.0 ml (*L. corniculatus*, *L. leucocephala*) aliquots of the suspension were used to inoculate each tube. Seedlings were grown under artificial illumination (12 h or 16 h

photoperiod) at 22°C for 4-6 weeks (*L. pedunculatus*), 6-8 weeks (*L. corniculatus*), or 6-12 weeks (*L. leucocephala*). *L. pedunculatus* seedlings were watered regularly with sterile MilliQ water. *L. corniculatus* and *L. leucocephala* seedlings were watered regularly with Hoagland's Medium (Section 2.5.6).

## 2.4.2 Determination of Nitrogen Fixation

Nitrogen fixation was determined by examination of plants, with plants showing stunted growth and yellowing of leaves indicating an ineffective symbiosis, and by acetylene reduction to ethylene.

### 2.4.2.1 Acetylene Reduction

The root system of test and control plants was removed, by excision with a razor blade, and placed separately in small (16 x 150 mm) Bell test tubes, which were then sealed individually with a gas impermeable bung.

Air (1.0 ml) was withdrawn (26 gauge needle, Terumo, Tuberculin syringe) from each tube and 1 ml acetylene (instrument grade, NZ Industrial Gases Ltd., which had been bubbled through concentrated sulphuric acid) was injected (26 gauge needle). After 1 hour, 1 ml of air was withdrawn from each tube into the same syringe, which was delivered immediately to James Evans (DSIR, Palmerston North, New Zealand) who performed the column injection.

0.5 ml of the withdrawn sample volume was analysed on a Pye Unicam 204 gas chromatograph, with flame ionization detector, and a column (900 mm x 4 mm internal diameter) packed with Poropack T or Poropack N under the following operating conditions: injection temperature 125°C; column temperature 125°C; detector temperature 150°C; carrier gas, oxygen free nitrogen (83 ml/min); ignition gases, dry hydrogen (42 ml/min), dry air (115 ml/min). Use of these operating parameters gives elution times of 0.35 min for ethylene and 0.6 min for acetylene.

The gas chromatograph was calibrated for each run with an acetylene standard.

## 2.4.3 Reisolation of Bacteria From Nodules

Nodules, with a small section of root remaining to aid handling, were excised with

the aid of a sterile scalpel blade and sterilized in the same manner as that described for the surface sterilization of seeds (Section 2.4.1). Surface sterilized nodules were aseptically crushed and the fluid released was plated onto YM agar (Section 2.5.3). The plates were incubated for two to six days at 30°C, then the resultant colonies were single colony purified (Section 2.2).

## **2.4.4 Light and Electron Microscopy**

### **2.4.4.1 Sections Stained with Toluidine Blue for Light Microscopy, and Thin Sections for Electron Microscopy**

Root nodules for study were removed by excision with a scalpel blade at room temperature under fixative (2% [v/v] formaldehyde, 3% [v/v] glutaraldehyde, in phosphate buffer [0.1 M disodium hydrogen orthophosphate, pH 7.2]). These nodules were cut, under fixative at room temperature, with a razor blade into either longitudinal or transverse sections. Cut sections were transferred to glass vials containing fresh fixative, in which they were fixed at room temperature for a further 1.5 h. The tissue samples were then washed three times in phosphate buffer, then postfixed for 30 min in 1% (w/v) osmium tetroxide in phosphate buffer. The samples were then dehydrated through an acetone series, with the two final steps in 100% acetone. The samples were then infiltrated overnight in 1:1 (v/v) mixture of acetone and Polarbed 812 resin (Biorad), and in 100% Polarbed 812 for a further 8 h. Samples were then embedded in fresh Polarbed 812 using silicone rubber moulds and left to cure at 60°C for 48 h (Craig and Williamson, 1972).

For light microscopy, 1 µm sections were stained with 0.5% (w/v) toluidine blue in phosphate buffer (described above). For electron microscopy thin sections were stained with saturated uranyl acetate in 50% (v/v) ethanol, and then with lead citrate (Craig and Williamson, 1972), and were examined in a Philips EM201 transmission electron microscope.

Fixation was performed either by the Author, or by Douglas Hopcroft (Electron Microscope Unit, DSIR, Palmerston North, New Zealand).

Embedding, sectioning and staining were performed by either Douglas Hopcroft or Raymond Bennett (Electron Microscope Unit, DSIR, Palmerston North, New

Zealand).

Examination of sections and photography were performed by the Author.

#### **2.4.4.2 Sections Stained with Calcofluor White and Acridine Orange**

Root nodules for study were removed by excision with a scalpel blade at room temperature. These nodules were cut at room temperature with a razor blade into thin longitudinal sections, either under fixative (in which they were fixed for a further 1.5 h at room temperature, Section 2.4.4.1) or under water. These longitudinal sections were stained directly with 0.1% (w/v) Calcofluor (Calcofluor White, Sigma) and 0.01% (w/v) acridine orange (BDH) in water and examined under epifluorescence microscopy.

### **2.5 MEDIA**

All media were sterilized at 121°C for 15 min prior to use. Liquid media were cooled to the desired growth temperature before addition of antibiotic(s) and inoculation. Solid media were cooled to 50°C prior to antibiotic addition and pouring. Uninoculated plates were stored at 4°C. When stored plates were used they were removed from storage 1 h or more before use and dried for approximately 30 min in a laminar flow cabinet to prevent "sweating".

Calcofluor (Cellufluor, Polysciences or Calcofluor White, Sigma) was included in solid media, where required, to 0.02% (w/v). The appropriate quantity of Calcofluor was dissolved in MilliQ water, sterilized using a Millipore filter (0.45 µm pore size), then added to the sterilized media.

Congo Red (BDH) was included in solid media, where required, to 0.005% (w/v). The appropriate quantity of a sterile stock solution (0.25 g Congo Red in 100 ml MilliQ water) was added to sterilized media.

#### **2.5.1 LB Medium**

LB media (Miller, 1972) contained (g/l): tryptone (Difco), 10.0; yeast extract (Difco), 5.0; sodium chloride, 5.0. The pH was adjusted to 7.0 prior to autoclaving. For solid media, agar (Davis) was added to 15.0 g/l.

### 2.5.2 TY Medium

TY media (Beringer, 1974) contained (g/l): tryptone (Difco), 5.0; yeast extract (Difco), 3.0; calcium chloride ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ), 1.0. For solid media agar (Davis) was added to 15.0 g/l.

### 2.5.3 YM Medium

YM media ( $\text{M}^+$  medium, Pankhurst, 1977) contained (g/l): potassium dihydrogen orthophosphate, 0.3; disodium hydrogen orthophosphate, 0.3; magnesium sulphate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ), 0.1; calcium chloride ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ), 0.05; ferric chloride, 0.001; yeast extract (Difco) 1.0; mannitol, 5.0. For solid media agar (Davis) was added to 15.0 g/l. The pH was adjusted to 6.8 prior to autoclaving.

### 2.5.4 S20 Medium

S20 media (Modified M9 media, Chua *et al.*, 1985) contained (g/l): potassium dihydrogen orthophosphate, 3.0; disodium hydrogen orthophosphate, 6.0; sodium chloride, 0.5; ammonium chloride, 1.0. The following were added as sterile solutions after autoclaving (ml/l): 10% (w/v) magnesium sulphate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ), 2.5; 1% (w/v) calcium chloride ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ), 1.5; 20% (w/v) sucrose, 12.0; and (500  $\mu\text{g/ml}$ ) biotin, 1.0. Nitrogen-free S20 media omitted addition of ammonium chloride. For solid media, agar (Davis) was added to 15.0 g/l.

For the preparation of solid media (per litre) the agar was autoclaved separately (in 700 ml MilliQ water) to the salts (in 283 ml MilliQ water).

For solid media containing Calcofluor, the appropriate quantity of Calcofluor (Section 2.5) was added to the autoclaved agar solution, any required antibiotics added to the salts solution, then the two solutions were combined. This procedure is required to prevent precipitation of the Calcofluor.

### 2.5.5 Thornton's Medium

Thornton's Medium (Thornton, 1930) was prepared from the following stock solutions.

Potassium phosphate stock solution (g/l): potassium dihydrogen orthophosphate, 54.6; dipotassium hydrogen orthophosphate, 30.0.

Magnesium sulphate/sodium chloride stock solution (g/l):  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 40.0; NaCl, 20.0.

Ferric chloride stock solution (g/l):  $\text{FeCl}_3$ , 100.0

Thornton's Medium consisted of (ml/l): Potassium phosphate stock solution, 2.0; magnesium sulphate/sodium chloride stock solution, 5.0; ferric chloride stock solution, 0.1; Hoagland's trace element solution (see section 2.5.6) 1.0; calcium orthophosphate, 1.0 g/l; ferric phosphate, 1.0 g/l; agar (Davis), 6.0 g/l.

### 2.5.6 Hoagland's Medium

Hoagland's medium was prepared from the following stock solutions.

Potassium phosphate stock solution (g/l): potassium dihydrogen orthophosphate, 122.5; dipotassium hydrogen orthophosphate, 17.4.

Magnesium sulphate/sodium chloride stock solution (g/l):  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 246.49; NaCl, 14.61.

Potassium sulphate stock solution (g/l):  $\text{K}_2\text{SO}_4$ , 87.13.

Calcium chloride stock solution (g/l):  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 147.02.

Ferric chloride/EDTA stock solution:  $\text{Na}_2\text{EDTA}$  5.0 g/l;  $\text{FeCl}_3$ , 5.0 ml/l of a 60% (w/v) solution.

Hoagland's trace element stock solution: (g/l): boric acid, 2.86; manganous chloride ( $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ), 0.22; copper sulphate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ), 0.08; cobaltous sulphate ( $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ ), 0.095; sodium molybdate ( $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ ), 0.054.

Hoagland's medium consisted of (ml/l): potassium phosphate stock solution, 1.0; magnesium sulphate stock solution, 2.0; potassium sulphate stock solution, 4.5; calcium chloride stock solution, 4.5; ferric chloride/EDTA stock solution, 2.5;



Hoagland's trace elements, 1.0.

## 2.6 BUFFERS AND SOLUTIONS

**2.6.1 TBE Buffer** contained 89 mM Tris-HCl, 2.5 mM Na<sub>2</sub>EDTA, and 89 mM boric acid, pH 8.3. A ten times concentrated stock solution was prepared by dissolving 107.7 g Tris, 9.3 g Na<sub>2</sub>EDTA and 55 g boric acid in 1 litre of MilliQ water, pH 8.3 (Maniatis *et al.*, 1982).

**2.6.2 STET Buffer** contained 8% (w/v) sucrose, 5% (v/v) triton X-100, 50 mM Na<sub>2</sub>EDTA (pH 8.0) and 50 mM Tris.HCl (pH 8.0).

**2.6.3 HaeIII (Universal) Buffer** was prepared at ten times the working concentration and contained 100 mM mercaptoethanol, 60 mM Tris-HCl (pH 7.6) and 100 mM magnesium chloride.

**2.6.4 SDS Loading Buffer** contained 1% (w/v) sodium dodecyl sulphate (SDS), 0.02% (w/v) bromophenol blue, 20% (w/v) sucrose, and 5 mM Na<sub>2</sub>EDTA (pH 8.0).

**2.6.5 TE Buffer** was prepared to the required concentration from 1M Tris-HCl (pH 8.0) and 250 mM Na<sub>2</sub>EDTA (pH 8.0) stock solutions.

**2.6.6 TEC Buffer** contained 10 mM Tris-HCl (pH 8.0), 0.25 mM Na<sub>2</sub>EDTA (pH 8.0), and 30 mM calcium chloride

**2.6.7 Hybridization Buffer** (Southern, 1975) contained (per litre): 50 ml 1M Hepes (Sigma), pH 7.0; 150 ml 20 x SSC (Section 2.6.10); 6 ml phenol extracted herring sperm DNA (3 mg/ml, Sigma); 5 ml 20% (w/v) SDS; 2.2 g Ficoll (Sigma 70); 2 ml *Escherichia coli* transfer RNA (10 mg/ml, Sigma); 2 g Bovine Serum Albumin; 2 g Polyvinylpyrrolidone(Sigma PVP-10); MilliQ water to 1.0 litre.

**2.6.8 Tris-Equilibrated Phenol** (Maniatis *et al.*, 1982) was prepared by melting solid phenol in a 50°C waterbath. Hydroxyquinoline was added to a final concentration of 0.1% (w/v). An equal volume of 1.0 M Tris-HCl (pH 8.0) was added at room temperature and stirred for 15 min. The phenolic phase was retained and washed 2-3 times with 1.0 M Tris-HCl (pH 8.0), essentially until the

pH of the phenolic phase was  $> 7.8$  (DNA will partition into the organic phase if the pH is acidic). After equilibration the phenolic phase was retained and washed 2-3 times with 100 mM Tris-HCl (pH 8.0) containing 0.02%  $\beta$ -mercaptoethanol. This equilibrated phenol solution was stored under 100 mM Tris-HCl (pH 8.0) in a brown bottle at 4°C.

**2.6.9 5 x Ligation Buffer** contained 250 mM Tris-HCl (pH 7.6), 50 mM magnesium chloride, 25% (w/v) polyethylene glycol 8000 (Sigma), 5 mM ATP, 5 mM dithiothreitol.

**2.6.10 20 x SSC** contained 3.0 M sodium chloride and 0.3 M sodium citrate.

**2.6.11 2 x SSC** was prepared by dilution of 20 x SSC 10 fold.

**2.6.12 TES (10/1/100)** contained 10 mM Tris-HCl (pH 8.0), 1.0 mM  $\text{Na}_2\text{EDTA}$  (pH 8.0) and 100 mM sodium chloride.

**2.6.13 Phage Dilution Buffer** contained 7.3 mM sucrose (2.5 g/l), 2.87 mM dipotassium hydrogen orthophosphate (0.5 g/l), 1.71 mM sodium chloride (0.1 g/l), and 0.5 g/l yeast extract (Difco).

**2.6.14 Phosphate buffered saline** contained 3.16 mM potassium dihydrogen orthophosphate (0.43 g/l), 9.65 mM dipotassium hydrogen orthophosphate (1.68 g/l), and 123 mM sodium chloride (7.2 g/l). The pH was adjusted to 7.2.

## 2.7 DNA ISOLATION

### 2.7.1 Isolation of Total DNA from *Rhizobium*

The method used was based upon that of Fischer and Lerman (1979) as described by Chua *et al.* (1985).

*Rhizobium* cultures (5 ml) were shaken for 40 h at 28-30°C in TY broth (Section 2.5.2) containing appropriate antibiotics (Section 2.2). The cells (2 x 1.5 ml) were harvested by centrifugation in a microcentrifuge for 5 min. The pellet was washed once by suspension in 500  $\mu\text{l}$  TE buffer (50/20: 50 mM Tris-HCl [pH 8.0], 20 mM  $\text{Na}_2\text{EDTA}$  [pH 8.0]) containing 0.1% (w/v) sarcosyl (N-lauroyl sarcosine, Sigma).

The suspensions were combined (to give 1.0 ml) and the cells were pelleted by centrifugation in a microcentrifuge for 5 min. The cell pellet was resuspended in TE buffer (50/20) and the cells were pelleted by centrifugation in a microcentrifuge for 5 min. The cell pellet was then resuspended in 500  $\mu$ l TE buffer (50/20), lysozyme (Sigma) was added to a final concentration of 300  $\mu$ g/ml, and the mixture was incubated at 37°C for 30 min. Proteinase K (Boehringer Mannheim) was added to a final concentration of 300  $\mu$ g/ml and sarcosyl to a final concentration of 1% (w/v). This mixture was incubated overnight at 50°C, cooled to room temperature and phenol/chloroform extracted as described in Section 2.8. The DNA was precipitated by the addition of ethanol or isopropanol as described in Section 2.9.

### 2.7.2 Plasmid Isolation by the Rapid Boiling Method (Holmes and Quigley, 1981)

The cells from 1.5 ml of an overnight *E. coli*, LB broth culture (Section 2.5.1), containing appropriate antibiotics (Section 2.2) and shaken at 30°C, were pelleted by centrifugation in a 1.5 ml microcentrifuge tube. The supernatant was drained and the pellet was resuspended in 350  $\mu$ l STET buffer (Section 2.6.2). Lysozyme (25  $\mu$ l of a 10 mg/ml solution) was added and the tube was placed in a boiling waterbath for 40 sec. The tube was then centrifuged for 10 min in a microcentrifuge and the resulting gelatinous pellet was removed with a sterile tooth pick. The DNA was precipitated by the addition of an equal volume (usually 300  $\mu$ l) of isopropanol. The contents were mixed by inversion and the tube was allowed to stand at -20°C for 10-20 min. The plasmid DNA was pelleted by centrifugation for 5 min in a microcentrifuge. The plasmid DNA pellet was washed once with 95% ethanol at room temperature and dried under a vacuum for 15 to 30 min prior to resuspension in 30  $\mu$ l (pLAFR1 cosmids) to 50  $\mu$ l (pUC plasmids) MilliQ water. pLAFR1 cosmid DNA preparations were almost invariably phenol/chloroform extracted (Section 2.8).

pLAFR1 cosmids were isolated from *E. coli* HB101, in which they were maintained. HB101 expresses endonuclease A (*endA*<sup>+</sup>), which is not completely inactivated during the boiling procedure. As a result, DNA degradation is reported to occur (Sambrook *et al.*, 1989) during digestion with restriction enzymes (in the presence of Mg<sup>2+</sup>, see Sections 2.11 and 2.6.3). Degradation did not, however, appear to be a significant problem. This may be due to the incorporation of a

phenol/chloroform extraction step in most pLAFR1 cosmid preparations (see above), however, preparations which were not extracted did not appear to suffer from significant degradation, even upon long term storage.

Furthermore, it is generally recommended that larger plasmids (greater than 15 kb, eg, pLAFR1 cosmids) be released by gentle lysis (eg by SDS lysis [methods based upon Godson and Vapnek, 1973]). However, the rapid boiling method gave satisfactory yields of apparently undamaged pLAFR1 cosmids suitable for restriction enzyme digestion, and amenable to subfragment isolation and cloning.

### **2.7.3 Plasmid Isolation by the Alkaline Lysis Method : Miniprep Cosmid Isolation**

Initially, pLAFR1 cosmids were isolated by the method of Ish-Horowicz and Burke (1981) and Maniatis *et al.* (1982), however, once the rapid boiling method (Section 2.7.2) was found to give satisfactory yields, it was used almost exclusively.

The cells from 2 x 1.5 ml of an overnight *E. coli*, LB broth (Section 2.5.1) culture, containing appropriate antibiotics and shaken at either 30°C or 37°C, were pelleted by centrifugation in a microcentrifuge. The supernatant was drained and the pellets were combined by resuspension in 100 µl of a solution containing 50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM Na<sub>2</sub>EDTA (pH 8.0) and 0.5 mg lysozyme (Sambrook *et al.*, 1989 have since reported that the inclusion of lysozyme in the buffer is unnecessary). The mixture was allowed to stand at room temperature for 5 min. 200 µl of a solution containing 0.2 N sodium hydroxide and 1% (w/v) SDS was added, mixed by rapid inversion several times, and the mixture was allowed to stand on ice for 5 min. 150 µl of potassium acetate solution (29.44 g potassium acetate and 11.5 ml glacial acetic acid per 100 ml, pH 4.8) was added, mixed briefly by vortexing, and the mixture was allowed to stand on ice for 5 min. The resulting precipitate was pelleted by centrifugation in a microcentrifuge for 3 min and the supernatant was transferred to a fresh 1.5 ml microcentrifuge tube. The DNA was precipitated by addition of 0.6 volumes of isopropanol, allowing the tube to stand 15-30 min at room temperature. Subsequent steps were as described in Section 2.9.

## 2.8 PURIFICATION OF DNA BY PHENOL/CHLOROFORM EXTRACTION

The method used was as described in Maniatis *et al.* (1982) and more recently in Sambrook *et al.* (1989).

DNA samples isolated by methods described in Section 2.7 were extracted two times with equal volumes of Tris-equilibrated phenol (Section 2.6.8) and chloroform (deproteinisation is more efficient when two organic solvents are used), and once with an equal volume of chloroform. The DNA was then precipitated by ethanol/isopropanol precipitation as described in Section 2.9.

## 2.9 PRECIPITATION OF DNA WITH ETHANOL OR ISOPROPANOL

The method used was as described in Maniatis *et al.* (1982) and more recently in Sambrook *et al.* (1989).

1/10 volume of 3.0 M sodium acetate and either 2.5 volumes of 95% ethanol or, occasionally, 0.6 volumes of isopropanol, were added to a tube containing DNA to be precipitated. The solution was mixed by inversion and the mixture was allowed to stand at -20°C for at least 2 h after which time the DNA was pelleted by centrifugation in a microcentrifuge for 5-10 min. The pellet was washed once with 70% ethanol and dried under vacuum for 15-30 min before resuspension in MilliQ water, except that solutions containing small DNA fragments (less than 1 kb in size) were dried in a fume hood (Svaren *et al.* [1987] have reported that vacuum drying causes denaturation of small DNA fragments). In addition Sambrook *et al.* (1989) have reported that the recovery of larger fragments is greatly reduced.

As noted above, 0.6 volumes of isopropanol was occasionally used in place of ethanol, however, ethanol was used preferentially as it is more volatile and solutes (eg sodium chloride) are less easily coprecipitated, thereby minimising coprecipitation of components that may prevent redissolution of the DNA.

## 2.10 DNA QUANTIFICATION

The methods used were as described in Maniatis *et al.* (1982) and more recently in Sambrook *et al.* (1989).

DNA was quantified by two methods, spectrophotometrically for pure solutions of high concentration, and by intensity of ethidium bromide fluorescence for impure samples of low concentration.

### 2.10.1 Spectrophotometric Determination of DNA Concentration

Concentrated DNA solutions were diluted appropriately and the absorbance of the solutions in 1 cm light path, quartz cuvettes was determined at both 260 nm and 280 nm. The reading at 260 nm allowed calculation of the concentration of nucleic acid present in the sample since an OD of 1 corresponds to approximately 50 µg/ml double stranded DNA. The ratio of readings at 260 nm and 280 nm ( $OD_{260}/OD_{280}$ ) was used as an estimate of the DNA purity where pure DNA has an  $OD_{260}/OD_{280}$  value of 1.8.

### 2.10.2 Minigel Method for Determination of DNA Concentration

A sample from the DNA solution of interest was examined by electrophoresis through an agarose gel (Section 2.12) together with a series of standard DNA solutions of known concentration. After the bromophenol blue dye front had migrated approximately 2 cm the gel was stained with ethidium bromide, destained, and photographed (Section 2.12). The intensity of fluorescence of the unknown DNA sample was then compared to that of the known DNA standards.

## 2.11 RESTRICTION ENDONUCLEASE DIGESTION OF DNA

All restriction endonuclease digests were carried out in *Hae*III buffer (Section 2.6.3) with the salt concentration adjusted for each enzyme with a sterile solution of 1.0 M sodium chloride in accordance with the manufacturer's recommendations. Digestions were performed at 37°C for 1 h and stored, either on ice or at -20°C, while an aliquot was checked on an agarose gel (Section 2.12) to ensure that digestion had proceeded to completion. Digestions were stopped by addition of 1/5 volume SDS dye mix (Section 2.6.4). In the event that a digestion was incomplete the solution was diluted two fold with MilliQ water and the appropriate quantity of *Hae*III buffer and 1 M sodium chloride were added. Fresh enzyme was added and the mixture was incubated a further 1 h. In the event that the DNA was still not digested to completion, the DNA stock was further purified, by phenol/chloroform extraction (Section 2.8), and digestion repeated.

## 2.12 AGAROSE GEL ELECTROPHORESIS OF DNA

Horizontal agarose gel electrophoresis was performed either in a Mini-gel apparatus for 1-2 h or in a Biorad DNA Sub-Cell (150 x 200 mm gel bed) overnight. Agarose concentrations varied from 0.7% to 1.2%. The appropriate quantity of agarose was added to 200 ml TBE electrophoresis buffer (Section 2.6.1) and the agarose was melted in a microwave. After cooling to 50°C in a water bath, the gel was poured and allowed to set. DNA samples (with addition of 1/5 volume SDS sample buffer, Section 2.6.4) were loaded and the fragments were separated by electrophoresis at 80 V to 100 V (Minigels) or 30 V (Biorad Sub Cell). After electrophoresis, gels were stained with ethidium bromide for approximately 5 min, washed with MilliQ water, visualised on a UV transilluminator and photographed on Polaroid type 667 film.

DNA fragment sizes (in kilobases, kb) were determined, after agarose gel electrophoresis, by measuring the distance a fragment had migrated from the well in the gel. The molecular weight was then calculated by comparison with a plot of the distance migrated in the same gel by standard phage lambda DNA fragments (generated by *Hind*III digestion of phage lambda DNA), against the logarithm of the molecular weight (kb) of the standard phage lambda fragments. All gels (and Southern blots of gels probed with <sup>32</sup>P-labelled DNA fragments, Sections 2.16 and 2.17) shown in this thesis show the molecular weight (in kb) of the standard phage lambda DNA fragments.

## 2.13 RECOVERY OF DNA FROM AGAROSE GELS

DNA was recovered from SeaPlaque agarose gels (0.7% to 1.2% in TBE [Section 2.6.1] or TAE electrophoresis buffer [40 mM Tris-HCl, 20 mM glacial acetic acid and 2 mM Na<sub>2</sub>EDTA, pH 8.2]) by phenol freeze extraction (method based upon Thuring *et al.*, 1975).

After gel electrophoresis to separate DNA fragments, the DNA fragment(s) of interest were excised from the gel with the minimum amount of excess agarose and placed in 1.5 ml microcentrifuge tubes. The agarose was melted at 65°C, covered with Tris-equilibrated phenol (Section 2.6.8), mixed by inversion, and the mixture was frozen (generally) overnight at -20°C. The tube was then centrifuged in a microcentrifuge for 10 min. The aqueous phase was recovered, extracted with

phenol/chloroform (Section 2.8) and precipitated with ethanol (Section 2.9).

## **2.14 DNA LIGATIONS**

### **2.14.1 CAP-Treating of Vector DNA**

Approximately 1.0 µg vector DNA was digested to completion by the appropriate restriction endonuclease (Section 2.11). 1.0 µl calf alkaline phosphatase (CAP) was added and the mixture was incubated for 30 min at 37°C. An additional 1.0 µl CAP was added and the mixture was incubated for an additional 30 min at 37°C. The mixture was heated at 65°C for 5 min, then phenol/chloroform extracted (Section 2.8), and the precipitated DNA (Section 2.9) resuspended in MilliQ water.

### **2.14.2 Ligation**

Ligation mixtures contained 4.0 µl of 5 x ligation buffer (Section 2.6.9), an equimolar ratio insert:vector, (at least 20 ng DNA insert and at least 20 ng vector DNA, generally pretreated with calf alkaline phosphatase, Section 2.14.1), 1.0 µl of 1/10 or 1.0 µl of undiluted T4-DNA ligase (New England Biolabs), and MilliQ water (to 20 µl). Ligation mixtures were left in a refrigerator (10-14°C) overnight.

To check that ligation had occurred, 2.0 µl of the ligation mix was removed prior to addition of T4-DNA ligase. 2.0 µl of SDS sample buffer (Section 2.6.4) was added and the sample was examined on an agarose gel (Section 2.12) alongside a 2.0 µl sample (with 2.0 µl SDS sample buffer) removed after addition of T4-DNA ligase and overnight ligation.

## **2.15 PREPARATION AND TRANSFORMATION OF COMPETENT CELLS**

An overnight, LB broth (Section 2.5.1) culture (30°C) of *E. coli* strains HB101 or MC1022 was diluted 1/100 into 25 ml LB broth, then incubated with shaking at 37°C for 2 h (to an OD<sub>600</sub> of approximately 0.4). Cells were harvested by centrifugation at 3000 x g for 10 min at 4°C in glass Corex tubes. The pellet was carefully resuspended in 10 ml ice cold (4°C) 60 mM calcium chloride (suspension was performed in the Corex tube) and stored on ice for 20 min. The cells were pelleted at 3000 x g for 10 min at 4°C and carefully resuspended in 250 µl ice cold



(4°C) 60 mM calcium chloride. All procedures, except, centrifugation were performed in a cold room (approximately 4-5°C).

50 µl of competent cells prepared as described above were mixed with 5 µl ligated DNA (Section 2.14.2) and 45 µl TEC buffer (Section 2.6.6) in an ice cold (4°C) microcentrifuge tube and placed on ice for 1 h. The cells were then heat-shocked at 42°C for 2.5 min and placed on ice for a further 15 min. 0.9 ml of LB broth (Section 2.5.1) was added and the cells were shaken within the microcentrifuge tube at 37°C for 90 min. 100 µl aliquots were plated out onto appropriate selective media (Section 2.2). Plates were incubated overnight and several (generally 10) transformants were single colony purified. Single colony purified transformants were checked for the presence of the appropriate plasmid by DNA isolation (Section 2.7.2), digestion of the isolated DNA with (an) appropriate restriction endonuclease(s) (Section 2.11) and examination of the digestion fragments for the presence of both vector and insert by agarose gel electrophoresis (Section 2.12).

In addition to ampicillin (Section 2.2), plates used in the selection for transformants of MC1022 (used where the vector was pUC118) were spread with 20 µl of a 20 mg/ml solution of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, BRL). White (recombinant) colonies were selected for single colony purification and plasmid identification as described in the previous paragraph.

## 2.16 SOUTHERN BLOT TECHNIQUE

DNA to be transferred was separated by overnight electrophoresis, stained, visualised and photographed as describe in Section 2.12 and the gel dimensions were measured after removal of the gel portion "above" the wells.

The gel was placed in a dish containing 0.25 M hydrochloric acid and gently agitated for 15 min. The hydrochloric acid solution was poured off and the gel was immersed in 0.5 M sodium hydroxide-0.5 M sodium chloride, with gentle agitation, for 30 min. The NaOH-NaCl solution was drained and the gel was immersed in 0.5 M Tris (pH 7.4)-2.0 M sodium chloride, with gentle agitation, for 15 min. The gel was finally washed for 2 min in 2 x SSC (Section 2.6.11).

While the gel was being treated, a plastic trough with wells at each end was prepared by placing two sheets of 20 x SSC (Section 2.6.10) soaked Whatman

3MM chromatography paper in it such that the ends of the paper projected into the wells. The wells were then filled with 20 x SSC to just below the horizontal surface of the paper between the wells. A sheet of Gladwrap was placed over the trough and pressed flat. A grid 2 mm less than the gel size was marked on the Gladwrap and removed. The treated gel was placed, inverted, over the grid such that the edges of the gel overlapped the edges of the grid. A piece of nitrocellulose or nylon membrane (Hybond-C extra or Hybond-N, Amersham), cut to 2 mm greater than the gel size and presoaked in 2 x SSC, was placed over the gel ensuring that no air bubbles were present. Two pieces of Whatman 3MM chromatography paper, cut 2 mm less than the gel size and presoaked in 2 x SSC, were placed over the membrane. Two pieces of Whatman 3MM chromatography paper (unsoaked) were placed upon the two soaked pieces of 3MM paper. A stack of paper towels approximately 50 mm deep was placed upon the chromatography paper, followed by a flat metal or plastic tray and a weight sufficient to keep the entire stack flat.

After overnight transfer, the apparatus was disassembled and the membrane was washed for 5 min in 2 x SSC, then baked under vacuum at 80°C for 2 h.

## 2.17 HYBRIDIZATION

### 2.17.1 Preparation of <sup>32</sup>P-Labelled Probe DNA

DNA to be labelled was digested for 30 min at 37°C with *Hae*III in a 25 µl reaction mixture containing 200-250 ng DNA, 2.5 µl *Hae*III buffer (Section 2.6.3), and MilliQ water to 25 µl. 4.0 µl random primers (100 µg) obtained from Professor Barry Scott were added and the mixture was boiled for 2 min, then chilled rapidly on ice. The following reagents were then added in the listed order: 2.5 µl MilliQ water; 1.5 µl *Hae*III buffer; 1.0 µl 20 mM dGTP; 1.0 µl 20 mM dATP; 1.0 µl 20 mM dTTP; 3.0 µl [ $\alpha$ -<sup>32</sup>P] dCTP (3000 Ci/mmol, New England Nuclear); and 1.0 µl DNA polymerase I (Klenow fragment, Boehringer Mannheim). The mixture was incubated at 37°C for 30 min to 2 h, then the reaction was stopped by the addition of 3 µl 250 mM Na<sub>2</sub>EDTA (pH 8.0), followed by phenol/chloroform extraction (Section 2.8).

Free nucleotides were separated from labelled DNA either on a 6 cm Sephadex G-50 column (Section 2.17.2) equilibrated with TES (10/1/100, Section 2.6.12), or

on a minispin Sephadex G-50 column (Section 2.17.3) equilibrated with TES (10/1/100).

### **2.17.2 Probe DNA/Free Nucleotide Separation by Conventional Column Chromatography**

Sephadex G-50 columns were constructed by plugging a 10 cm glass pipette with sterile glass wool. A slurry of Sephadex G-50 resin in TES (10/1/100, Section 2.6.12) was added until it had packed to approximately 6 cm. The column was then washed several times with TES (10/1/100). The  $^{32}\text{P}$ -labelled DNA sample was applied in a volume of 200  $\mu\text{l}$  to the top of the column. When the DNA had entered the column, elution using TES (10/1/100) was initiated. 5-drop fractions (approximately 200-250  $\mu\text{l}$ ) were collected in 1.5 ml microcentrifuge tubes. The radioactivity of each tube was measured with a hand held monitor. The leading peak (generally 3-4 tubes) of radioactivity was retained (probe DNA). The trailing peak, consisting of unincorporated [ $\alpha$ - $^{32}\text{P}$ ]dCTP (and other dNTP's) was discarded.

### **2.17.3 Probe DNA/Free Nucleotide Separation by Minispin Column Chromatography**

Minispin columns were constructed by plugging the bottom of a 1 ml plastic, disposable, Terumo, Tuberculin syringe with sterile glass wool. The syringe was filled with Sephadex G-50 resin, equilibrated in TES (10/1/100, Section 2.6.12). Additional resin was added until the syringe was full to the 1 ml mark. The syringe was inserted into a disposable plastic tube and centrifuged at speed 3 in a BTL bench centrifuge (approximately 1500 x g) for 4 min at room temperature in a swinging bucket rotor (all subsequent centrifugation steps described were also at the same speed and duration). Additional resin was added until the volume of resin in the syringe, after centrifugation, was unchanged at 0.9 ml. 100  $\mu\text{l}$  of TES (10/1/100) was added to the column, which was recentrifuged. This step was repeated twice. The radiolabelled DNA was added to the column in 100  $\mu\text{l}$  of TES (10/1/100). The end of the syringe was inserted into the perforated cap of a 1.5 ml microcentrifuge tube. The assembly was replaced in the disposable plastic tube and recentrifuged. The 100  $\mu\text{l}$  of TES (10/1/100) in the bottom of the microcentrifuge tube, containing the radiolabelled DNA, was transferred to a capped 1.5 ml microcentrifuge tube.

#### 2.17.4 Hybridization of Probe DNA to Southern Blots

The Southern blot (Section 2.16) to be probed was prehybridized for at least 2 h at 65°C in hybridization buffer (Section 2.6.7) in either a sealed plastic bag with the air excluded or a sealed glass tube. After prehybridization, most of the hybridization buffer was poured off and boiled  $^{32}\text{P}$ -labelled probe was added, to a concentration of no greater than 25 ng probe DNA/ml hybridization buffer.

After overnight hybridization at 65°C, the filter was removed and washed three times for 15 min at room temperature in 2 x SSC. The washed filter was then blotted almost dry, backed with a sheet of Whatman 3MM paper, covered with Gladwrap, and exposed, in the presence of (a) Cronex intensifying screen(s), to a sheet of Fuji Medical X-ray film in a X-ray cassette. After exposure for 5 h to 7 days at -70°C, the film was developed in a Kodak X-Omat automatic processor.

### 2.18 BACTERIAL CROSSES

Crosses were carried out by the patch plate method (Dixon *et al.*, 1976) on TY agar (Section 2.5.2).

#### 2.18.1 Tn5 Mutagenesis - Isolation of EPS Mutants

Recipient *Rhizobium loti* strains (PN184 or PN4115) were shaken for 2 days at 30°C in 5 ml of TY broth (Section 2.5.2). The Tn5 donor *Escherichia coli* strain, PN302 (*E. coli* SM10, carrying pSUP1011), was shaken overnight at 30°C in LB broth (Section 2.5.1), diluted 1/10 in fresh LB broth and shaken for 4 h at 37°C. 50 µl aliquots of donor and recipient were mixed on a TY-agar plate and left to dry down in a Laminar flow cabinet. The plates were incubated overnight at 30°C and the cross was then eluted from the plate with 5 ml sterile MilliQ water.

For the isolation of EPS mutants derived from PN184, serial dilutions of the cell suspensions were plated onto nitrogen-free S20 agar (Section 2.5.4) containing neomycin, streptomycin (Section 2.2) and 0.02% Calcofluor (Sections 2.5 and 2.19.1) and incubated for 5 days at 30°C. Colonies which failed to fluoresce when the plates were exposed to long wave UV light were single colony purified through two rounds on TY agar (Section 2.5.2) containing neomycin and streptomycin. Single colony purified transconjugants were streaked onto YM agar (Section 2.5.3)

containing neomycin and streptomycin and incubated for 5 days. The YM agar-grown transconjugants were tested for lack of fluorescence by plating onto S20, and nitrogen-free S20 agars containing neomycin, streptomycin, and 0.02% Calcofluor. After demonstrating that the non-fluorescent characteristic of the mutants was retained, the YM agar-grown transconjugants were used for strain maintenance.

For the isolation of EPS mutants derived from PN4115, serial dilutions of the cell suspensions were plated onto nitrogen-free S20 agar (Section 2.5.4), YM agar (Section 2.5.3), and TY agar (Section 2.5.2), all containing neomycin, streptomycin (Section 2.2), and 0.02% Calcofluor (Section 2.5). Colonies with altered mucoidy or colour were single colony purified through two rounds on S20 agar containing neomycin and streptomycin. Single colony purified transconjugants were streaked onto YM agar containing neomycin and streptomycin, incubated for 5 days, and these YM agar grown cultures used for strain maintenance.

## **2.18.2 Isolation of Complementing Cosmids**

### **2.18.2.1 Cosmids Complementing the Mutation Carried by Each of the PN184-Derived EPS Mutants**

The construction of a genomic library to NZP2037 has been previously described (Chua *et al.*, 1985). 100 µl of a two day TY broth (Section 2.5.2) culture, shaken at 30°C, of the recipient *Rhizobium* mutant was mixed on a TY agar plate together with 50 µl of an overnight LB broth (Section 2.5.1) culture, shaken at 30°C, of *E. coli* strain PN362 (to provide the Tra<sup>+</sup> helper plasmid), and 50 µl of an overnight LB broth culture, shaken at 30°C, of the *E. coli* HB101/pLAFR1 gene library to NZP2037 (Chua *et al.*, 1985). The cross was incubated overnight at 30°C. After overnight incubation, the cross was eluted from the plate with 5 ml sterile MilliQ water. Serial dilutions of the cell suspensions were plated onto nitrogen-free S20 agar (Section 2.5.4) containing neomycin, streptomycin, tetracycline (Section 2.2), and 0.02% Calcofluor (Section 2.5). The plates were incubated at 30°C for 5 days after which time the plates were examined for restoration of mucoidy and/or restoration of fluorescence (see Section 2.19.1).

Complemented colonies were single colony purified on TY agar (Section 2.5.2) containing streptomycin, neomycin and tetracycline (Section 2.2). A working

culture for strain maintenance was obtained by plating each single colony purified, complemented transconjugant onto YM agar (Section 2.5.3) containing streptomycin, neomycin, and tetracycline.

50  $\mu$ l of a two day TY broth (Section 2.5.2) culture, shaken at 30°C, of each YM-maintained, complemented transconjugant was mixed on a TY agar plate together with 50  $\mu$ l of an overnight LB broth (Section 2.5.1) culture, shaken at 30°C, of *E. coli* strain PN362 (to provide the Tra<sup>+</sup> helper plasmid) and 100  $\mu$ l of an overnight LB broth (Section 2.5.1) culture, shaken at 30°C, of *E. coli* strain HB101. After overnight incubation a loop of the cross was streaked out onto LB agar containing tetracycline (Section 2.2). The plate was incubated overnight and the resultant colonies were single colony purified on LB agar containing tetracycline.

To confirm that the original complemented mutants were not revertants, a single colony purified HB101 strain carrying a representative complementing cosmid for each mutant (see Table 2) was crossed with each original mutant as described in the first paragraph of this section, but replacing HB101/pLAFR1 with the appropriate single colony purified HB101 strain. The complemented colonies were single colony purified, and the cosmid carried by each complemented colony was transferred back into *E. coli* HB101 as described in the second, and third paragraphs of this section. In this way cosmids were identified which were stable even after repeated transfer.

#### **2.18.2.2 Cosmid Cross-Complementation**

A representative cosmid (Section 2.18.2.1, and Table 2) complementing each (rough, see Section 3.2.5.1) PN184-derived EPS mutant (isolated as described in Section 2.18.1), was used in cross complementation experiments with all other PN184-derived EPS mutants.

The methods used were as described in Section 2.18.1, but replacing the HB101/pLAFR1 gene library to NZP2037 with each representative cosmid.

#### **2.18.2.3 Cosmids Complementing the Mutation Carried by Each of the PN4115-Derived EPS Mutants**

An attempt was made to isolate cosmids which stably complemented the mutations carried by the PN4115-derived EPS mutants by the same method described in

Section 2.18.2.1, but with the following modifications. An *E. coli* HB101/pLAFR1 gene library to NZP2213 (obtained from Prof. D B Scott) was used, instead of the HB101/pLAFR1 gene library to NZP2037 (Chua *et al.*, 1985). Single colony purification of PN4115-derived strains was performed on S20 agar (Section 2.5.4) containing appropriate antibiotics (Section 2.2).

### **2.18.3 Tn5 Mutagenesis - Isolation of LPS Mutants**

The recipient *R. loti* strain, PN184, was shaken for 2 days at 30°C in 5 ml TY broth (Section 2.5.2). The Tn5 donor *E. coli* strain, PN302, was shaken overnight at 30°C in LB broth (Section 2.5.1), diluted 1/10 in fresh LB broth and shaken for 4 h at 37°C. 50 µl aliquots of donor and recipient were mixed on a TY agar plate and left to dry down in a laminar flow cabinet. The plates were incubated overnight at 30°C and the cross was then eluted from the plate with 5 ml sterile MilliQ water.

Serial dilutions of the cell suspensions were plated onto TY agar (Section 2.5.2) containing neomycin and streptomycin (Section 2.2). The plates were incubated for 5 days at 30°C. Colonies showing reduced mucoidy were single colony purified through two rounds on TY agar containing neomycin and streptomycin, and through one round on S20 agar (Section 2.5.4) containing neomycin and streptomycin. Single colony purified transconjugants were streaked onto a YM agar (Section 2.5.3) plate containing neomycin and streptomycin, and incubated for 5 days. These YM agar cultures were then used for strain maintenance after testing each YM grown strain for reduced mucoidy on TY agar containing neomycin and streptomycin.

## **2.19 DETECTION, ISOLATION AND ANALYSIS OF EXOPOLYSACCHARIDE**

### **2.19.1 Detection of EPS Production on Solid Media**

A mucoid colony phenotype on S20-, nitrogen-free S20- or YM-agars (Sections 2.5.4 and 2.5.3) indicated the production of EPS. The production of Calcofluor-binding EPS was demonstrated on S20-, nitrogen-free S20-, YM- and TY-agar (Section 2.5.2) plates by adding Calcofluor to 0.02% (Section 2.5) and viewing colonies under long wave ultra violet light (Calcofluor-bright phenotype).

## 2.19.2 EPS Isolation from Broth Culture

Exopolysaccharide was isolated by two methods, cetrimide (hexadecyltrimethylammonium bromide, Sigma) precipitation, and ethanol precipitation.

### 2.19.2.1 Cetrimide Precipitation of EPS

EPS was cetrimide precipitated from nitrogen-free S20 broth (Section 2.5.4) and YM broth (Section 2.5.3) culture supernatants of PN184, PN4115 and the PN184-derived, and PN4115-derived, EPS mutants.

For the isolation of EPS from nitrogen-free S20 broth (Section 2.5.4) cultures, a loop of the YM agar (Section 2.5.3) maintained strain was used to inoculate 2 ml of TY broth (Section 2.5.2) containing appropriate antibiotics (Section 2.2). After 2 days shaking at 30°C, the culture was diluted 1/100 into 20 ml S20 broth containing appropriate antibiotics. After 2 days shaking at 30°C this culture was diluted 1/100 and grown, with shaking at 30°C, to mid-late log phase in 500 ml of nitrogen-free S20 broth containing appropriate antibiotics. A loop of the broth culture was streaked out onto a TY agar plate (no antibiotics) to check for possible contamination. The cells were pelleted by centrifugation (20 min, 6000 x g, 20°C).

For the isolation of EPS from YM broth (Section 2.5.3) cultures, a loop of the YM agar maintained strain was used to inoculate 5 ml of TY broth (Section 2.5.2) containing appropriate antibiotics (Section 2.2). After two days shaking at 30°C, the culture was diluted 1/100 into 500 ml YM broth and grown, with shaking at 25°C, to mid-late log phase. A loop of the broth culture was streaked onto a TY agar plate (no antibiotics) to check for possible contamination. 500 ml of MilliQ water was added to reduce the viscosity of the culture, then the cells were pelleted by centrifugation (20 min, 6000 x g, 20°C).

EPS was then cetrimide precipitated from each culture supernatant by the method of Robertsen *et al.* (1981). The supernatant was concentrated by rotary evaporation (40°C) to approximately 50 ml. The evaporated supernatant was dialysed (dialysis tubing was prepared as described in Maniatis *et al.*, 1982) for 24 h against running tap water in a cold room (4°C), and for at least a further 24 h at 4°C against MilliQ water. The dialysed supernatant was then centrifuged (20 min, 6000 x g, 10 °C) to remove any precipitated material. Sodium sulphate was added



to a final concentration of 10 mM, and the (acidic) EPS was precipitated by the addition of 50 ml of 3% (w/v) cetrимide over 2 h at 37°C, with occasional stirring. The precipitate of EPS was collected by centrifugation (20 min, 6000 x g, 20°C) and washed by resuspension in MilliQ water, followed by centrifugation (20 min, 6000 x g, 20°C) 3 times.

For initial preparations, the cetrимide precipitated, water washed, EPS was dissolved in 100 ml 10% (w/v) sodium chloride, and collected by centrifugation (20 min, 6000 x g, 20°C), after the addition of 2 volumes acetone. This procedure was repeated twice. After addition of acetone, the sodium chloride/acetone solution was occasionally biphasic, rather than being monophasic as desired. In this case, a small amount of MilliQ water was added, to cause a biphasic to monophasic solution transition. The acetone precipitated EPS was redissolved in 100 ml of 10% (w/v) sodium chloride, dialysed for 24 h against 1% (w/v) sodium chloride, then dialysed extensively (several days with frequent changes) against MilliQ water, and finally lyophilized. It was, however, found that some cetrимide occasionally remained in the sample using this method.

For subsequent preparations a modified procedure was therefore used. In this method the cetrимide precipitated EPS was washed only once with MilliQ water, thereby minimising the losses which normally occurred during this step. The washed EPS was dissolved in 200 ml of 10% (w/v) sodium chloride and reprecipitated with the addition of two volumes acetone. The precipitated EPS, which floats at the surface, was then spooled out and redissolved in 200 ml of 10% (w/v) sodium chloride. This procedure was repeated three times. This procedure substantially reduced carry over of cetrимide, particularly in large lumps of EPS which are collected, in the first method, by centrifugation. These lumps of EPS, which are dense and sink, are not collected by spooling out the EPS floating at the surface. After the final acetone precipitation, the precipitated EPS was collected by spooling, dissolved in 100 ml of 10% (w/v) sodium chloride, dialysed against 1% (w/v) sodium chloride for 24 h, then extensively against MilliQ water. The dialysed EPS was then lyophilized.

#### **2.19.2.2 Ethanol Precipitation of EPS**

EPS was ethanol precipitated from YM broth (Section 2.5.3) culture supernatants of PN184 and the PN184-derived EPS mutants.

A loop of the YM agar (Section 2.5.3) maintained strain was used to inoculate 5 ml of TY broth (Section 2.5.2), containing appropriate antibiotics (Section 2.2). After 2 days shaking at 30°C, the culture was diluted 1/100 into 500 ml YM broth, and grown, with shaking at 25°C, to mid-late log phase. A loop of the broth culture was streaked out onto a TY agar plate (no antibiotics) to check for possible contamination. 500 ml of MilliQ water was added to reduce the viscosity of the culture. The cells were then pelleted by centrifugation (20 min, 6000 x g, 20°C).

The culture supernatant was first concentrated by rotary evaporation at 40°C to approximately 50 ml, dialysed for 24 h against running tap water in a cold room (4°C), and then for at least 24 h at 4°C against MilliQ water. The dialysed supernatant was then centrifuged (20 min, 6000 x g, 4°C) to remove any precipitated material. The total volume of the dialysed supernatant was brought to 100 ml with MilliQ water. 4 volumes of ice cold ethanol were added and the mixture was left to stand for 16 h at 4°C. The precipitate of EPS was collected by centrifugation (20 min, 6000 x g, 4°C), washed by resuspension in MilliQ water, followed by centrifugation (20 min, 6000 x g, 4°C) 3 times, and finally lyophilized. The supernatant was retained after addition of the 4 volumes ethanol, and subsequent collection of EPS. This supernatant was reconcentrated, by rotary evaporation at 40°C, to 100 ml. 10 volumes of ethanol was then added to precipitate any  $\beta$ -glucan and/or oligosaccharide present in the supernatant.

### 2.19.3 Examination of EPS by $^1\text{H}$ Nuclear Magnetic Resonance Spectroscopy

EPS Samples (generally 10 mg) for  $^1\text{H}$ -nuclear magnetic resonance spectroscopy ( $^1\text{H}$ -NMR spectroscopy) were dissolved with the aid of sonication in  $\text{D}_2\text{O}$  (99.8%, Aldrich, or 99.9% Sigma) and then lyophilized. The sample was redissolved with the aid of sonication in  $\text{D}_2\text{O}$ , lyophilized for at least 16 h and redissolved with the aid of sonication in 1.0 ml  $\text{D}_2\text{O}$  (normally 99.9%, Sigma).  $^1\text{H}$ -NMR spectra were recorded at 80°C using a JEOL GX270 spectrometer operating at 270 MHz with a  $^1\text{H}$  pulse width of 12  $\mu\text{s}$  (90° pulse). The free induction decay signal was sampled using 16 k data points over a 3 kHz spectral width giving a resolution, after Fourier transformation, of 0.37 Hz per data point. Typically 500-1000 transients were recorded at a pulse repetition time of 2.8 s (approximately 4.3 times the longest  $T_1$  of the EPS sugar residue protons). The longest  $T_1$  s arose from relaxation of ring and *O*-acetate methyl protons, both of which had a  $T_1$  of approximately 650 ms. The signal(s) arising from the glycosyl residue ring protons were integrated for

each spectrum in comparison to the signal(s) arising from the methyl protons of the *O*-acetate modifications, which were assigned a reference value of 1.0.

#### 2.19.4 Paper Chromatography of EPS

A sample of cetrimide precipitated, lyophilized EPS (5 mg), was hydrolysed in 2 M hydrochloric acid for 5 h at 100°C using a reflux apparatus and boiling water bath. The hydrolysate was dried under vacuum in the presence of sodium hydroxide pellets, redissolved in MilliQ water, and dried again in the presence of sodium hydroxide pellets.

Hydrolysed samples were dissolved in MilliQ water and applied (150 µg per lane) to Whatman No 1 chromatography paper together with 50 µg samples of sugar standards dissolved in water. Chromatograms were double developed vertically in either 1-butanol-pyridine-water (6:4:3) or 1-butanol-acetic acid-water (4:1:5). Sugars were visualized by spraying the chromatogram with 1.23% (w/v) *p*-anisidine-0.116% (w/v) phthalic acid in absolute ethanol and heating the chromatogram to 105°C for 5 mins. Chromatograms were further examined by exposure to long wave ultra violet light (Leigh *et al.*, 1985; Macek, 1963).

#### 2.19.5 Column Chromatography of EPS

Approximately 25 ml of DEAE-Sephacel (exclusion limit  $1 \times 10^6$  Da, Sigma) slurry was mixed with 50 ml of 1.0 mM sodium chloride and allowed to settle. The liquid layer, including any fines was poured off, a further 50 ml of 1.0 mM sodium chloride was added and the procedure was repeated. 25 ml of 1.0 mM sodium chloride was then added and the solution was mixed to form a slurry. This slurry was used to gravity pack a 6.5 cm x 1.5 cm column. 500 ml of 1.0 mM sodium chloride was run through the column prior to loading the EPS sample.

A sample (generally 10 mg) of lyophilized EPS was dissolved in 2 ml of 1.0 mM sodium chloride. The sample was then loaded onto the equilibrated column, which was then washed with 100 ml of 1.0 mM sodium chloride to elute any neutral or high molecular weight EPS. Thirty three 3 ml drop fractions were collected. EPS which bound to the column was eluted with 130 ml (65 ml of 1.0 mM sodium chloride, 65 ml of 1.0 M sodium chloride) of a linear sodium chloride gradient (1.0 mM to approximately 800 mM). Forty three 3 ml drop fractions were

collected.

The sodium chloride concentration in the 3 ml fractions was determined by comparison of the conductivity of each fraction with a standard conductivity curve for 1.0 mM to 1.0 M sodium chloride.

Fractions were tested for glucose equivalents by the anthrone method (Section 2.19.6).

#### **2.19.6 Determination of Glucose Equivalents by the Anthrone Method**

Glucose equivalents were determined by the anthrone method as described by Spiro (1962). The anthrone reaction is based upon the formation, in concentrated sulphuric acid, of furfural derivatives which react with anthrone to form a blue-green colour (Spiro, 1962).

The anthrone reagent was prepared by adding 720 ml of analytical grade sulphuric acid to 280 ml MilliQ water. While the reagent was still warm, 500 mg of anthrone and 10 g of thiourea were added and mixed until dissolved. The reagent was stored at 4-5°C in a cold room.

To assay for glucose equivalents, 5 ml of anthrone reagent was added to glass test tubes containing 200 µl of each 3 ml column fraction (Section 2.19.5) and 800 µl MilliQ water. The tubes were vortexed, capped with a glass reagent bottle lid, and heated in a boiling water bath for 15 min. After 15 min the tubes were cooled in a water bath to 20°C, and the absorbance at 620 nm was determined, after equal periods of cooling for each tube. A negative control (blank) containing 1 ml MilliQ water, but otherwise treated as described above was used to zero the spectrophotometer. Alternatively, MilliQ water can be used to zero the spectrophotometer, with absorbance corrected against the blank.

A set of glucose standards (0-200 µg/assay) was used to generate a standard curve (graphed in terms of µg glucose/assay). Glucose equivalents in each 3 ml fraction were therefore easily determined by multiplying µg glucose equivalents/assay by 15.

## 2.20 ISOLATION AND ANALYSIS OF LIPOPOLYSACCHARIDE

### 2.20.1 LPS Isolation

LPS was isolated by the hot phenol-water method (Westphal and Jann, 1965) as described in Noel *et al.* (1986).

A loop of the YM agar maintained strain was suspended in 5 ml of TY broth (Section 2.5.2), containing appropriate antibiotics (Section 2.2), and shaken at 30°C for 2 days. The bacteria were diluted 1/100 into 500 ml of TY broth, containing appropriate antibiotics, and shaken at 25°C for 2 days. The cells were pelleted by centrifugation (15 min, 6000 x g, 10°C) and washed 3 times by suspension in 1% (w/v) sodium chloride, followed by centrifugation (20 min, 6000 x g, 10°C) and twice by resuspension in MilliQ water, followed by centrifugation (20 min, 6000 x g, 10°C). The washed pellet can be stored at -20°C if necessary. For extraction, the pellet was suspended in 9 ml MilliQ water and put at 65°C in a water bath. 10 ml of 90% (v/v melted phenol) phenol at 65°C was added with vigorous mixing. After 15 min incubation at 65°C, the mixture was cooled to 10°C in a waterbath and centrifuged (30 min, 6000 x g, 10°C). The aqueous phase (top) was dialysed exhaustively against repeated changes of MilliQ water and lyophilized.

To confirm that LPS was present in the crude LPS preparations, 2-keto-3-deoxyoctanoic acid (KDO) was determined by the method of Karkhanis *et al.* (1978). 1 ml of 0.2 N sulphuric acid was added to a test tube containing 2 mg lyophilized material. The mixture was heated in a boiling water bath for 30 min, cooled to room temperature, and the sample was centrifuged in a microcentrifuge for 5 min. 500 µl of the clear solution was removed and added to a glass test tube. 250 µl of 0.04 M periodic acid in 0.125 N sulphuric acid was added, the mixture was then vortexed and allowed to stand for 20 min at 25 °C. 250 µl of 2.6% (w/v) sodium arsenite in 0.5 N hydrochloric acid was added, the mixture was vortexed, and, after the brown colour had disappeared, 500 µl of 0.6% (w/v) thiobarbituric acid was added. The mixture was vortexed, then heated at 100°C in a boiling water bath for 15 min. After boiling, and while the mixture was still hot, 1.0 ml of dimethyl sulphoxide was added. The mixture was allowed to cool to room temperature and its optical density was recorded at 548 nm against a blank, treated as described above, but without added lyophilized material.

### 2.20.2 LPS Analysis

The lyophilized material from phenol-water extracts was examined by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (Section 2.23). For each preparation, either 10 µg, or 1.0 mg of lyophilized material was examined by electrophoresis on SDS-polyacrylamide gels (12.5%, 15% and 20%, Section 2.23) (Laemmli, 1970) and stained with either periodic acid (PA)-silver (10 µg sample, Hitchcock and Brown, 1983; Tsai and Frasch, 1982) or PA-Schiff (1.0 mg sample, Fairbanks *et al.*, 1971), as described in Sections 2.20.2.1 and 2.20.2.2.

#### 2.20.2.1 Periodic Acid-Silver Staining

Phenol-water extracts (Section 2.20.1) were suspended in loading buffer (Section 2.23) and boiled for 3 min. After brief centrifugation, volumes (20 µl) containing 10 µg phenol-water extract were loaded onto a SDS-polyacrylamide gel (Section 2.23) with a Hamilton microlitre syringe and subjected to electrophoresis for approximately 3 h (until the dye front was approximately 1 cm from the bottom of the gel) as described in Section 2.23. In the following steps pyrex staining dishes were used and the gel was touched only when wearing washed vinyl gloves.

The gel was removed and fixed overnight in 40% (v/v) ethanol-5% (v/v) glacial acetic acid. The fixative was drained and oxidation performed for 5 min in 40% (v/v) ethanol-5% (v/v) glacial acetic acid-0.7% (w/v) periodic acid. The gel was washed with large volumes of MilliQ water (500 ml) 5-8 times over 2 h. The water was drained and the gel was agitated for 10 min in staining reagent. (To 2 ml of concentrated ammonium hydroxide, added to 28 ml of 0.1 N sodium hydroxide, was added 5 ml of 20% [w/v] silver nitrate, with continuous stirring. A transient brown precipitate formed but rapidly disappeared. 115 ml of MilliQ water was then added to make 150 ml reagent). The gel was washed 3 times with 200 ml MilliQ water, then the water was drained and replaced with 200 ml developer (developer contained [per 1.0 litre] 50 mg citric acid and 0.5 ml 37% (w/v) formaldehyde). The LPS was stained dark brown within 5 min, at which time development was stopped by agitating the gel for 1 h in 200 ml MilliQ water containing 10 ml of 7% (v/v) glacial acetic acid. The stop solution was drained and the gel was washed in MilliQ water until photographed in the wet state.

After photography the gel was removed from its storage tray and placed, inverted,

on a sheet of Gladwrap. A sheet of Whatman 3MM paper (cut approximately 2 cm larger than the gel) was pressed gently onto the gel. The piece of Whatman 3MM paper, with the gel stuck to its underside was inverted, so that the gel was uppermost. A fresh sheet of gladwrap (precut to the same dimensions as the Whatman 3MM paper) was placed over to gel. The gel sandwich was immediately placed in a preheated (60°C) gel dryer (BioRad Model 483 Slab Gel dryer, or BioRad Model 583 Gel Dryer), vacuum was applied, and the gel was dried for approximately 2 h.

### **2.20.2.2 Periodic Acid-Schiff Staining**

1 mg of phenol-water extract (Section 2.20.1) was suspended in 50 µl loading buffer (Section 2.23) and boiled for 3 min. The entire volume was applied to a SDS-polyacrylamide gel (Section 2.23) with a Hamilton microlitre syringe and electrophoresis was performed as described in Sections 2.20.2.1 and 2.23. In the following steps pyrex staining dishes were used and the gel was touched only when wearing washed vinyl gloves.

The gel was removed and gently agitated in (the following order): 1) 25% (v/v) isopropanol-10% (v/v) glacial acetic acid, overnight; 2) 10% (v/v) isopropanol-10% (v/v) glacial acetic acid, 6 h; 3) 10% (v/v) glacial acetic acid, overnight; 4) 10% (v/v) glacial acetic acid, several hours; 5) 0.5% (w/v) periodic acid, 2 h; 6) 0.5% (w/v) sodium arsenite-5% (v/v) glacial acetic acid, 30 min; 7) 0.1% (w/v) sodium arsenite-5% glacial acetic acid, 20 min; 8) glacial acetic acid, 20 min; 9) Schiff's reagent (5 g sodium metabisulphite and 50 ml 1 N hydrochloric acid was added to a solution containing 2.5 g basic fuchsin dissolved in 500 ml MilliQ water. The solution was stirred for several hours and decolourised with 2 g activated charcoal), overnight; 10) 0.1% (w/v) sodium metabisulphite-0.01 N hydrochloric acid over several hours. Step 10 was repeated until the rinse solution failed to turn pink upon addition of several drops of 37% (w/v) formaldehyde. Pink patterns appeared during step 9, while subsequent steps removed excess reagent, intensified the pattern and retarded fading. Gels were dried as described in section 2.20.2.1.

## **2.21 ISOLATION OF CAPSULAR POLYSACCHARIDE**

Capsular polysaccharide was isolated as described in Mort and Bauer (1980).

A loop of the YM agar (Section 2.5.3) maintained strain was used to inoculate 5 ml TY broth (Section 2.5.2) containing appropriate antibiotics (Section 2.2). After 2 days shaking at 30°C the culture was diluted 1/100 into 500 ml YM broth, containing appropriate antibiotics, and grown to mid-late log phase at 30°C, with shaking. A loop of the broth culture was streaked out onto a TY agar plate (no antibiotics) to check for possible contamination.

The cells were pelleted by centrifugation (10 min, 10,000 x g, 10°C). The cell pellet was washed three times by resuspension in sterile phosphate buffered saline (Section 2.6.14), followed by centrifugation (10 min, 10,000 x g, 10°C). The cells were then suspended in 50 ml of sterile phosphate buffered saline and left 6 days at 4°C to solubilize the capsule. The cells were removed by centrifugation (10 min, 10,000 x g, 4°C). Na<sub>2</sub>EDTA (pH 8.0) was added to the supernatant to 10 mM, and the supernatant was dialysed extensively against MilliQ water, then lyophilized.

## 2.22 ANALYSIS OF TOTAL PROTEIN BY SDS-PAGE

A loop of the YM agar (Section 2.5.3) maintained strain was suspended in 5 ml TY broth (Section 2.5.2) containing appropriate antibiotics (Section 2.2). After 2 days shaking at 30°C, the cells from a 1.5 ml culture volume were harvested by centrifugation in a microcentrifuge for 5 min. The supernatant was discarded and the cells were washed, by resuspension in fresh TY broth followed by recentrifugation in a microcentrifuge. The washed cells were resuspended in 200 µl loading buffer (Section 2.23), boiled for 3 min, and centrifuged briefly in a microcentrifuge. 20 to 25 µl of each sample was applied to a SDS-polyacrylamide gel (Section 2.23) of the desired percentage with a Hamilton microlitre syringe.

After electrophoresis, gels were stained with Coomassie Brilliant Blue (Section 2.22.1).

### 2.22.1 Coomassie Brilliant Blue Staining

Polyacrylamide gels were stained for protein with Coomassie Brilliant Blue according to a method based upon that of Fairbanks *et al.* (1971).

The gels were carefully placed in plastic trays containing approximately 400 ml of the following solutions in the order listed: 1) 25% (v/v) isopropyl alcohol-10%



(v/v) glacial acetic acid-0.05% (w/v) Coomassie Brilliant Blue, overnight; 2) 10% (v/v) isopropyl alcohol-10% (v/v) glacial acetic acid-0.005% (w/v) Coomassie Brilliant Blue, 6 hours; 3) 10% (v/v) glacial acetic acid, overnight; 4) several washes in 10% (v/v) glacial acetic acid until the background was clear.

Gels were dried as described in Section 2.20.2.1.

## 2.23 SODIUM DODECYL SULPHATE (SDS)-POLYACRYLAMIDE GELS

SDS polyacrylamide gels, 1.0 mm in thickness, and of the desired percentage were prepared from the stock solutions presented in Table 3. Gels were run under constant current conditions, 16 mA through the stacking gel, and 24 mA through the resolving gel. Voltage was made unlimiting and gradually increased during the run.

## 2.24 BACTERIOPHAGE $\Phi$ 2037/1 SENSITIVITY TESTS

*Rhizobium loti* bacteriophage  $\Phi$ 2037/1 (Patel, 1976) was obtained from Dr J. Patel (DSIR, Palmerston North, New Zealand).

### 2.24.1 Purification of Bacteriophage $\Phi$ 2037/1

A loop of YM agar (Section 2.5.3) maintained PN184 was used to inoculate 5 ml TY broth (Section 2.5.2). After 2 days shaking at 30°C, 300  $\mu$ l aliquots were removed to separate sterile test tubes. 100  $\mu$ l aliquots of serial (100 fold) dilutions (in phage dilution buffer, Section 2.6.13) of  $\Phi$ 2037/1 were added and the mixture was incubated, without shaking, at 30°C for 30 min. 3 ml of TY top agar (TY agar [Section 2.5.2] but with 0.01 g calcium chloride and 8.0 g agar per litre) was added to each tube. The mixture was briefly vortexed, then poured onto modified YM agar (YM agar [Section 2.5.3] with mannitol replaced with 2.4 g sucrose per litre) plates. Modified YM agar was essentially YS agar (Vincent, 1970). The plates were incubated at 30°C until plaques appeared. A single plaque was picked off with a sterile toothpick and the phage were eluted into 400  $\mu$ l of phage dilution buffer by standing 2 h at 4°C. Serial dilutions of the eluted phage were prepared and the procedure described above was repeated twice to obtain a purified phage.

Table 3. Solutions used in the preparation of SDS-polyacrylamide gels.

Solution		g/100 ml
A.	Acrylamide	30.0
	Bis	0.8
B.	1.5 M Tris-HCl, pH 8.8	18.16
C.	10% SDS	10.0
D.	0.25 M Tris-HCl, pH 6.8	3.0
E.	40% Acrylamide	40.0
F.	1% Bis	1.0

Separation gels contained:

	Approximate % polyacrylamide		
	12.5%	15%	20%
Solution E	18.75 ml	22.50 ml	30.00 ml
Solution F	4.35 ml	5.22 ml	6.96 ml
Solution B	15.00 ml	15.00 ml	15.00 ml
Solution C	0.60 ml	0.60 ml	0.60 ml
MilliQ water	19.14 ml	16.68 ml	10.50 ml
Temed	15.00 $\mu$ l	15.00 $\mu$ l	15.00 $\mu$ l
10% Ammonium persulphate	240.00 $\mu$ l	240.00 $\mu$ l	240.00 $\mu$ l

Stacking gels contained:

Solution A	1.00 ml
Solution D	2.50 ml
Solution C	0.10 ml
MilliQ water	6.25 ml
Temed	10.00 $\mu$ l
10% Ammonium persulphate	100.00 $\mu$ l

Loading buffer contained:

Solution D	2.5 ml
Solution C	4.0 ml
$\beta$ -mercaptoethanol	1.0 ml
Glycerol	2.0 ml
Bromophenol blue (0.02%)	0.5 ml
MilliQ water	0.4 ml

Running buffer contained (per 2 l):

Tris	6.0 g (25 mM)
Glycine	28.8 g (190 mM)
Solution C	20.0 ml
MilliQ water to 2 l	
pH adjusted to 8.8	

### **2.24.2 Preparation of a Liquid Lysate**

After the third round of purification (Section 2.24.1) a working lysate was prepared as follows. A loop of YM agar (Section 2.5.3) maintained PN184 was used to inoculate 2 ml TY broth (Section 2.5.2). After 2 days shaking at 30°C, 100 µl of the culture was diluted 100 fold into 9.9 ml of modified YM broth (Section 2.24.1). After 2 days shaking at 30°C this culture was inoculated with 100 µl of a third round purified  $\Phi$ 2037/1 plaque after it had been eluted into phage dilution buffer (see Section 2.24.1). The mixture was incubated, without shaking, at 30°C for 30 min, then added to 40 ml fresh modified YM broth and shaken at 30°C until lysis occurred. A few drops of chloroform were added to lyse any remaining bacterial cells, then the cell debris were pelleted by centrifugation (20 min, 9000 x g, 10°C). The lysate was retained and the titre was determined by examining PN184 for lysis as described in Section 2.24.1.

### **2.24.3 Sensitivity of the PN184-Derived EPS Mutants to $\Phi$ 2037/1**

Sensitivity tests were performed by plaque formation and by spot tests.

#### **2.24.3.1 Plaque Formation**

Each strain was tested against serial dilutions of phage lysate (Section 2.24.2) as described for a single round of  $\Phi$ 2037/1 purification (Section 2.24.1), but replacing PN184 with each PN184-derived EPS mutant.

#### **2.24.3.2 Spot Tests**

Each strain was tested against serial dilutions of phage lysate (Section 2.24.2) as described for a single round of  $\Phi$ 2037/1 purification (Section 2.24.1) with the following changes: the TY top agar was added directly to 300 µl of the modified YM broth-grown test strain (rather than TY broth-grown); the mixture was poured onto a modified YM agar plate, and the plates were incubated at 30°C overnight; these plates were spotted with 20 µl of phage lysate (Section 2.24.2) dilutions (100 fold); the spots were air dried in a laminar flow cabinet and the plates were incubated for 2 days at 30°C, then examined for lysis.

## Chapter 3. RESULTS

### 3.1 FLUORESCENCE OF *R. LOTI* STRAINS ON CALCOFLUOR AGAR

Preliminary experiments demonstrated that NZP2037 and its Sm<sup>r</sup> derivative PN184 (Chua *et al.*, 1985), fluoresced blue-white on S20-, nitrogen-free S20-, and YM-Calcofluor agars (Section 2.19.1), and blue-green, changing to blue-white (after two days incubation) on TY-Calcofluor agar, upon exposure to long wave UV light (Fig. 4). Neither strain exhibited a fluorescent halo on any of the Calcofluor agars. In addition, PN4010 (Pankhurst *et al.*, 1986), a plasmid cured derivative of PN184, also fluoresced, to the same extent as PN184, on S20-, nitrogen-free S20-, and TY-Calcofluor agars, upon exposure to long wave UV light. Fluorescence on YM-Calcofluor agar was not examined. PN4010 did not exhibit a fluorescent halo. NZP2037, PN184, and PN4010 are therefore "Calcofluor-bright". NZP2037 was not further examined.

In addition, a series of *L. pedunculatus* infection mutants (PN1018, PN1019, and PN1027), derived from PN184 by Tn5 mutagenesis (Chua *et al.*, 1985), were also found to fluoresce on S20-, nitrogen-free S20-, YM-, and TY-Calcofluor agars (Section 2.19.1), upon exposure to long wave UV light (Fig. 5). None of the strains exhibited a fluorescent halo.

In contrast, NZP2213 and its Sm<sup>r</sup> derivative PN4115, failed to fluoresce on S20, nitrogen-free S20-, YM-, and TY-Calcofluor agars (Section 2.19.1) upon exposure to long wave UV light (Fig. 4). NZP2213 and PN4115 are therefore "Calcofluor-dark". NZP2213 was not further examined except as described in Sections 3.2.1. and 3.2.10.

When PN184, PN1018, PN1019, and PN1027 were streaked onto S20-, nitrogen-free S20-, and YM-agars (with or without Calcofluor, Section 2.5), all streaks grew white and mucoid (Figs. 6 and 7). The mucoid growth form was suppressed on TY agar (with or without Calcofluor), although the streaks still retained a white colouration. PN4010 was also found to be white and mucoid when streaked onto S20-, nitrogen-free S20-, and YM-agars (with or without Calcofluor). The mucoid growth form was suppressed on TY agar (with or without Calcofluor) to the same extent as PN184, and the streaks retained the white colouration.

Figure 4:

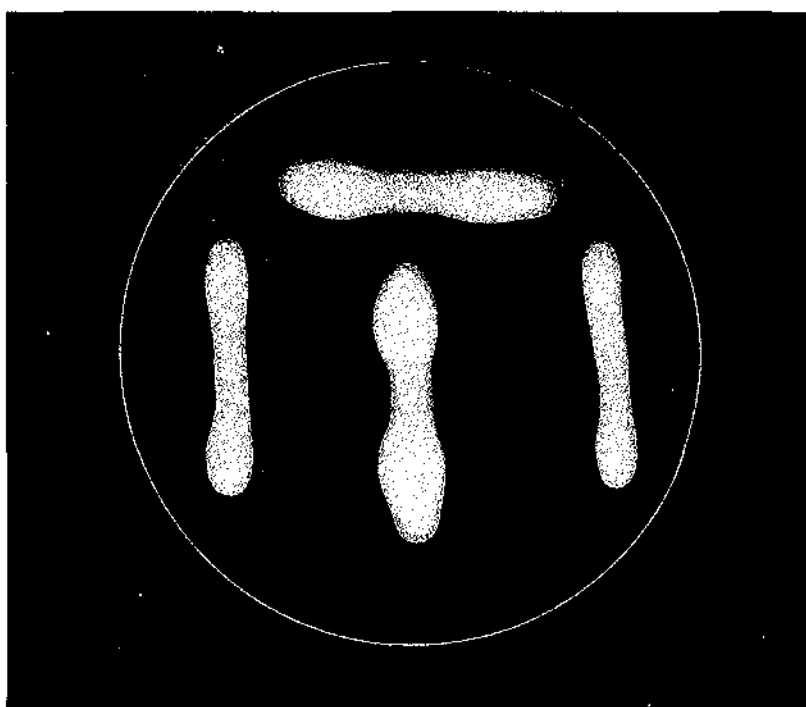
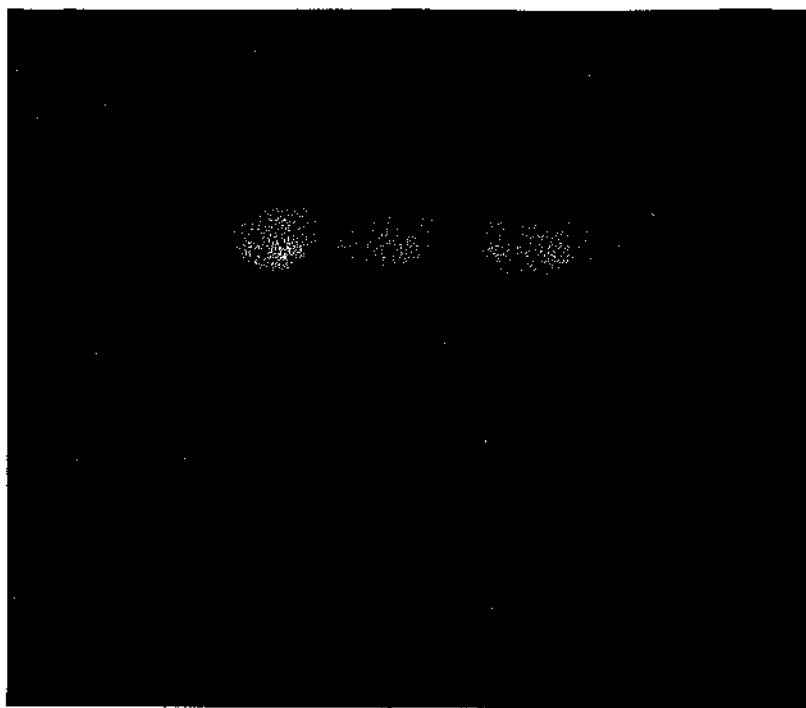
Fluorescence of PN184, and non-fluorescence of PN4115 on YM-Calcofluor agar.

Fluorescence is exhibited upon exposure to long wave UV light. PN184 (top), and PN4115 (bottom).

Figure 5:

Fluorescence of PN184, and the PN184-derived *L. pedunculatus* infection mutants PN1018, PN1019, and PN1027 on YM-Calcofluor agar.

Fluorescence is exhibited upon exposure to long wave UV light. PN184 (top) and bottom (left to right) PN1018, PN1019 and PN1027.



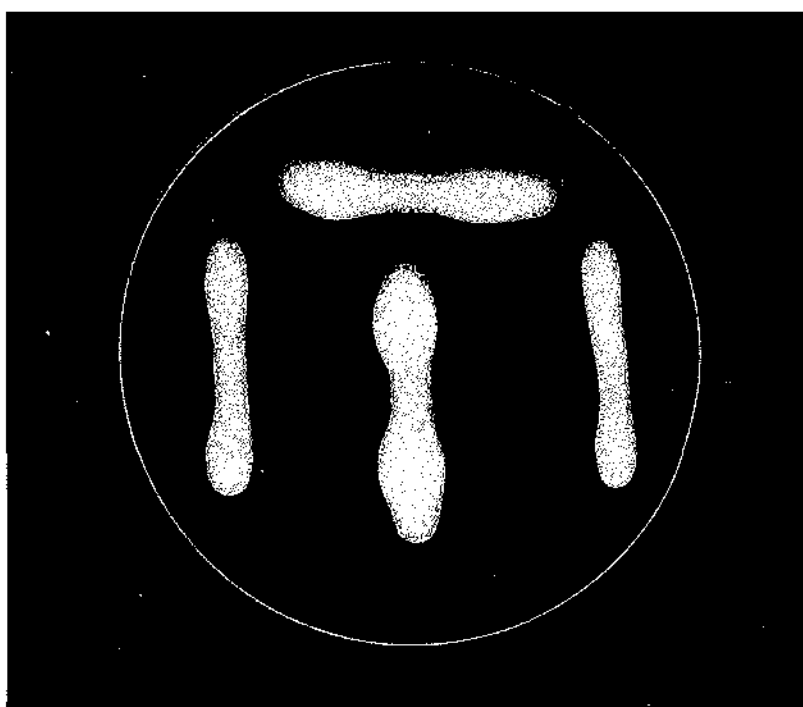
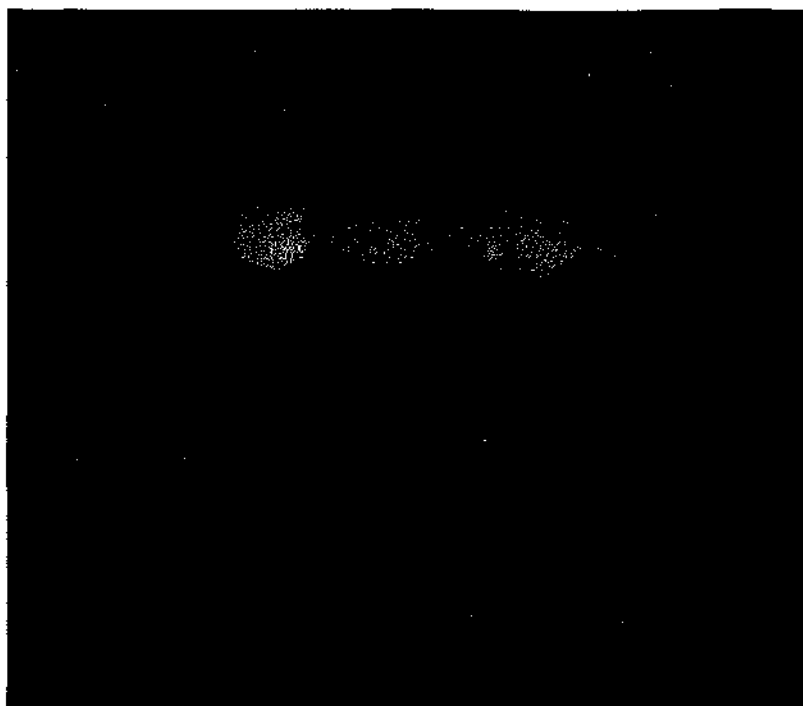




Figure 6:

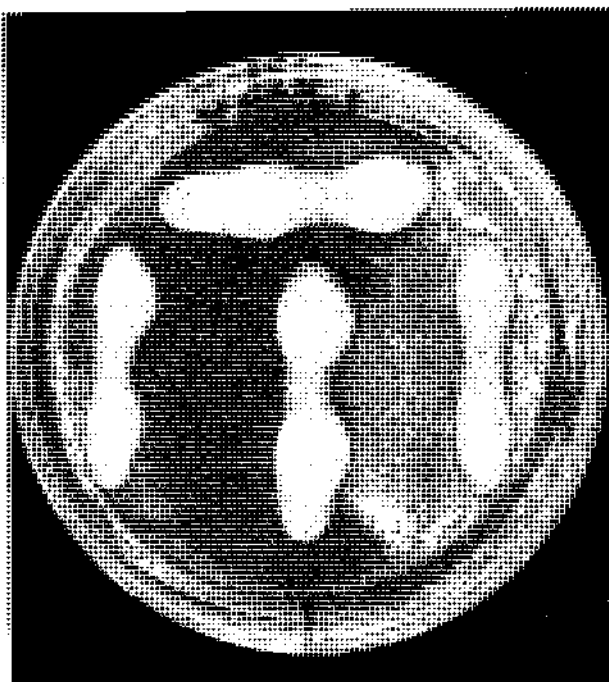
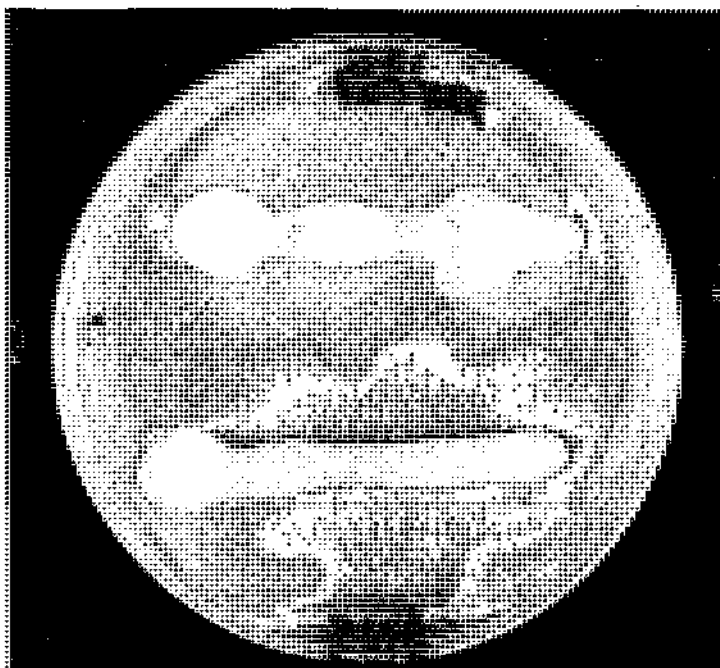
Mucoid, white, phenotype of PN184 and mucoid, cream, phenotype of PN4115 on YM-Calcofluor agar.

The figure shows the same agar plate shown in Fig. 4, but with an additional 2 days growth. PN184 (top) and PN4115 (bottom).

Figure 7:

Mucoid phenotypes of PN184 and the PN184-derived, *L. pedunculatus*, infection mutants PN1018, PN1019, and PN1027 on YM-Calcofluor agar.

The figure shows the same agar plate shown in Fig. 5, but with an additional 2 days growth. PN184 (top) and bottom (left to right) PN1018, PN1019, and PN1027.



In contrast, when PN4115 was streaked onto S20-, nitrogen-free S20-, or YM-agars (with or without Calcofluor, Section 2.5) the streaks grew yellow-cream coloured and mucoid (Fig. 6). The mucoid growth form was suppressed on TY agar (with or without Calcofluor), although to a lesser extent than for PN184, and the yellow-cream colouration was retained.

The mucoid growth form of PN184 and PN4115 on S20-, nitrogen-free S20-, and YM-agars suggested that these strains produce EPS. Given that PN184 fluoresced in the presence of Calcofluor and that, for example, *R. meliloti* EPSI binds Calcofluor (Finan *et al.*, 1985; Leigh *et al.*, 1985), it was apparent that EPS mutants derived from PN184 might be selected by Tn5 mutagenesis, followed by selection for non-fluorescence on Calcofluor agar (Section 2.19.1).

In contrast, it was apparent that selection for EPS mutants derived from PN4115 would require selection for either non-mucoid (EPS<sup>-</sup>) colonies, or colonies with an increased production of EPS (EPS overproducing).

## 3.2 THE ISOLATION AND CHARACTERIZATION OF EPS MUTANTS DERIVED FROM NZP2037

### 3.2.1 The Isolation of EPS Mutants by Tn5 Mutagenesis of PN184

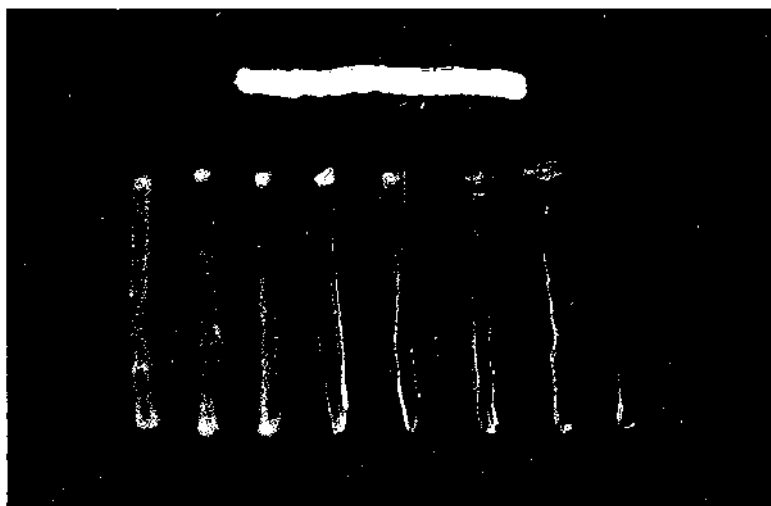
To isolate EPS mutants of *R. loti* NZP2037, Tn5 mutagenesis was carried out by crossing *E. coli* SM10 (Simon *et al.*, 1983), carrying pSUP1011 (Simon *et al.*, 1983), with *R. loti*, strain PN184. EPS negative or EPS defective transconjugants were obtained by plating onto nitrogen-free S20 agar containing neomycin, streptomycin and 0.02% Calcofluor (Section 2.18.1). Nm<sup>r</sup>Sm<sup>r</sup> transconjugants were obtained at a frequency of approximately 10<sup>-5</sup>. Of the 12,000 Nm<sup>r</sup>Sm<sup>r</sup> transconjugants examined, eight (henceforth designated PN184-derived EPS mutants) were nonfluorescent (Calcofluor dark) when exposed to long wave UV light (Fig. 8). An additional non-fluorescent mutant was isolated, but, was subsequently found to contain two *Eco*RI fragments hybridizing to a pKan2 DNA probe (described in Section 3.2.4.1). As a result this strain was not examined further, and is not described subsequently, except in Section 3.2.4.1.

To verify that the PN184-derived EPS mutants were PN184 derivatives, both the total protein profiles (Section 2.22) of PN184 and of each mutant, and the *Eco*RI

Figure 8:

Fluorescence of PN184 and non-fluorescence of the PN184-derived EPS mutants on S20-Calcofluor agar.

Fluorescence is exhibited upon irradiation with long wave UV light. PN184 (top) and bottom (left to right) PN1177, PN1178, PN1179, PN1180, PN1181, PN1182, PN1183 and PN1184.



restriction endonuclease digestion patterns (Section 2.11) of total DNA (Section 2.7.1) preparations from PN184 and each mutant, were examined and compared. The protein profiles and *Eco*RI digestion patterns of each mutant were found to be identical to those of PN184 (not shown).

During the course of the verification experiments, the protein profiles (Section 2.22) of the PN184-derived infection mutants PN1018, PN1019, and PN1027, together with the protein profile of NZP2213, were also examined (Fig. 9). While the protein profiles of PN1018 and PN1019 are very similar to the protein profile of PN184, the protein profile of PN1027 shows a highly expressed protein (of approximately 43 kD) not visible by Coomassie Brilliant Blue staining (Section 2.22.1) in the profiles of PN184, PN1018 or PN1019. The total protein profile of NZP2213 differed substantially from the total protein profile of PN184.

### 3.2.2 Colony Characteristics of the PN184-Derived EPS Mutants

The Calcofluor-dark phenotype of the PN184-derived EPS mutants PN1177, PN1178, PN1179, PN1180, PN1181, PN1182, PN1183, and PN1184 on Calcofluor agar has been described in Section 3.2.1 (Fig. 8).

Five of the eight EPS mutants, strains PN1180, PN1181, PN1182, PN1183, and PN1184 produced rough (non-mucoid) colonies, this being most pronounced on YM- (Fig. 10A), S20-, and nitrogen-free S20-agars, suggesting that these strains produce little or no EPS (Section 2.19.1). The remaining three EPS mutants, strains PN1177, PN1178, and PN1179, produced smooth (mucoid) colonies (Fig. 10A). This suggests that the smooth PN184-derived EPS mutants produce an EPS that does not bind Calcofluor (Section 2.19.1). All the EPS mutants produced yellow-cream coloured colonies on TY agar, identical in colour to PN4115 (Fig. 6).

When PN184 and the PN184-derived EPS mutants were streaked onto YM-Congo Red agar (Section 2.5), the dye was taken up to produce deep orange coloured streaks after 5 days growth (Fig. 10B). There was no apparent difference between PN184 and the PN184-derived EPS mutants, except that the streaks of the rough mutants PN1180, PN1181, PN1182, PN1183, and PN1184 were a deeper orange, consistent with the mucoid phenotype of PN184 and the smooth PN184-derived EPS mutants effectively diluting the intensity of the deep orange colouration.

Figure 9:

Total protein profiles of PN184, NZP2213, and the PN184-derived *L. pedunculatus* infection mutants PN1018, PN1019, and PN1027.

SDS-PAGE (12.5% gel) of lanes: 1. PN1027; 2, PN1019; 3, PN1018; 4, NZP2213; 5, PN184; 6, blank; 7, protein molecular weight markers (Bio-Rad). The molecular weight of the protein molecular weight markers is given in kilodaltons (kD).

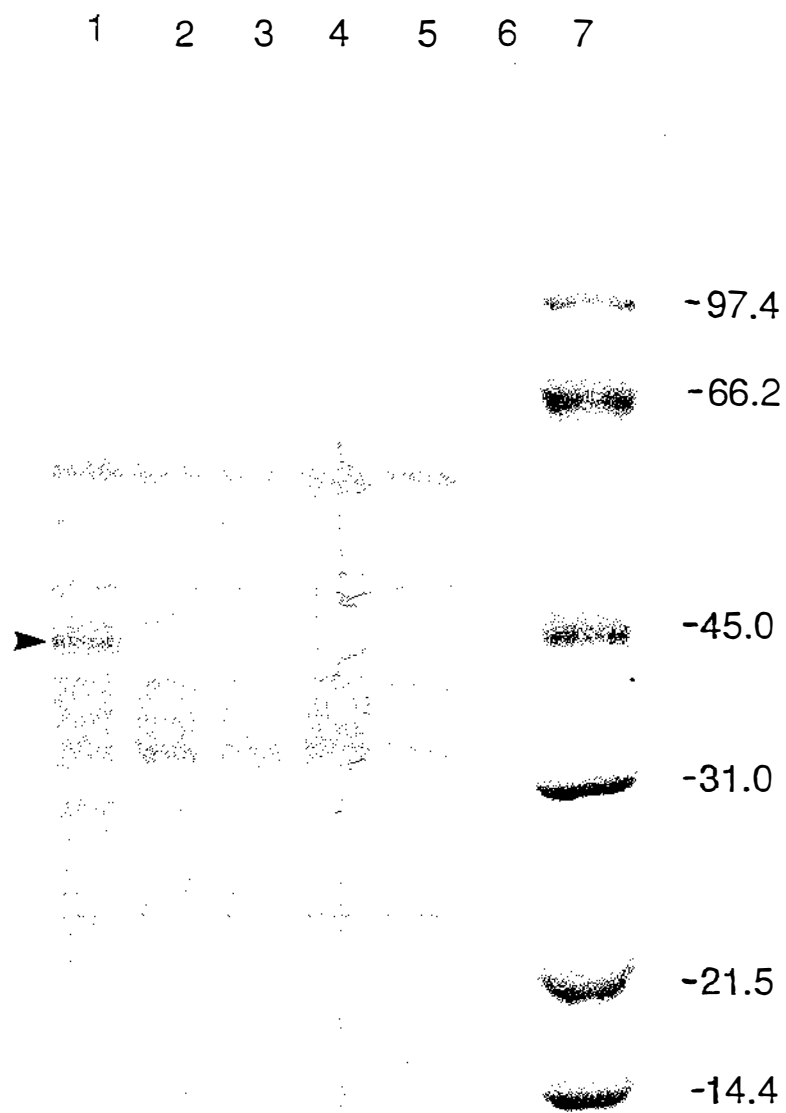




Figure 10A:

Smooth or rough phenotypes of PN184 and the PN184-derived EPS mutants on YM agar.

Smooth phenotype of PN184 (top), and bottom (left to right), smooth phenotypes of the PN184-derived EPS mutants PN1177, PN1178, and PN1179, and rough phenotypes of the PN184-derived EPS mutants PN1180, PN1181, PN1182, PN1183, and PN1184.

Figure 10B:

Orange colouration of PN184, and the PN184-derived EPS mutants on YM Congo Red agar.

PN184 (top), and bottom (left to right), PN184-derived EPS mutants PN1177, PN1178, PN1179, PN1180, PN1181, PN1182, PN1183, and PN1184.

**A****B**

### 3.2.3 Resistance of the PN184-Derived EPS Mutants to $\Phi$ 2037/1

Patel (1976) has described the isolation of a series of bacteriophages virulent to *R. loti* NZP2037. To investigate whether the PN184-derived EPS mutants had acquired resistance to one of these bacteriophages ( $\Phi$ 2037/1), phage lysates were prepared as described in Section 2.24.2 and used as stock phage solutions to test for resistance.

In preliminary experiments, phage lysates containing up to  $10^8$  plaque forming units/ml (PFU/ml) were prepared. Spot tests, performed as described in Section 2.24.3.2, demonstrated that the PN184-derived EPS mutants were resistant to lysis, while PN184 was confluentlly lysed over the area of the spot (not shown).

Subsequently, plating for plaques was performed, as described in Section 2.23.3.1, using a phage lysate containing  $10^6$  PFU/ml, together with 100 fold, and 1000 fold dilutions of this lysate. The concentrated lysate gave confluent lysis for PN184, but no lysis for the PN184-derived EPS mutants. The 100 fold and 1000 fold dilutions gave non-confluent lysis for PN184 (individual plaques visible in Fig. 11), while no plaques were observed for the PN184-derived EPS mutants (Fig. 11).

### 3.2.4 Confirmation of the Presence of Tn5 Sequences

#### 3.2.4.1 Confirmation of a Single Tn5 Insertion

To confirm the presence of a single Tn5 insertion in each PN184-derived EPS mutant, total DNA from each mutant, and total DNA from PN184, isolated as described in Section 2.7.1, was digested to completion with *Eco*RI (Section 2.11). Digestion to completion was checked on a 0.7% agarose minigel, then the samples were run overnight (approximately 16 h) on a 0.7% agarose gel (Section 2.12). A Southern blot (Section 2.16) of this gel was probed with  $^{32}$ P-labelled (Section 2.17) pKan2 DNA, which is pBR322 carrying the 3.5-kb *Hind*III fragment of Tn5 (Scott *et al.*, 1982). For all mutants a single hybridizing band (Tn5 has no *Eco*RI sites) was observed (Fig. 12A.). The size in kilobases of the hybridizing fragments for each mutant, together with the corrected size of the wild type (PN184) fragment (that is subtracting 5.7 kb for the size of Tn5), is shown in Table 4.

In addition to the eight mutants described, an additional smooth, non-fluorescent

Figure 11:

Resistance of the PN184-derived EPS mutants to  $\Phi$ 2037.

Lysis of a PN184 lawn (A) and non-lysis of lawns of PN1177, PN1178, PN1179 (B, left to right), PN1180, PN1181, PN1184 (C, left to right), and PN1182 and PN1183 (D, left to right) by a 1000 fold dilution of a  $10^6$  PFU/ml,  $\Phi$ 2037/1 lysate. Lysis is indicated by the presence of plaques.

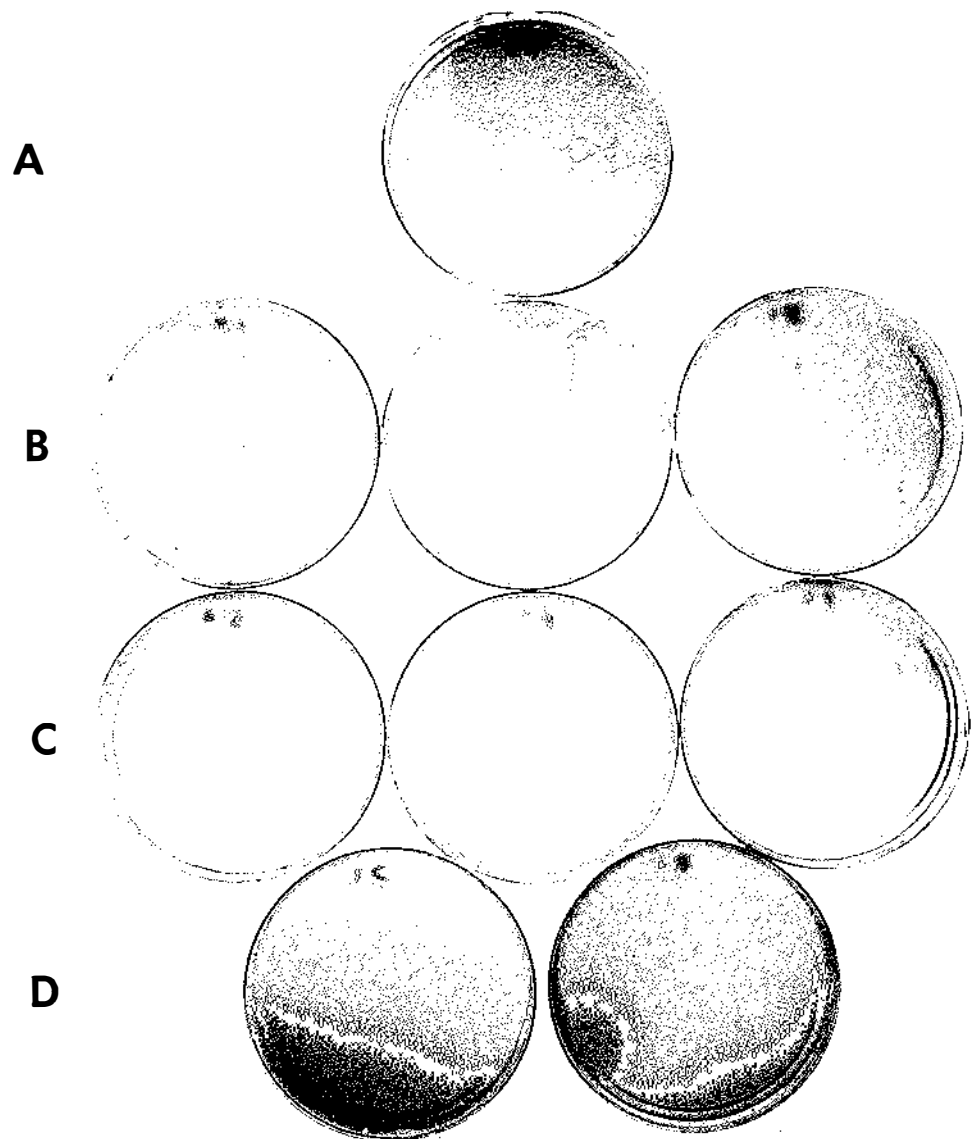


Figure 12A:

Confirmation of a single Tn5 insertion in each PN184-derived EPS mutant.

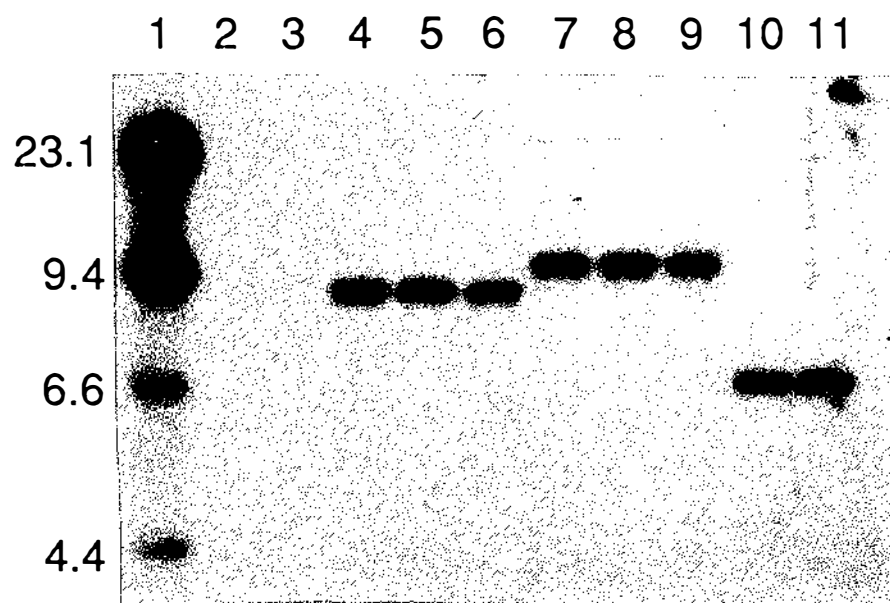
Autoradiogram of a Southern blot of *Eco*RI digested total DNA from PN184 and the PN184-derived EPS mutants probed with <sup>32</sup>P-labelled, pKan2 DNA. Lanes: 1, <sup>32</sup>P-labelled, *Hind*III-digested, phage lambda DNA; 2, blank; 3, PN184; 4, PN1177; 5, PN1178; 6, PN1179; 7, PN1180; 8, PN1181; 9, PN1184; 10, PN1182; 11, PN1183.

Figure 12B:

The PN184-derived EPS mutant GH4 shows two *Eco*RI fragments hybridizing to <sup>32</sup>P-labelled pKan2 DNA.

Autoradiogram of a Southern blot of *Eco*RI digested total DNA from PN184, and the PN184-derived EPS mutant GH4 probed with <sup>32</sup>P-labelled, pKan2 DNA. Lanes: 1, <sup>32</sup>P-labelled, *Hind*III digested, phage lambda DNA; 2, blank; 3, PN184; 4, GH4.

A



B

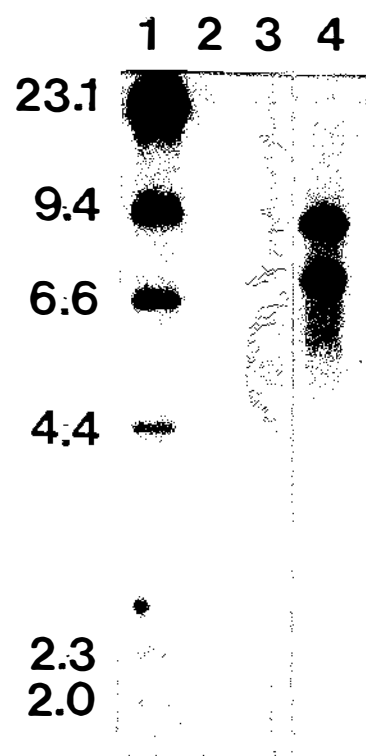


Table 4. *Eco*RI digestion fragments of the PN184 derived EPS mutants containing a Tn5 insertion.

Strain	Size of <i>Eco</i> RI fragment containing Tn5 (kb)	Predicted size <sup>1</sup> of <i>Eco</i> RI fragment in PN184 (kb)
PN1177	9.0	3.3
PN1178	9.0	3.3
PN1179	9.0	3.3
PN1180	9.8	4.1
PN1181	9.8	4.1
PN1182	6.7	1.0
PN1183	6.7	1.0
PN1184	9.8	4.1

<sup>1</sup> Obtained by subtracting 5.7 kb (for Tn5) from the size of the *Eco*RI fragment carrying Tn5 in each mutant.



PN184-derived EPS mutant was also isolated. However, two bands hybridized (Fig. 12B) under the conditions described above, and for this reason this mutant was not further examined.

#### **3.2.4.2 Confirmation that Each Mutation Arose from an Independent Tn5 Insertion**

To confirm that each mutation arose from an independent insertion event, total DNA from each PN184-derived EPS mutant, and total DNA from PN184, isolated as described in Section 2.7.1, was digested to completion with *Bam*HI (Section 2.11). Digestion to completion was checked on a 0.7% agarose minigel, then the samples were run overnight (approximately 16 h) on a 0.7% agarose gel (Section 2.12). A Southern blot (Section 2.16) of this gel was probed with <sup>32</sup>P-labeled (Section 2.17) pKan2 DNA. In all cases, two fragments hybridized as expected (Tn5 has a single *Bam*HI site) and each hybridization pattern was unique (Fig. 13).

#### **3.2.5 The Isolation and Characterization of Cosmids Restoring the Calcofluor-Bright Phenotype of PN184**

##### **3.2.5.1 Cosmid Isolation**

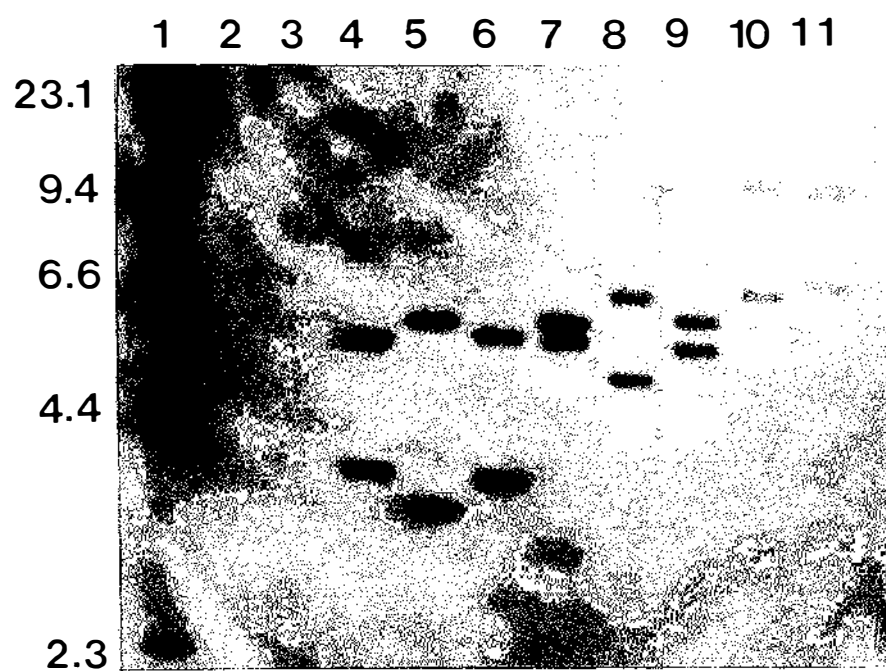
Cosmids were isolated which stably complemented the mutations in the five rough, Calcofluor-dark EPS mutants PN1180, PN1181, PN1182, PN1183, and PN1184 by crossing the NZP2037 (pLAFR1) gene library (residing in *E. coli* HB101, Chua *et al.*, 1985) *en masse* with each mutant, using pRK2013 as the helper plasmid, and selecting for restoration of fluorescence on nitrogen-free S20-Calcofluor agar containing streptomycin, neomycin, and tetracycline (Section 2.18.2.1).

Cosmids could not be isolated which stably complemented the mutations carried by the smooth, Calcofluor-dark EPS mutants PN1177, PN1178, and PN1179, despite repeating the experiment (Section 2.18.2.1) three times. Several non-overlapping cosmids were isolated for each mutant which, initially, appeared to complement the mutations. However, complementation did not persist through the "back cross" procedure (Section 2.18.2.1) used to identify stably complementing cosmids.

Figure 13:

Confirmation that each PN184-derived EPS mutant arose from an independent Tn5 insertion.

Autoradiogram of a Southern blot of *Bam*HI digested total DNA from PN184 and the PN184-derived EPS mutants probed with <sup>32</sup>P-labelled, pKan2 DNA. Lanes: 1, <sup>32</sup>P-labelled, *Hind*III digested, phage lambda DNA; 2, blank; 3, PN184; 4, PN1177; 5, PN1178; 6, PN1179; 7, PN1180; 8, PN1181; 9, PN1184; 10, PN1182; 11, PN1183.



### 3.2.5.2 The *Eco*RI Digestion Profiles of Complementing Cosmids

For each rough PN184-derived EPS mutant, several complementing cosmids were isolated which had overlapping restriction endonuclease digestion fragments. The restriction endonuclease digestion profiles of cosmids complementing each rough EPS mutant were obtained by digesting cosmid DNA, isolated by the rapid boiling method (Section 2.7.2), with *Eco*RI (Section 2.11). Digestion to completion was checked on 0.7% agarose minigels, and the digest of a representative cosmid complementing each rough mutant was run overnight (approximately 16 h) on a 0.7% agarose gel (Section 2.12). *Eco*RI digests of representative cosmids complementing the mutation carried by each rough PN184-derived EPS mutant are shown in Fig. 14. For cosmid designations see the legend to Fig. 14 and Table 2.

Each complementing cosmid was found to contain an *Eco*RI fragment from NZP2037 (PN184, arrowed in Fig. 14 for each set) which, with the addition of a Tn5 insertion (5.7 kb), would match the size of the relevant *Eco*RI fragment hybridizing to <sup>32</sup>P-labelled pKan2 in the *Eco*RI digests of total DNA from each mutant (Fig. 12A) as summarized in Table 4.

Mutants PN1180, PN1181, and PN1184 were complemented by a group of cosmids (pPN31, pPN32, and pPN35) with *Eco*RI digestion fragments in common, while mutants PN1182 and PN1183 were complemented by a second group of cosmids (pPN33 and pPN34) with *Eco*RI digestion fragments in common (Fig. 14).

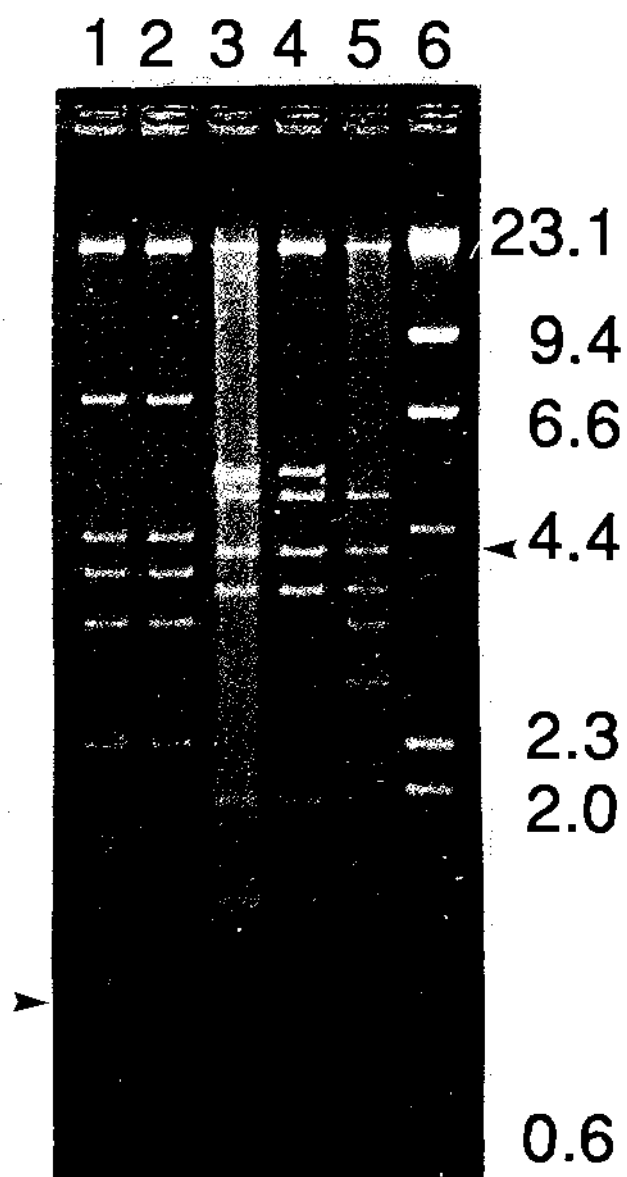
Examination of the *Eco*RI digestion profiles of the two sets of cosmids (Fig. 14) indicates that there are no common fragments between the two sets. This was confirmed by probing a Southern blot (Section 2.16) of the gel shown in Fig. 14 with <sup>32</sup>P-labelled (Section 2.17) pPN32. Hybridization was observed to the common *Eco*RI digestion fragments from pPN31, pPN32, and pPN35 but was observed only for the pLAFR1 fragment from pPN33 and pPN34 (not shown). These results suggest that the gene(s) present on the two sets of cosmids are not closely linked.

On the basis of the *Eco*RI digestion patterns of cosmids complementing the rough EPS mutants (Fig. 14) and considering the hybridization patterns of *Eco*RI-digested total DNA from each mutant probed with <sup>32</sup>P-labeled pKan2 DNA (Fig. 12A, see also Table 4), the mutants were classified into three groups: smooth

Figure 14:

*Eco*RI digestion profiles of cosmids complementing the rough PN184-derived EPS mutants.

Lanes: 1, pPN34, complementing PN1183; 2, pPN33, complementing PN1182; 3, pPN35, complementing PN1184; 4, pPN32, complementing PN1181; 5, pPN31, complementing PN1180; 6, *Hind*III digested phage lambda DNA.



mutants PN1177, PN1178, and PN1179 (Group 1); rough mutants PN1180, PN1181, and PN1184 (Group 2); and rough mutants PN1182 and PN1183 (Group 3).

### 3.2.5.3 Cosmid Cross-Complementation

The representative cosmids pPN31, pPN32, pPN33, pPN34, and pPN35 (Fig. 14) were isolated by complementation of the mutation carried by PN1180, PN1181, PN1182, PN1183, and PN1184, respectively.

Each of these representative cosmids was also crossed, in a triparental mating, using pRK2013 as the helper plasmid (Section 2.18.2.2), with all the other PN184-derived EPS mutants to define the cosmid complementation groups. The results are summarized in Table 5. None of the cosmids complemented any of the mutations carried by the smooth (Group 1) PN184-derived EPS mutants. The cosmid complementing the mutation carried by a PN184-derived EPS mutant within one of the two groups of rough EPS mutants (Groups 2 and 3), cross complemented within each group, but did not cross complement between the groups.

These results provide further evidence that the mutations carried by the Group 1, Group 2, and Group 3 PN184-derived EPS mutants are located in (at least) three separate loci, and that these three loci are not closely linked. The three loci into which Tn5 has inserted, therefore, presumably represent three genes or gene regulatory regions, functional copies of which are required for the production of the Calcofluor binding EPS synthesized by PN184.

### 3.2.5.4 Complementation Tests with *R. meliloti* Cosmids

Cosmids (pD34, pD56, and pEx312, Table 2) isolated from a *R. meliloti* gene library, that complement *exo* mutations mapping to pRmeSU47b (Leigh *et al.*, 1985, Long *et al.*, 1988) were transferred separately into each of the PN184-derived EPS mutants in a triparental mating (as described for cosmid cross complementation, Section 2.18.2.2). However, none of the cosmids complemented the mutations carried by the PN184-derived EPS mutants.

Table 5. Cosmid cross complementation.

Mutant	Cosmid				
	pPN31	pPN32	pPN33	pPN34	pPN35
PN1177	-	-	-	-	-
PN1178	-	-	-	-	-
PN1179	-	-	-	-	-
PN1180	+	+	-	-	+
PN1181	+	+	-	-	+
PN1182	-	-	+	+	-
PN1183	-	-	+	+	-
PN1184	+	+	-	-	+
+ complementation observed					
- complementation not observed					



### 3.2.6 Cloning of the Wild Type Fragments Predicted to Carry a Tn5 Insertion in Each Rough PN184-Derived EPS Mutant

DNA (isolated as described in Section 2.7.2) from cosmids pPN32 (cosmid complementing PN1181, Group 2) and pPN33 (cosmid complementing PN1182, Group 3) was digested to completion with *Eco*RI (Section 2.11). Digestion to completion was checked on a 0.7% agarose minigel (pPN32) or a 1.0% agarose minigel (pPN33) as described in Section 2.12. The fragments were then separated on a 0.7% agarose (Seaplaque) minigel (pPN32) or a 1.0% agarose (Seaplaque) minigel (pPN33). The wild type *Eco*RI fragments (4.1 kb from pPN32, and 1.0 kb from pPN33) into which Tn5 had putatively inserted to generate mutants PN1181 and PN1182 (respectively), were isolated as described in Section 2.13. Each fragment was ligated into the *Eco*RI site of CAP-treated pUC118 (Section 2.14). The ligation mixture contained an approximate 1:1 molar ratio of insert to vector (based upon approximately 50 ng vector) in a 20  $\mu$ l volume.

Competent MC1022 cells were transformed with 5  $\mu$ l of the ligation mix and plated onto LB agar supplemented with ampicillin, and spread with 20  $\mu$ l of X-Gal (Section 2.15).

Several white colonies were selected for each of the two ligation transformations. Miniprep plasmid DNA was isolated (Section 2.7.2), digested with *Eco*RI to completion (Section 2.11), and the presence of the correct fragment (1.0 kb or 4.1 kb) was checked on a 1.0% agarose minigel (Section 2.12). A clone containing each fragment was retained.

### 3.2.7 Confirmation that the Cloned Wild Type *Eco*RI Fragment Corresponds to the *Eco*RI Fragment in Each Rough Mutant Carrying a Tn5 Insertion

The expected size of the wild type (PN184) *Eco*RI fragment into which Tn5 had inserted to generate the PN184-derived EPS mutants PN1182 and PN1183 was 1.0 kb (see Section 3.2.4.1, Fig. 12A, and Table 4). The <sup>32</sup>P-labelled, 1.0 kb *Eco*RI fragment, isolated from pPN33 (also present in pPN34) as described in Section 3.2.6, was used to probe a Southern blot (Section 2.16) from an overnight, 1.0% agarose gel (Section 2.12) of *Eco*RI digested (Section 2.11) total DNA (Section 2.7.1) from PN184, PN1182, and PN1183, and *Eco*RI digested cosmids (Section 2.7.2) pPN33, and pPN34. If the 1.0 kb fragment was the correct wild type

fragment into which Tn5 had inserted to generate the mutations carried by PN1182 and PN1183, then a hybridizing fragment of 1.0 kb should have been present in the lanes containing *Eco*R1 digested DNA from PN184, pPN33, and pPN34. In contrast, a hybridizing fragment of 6.7 kb (1.0 kb + 5.7 kb for Tn5) should have been present in the lanes containing *Eco*R1 digested DNA from PN1182 and PN1183. Hybridization, however, was observed to a 1.0 kb *Eco*R1 fragment in all cases (not shown).

It was possible, however, that differential mobility had occurred between the *Hind*III digested, phage lambda DNA, size markers and the *Eco*R1 digested total DNA samples from each mutant in the gel which was blotted to generate the autoradiograph shown in Figure 12A. If the *Hind*III digested phage lambda DNA size markers had migrated slightly further than equivalent sized *Eco*R1 fragments in the lanes containing the total DNA, then the true size of the wild type (PN184) *Eco*R1 fragment into which Tn5 inserted in mutants PN1182 and PN1183 would be less than the predicted size.

An *Eco*R1 fragment of 0.8 kb was also common to *Eco*R1 digests of cosmids pPN33 and pPN34 (Fig. 14). To isolate this fragment, DNA from cosmid pPN33, isolated as described in Section 2.7.2, was digested to completion with *Eco*R1 (Section 2.11). The 0.8 kb *Eco*R1 fragment was isolated, ligated into the *Eco*R1 site of CAP treated PUC118 (Section 2.14). Competent MC1022 cells were transformed with 5 µl of the ligation mix (Section 2.15), several white colonies were checked for the presence of the 0.8 kb *Eco*RI insert (Sections 2.11 and 2.12), and a clone was retained. Confirmation that the 0.8 kb *Eco*R1 fragment from pPN33, cloned in plasmid pPN47, and residing in strain PN1315 is the correct wild type (PN184) *Eco*R1 fragment into which Tn5 had inserted in mutants PN1182 and PN1183 is described in Section 3.2.7.1.

Confirmation that the 4.1 kb *Eco*R1 fragment from pPN32, cloned in plasmid pPN48, and residing in strain PN1316, is the correct wild type (PN184) *Eco*R1 fragment into which Tn5 had inserted in mutants PN1180, PN1181, and PN1184, is described in Section 3.2.7.2.

### 3.2.7.1 The 0.8 kb Fragment Present in Cosmids pPN33 and pPN34

The following experiment was performed to confirm that cosmids pPN33 and

pPN34, complementing the mutations carried by the rough (Group 3) PN184 derived EPS mutants PN1182 and PN1183 respectively, contained a wild type *EcoRI* fragment, of 0.8 kb, into which Tn5 had inserted in each mutant. A Southern blot (Section 2.16) from an overnight 1.0% agarose gel (Section 2.12) of *EcoRI*-digested (Section 2.11) total DNA (Section 2.7.1) from PN184, PN1182, and PN1183, and *EcoRI* digested cosmids (Section 2.7.2) pPN33 and pPN34 was probed with <sup>32</sup>P-labelled pPN47 (containing the 0.8 kb *EcoRI* fragment from pPN33) DNA (Sections 2.7.2 and 2.17). The blot also included total *EcoRI*-digested DNA (Sections 2.7.1 and 2.11) from PN4010 (see Section 3.2.8).

If the 0.8 kb fragment is the correct wild type fragment into which Tn5 had inserted to generate the mutations carried by strains PN1182 and PN1183, then a hybridizing fragment of 0.8 kb should be present in the lanes containing *EcoRI*-digested PN184, pPN33 and pPN34 DNA, but a hybridizing fragment of approximately 6.5 kb (that is 0.8 kb + 5.7 kb for Tn5) should be present in the lanes containing total *EcoRI*-digested DNA from PN1182 and PN1183. Figure 15 shows that this was the result obtained.

The observation that the cosmids complementing the mutations in PN1182 and PN1183 contain a wild type *EcoRI* fragment into which Tn5 has inserted in each mutant, suggests that the EPS mutation present in each of PN1182 and PN1183 derives from the Tn5 insertion, rather than, for example, an independent point mutation, or insertion of an endogenous transposable element into a gene, expression of which is required for EPS synthesis.

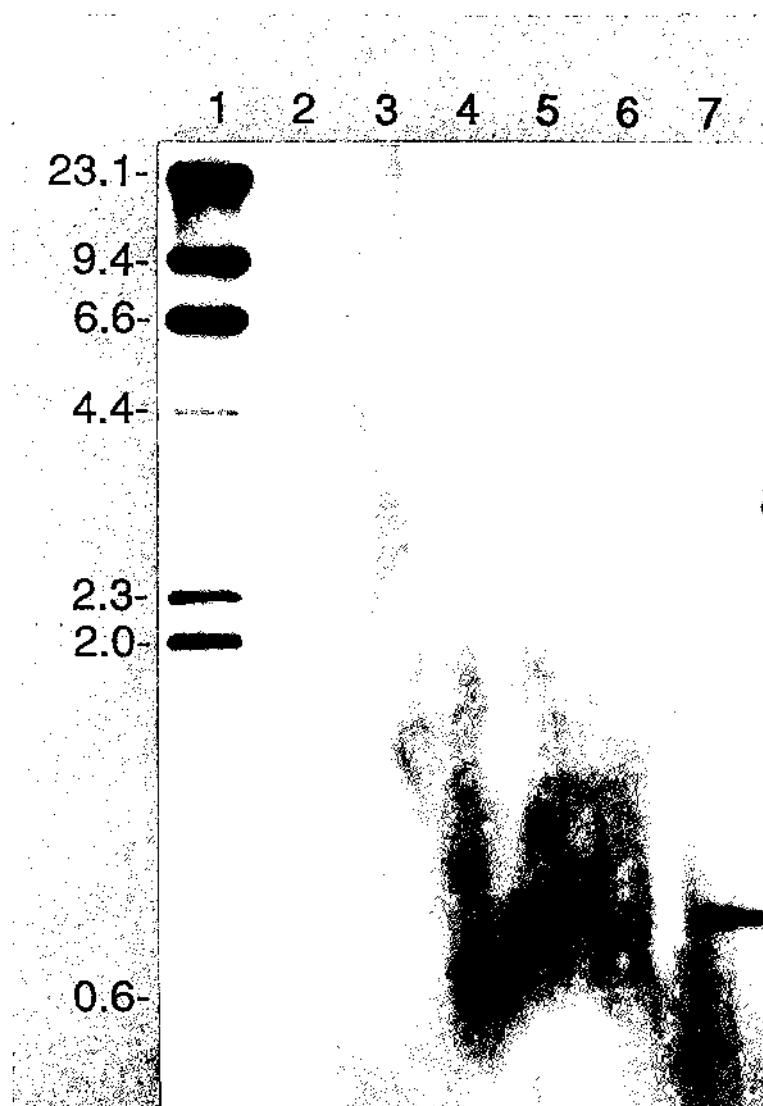
### 3.2.7.2 The 4.1 kb Fragment Present in Cosmids pPN31, pPN32 and pPN35

The following experiment was performed to confirm that cosmids pPN31, pPN32, and pPN35, complementing the mutations carried by the rough (Group 2) EPS mutants PN1180, PN1181, and PN1184 respectively, contained a wild type *EcoRI* fragment, of 4.1 kb, into which Tn5 had inserted in each mutant. A Southern blot (Section 2.16) from an overnight 0.7% agarose gel (Section 2.12) of *EcoRI* digested (Section 2.11) total DNA (Section 2.7.1) from PN184, PN1180, PN1181, and PN1184, and *EcoRI* digested cosmids (Section 2.7.2) pPN31, pPN32, and pPN35 was probed with the <sup>32</sup>P-labelled (Section 2.17) 4.1 kb *EcoRI* fragment from pPN48 (isolated as described in Section 2.13). The blot also included total *EcoRI*-digested DNA (Sections 2.7.1 and 2.11) from PN4010 (see Section 3.2.8).

Figure 15:

Insertion of Tn5 into a 0.8 kb wild type *Eco*RI fragment in the PN184-derived EPS mutants PN1182 and PN1183.

Autoradiogram of a Southern blot of *Eco*RI digested DNA from Lane: 1, <sup>32</sup>P-labelled, *Hind*III-digested, phage lambda DNA; 2, PN4010; 3, PN184; 4, PN1182; 5, pPN33; 6, PN1183; and 7, pPN34, probed with <sup>32</sup>P-labelled pPN47 DNA.



If the 4.1 kb fragment is the correct wild type fragment into which Tn5 had inserted to generate the mutations carried by strains PN1180, PN1181, and PN1184, then a hybridizing fragment of 4.1 kb should be present in the lanes containing *Eco*RI-digested PN184, pPN31, pPN32, and pPN35 DNA, but a hybridizing fragment of 9.8 kb (that is 4.1 kb + 5.7 kb for Tn5) should be present in the lanes containing total *Eco*RI-digested DNA from PN1180, PN1181, and PN1184. Figure 16 shows that this was the result obtained.

The observation that the cosmid pPN31, pPN32 and pPN35, complementing the mutations in PN1180, PN1181, and PN1184 respectively, contain a wild type *Eco*RI fragment into which Tn5 has inserted in each mutant, suggests that the EPS mutation present in each of PN1180, PN1181, and PN1184 derives from the insertion of Tn5, rather than, for example, an independent point mutation, or insertion of an endogenous transposable element into a gene, expression of which is required for EPS synthesis.

### 3.2.8 Identification of the Location of the Wild Type DNA Sequences

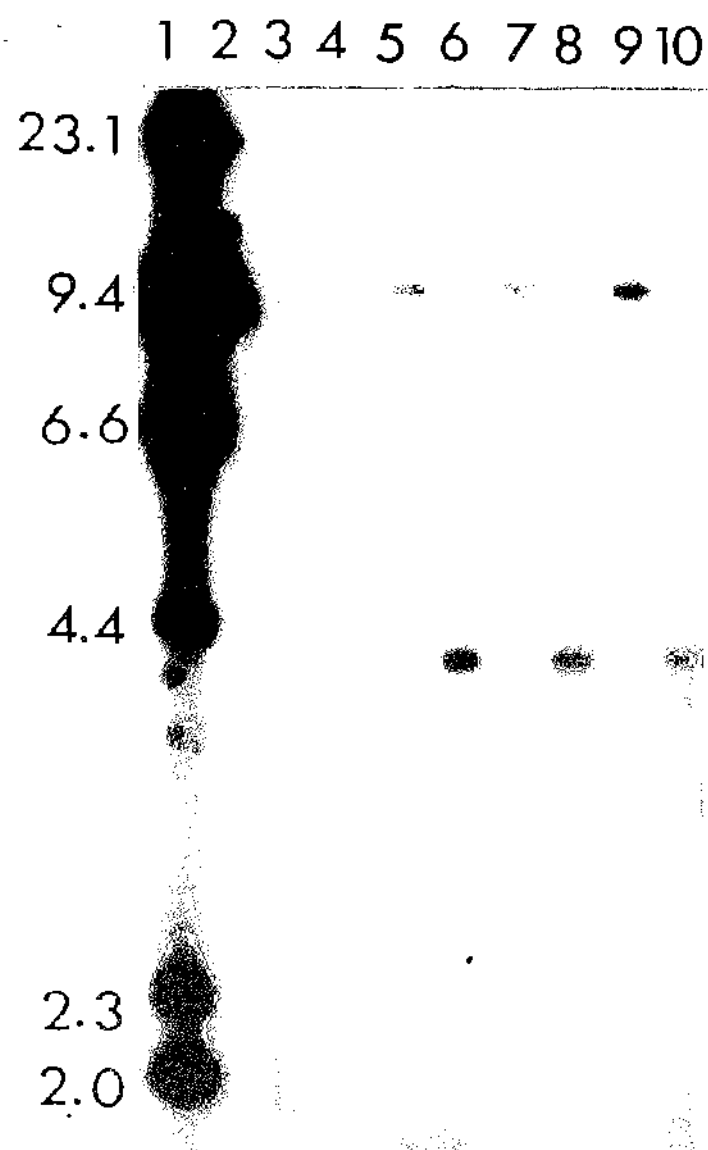
To identify the location (plasmid or chromosome) of the wild-type DNA from each complementing cosmid, Southern blots (Section 2.16) from overnight, 0.7% agarose gels (Section 2.12) of *Eco*RI-digested (Section 2.11) total DNA (Section 2.7.1) from *R. loti* PN184 and *R. loti* PN4010, a plasmid-cured derivative of *R. loti* NZP2037 (Pankhurst *et al.*, 1986), were probed with either <sup>32</sup>P-labeled pPN32 or <sup>32</sup>P-labeled pPN33 (Sections 2.7.2 and 2.17). In both cases hybridization was observed to the appropriate *Eco*RI digestion fragments in both lanes, indicating that the cosmid contains chromosomal DNA (not shown).

In a refinement of this experiment, Southern blots (Sections 3.2.7.1 and 3.2.7.2) of *Eco*RI-digested total DNA from PN184 and PN4010 were probed with either <sup>32</sup>P-labelled pPN47 (Fig 15), or the <sup>32</sup>P-labelled 4.1 kb *Eco*RI fragment from pPN48 (Fig 16), as appropriate. In both cases hybridization was observed to both PN184 and PN4010 DNA, indicating that the cosmid contains chromosomal DNA.

Figure 16:

Insertion of Tn5 into a 4.1 kb wild type *EcoRI* fragment in the PN184-derived EPS mutants PN1180, PN1181, and PN1184.

Autoradiogram of a Southern Blot of *EcoRI* digested DNA from lanes: 1,  $^{32}\text{P}$ -labelled, *HindIII*-digested, phage lambda DNA; 2, Blank; 3, PN4010; 4, PN184; 5, PN1180; 6, pPN31; 7, PN1181; 8, pPN32; 9, PN1184; and 10, pPN35, probed with the  $^{32}\text{P}$ -labelled 4.1 kb *EcoRI* fragment from pPN48.





### 3.2.9 Exopolysaccharide Isolation and Characterization

#### 3.2.9.1 EPS Isolation

EPS could be cetrимide precipitated (Section 2.19.2.1) from YM- and nitrogen-free S20-broth culture supernatants for PN184 and for the smooth PN184-derived EPS mutants PN1177, PN1178, and PN1179. However, under conditions identical to those used to isolate the cetrимide precipitate of EPS from PN184, no precipitate was obtained from the five rough PN184-derived EPS mutants PN1180, PN1181, PN1182, PN1183, and PN1184.

A precipitate of EPS was also obtained after addition of 4 volumes of ethanol (Section 2.19.2.2) to YM culture supernatants of PN184 and the smooth PN184-derived EPS mutants PN1177, PN1178, and PN1179. Addition of additional ethanol to 10 volumes (Section 2.19.2.2) did not result in the precipitation of any additional polysaccharide. Under identical conditions to those used to isolate the ethanol precipitate of EPS from PN184, no precipitate was obtained from the five rough PN184-derived EPS mutants PN1180, PN1181, PN1182, PN1183, and PN1184.

In addition, a cetrимide precipitate of EPS was also obtained from nitrogen-free S20 broth culture supernatants for the *L. pedunculatus* infection mutants PN1018, PN1019, and PN1027 (Chua *et al.*, 1985). The precipitation of EPS with ethanol was not examined.

#### 3.2.9.2 $^1\text{H}$ -NMR Spectroscopy of PN184 EPS

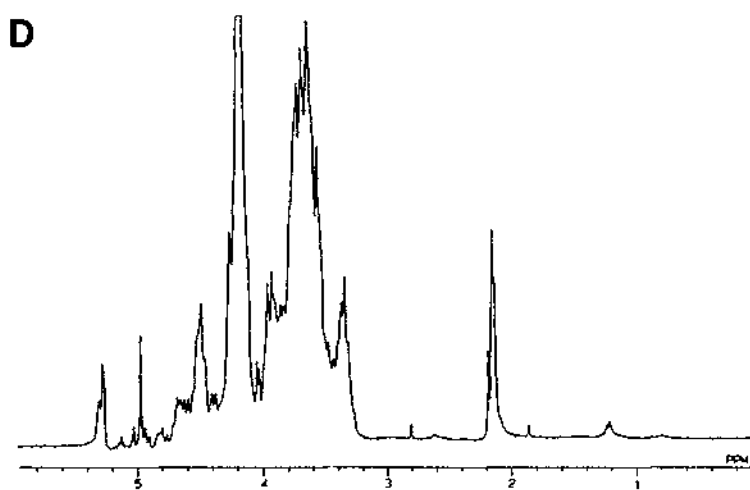
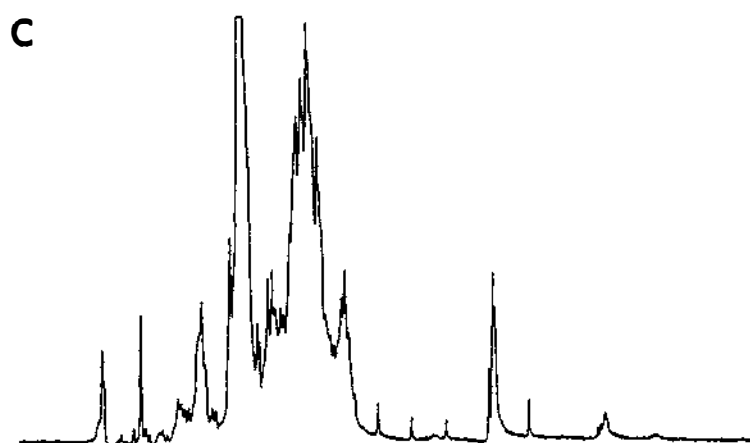
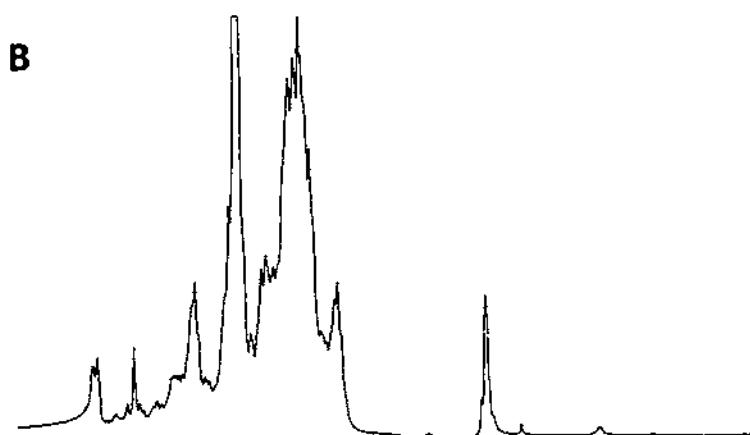
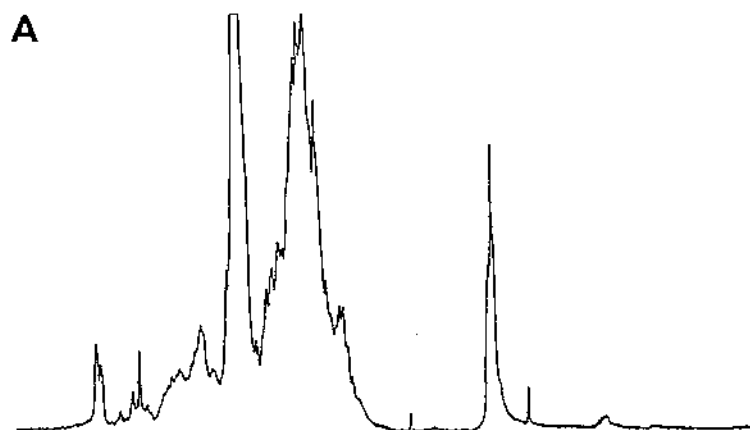
PN184 EPS, cetrимide precipitated (Section 2.19.2.1) from nitrogen-free S20 broth culture supernatants in mid log phase was examined by  $^1\text{H}$ -NMR spectroscopy (Section 2.19.3, Fig. 17A).

While the structure of the EPS from *R. loti* PN184 has not previously been examined in detail (Bailey *et al.*, 1971; Pankhurst *et al.*, 1982), the  $^1\text{H}$ -NMR spectrum of PN184 cetrимide precipitated EPS has been interpreted as follows. An envelope of signals (4.35-4.8 ppm) is present in the  $\beta$ -anomeric proton region (Kotowycz and Lemieux, 1973) together with a prominent doublet (5.3 ppm) in the  $\alpha$ -anomeric proton region (Kotowycz and Lemieux, 1973), suggesting the presence

Figure 17:

$^1\text{H}$ -NMR spectra of EPS from PN184 and the smooth PN184-derived EPS mutants.

10 mg cetrinide precipitated EPS from: A, PN184; B, PN1177; C, PN1178; and D, PN1179.



of both  $\alpha$ - and  $\beta$ -anomeric glycosidic linkages. The envelope of signals between 3.2 and 4.0 ppm results from the ring protons of the glycosyl residues. The multiplet at 2.15 ppm results from the methyl protons of *O*-acetate modifications (Aman *et al.*, 1981; Djordjevic *et al.*, 1986; Hollingsworth *et al.*, 1988; Kuo and Mort, 1986; McNeil *et al.*, 1986). A signal resulting from the methyl protons of pyruvate modifications linked to O-4 and O-6 of a glycosyl residue is frequently present between 1.44 and 1.55 ppm in the spectra of EPS from other *Rhizobium* species investigated (Aman *et al.*, 1981; Djordjevic *et al.*, 1986; Hollingsworth *et al.*, 1988; Kuo and Mort, 1986; McNeil *et al.*, 1986). This signal is not present in the spectrum of PN184 cetrimide precipitated EPS, however, a signal is present at 1.24 ppm. This signal may result from a pyruvate modification linked to O-3 and O-4 of a glycosyl residue (Garegg *et al.*, 1980). However, the presence of a signal at 2.62 ppm suggests that the signals at 1.24 and 2.62 ppm may result from the methyl and methylene protons, respectively, of a 3-hydroxybutanolyl modification (Hollingsworth *et al.*, 1984; Hollingsworth *et al.*, 1988; Kuo and Mort, 1986; McNeil *et al.*, 1986). The methylene protons of succinate modifications result in two triplets at 2.47 and 2.63 ppm in the spectrum of the acidic Calcofluor-binding EPS (EPSI) of *R. meliloti* (Aman *et al.*, 1981). While a doublet (assignable to 3-hydroxy butanoic acid) is present in the spectrum of PN184 cetrimide precipitated EPS at 2.62 ppm, no signal is present at 2.47 ppm, suggesting that PN184 EPS is not succinylated. Signals between 4.8 and 5.2 ppm are consistent with the presence of modified (acetate, 3-hydroxybutanoate) glycosyl residues (Kuo and Mort, 1986). The signal between 0.75 and 0.85 ppm has not been identified.

Comparison, by relative integration, of the magnitude of the signals arising from the ring protons of the glycosyl residues (3.2-4.0 ppm) to the magnitude of the signals arising from the methyl protons of the *O*-acetate residues (2.15 ppm) gave a ratio of 8.1:1.0.

The  $^1\text{H}$ -NMR spectra for PN184 EPS, cetrimide precipitated from nitrogen-free S20 broth and from YM broth (Section 2.19.2.1), and ethanol precipitated from YM broth (Section 2.19.2.2), were compared and were found to be essentially identical (compare, for example Fig. 17A and Fig. 18A).

PN184 EPS, cetrimide precipitated from YM broth culture supernatants (Section 2.19.2.1) harvested from mid log phase (2 days) to late stationary phase (8 days) was examined by  $^1\text{H}$ -NMR spectroscopy. Signal assignments are as described for

the  $^1\text{H}$ -NMR spectrum of PN184 EPS, cetrимide precipitated from nitrogen-free S20 broth culture supernatants (above). While the  $^1\text{H}$ -NMR spectra obtained were essentially identical for all culture ages (Fig. 18A to D), some variation in the glycosyl residue ring proton:*O*-acetate methyl proton ratio, depending on culture age, was observed. This range was from a ratio of 8:1 at 2 days to 6.6:1 at 4 and 6 days and 7.7:1 at 8 days. Hence the variation observed was toward an increase in *O*-acetylation. In addition, the glycosyl residue ring proton:*O*-acetate methyl proton ratios for PN184 EPS samples, cetrимide precipitated from additional, independent mid-late log phase cultures (nitrogen-free S20- and YM-broths) were invariably in this same range.

### 3.2.9.3 $^1\text{H}$ -NMR Spectroscopy of EPS from the PN184-Derived EPS Mutants PN1177, PN1178, and PN1179

The structure of PN1177, PN1178, and PN1179 EPS, cetrимide precipitated (Section 2.19.2.1) from nitrogen-free S20 culture supernatants, harvested at mid log phase, was examined by  $^1\text{H}$ -NMR spectroscopy (Fig 17B, C, and D) and the spectra obtained were compared to the spectrum obtained for PN184 EPS (Fig 17A). Signal assignments were as previously described for the  $^1\text{H}$ -NMR spectrum of PN184 EPS (Section 3.2.9.2, Fig.17A).

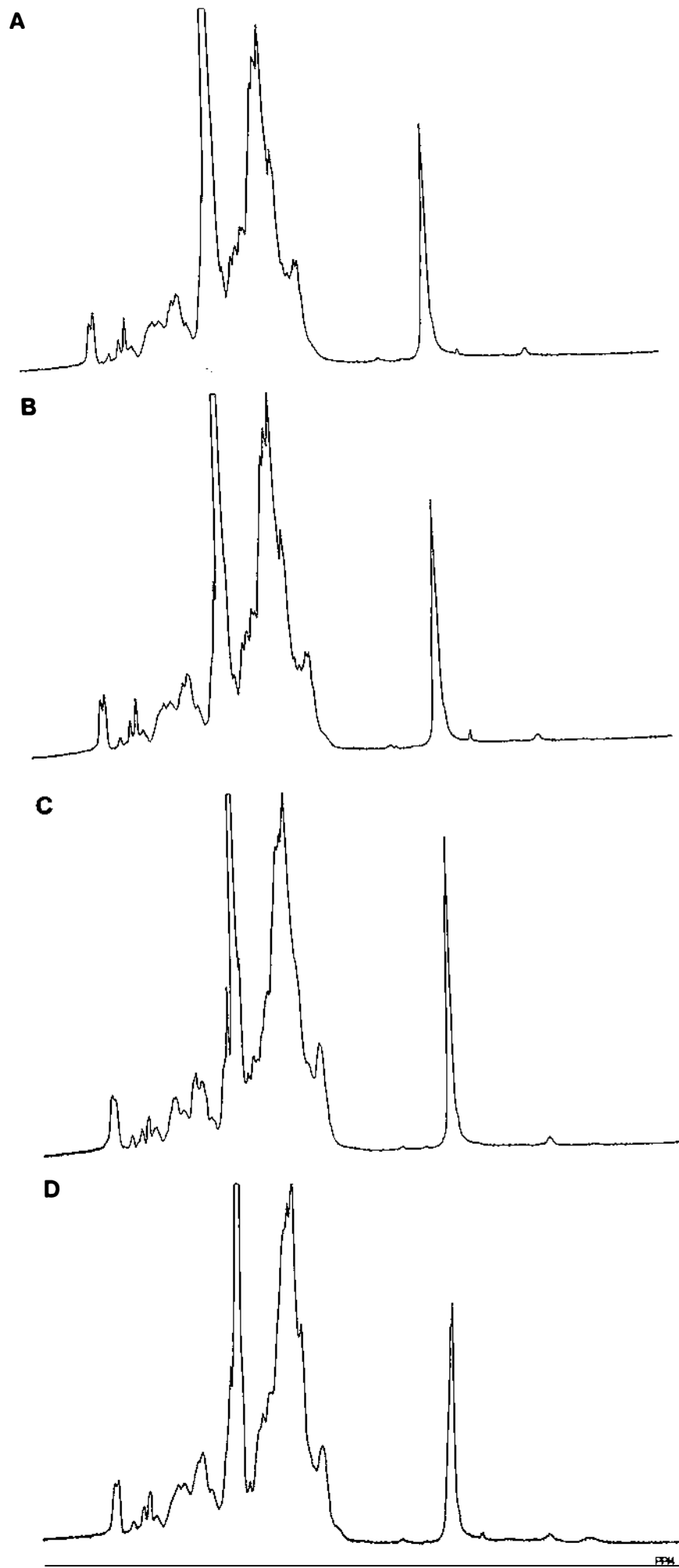
The  $^1\text{H}$ -NMR spectra of cetrимide precipitated EPS from PN1177, PN1178, and PN1179 (Figs. 17B to D) differed in several respects from the  $^1\text{H}$ -NMR spectrum of cetrимide precipitated EPS from PN184 (Fig. 17A). The most prominent difference was in the magnitude of the signals arising from the methyl protons of *O*-acetate modifications (2.15 ppm). These signals were integrated relative to the signals arising from the ring protons of the glycosyl residues (3.2-4.0 ppm). The respective ratios of integrals obtained, glycosyl residue ring protons:*O*-acetate methyl protons, were: PN1177, 19.0:1.0; PN1178, 16.1:1.0; and PN1179, 15.2:1.0.

Additional differences between the  $^1\text{H}$ -NMR spectrum of PN184 EPS and the  $^1\text{H}$ -NMR spectra of EPS from PN1177, PN1178, and PN1179 were also observed, suggestive of identical structural modifications to the EPS produced by each mutant. The magnitude of the signals in the  $\beta$ -anomeric proton region (4.35-4.75 ppm) is consistently altered and, in addition, four newly resolved signals were present between 4.75 and 4.90 ppm. Signals between 3.3 ppm and 3.45 ppm, and signals between 3.9 ppm and 4.0 ppm are more defined than the equivalent regions

Figure 18:

$^1\text{H}$ -NMR spectra of PN184 EPS, isolated from the supernatants of YM broth cultures at different growth stages.

10 mg of cetrinide precipitated EPS from PN184 at culture ages: A, 2 days (mid to late log phase); B, 4 days (late log phase); C, 6 days (early stationary phase); and D, 8 days (late stationary phase).



in the spectrum of PN184 EPS. Additional sharp peaks in the spectra at 1.85, 2.53, 2.81, and 3.08 ppm are unidentified, but probably represent contaminating signals.

#### 3.2.9.4 $^1\text{H}$ -NMR Spectroscopy of EPS from the *L. pedunculatus* Infection Mutants PN1018, PN1019, and PN1027

The structure of PN1018, PN1019, and PN1027 EPS, cetrимide precipitated (Section 2.19.2.1) from YM broth culture supernatants harvested at mid-log phase, was examined by  $^1\text{H}$ -NMR spectroscopy (Figs. 19B, C, and D respectively). The spectra obtained were compared to the spectrum of PN184 EPS (Fig. 19A), cetrимide precipitated from a YM-broth culture supernatant, harvested at mid-log phase. Signal assignments were as previously described for the  $^1\text{H}$ -NMR spectrum of PN184 EPS, cetrимide precipitated from nitrogen-free S20 culture supernatants (Section 3.2.9.2).

The  $^1\text{H}$ -NMR spectrum of PN1018 EPS (Fig. 19B) differs to that of PN184 in the magnitude of the peak arising from the methyl protons of *O*-acetate modifications (2.15 ppm). This signal was integrated relative to the signals arising from the ring protons of the glycosyl residues (3.2-4.0 ppm), to give a ratio of 9.8:1.0 (ring protons:*O*-acetate region), beyond the highest relative integral recorded for PN184 EPS (8.1:1.0), but less than the ratios observed for EPS isolated from the smooth PN184-derived EPS mutants (Section 3.2.9.3). Otherwise, the  $^1\text{H}$ -NMR spectrum of PN1018 EPS closely resembles the  $^1\text{H}$ -NMR spectrum of PN184 EPS. The  $^1\text{H}$ -NMR spectra of PN1019 EPS and PN1027 EPS closely resemble the  $^1\text{H}$ -NMR spectrum of PN184 EPS. The ratios of the integrals (glycosyl residue ring protons:*O*-acetate methyl protons) for PN1019 and PN1027 were, respectively 8.2:1.0, and 6.8:1.0, close to and within (respectively) the range observed for PN184 EPS (Section 3.2.9.2).

The increased magnitude of the signals in the spectrum of PN1027 EPS (Fig. 19D) at 1.25 ppm and the presence of a broad peak at both 0.85 ppm and 3.15 ppm derive from a small amount of cetrимide remaining in the EPS sample after acetone precipitation (see Section 2.19.2.1).

#### 3.2.9.5 Analysis of PN184 EPS by Paper Chromatography

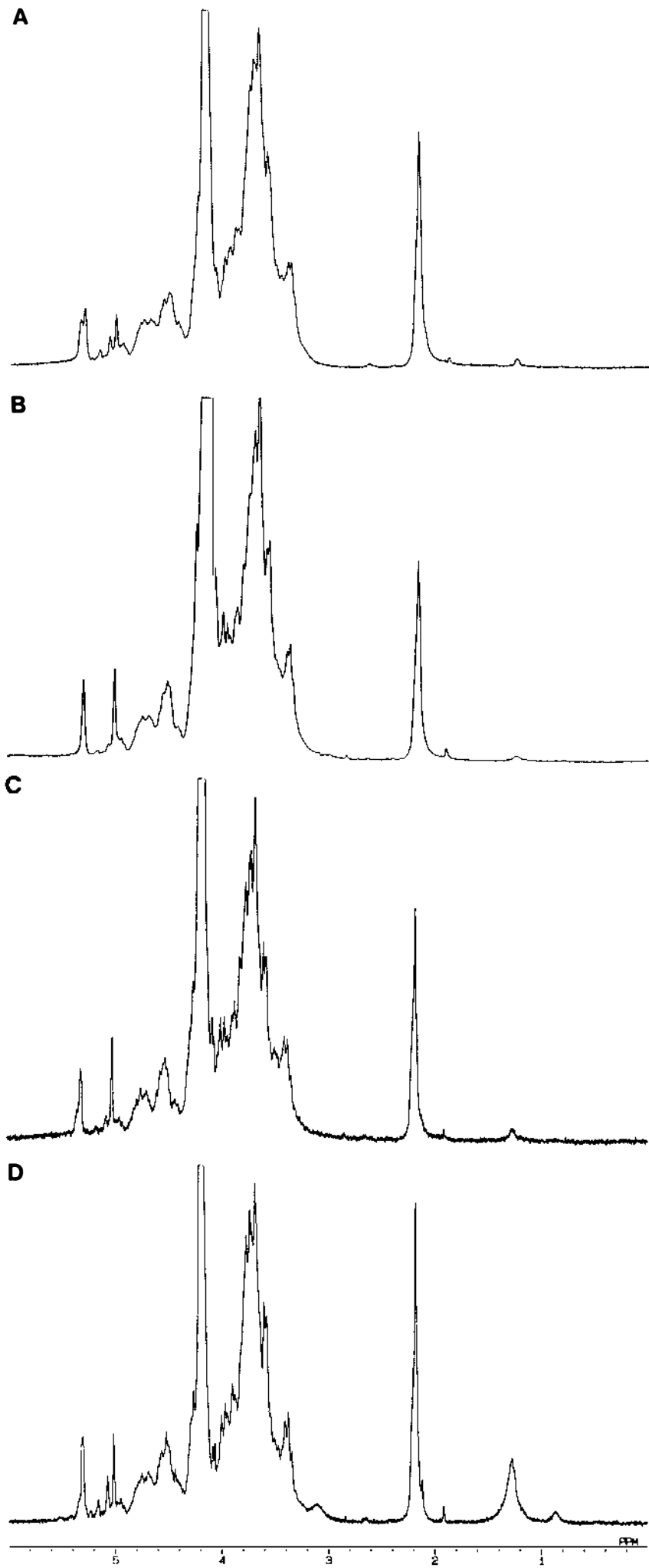
Bailey *et al.* (1971) and Pankhurst *et al.* (1982) have previously identified glucose,



Figure 19:

$^1\text{H}$ -NMR spectra of EPS from PN184 and the PN184-derived *L. pedunculatus* infection mutants PN1018, PN1019 and PN1027.

10 mg of cetrimide precipitated EPS from: A, PN184; B, PN1018; C, PN1019; and D, PN1027.



galactose and uronic acid as constituents of PN184 EPS. Here the constituent sugars of PN184 EPS were examined by paper chromatography.

Acid hydrolysates (Section 2.19.4) of PN184 EPS, cetrимide precipitated from nitrogen-free S20 broth supernatants at mid-log phase (Section 2.19.2.1), were examined by paper chromatography (Section 2.19.4). Figures 20A and 20B show chromatography of PN184 acid hydrolysed EPS, together with selected sugar standards, using the butanol:pyridine:water solvent system (Section 2.19.4). Visible are an intense yellow-brown spot ( $R_{\text{Glu}}$  1.02), fluorescing under long wave UV light, comigrating with glucose ( $R_{\text{Glu}}$  1.0), a less intense yellow-brown spot ( $R_{\text{Glu}}$  0.93), fluorescing under long wave UV light, comigrating with galactose ( $R_{\text{Glu}}$  0.92), and a weak red spot ( $R_{\text{Glu}}$  1.21), not fluorescing under UV light, migrating close to ribose ( $R_{\text{Glu}}$  1.22). A weak yellow-brown spot ( $R_{\text{Glu}}$  1.69), fluorescing under UV light, migrating close to glucuronic acid ( $R_{\text{Glu}}$  1.73) has also been observed on chromatograms, loaded with a greater concentration of hydrolysed EPS, and run using the butanol:acetic acid:water solvent system (Section 2.19.4). An equivalent spot was not readily visible at the loading used for the chromatogram shown in Figs. 20A and B (which does not include a glucuronic acid standard). Several spots with mobilities lower than galactose were also observed and were probably either oligosaccharides resulting from some incompletely hydrolysed EPS remaining in the sample, and/or oligosaccharides generated during hydrolysis. The same pattern of spots was observed for EPS cetrимide precipitated from YM broth supernatants (Section 2.19.2.1).

### **3.2.9.6 Analysis of Cetrимide Precipitated EPS for Fluorescence on Calcofluor Agar**

To determine if Calcofluor fluorescence was associated with PN184 EPS, 2 mg of PN184 EPS and 2 mg PN1177 EPS, both samples cetrимide precipitated from nitrogen-free S20 broth supernatants harvested at mid log phase (Section 2.19.2.1), were dissolved in sterile MilliQ water to give a highly viscous solution. The viscous solutions were spotted onto a TY Calcofluor agar plate (Sections 2.5, 2.5.2, and 2.19.1) and examined over several days. The PN184 EPS sample was found to fluoresce (Fig. 21), while the PN1177 EPS sample showed no fluorescence (Fig. 21). In addition, the PN184 EPS sample initially fluoresced a blue-green colour, but after several days fluoresced blue-white. Similarly, both PN1178 EPS and PN1179 EPS also failed to fluoresce.

Figure 20A:

Paper chromatography of PN184 EPS.

Ascending chromatography of acid-hydrolysed, cetrinide precipitated, PN184 EPS on Whatman #1 Chromatography paper using the 1-butanol:pyridine:water (6:4:3) solvent system. Lanes: 1, PN184 EPS; 2, Glucose; 3, Galactose; 4, Rhamnose; 5, Fucose; 6, PN184 EPS; 7, Arabinose; 8, Xylose; 9, Ribose; 10, PN184 EPS.

Figure 20B:

Fluorescence of sugars upon exposure to long wave UV light.

Chromatogram described in Fig. 20A irradiated with long wave UV light.

A



B

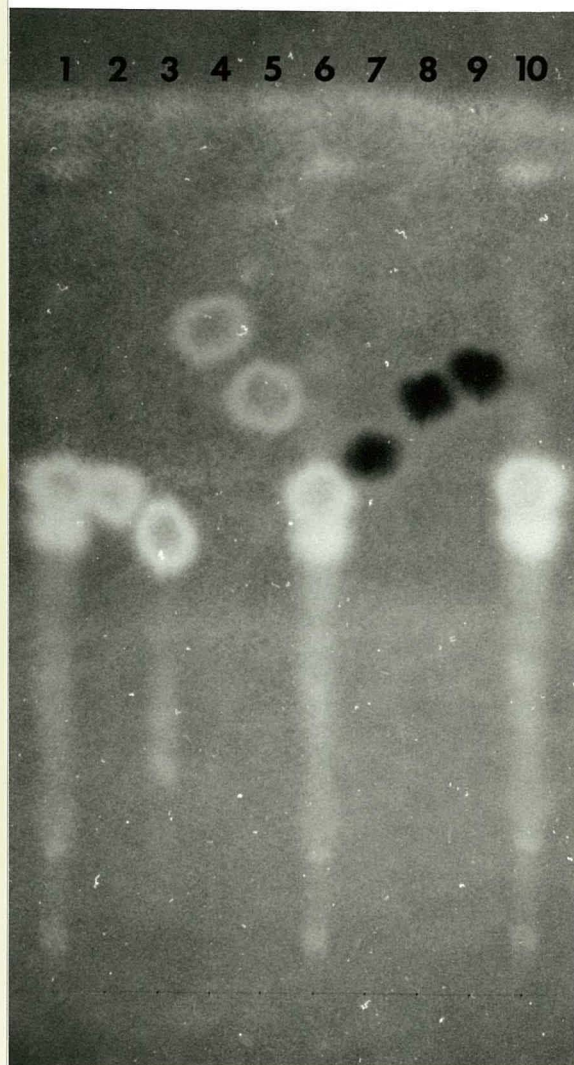
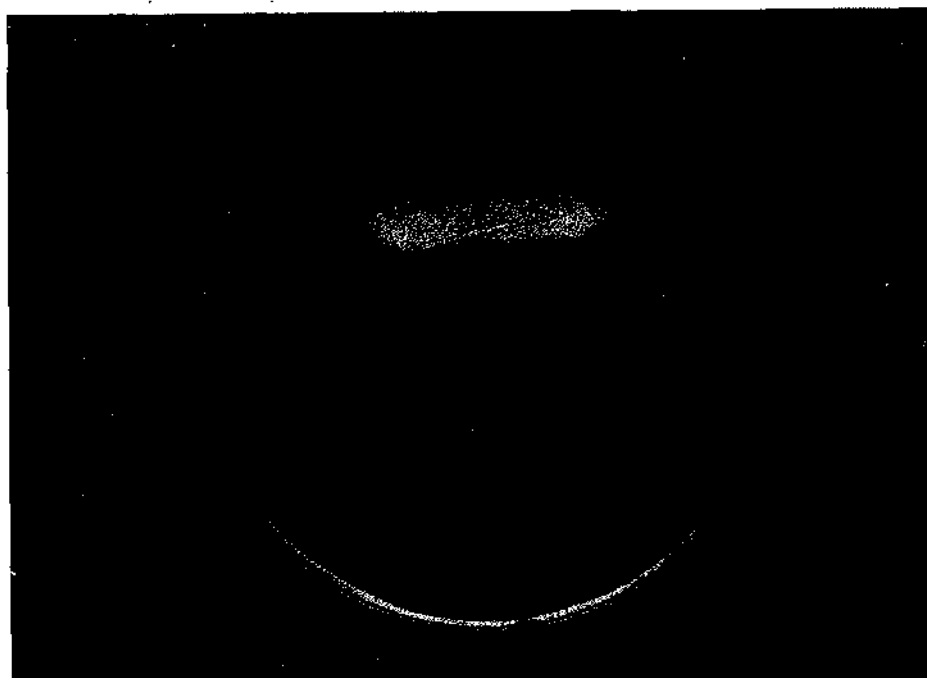


Figure 21:

Calcofluor-induced fluorescence of PN184 EPS and non-fluorescence of PN1177 EPS.

Cetrimide precipitated PN184 EPS (top) and PN1177 EPS (bottom) on TY-Calcofluor agar. Fluorescence is observed upon irradiation with long wave UV light.



### 3.2.9.7 Column Fractionation of Cetrimide Precipitated EPS

Cetrimide precipitated EPS samples from YM broth supernatants (Section 2.19.2.1) of PN184 and PN1177 were fractionated through DEAE-Sephacel (exclusion limit approximately  $1 \times 10^6$  Da) using a linear sodium chloride gradient (Sections 2.19.5 and 2.19.6).

#### 3.2.9.7.1 Column Fractionation of PN184 EPS

Column fractionation of 10 mg of PN184 EPS was found to yield two anthrone reactive, polysaccharide fractions, a major fraction with a peak elution at 330 mM sodium chloride and a minor fraction eluting in 1 mM sodium chloride (Fig. 22). Approximately 82% of the material loaded onto the column shown in Figure 22 eluted. These results suggest the presence of either high (1 mM NaCl fraction) and low (330 mM NaCl fraction) molecular weight fractions of the same EPS, or the presence of acidic (330 mM NaCl fraction) and neutral (1 mM NaCl fraction) EPS fractions. The peak ratios (330 mM NaCl fraction:1 mM NaCl fraction) were 8.2:1.0 (fractions 46-61:fractions 6-13). The material eluting in fractions 6 to 13 (1 mM NaCl fraction), and the material eluting in fractions 49 to 57 (330 mM NaCl fraction) were pooled separately, dialysed extensively against MilliQ water, then lyophilized.

The lyophilized fractions were examined by  $^1\text{H}$ -NMR spectroscopy (Fig. 23) with the two fractions producing essentially identical  $^1\text{H}$ -NMR spectra. This suggests that the minor (eluting in 1 mM NaCl) and major (with a peak elution at 330 mM NaCl) fractions represent high and low molecular weight fractions, respectively, of the same EPS, rather than fractions arising from the elution of separate neutral and acidic exopolysaccharides.

1 mg of each lyophilized fraction (minor and major peak), together with 1 mg of the unfractionated material were dissolved in 50  $\mu\text{l}$  sterile MilliQ water and spotted onto a TY-Calcofluor agar plate (Sections 2.5, 2.5.2, and 2.19.1). Both fractions fluoresced, but with a reduced intensity compared to the intensity of fluorescence of the unfractionated material.

In addition, it was found that the minor (high molecular weight) EPS fraction, when dissolved in either MilliQ water, or 1 mM sodium chloride, was considerably



Figure 22:

DEAE-Sephacel column fractionation of PN184 EPS.

- Glucose equivalents
- NaCl gradient

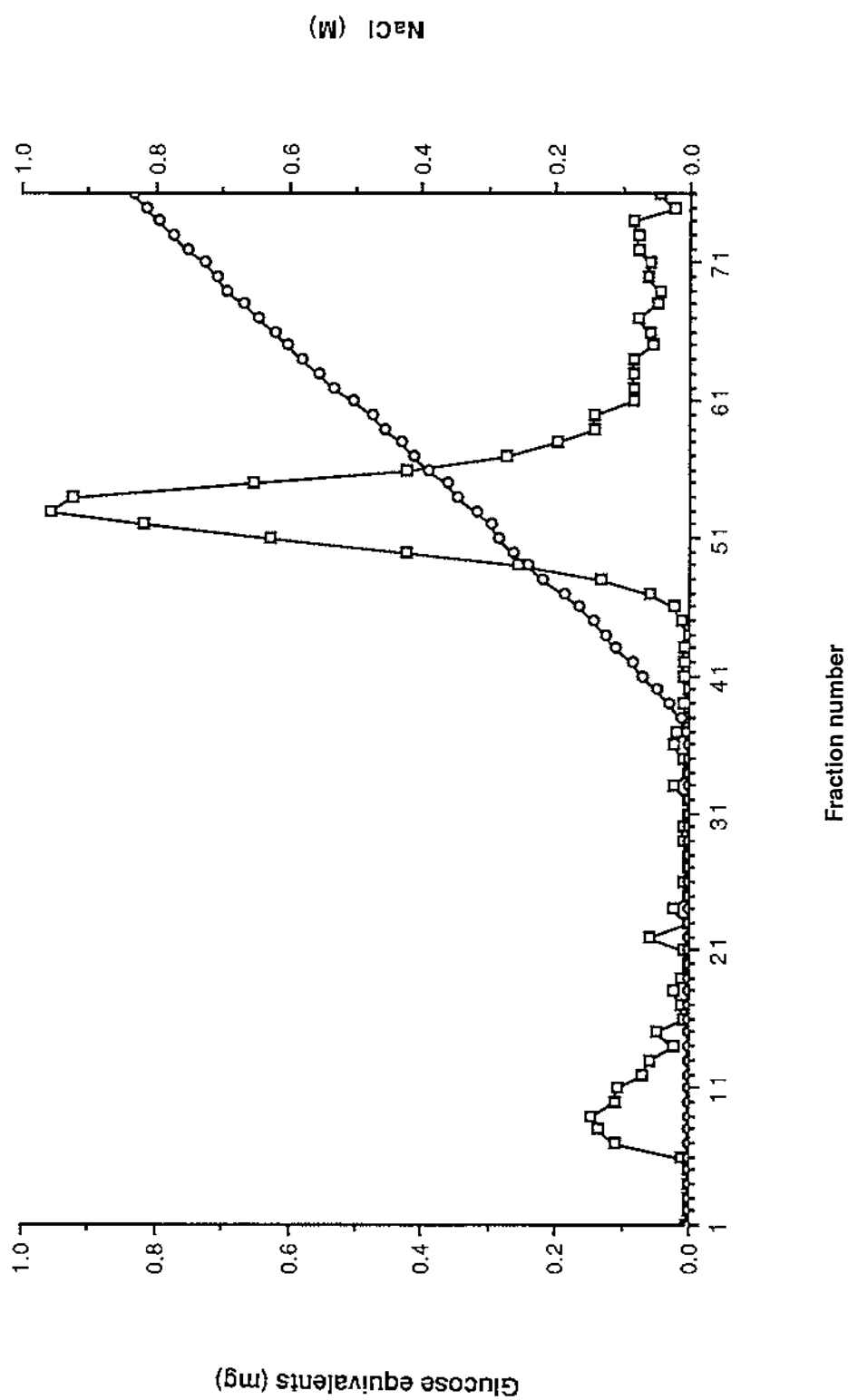


Figure 23:

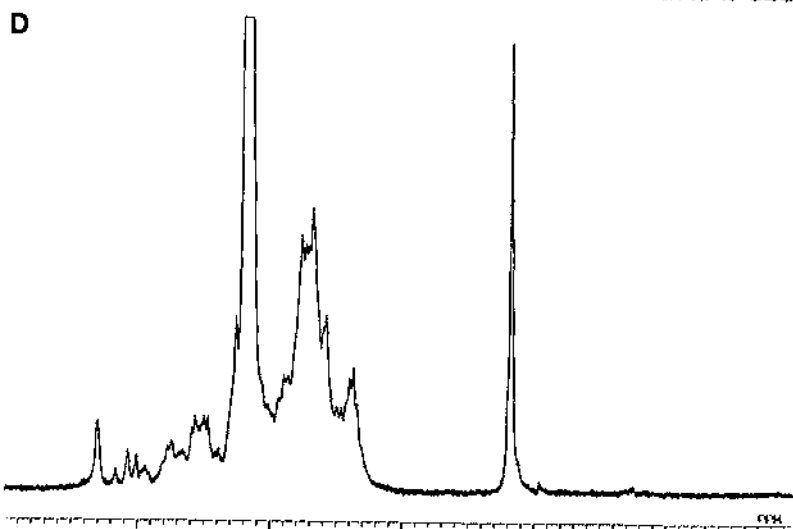
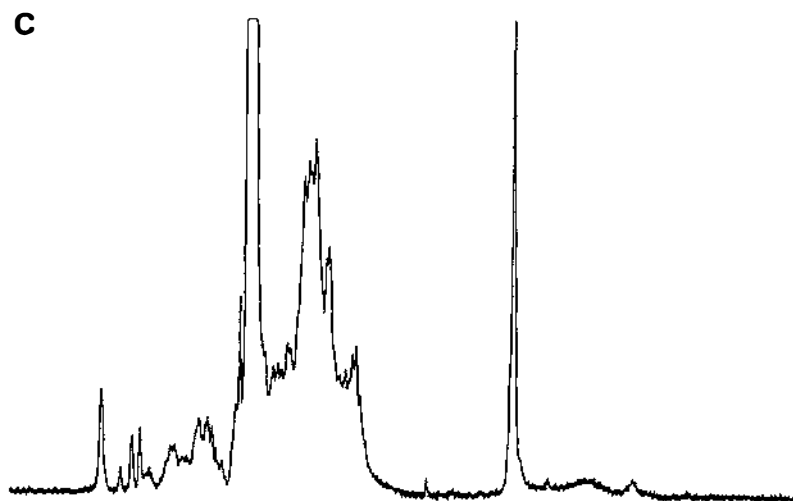
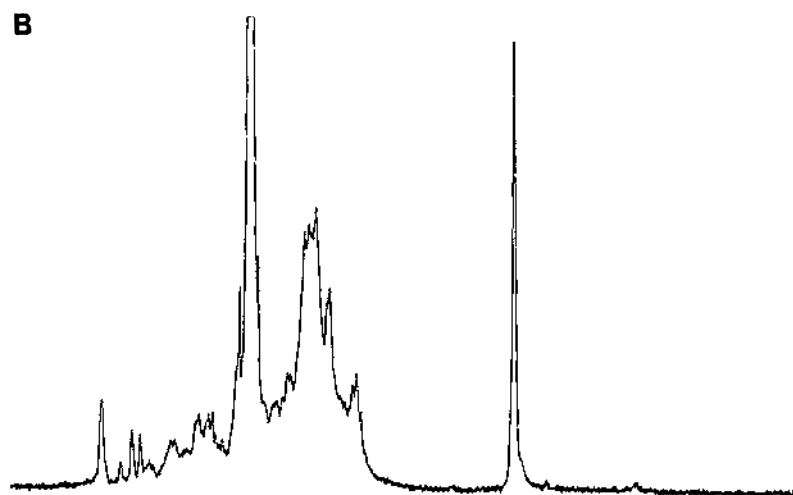
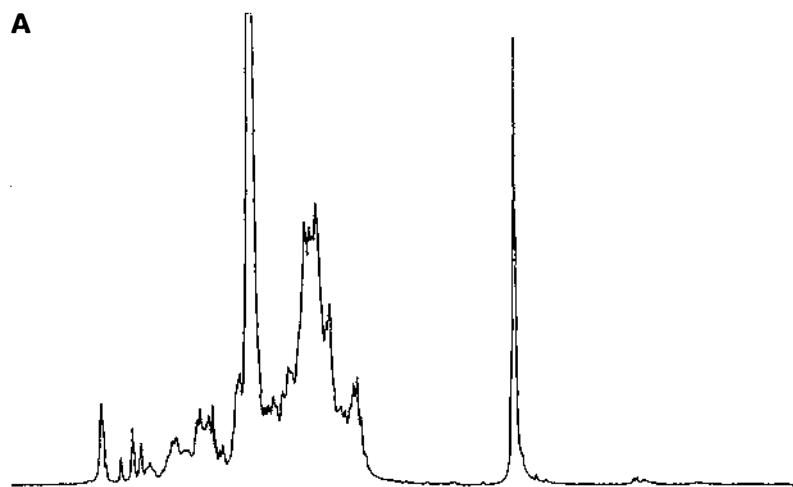
$^1\text{H}$ -NMR spectra of high and low molecular weight PN184 EPS fractions separated by DEAE-Sephacel column chromatography.

A) 10 mg PN184 EPS from the sample applied to the DEAE-Sephacel column shown in Fig. 22;

B) 2 mg PN184 EPS from the sample applied to the DEAE-Sephacel column shown in Fig. 22;

C) 2 mg EPS from the dialysed, lyophilized fraction eluting in 1 mM sodium chloride (high molecular weight) in Fig. 22;

D) 2 mg EPS from the dialysed, lyophilized fraction with a peak elution at 330 mM sodium chloride (low molecular weight) in Fig. 22.



more viscous than the major (low molecular weight) EPS fraction.

### 3.2.9.7.2 Column Fractionation of PN1177 EPS

PN1177 EPS was considerably more viscous than PN184 EPS when dissolved in 1 mM sodium chloride. Due to the increased viscosity observed, only 3-5 mg of cetrimide precipitated material could be loaded onto each column, compared to 10 mg for PN184 EPS. The column shown in Figure 24 was loaded with 3 mg of PN1177 EPS.

Column fractionation of PN1177 cetrimide precipitated EPS was found to yield a major fraction with a peak elution at 200 mM sodium chloride and a small fraction eluting in 1 mM sodium chloride (Fig. 24). Approximately 62% of the material loaded onto the column shown in Figure 24 eluted. The peak ratios (200 mM NaCl fraction:1 mM NaCl fraction) were 11.8:1.0 (fractions 40-48:fractions 3-7) or 17.4:1.0 (fractions 40-54:fractions 3-7), compared to 8.2:1.0 for the equivalent peaks for PN184 EPS (Section 3.2.9.7.1). PN1177 may, therefore, produce only a small amount of high molecular weight EPS (eluting in 1 mM NaCl) compared to PN184.

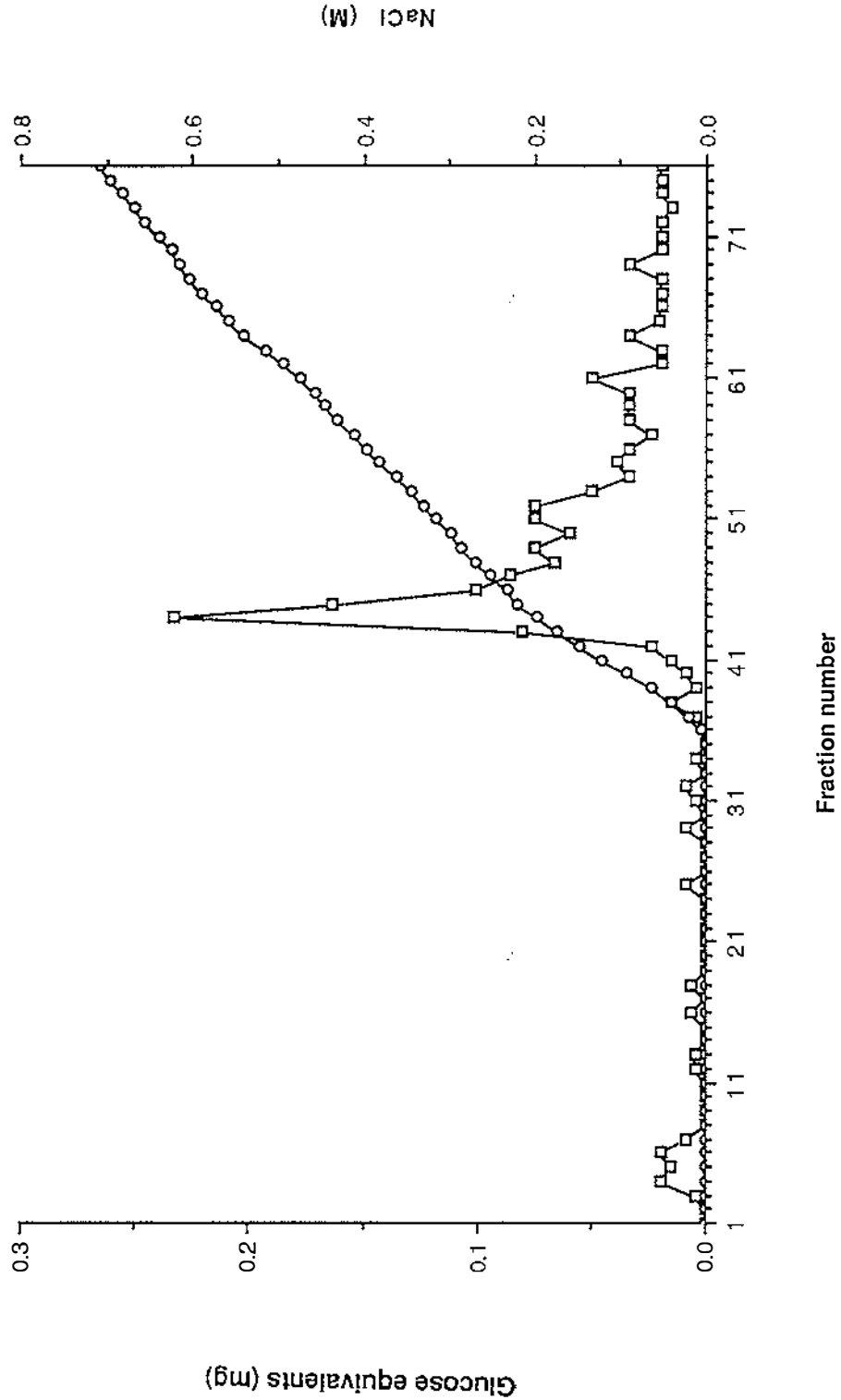
The material eluting in fractions 3 to 5 (1 mM NaCl fraction), and in fractions 42 to 46 (200 mM NaCl fraction) was pooled separately, dialysed extensively against MilliQ water, then lyophilized. The lyophilized fraction containing the major peak (200 mM NaCl fraction) was examined by  $^1\text{H}$ -NMR spectroscopy. The  $^1\text{H}$ -NMR spectrum obtained was essentially identical to that obtained for unfractionated PN1177 EPS (not shown). There was insufficient lyophilized material to examine the pooled minor fraction (1 mM NaCl) by  $^1\text{H}$ -NMR spectroscopy.

During the course of these experiments it was observed that dissolving PN1177 EPS in sodium chloride of higher ionic strength (50 mM NaCl and above) resulted in a dramatic decrease in the viscosity of the EPS solution. Equivalent experiments with PN184 EPS did not result in a noticeable decrease in viscosity. In addition, overnight "incubation", at room temperature, of PN1177 EPS in sodium chloride resulted in increasing gelation of the EPS with increasing ionic strength. Gelation was not observed in equivalent experiments with PN184 EPS. Analysis of PN1177 EPS by  $^1\text{H}$ -NMR spectroscopy has shown that PN1177 EPS carries fewer *O*-acetate modifications than PN184 EPS (Sections 3.2.9.2 and 3.2.9.3). Atkins

Figure 24:

DEAE-Sephacel column chromatography of PN1177 EPS.

- Glucose equivalents
- NaCl gradient



(1986) has similarly reported gelation, in the presence of inorganic salts, of deacetylated *Klebsiella aerogenes* serotype K54 capsular polysaccharide.

### 3.2.10 Lipopolysaccharide Analysis

Lipopolysaccharide was isolated from PN184 and the PN184-derived EPS mutants (Section 2.20.1) and analysed by SDS-PAGE (Section 2.20.2).

SDS-PAGE of hot phenol-water extracted LPS from PN184 and the PN184 derived EPS mutants resulted in the separation of the LPS into a ladderlike pattern of doublet bands of increasing molecular weight. Both bands of each doublet are sensitive to periodate oxidation (Section 2.20.2.1 and 2.20.2.2), and stain brown to yellow-brown with PA-silver (Section 2.20.2.1, Fig. 25A) depending on the staining intensity. PA-Schiff staining (2.20.2.2, Fig. 25B) did not resolve the doublets, and stained the fastest migrating (doublet) band visible after PA-silver staining (Fig. 25A, arrowed) only poorly (Fig. 25B, arrowed). The observation that the upper band of each doublet stains more intensely with PA-silver suggests that, either this band contains a larger quantity of LPS, or that this band contains LPS equivalent in amount to that in the lower band, but with more groups sensitive to the staining reaction.

The fastest migrating doublet of bands (of lowest molecular weight) migrates immediately behind the dye front, and presumably represents either the LPS core (lipid A and core oligosaccharide) lacking O-antigen, or represents the LPS core carrying minimal O-antigen.

Migrating behind this doublet are a series of five or more doublet bands of increasing molecular weight and reducing intensity of staining. These doublet bands are separated by a large molecular weight interval (averaging approximately 5 kDa based upon comparative mobility of protein molecular weight markers), while the bands of each doublet are separated by a comparatively small molecular weight interval (averaging less than 1 kDa based upon comparative mobility of protein molecular weight markers). Protein molecular weight markers are either not stained, or stained only very weakly by the PA-silver staining method described in Section 2.20.2.1. The gel shown in Fig. 25B was double stained, first with PA-Schiff (Section 2.20.2.2, which does not reveal the protein molecular weight markers), and then with Coomassie brilliant blue (Section 2.22.1) to reveal



Figure 25A:

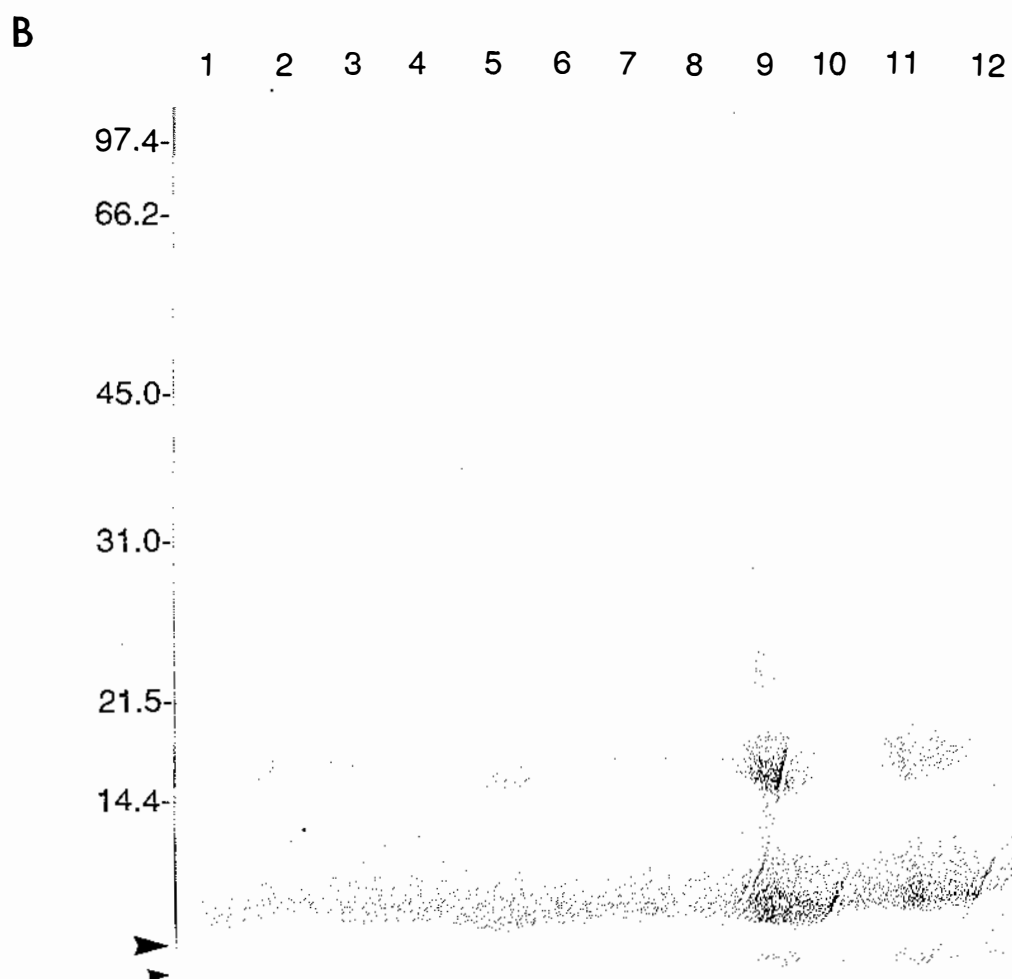
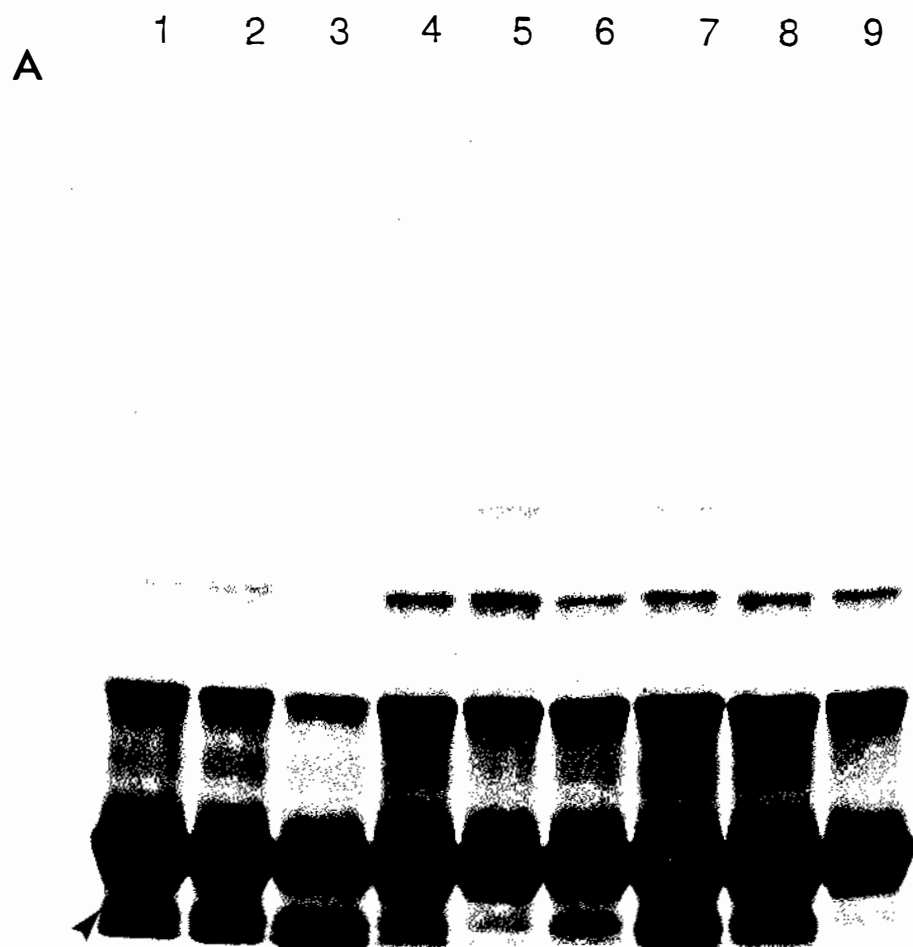
SDS-PAGE profiles of LPS from PN184, and the PN184-derived EPS mutants.

SDS-PAGE (0.1% SDS, 15% acrylamide, PA-silver stain) of hot phenol-water extracted LPS from lane: 1, PN184; 2, PN1177; 3, PN1178; 4, PN1179; 5, PN1180; 6, PN1181; 7, PN1184; 8, PN1182; 9, PN1183. The fastest migrating doublet of bands is arrowed.

Figure 25B:

SDS-PAGE profiles of LPS from PN184, NZP2213, the PN184-derived EPS mutants, and PN184-derived *L. pedunculatus* infection mutants PN1018 and PN1027.

SDS-PAGE (0.1% SDS, 12.5% acrylamide, PA-Schiff stain) of hot phenol-water extracted LPS from lane: 1, NZP2213; 2, PN184; 3, PN1177; 4, PN1178; 5, PN1179; 6, PN1180; 7, PN1181; 8, PN1182; 9, PN1183; 10, PN1184; 11, PN1018; 12, PN1027. The position of protein molecular weight markers ( $M_r$  shown in kD) is given for comparison of relative migration. Bold arrow (►), the fastest migrating pink-staining bands. Light arrow (▶), blue staining bands.



the protein molecular weight markers.

KDO determination (Section 2.20.1) invariably resulted in very low OD<sub>548</sub> values, typically 0.05 to 0.08 for 2 mg starting material. This equates to approximately 0.04% to 0.07% KDO. This may reflect a large amount of O-polysaccharide (antigen) or may reflect the crude nature of the starting material.

No substantial differences, as defined by SDS-PAGE, were detected between the LPS produced by PN184 and the LPS produced by the PN184-derived EPS mutants. All doublet bands present for PN184 (lane 1) were also present for the PN184-derived EPS mutants, although differences in the staining intensity of specific bands was observed, in particular the fastest migrating doublet of bands.

During the course of this work LPS, isolated from the PN184-derived infection mutants PN1018 and PN1027 (Chua *et al.*, 1985) and from NZP2213, was also examined (Fig. 25B). No substantial differences, as defined by SDS-PAGE, were detected between the LPS produced by PN184 and the LPS produced by PN1018 and PN1027. The percentage KDO values recorded (0.04% to 0.09%, determined as described in Section 2.20.1) were similar to those recorded for LPS from PN184 and the PN184-derived EPS mutants. In contrast, all the NZP2213 LPS bands migrated more rapidly than the equivalent bands in the lanes containing LPS from any of the other *R. loti* strains examined (Fig. 25B). This suggests that NZP2213 LPS differs in structure to the LPS produced by PN184, and the PN184-derived mutants.

In addition to the pink-staining bands present on LPS gels stained with PA-Schiff (Section 2.20.2.2), blue bands (Fig. 25B, arrowed), visible after Coomassie Brilliant Blue staining (2.22.1), were also observed at the dye front. These blue staining bands were present in all lanes, but were reduced in the lane containing LPS from NZP2213. The origin of these bands is unknown.

### 3.2.11 Examination of Capsular Polysaccharide for Calcofluor Fluorescence

Capsular polysaccharide was isolated from PN184 as described in Section 2.21. 15 mg of lyophilised material was isolated. 2 mg of this material was dissolved in 50 µl MilliQ water and spotted onto a TY-Calcofluor agar plate. No fluorescence was observed.

### 3.2.12 Nodulation Tests

To investigate the symbiotic effect of the EPS mutations, nodulation tests (Section 2.4) were performed on both *Lotus pedunculatus*, a determinate nodulating legume, and *Leucaena leucocephala*, an indeterminate nodulating legume. The results reported derive from either two independent trials (*L. pedunculatus* tests) or three independent trials (*L. leucocephala* tests).

The root nodules formed on *L. pedunculatus* plants, examined six weeks after inoculation with PN184, were found to be fully effective (Nod<sup>+</sup>Fix<sup>+</sup>), with 6-10 spherical, pink-brown nodules, 1.5-2.0 mm in diameter present on the roots of each plant. Control plants inoculated with NZP2213 formed small, white-brown, ineffective (Nod<sup>+</sup>Fix<sup>-</sup>), nodular swellings as expected. Uninoculated plants were uniformly Nod<sup>-</sup>. All the PN184-derived EPS mutants induced the formation of fully effective (Nod<sup>+</sup>Fix<sup>+</sup>) nodules, which were indistinguishable from those formed after inoculation with PN184, and at the same frequency per plant. Plants inoculated with the PN184-derived EPS mutants grew to the same size as those inoculated with PN184 and showed no signs of nitrogen deficiency.

The nodules induced by the PN184-derived EPS mutants were crushed and bacteria were isolated as described in Section 2.4.3. 20-30 colonies arising from bacteria isolated from each nodule were single colony purified. All bacteria isolated from the nodules retained the appropriate mutant phenotype (Calcofluor-dark, neomycin resistant, mucoid/non-mucoid as appropriate).

*L. leucocephala* plants examined eight to twelve weeks after inoculation with PN184 were found to bear fully effective (Nod<sup>+</sup>Fix<sup>+</sup>) nodules, with two to five cylindrical, brown nodules, 2.0 to 3.0 mm in length present on the roots of each plant. Examination of nodules by light and electron (Fig. 26) microscopy (Section 2.4.4.1) demonstrated that invasion had occurred and that bacteroids were present within each nodule. Tannin (flavolan) deposits were restricted to the periphery of each nodule. Hand sections of nodules were stained with Calcofluor and acridine orange and examined by epifluorescence microscopy (Section 2.4.4.2). While plant cell walls and nuclei were stained as expected bacteroid infected plant cells were also found to fluoresce intensely (Fig 27A and 27B). The nodules formed on control plants inoculated with NZP2213 were ineffective (Nod<sup>+</sup>Fix<sup>-</sup>), with either very small nodular swellings, or large tumor-like structures being formed.

Figure 26:

Electron micrograph of bacteroids in an infected cell of a *L. leucocephala* nodule, after inoculation with PN184.

The bar represents 1  $\mu\text{m}$ . Abbreviations: B, bacteroid.

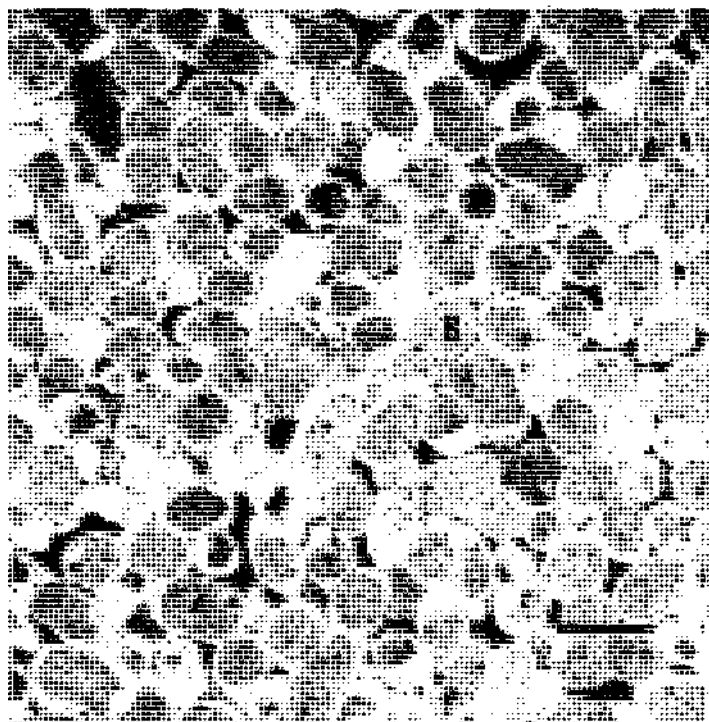
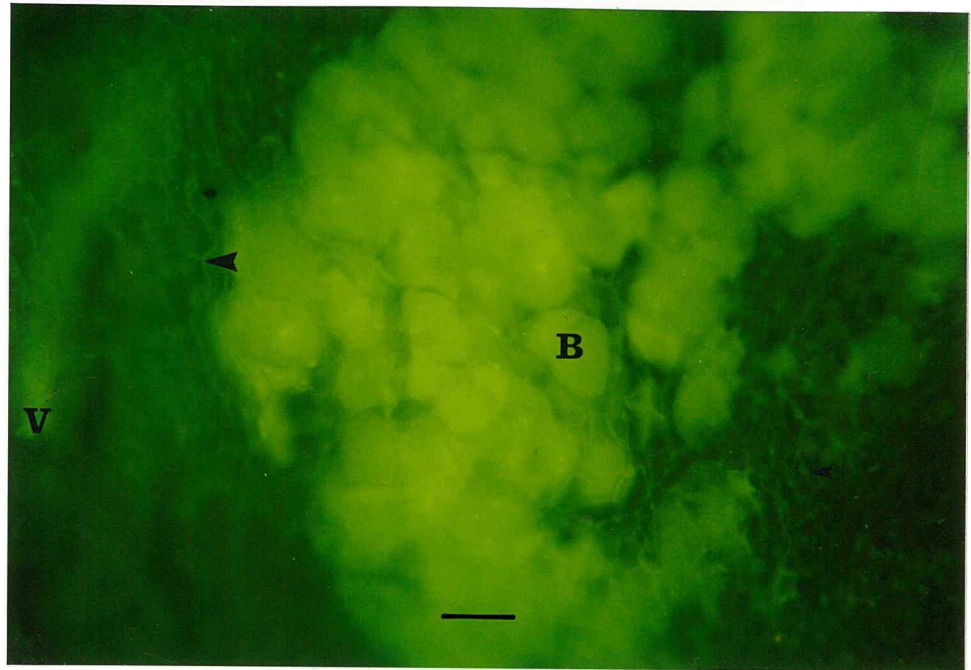


Figure 27A and B:

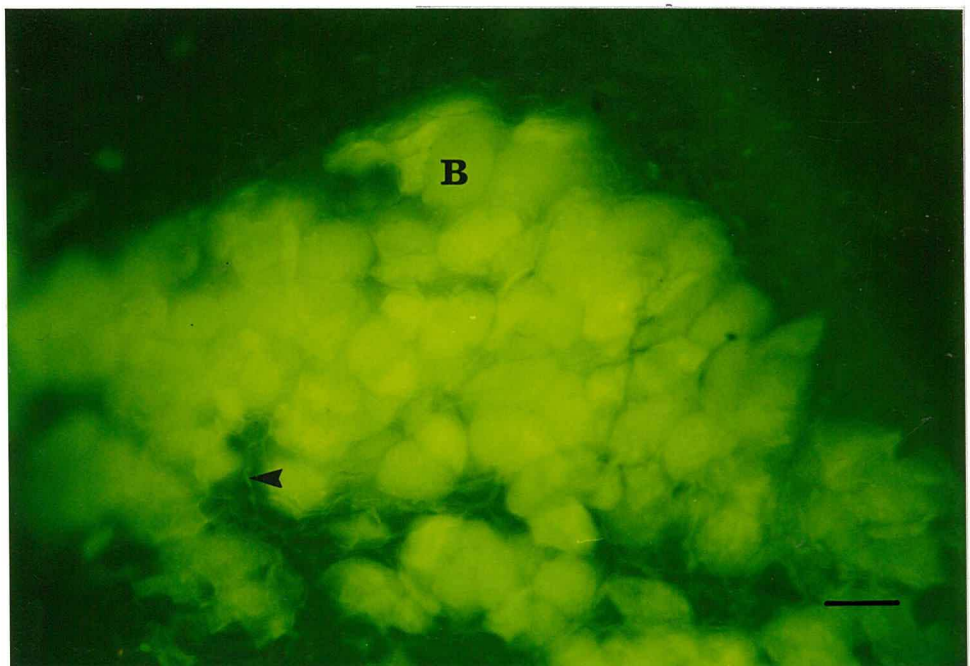
Fluorescence of *L. leucocephala* cells containing PN184 bacteroids in Calcofluor-acridine orange stained nodule sections examined by epifluorescence illumination.

The bar represents 100  $\mu\text{m}$ . Abbreviations: B, bacteroid filled plant cells; ►, plant cell wall; V, vascular tissue; ►, plant cell nucleus.

A



B





Uninoculated plants were uniformly Nod<sup>-</sup>. The small, white-brown, spherical, nodular swellings formed on the roots of plants inoculated with PN184-derived EPS mutant PN1182 were found to be Fix<sup>-</sup>. In addition, one plant of those examined was Nod<sup>-</sup>. The small, white-brown, spherical, nodular swellings formed on the roots of plants inoculated with the remaining PN184-derived EPS mutants (PN1177, PN1178, PN1179, PN1180, PN1181, PN1183, and PN1184) were found to be Fix<sup>-</sup>. Figure 28 shows nodular swellings, formed after inoculation of *L. leucocephala* with PN1183, typical of those formed by all the PN184-derived EPS mutants. Examination, by light and electron microscopy, of the nodular swellings formed on plants inoculated with the PN184-derived EPS mutants, demonstrated that invasion had not occurred, and that the nodular swellings formed contained dense tannin (flavolan) deposits throughout the structure (Fig. 29). In addition, tannin deposits are also visible in the outer wall (or intercellular spaces), and occasionally the inner wall (or intercellular spaces), of cells at, or near, the nodule surface (Fig. 29), which are likely to have come into contact with the inoculated bacteria.

The nodules induced by the PN184-derived EPS mutants were crushed and the bacteria were isolated as described in Section 2.4.3. Bacteria were, however, obtained only very rarely and presumably represent bacteria, which were present in the intercellular spaces of the outer cell layers of the nodule, that were protected during surface sterilization. Nevertheless, all bacteria which were isolated from the nodules retained the appropriate mutant phenotype (Calcofluor-dark, mucoid/non-mucoid as appropriate, and neomycin resistant).

When the rough PN184-derived EPS mutants carrying their respective complementing cosmids were inoculated onto *L. leucocephala*, wild type nodulation and nitrogenase activity, as defined by acetylene reduction (Section 2.4.1), was restored. Figure 30 shows a fully effective nodule formed on a plant inoculated with PN1183, carrying its complementing cosmid pPN34, which is typical of the fully effective nodules formed on plants inoculated with both PN184, and the remaining PN184-derived EPS mutants carrying their complementing cosmids. Examination of these effective nodules by light microscopy revealed that tannin (flavolan) deposits were infrequent and restricted to cells near the periphery of each nodule (Fig. 31). Similarly, tannin deposits were only rarely observed in the walls (or intercellular spaces) of cells at the nodule surface (Fig. 31). Examination of these effective nodules by electron microscopy demonstrated that

Figure 28:

Ineffective nodular swellings formed on *L. leucocephala* after inoculation with the PN184-derived EPS mutant, PN1183.

The bar represents 500  $\mu\text{m}$ .

Figure 29:

Light micrograph of a transverse section through a nodule formed after inoculation of *L. leucocephala* with the PN184-derived EPS mutant, PN1183.

The bar represents 100  $\mu\text{m}$ . Abbreviations: T, tannin filled plant cells; V, vascular trace; ►, tannin deposits in the cell walls (or intercellular spaces) of cells at the nodule surface.

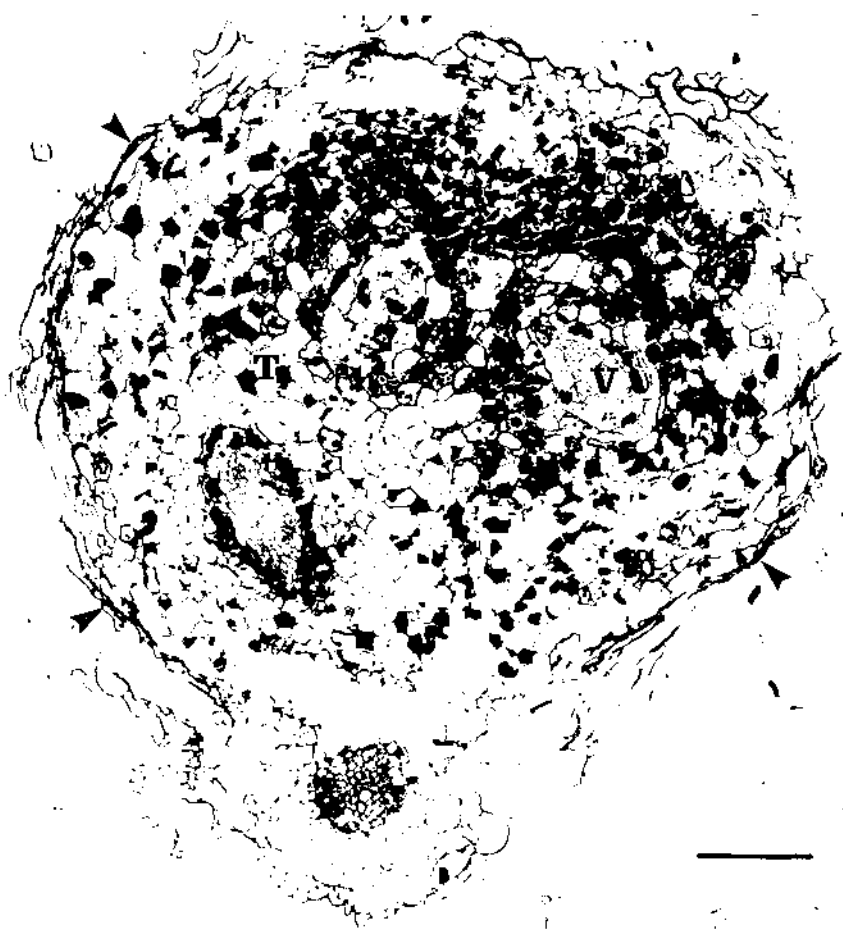


Figure 30:

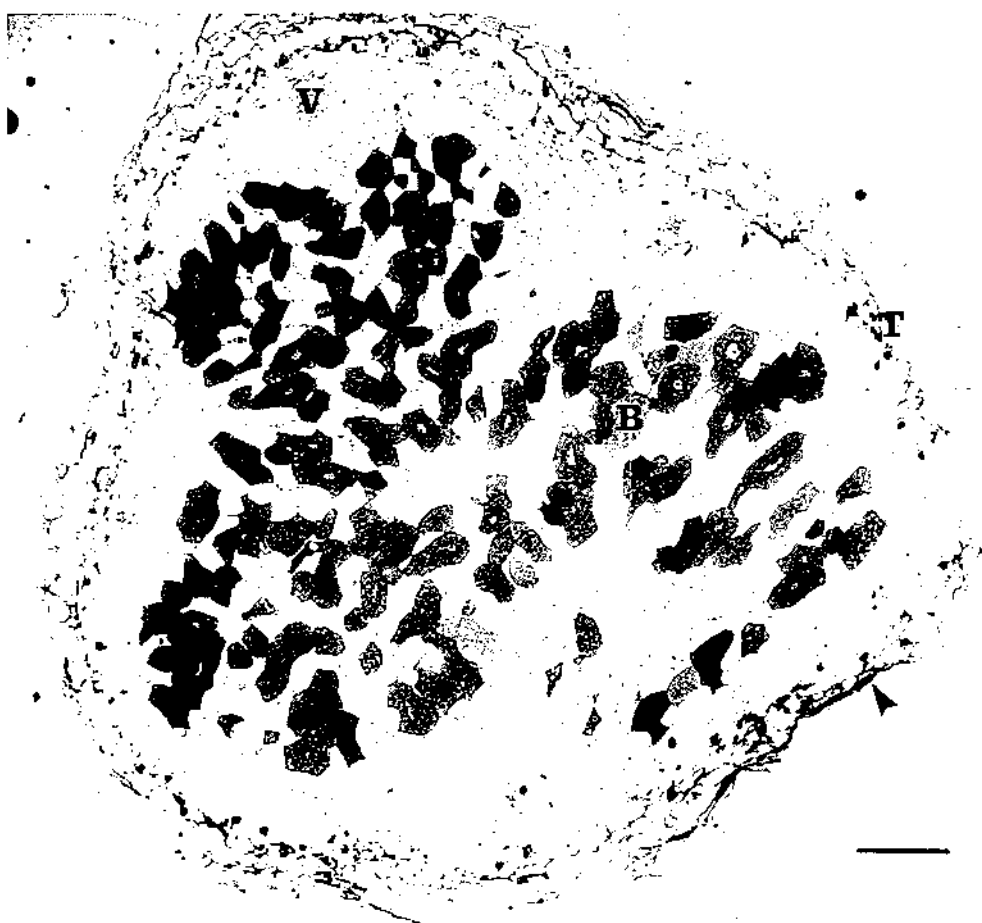
Effective nodule formed on *L. leucocephala* after inoculation with the PN184-derived EPS mutant PN1183, carrying its complementing cosmid pPN34.

The bar represents 500  $\mu\text{m}$ .

Figure 31:

Light micrograph of a transverse section through a nodule formed after inoculation of *L. leucocephala* with the PN184-derived EPS mutant PN1182, carrying its complementing cosmid pPN33.

The bar represents 100  $\mu\text{m}$ . Abbreviations: T, tannin filled plant cells; V, vascular trace; B, bacteroid filled plant cells; ►, tannin deposits in the cell walls (or intercellular spaces) of cells at the nodule surface.



invasion via infection threads, which contained a dense fibrous matrix, had occurred (Fig 32), and that bacteroids were present within each nodule (Fig. 33).

The nodules induced by the rough PN184-derived EPS mutants carrying their respective complementing cosmids were crushed and the bacteria were isolated as described in Section 2.4.3. All the bacteria isolated from each nodule retained the complemented phenotype (Calcofluor-bright, mucoid, and tetracycline resistant), with the exception of several colonies derived from nodules inoculated with PN1180 carrying its complementing cosmid pPN31, PN1183 carrying its complementing cosmid pPN34, and PN1184 carrying its complementing cosmid pPN35. These colonies exhibited the characteristics of the original EPS mutant (Calcofluor-dark, non-mucoid, tetracycline sensitive, neomycin resistant), consistent with loss of the complementing cosmid.

### 3.3 THE ISOLATION OF LPS MUTANTS DERIVED FROM PN184

To isolate LPS mutants of *R. loti* NZP2037, Tn5 mutagenesis was carried out by crossing *E. coli* SM10 (Simon *et al.*, 1983), carrying pSUP1011 (Simon *et al.*, 1983), with *R. loti* PN184, a Sm<sup>r</sup> derivative of NZP2037 (Section 2.18.3). In an attempt to isolate LPS-defective transconjugants the cross was plated out onto TY agar containing neomycin and streptomycin, as growth on TY agar retards production of EPS, thereby potentially allowing identification of LPS mutants by their expected rough colony phenotype (Section 2.18.3).

Nm<sup>r</sup>Sm<sup>r</sup> transconjugants were obtained at a frequency of approximately  $10^{-5}$ . Of approximately 60,000 Nm<sup>r</sup>Sm<sup>r</sup> transconjugants examined, 21 appeared to have a rough colony phenotype. After single colony purification, 14 of these continued to show a rough colony phenotype on TY agar (Section 2.5.2), and in addition showed a smooth (mucoid) and Calcofluor-bright colony phenotype, indicating EPS production, on YM Calcofluor agar (Section 2.19.1).

LPS was isolated from these putative LPS mutants by the hot phenol-water method (Section 2.20.1) and was examined by SDS-PAGE as described in Section 2.20.2. Some minor differences in the mobility of equivalent LPS bands was observed between PN184 and the putative PN184-derived LPS mutants which may indicate the presence of LPS with different aggregate forms (not shown). However, increasing the SDS concentration to 0.5% (w/v) in both the gel and the running

Figure 32:

Electron micrograph of an infection thread formed on *L. leucocephala* after inoculation with the PN184-derived EPS mutant PN1184, carrying its complementing cosmid pPN35.

The bar represents 5  $\mu\text{m}$ . Abbreviations: B, bacterial cell; P, polyhydroxybutyrate; M, peribacteroid membrane; ►, infection thread; F, fibrous matrix.

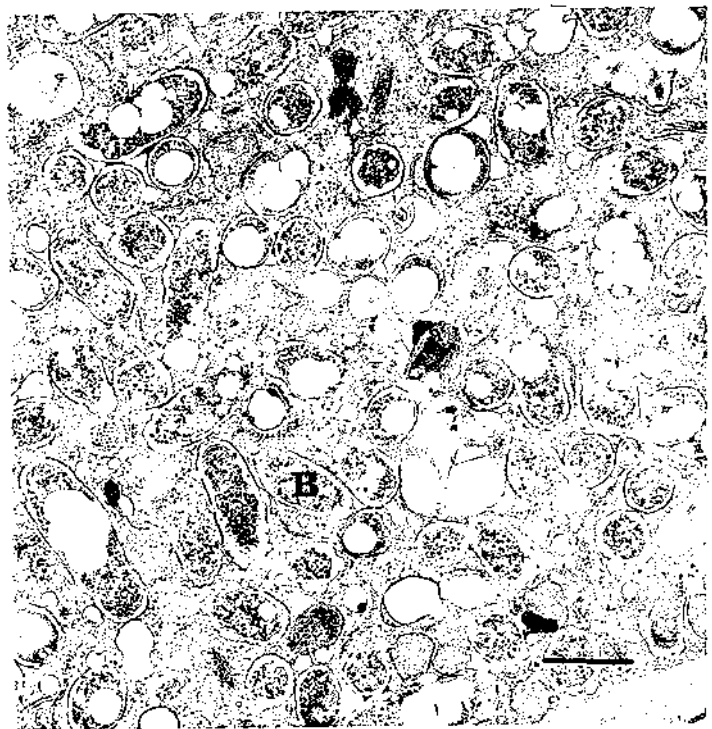


Figure 33:

Electron micrograph of bacteroids in an infected cell of a *L. leucocephala* nodule formed after inoculation with the PN184-derived EPS mutant PN1183, carrying its complementing cosmid pPN34.

The bar represents 1  $\mu\text{m}$ . Abbreviations: B, bacteroid.







buffer eliminated these mobility differences (Fig 34), although minor differences in the staining intensity of the fastest migrating band (arrowed in Fig. 34) were still observed, particularly in the lanes containing LPS from mutants LPS6, LPS7, LPS10 and LPS12. Similar differences in the staining intensity of the fastest migrating band(s) were also observed for LPS from several of the PN184-derived EPS mutants (Section 3.2.10, Fig. 25A, lanes 4, 5, 6 and 9), but did not result in an ineffective phenotype on the determinate nodulating legume *L. pedunculatus* (Section 3.2.12). The formation of ineffective nodules on *L. pedunculatus* might be expected if the differences in staining intensity observed are correlated with major LPS changes (Noel *et al.*, 1986).

Examination of the amount of LPS isolated from each mutant (Table 6), showed that the putative PN184-derived LPS mutants produced uniformly less LPS than PN184, particularly mutants LPS1, LPS7, LPS9, LPS10, and LPS12. It should be noted, however that the amount of LPS isolated from PN184 can also vary substantially (Table 6).

The percentage KDO values recorded (0.05% to 0.09%), determined as described in Section 2.20.1) were similar to those previously described for LPS isolated from PN184 (Section 3.2.10).

Since none of the putative LPS mutants showed the substantive differences in LPS structure, as defined by SDS-PAGE, that were desired (complete loss of one or more bands), they were not examined further. Nevertheless it is evident that further investigation may be warranted for several of the putative LPS mutants, particularly LPS7, LPS10, and LPS12, which show both a reduction in the staining intensity of the fastest migrating LPS band after SDS-PAGE (Fig. 34), and reduced production of LPS (Table 6).

Figure 34:

SDS-PAGE profiles of LPS from PN184, and the putative PN184-derived LPS mutants.

SDS-PAGE (0.5% SDS, 15% acrylamide, PA-silver stain) of hot phenol-water extracted LPS from lane: 1, PN184; 2, LPS1; 3, LPS2; 4, LPS3; 5, LPS4; 6, LPS5; 7, LPS6; 8, LPS7; 9, LPS8; 10, LPS9; 11, LPS10; 12, LPS11; 13, LPS12; 14, LPS13; 15, LPS14. The fastest migrating band is arrowed.

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



Table 6. Isolation of LPS from *R. loti* PN184 and from the putative PN184-derived LPS mutants.

Strain	LPS (mg freeze dried LPS/500ml culture)
PN184	28.0, 39.8 <sup>1</sup>
LPS1	7.5
LPS2	12.6
LPS3	13.7
LPS4	7.5
LPS5	10.6
LPS6	17.5
LPS7	24.1
LPS8	20.1
LPS9	7.3
LPS10	5.3
LPS11	19.5
LPS12	1.3
LPS13	14.6
LPS14	18.9

<sup>1</sup> Results from a previous isolation (28.0 mg) and an isolation performed at the same time as the putative LPS mutants (39.8 mg)

### 3.4 THE ISOLATION AND CHARACTERIZATION OF EPS MUTANTS DERIVED FROM NZP2213

To extend the observations described above to a second strain of *R. loti*, EPS mutants were also obtained by Tn5 mutagenesis (Section 2.18.1) of *R. loti*, strain PN4115, a streptomycin resistant derivative of *R. loti* strain NZP2213.

#### 3.4.1 The Isolation of EPS Mutants by Tn5 Mutagenesis of PN4115

Preliminary experiments had demonstrated that PN4115 failed to fluoresce (was Calcofluor-dark) on S20, nitrogen-free S20-, YM-, or TY-Calcofluor agars (Fig. 4). Therefore a strategy to identify PN4115-derived EPS mutants based upon selection for non-mucoidy, was attempted, rather than selection for non-fluorescence in the presence of Calcofluor (Section 2.19.1), the strategy used to isolate the PN184-derived EPS mutants.

To isolate EPS mutants of *R. loti* NZP2213, Tn5 mutagenesis was carried out by crossing *E. coli* SM10 (Simon *et al.*, 1983), carrying pSUP1011 (Simon *et al.*, 1983), with *R. loti* PN4115, a Sm<sup>r</sup> derivative of NZP2213 (Section 2.18.1). The resulting transconjugants were plated onto either nitrogen-free S20- or YM-Calcofluor agars (Sections 2.5.4, 2.5.3, and 2.19.1) containing neomycin and streptomycin (Section 2.2). Calcofluor was included to allow the possible identification of any Calcofluor-bright mutants. Nm<sup>r</sup>Sm<sup>r</sup> transconjugants were obtained at a frequency of approximately 10<sup>-5</sup>. Approximately 120,000 Nm<sup>r</sup>Sm<sup>r</sup> transconjugants were plated out onto nitrogen-free S20 Calcofluor agar. However, no non-mucoid transconjugants could be identified. Approximately 150,000 Nm<sup>r</sup>Sm<sup>r</sup> transconjugants were plated out onto YM Calcofluor agar. Six identifiable mutants with altered mucoidy were isolated.

To verify that the six EPS mutants were PN4115 derivatives, both the total protein profiles of PN4115 and each PN4115-derived EPS mutant (Section 2.22), and the *Eco*RI restriction endonuclease digestion patterns of total DNA preparations from PN4115 and from each PN4115-derived EPS mutant (Sections 2.7.1, 2.11 and 2.12), were examined and compared. The protein profiles and *Eco*RI digestion patterns of each mutant were found to be identical to those of PN4115 (not shown).

### 3.4.2 Colony Characteristics of the PN4115-Derived EPS Mutants

Each of the six PN4115-derived EPS mutants was non-mucoid (rough) when streaked onto S20 agar (Section 2.5.4) and YM agar (Section 2.5.3), as shown in Figure 35.

All the PN4115-derived EPS mutants were found to be Calcofluor-bright (Section 2.19.1). Figure 36A shows the fluorescence of PN4115-derived EPS mutants PN1312, PN1313, and PN1314 on YM-Calcofluor agar. The PN4115-derived EPS mutants GH106, GH180, and GH214 were also found to fluoresce on YM- and TY-Calcofluor agars (not shown). However, GH180 showed a high rate of reversion to mucoidy (Fig. 35) and was not examined further, while GH106 and GH214 were subsequently shown to possess two or more hybridizing fragments when total *Eco*RI digested DNA from each mutant was probed with <sup>32</sup>P-labelled pKan2 DNA (Section 3.4.3). GH106 and GH214 were therefore not examined further, except as described in Section 3.4.3.

The PN4115-derived EPS mutants PN1312, PN1313 and PN1314 were found to form yellow-cream coloured colonies on YM- and TY-agars, identical in colour to the colonies formed by PN4115 (see Fig. 6).

The PN4115-derived EPS mutants PN1312, PN1313, and PN1314 were streaked out onto YM-Congo red agar (Section 2.5), with all three mutants staining deep red (Fig. 36B), suggesting that these strains may be producing cellulose, or that non-production of EPS has unmasked cellulose to the stain (Kneen and LaRue, 1983).

In addition to the non-mucoid PN4115-derived EPS mutants described here, a large number of additional mutants were isolated, based upon the dark colouration of their colonies. These mutants were not further examined.

### 3.4.3 Confirmation of the Presence of Tn5 Sequences

#### 3.4.3.1 Confirmation of a Single Tn5 Insertion

To confirm the presence of a single Tn5 insertion in each mutant, total DNA from each mutant, and total DNA from PN4115 (Section 2.7.1), was digested to completion with *Eco*RI (Section 2.11). Digestion to completion was checked on a

Figure 35:

Mucoid phenotype of PN4115 and non-mucoid phenotypes of the PN4115-derived EPS mutants on YM agar.

PN4115 (top), and bottom (left to right) GH106, PN1312, PN1313, GH180, GH214, and PN1314. The PN4115-derived EPS mutant GH180 shows a high rate of reversion to mucoidy.

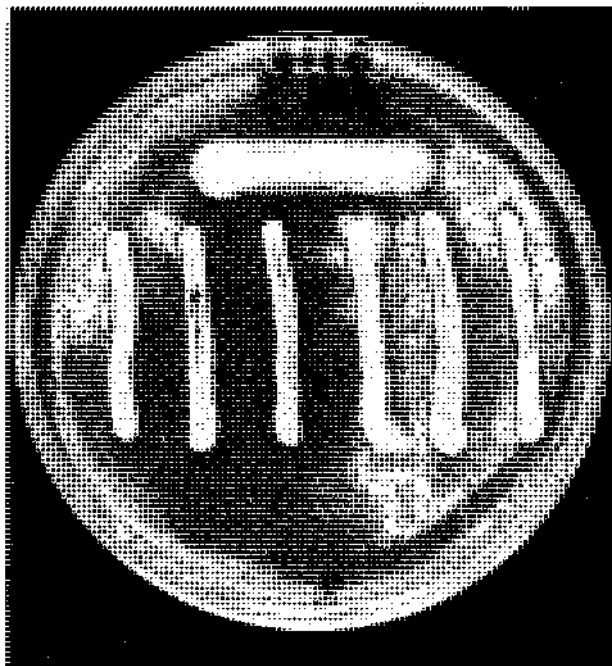




Figure 36A:

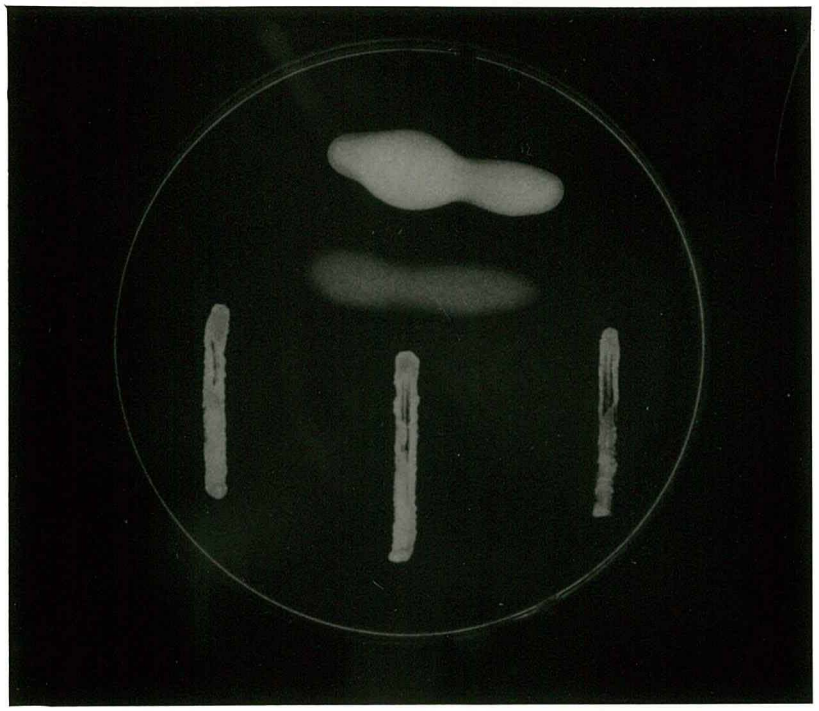
Fluorescence of the PN4115-derived EPS mutants on YM-Calcofluor agar.

Fluorescence is exhibited upon exposure to long wave UV light. PN184 (top), PN4115 (middle), and bottom (left to right) PN1312, PN1313, and PN1314.

Figure 36B:

Red phenotype of the PN4115-derived EPS mutants and orange phenotype of PN4115 on YM-Congo red agar.

PN4115 (top), and bottom (left to right) PN1312, PN1313, and PN1314.

**A****B**

0.7% agarose minigel, then the samples were run overnight (approximately 16 h) on a 0.7% agarose gel (Section 2.12). A Southern blot (Section 2.16) of this gel was probed with  $^{32}\text{P}$ -labelled (Section 2.17) pKan2 DNA, which is pBR322 carrying the 3.5-kb *Hind*III fragment of Tn5 (Scott *et al.*, 1982). For mutants PN1312, PN1313, and PN1314 a single hybridizing fragment (Tn5 has no *Eco*RI sites) of identical size was observed (Figure 37A). The size in kilobases of the hybridizing fragments for mutants PN1312, PN1313, and PN1314, together with the corrected size of the equivalent wild type (PN4115) fragment (that is subtracting 5.7 kb for the size of Tn5) is shown in Table 7.

In addition to the PN4115-derived EPS mutants PN1312, PN1313, and PN1314, two additional mutants, GH106 and GH214, were also examined for Tn5 insertion. However, under the conditions described above, two fragments hybridized for GH106, and three fragments hybridized for GH214 (Fig. 37B). As a result, these mutants were not further examined.

#### **3.4.3.2 Confirmation that Each Mutation Arose from an Independent Tn5 Insertion**

To confirm that each mutation arose from an independent insertion event, total DNA from PN4115, and total DNA from PN1312, PN1313, and PN1314 (Section 2.7.1) was digested to completion with *Bam*HI (Section 2.11). Digestion to completion was checked on a 0.7% agarose minigel, then the samples were run overnight (approximately 16 h) on a 0.7% agarose gel (Section 2.12). A Southern blot (Section 2.16) of this gel was probed with  $^{32}\text{P}$ -labelled (Section 2.17) pKan2 DNA. In all cases, two fragments hybridized as expected (Tn5 has a single *Bam*HI site) and each hybridization pattern was unique (Fig. 38).

Considered alongside the observation (Section 3.4.3.1) that each mutant carries a Tn5 insertion in a single *Eco*RI fragment, and that the size of this *Eco*RI fragment is identical for each mutant, these results are consistent with independent insertion of Tn5 into the same wild type *Eco*RI fragment, potentially into different regions of the same gene.

## Figure 37A:

Confirmation of a single Tn5 insertion in the PN4115-derived EPS mutants PN1312, PN1313, and PN1314.

Autoradiogram of a Southern blot of *Eco*RI digested total DNA from PN4115 and the PN4115-derived EPS mutants PN1312, PN1313, and PN1314, probed with <sup>32</sup>P-labelled pKan2 DNA. Lanes: 1, <sup>32</sup>P-labelled, *Hind*III digested, phage lambda DNA; 2, blank; 3, PN4115; 4, PN1312; 5, PN1313; 6, PN1314.

## Figure 37B:

The PN4115-derived EPS mutants GH106 and GH214 show multiple *Eco*RI fragments hybridizing to <sup>32</sup>P-labelled pKan2 DNA.

Autoradiogram of a Southern blot of *Eco*RI digested total DNA from PN4115, and the PN4115-derived EPS mutants GH106 and GH214, probed with <sup>32</sup>P-labelled pKan2 DNA. Lanes: 1, <sup>32</sup>P-labelled, *Hind*III digested, phage lambda DNA; 2, blank; 3, PN4115; 4, GH106; 5, GH214.

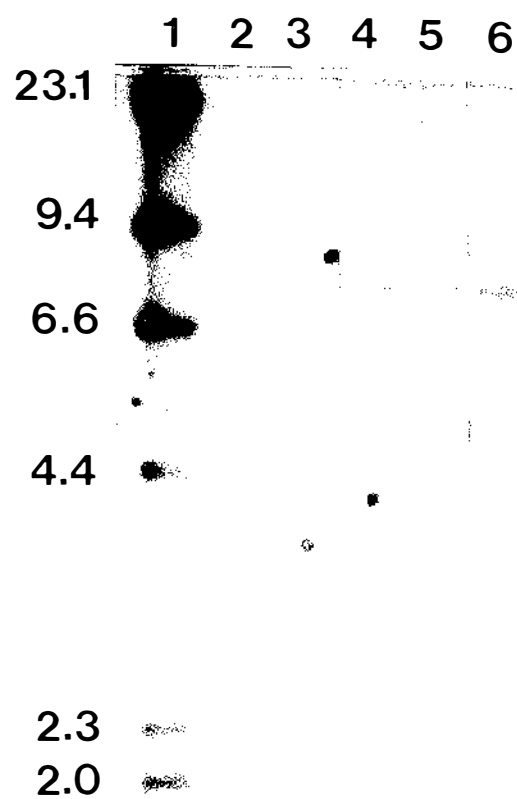
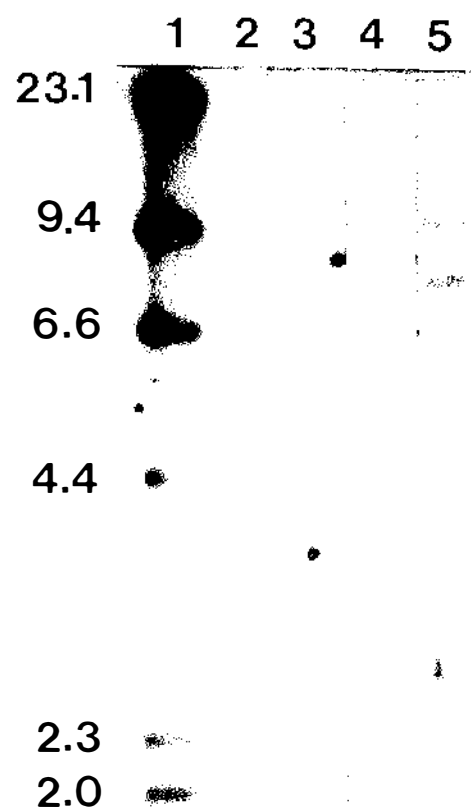
**A****B**

Table 7. *Eco*RI digestion fragments of the PN4115-derived EPS mutants containing a Tn5 insertion.

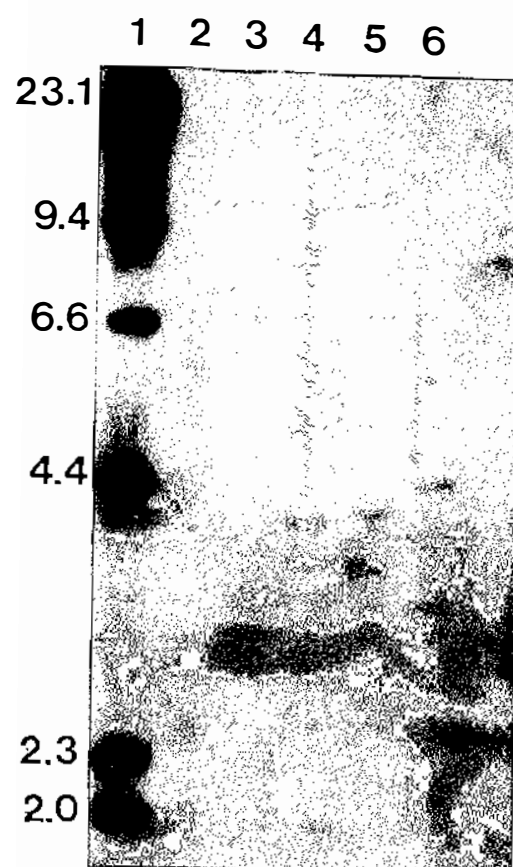
Strain	Size of <i>Eco</i> RI fragment carrying Tn5 (kb)	Predicted size <sup>1</sup> of <i>Eco</i> RI fragment in PN4115 (kb)
PN1311	7.5	1.8
PN1312	7.5	1.8
PN1313	7.5	1.8

<sup>1</sup> Obtained by subtracting 5.7 kb (for Tn5) from the size of the *Eco*RI fragment carrying Tn5.

Figure 38:

Confirmation that the PN4115-derived EPS mutants PN1312, PN1313, and PN1314, arose from independent Tn5 insertions.

Autoradiogram of a Southern blot of *Bam*HI digested total DNA from PN4115 and PN4115-derived EPS mutants PN1312, PN1313, and PN1314 probed with <sup>32</sup>P-labelled pKan2 DNA. Lanes: 1; <sup>32</sup>P-labelled, *Hind*III-digested, phage lambda DNA; 2, blank; 3; PN4115; 4, PN1312; 5, PN1313; 6, PN1314.





### 3.4.4 The Attempted Isolation of Cosmids Restoring the Mucoid, Calcofluor-Dark Phenotype of PN4115

Cosmids could not be isolated which stably complemented the mutations carried by PN4115-derived EPS mutants PN1312, PN1313, and PN1314. Several non-overlapping cosmids were isolated for each mutant which, initially, appeared to complement the mutation carried by each mutant. However, complementation did not persist through the "back cross" procedure (Section 2.18.2.2) used to identify stably complementing cosmids.

### 3.4.5 Exopolysaccharide Isolation and Characterization

#### 3.4.5.1 EPS Isolation

EPS could be cetrимide precipitated (Section 2.19.2) from YM- and nitrogen-free S20 broth culture supernatants of PN4115, however, EPS was regularly obtained from YM-culture supernatants as PN4115 was found to grow only poorly in nitrogen-free S20 broth.

Under identical conditions to those used to obtain a cetrимide precipitate of PN4115 EPS, no precipitate could be obtained from the PN4115-derived EPS mutants PN1312, PN1313, and PN1314.

#### 3.4.5.2 $^1\text{H}$ -NMR Spectroscopy of PN4115 EPS

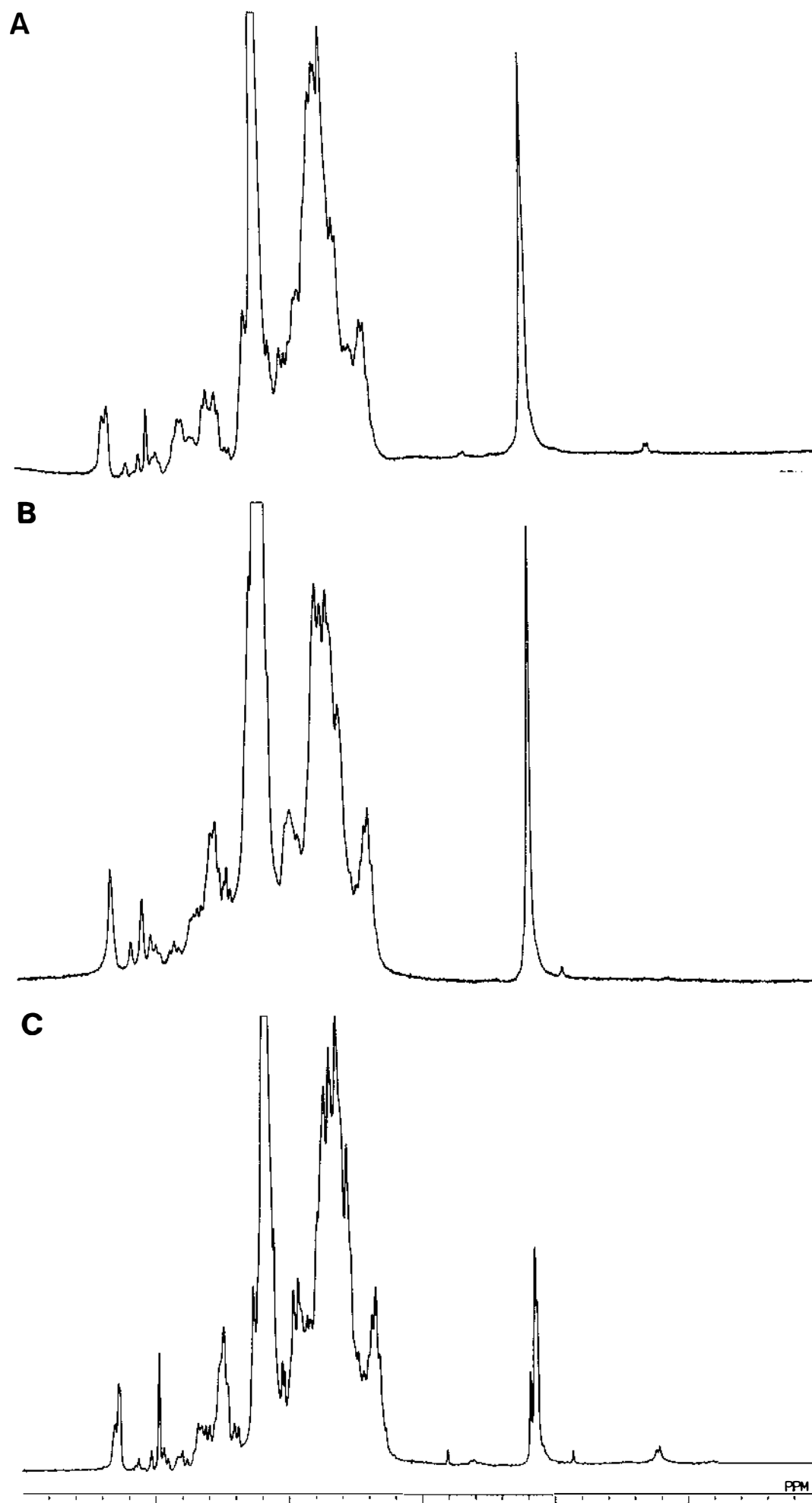
PN4115 EPS, cetrимide precipitated (Section 2.19.2) from YM culture supernatants in mid-log phase was examined by  $^1\text{H}$ -NMR spectroscopy (Fig. 39B).

While the structure of the EPS from PN4115 has not previously been examined, the  $^1\text{H}$ -NMR spectrum of PN4115 cetrимide precipitated EPS has been interpreted as follows. An envelope of signals (4.35 to 4.75 ppm) is present in the  $\beta$ -anomeric proton region (Kotowycz and Lemieux, 1973), together with a prominent peak (5.3 ppm) in the  $\alpha$ -anomeric proton region (Kotowycz and Lemieux, 1973), suggesting the presence of both  $\alpha$ - and  $\beta$ -anomeric glycosidic linkages. The envelope of signals between 3.2 and 4.0 ppm results from the ring protons of the glycosyl residues. The peak at 2.15 ppm results from the methyl protons of *O*-acetate modifications (Aman *et al.*, 1981; Djordjevic *et al.*, 1986; Hollingsworth *et al.*,

Figure 39:

$^1\text{H}$ -NMR spectra of EPS from PN184, PN4115 and PN1177.

10 mg cetrimide precipitated EPS from: A, PN184; B, PN4115; and C, PN1177.



1988; Kuo and Mort, 1986; McNeil *et al.*, 1986). Unlike PN184 EPS, there are no signals (at 1.24 and 2.62 ppm) consistent with the presence of 3-hydroxybutanoic acid modifications (see Section 3.2.9.2). In addition, there are no signals present at 2.47 ppm and 2.63 ppm, and between 1.44 ppm and 1.55 ppm, consistent with the presence of succinyl or pyruvyl modifications, respectively (see Section 3.2.9.2). Therefore, PN4115 EPS, like PN184 EPS (see Section 3.2.9.2), is not modified with either succinate or pyruvate. Signals between 4.9 ppm and 5.2 ppm are consistent with the presence of *O*-acyl (*O*-acetate) modified glycosyl residues (Kuo and Mort, 1986; McNeil *et al.*, 1986).

Comparison, by relative integration, of the magnitude of the signals arising from the ring protons of the glycosyl residues to the magnitude of the signal arising from the methyl protons of the *O*-acetate modifications gave a ratio of 6.4:1.0.

The  $^1\text{H}$ -NMR spectrum of PN4115 EPS, therefore, shows similarities and differences to the  $^1\text{H}$ -NMR spectra of EPS from PN184 (Fig. 39A, see also Section 3.2.9.2) and the smooth PN184-derived EPS mutants (Fig. 39C, the figure shows the  $^1\text{H}$ -NMR spectrum of PN1177 EPS reproduced, for comparison, from Fig. 17B, see also Section 3.2.9.3). As described above, PN4115 shows no signals (at 1.24 and 2.62 ppm) consistent with the presence of 3-hydroxybutanoic acid modifications. Signals consistent with the presence of 3-hydroxybutanoic acid modifications are present in the  $^1\text{H}$ -NMR spectra of EPS from both PN184 and PN1177. The glycosyl residue ring proton:*O*-acetate methyl proton ratio of PN4115 EPS (6.4:1.0) is similar to the range observed for PN184 EPS (6.6:1.0 to 8.1:1.0, Section 3.2.9.2), indicating a similar level of *O*-acetylation. In comparison, the ring proton:*O* acetate ratio for PN1177 EPS was 19.0:1.0, indicating *O*-acetylation of PN1177 EPS to a significantly reduced level. However, the  $^1\text{H}$ -NMR spectrum of PN4115 EPS shows several similarities to the  $^1\text{H}$ -NMR spectrum of PN1177 EPS, including the magnitude of the signals between 3.3 and 3.45 ppm, the magnitude of the signals between 3.9 and 4.0 ppm, and the relative magnitude of the signals in the  $\beta$ -anomeric proton region (4.35-4.75 ppm). In addition, a multiplet is present between 4.75 ppm and 4.90 ppm in the  $^1\text{H}$ -NMR spectra of both PN4115 EPS and PN1177 EPS.

### 3.4.5.3 Analysis of Cetrimide Precipitated EPS for Fluorescence on Calcofluor Agar

2 mg of PN184 EPS and 2 mg of PN4115 EPS, cetrimide precipitated from YM culture supernatants harvested at mid log phase (Section 2.19.2.1), were dissolved in water to give highly viscous solutions, which were spotted onto a TY Calcofluor agar plate (Section 2.19.1). Upon irradiation with UV light the PN184 EPS sample was found to fluoresce, while, in contrast the PN4115 EPS sample showed no fluorescence.

### 3.4.5.4 Column Fractionation of PN4115 EPS

DEAE-Sephacel column fractionation (Sections 2.19.5 and 2.19.6) of PN4115 EPS, cetrimide precipitated from YM broth supernatants at mid log phase (Section 2.19.2.1) was found to yield two anthrone reactive, polysaccharide fractions, a major fraction with a peak elution at 330 mM sodium chloride and a broad minor fraction eluting in 1 mM sodium chloride (Fig. 40). Approximately 64% of the material loaded onto the column shown in Figure 40 eluted. The peak ratios (330 mM NaCl fraction:1 mM NaCl fraction) were 22.0:1.0 (fractions 49-65:fractions 11-19). These results suggest that PN4115 produces little high molecular weight EPS (eluting in 1 mM NaCl).

The material eluting in fractions 11 to 19 (1 mM NaCl fraction), and 51 to 59 (330 mM NaCl fraction) were pooled separately, dialysed extensively against MilliQ water, then lyophilized.

The lyophilized 330 mM NaCl fraction was examined by  $^1\text{H}$ -NMR spectroscopy. The resultant spectrum was essentially identical to that obtained for unfractionated PN4115 EPS (not shown). There was insufficient lyophilised material to examine the 1 mM NaCl fraction by  $^1\text{H}$ -NMR spectroscopy.

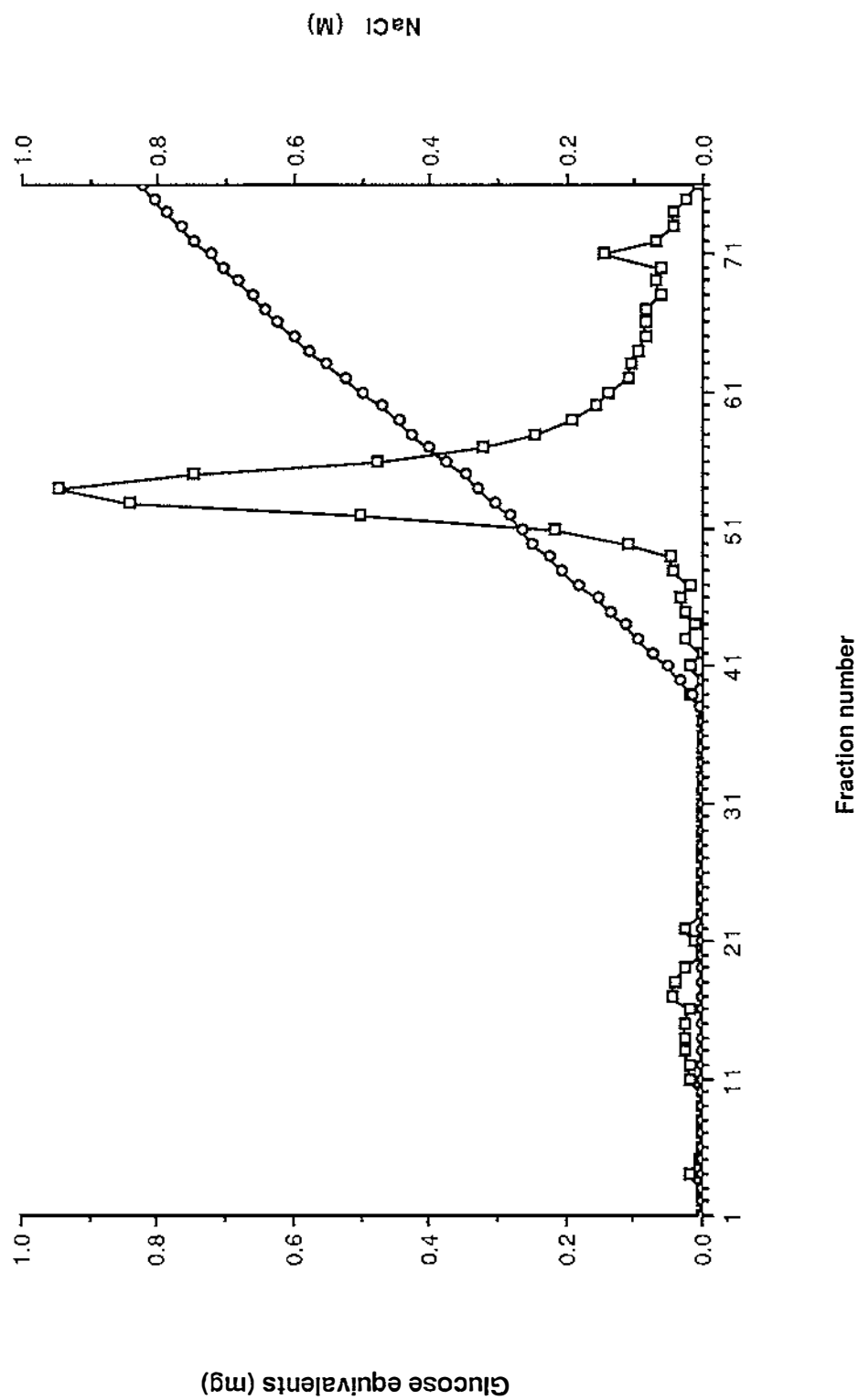
### 3.4.6 Nodulation Tests

To investigate the symbiotic effect of the mutations carried by the PN4115-derived EPS mutants, nodulation tests (Section 2.4) were performed on both *Lotus corniculatus* (var. *cree*), a determinate nodulating legume, and *Leucaena leucocephala*, an indeterminate nodulating legume.

Figure 40:

DEAE-Sephacel column chromatography of PN4115 EPS.

- Glucose equivalents
- NaCl gradient



The root nodules formed on *L. corniculatus* plants, examined six weeks after inoculation with PN4115, were found to be fully effective (Nod<sup>+</sup>Fix<sup>+</sup>), with 5-12 spherical, white-brown nodules, 1.0-1.5 mm in diameter, present on the roots of each plant. Examination of nodule sections by epifluorescence microscopy (Fig. 41) and by electron microscopy (Fig. 42) demonstrated that invasion had occurred and that bacteroids were present within each nodule. Similarly, control plants inoculated with PN184 were also fully effective (Fig. 43). Uninoculated plants were uniformly Nod<sup>-</sup>. The PN4115-derived EPS mutants PN1312, PN1313, and PN1314 all induced the formation of fully effective root nodules, the nodules formed being indistinguishable from those formed after inoculation with PN4115, and at the same frequency per plant. Plants inoculated with the PN4115-derived EPS mutants grew to the same size as those inoculated with PN4115 and showed no signs of nitrogen deficiency.

The nodules induced by the PN4115-derived EPS mutants were crushed and bacteria were isolated as described in Section 2.4.3. 20-30 colonies arising from bacteria present in each nodule were single colony purified and examined for retention of the mutant phenotypes. All bacteria isolated were found to retain the mutant phenotypes (neomycin resistance, non-mucoid, Calcofluor-bright, red colonies on Congo Red agar).

The nodular structures formed on *L. leucocephala* plants examined six weeks after inoculation with PN4115 were found to be ineffective (Nod<sup>+</sup>Fix<sup>-</sup>), with 4-10 nodule-like structures present on the roots of each plant. The nodular structures formed varied from small, white-brown, nodular swellings (Fig. 44A) to large, brown, tumour-like structures (Fig. 44B, and C). Examination of the nodular structures by light, epifluorescence (Fig. 45) and electron microscopy (Fig. 46) showed that tannin deposits (flavolans) were distributed throughout the structures, that the plant cells had been invaded, and that infection threads were present (Fig. 47A and B). Plant cell invasion was, however, irregular (compare, for example, Figs. 41 and 45). The bacteria present in invaded cells were surrounded by a dense fibrous matrix (Fig. 46), apparently identical to the fibrous matrix surrounding the bacteria in infection threads (Figs 47A and 47B). Fibrous material was also present outside the infection thread membrane (Fig. 47B). The fibrous matrix was not observed in infected cells of control plants inoculated with the effective *R. loti* strain PN184 (see for comparison Fig. 26). In addition, the bacteria present in PN4115-infected cells show evidence of partial degeneration or senescence (Fig.



Figure 41:

Fluorescence of *L. corniculatus* cells containing PN4115 bacteroids in Calcofluor-acridine orange stained nodule sections examined by epifluorescence illumination.

The bar represents 100  $\mu\text{m}$ . Abbreviations: B, bacteroid filled plant cells; ►, plant cell wall; ►, plant cell nucleus.

Figure 42:

Electron micrograph of bacteroids in an invaded cell of a *L. corniculatus* nodule formed after inoculation with PN4115.

The bar represents 1  $\mu\text{m}$ . Abbreviations: B, bacteroid; M, peribacteroid membrane.

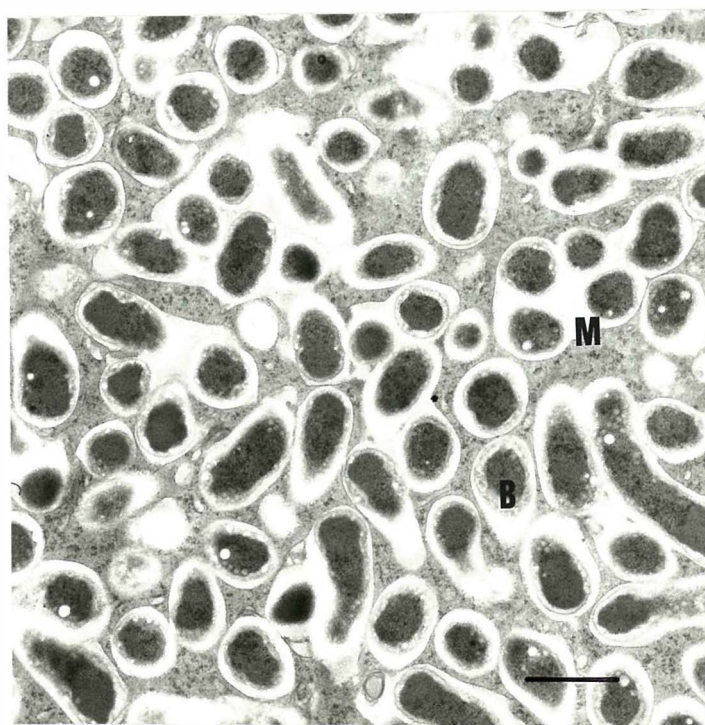
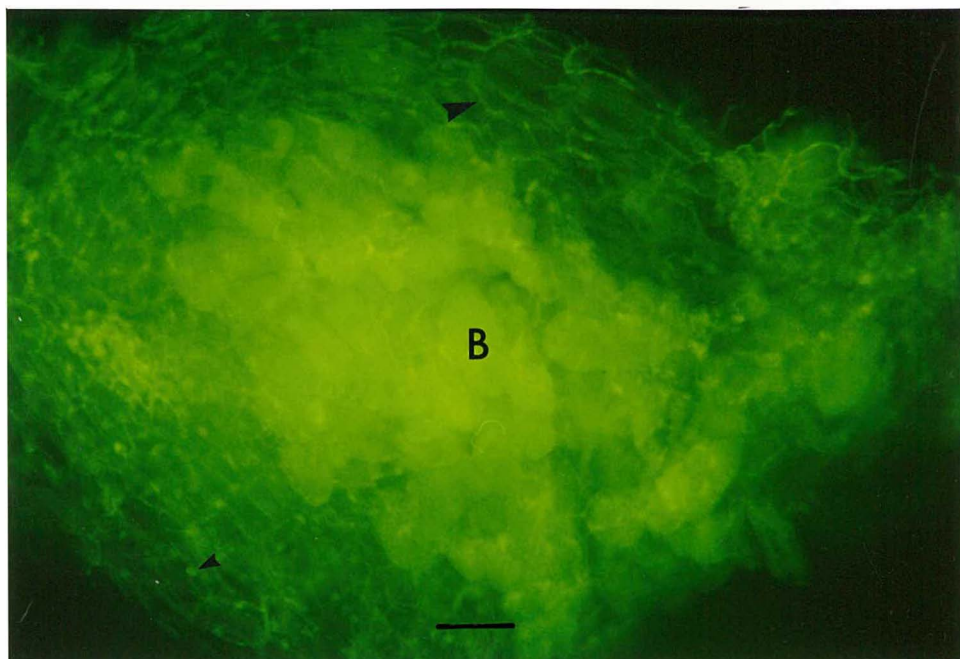


Figure 43:

Electron micrograph of bacteroids in an invaded cell of a *L. corniculatus* nodule formed after inoculation with PN184.

The bar represents 1  $\mu\text{m}$ . Abbreviations: B, bacteroid; ►, peribacteroid membrane.

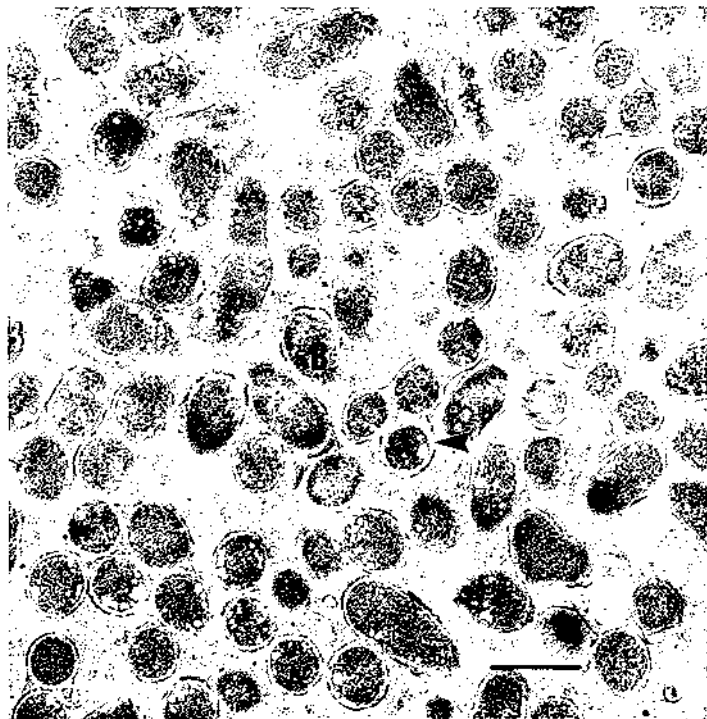


Figure 44:

Light micrographs of (A) a small, ineffective, nodular swelling, and (B and C) large, tumour-like, nodular structures formed on *L. leucocephala* roots after inoculation with PN4115.

The bar represents 500  $\mu\text{m}$ .

**A****B****C**

Figure 45:

Irregular fluorescence of *L. leucocephala* cells invaded by PN4115 in a Calcofluor-acridine orange stained section of a tumour-like nodule, examined by epifluorescence illumination.

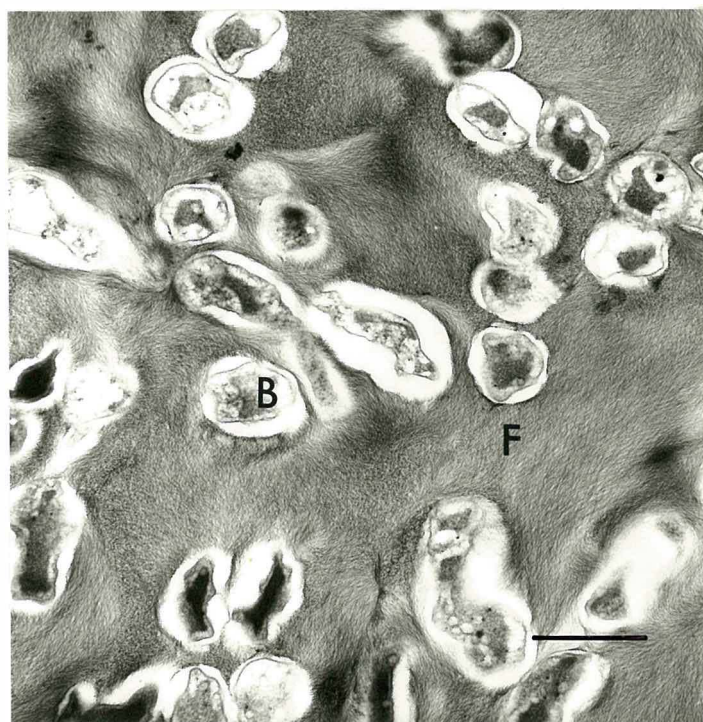
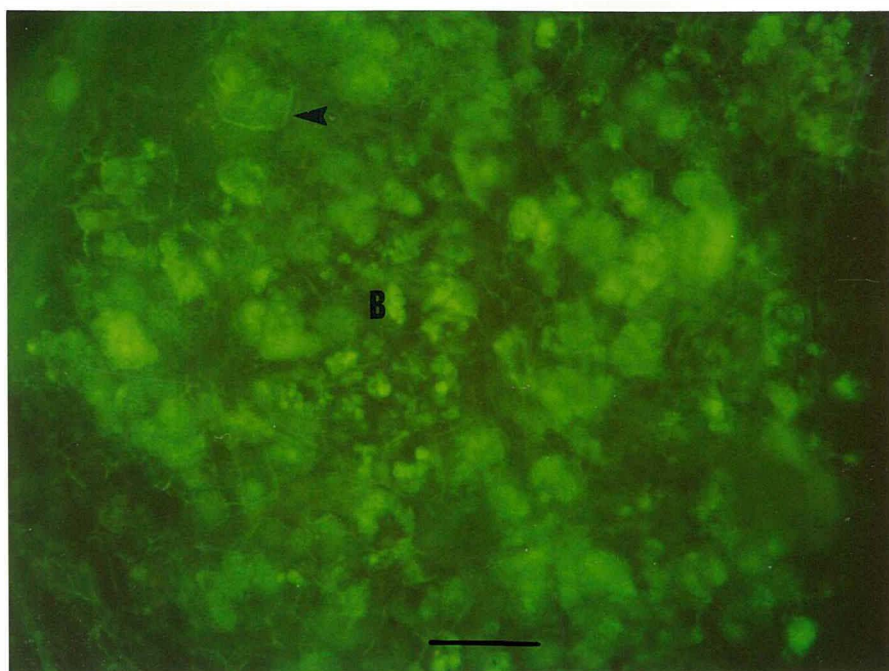
The bar represents 100µm. Abbreviations: B, bacteria and fibrous material in a plant cell; ►, plant cell wall.

Figure 46:

Electron micrograph of bacteria and fibrous material in a *L. leucocephala* cell after invasion by PN4115.

The bar represents 1 µm. Abbreviations: B, bacterium; F, fibrous material.







## Figure 47A:

Electron micrograph of infection threads formed after inoculation of *L. leucocephala* with PN4115.

The bar represents 2  $\mu\text{m}$ . Abbreviations: B, bacterial cell; F, fibrous material.

## Figure 47B:

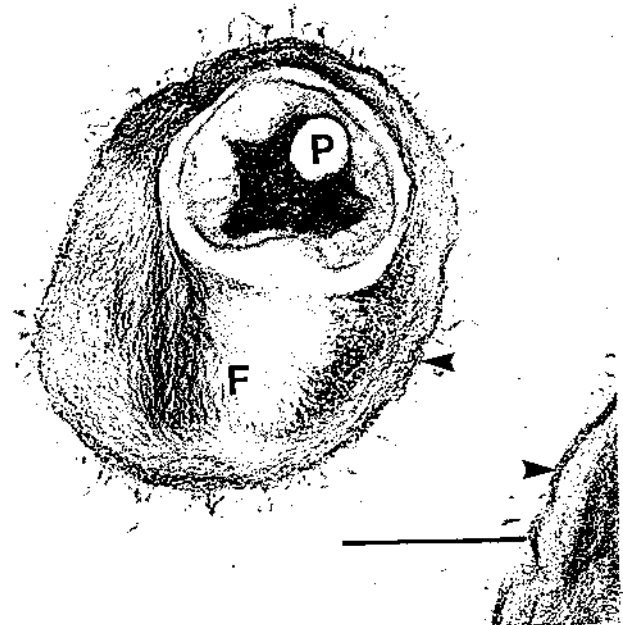
Electron micrograph of an infection thread formed after inoculation of *L. leucocephala* with PN4115.

The bar represents 0.5  $\mu\text{m}$ . Abbreviations: B, bacterial cell; P, polyhydroxybutyrate; F, fibrous material; ►, infection thread membrane.

A



B



46). Occasionally, nodules capable of a low level of nitrogen fixation were observed, indicating that at least some bacteria differentiate into bacteroids.

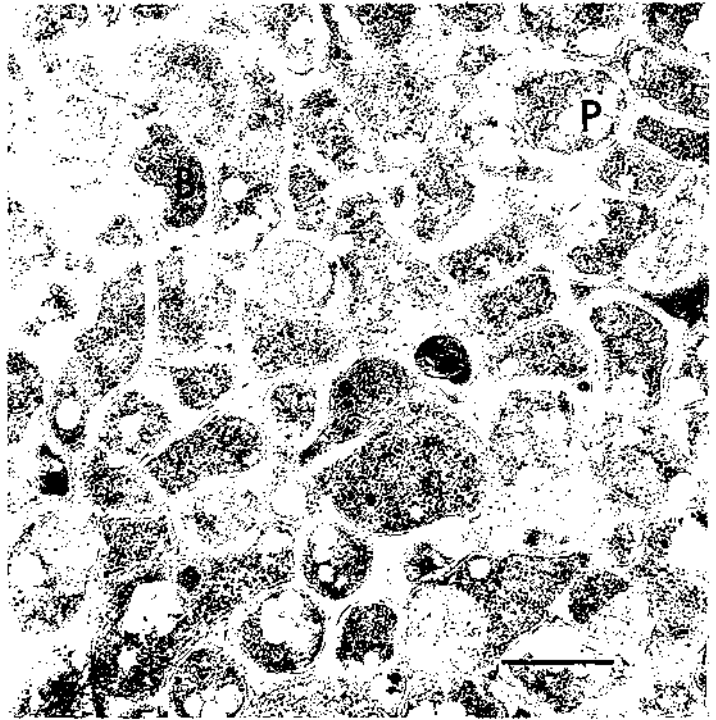
The small, white-brown, spherical, nodular swellings formed on the roots of *L. leucocephala* plants inoculated with the PN4115-derived EPS mutants PN1312, PN1313 and PN1314 were all Fix<sup>-</sup>, and were identical in appearance to those shown for PN4115 in Figure 44A. However, in contrast to plants inoculated with PN4115, no large, tumour-like, nodular structures were observed (see Figs. 44B and 44C). Examination of the nodular swellings formed on plants inoculated with PN1312, PN1313, or PN1314 by light and electron microscopy demonstrated that tannin deposits were distributed throughout the nodular swellings. In addition, several cells in the nodular swellings formed on plants inoculated with PN1312 had been invaded (Fig. 48). However, the extensive, irregular, invasion characteristic of PN4115 (Fig. 41) was not observed. In addition, the dense threadlike matrix present in invaded cells of plants inoculated with PN4115 was not present (Fig. 48). Furthermore nitrogen fixation, as defined by acetylene reduction (Section 2.4.2.1), was not observed. No invaded cells were observed in the nodular swellings formed on plants inoculated with either PN1313 or PN1314.

The nodular swellings induced by the PN4115-derived EPS mutants were crushed and the bacteria were isolated as described in Section 2.4.3. No bacteria were isolated from the nodular swellings induced on plants inoculated with PN1313 or PN1314, however, bacteria retaining the mutant phenotypes (neomycin resistance, Calcofluor-bright, non-mucoid, red colonies on YM Congo Red agar) were isolated from nodules inoculated with PN1312.

Figure 48:

Electron micrograph of bacteria in an invaded *L. leucocephala* cell after inoculation with the PN4115-derived EPS mutant PN1312.

The bar represents 1  $\mu\text{m}$ . Abbreviations: B, bacterium; P, polyhydroxybutyrate.



## Chapter 4. DISCUSSION

A number of previous studies have suggested that exopolysaccharide synthesis, by the invading *Rhizobium* strain, is required for effective nodulation of indeterminate nodulating legumes (Borthakur *et al.*, 1986; Chakravorty *et al.*, 1982; Chen *et al.*, 1985; Diebold and Noel, 1989; Finan *et al.*, 1985; Leigh *et al.*, 1985; Napoli and Albersheim, 1980) but is not required for effective nodulation of determinate nodulating legumes (Borthakur *et al.*, 1986; Diebold and Noel, 1989; Kim *et al.*, 1989; Ko and Gayda, 1990), although exceptions among determinate nodulating legumes exist (Chen *et al.*, 1985; Law *et al.*, 1982).

The most persuasive approaches used in previous studies suggesting a nodule-type specific requirement for EPS have involved, either introducing EPS (*pss*) mutations by marker exchange into two strains of *R. leguminosarum* bv. *phaseoli* differing only in their symbiotic plasmids and hence their host legumes (Borthakur *et al.*, 1986), or utilizing haploid recombination to transfer EPS mutations between *R. leguminosarum* bv. *phaseoli* and either *R. leguminosarum* bv. *viciae* or *R. leguminosarum* bv. *trifolii* (Diebold and Noel, 1989). As identified (Diebold and Noel, 1989), both approaches have intrinsic weaknesses.

Ideally, one would wish to study the effect of EPS mutations in a single strain of a *Rhizobium* species that is capable of effectively nodulating both determinate and indeterminate nodulating legumes. Chen *et al.* (1985) have isolated 90 mutants with altered EPS synthesis derived from *Rhizobium* sp. NGR234, a broad-host range species satisfying this criterion. However, although, for example, the EPS<sup>-</sup> (unconditional rough) mutants isolated were invariably symbiotically defective on *L. leucocephala*, an indeterminate nodulating host (most mutants inducing the formation of small callus-like structures), less extensive inhibition of effective nodulation was also observed for some mutants on determinate nodulating hosts, such as siratro (Chen *et al.*, 1985). Furthermore, coinoculation of EPS with an EPS<sup>-</sup> mutant restored the mutant to effective nodulation on both *L. leucocephala* and siratro (Djordjevic *et al.*, 1987).

Strains of *R. loti* are able to nodulate both determinate nodulating legumes such as *Lotus pedunculatus* (PN184 [N2P2037 *str*-1] effective, PN4115 [N2P2213 *str*-1] ineffective) and *Lotus corniculatus* (both PN184 and PN4115 effective), and

indeterminate nodulating legumes such as *Leucaena leucocephala* (PN184 effective, PN4115 ineffective). *R. loti*, therefore, constitutes a model species for investigating the requirement and ultimately the role of EPS during invasion of indeterminate versus determinate nodulating legumes.

The work described in this Thesis was therefore aimed primarily at elucidating the potential for using *R. loti* as a model *Rhizobium* species for studying the requirement for, and ultimately the role of, EPS in invasion of indeterminate versus determinate nodulating legumes. To this end, EPS mutants were isolated from both the broad host range *R. loti* strain, PN184, and the restricted host range *R. loti* strain, PN4115. The mutants were biochemically characterized, and their invasion properties on indeterminate and determinate nodulating host legumes were determined.

In addition, three infection mutants, PN1018, PN1019, and PN1027, isolated previously (Chua *et al.*, 1985), which are ineffective (PN1018, PN1027), or Nod<sup>-</sup> (PN1019) on the determinate nodulating legume *L. pedunculatus*, were also examined.

#### 4.1 THE SYMBIOTIC EFFECTIVENESS OF *R. LOTI* EPS MUTANTS

The work described in this thesis has shown that selection for a Calcofluor-dark phenotype, after Tn5 mutagenesis, allows the isolation of EPS mutants derived from *R. loti* strain PN184 (Fig. 8). The PN184-derived EPS mutants fell into two classes based upon colony characteristics. Strains PN1177, PN1178, and PN1179 were mucoid (smooth), while strains PN1180, PN1181, PN1182, PN1183, and PN1184 were non-mucoid (rough, Fig. 10A). The rough PN184-derived EPS mutants failed to produce any EPS (Section 3.2.9.1), while, in contrast, the smooth PN184-derived EPS mutants each produce an EPS which shows reduced *O*-acetylation compared to the EPS produced by PN184 (Fig. 17, Section 3.2.9.3).

All of the PN184-derived EPS mutants were ineffective on *L. leucocephala*, an indeterminate nodulating legume, but were fully effective on *L. pedunculatus*, a determinate nodulating legume (Section 3.2.12). Small nodular swellings formed on *L. leucocephala* plants inoculated with all the PN184-derived EPS mutants (Figs. 28 and 29), but no evidence for infection thread formation or invasion has been found. The nodular swellings induced by the PN184-derived EPS mutants

contained tannin (flavolan) deposits distributed throughout the swellings, together with tannin deposits in the walls of cortical and epidermal cells which were likely to be in contact with the inoculated bacteria (Fig. 29). The distribution of tannins throughout the nodular swellings resembled the distribution of tannin deposits in nodular swellings formed on *L. leucocephala* after inoculation with either *Rhizobium* sp NGR234 EPS mutants (Chen *et al.*, 1985; Djordjevic *et al.*, 1987), NZP2213 (Pankhurst *et al.*, 1987), or PN4115. Similarly, the presence of tannin deposits in the walls of cortical and epidermal cells of the nodular swellings formed on *L. leucocephala* after inoculation with the PN184-derived EPS mutants resembled the presence of phenolic compounds in the outer cortical cells of pseudonodules formed after inoculation of alfalfa with an EPS mutant (Rm0540) of *R. meliloti* that fails to produce EPSI (Puhler *et al.*, 1991). These results suggest that a plant defense response is elicited upon inoculation of *L. leucocephala* and alfalfa with *Rhizobium* EPS mutants.

When either PN184, or the PN184-derived EPS mutants carrying their respective complementing cosmids, were used to inoculate *L. leucocephala*, fully effective nodules were induced (Figs. 30 and 31). Infection threads were present and contained a dense fibrous matrix (Fig. 32), likely to be polysaccharide, which may reflect EPS production by the inoculated *R. loti* strain. This fibrous material was not observed in bacteroid-containing cells. A fibrous or gum-like matrix has also been observed in infection threads formed after inoculation of alfalfa with *R. meliloti* (Jordan *et al.*, 1963; Patel and Yang, 1981) and after inoculation of pea with *R. leguminosarum* bv. *viciae* (Newcomb, 1976). Such deposits are generally considered to be EPS derived from the bacterium (Dart, 1977) although, alternatively, the fibrous material may be polysaccharide produced by the plant, presumably in response to the invading rhizobia. The fibrous matrix present in infection threads is not observed to surround mature bacteroids after inoculation of alfalfa with *R. meliloti* (Jordan *et al.*, 1963; Patel and Yang, 1981) and after inoculation of pea with *R. leguminosarum* bv. *viciae* (Newcomb, 1976).

To extend the results discussed above to a second strain of *R. loti*, EPS mutants were isolated from PN4115 by Tn5 mutagenesis, followed by selection for a non-mucoid colony phenotype (Fig. 35). Interestingly, while these mutants were non-mucoid they were found to fluoresce when streaked onto Calcofluor-agar plates (Fig. 36A). When these EPS mutants were tested on *L. corniculatus*, a determinate nodulating legume, they, like PN4115, were fully effective (Section 3.4.6).



PN4115 is normally ineffective on the indeterminate nodulating legume *L. leucocephala*, although infection threads form, plant cells are invaded and, occasionally, nodules capable of a low level of nitrogen fixation are observed. However, while PN4115 induced the formation of both small, regular, ineffective nodular swellings (Fig. 44A) and larger, irregular, tumor-like nodules (Figs. 44B and C), which occasionally contained nitrogen-fixing bacteroids, the PN4115-derived EPS mutants induced the formation of only small, regular, ineffective nodular swellings, with either no (PN1313 and PN1314), or a reduced level (PN1312, Fig. 48) of invasion. This suggests that the step leading from the small, ineffective nodular swellings to the tumor-like nodules might require the production of EPS, while the Calcofluor-binding polysaccharide produced by the mutants may be able to partially substitute for EPS to allow the limited invasion, of the small, regular, ineffective nodular swellings by PN1312. However, the bacteria present in PN1312-invaded nodular swellings did not fix nitrogen.

Examination, by electron microscopy, of thin sections of *L. leucocephala* nodules formed after inoculation of plants with PN4115, revealed the presence of a large amount of fibrous material, presumably polysaccharide, surrounding the bacterial cells in both infection threads and invaded plant cells (Figs. 46, 47A, and 47B; previously described by Pankhurst *et al.*, 1987). No infection threads were identified in the nodular swellings formed on *L. leucocephala* after inoculation with the PN4115-derived EPS mutants. In addition, no fibrous material was present in *L. leucocephala* cells that had been invaded by the PN4115-derived EPS mutant PN1312 (Fig. 48). This might suggest that the fibrous material present in *L. leucocephala* nodules formed on plants inoculated with PN4115 is EPS derived from the bacterium. Alternatively, however, PN1312 may not be capable of synthesizing a (signal) molecule which induces polysaccharide production by the invaded plant cell.

## 4.2 COMPLEMENTATION OF THE MUTATIONS CARRIED BY THE PN184-DERIVED EPS MUTANTS

The construction of a gene library to the broad host range *R. loti* strain NZP2037 in the broad host range cosmid vector pLAFR1 (Friedman *et al.*, 1982) has been previously described (Chua *et al.*, 1985). To isolate pLAFR1 cosmids which complemented the mutation carried by each of the PN184-derived EPS mutants, the NZP2037-pLAFR1 gene library was crossed *en masse* with each mutant in a

triparental mating, and complemented colonies were selected by restoration of the Calcofluor-bright (and mucoid) phenotype on S20-Calcofluor agar. While no cosmids could be isolated which stably complemented the smooth PN184-derived EPS mutants PN1177, PN1178, and PN1179, a series of overlapping cosmids were isolated which complemented the mutation in each rough PN184 -derived EPS mutant (Section 3.2.5.1). A representative cosmid was retained for each rough EPS mutant (Fig. 14).

The PN184-derived EPS mutants were initially divided into two groups based upon colony characteristics, either smooth or rough. The rough PN184-derived EPS mutants were subdivided into two groups based upon the size of the *Eco*RI fragment hybridizing to <sup>32</sup>P-labelled pKan2 DNA in each case, 9.8 kb for the Group 2 mutants PN1180, PN1181, and PN1184, and 6.7 kb for the Group 3 mutants PN1182 and PN1183 (Fig. 12A, Table 4). The smooth PN184-derived EPS mutants PN1177, PN1178, and PN1179 (Group 1) all possessed a 9.0 kb *Eco*RI fragment hybridizing to <sup>32</sup>P-labelled pKan2 DNA (Fig. 12A, Table 4). In addition, each of the PN184-derived EPS mutants was shown to contain an independent Tn5 insertion (Fig. 13). Examination of the *Eco*RI digestion profiles of the cosmids complementing each rough PN184-derived EPS mutant demonstrated that common *Eco*RI fragments were present in the cosmids complementing the strains within each of Groups 2 and 3 (Fig. 14). However, no common *Eco*RI fragments were observed between the two groups of cosmids, confirming that two distinctive regions have been defined into which Tn5 had inserted. The observation that there were no common *Eco*RI digestion fragments between the two sets of cosmids suggests that the two genetic regions so identified, are not closely linked.

Each representative cosmid, complementing the mutation carried by a rough, PN184-derived EPS mutant, was used in cross complementation experiments with all the other PN184-derived EPS mutants (Table 5). The representative cosmids cross complemented the mutations carried by the PN184-derived EPS mutants within their individual group, both to a Calcofluor-bright, mucoid colony phenotype, and to fully effective nodulation on *L. leucocephala*, but did not complement the mutations carried by the PN184-derived EPS mutants in each of the other two groups. None of the cosmids complemented the mutations carried by the smooth PN184-derived EPS mutants (Group 1).

The representative cosmids (Fig. 14), complementing the mutations carried by each mutant in Group 2, contain a common, wild-type (NZP2037/PN184), *EcoRI* fragment, which, with the addition of Tn5, would match the size of the *EcoRI* fragment carrying Tn5 in each mutant. Similarly, the representative cosmids (Fig. 14), complementing the mutations carried by each mutant within Group 3, contain a common, wild type (NZP2037/PN184), *EcoRI* fragment, which, with the addition of Tn5, would match the size of the *EcoRI* fragment carrying Tn5 in each mutant. These common *EcoRI* fragments, from a cosmid (pPN32 and pPN33) complementing a member (PN1181 and PN1182) of each group have been cloned into the *EcoRI* site in pUC118 to generate plasmids pPN48 and pPN47 respectively. That the subcloned *EcoRI* fragments from the two cosmids do correspond to the wild type *EcoRI* fragments into which Tn5 has inserted in each mutant within the two groups, has been verified by the demonstration of appropriate mobility shifts after hybridization of the appropriate *EcoRI* subfragment to Southern blots of *EcoRI* digested total DNA from each mutant within the two groups, and *EcoRI* digested DNA from each complementing cosmid (Figs. 15 and 16).

In addition, the hybridization of the *EcoRI* fragment, subcloned in pPN47 and pPN48, to an *EcoRI* fragment of identical size in a digest of total DNA from *R. loti* strain PN4010, a plasmid cured derivative of NZP2037, demonstrated that the two presumptive genes (or gene regulatory regions) identified are located on the chromosome (Section 3.2.8, Figs. 15 and 16). In addition, work described in this thesis has also shown that PN4010 is Calcofluor-bright (Section 3.1), further demonstrating that the genes required for the synthesis of the Calcofluor-binding EPS are located on the chromosome. Chromosomally located genes required for EPS synthesis have similarly been reported for a number of *Rhizobium* species, including *R. meliloti* (Finan *et al.*, 1986, Leigh *et al.*, 1985, Doherty *et al.*, 1988) and *R. leguminosarum* bv. *phaseoli* (Diebold and Noel, 1989).

Cosmids complementing the mutations carried by each of the smooth, PN184 derived EPS mutants (Group 1) could not be isolated (Section 3.2.5.1). Several potential reasons exist to explain non-complementation. For example, the library may be incomplete, the mutations may be dominant, or expression of the gene(s) on pLAFR1 may result in imbalances in enzyme concentrations which are lethal or which interfere with EPS synthesis (Diebold and Noel, 1989). Nevertheless, the observations that the *EcoRI* fragment carrying Tn5 from each smooth mutant is of

identical size (Fig. 12A), and that the  $^1\text{H}$ -NMR spectra of the EPS isolated from each smooth mutant are essentially identical (Fig. 17), suggest that the respective mutations carried by the three mutants in this group are in the same presumptive gene, or gene regulatory region.

These results, therefore, suggest that Tn5 insertion has resulted in mutations at three distinct loci, all three showing a correlation between mutation, defective EPS synthesis, and ineffective nodulation of *Leucaena leucocephala*. Since the cosmids complementing the mutations carried by the Group 2 and Group 3 rough mutants restored both EPS production and the wild type proficiency for effective nodulation, it is clear that the mutations causing the EPS<sup>-</sup> phenotype of these mutants were responsible for ineffective nodule development on *L. leucocephala*. It is possible the the mutations have a pleiotropic effect on polysaccharides additional to EPS. However, the correlation observed strongly suggests a requirement for EPS synthesis for effective nodulation of *L. leucocephala*, and conversely that EPS synthesis is not required for effective nodulation of *Lotus pedunculatus*.

#### 4.3 THE BIOCHEMICAL CHARACTERIZATION OF THE MUTATIONS

Preliminary experiments demonstrated that *R. loti* strain PN184 was mucoid and Calcofluor-bright (Fig.4). Subsequently, precipitated PN184 EPS was also shown to fluoresce in the presence of Calcofluor (Fig. 21). Current evidence therefore suggests that PN184, like *R. meliloti* strain Rm1021 (Finan *et al.*, 1985; Leigh *et al.*, 1985), synthesizes a Calcofluor-binding EPS. In contrast, neither the PN184-derived EPS mutants (Fig. 8), nor precipitated EPS from the PN184-derived EPS mutants (Fig. 21, Section 3.2.9.6) fluoresced on Calcofluor agar.

Interestingly, while both PN4115 (Fig. 4), and precipitated PN4115 EPS (Section 4.5.3), failed to fluoresce on Calcofluor agar, the PN4115-derived EPS mutants PN1312, PN1313, and PN1314 were all Calcofluor-bright (Fig. 36A). The three mutants, however, failed to produce any cetrinide precipitable EPS in broth culture (Section 3.4.5.1) and are non-mucoid on agar plates (Fig. 35). This suggests that the polysaccharide responsible for the Calcofluor-bright phenotype of the PN4115-derived EPS mutants might be quite different to the cetrinide precipitable, Calcofluor-binding EPS of PN184. Smit *et al.* (1987) have reported the isolation of cellulose fibril overproducing strains of *R. leguminosarum* strain 248 which

fluoresce on Calcofluor agar (the parent strain shows low fluorescence). Kneen and LaRue (1983) have shown that cellulose producing *Rhizobium* and *Agrobacterium* strains grow with an intense red colour on Congo red agar. The intense red staining of the PN4115-derived EPS mutants on Congo red-agar (Fig. 36B) therefore suggests that they may be producing cellulose fibrils. The production of cellulose fibrils by the PN4115-derived EPS mutants would explain their Calcofluor-bright phenotype. Neither PN4115 (Fig. 36B), nor PN184 (nor the PN184-derived EPS mutants, Fig. 10B) stained red in the presence of Congo red, suggesting that these strains do not produce cellulose. Current evidence, therefore, suggests that the Calcofluor-binding polysaccharides produced by PN184 (EPS) and by the PN4115-derived EPS mutants (putatively cellulose) are quite different.

The EPS produced by PN184 has been previously examined by Pankhurst *et al.* (1982) and Bailey *et al.* (1971). These studies have identified glucose, galactose, and uronic acid as components of PN184 EPS. The same components were identified in this study (Section 3.2.9.5), with the addition of a component co-migrating with ribose in a butanol:pyridine:water solvent system (Figs. 20A and 20B).

The EPS produced by PN184 and the EPS produced by each of the smooth, PN184-derived EPS mutants (Group 1, PN1177, PN1178, and PN1179) was examined by  $^1\text{H}$ -NMR spectroscopy. The most prominent difference in the spectra was the significant reduction in the magnitude of the signals arising from the methyl protons of *O*-acetate modifications to the EPS produced by the smooth PN184-derived EPS mutants (Fig. 17, Section 3.2.9.3). This suggests that there may be a requirement for a specific degree of acetylation, or acetylation of specific sugar residues for EPS to function in the *R. loti*-*L. leucocephala* symbiosis. These implications are discussed more fully in Section 4.4.

Examination of the  $^1\text{H}$ -NMR spectra of EPS from PN184 and EPS from the smooth, PN184-derived EPS mutants has also shown that, in addition to a reduction in *O*-acetylation, further differences are also observed (Section 3.2.9.3). These include, the relative magnitude of the signals in the  $\beta$ -anomeric proton region, and the presence of a group of signals between 4.75 ppm and 4.90 ppm in the  $^1\text{H}$ -NMR spectra of EPS from the smooth, PN184-derived EPS mutants. However, the structure of the EPS produced by each strain has not been characterized sufficiently to assign all the  $^1\text{H}$ -NMR signals to specific structural

features of each EPS. Determination of the complete structure of the EPS produced by PN184 will be required to allow significant expansion of this work.

The  $^1\text{H}$ -NMR spectrum of PN4115 EPS (Fig. 39B), while indicating *O*-acetylation to approximately the same extent as PN184 EPS (Fig. 39A), shows other features similar to the  $^1\text{H}$ -NMR spectra of EPS produced by the smooth, PN184-derived EPS mutants (Fig. 39C, Section 3.4.5.2). These features include, the relative magnitude of signals in the  $\beta$ -anomeric proton region, and the presence of a group of signals between 4.75 ppm and 4.90 ppm. The  $^1\text{H}$ -NMR spectrum of PN4115 EPS differs, however, to the  $^1\text{H}$ -NMR spectra of both PN184 EPS and EPS from the smooth, PN184-derived EPS mutants in lacking signals at 1.24 ppm and 2.62 ppm, suggesting that EPS from PN4115 does not carry the 3-hydroxybutanoic acid residues tentatively identified as modifications to EPS from PN184 and the smooth, PN184-derived EPS mutants.

The EPS produced by each of three PN184-derived *L. pedunculatus* infection mutants (PN1018, PN1019, and PN1027, Chua *et al.*, 1985) was also examined (Fig. 19, Section 3.2.9.4). All three strains were Calcofluor-bright, indicating that all three strains are capable of producing a Calcofluor-binding EPS. Examination of the  $^1\text{H}$ -NMR spectra of PN1019 EPS and PN1027 EPS, has failed to identify any significant differences to the EPS produced by PN184. Indeed, given that the PN184-derived EPS mutants are fully effective on *L. pedunculatus*, differences in EPS structure between PN184 and the PN184-derived *L. pedunculatus* infection mutants would not be expected. However, examination of the  $^1\text{H}$ -NMR spectrum of EPS from PN1018 indicated that the EPS produced by this strain was less acetylated than the EPS produced by PN184, although the reduction in *O*-acetylation was not to the same extent as that observed for the smooth, PN184-derived EPS mutants. Otherwise,  $^1\text{H}$ -NMR spectra of PN1018 EPS resembled  $^1\text{H}$ -NMR spectra of EPS from PN184, rather than the  $^1\text{H}$ -NMR spectra of EPS from either the smooth, PN184-derived EPS mutants, or PN4115. For example, the relative magnitude of the signals in the  $\beta$ -anomeric proton region for PN1018 EPS was essentially identical to the relative magnitude of the signals in the  $\beta$ -anomeric proton region for PN184 EPS.

These results suggest that the reduction in *O*-acetylation observed for the smooth PN184-derived EPS mutants is not responsible, in itself, for the inability of these strains to bind Calcofluor, since PN1018 EPS also shows reduced *O*-acetylation,

but binds Calcofluor. Reduced *O*-acetylation of PN1018 EPS may, however, play some role in the Nod<sup>-</sup> phenotype of this strain on *L. leucocephala*. In addition, reduced *O*-acetylation is consistent with PN1018 carrying a mutation with pleiotropic effects, possibly affecting the *O*-acetylation of several polysaccharides (discussed more fully later in this Section).

Jones *et al.* (1987) have shown that *R. loti* strain NZP2037 (the streptomycin sensitive parent strain to PN184) produces a peptidoglycan-bound polysaccharide (PBP) which is associated with the resistance of this strain to prodelphinidin-rich flavolans (tannins). Prodelphinidin-rich flavolans are present in the roots of, for example, *L. pedunculatus* and *L. leucocephala*, but are not present in the roots of *L. corniculatus* var. *cree* (Pankhurst *et al.*, 1987). In contrast, NZP2213 (the streptomycin sensitive parent strain to PN4115) and PN1018 do not produce the PBP and are sensitive to prodelphinidin-rich flavolans (Jones *et al.*, 1987). NZP2213 is ineffective on both *L. pedunculatus* and *L. leucocephala*, and is fully effective on *L. corniculatus* var. *cree* as expected if resistance to prodelphinidin-rich flavolans is required *in planta* (Pankhurst *et al.*, 1987). PN1018, however, is ineffective on both *L. pedunculatus* and *L. corniculatus* (var *cree*), and is Nod<sup>-</sup> on *L. leucocephala* (G. Hotter, D. B. Scott, C. Young, unpublished observations) suggesting that, either the mutation in PN1018 has a pleiotropic effect (as mentioned above), or that the hypothesis of PBP requirement is invalid. In any case the work described in this thesis suggests that, if resistance to prodelphinidin-rich flavolans is required, then this requirement occurs after the requirement for EPS, since PN4115 is able to invade *L. leucocephala* nodules. Subsequent development may then be inhibited by the sensitivity of PN4115 to the prodelphinidin-rich flavolans present in *L. leucocephala* cells.

Examination of the <sup>1</sup>H-NMR spectra of EPS from all the *R. loti* strains examined in this Thesis indicated that signals consistent with the modification of the EPS produced by each strain with succinate were lacking. Additional support for the absence of succinate modifications to *R. loti* EPS may derive from the observation that *exoH* mutants of *R. meliloti* Rm1021, which produce EPSI lacking succinate modifications, lack the fluorescent halo observed for the parental strain on Calcofluor agar (Leigh *et al.*, 1987). Similarly, all the Calcofluor-bright *R. loti* strains examined here also lack a fluorescent halo on Calcofluor agar (Figs. 4, 5, and 36A). Leigh *et al.* (1987) have suggested that the haloless phenotype of *R. meliloti* *exoH* mutants may arise due to the failure of an endoglycosidase to degrade the non-succinylated EPS to diffusable Calcofluor-binding subfragments.

Subsequently, Leigh and Lee (1988) have shown that *R. meliloti* produces both high and low molecular weight forms of EPSI, and that *exoH* mutants produce little low molecular weight EPSI, but have also shown that the low molecular weight form of EPSI binds Calcofluor only weakly.

Similarly, examination of the  $^1\text{H}$ -NMR spectra of EPS from all the *R. loti* strains examined in this Thesis indicated that signals consistent with the modification of the EPS produced by each strain with pyruvate were lacking. Pyruvate modifications to EPS have been identified for all other *Rhizobium* species examined (Aman *et al.*, 1981; Canter-Cremers *et al.*, 1991; Djordjevic *et al.*, 1986; Hollingsworth *et al.*, 1988; Kuo and Mort, 1986; McNeil *et al.*, 1986). However, pyruvate modifications are not present on the EPS produced by *B. japonicum* USDA110 (Puvanesarajah *et al.*, 1987).

It is possible that the EPS mutations selected by Tn5 mutagenesis of PN4115 and PN184, may affect the synthesis of more than one (exo)polysaccharide. For example, it is possible that Calcofluor, rather than being bound by the cetrimide-precipitable EPS produced by PN184, is bound by a neutral (capsular) polysaccharide such as cellulose. This work has, however, shown that the cetrimide precipitated EPS produced by PN184 fluoresces when dissolved in water and applied to a Calcofluor-agar plate (Fig. 21). Nevertheless, a precedent for the synthesis of more than one EPS exists for *R. meliloti* SU47, which produces an acidic, Calcofluor-binding EPS (EPSI or EPSa) and a second acidic EPS (EPSII or EPSb), which can substitute for EPSI on some hosts (Glazebrook and Walker, 1989; Zhan *et al.*, 1989).

Fractionation of the cetrimide precipitated EPS from PN184 on DEAE-Sephacel columns resulted in the separation of this material into two anthrone reactive, polysaccharide fractions, the first eluting in 1 mM sodium chloride, and the second with a peak elution in 330 mM sodium chloride (Fig. 22, Section 3.2.9.7.1). The EPS present in these fractions was examined by  $^1\text{H}$ -NMR spectroscopy, with the fractions producing very similar spectra. DEAE-Sephacel has an exclusion limit of approximately  $1 \times 10^6$  Da suggesting that the polysaccharide fractions obtained represent high molecular weight (eluting in 1 mM sodium chloride) and low molecular weight (eluting in 330 mM sodium chloride) fractions of a single EPS, where the low molecular weight fraction corresponds to the repeating subunit of the high molecular weight polymer. These results also indicate that PN184



produces more low molecular weight material than high molecular weight material (fraction ratio 8.2:1.0). Fractionation of EPS from *R. meliloti*, strain Rm1021, (Leigh and Lee, 1988), and from *Rhizobium* sp. NGR234 (Gray *et al.*, 1991), on Biogel-A5m (exclusion limit approximately  $5 \times 10^6$  Da) has also identified high and low molecular weight anthrone reactive material, with essentially identical  $^1\text{H}$ -NMR spectra. The low molecular weight *R. meliloti* material, which was the dominant fraction, corresponded to the repeat units of the high molecular weight polymer (Leigh and Lee, 1988), and can be separated into the monomer, trimer and tetramer of the repeating unit (Gray *et al.*, 1991).

When dissolved in water and applied to a TY Calcofluor-agar plate, both the high and low molecular weight fractions of PN184 EPS fluoresce, but neither fraction as intensively as unfractionated EPS (Section 3.2.9.7.1). Leigh and Lee (1988) have shown that when the high and low molecular weight fractions of *R. meliloti* Rm1021 EPS are similarly spotted onto a LB Calcofluor agar plate, only the high molecular weight fraction fluoresces brightly, while the low molecular weight fraction fluoresces only weakly, with the fluorescence of the low molecular weight fraction fading over time.

Since capsular polysaccharide isolated from PN184 fails to fluoresce when dissolved in water and applied to a TY-Calcofluor agar plate (Section 3.2.11), the cetrimide precipitated EPS (and its constituent high and low molecular weight fractions separated by DEAE-Sephacel chromatography) remains the only polysaccharide yet isolated from PN184 which has been shown to bind Calcofluor.

Cetrimide precipitated EPS from PN1177 was also fractionated through DEAE-Sephacel columns, resulting in the separation of this material into a major fraction with a peak elution at 200 mM sodium chloride (low molecular weight) and a minor fraction eluting in 1 mM sodium chloride (high molecular weight, Fig. 24, Section 3.2.9.7.2). PN1177, however, produced little high molecular weight material in comparison to PN184 (ratio, low  $M_r$  fraction:high  $M_r$  fraction 11.8:1.0). Column retention was, however, more extensive than observed for PN184 EPS, with elution of only 62% of the material loaded onto the column shown in this Thesis, compared to 82% for PN184 EPS. The change in the peak elution position of the low molecular weight EPS fraction (PN1177, 200 mM sodium chloride; PN184, 330 mM sodium chloride) is consistent with the reduction in *O*-acetate modifications observed for PN1177 EPS, which might be expected to

result in a reduced charge compared to PN184 EPS. Additional differences between the  $^1\text{H}$ -NMR spectra of PN1177 EPS and PN184 EPS (as discussed earlier in this Section) may also be correlated with altered charge. However, the observation that PN4115 EPS has a similar  $^1\text{H}$ -NMR spectrum to PN1177 EPS, with the exception of the degree of *O*-acetylation, which is in the same range as that observed for PN184 EPS, and that the low molecular fraction of PN4115 EPS eluting after DEAE-Sephacel chromatography, like that of PN184 EPS, has a peak elution at 330 mM sodium chloride, argue against this possibility. It therefore appears likely that the reduction in *O*-acetylation alone may account for the reduced charge of PN1177 EPS.

As mentioned above, DEAE-Sephacel chromatography of PN4115 EPS resulted in fractionation of the EPS into a major fraction eluting at 330 mM sodium chloride (low molecular weight fraction) and a small fraction eluting in 1 mM sodium chloride (high molecular weight fraction, Fig. 40, Section 3.4.5.4). PN4115, therefore, unlike PN184, and like PN1177 produces only a small amount of high molecular weight EPS (ratio, low  $M_r$  fraction:high  $M_r$  fraction 22.0:1.0). Like PN1177 EPS, however, column retention of PN4115 EPS was higher than that observed for PN184 EPS, with elution of only 64% of the material loaded onto the column shown in this Thesis. The EPS profile (considering non-fluorescence on Calcofluor agar,  $^1\text{H}$ -NMR spectra of precipitated EPS, and DEAE-Sephacel fractionation of precipitated EPS) of PN4115 therefore resembles the EPS profile of the smooth, PN184-derived EPS mutants, with the exception of the comparatively high degree of *O*-acetylation of PN4115 EPS and its apparent lack of modification with hydroxybutanoic acid. Furthermore, both PN4115, and all the PN184-derived EPS mutants produce yellow-cream coloured colonies on TY- and YM-agars (Fig. 6, Section 3.2.2). In contrast, PN184 produces white coloured colonies (Fig. 6). These results suggest that the white colouration of colonies may require specific structural characteristics of EPS.

The biochemical effect of the EPS mutation carried by each of the EPS mutants described in this Thesis has, therefore, been (at least partially) defined. It is not possible, however, to define the point in the biochemical pathway for EPS synthesis, where each mutation has effect. Some general comments are, however, appropriate, after first considering, briefly, the biochemical pathway for EPS synthesis.

Many bacterial exopolysaccharides, including xanthan gum and *R. meliloti* EPSI, are synthesized on isoprenoid lipid carriers in the cytoplasmic membrane as oligosaccharide subunits which are subsequently polymerized (Shibaev, 1986; Sutherland, 1985). *Xanthomonas campestris* *gumD* mutants, for example, fail to add glucose, which is the first sugar residue in xanthan gum unit biosynthesis, to the lipid carrier (Reed *et al.*, 1991). Reed *et al.* (1991) have reported that ExoY from *R. meliloti* and *Rhizobium* sp. NGR234, and Pss2 from *R. leguminosarum* bv. *phaseoli* are related to GumD, and may therefore have a similar function. An additional point of interest is that the *R. meliloti* EPSI subunit octasaccharide can be substituted with either pyruvyl or acetyl residues before polymerization (Tolmasky *et al.*, 1982). Furthermore, both peptidoglycan polysaccharides, and lipopolysaccharides are also synthesized on the same isoprenoid lipid carriers (Sutherland, 1982, 1985).

Given that the *R. meliloti* octasaccharide can be substituted with acetyl residues prior to polymerization, reduced *O*-acetylation of the EPS produced by each smooth, PN184-derived EPS mutant (Fig. 17, Section 3.2.9.3) may conceivably reduce the efficiency of polymerization. Interestingly, PN1177 appears to produce only a small amount of high molecular weight EPS (Fig. 24, Section 3.2.9.7.2). The reduction in *O*-acetylation observed may be caused by insertion of Tn5 into a gene encoding an acetyl transferase. Alternatively, the decrease in *O*-acetylation may be caused by the inability of the smooth, PN184-derived EPS mutants to add either a (or more than one) side chain glycosyl residue, or a main chain glycosyl residue where that glycosyl residue is *O*-acetylated. Loss of an *O*-acetylated glycosyl residue from either the main or side chain, but particularly loss from the main chain, could inhibit polymerization. It appears likely, after consideration of similarities (previously discussed) in the <sup>1</sup>H-NMR spectra of EPS from PN4115 and the smooth PN184-derived EPS mutants, that the EPS's produced by these strains have, at least, a very similar main chain and side chain structure. Furthermore, both PN1177 and PN4115 produce little high molecular weight EPS. This observation may add weight to the possibility that the mutation carried by each of the smooth PN184-derived EPS mutants, results in the inability of these strains to add a main chain, or side chain glycosyl residue to the oligosaccharide, which results in the inhibition of polymerization.

The rough, PN184-derived EPS mutants, and the PN4115-derived EPS mutants, fail to synthesize any EPS. Possible activities for the proteins encoded or controlled

by the genetic regions identified by Tn5 insertion in each of the mutants include functions prior to the first glycosyl transferase reaction or functions as glycosyl transferases, possibly including the initial glycosyl transferase which would transfer the first glycosyl residue to the isoprenoid lipid carrier. It is, however, not possible, from the results presented in this Thesis, to distinguish between these and other possibilities. However, the observation that the PN4115-derived EPS mutants are Calcofluor bright and may produce cellulose, suggests that PN4115 might carry a gene encoding a product responsible, both for inhibiting the production of a Calcofluor binding polysaccharide associated with the capsule (possibly cellulose), and also responsible for the positive regulation of EPS synthesis. Alternatively, the blocking of the biochemical pathway for EPS biosynthesis might allow the redirection of component sugar molecules to an alternative pathway (of cellulose biosynthesis) which is normally repressed.

The *L. pedunculatus* infection mutant PN1018 also shows reduced *O*-acetylation of its EPS (Fig. 19, Section 3.2.9.4). As previously discussed, additional features of the  $^1\text{H}$ -NMR spectrum of PN1018 EPS resemble the  $^1\text{H}$ -NMR spectrum of PN184 EPS rather than the  $^1\text{H}$ -NMR spectra of EPS from the smooth, PN184-derived EPS mutants. This suggests that the reduced *O*-acetylation of PN1018 EPS, and reduced *O*-acetylation of EPS from the smooth, PN184-derived EPS mutants, has been caused by insertion of Tn5 into (at least) two separate genetic regions. Furthermore, the fact that both EPS and peptidoglycan polysaccharides are both synthesized on isoprenoid lipid carriers may offer a logical basis for the possibility that the mutation carried by PN1018 affects the synthesis of several polysaccharides, including the PBP required for resistance to plant flavonols (Jones *et al.*, 1987).

#### 4.4 THE REQUIREMENTS FOR FUNCTIONAL EPS

Both PN184 and PN4115 are capable of inducing the formation of infection threads on *L. leucocephala*, invading the plant cells, proliferating within the invaded plant cells, and at least in the case on PN184, differentiating into bacteroids (Sections 3.2.12 and 3.4.6). Release of PN4115 into plant cells is however abnormal (this work, Pankhurst *et al.*, 1987). Both PN184 and PN4115 produce a highly acetylated EPS in comparison to the reduced level of acetylation observed for the EPS produced by each of the smooth, PN184-derived EPS mutants. The PN184-derived EPS mutants are apparently incapable of inducing

the formation of infection threads on *L. leucocephala*, and, therefore, subsequent invasion of plant cells. This suggests that a highly acetylated EPS, possibly modified with *O*-acetyl residues at specific locations, may be required for EPS function during infection thread formation and invasion of *L. leucocephala* nodules. Similarly, specific, non-carbohydrate modifications to the repeating unit of *R. meliloti* EPSI are required for effective nodulation to proceed. *ExoH* mutants of *R. meliloti* synthesize EPSI lacking succinate modifications and induce ineffective nodules on alfalfa (Leigh *et al.*, 1987). Infection threads are observed but abort in the nodule cortex (Leigh *et al.*, 1987). Subsequent work (Leigh and Lee, 1988) has, however, shown that *exoH* mutants, in addition to lacking succinate modifications, also produce only a small amount of low molecular weight EPS. In addition, Muller *et al.* (1988) have isolated a *R. meliloti* mutant that synthesises EPSI lacking pyruvate modifications which also induces ineffective nodules on alfalfa. In contrast to *R. meliloti* *exoH* mutants, however, these mutants fail to induce the formation of infection threads (Muller *et al.*, 1988). However, while Skorupska *et al.* (1985) and Philip-Hollingsworth *et al.*, (1989) have presented evidence suggesting that *O*-acetate modifications might be important in the *R. leguminosarum* bv. *trifolii*-clover symbiosis, *R. meliloti* EPSI mutants with an altered frequency of *O*-acetylation have not been reported.

The apparent requirement for specific non-carbohydrate modifications to EPS suggests that EPS (presumably a low molecular weight oligosaccharide subunit) may function as a signal molecule during nodule invasion (and possibly during early nodule development, after nodule initiation). That *Rhizobium* produced oligosaccharide signal molecules are involved in the symbiosis has been established for a sulphated, acylated, glucosamine oligosaccharide (lipo-oligosaccharide) signal molecule produced by *R. meliloti* that is required for root hair curling and cortical cell division on alfalfa (Lerouge *et al.*, 1990; Truchet *et al.*, 1991). Interestingly, Truchet *et al.* (1991) have shown that *R. meliloti* culture supernatants contain two forms of the lipo-oligosaccharide signal molecule, NodRm-1, the form shown in Figure 2A of this thesis, and Ac-NodRm-1, which is *O*-acetylated at carbon 6 of the terminal non-reducing sugar. Truchet *et al.* (1991) have also shown that a mixture of NodRm-1 and Ac-NodRm-1 is more active in eliciting nodule formation than NodRm-1 alone, suggesting that Ac-NodRm-1 may be more active than NodRm-1. Similarly, a *R. meliloti* *nodL* mutant exhibits delayed nodulation and produces NodRm-1 but not Ac-NodRm-1 (Truchet *et al.*, 1991), consistent with the observation that the putative *nodL* gene product has

sequence similarity with bacterial acetyl transferases (Downie, 1989). More recently, Spaink *et al.* (1991) have shown that *R. leguminosarum* bv. *viciae* also produces lipo-oligosaccharide signal molecules. Spaink *et al.* (1991) have shown that the *R. leguminosarum* bv. *viciae* NodL protein, like that of *R. meliloti* (Truchet *et al.*, 1991), is involved in the addition of an *O*-acetyl group to carbon 6 of the terminal non-reducing sugar. In addition, Spaink *et al.* (1991) have also shown that the NodE protein is required for the production of the highly unsaturated fatty acid moiety present on the lipo-oligosaccharide. In the absence of NodE a monounsaturated fatty acid was present instead (Spaink *et al.*, 1991). Spaink *et al.* (1991) have shown that the *O*-acetyl modification and the presence of the highly unsaturated fatty acid moiety are required for the lipo-oligosaccharide to have nodule meristem-inducing activity at nanomolar concentrations, while all lipo-oligosaccharides tested were able to elicit root hair deformation at nanomolar concentrations.

A requirement for another signal molecule during invasion (and subsequent nodule development) may therefore not be surprising, and indeed the mutation in the smooth, PN184-derived EPS mutants may also be in a gene encoding an acetyl transferase. In addition, Puhler *et al.* (1991) have recently reported that an EPS mutant of *R. meliloti* induces a localised plant defense response, and have suggested that EPS-derived oligosaccharides may act to suppress plant defense responses. Furthermore, coinoculation of *Rhizobium* sp. NGR234 EPS mutants with either *Rhizobium* sp. NGR234 EPS, or the repeat unit of *Rhizobium* sp. NGR234 EPS, enabled the EPS mutants to induce the formation of fully effective nodules on *L. leucocephala* (Djordjevic *et al.*, 1987).

Interestingly, both PN4115 and the smooth PN184-derived EPS mutants show consistent, similar features in the  $^1\text{H}$ -NMR spectra of their cetrinide precipitated EPS, suggestive of structural similarity. As discussed in Section 4.3, these features include the relative magnitude of signals in the  $\beta$ -anomeric proton region, and the presence of a group of signals between 4.75 ppm and 4.9 ppm. The failure of the EPS isolated from the smooth, PN184-derived EPS mutants, and of EPS isolated from PN4115, to bind Calcofluor is consistent with these structural similarities. However, the differences observed between these regions in the  $^1\text{H}$ -NMR spectrum of PN184 EPS, compared to these regions in the  $^1\text{H}$ -NMR spectra of EPS from the smooth, PN184-derived EPS mutants and EPS from PN4115, are not apparently related to the inability of the smooth PN184-derived EPS mutants to

induce the formation of infection threads on (and invade) *L. leucocephala* since PN4115 does induce the formation of infection threads, and invades *L. leucocephala*. These features may, however, be related to the much reduced ability of PN4115 to proceed past invasion, to fully effective nodulation. This must, however, be balanced against the observation that NZP2213 (the streptomycin sensitive parent strain to PN4115) fails to produce a PBP which is required for protection against prodelphinidin rich flavolans present in the plant cell (Jones *et al.*, 1987). The PBP may, therefore, be required for effective nodulation of both determinate and indeterminate nodulating hosts which produce prodelphinidin rich flavolans (Jones *et al.*, 1987).

It is also apparent that effective nodulation (invasion) of indeterminate nodulating legumes requires the production of both high and low molecular weight EPS. *ExoG* and *exoJ* mutants of *R. meliloti* produce no, or little, high molecular weight EPS respectively, and form effective nodules at low frequency compared to the wild type strain (Long *et al.*, 1988), while, in contrast, EPS<sup>-</sup> mutants form effective nodules only at a very low frequency. Similarly, both PN4115 and PN1177 produce only a small amount of high molecular weight EPS (Sections 3.4.5.4 and 3.2.9.7.2). PN4115 is able to effectively nodulate *L. leucocephala* only at a low frequency, while PN1177 is unable to invade *L. leucocephala*.

The observation that, after invasion by PN4115, *L. leucocephala* cells contain a large amount of fibrous material (Figs. 46, 47A, and 47B), which is not present in cells containing PN184 bacteroids, suggests that PN4115 might be unable to correctly regulate the production of its EPS during invasion. This fibrous material is present only in infection threads of *L. leucocephala* nodules formed after inoculation with PN184 or the PN184-derived EPS mutants carrying their complementing cosmids (Fig 32). As discussed in Section 4.1, a fibrous matrix has also been observed in infection threads formed after inoculation of alfalfa with *R. meliloti* and after inoculation of pea with *R. leguminosarum* bv. *viciae*, but is not observed to surround bacteroids (Jordan *et al.*, 1963; Patel and Yang, 1981; Newcomb, 1976). The PN4115-derived EPS mutant PN1312 is able to partially invade cells within the nodular swellings formed on *L. leucocephala* after inoculation with this strain, but no fibrous material is observed (Fig. 48), consistent with the EPS<sup>-</sup> phenotype of this strain. Similarly, Borthakur *et al.* (1985) have suggested that repression of the synthesis of *R. leguminosarum* bv. *phaseoli* EPS (by *psi*) may be required in the bacteroid state for effective nodulation of

*Phaseolus* bean, even though *Phaseolus* bean is a determinate nodulating legume not requiring the production of EPS by *R. leguminosarum* bv. *phaseoli* for effective nodulation (Borthakur *et al.*, 1986). More recently, Latchford *et al.* (1991) have shown that *psi* is indeed expressed by bacteroids in *Phaseolus* nodules. *ExoX* of *R. meliloti* Rm1021 resembles *psi*, in that, when it is present in multiple copies, its activity results in inhibition of EPSI production (Zhan and Leigh, 1990). However, *exoX* mutants form normal nodules, suggesting that inhibition of EPSI synthesis by *exoX* in the nodule is not required for effective nodulation (Zhan and Leigh, 1990). More recently Reuber *et al.* (1991) have shown that (at least) several *R. meliloti* Rm1021 *exo* genes (*exoF*, *exoA*, and *exoP*) are highly expressed in the early symbiotic zone, but are expressed at either a low level, or not at all, in both the late symbiotic zone and the bacteroid zone, of alfalfa nodules. Similarly, *pssA* of *R. leguminosarum* bv. *phaseoli* is not expressed by bacteroids in *Phaseolus* nodules (Latchford *et al.*, 1991). In addition, *exoR94* strains of *R. meliloti* Rm1021, which overproduce EPSI, are Fix<sup>-</sup> on alfalfa unless they carry additional mutations suppressing EPSI synthesis (Doherty *et al.*, 1988), so it is possible that *exoR* is involved in suppressing the synthesis of EPSI in the nodule. Furthermore, Tully and Terry (1985) have shown that *B. japonicum* bacteroids produce little or no EPS, and that this reduction in EPS synthesis might be in response to the low free oxygen content of soybean nodules. Together, these results suggest that EPS synthesis is only required during nodule invasion, and that EPS, therefore, has a specific role during this stage. These results correlate well with the observation that EPS mutants are typically blocked in nodulation at the point of invasion of their indeterminate nodulating host legumes.

#### 4.5 LIPOPOLYSACCHARIDE ANALYSIS

During the course of this work lipopolysaccharide synthesis, by both wild type and mutant *R. loti* strains, was examined by SDS-PAGE of phenol water extracts.

Considerable variation in the SDS-PAGE profiles of LPS from different *Rhizobium* species has been observed. After electrophoresis through 0.1% SDS-polyacrylamide gels, and PA-silver staining, PN184 LPS is observed as a ladder of doublet bands ("rungs") of increasing molecular weight (Fig. 25A), although gels with only singlet bands have been obtained. The upper band of each doublet is invariably more intensively stained than the lower band. Increasing the SDS concentration to 0.5% eliminates the doublets, and instead a ladder of singlet bands



("rungs") of increasing molecular weight is observed (Fig. 34). This banding pattern closely resembles that of LPS from *R. leguminosarum* bv. *viciae* 128C563 and *R. leguminosarum* bv. *trifolii* 0403 (Carlson, 1984). Similarly, the banding pattern observed resembles that obtained for LPS from *B. japonicum*, except that for *B. japonicum* a ladder of triplet bands of increasing molecular weight was observed in 0.1% SDS gel systems, which was reduced to a ladder of singlet bands of increasing molecular weight upon increasing the SDS concentration to 1.0 % (Puvanesaragah *et al.*, 1987). This triplet to singlet transition upon increasing the SDS concentration from 0.1% to 1.0% suggests that the triplets arose from different aggregation states of the LPS (multimeric forms of LPS), rather than sequentially increasing substitution of core LPS with O-antigen repeats (Puvanesaragah *et al.*, 1987). Similarly, increasing the SDS concentration from 0.1% to 0.5% resulted in the elimination of apparent high molecular weight aggregate bands during SDS-PAGE of LPS from *E. coli* 00114 (Peterson and McGroarty, 1985).

Sindhu *et al.* (1990) have shown that *R. leguminosarum* bv. *phaseoli* 8002 LPS migrates as a ladder of doublet bands, while *R. leguminosarum* bv. *phaseoli* CFN42 LPS migrates as two primary bands, corresponding to the complete LPS and LPS lacking the O-antigen (Cava *et al.*, 1989). Sindhu *et al.* (1990) have suggested that the doublet bands may arise due to modifications to the non-reducing end of the O-antigen region of the LPS. Alternatively, there may be two different forms of core polysaccharide which confer different mobilities when the same number of O-antigen groups are attached, thereby generating two families of distinctive LPS molecules (Sindhu *et al.*, 1990).

The large molecular weight interval between the "rungs" of the ladder of PN184 LPS bands (Figs. 25A and 34) is quite unlike that obtained for LPS carrying sequentially increasing substitution with O-antigen repeats. Where a ladder of LPS bands of increasing molecular weight is observed due to the substitution of core LPS with discrete O-antigen repeats, the molecular weight intervals observed after SDS-PAGE are considerably less (see for example Carlson, 1984; and Peterson and McGroarty, 1985) than those observed after SDS-PAGE of LPS from PN184.

The most likely explanation for the overall banding pattern of PN184 LPS, therefore, is that the doublet bands represent different aggregate forms of LPS, where the LPS has a complex, and large, O-antigen polysaccharide rather than a

repeating O-antigen oligosaccharide, resulting in a large molecular weight interval between the "rungs" of the ladder. The observation that PN184 LPS has a very low level of 2-keto-3-deoxyoctanoic acid (Section 3.2.10) may similarly suggest that PN184 LPS carries a substantial amount of O-antigen.

Ignoring minor variations in the intensity of band staining, there were no gross differences in LPS profiles between PN184 and the PN184-derived EPS mutants (Fig. 25A, Section 3.2.10). However, all the PN184-derived EPS mutants were resistant to the virulent *R. loti* bacteriophage  $\Phi$ 2037/1 (Fig. 11). Resistance of certain *R. meliloti* EPS mutants (*exoB*, *exoC*, and *exoE*) to virulent *R. meliloti* bacteriophages has similarly been described (Finan *et al.*, 1985, Leigh *et al.*, 1985). However, subsequent work has demonstrated that LPS from each of these mutants has different banding patterns to the LPS from the parent strain (Leigh and Lee, 1988; Clover *et al.*, 1989), suggesting that the bacteriophage resistance shown by these strains was due to altered LPS. Recently, Buendia *et al.* (1991) have shown that *R. meliloti* ExoB functions as a UDP-glucose 4-epimerase (which interconverts UDP-glucose and UDP-galactose), fully accounting for the multiple surface carbohydrate defects shown by *exoB* mutants (including phage resistance properties) since galactose is present in EPSI (Aman *et al.*, 1981), EPSII (Glazebrook and Walker, 1989; Zhan *et al.*, 1989), and LPS (reported in Buendia *et al.*, 1991). It is possible, therefore, that, while no gross differences in the overall LPS band profile are evident between PN184 and the PN184-derived EPS mutants (Fig. 25A), the differences in band staining intensity observed may reflect LPS differences, which are minor in comparison to those observed for *R. meliloti* *exoB*, *exoC*, or *exoE* mutants, but which are sufficient to result in resistance to  $\Phi$ 2037/1. However, more extensive differences in the staining intensity of LPS than those observed between PN184 and the PN184-derived-EPS mutants have been disregarded (Leigh and Lee, 1988), and are not associated with bacteriophage resistance (Finan *et al.*, 1985; Leigh *et al.*, 1985).

An attempt was made to isolate LPS mutants derived from PN184 by Tn5 mutagenesis, followed by selection for non-mucoid colonies on TY-agar, a medium which suppresses the production of EPS (Section 3.3). While potential quantitative LPS mutants were isolated (Table 6), and mutants with an altered intensity of band staining after SDS-PAGE were observed (Fig. 34), no LPS mutants were isolated which showed the desired mutant phenotype, that is, complete or significant loss of bands corresponding to O-antigen polysaccharide

modified LPS, despite screening 60,000 Nm<sup>r</sup> Sm<sup>r</sup> transconjugants. Some differences in the mobility of bands in 0.1% SDS-polyacrylamide gels was observed, however, these mobility differences were eliminated by increasing the SDS concentration to 0.5%. The doublet bands were not observed when LPS was examined on 0.5% SDS- polyacrylamide gels, although LPS migrated as doublet bands only rarely in 0.1% SDS-polyacrylamide gels of LPS from PN184 and the putative LPS mutants. These mutants were not examined further.

Interestingly, LPS from NZP2213, when examined by SDS-PAGE, was also found to migrate as a ladder with large molecular weight intervals between the "rungs", also inconsistent with sequential substitution with increasing O-antigen repeats. However, the molecular weight of each "rung" in the ladder of NZP2213 LPS is less than the molecular weight of the corresponding "rung" for PN184 LPS (Fig. 25B). This suggests that NZP2213 LPS is substituted with a smaller O-antigen polysaccharide than PN184 LPS. The significance of this observation has, however, not been determined. It is notable, however, that LPS mutants of *R. leguminosarum* bv. *phaseoli*, which lack the O-antigen are ineffective on the determinate nodulating *Phaseolus* bean (Noel *et al.*, 1986, Carlson *et al.*, 1989). Therefore, reduced O-antigen polysaccharide on NZP2213 LPS may in part be responsible for the ineffective phenotype of this strain on (at least) its determinate nodulating host *L. pedunculatus*.

## Chapter 5. SUMMARY and CONCLUSIONS

PN184, a streptomycin resistant, Calcofluor-bright, derivative of the broad host range, Calcofluor-bright, *R. loti* strain NZP2037, is able to effectively nodulate both determinate nodulating legumes such as *L. pedunculatus* and indeterminate nodulating legumes such as *L. leucocephala*. This work has shown that selection for a Calcofluor-dark phenotype, after Tn5 mutagenesis, allows the isolation of PN184-derived EPS mutants. The PN184-derived EPS mutants were ineffective on *L. leucocephala*, but were fully effective on *L. pedunculatus*. The mutants either fail to produce a Calcofluor-binding EPS with both high and low molecular weight forms (the rough, PN184-derived EPS mutants) or produce a non-Calcofluor-binding EPS which shows a reduction in *O*-acetylation compared to the EPS produced by PN184 (the smooth, PN184-derived EPS mutants). In addition, the production of high molecular weight EPS by the smooth, PN184-derived EPS mutant PN1177, is reduced compared to PN184.

PN4115, a streptomycin resistant, Calcofluor-dark, derivative of the restricted, effective host range *R. loti* strain NZP2213, is able to effectively nodulate only a restricted range of determinate nodulating legumes, including *L. corniculatus*. The extension of the observations reported above for the PN184-derived EPS mutants to PN4115-derived EPS mutants required mutant identification, after Tn5 mutagenesis, by selection for non-mucoid colony phenotypes. The non-mucoid PN4115-derived EPS mutants isolated failed to produce EPS, and were fully effective on *L. corniculatus* a determinate nodulating legume (PN4115 is ineffective on *L. pedunculatus*). PN4115 is normally ineffective on *L. leucocephala*, but induces the formation of both large, tumour-like, ineffective, nodular structures, and small, ineffective, nodular swellings. The PN4115-derived EPS mutants, in contrast, only induced the formation of small, ineffective, nodular swellings. All the PN4115-derived EPS mutants were found to produce a Calcofluor-binding polysaccharide which appears to be quite different to the Calcofluor-binding polysaccharide (EPS) produced by PN184. This polysaccharide (possibly cellulose), may be able to partially substitute for EPS in invasion, since one of the PN4115-derived EPS mutants, PN1312, was able to invade *L. leucocephala*.

The partial biochemical characterization of EPS from PN184 and the PN184-derived EPS mutants is consistent with infection thread induction on, and invasion

of, *L. leucocephala* requiring the production, by the invading *R. loti* strain, of a highly acetylated EPS, possibly with *O*-acetyl modifications linked to specific glycosyl residues. This work is, therefore, the first study reporting strong evidence of a requirement for specific acetylation of EPS for effective nodulation (invasion) of indeterminate nodulating legumes.

The observations that the PN184-derived EPS mutants show either non-production of EPS, or production of an EPS with altered structure, including significantly reduced *O*-acetylation, compared to EPS from PN184, should have predictive value. Based upon these observations, therefore, one would predict that PN4115, which is able to invade *L. leucocephala* nodules, should produce an EPS which is *O*-acetylated to a similar extent to EPS from PN184, as observed. Similarly, the *L. pedunculatus* infection mutants PN1018, PN1019, and PN1027 might be predicted to show no changes in EPS structure, as results from nodulation tests with the PN184-derived EPS mutants indicate that EPS is not required for effective nodulation of *L. pedunculatus*. As predicted, both PN1019 and PN1027 produce an EPS which is essentially identical to the EPS produced by PN184. PN1018, however, produces an EPS which is less acetylated than the EPS produced by PN184, but *O*-acetylation is not reduced to the same extent as that observed for the smooth, PN184-derived EPS mutants. This observation may, however, be partially consistent with the Nod<sup>-</sup> phenotype of PN1018 on *L. leucocephala* if the mutation carried by PN1018 has a pleiotropic effect on several polysaccharides, including a peptidoglycan bound polysaccharide (Jones *et al.*, 1987). In addition, one might predict that *Rhizobium* sp. strain NGR234, which is able to effectively nodulate *L. leucocephala*, should produce a highly acetylated EPS, as observed (Gray *et al.*, 1991).

It is clear from the results reported in this Thesis that the extracellular polysaccharide profile of PN4115 (and NZP2213) differs substantially to the extracellular polysaccharide profile of PN184 (and NZ2037). NZP2213 fails to produce a peptidoglycan bound polysaccharide required for resistance to prodelphinidin-rich flavolans produced by some host legumes (Jones *et al.*, 1987). In addition, both the EPS produced by PN4115 and the LPS produced by NZP2213 differ significantly to EPS and LPS produced by PN184.

Interestingly, the <sup>1</sup>H-NMR spectrum of PN4115 EPS, closely resembles, with the exception of the degree of *O*-acetylation, the <sup>1</sup>H-NMR spectra of EPS from the

smooth, PN184-derived EPS mutants, suggestive of structural similarity. In addition, both PN4115 and PN1177 produce only a small amount of high molecular weight EPS. Furthermore, both PN4115 and the PN184-derived EPS mutants grow as yellow-cream coloured colonies, while PN184 grows as white coloured colonies.

It must remain a possibility that EPS is not required for invasion of *L. leucocephala* by *R. loti* strains, and that another, as yet unidentified (exo)polysaccharide is required. However, the observation that EPS mutants of *Rhizobium* species NGR234 fail to effectively nodulate *L. leucocephala* (Chen *et al.*, 1985; Djordjevic *et al.*, 1987) suggests that the production of an EPS by *R. loti* would be a likely requirement for effective nodulation. In addition, all the *R. loti* EPS mutants described here show a correlation between mutation, defective EPS synthesis, and ineffective nodulation on *L. leucocephala*. Furthermore, this work has shown that the mutations causing the EPS<sup>-</sup> phenotype of the rough PN184-derived EPS mutants were responsible for ineffective nodule development on *L. leucocephala*.

The results reported in this Thesis are consistent with supporting the hypothesis that EPS is not required for effective nodulation of determinate nodulating *R. loti* host legumes (*L. pedunculatus* and *L. corniculatus* var. *cree*), and are consistent with refuting the hypothesis that EPS is not required for the effective nodulation of the indeterminate nodulating *R. loti* host legume *L. leucocephala*.

Portions of this work have been published (Hotter and Scott, 1991; Appendix 1), the paper being written by the author, while a second publication is currently in preparation.

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## APPENDIX 1

Published Paper.

**Hotter, G. S., and D. B. Scott.** 1991. Exopolysaccharide Mutants of *Rhizobium loti* are Fully Effective on a Determinate Nodulating Host but Are Ineffective on an Indeterminate Nodulating Host. *J. Bacteriol.* 173:852-859.



## ERRATA

piii	line 5	'EPS' should be 'polysaccharide'.
pxiv	Fig. 27A	'epifluorecence' should be 'epifluorescence'.
pxvi	Fig. 44	'tumor' should be 'tumour'.
p42	line 4	'was' should be 'were'.
p52	line 23	'was' should be 'were'.
p54	line 12	'transiluminator' should be 'transilluminator'.
p58	line 22	'cetrifuged' should be 'centrifuged'.
p58	line 32	'transfered' should be 'transferred'.
p65	line 19	'was' should be 'were'.
p89	line 4	'aquired' should be 'acquired'.
p131	line 27	'noticable' should be 'noticeable'.
p202	line 24	'resitance' should be 'resistance'.
p209	line 2	'was' should be 'were'.
p211	line 27	'sustantially' should be 'substantially'.
p211	line 34	'theexception' should be 'the exception'.
p212	line 2	'adition' should be 'addition'.